Inhibition of Cell Proliferation by \textit{Rana catesbeiana} and \textit{Rana japonica} Lectins Belonging to the Ribonuclease Superfamily\textsuperscript{1}

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\textbf{ABSTRACT}

Two frog egg lectins [\textit{Rana catesbeiana} lectin (SBL-C) and \textit{Rana japonica} lectin] preferentially agglutinate a large variety of human and animal tumor cells but not blood cells, lymphocytes, or fibroblasts. These lectins belong to the superfamily of pyrimidine base-specific RNases. The two lectins bound to a heparin-Sepharose column and were eluted from the column by an increase of NaCl molarity. Both their tumor cell-agglutinating activity and RNase activity were inhibited by heparin, and also by polyamines, such as spermine. Both lectins inhibited P388 leukemia cell proliferation. The inhibitory activity of SBL-C was blocked by addition of heparin. SBL-C inhibited protein synthesis by P388 cells, but RNase A did not. No lectin-induced antiproliferative effect was observed after sialidase treatment of cells. The antiproliferative activity of SBL-C was also inhibited by ammonium chloride treatment. These results suggest that internalization of the lectin by lectin receptor (sialo-glycoconjugate)-mediated endocytosis is followed by cell death due to inhibition of protein synthesis. Administration of SBL-C i.p. delayed time to death in mice receiving i.p. transplants of Sarcoma 180 and Mep II cells.

\textbf{INTRODUCTION}

Two frog egg lectins, isolated from \textit{Rana catesbeiana} (SBL-C) and from \textit{Rana japonica} (SBL-J), have been found to agglutinate a large variety of tumor cells, and this agglutinating activity is inhibited by sialoglycoproteins and gangliosides (1–3). The complete amino acid sequences and intramolecular disulfide bond pairs of SBL-C and -J have been elucidated (4–6). These lectins are carbohydrate-free single-chain basic proteins consisting of 111 amino acid residues and 4 disulfide bonds. Their sequences, including attachments of disulfide bond pairs, are highly homologous to that of a RNase isolated from \textit{R. catesbeiana} (7), and also relatively similar to those of human angiogenin (8), onconase (P-30 protein) isolated from \textit{R. pipiens} eggs (9, 10), and a pancreatic RNase family (11). Since the two lectins act as RNases, they are classed as members of the RNase superfamily. Diphtheria toxin and other cytotoxic proteins bind to their respective receptors on the cell surface membrane and undergo internalization and a translocation process, whereby at least the enzymatically active portion of the toxin penetrates to the cytosol compartment and catalyzes transfer of the ADP-ribose moiety of NAD into covalent linkage with elongation factor 2, thus blocking protein synthesis and causing cell death (12, 13). As reported here, SBL-C and -J inhibit cell proliferation, and it is possible that their cytotoxicity results from receptor (sialo-glycoconjugate)-mediated endocytosis followed by intracellular biological activity.

In the present report, we: (a) determine the affinity of SBL-C and -J for a heparin column, effects of heparin and polyamines on tumor cell-agglutinating and RNase activities of these lectins, and mediation of their cytotoxic activity by receptor recognition; (b) compare the action of these lectins with that of RNase A; and (c) demonstrate that SBL-C is effective for \textit{in vivo} therapy of tumor-bearing mice.

\textbf{MATERIALS AND METHODS}

\textbf{Materials and Cell Lines.} Bovine pancreatic RNase A (type IIIA) and \textit{Torula} yeast RNA (type VI) were obtained from Sigma Chemical Co. (St. Louis, MO). Sialidase from \textit{Arthrobacter ureafaciens} was purchased from Yacobi Tai (Kyoto, Japan). Sialidase inhibitor (2,3-dehydro-2-deoxy-N-acetylenuraminic acid) was from Boehringer Mannheim. Heparin-Sepharose and S-Sepharose were obtained from Pharmacia-LKB. P388 and L1210 murine leukemia cells were obtained from the Japanese Cancer Research Resources Bank. Rat (Donryu) ascites hepatoma AH109A cells were originally donated from the Institute of Development, Aging and Cancer, Tohoku University. Sarcoma 180 cells and Ehrlich cells were propagated as ascites in ddY mice, and Mep II ascites cells were grown in ddY mice. These cell lines were also donated from the Cancer Cell Repository, the Institute of Development, Aging and Cancer, Tohoku University.

\textbf{Purification of Lectins by Heparin-Sepharose Affinity Chromatography.} “Fraction II” from \textit{R. catesbeiana} eggs was isolated by sequential chromatographies on Sephadex G-75, DEAE-cellulose, hydroxyapatite, and carboxymethyl cellulose columns as described previously (3). Applied to a heparin-Sepharose column and eluted with a linear NaCl gradient as described in the legend to Fig. 1A. The active fractions indicated by the bar in Fig. 1A were pooled and designated as SBL-C.

\textbf{SBL-J} was obtained from \textit{R. japonica} by a modification of the method of Sakakibara et al. (2), i.e., sequential chromatographies on Sephadex G-75, DEAE-cellulose, heparin-Sepharose, and S-Sepharose columns (see Fig. 1, B and C).

\textbf{Preparation of Modified SBL-Cs.} Modified SBL-Cs were prepared by acetylation (14), carbamylation (15), maleylation (16), reductive methylation (17), succinylation (18), and 2,3-butanediol modification (19) as described previously (3, 4). Polyacrylamide Gel Electrophoresis and High Performance Liquid Chromatography. Sodium dodecyl sulfate-PAGE was performed on a slab gel apparatus using the method of Laemmli (20). Nondenaturing PAGE was performed in 7% gel with \(\beta\)-alanine/acetic acid (pH 4.5) according to the method of Reisfeld et al. (21). Protein content of samples was determined by fluoroscinic acid (22) with bovine serum albumin as the standard.

The size exclusion chromatography using a TSK gel G3000 SW was performed with phosphate-buffered saline (10 mM, pH 7.4) as solvent, with a flow rate of 1.0 ml/min.

\textbf{Agglutination and Inhibition Assay.} Tumor cell agglutination was assessed using Ehrlich ascites carcinoma cells, AH109A cells, and F388 leukemia cells as described previously (3). Inhibition of lectin-induced AH109A agglutination was evaluated based on effects of serial dilutions of each potential inhibitor (3).

\textbf{RNase Activity and Enzyme Inhibition Assay.} Standard assay was performed according to the method of Reddi (23) with some modifications. Enzyme solution (20 \(\mu\)l) was added to 180 \(\mu\)l of RNA solution consisting of 50 mM acetate buffer (pH 6.0) and 0.1% RNA, and the mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 200 \(\mu\)l of ice-cold
Fig. 1. Preparation of SBL-C and SBL-J. A, heparin-Sepharose affinity chromatography of “Fraction II” (3) obtained from R. catesbeiana eggs. “Fraction II” (33 mg) was applied to a heparin-Sepharose column (1.0 × 20 cm) equilibrated with 50 mM acetate buffer (pH 6.0). After a washing with 300 ml of equilibrating buffer, the column was developed with a linear 0–0.8 M NaCl gradient in the same buffer (total volume, 600 ml) (the gradient began at Tube 25). Fractions of 5.3 ml were collected, and each fraction was assayed for protein (absorbance at 280 nm) and RNase activity. RNase activity determined by the method of Reddi (23) was expressed as absorbance at 260 nm. The appropriate fraction (Tubes 47–54) indicated by the bar was pooled, dialyzed against distilled water, and lyophilized. Using lyophilized fractions 47–54 (in protein solution with a concentration of 50 μg/0.2 ml), obvious agglutination of 10^6 tumor cells (i.e., Ehrlich ascites cells, AH109A ascites cells, or P388 cells) was indicated as +. B, heparin-Sepharose affinity chromatography of “D1 fraction” (2) from R. japonica eggs. “D1 fraction” (107 mg) was applied to a heparin-Sepharose column and eluted under the same conditions described in A. “HS-II fraction” (Tubes 48–53) indicated by the bar was treated and assayed for RNase and tumor cell-agglutinating activities as described in A. C, S-Sepharose chromatography of the “HS-II fraction.” “HS-II fraction” (9.8 mg) was applied to an S-Sepharose column (1.0 × 30 cm) equilibrated with 50 mM acetate buffer (pH 5.0) and eluted by the same gradient system as described in A. Fractions of 4.3 ml were collected. The appropriate fraction (Tubes 46–49), indicated by the bar, was treated and assayed for RNase and tumor cell-agglutinating activities as described in A.
5% perchloric acid containing 0.25% uranyl acetate. The acidified reaction mixture was then chilled with ice for 30 min and centrifuged at 2000 × g for 10 min. A 300-μl aliquot of the supernatant was diluted with 1.2 ml of distilled water, and the absorbance of the solution was measured at 260 nm. Enzyme inhibition assay was performed as follows: (a) inhibitors were added to the enzyme solution; (b) the mixture was incubated at room temperature for 30 min; and (c) residual activity was measured essentially as described above.

Antiproliferative Activity of the Lectins, and Its Inhibition. P388 and L1210 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. The cells were diluted with medium for passageing, and 2 × 10^5 cells/well were seeded in 96-well plates. Sialidase-treated cells were prepared by adding A. ureafaciens sialidase to cell suspensions. Plates with added SBL-C, SBL-J, or RNase A were incubated in a CO₂ incubator for 48 h, and the number of cells in each well was counted. Cell proliferation was determined as percentage increase in cell number: [(No. of cells at time 0 - No. of cells at time zero)/No. of cells at time zero] × 100. Growth of cultures without added lectin was normalized to 100% (0% inhibition), and proliferation in experimental cultures was expressed as a percentage of this control growth (24). The number of cells at 48 h in all cultures without lectin was at least 4 times that at 0 h. Assay of inhibition of the lectin cytotoxicity was performed as follows: (a) inhibitor was added to lectin solution; (b) the mixture was incubated at 37°C for 30 min; and (c) cell proliferation was determined as described above. Data were expressed as the percentage of residual antiproliferative activity.

Effect of pH on Ability of Ammonium Chloride to Protect P388 Cells against SBL-C and SBL-J. SBL-C or SBL-J plus 10 mM NH₄Cl were added to cells growing in 96-well plates, followed by incubation at 37°C for 2, 4, or 6 h. Medium (and unbound lectin) was removed, phosphate buffer at various pHs was added, and cells were again incubated at 37°C for 30 min. Buffer was removed and growth medium containing 10 mM NH₄Cl was added. After incubation for 18 h, the number of cells in each well was counted. The number of cells at 18 h in all cultures without lectin was 1.6-2.0 times that at 0 h.

Assay of Protein Synthesis by Cells Cultured with SBL-C and RNase A. Protein synthesis was measured at the end of each experiment. SBL-C-, SBL-J-, or RNase A-treated P388 cells were further incubated for 60 min in medium containing [3H]leucine. Cells were then washed with phosphate-buffered saline (pH 7.2) and dissolved in 0.1 M KOH. Trichloroacetic acid was added to a final concentration of 10% (v/v), and acid-precipitable radioactivity was measured (25).

In Vivo Antitumor Activity Assay of SBL-C and Modified SBL-Cs. Mice were given i.p. injections of Sarcoma 180 cells, Mep II cells, or Ehrlich cells (1 × 10⁶ cells/mouse) on Day 0. The Sarcoma 180- bearing ddY or Mep II-bearing ddY mice were divided into two groups, one given a single i.p. injection of SBL-C or Day 1 and the other given repeated injections of SBL-C every 10 days from Day 1 to Day 10. The Ehrlich-bearing ddY mice were given injections of modified SBL-C on Days 1, 2, and 3. In a separate experiment, Sarcoma 180 cells (1 × 10⁶ cells/200 μl) were incubated in the presence of SBL-C (1000, 100, or 10 μg) at 37°C for 30 min and then were transplanted i.p. into ddY mice. Survival time was recorded in days.

RESULTS

Heparin-Sepharose Affinity Chromatography. When “Fraction II” from R. catesbeiana eggs was applied to a heparin-Sepharose column and eluted with a linear gradient of increasing NaCl molarity, both lectin and RNase activities were reproducibly found in only a single peak eluted over the range of 0.20-0.26 M NaCl (taken as SBL-C) (Fig. 1A).

For purification of R. japonica lectin, the active fraction (D1) pooled from DEAE-cellulose column (2) was chromatographed directly on a heparin-Sepharose column as described above. Both lectin and RNase activities eluted in the range of 0.22-0.26 M NaCl (HS-II). The HS-II fraction was further purified on an S-Sepharose column eluted with a linear gradient of increasing NaCl molarity. Both activities were found in fractions eluting over the range of 0.33-0.35 M NaCl (taken as SBL-J) (Fig. 1, B and C).

On high performance liquid chromatography (TSK gel G 3000 SW), both SBL-C and SBL-J eluted slightly behind cytochrome c (Mᵦ 12,400), and both migrated as a single band on sodium dodecyl sulfate-PAGE (Mᵦ ~13,000) and on non-denaturing PAGE (toward the cathode) in the same manner as described previously (2, 3) (data not shown).

Selection of Cell Lines Used for Agglutination Assay, Inhibition of Agglutination, and Antitumor Activity Assay in Vivo and in Vivo. We have found previously that rat ascites hepatoma cells AH109A and Ehrlich ascites cells showed high susceptibility of agglutination by SBL-C and -J, i.e., agglutinated by 0.5-2.0 μg/200 μl (0.2-0.8 μM) SBL-C and -J, respectively (see Refs. 1-3). Accordingly, lectin activity was determined by agglutination assay using AH109A and Ehrlich cells as a standard assay system. For inhibition of agglutination, we used AH109A cells which, of various ascites form tumor cells tested, were most highly agglutinated by SBL-C and -J. For evaluation of antiproliferative activity of SBL-C and -J, suppression of antiproliferative effect by SBL-C inhibition, and inhibition of protein synthesis, we used well-established cultured cell lines, such as P388 and L1210. SBL-C and -J also agglutinated P388 and L1210 cells at a concentration of 0.02-0.4 μg/200 μl (0.008-0.16 μM). To determine the antiproliferative activity of SBL against tumor cells, we used a new SBL-C-resistant P388 variant cell line, RC-150, the establishment of which is described elsewhere (26). For evaluation of in vivo antitumor activity, Ehrlich, Sarcoma 180, and Mep II cells propagated as ascites formed in mice were used. SBL-C not only produced agglutination of these ascites tumor cells but also showed inhibition of tumor cell growth in mice in vivo. Only the in vivo antitumor activity of the SBL-Cs was examined because the water solubility of amino group-modified SBL-Cs is low.

Inhibition of Lectin-induced Tumor Cell Agglutination by Glycosaminoglycans and Polyamines. Agglutination of AH109A cells induced by SBL-C or -J was inhibited by heparin (Table 1) but not by much higher concentrations of other glycosaminoglycans (e.g., chondroitin sulfate A, chondroitin sulfate C, keratan sulfate, hyaluronic acid) or colominic acid (α-2,8-poly-N-acetylmuramid acid) (data not shown). Bovine submaxillary mucin inhibited the agglutination

### Table 1 Inhibition of SBL-C- and SBL-J-induced tumor cell agglutination by glycosaminoglycans, glycoproteins, and polyamines

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration inhibiting 100% of lectin activity</th>
</tr>
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<tbody>
<tr>
<td>Heparin</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td>Chondroitin sulfate B⁺</td>
<td>17-35</td>
</tr>
<tr>
<td>Mucin</td>
<td>3.2-6.4 μg/ml</td>
</tr>
<tr>
<td>(Bovine submaxillary)</td>
<td>110 µg/ml</td>
</tr>
<tr>
<td>(Porcine stomach)</td>
<td>57 µg/ml</td>
</tr>
<tr>
<td>Asialomucin</td>
<td>70-140 µg/ml</td>
</tr>
<tr>
<td>Fucosyl</td>
<td>125-250 µg/ml</td>
</tr>
<tr>
<td>Asialofucosyl</td>
<td>600-1000 µg/ml</td>
</tr>
<tr>
<td>Potassium carbonate</td>
<td>25-50</td>
</tr>
<tr>
<td>Spermidine</td>
<td>12.5</td>
</tr>
<tr>
<td>Spermine</td>
<td>3.1</td>
</tr>
</tbody>
</table>

| From porcine skin.      |
| Bovine submaxillary mucin (type I) from Sigma.|
| Data are the same as those in Ref. 3.|
| Bovine submaxillary mucin from Worthington Biochemical Co.|
| Porcine stomach mucin (type III) from Sigma.|
| NT, not tested.        |
reactions, but porcine stomach mucin did not (Table 1). Spermine, spermidine, and putrescine inhibited the agglutination reactions in that order (Table 1).

**Inhibition of Lectin RNase Activity by Glycosaminoglycans and Polyamines.** SBL-C and -J hydrolyzed RNA in a manner similar to that of RNase A, with preferential hydrolytic cleavage of poly(U) over poly(C) (4) and of uridylyl (3'-5')guanosine over cytidylyl (3'-5')guanosine (27). Specific activities of SBL-C and -J were 10.9 and 10.2%, respectively, of RNase A activity (27).

RNase activity of SBL-C and -J was inhibited by heparin (Fig. 2A) but not by chondroitin sulfates A, B, or C; keratan sulfate; or colominic acid (data not shown). Heparin also inhibited the activity of RNase A (Fig. 2A).

RNase activity of SBL-J was inhibited by spermine (Fig. 2B), spermidine, and putrescine (data not shown), in that order. Spermine also inhibited RNase activity of SBL-C (Fig. 2B), but putrescine and spermidine did not (data not shown). None of these polyamines significantly inhibited the activity of RNase A (Fig. 2B).

**Inhibition of Cell Proliferation by Lectins.** Both SBL-C and -J inhibited proliferation of P388 murine leukemia cells and L1210 cells. Addition of lectin solutions at 1.56–6.25 μM (final concentration) resulted in 50% inhibition of P388 cell proliferation (Fig. 3). L1210 cells were more sensitive than P388 cells; inhibition of L1210 prolif-

Fig. 2. Inhibition of RNase activity by heparin and spermine. Enzyme solution was incubated with inhibitors at the indicated concentrations for 30 min, and residual activity was measured. Residual enzymatic activity was expressed as a percentage, with activity of frog lectins or RNase A without inhibitor defined as 100%. A, heparin; B, spermine.

Fig. 3. Inhibition of cell proliferation by lectins. P388 and L1210 cell lines were used. Cells were treated by SBL-C, SBL-J, or RNase A at the indicated concentrations for 48 h. Cell proliferation without added lectin was normalized to 100% (0% inhibition). Antiproliferative effect of the lectins was expressed as percentage of inhibition.

Fig. 4. Differences in antiproliferative effects of frog lectins on intact and sialidase-treated P388 cells. A, SBL-C; B, SBL-J. A 6.25 μM concentration of lectin was used. A. ureafaciens sialidase was added to the cell suspension (2.5 × 10⁶/ml) and incubated at 37°C for 30 min. The sialidase-treated cells were washed with medium and incubated with lectin for 48 h. To assess the effects of an inhibitor, sialidase inhibitor was mixed with sialidase and allowed to stand for 15 min, and cells were treated with the mixture as described above. The antiproliferative effect of the lectins was expressed as percentage of inhibition as in Fig. 3.

In contrast, RNase A did not inhibit cell proliferation even at a final concentration of 40 μM (Fig. 3).
Inhibition of P388 proliferation by SBL-C and -J was greatly reduced by pretreatment of cells with A. ureafaciens sialidase. This reduction of inhibition was blocked in a dose-dependent manner when the pretreatment was carried out in the presence of the sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (28) (Fig. 4).

The antiproliferative effect of SBL-C against P388 cells was suppressed by heparin and putrescine (Fig. 5), although putrescine did not inhibit the RNase activity of SBL-C. Spermidine and spermine inhibited proliferation at concentrations above 0.125 mM (data not shown).

Effect of Ammonium Chloride on Antiproliferative Effects of SBL-C and -J. Antiproliferative effect of SBL-C was inhibited by ammonium chloride at neutral pH, but brief treatment at pH 5.0 completely abolished this inhibitory effect (Fig. 6). By analogy with the effect of acidotropic drugs on cytotoxicity of diphtheria toxin, the effect of ammonium chloride may be to inhibit transport of lectins from the cell surface into the cytoplasm. Our observations suggest that SBL-C and SBL-J require a low pH to penetrate the cell membrane and that their cytotoxic action is exerted intracellularly.

Inhibition of Protein Synthesis. Protein synthesis by P388 cells was inhibited by 1 h of exposure to 30 \( \mu \text{g} \) SBL-C (Fig. 7) and SBL-J (data not shown) but was unaffected by RNase A.

Increase of Survival Rate of Tumor-bearing Mice Treated with SBL-C. The survival time of mice inoculated with SBL-C-treated Sarcoma 180 cells was prolonged, compared to untreated control

Fig. 6. Effect of pH on the ability of ammonium chloride to protect P388 cells against SBL-C and SBL-J. Experimental conditions are described in the text. After growth medium containing SBL-C (6.25 \( \mu \text{M} \)) or -J (6.25 \( \mu \text{M} \)) plus 10 mM NH\(_4\)Cl was removed, cells were treated in phosphate buffer adjusted to pH 5 or 7 at 37\(^\circ\)C for 30 min. Residual antiproliferative activity was expressed as a percentage as in Fig. 5.

Fig. 7. Effect of SBL-C on protein synthesis by P388 cells. Cells were treated by SBL-C and RNase A at the indicated concentrations for 48 h. Incorporation of [\(^{3}\)H]leucine into SBL-C- and RNase A-treated cells was performed as described in "Materials and Methods.

Sarcoma 180-bearing mice (Fig. 8). Mice inoculated with Sarcoma 180 cells treated with 1000, 100, and 10 \( \mu \text{g} \) SBL-C (corresponding to 402, 40, and 4.0 \( \mu \text{M} \) concentrations) had 100, 75, and 50% survival, respectively, after 45 days (\( n = 6 \) or 8). We attempted to determine whether SBL-C has antitumor activity against viable tumor cells in the intraperitoneal cavity. Sarcoma 180-bearing mice given single injections of 10, 5, and 2.5 mg/kg of SBL-C had 17, 50, and 33% survival, respectively, and Mep II-bearing mice given single injections of 10 and 5 mg/kg of SBL-C had 50 and 17% survival, respectively, after 45 days (\( n = 6 \) or 8) (Fig. 9, a and c). For 10 days of continuous SBL-C injection, Sarcoma 180- or Mep II-bearing mice receiving injection of 2, 1, and 0.5 mg/kg of SBL-C had 100, 100, and 67% survival and
treated S-180 cells were transplanted i.p. into ddY mice, and survival time was recorded in days. □, 1000 μg SBL-C (n = 6); △, 100 μg SBL-C (n = 8); ▲, 10 μg SBL-C (n = 8); ○, untreated control.

DISCUSSION

Restriction enzymes which bind to nucleic acids apparently recognize heparin as a nucleic acid analogue and bind to a heparin-Sepharose (agarose) column because of this affinity rather than by ion-exchange (29). SBL-C and SBL-J are both heparin-binding proteins, because these two lectins bound to a heparin-Sepharose column and were eluted by 0.20–0.26 M NaCl (Fig. 1). Because (a) primary amino acid sequences of SBL-C and -J exhibit high homology with *R. catesbeiana* hepatic RNase, onconase (P-30 protein), pancreatic RNase(s), and angiogenin, (b) all these proteins have similar S-S loop structures (4–9), and (c) both lectins have RNase activity, and onconase and angiogenin also have ribonucleolytic activity (9, 30), we consider SBL-C and -J to be members of the RNase superfamily.

SBL-C and -J agglutinate tumor cells (1–3), whereas bovine pancreatic RNase A and *R. catesbeiana* hepatic RNase [also purified on heparin-Sepharose column (7)] do not (4, 27). Lectin-induced tumor cell agglutination is inhibited by sialoglycoconjugates (2, 3) and also by heparin and polyamines (see Table 1). These results suggest that SBL-C and -J recognize cell surface sialoglycoconjugates, but RNase A and *R. catesbeiana* hepatic RNase do not.

Base specificity of SBL-C and -J and *R. catesbeiana* hepatic RNase has been studied by analysis of digestion products of polynucleotides. The susceptibility of nucleotidyl linkages was found to decrease in the order poly(U) > poly(C) > poly(A), poly(I) (4). Enzymatic activities of SBL-C and -J using yeast RNA as substrate were inhibited by not only heparin (Fig. 2A) but also spermine (Fig. 2B). In contrast, the activity of RNase A was inhibited by heparin but not by spermine (Fig. 2).

Since heparin inhibited tumor cell agglutination reaction and RNase activity of SBL-C and -J, we hypothesize that heparin, as a substrate analogue, binds to a nucleotide recognition site or cell binding site of the lectins. If the inhibition of tumor cell agglutination by polyamines results from interaction with negatively charged cell surface components, it seems reasonable to assume that cell membrane carbohydrate structures including sialosyl residues (i.e., lectin-binding region) are influenced by treatment with polyamines. Inhibition of RNase activity of lectins by polyamines may occur via polyamine binding as follows. Polyamines can bind to RNA, DNA, and phospholipids and consequently stimulate protein synthesis (31). Polyamines also bind preferentially to poly(C) and poly(U) (32). Substrate specificity of RNases (e.g.; SBL-C and -J) which hydrolyze RNA at linkages between pyrimidine 3'-nucleotides and adjacent nucleotides may be altered by polyamines after their binding to RNA.

Based on the findings that heparin and polyamines inhibited RNase activity of SBL-C and -J by ~50% even at concentrations of 0.2 mg/0.2 ml and 60 μM, respectively (Fig. 2), and that bovine submaxillary mucin inhibited RNase activity of SBL-C but porcine stomach mucin did not, which will be described in detail elsewhere, we suggest that the lectins have functional site(s) for the tumor cell-agglutinating and RNase activities and that each activity is influenced by mutual interaction of the active site(s).

The lectins had antiproliferative effects on intact P388 and L1210 cells, but RNase A did not (Fig. 3). Sialidase-treated cells were immune to the antiproliferative effect of the lectins, and this immunity was almost abolished when the cells were incubated simultaneously with sialidase and a sialidase inhibitor (Fig. 4). The ability of heparin and putrescine to suppress the antiproliferative effect of SBL-C depended on inhibition of lectin binding to cell surface lectin receptors (Fig. 5). These three findings suggest that the antiproliferative effect of the lectins is mediated via recognition of lectin receptor(s).

When diphtheria toxin and Semliki Forest virus reach the cytosol via the lysosome or endosome, the low pH in the lysosome or endosome is important to trigger the translocation across the membrane (33, 34). Acidotropic (lysosomotropic) drugs, such as ammonium chloride, chloroquine, and methylamine, are accumulated in lysosomes or endosomes, neutralize the pH in the lysosomes or endosomes, and cause lysosomal vacuolation, thus protecting cells against the cytotoxic effects of toxins like diphtheria toxin and Shiga toxin (35, 36). The antiproliferative effect of SBL-C and -J was inhibited by ammonium chloride treatment (Fig. 6), suggesting that the lectins are internalized into cells by the same mechanism as described above. Furthermore, we have tried to circumvent the protection afforded by ammonium chloride by incubating SBL-treated cells at a low pH for a short time (see Ref. 33). The result suggests that low pH is necessary for the transfer of SBLs to cytosol (Fig. 6).

Results of the present study suggest that SBL-C and -J bind to sialosyl residues on the tumor cell surface to induce tumor cell agglutination, that the lectin-lectin receptor complex undergoes endocytosis, and that the internalized lectin subsequently inhibits protein synthesis (Fig. 7) and cell proliferation (Fig. 3). SBL-C was also effective in vivo in killing ascites tumor cells (Figs. 8–10). Bovine seminal RNase (37, 38), onconase (10), and RNase hybrid proteins (39, 40) have cytotoxic effects on tumor cells. Mammalian RNases coupled to antibodies as the targeting moiety have been utilized as cell type-selective cytotoxins (40), and the effect of SBL-C is receptor mediated. Therefore SBL-C has potential therapeutic applications, similar to antibody-RNase conjugates.

SBL-C and -J induced tumor cell agglutination, were cytotoxic, and had RNase activity, whereas RNase A did not exhibit cell agglutination or cytotoxicity. Apparently, the lectins recognize both carbohydrate structures containing sialosyl residues and RNA structure or
RNA-protein complexes. We conclude that these two lectins are bifunctional molecules possessing both sialosyl recognition and nucleotide recognition sites.

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