Effects of Polynucleotides on Production and Activity of Streptolysin S

Yoriko Taketo and Akira Taketo

Departments of Pharmacology and Biochemistry, School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920, Japan

Z. Naturforsch. **35 c,** 578–584 (1980); received March 10, 1980

Streptolysin S, RNA, Polyguanylic Acid, *Streptococcus pyogenes*

Activity of various natural and synthetic polynucleotides as a carrier for streptolysin S was tested in a resting cell system. As the carrier, intact molecules of MS2 RNA, *E. coli* tRNA or rat liver RNA were almost inactive, whereas RNase I core of these RNAs, especially the core fractions eluted from DEAE-cellulose column at higher NaCl concentrations, effectively induced production of the extracellular hemolysin. The carrier activity of yeast RNA was significantly enhanced by simultaneous addition of RNase I, to the streptococcal suspension. Preincubation of yeast RNA with growing streptococci or a protein fraction from the bacterial culture supernatant increased its carrier activity. Evidences were obtained suggesting involvement of streptococcal nuclease in the enhancement of the carrier effect. Production of the streptococcal hemolysin was markedly promoted by polyguanylic acid but neither by polyadenylic acid, polycytidylic acid nor by polyuridylic acid.

Like trypan blue, polyguanylic acid exerted potent inhibitory effect on hemolytic activity of streptolysin S complex. Similar but less marked effect was observed with certain RNase I core fractions of rat liver RNA. Some data concerning effect of polynucleotides on stability of the hemolysin were also presented.

Introduction

Streptolysin S is an oxygen-stable cytolytic exotoxin of hemolytic streptococci. For production of this toxin, addition of certain carrier (or inducing) substance is essential, and the active toxin is released into medium as a complex composed of a polypeptide (apotoxin) and the carrier [1]. Since Okamoto's original finding that yeast RNA enhanced SLS production [2], RNA preparations from various sources have been tested for their effect on the toxin synthesis. Although most RNA preparations were effective, certain RNA species were inactive: thus according to Bernheimer and Rodbart, SLS-inducing effect of tobacco mosaic virus RNA was feeble [3], whereas Kadono reported that rat liver RNA was deficient in SLS-inducing activity when tested in a resting cell system [4]. In addition, ribosomal RNA of *Escherichia coli* could not induce SLS production in the resting cell system [5]. The SLS-inducing effect (or the carrier activity for SLS) of RNA is increased by treatment with RNase I and the activity is contained in the nuclease-resistant core fraction precipitable with 29–40% ethanol [3]. Although *E. coli* ribosomal RNA yielded potent core fraction [5], tobacco mosaic virus RNA and rat liver RNA remained inactive even after RNase I treatment [3, 4].

In order to elucidate role of polynucleotide in SLS production, we have tested the carrier activity of various RNA preparations and synthetic homoribopolymers. In addition, we have investigated effects of polynucleotides on hemolytic activity and stability of the streptococcal exotoxin.

Abbreviations: AF, active fraction of RNase I core of yeast RNA (Guanylic acid-rich oligonucleotides with potent carrier activity for SLS); BBM, Bernheimer's basal medium; HU, hemolytic unit; polyA, polyadenylic acid; polyC, polycytidylic acid; polyG, polyguanylic acid; polyU, polyuridylic acid; SLS, streptolysin S; tRNA, transfer RNA.

Reprint requests to Dr. Akira Taketo.

0341-0382/80/0700-0578 $ 01.00/0

Materials and Methods

Bacteria, media and culture technique

Strain of *Streptococcus pyogenes* used were Sa [6] and C203U [7]. The bacteria were grown in a peptone-meat infusion broth at 37 °C for 16–18 h, without aeration. For resting cell experiments, the bacteria were collected by centrifugation, washed twice with 0.15 M saline and suspended in Bernheimer's basal medium [7]. The bacterial concentration was determined by measuring turbidity at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer.

Polynucleotides and enzymes

Yeast RNA was obtained from Merck Co., Darmstadt or Kohjin Co., Tokyo. PolyG, polyA, polyC and polyU were purchased from Boehringer, Mannheim. AF was prepared from RNase I core of yeast RNA by DEAE-cellulose column chromatography [8]. MS2 phage RNA was extracted from purified MS2 particles by phenol method [9]. For preparation of *E. coli* tRNA, washed cells of strain C were extracted with phenol [10] and the tRNA was collected by ethanol precipitation. The RNA dissolved in 0.1 M KCl −0.02 M potassium acetate (pH 5.5) was applied on a DEAE-cellulose column and eluted with linear KCl gradients of 0.1 to 1.5 M in 0.02 M potassium acetate (pH 5.5). The peak fractions were joined and the tRNA was collected by precipitation with two volumes of ethanol. Wistar rat liver RNA was prepared by the procedure of Kirby [11]. RNase I was purchased from Worthington Biochemical Corp. and pronase P was a product from Kaken Co., Tokyo. For preparation of extracellular streptococcal nuclease, 360 g of ammonium sulfate was added per 600 ml of chilled Sa culture supernatant and the precipitate collected by centrifugation was dissolved in a small volume of 0.02 M potassium acetate, pH 4.5, filtrated through a Sephadex G-25 column, and the effluent (total volume adjusted to 10 ml) was used as the crude nuclease preparation. For further purification, the crude nuclease was passed through a DEAE-cellulose column preequilibrated with the acetate buffer. The effluent was applied on a CM-cellulose column and eluted with gradients of 0.02 M potassium acetate (pH 4.5) −0.2 M potassium acetate (pH 6.5). The peak fractions were joined, diluted with 0.02 M potassium acetate (pH 4.5) and applied on a CM-Sephadex C-25 column. The column was developed with 0.2 M glycine-KOH buffer (pH 9.5) and the peak fraction was preserved at 0 °C. Details of purification and properties of the nuclease will be published elsewhere. To obtain RNase I-resistant core, each RNA (50 OD_{260} units/ml) was incubated with 100 µg/ml of RNase I at 37 °C for 24 h in 0.05 M Tris-HCl (pH 7.2) containing 5 mM EDTA and trace amount of chloroform. After digestion, the core was collected by ethanol precipitation and fractionated on a DEAE-cellulose column as previously described [5].

Table I. Carrier activity of various RNAs for SLS. Washed cells of strain Sa were incubated with each RNA or AF, in BBM at 37 °C. After 60 min, the mixture was centrifuged in the cold and SLS activity in the supernatant was assayed.

RNA	Concentration [mg/ml]	SLS formed [HU/ml]
Yeast RNA	5	320
	10	490
E. coli tRNA	0.1	12
	2.5	23
MS2 RNA	0.1	13
	2.5	80
Rat liver RNA	0.1	12
	2.5	45
AF	0.1	15000

Determination of the carrier activity for SLS

The carrier activity of each polynucleotide preparation was determined from its capacity to induce SLS production in a resting cell system [6]. Titration of SLS and definition of hemolytic unit were the same as those described previously [6, 8].

Nucleotide analysis

Each RNA was hydrolyzed into mononucleotides by incubation in 0.3 N KOH at 37 °C for 15−20 h. After chilling, the mixture was neutralized by perchloric acid and precipitated potassium perchlorate was removed by centrifugation. Nucleotides in the supernatant were then analyzed by Dowex-1 column chromatography [12].

Results

Activity of various RNA preparations to promote SLS formation

When tested in a resting cell system, SLS-inducing effect of *E. coli* tRNA, MS2 RNA and rat liver RNA was negligibly feeble (Table I). Although the carrier activity of yeast RNA preparation was not so high, AF of the RNA exerted remarkable SLS-inducing effect under the experimental conditions. In contrast with intact molecules, RNase I core preparations of *E. coli* tRNA and MS2 RNA efficiently induced SLS production in resting streptococcal suspension (Table II). Moreover, the core fraction of rat liver RNA exhibited potent activity for SLS induction, differing widely from the results reported by Kadono [4]. As shown in Table III, guanylic acid content was markedly high in the RNase I core of rat liver RNA as

Table II. SLS-inducing activity of DEAE fractions of RNase I core. Streptococcal cells were incubated with each sample (2 OD_{260} units/ml) in BBM at 37 °C for 60 min and extracellular SLS activity was determined.

Fraction	SLS formed (HU/ml)		
	E. coli tRNA	MS2 RNA	Rat liver RNA
Original core	0.97×10^3	1.1×10^3	2.9×10^3
Pass through	0.015×10^3	0.011×10^3	0.013×10^3
0.25 M eluate	0.022×10^3	0.074×10^3	0.012×10^3
0.50 M eluate	1.9×10^3	0.95×10^3	2.2×10^3
0.75 M eluate	2.3×10^3	1.2×10^3	4.0×10^3
1.00 M eluate	2.1×10^3	2.4×10^3	5.8×10^3

Table III. Nucleotide composition.

RNA	mol %			
	AMP	GMP	CMP	UMP
Yeast RNA	25.6	27.5	20.5	26.4
Rat liver RNA	17.9	32.4	27.7	22.1
RNase I core of rat liver RNA	14.1	57.5	24.6	3.9
AF	20.4	67.3	5.5	6.8

well as in AF of yeast RNA. When the core of rat liver RNA was subjected to photooxidation in the presence of methylene blue, the SLS-inducing activity was reduced by one half (data not shown). RNase I core of rat liver RNA still contained a large amount of oligonucleotides devoid of SLS-inducing activity. Upon DEAE-cellulose chromatography, these inactive fractions were eluted earlier, whereas oligonucleotides with distinct carrier activity for SLS were eluted at NaCl concentrations of 0.5 to 1.0 M (Table II). In this chromatographic property, RNase I core preparations of *E. coli* tRNA, MS2 RNA and rat liver RNA closely resembled those of yeast RNA or *E. coli* ribosomal RNA [5].

SLS-inducing effect of homoribopolymers

The fact that RNase I core or AF manifests SLS-inducing effect indicates a critical role of guanylic residue in the carrier molecules. Because significance of other nucleotide residues of AF in SLS production was uncertain, experiments were performed using simple polynucleotides with definite sequences. As presented in Table IV, yield of the toxin was conspicuously increased by polyG, but not by polyA, polyC or polyU. In addition, SLS-inducing effect of polyG was not significantly reinforced by simultaneous addition of polyA, polyC or polyU. It is thus clear that guanylic acid cluster *per se* is sufficient for the carrier activity.

Table IV. Carrier activity of polynucleotides for SLS.

Polynucleotide [a]	SLS formed (HU/ml)
Yeast RNA	0.061×10^3
AF	18×10^3
PolyU	0.0045×10^3
PolyC	0.0029×10^3
PolyA	0.015×10^3
PolyG	11×10^3
PolyG and polyU	12×10^3
PolyG and polyC	14×10^3
PolyG and polyA	13×10^3

[a] Each polynucleotide was added at a concentration of 100 µg/ml.

Table V. Effect of streptococcal product on the carrier activity of RNA. Yeast RNA was dissolved, at 10 mg/ml, in deionized water, peptone-meat infusion broth or extracellular protein fraction. The RNA-containing broth was distributed into three tubes and the two tubes were inoculated with a loopful of strain Sa or C203U. Each mixture was incubated at 37 °C for 20 h, chilled, and centrifuged at 15 000 r.p.m. for 20 min. The RNA in the supernatant was precipitated with three volumes of ethanol, dialyzed against deionized water and its carrier activity was tested.

RNA incubated in	SLS formed	
	HU/ml	HU/OD_{260}
Deionized water	3.2×10^3	16
Peptone-meat infusion broth	3.5×10^3	19
Sa culture	12×10^3	84
C203U culture	13×10^3	90
Extracellular protein fraction [a]	22×10^3	204

[a] The fraction was obtained from 20 h-culture supernatant of strain Sa by precipitation with 0.6 g/ml of ammonium sulfate, followed by dialysis against 0.02 M potassium acetate, pH 6.5. The volume was 1/30 of the original culture supernatant. When RNA was incubated in the fraction, 1/100 volume of 0.5 M $MgSO_4$ and a few drops of chloroform were added.

Role of streptococcal nuclease in processing of the carrier RNA

During SLS production in resting streptococci, yeast RNA added as the carrier was degradated in part (unpublished observation). It seems therefore probable that the carrier activity of RNA is modified by certain function of streptococci. In order to verify this possibility, hemolytic streptococci were

grown in a peptone-meat infusion broth supplemented with 1% yeast RNA and the RNA was collected from the culture supernatant by ethanol precipitation. As shown in Table V, the RNA reisolated from the spent medium was more active in SLS induction than untreated RNA. Simple incubation of RNA in a peptone-meat infusion broth had no effect on the carrier activity. Addition of yeast RNA into a culture of streptococcal strain Sa naturally resulted in production of a significant amount of SLS. The exotoxin formation *per se* is, however, not related to the observed increase in the carrier activity. Thus, RNA preincubated with a SLS-negative strain C203U was as active as RNA exposed to Sa culture. Moreover, the carrier activity of yeast RNA was distinctly enhanced by preincubation with a protein fraction of the culture supernatant, suggesting involvement of streptococcal extracellular product, possibly nuclease. Enhancement of the carrier activity did not occur, when a culture supernatant subjected to pronase treatment was used. As shown in Table VI increment of the carrier activity of yeast RNA treated with partially purified streptococcal nuclease was nearly parallel to a degree of hydrolysis by the enzyme. Owing to crudeness of the nuclease preparation, requirements for processing of carrier RNA was not so clear. Sensitivity to EDTA, however, suggests that the nuclease action depends on divalent metal ion such as Mg^{2+}, Ca^{2+} or Mn^{2+}.

Regardless of source, the carrier activity of intact RNA is negligibly low in the resting system, sug-

Table VI. Carrier activity of RNA treated with streptococcal nuclease.

Condition of treatment	Ethanol-soluble fraction OD_{260}	Carrier activity HU/mg Ethanol-insoluble fraction
Complete [a]	59	45×10^3
− Enzyme	6.6	0.44×10^3
− Mg^{2+}	36	28×10^3
− Ca^{2+}	57	30×10^3
− Mg^{2+}, − Ca^{2+}	33	14×10^3
+ 5 mM EDTA	12	0.72×10^3

[a] Yeast RNA was incubated with the crude nuclease preparation in 0.05 M potassium phosphate buffer (pH 7.0) containing 5 mM $MgSO_4$ and 5 mM $CaCl_2$ at 37 °C for 10 h. Two volumes of chilled ethanol were added and the mixture was centrifuged at 15 000 r.p.m. for 20 min. The supernatant was subjected to measurement of optical ensity at 260 nm, while the precipitate was dried in vacuo and its carrier activity was tested in the resting cell system.

Table VII. Effect of medium on activity of streptococcal nuclease. Yeast RNA (175 OD_{260} units) was incubated with or without streptococcal nuclease (25 µg/ml) at 37 °C for 25 h, in the presence of trace amount of chloroform. Each mixture was chilled, acidified with 1/13 volume of ice-cold 5 N perchloric acid, centrifuged and optical density of the supernatant was measured at 260 nm. The acid-insoluble fraction was dissolved in 0.2 M glycine-NaOH buffer (pH 9.5) and mixed with 4 volumes of ethanol. The mixture was kept at −20 °C overnight and the precipitate was collected by centrifugation. After washing with ethanol-ether mixture, the acid-insoluble fraction was dissolved in deionized water and its carrier activity as well as the optical density at 260 nm was measured. The value of each control mixture (incubated without nuclease) was subtracted.

Medium	RNA hydrolyzed Acid soluble OD_{260}	Carrier activity HU/Acid insoluble OD_{260}
Glycine buffer [a]	140	1.3×10^3
Peptone-meat infusion broth	127	0.72×10^3
BBM	48	0.08×10^3

[a] 0.1 M glycine-KOH buffer (pH 9.0) containing 1 mM $MgSO_4$ and 1 mM $CaCl_2$.

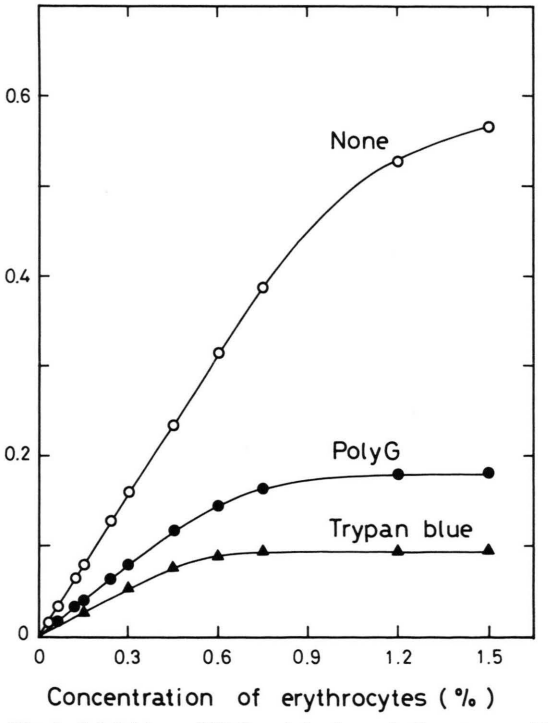

Fig. 1. Inhibition of SLS activity by polyG or trypan blue. Varying amounts of erythrocytes were incubated with a definite concentration of AF-SLS complex, in the presence or absence of polyG (1 µg/ml) or trypan blue (0.015 µg/ml) at 37 °C for 30 min. After addition of chilled 0.15 M saline, each mixture was centrifuged and hemoglobin released into the supernatant was measured with a Hitachi UV-VIS spectrophotometer.

Table VIII. Effect of polynucleotides on hemolytic activity of SLS. Washed streptococcal cells were incubated with yeast RNA (10 mg/ml), AF (0.1 mg/ml) or polyG (0.1 mg/ml) in BBM at 37 °C for 60 min. After removing the bacteria by centrifugation, each SLS complex released into the supernatant was partially purified by gel filtration through a Sephadex G-75 column. The SLS complex and erythrocytes were incubated in 0.15 M saline in the presence or absence of each polynucleotide (50 μg/ml) at 37 °C for 60 min.

Polynucleotide	Hemolytic activity (HU/ml)		
	RNA-SLS	AF-SLS	PolyG-SLS
None	0.69×10^3 (100) [a]	10×10^3 (100)	3.7×10^3 (100)
Yeast RNA	0.56×10^3 (81)	7.0×10^3 (70)	2.7×10^3 (73)
AF	0.47×10^3 (68)	6.9×10^3 (69)	2.7×10^3 (73)
PolyU	0.53×10^3 (77)	6.7×10^3 (67)	4.0×10^3 (108)
PolyC	0.58×10^3 (84)	6.7×10^3 (67)	5.2×10^3 (141)
PolyA	0.57×10^3 (83)	6.4×10^3 (64)	5.6×10^3 (151)
PolyG	0.00048×10^3 (0.069)	0.16×10^3 (1.6)	0.091×10^3 (2.5)

[a] Per cent.

gesting that either extracellular production of streptococcal nuclease is repressed or the enzymatic activity is inhibited in the system. In order to discriminate the alternatives, yeast RNA was digested by purified streptococcal nuclease in different media. As seen in Table VII, extent of degradation of RNA was low in the resting cell medium (BBM) and the acid-insoluble fraction obtained from BBM was, in SLS induction, less efficient than those from a glycine buffer or a meat-infusion broth. Even in BBM, resting streptococci produced an extracellular nuclease whose activity was masked in the medium but could easily be assayed in the glycine buffer (manuscript in preparation). On the other hand, addition of RNase I (100 μg/ml) into streptococcal suspension in BBM containing yeast RNA (10 mg/ml) resulted in 4.5-fold increase in SLS yield as compared with RNase I-unsupplemented control.

Inhibition of SLS by polyG

Effects of various polynucleotides on hemolytic activity of three SLS complexes are compared in Table VIII. Yeast RNA, polyU, polyC and polyA did not particularly affect the toxin-induced hemolysis. Regardless of the carrier moiety, hemolytic activity of SLS complexes was severely antagonized by polyG. A polyG preparation purified by filtration through a Sephadex G-75 column exhibited the same inhibitory effect as commercial products. In addition, GTP did not inhibit SLS activity. As shown in Fig. 1, mode of inhibition by polyG was rather similar to that of trypan blue, a specific inhibitor of SLS [13]. Similar SLS-inhibiting activity was associated with RNase I core preparations of *E. coli* ribosomal RNA [5], yeast RNA [14] and rat liver RNA. When the core of rat liver RNA was chromatographed on a DEAE cellulose column, the inhibitory activity was detected in fractions having potent SLS-inducing effect. Thus, hemolytic activity of 6.5×10^3 HU/ml of AF-SLS was reduced to 28% by the original core, 89% by 0.25 M NaCl eluate, 34% by 0.5 M eluate and 10% by 0.75 M eluate. (Each fraction was dialyzed against deionized water and then its SLS-inhibiting activity was tested at a concentration of 50 μg/ml.) On the other hand, hemolytic activity of the toxin was only slightly affected by AF. It is thus evident that the hemolysis-inhibiting effect of ribonucleotides is not always parallel with its carrier activity for SLS.

Effect of polynucleotides on stability of SLS

Like RNA-SLS or AF-SLS, the toxin induced by polyG was thermally unstable. Although polyU, polyC, and polyA were inactive as the carrier for SLS, these polynucleotides might stabilize the hemolysin. Therefore, streptococci were incubated with 100 μg/ml of polyU, polyC or polyA in BBM supplemented with 100 μg/ml of polyG. The hemolysin activity in the supernatant was, however, as heatlabile as that induced by polyG alone. Moreover, inactivation kinetics of polyG-SLS at 50 °C was not affected by a presence of 100 μg/ml of polyU, polyC or polyA (data not shown).

Discussion

The data presented above demonstrate that *E. coli* tRNA, phage MS2 RNA and rat liver RNA are feeble in promotion of SLS synthesis in a resting cell system. RNase I-resistant core fraction of these RNA, however, effectively induces production of the streptococcal exotoxin. These experimental results, together with previous observation on the carrier activity of *E. coli* ribosomal RNA [5] prove ineffectiveness of intact RNAs in SLS production. Although commercial preparation of yeast RNA is somewhat active in SLS synthesis, the RNA molecules are generally far from intact. (A considerable amount of oligonucleotides soluble in cold 0.5 N perchloric acid was detected in yeast RNA preparations used in present investigation.) On the other hand, Bernheimer and Rodbart described that, in a growing streptococcal culture, beef liver RNA effectively promoted SLS formation and the toxin-inducing effect was not enhanced by RNase I treatment [3]. Even in a growing culture, SLS-inducing activity of our rat liver RNA preparation was manifested only after treatment with RNase I: specific carrier activity of RNase I core of rat liver RNA was 157 times higher than that of the original RNA (unpublished observation). Data on nucleotide composition of the beef liver RNA preparations are lacking and direct comparison of Bernheimer and Rodbart's observation with ours is difficult. Previously, Kadono reported that SLS-inducing activity of rat liver RNA in a resting cell system was reduced by conversion into RNase I core [4]. Nucleotide composition of the core preparation was not determined and reasons for the reported ineffectiveness of RNase I treatment are quite unknown. It may be relevant to note that rat liver contains potent RNase inhibitor [15]. Moreover, RNase core fractions with the carrier activity exert an inhibitory effect toward SLS. At any rate, extensive digestion of rat liver RNA with RNase I does yield guanylic acid-rich core fraction with high carrier activity for the toxin.

SLS-inducing activity of polyG is nearly comparable to that of AF, whereas polyA, polyC and polyU are devoid of the activity. On the other hand, previous experiments have shown that oligoguanylic acid is, albeit active, far inferior to AF in SLS formation [16]. Whether the distinction is simply due to difference of chain length of the carrier nucleotides or not is to be settled. The finding that such simple homopolymer as polyG has strong carrier activity for SLS may afford a clue to elucidate mechanism of interaction between the toxin peptide and the carrier nucleotide.

Although the carrier activity of RNA is enhanced by treatment *in vitro* with RNase I, involvement of this pancreatic enzyme in SLS formation in affected tissue is improbable. SLS-inducing activity of yeast RNA is increased by incubation either in growing streptococcal culture or in the protein fraction of the culture supernatant. Further evidences obtained indicate participation of streptococcal extracellular nuclease in the "processing" of the carrier RNA. Occurrence of the nuclease is pathologically significant: RNAs released from damaged host tissues may be converted in situ into the carrier by the streptococcal nuclease and consequently promote formation of the cytolytic SLS complex. (Besides this, digested RNA may serve as a nutrient for streptococci.) Further work is in progress to clarify roles of streptococcal nuclease in the bacterial infection.

Interestingly enough, hemolytic activity of SLS is inhibited by polyG. It seems likely that carrier molecules with high affinity to SLS inhibit transfer of the apotoxin to its receptor site on erythrocyte surface. As reported by Ginsburg *et al.* [17], trypan blue serves as a carrier for the hemolysin. In the resting cell system, about 370 HU/ml of SLS was produced by addition of 10 μg/ml of the dye, and hemolytic effect of the toxin was also antagonized effectively by polyG (unpublished observation). On the other hand, AF does not exhibit such duality, indicating that SLS-inducing activity and the toxin-inhibiting effect are dissociable in a certain carrier substance. Although rich in guanylic acid residue, AF contains adenylic acid, cytidylic acid and uridylic acid units as well. Whether these nucleotide residues in AF suppress SLS-inhibiting activity of guanine cluster or not is now under investigation.

[1] I. Ginsburg, Microbial Toxins, **Vol. III,** (T. C. Montie, S. Kadis, and S. J. Ajl, eds.), pp. 90–171, Academic Press, New York 1970.
[2] H. Okamoto, Jap. J. Med. Sci. **IV,** 12, 167–208 (1940).
[3] A. W. Bernheimer and M. Rodbart, J. Exp. Med. **88,** 149–168 (1948).
[4] K. Kadono, Ann. Rept. Res. Inst. Tubercl. Kanazawa Univ. **23,** 67–76 (1965).
[5] Y. Taketo and A. Fujimura, Ann. Rept. Res. Inst. Tubercl. Kanazawa Univ. **21,** 143–148 (1963).
[6] A. Taketo and Y. Taketo, J. Biochem. **56,** 552–561 (1964).
[7] A. W. Bernheimer, J. Exp. Med. **90,** 373–392 (1949).
[8] Y. Taketo amd A. Taketo, J. Biochem. **60,** 357–362 (1966).
[9] A. Taketo, M. Ono, and H. Shibuya, J. Biochem. **57,** 488–496 (1965).
[10] G. Zubay, J. Mol. Biol. **4,** 347–356 (1962).
[11] K. S. Kirby, Biochem. J. **64,** 405–408 (1956).
[12] S. Osawa, K. Takata, and Y. Hotta, Biochim. Biophys. Acta **28,** 271–277 (1958).
[13] R. Ito, Folia Pharmacol. Japan. **30,** 124–140 (1940).
[14] Y. Taketo and A. Taketo, J. Biochem. **61,** 450–459 (1967).
[15] M. P. Scott, R. V. Storti, M. L. Pardue, and A. Rich, Biochemistry **18,** 1588–1594 (1979).
[16] K. Itagaki, Y. Kuriyama, Y. Shiobara, H. Hayashi, T. Yamagata, and F. Egami, J. Jap. Biochem. Soc. **37,** 217–225 (1965).
[17] I. Ginsburg, Z. Bentwich, and T. N. Harris, J. Exp. Med. **121,** 633–645 (1965).