Effects of Trypan Blue and Related Compounds on Production and Activity of Streptolysin S

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Most dyes related to trypan blue inhibited hemolytic activity of oligonucleotide-streptolysin S (SLS) complex, an exotoxin produced by *Streptococcus pyogenes*. Order of the inhibition was: trypan blue > benzo blue 2B > Congo red > Evans blue > benzo purpurine 4B > thiazine red > trypan red. When resting streptococcal cells were incubated with these dyes, significant amount of the hemolysin was produced. The carrier (or inducing) activity for SLS was further manifested in growing cell system and the potency of the compounds was as follows: Congo red > benzo blue 2B > trypan blue > Evans blue > Benzo purpurine 4B ≥ thiazine red > trypan red. In this system, Congo red was more effective than oligonucleotide fraction rich in guanyl residue. Chromotrope 2B, H acid and *o*-tolidine were ineffective, as the carrier as well as the inhibitor. Based on these results, structure-function relationship among SLS, the carrier and the inhibitor was discussed.

Introduction

Production of streptolysin S (SLS), a cytolytic exotoxin of hemolytic streptococci, is dependent on certain carrier (or inducing) substance which forms active complex with the toxin polypeptide [1]. Various substances are hitherto known as the carrier which include RNA [2], its RNase I-resistant oligonucleotide fraction [3, 4,], polyguanylic acid (polyG) [5], serum components [6, 7], certain detergents [8] and lipoteichoic acid [9]. These compounds are so divergent that it is hardly possible to deduce common chemical feature participated in the carrier activity.

On the other hand, trypan blue and its derivatives specifically inhibit hemolysis [10] and oncolysis [11, 12] caused by SLS complex. Moreover, at lower concentration, trypan blue serves as a carrier for SLS [5, 13]. PolyG, a potent carrier for SLS, also inhibits hemolysis caused by the toxin [5], and the inhibiting ability is retained even after dialysis against concentrated urea (unpublished observation). These results suggest existence of an intimate relationship between the carrier effect and the inhibitor activity.

Unlike complicated biogenic substances, trypan blue is structurally simple and several derivatives are commercially available. In order to elucidate chemical structures necessary for the inhibitor and the carrier activities, we have examined capacity of various chemicals for inhibition and production of SLS complex. Dyes of trypan blue series have distinct carrier activity for SLS and the potency is generally parallel to SLS-inhibiting activity of each compound. Both the carrier effect and the inhibitor activity are explicable by an unitary mechanism *i.e.* transfer of SLS polypeptide among different receptor substances.

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Materials and Methods

Chemicals

Trypan blue (3,3′-[3,3′-dimethyl[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt), Congo red (3,3′-[[biphenyl]-4,4′-diylbis(azo)]-bis[4-amino-1-naphthalenesulfonic acid] disodium salt), Evans blue (6,6′-[(3,3′-dimethyl[1,1′-biphenyl]-4,4′-diyl)bis(azo) bis[4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid] tetrasodium salt), thiazine red (2-[4-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]phenyl]-6-methyl-7-benzothiazolesulfonic acid disodium salt), Janus green (3-(dimethylamino)-7-[[*p*-(dimethylamino)-phenyl]azo]-5-phenylphenazinium chloride), *o*-tolidine dihydrochloride (3,3′-dimethyl-[1,1′-biphenyl]-4,4′-diamine dihydrochloride) and gentian violet ([4-[bis[*p*-(dimethylamino)phenyl]methylene]-2,5-cyclo-

hexadiene]-N-methylmethanaminium chloride) were purchased from Wako Pure Chemical Ind., Osaka. Benzo blue 2B (3,3′-[[biphenyl]-4,4′-diylbis-(azo)]-bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt), benzo purpurine 4B (3,3′-[(3,3′-dimethyl[1,1′-biphenyl]-4,4′-diyl)bis(azo)] bis[4-amino-1-naphthalenesulfonic acid] disodium salt), trypan red (4,4′-[(3-sulfo-4,4′-biphenylene)bis-(azo)]bis(3-amino-2,7-naphthalenedisulfonic acid) pentasodium salt) and H acid (1-naphthol-8-amino-3,6-disulfonic acid monosodium salt) were products from Tokyo Chemical Co., Tokyo and chromotrope 2B (4,5-dihydroxy-3-[(4-nitrophenyl)azo]-2,7-naphthalenedisulfonic acid disodium salt) from Chroma Gesellschaft, Stuttgart. Guanylic acid-rich oligonucleotide fraction (AF) of RNase core and the oligonucleotide-SLS complex were prepared as described previously [11, 14].

Assay of SLS-inhibiting activity

Oligonucleotide-SLS complex was accurately diluted with chilled 0.15 M saline in the presence or absence of each test compound. Hundred-fold and ten-fold dilution systems were used in combination with two-fold dilution system. (Two-fold serial dilution amplifies pippeting error and causes overestimation of SLS titer.) One ml of the diluted sample was mixed with one ml of chilled 3% rabbit erythrocyte suspension and incubated at 37 °C for 60 min. After addition of 2 ml of chilled 0.15 M saline, the mixture was centrifuged at 0 °C and hemoglobin released into the supernatant was determined by the optical density at 541 nm. Definition of hemolytic unit (HU) was the same as previously described [11, 14].

Determination of carrier activity for SLS

The carrier activity of each compound in resting cell system was determined, using *Streptococcus hemolyticus* strain Sa, as described previously [5]. For assay of the carrier activity in growing cell system, cells of strain Sa were cultured at 37 °C in peptone-meat infusion broth containing the test chemicals. After growing for 4 or 6 h, the mixture was centrifuged at 0 °C and hemolytic activity of the supernatant was determined. Growth of each culture was followed by measuring the turbidity at 660 nm, using a Bausch & Lomb Spectronic 20 spectrophotometer.

Results

Effect of various chemicals on hemolytic activity of SLS complex

Hemolytic activity of oligonucleotide-SLS complex was severely inhibited by derivatives of diphenyldiazo-bis-naphthalene sulfonate. As shown in Fig. 1, degree of the inhibition was: trypan blue > benzo blue 2B > Congo red > Evans blue > benzo purpurine 4B. Presence of methyl group in diazo-linked diphenyl portion was not crucial for the inhibitor activity: thus inhibitory effect of trypan blue (containing ditolyl group) and benzo blue (carrying diphenyl group) was not markedly different. Posi-

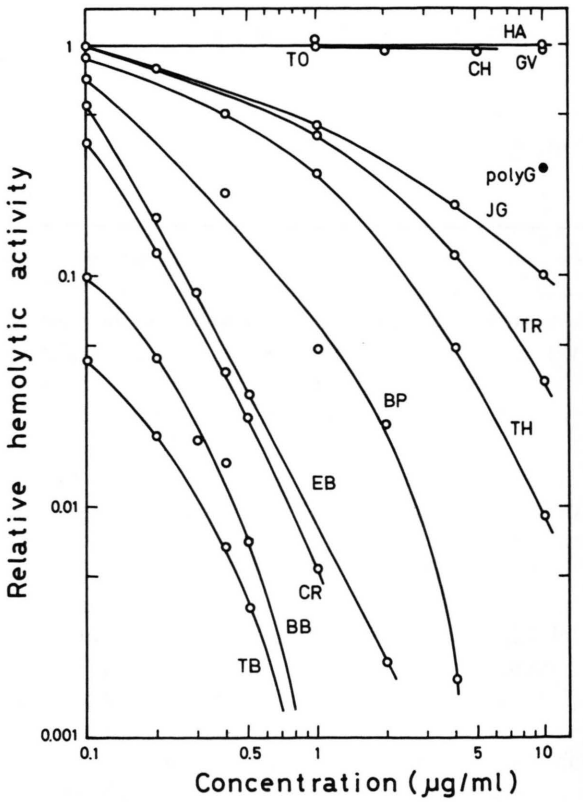

Fig. 1. Effect of various chemicals on the hemolytic activity of SLS complex. Oligonucleotide-SLS complex (1.5×10^4 HU/ml) was diluted with 0.15 M saline containing the indicated compound, then mixed with erythrocytes and incubated for 60 min at 37 °C. After centrifugation, amount of hemoglobin released into the supernatant was determined. Abbreviations: TB, trypan blue; BB, benzo blue 2B; CR, Congo red; EB, Evans blue; BP, benzo purpurine 4B; TH, thiazine red; TR, trypan red; JG, Janus green; TO, *o*-tolidine dihydrochloride; CH, chromotrope 2B; HA, H acid; GV, gentian violet.

tion of amino or hydroxy group in naphthalene ring as well as number and position of sulfonate group affected the SLS inhibiting activity to some extent. Chemicals which inhibited SLS were, however, not limited to bisazo dyes of benzidine series. Actually, thiazine red was more inhibitory than trypan red and Janus green was clearly obstructive to SLS-induced hemolysis. On the other hand, H acid, *o*-tolidine dihydrochloride and chromotrope 2B did not inhibit hemolytic activity of the streptococcal toxin, indicating that neither benzene-linked azo-naphthalene sulfonate moiety nor bitolyl group was sufficient for the inhibitory effect. Gentian violet, a dye structurally unrelated to trypan blue, was devoid of the SLS-inhibiting activity. These results confirm and extend qualitative observation of Ito [10] on SLS-inhibiting effect of trypan blue derivatives.

Carrier activity of trypan blue-related dyes for SLS

When washed streptococci suspended in Bernheimer's basal medium (BBM) [15] were incubated with certain chemicals of trypan blue series, considerable amount of SLS was produced extracellularly (Table I). For manifestation of the SLS-inducing effect, about 10 μg of each compound per ml of the incubation mixture was suitable. At higher concentrations, carrier activity for SLS of these dyes was hardly detectable, owing to masking of SLS activity by free dyes. The order of effectiveness as the SLS carrier was: trypan blue > benzo blue 2B > Evans blue > Congo red > benzo purpurine 4B > trypan red. Thiazine red, having diazo-naphthol-sulfonate moiety but considerably differing from trypan blue series, was significantly efficient in the toxin production. As the SLS carrier, these compounds were more active than yeast RNA, though less effective than AF. Chromotrope 2B, *o*-tolidine dihydrochloride and H acid were deficient in the carrier activity, whereas gentian violet and Janus green were rather inhibitory for production of the streptococcal toxin. It seems noteworthy that SLS-inducing (or carrier) activity of these chemicals was roughly parallel to their inhibitory effect on the hemolysin.

Table I. Effect of various chemicals on SLS production in resting cell system. Hemolytic streptococci suspended in BBM were incubated with the indicated reagent, at 37 °C for 60 min. After centrifugation, the hemolysin released into the medium was titrated.

Chemicals [10 μg/ml]	SLS produced [HU/ml]
None	7.2
Yeast RNA	7.8
AF	2662.7
Trypan blue	607.2
Benzo blue 2B	361.9
Evans blue	336.4
Benzo purpurine 4B	129.1
Congo red	224.7
Trypan red	55.2
Thiazine red	45.0
Chromotrope 2B	8.0
H acid	7.4
o-Tolidine dihydrochloride	7.6
Gentian violet	6.0
Janus green	1.4

Table II. Effect of various chemicals on SLS production and growth of hemolytic streptococci. Cells of strain Sa were cultured, at 37 °C for 6 h, in peptone-meat infusion broth containing the indicated reagent. The bacterial growth was followed by measuring turbidity at 660 nm, whereas the extracellular hemolysin was titrated after centrifugation.

Chemicals [10 μg/ml]	SLS formed [HU/ml]	Growth [OD_{660}]
None	22.5	0.268
Yeast RNA	23.7	0.270
AF	857.3	0.263
Trypan blue	751.2	0.272
Benzo blue 2B	875.4	0.252
Evans blue	605.7	0.262
Benzo purpurine 4B	315.9	0.260
Congo red	1182.6	0.272
Trypan red	148.8	0.265
Thiazine red	330.8	0.255
Chromotrope 2B	44.6	0.264
H acid	22.1	0.262
o-Tolidine dihydrochloride	20.2	0.264
Gentian violet	0.7	0.013
Janus green	< 0.1	0.011

The carrier activity of trypan blue derivatives was more pronounced in growing culture of hemolytic streptococci (Table II). This might be due to enhanced production of dye-SLS complex during longer incubation in enriched medium. In the growing cell system, Congo red, at 10 μg/ml was more active than AF. Even benzo blue exhibited the carrier activity comparable to that of AF. (Decreased yield of AF-induced SLS in the growing cell system may probably be due to inactivation during the prolonged incubation, rather than reduced synthesis.) The degree of effectiveness in the growing culture was as follows: Congo red > benzo blue 2B > trypan blue

Table III. Effect of antibiotics on SLS production induced by Congo red. Streptococci grown in peptone-meat infusion broth were collected and suspended, at $OD_{660} = 0.10$, in the fresh medium containing Congo red (10 μg/ml) and the indicated drug. The culture was incubated at 37 °C for 4 h and amount of extracellular SLS was determined.

Addition	Concentration	SLS formed [HU/OD_{660} of the culture]
None	–	3.1×10^3
Rifampicin	1	< 3
Chloramphenicol	100	< 3
Tetracycline hydrochloride	100	< 3

> Evans blue > thiazine red ⩾ benzo purpurine 4B > trypan red. This order is somewhat different from that in the resting cell system and might also reflect varying stability of each SLS complex. At any rate, dyes derived from diphenyl-diazo-bisnaphthylamine sulfonate exerted remarkable carrier effect on the toxin. In addition, chromotrope 2B revealed low but significant carrier activity in growing culture, whereas *o*-tolidine dihydrochloride and H acid remained inactive. By addition of 10 μg/ml of gentian violet or Janus green, the cellular growth was completely prevented and no SLS activity was detected in the medium. Other dyes, however, did not significantly affect growth of hemolytic streptococci.

As shown in Table III, Congo red-induced SLS production in growing streptococci was, like AF-dependent synthesis [16], extremely sensitive to rifampicin. Moreover, the toxin production was inhibited by chloramphenicol or tetracycline.

When the crude SLS induced by Congo red was passed through a Bio-Gel P-6 column with 0.1 M KCl, the active toxin was eluted nearly in a void volume. On the other hand, free Congo red was adsorbed by the matrix and 20% ethanol was required for its elution. Further purification and characterization of the dye-induced SLS is in progress.

Discussion

Bisazobenzidine dyes such as Congo red, benzo blue 2B, trypan blue and Evans blue exhibited dual activities for SLS: inhibition of its hemolytic function on the one hand and manifestation of its production on the other hand. These apparently paradoxical effects are explicable by mutal transfer of SLS polypeptide among carrier substances [17, 18]. Thus, after synthesis, the toxin polypeptide is transported into cell envelope and probably bound to an endogenous carrier site, transiently [19, 20]. Upon incubation with AF or azo dye having higher affinity, SLS is transferred to exogenous carrier and secreted into medium as the active exotoxin complex. Excess free dye, however, attracts SLS and consequently prevents transfer of the polypeptide to putative receptor site on erythrocyte surface.

For the carrier function of diphenyl-diazo-bisnaphthalene sulfonate derivatives, number and position of amino and/or hydroxy group and sulfonate group in naphthalene ring, as well as presence of methyl group in diphenyl portion, are not so crucial. Among these dyes, Congo red is the most simple and effective carrier for the toxin, at least in the growing cell system. On the other hand, thiazine red has a moderate effect and chromotrope 2B is slightly effective, whereas H acid and *o*-tolidine dihydrochloride are devoid of the carrier activity. These facts suggest that phenyl-azo-amino (or hydroxy) naphthalene sulfonate moiety is essential and its dimer is sufficient to induce proper conformation of SLS polypeptide. It might be more than fortuitous coincidence that guanylic acid as well bears amino and hydroxy groups and a negatively charged group (phosphate). In order to acquire the carrier activity, this nucleotide too must be polymerized.

Although hydrogen bond-dependent aggregation (or micelle formation) is thought to participate in the carrier activity of AF (probably polyG as well), ionic association may also be involved. As to binding of the carrier dyes to SLS polypeptide, at least three ways might be considered: hydrogen bonding through amino and/or hydroxy group, hydrophobic interaction mediated by benzene and/or naphthalene ring and electrostatic interaction through sulfonate group. Whether diverse carrier substances interact with SLS polypeptide by single chemical bonding or not is presently unknown. Obviously, information on structure of SLS polypeptide is also required for elucidation of the nature of bonding.

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