Molecular Nature of a Cell Membrane Antigen Specific for Human T-Cell Acute Lymphoblastic Leukemia

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ABSTRACT

In the present work, we characterized the molecular nature of a T-cell acute lymphoblastic leukemia (T ALL) specific antigen, termed TALLA, which is defined by monoclonal antibody SN1. SN1 shows an extremely high specificity for T ALL. In the present study, SN1 was further shown not to react significantly with various normal solid tissues. TALLA was determined to be a glycoprotein with an approximate molecular weight of 150,000. However, the molecular nature of TALLA is peculiar in that heating at 100°C for 2 min renders TALLA undetectable in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It should be noted that such heating is a common practice before analysis of proteins and glycoproteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No significant antigenic modulation of TALLA was observed when T ALL cells were reacted with SN1. Two new monoclonal antibodies, SN1a and SN1b, which show the same cell binding specificity as SN1 were also generated in the present work and compared to SN1. Competitive binding experiments showed that the epitopes on TALLA recognized by SN1, SN1a, and SN1b are sufficiently close to one another to allow complete reciprocal inhibition of antibody binding. These epitopes apparently became more exposed to antibody when T ALL cells were treated with neuraminidase; neuraminidase-treated T ALL cells bind 29–35% more SN1, SN1a, and SN1b as compared to the original T ALL cells.

INTRODUCTION

Previously, we have generated a McAb termed SN1 by using a human T leukemia antigen preparation isolated from T ALL cell membranes through a novel purification system (1). SN1 showed an exceedingly high specificity for T ALL (1, 2). SN1 did not react with any normal human cell specimens tested, either cultured or uncultured. These specimens include normal lymphoblastoid cell lines, thymocytes, bone marrow cells, spleen cells, lymph node cells, peripheral blood mononuclear cells, lymphocytes containing B- and T-cells, purified T-cells, monocytes, granulocytes, erythrocytes, and platelets. Furthermore, SN1 did not react with phytohemagglutinin-activated T-cells, nor with concanavalin A-activated T-cells. The results indicate that this new McAb SN1 defines a unique human leukemia antigen which is expressed on the cell surface of T-cell type ALL cells. We designated this antigen as TALLA, a T-cell ALL antigen. In the present work, the high specificity of SN1 was confirmed by its lack of reactivity with various normal tissues. Furthermore, we investigated the molecular nature and other properties of TALLA. In addition, we generated two new McAbs SN1a and SN1b and compared these McAbs with SN1.

MATERIALS AND METHODS

Cells. Established human cell lines were cultured in RPMI 1640 medium supplemented with 4–10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) (1, 3).

Immunofluorescence Chromatography of Cell Membrane Glycoproteins. Glycoproteins were isolated from cell membranes of MOLT-4, a T ALL cell line, as described before (4, 5). The glycoproteins were applied to a SN1-Sepharose column and the column was extensively washed with 20 mM Tris-HCl, pH 8.0, containing 0.5% taurocholate, 0.15 M NaCl, 2 mM EDTA, 0.03% NaN3, and 0.5 mM PMSF until the eluent showed no significant optical absorbance at 280 nm. The bound materials were eluted with 50 mM diethylamine-HCl, pH 11.3, containing 0.5% taurocholate, 2 mM EDTA, 0.03% NaN3, and 0.5 mM PMSF (6). The alkaline eluate was collected in tubes containing one-tenth volume of 0.5 M Tris-HCl, pH 7.1, to neutralize the eluate.

Radiodiodination. The immunofluorescence purified cell membrane antigen preparation was radiolabeled with 125I by two different procedures; a method using a 125I-labeled acylating agent (Bolton-Hunter reagent; 4, 7) and a method using Na125I and 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (IODO-GEN, Pierce Chemical Co.; 4, 8).

Radioimmunoprecipitation, SDS-PAGE, and Autoradiography. Immunoprecipitation of the 125I-labeled antigen preparations was carried out as previously described (3, 9). Briefly, the antigen preparations were pretreated by incubating for 1 h at 0°C with pansorbin (Calbiochem, San Diego, CA) which had been coated with affinity purified RaMIgG and control mouse IgG and suspended in Tris-HCl buffer, pH 8.0, containing aprotinin (100 kallikrein units/ml), 0.5% taurocholate, 2 mM EDTA, 0.05% NaN3, and 0.1% bovine serum albumin ("Tris-taurocholate"). For the specific immunoprecipitation, the radiolabeled samples were incubated, in duplicate, for 2 h at 0°C with pansorbin coated with RaMIgG and McAb SN1 (IgGl). Control immunoprecipitates were prepared by using pansorbin coated with RaMIgG and control mouse IgGl (MOPC 195 variant). The specific and control immunoprecipitates were washed twice with Tris-taurocholate. The immunoprecipitates were further washed twice with Tris-Resn 30 (Tris buffer containing 0.5% Renex 30, a nonionic detergent, instead of 0.5% taurocholate) and once with 0.0625 M Tris-HCl buffer (pH 6.8). The radiolabeled antigens of the washed immunoprecipitates were released from the pansorbin by two different procedures; by boiling for 2 min in the presence of 2% SDS (boiling condition) or by incubation at 37°C for 2 h in the presence of 6 M urea and 2% SDS (nonboiling condition). When samples were reduced, 10 mM diithiothreitol was added during the incubation. The released antigens were analyzed by SDS-PAGE as described before (3, 9) and autoradiography was prepared by using a Kodak X-OMAT AR film and X-Omatic intensifying screen.

Antigenic Modulation. Modulation of TALLA on human T leukemia cells was studied as previously described for GP37, another human T leukemia antigen (10).

Tissue. Various human normal tissues were obtained fresh from cadavers at RPMI. The capsule and fat of the individual organs were removed and the individual tissues were cut into small pieces using scissors. The tissue pieces were homogenized in 10 mM Tris-HCl buffer (pH 7.4) using a Teflon pestle in a Dounce homogenizer. The Tris-HCl buffer contained 0.15 M NaCl, 1 mM EDTA, aprotinin (100 kallikrein units/ml), 0.5 mM PMSF, and 0.1 mM leupeptin. Large particles were removed by centrifugation at 40 x g for 1 min. The homogenate was
washed twice with the Tris-HCl buffer by suspending and centrifuging at 35,000 × g for 20 min. The washed pellet was further homogenized with a Dounce homogenizer, divided in several tubes, and stored at −76°C. The protein content of the homogenate was estimated by a modification of the Lowry method as previously described (4). Antigens of the tissue homogenate were extracted with taurocholate, a detergent, as follows: the tissue homogenate was centrifuged at 100,000 × g for 1 h and an appropriate volume of 2% taurocholate in 50 mM Tris-HCl buffer (pH 7.5) was added to achieve approximately 10 mg protein/ml. The Tris-HCl buffer contained 0.13 mM NaCl, 0.03% NaN3, 2 mM EDTA, aprotinin (200 kallikrein units/ml), 0.2 mM leupeptin, and 2% taurocholate. The tissue suspension was homogenized in a Dounce homogenizer and shaken for 1 h in ice-water in a gyratory shaker. The mixture was centrifuged at 100,000 × g for 1 h and the supernatant was used for protein determination (see above) and solid phase RIA (see below). Portions of the supernatants of the individual tissue extracts were diluted 4- and 20-fold prior to use in a solid phase RIA.

All procedures for the tissue homogenization and detergent treatment were carried out at 0 or 4°C.

Solid Phase RIA. A solid phase RIA was utilized to determine the reactivity of McAbs with a detergent extract of various human tissues. Details of this assay were recently reported (6).

Generation of McAbs SN1a and SN1b. A BALB/c mouse was immunized with the immunoaffinity purified antigen preparation following our previously reported procedure (1). Cell fusion, hybridoma screening, cloning, specificity characterization, and McAb class determination were carried out as described before (1, 3). Ascites of hybridomas was prepared as previously described (1, 3).

Competitive Antibody Binding to TALLA on the Cells. This assay was carried out as previously described (11, 12). Briefly, 10 μl (2 × 10^9 cells) of JM (a TALLA-expressing T ALL cell line) cells in HEPES buffer were incubated at 4°C for 1 h with 20 μl of buffer (control) or 20 μl of serial dilutions of hybridoma ascites [SN1, SN1a, or SN1b (all IgG)] or of control ascites [McAb SN2 (IgG)] and MOPC 195 variant (IgG)] in individual wells of 96-well microtiter plates. The HEPES buffer (pH 7.3) consists of RPMI 1640 medium containing 25 mM HEPES, 0.1% NaNO3, 0.1% normal human IgG, 0.5% bovine serum albumin and aprotinin (50 kallikrein units/ml) (1). Ten μl (2 × 10^6 cpm) of 125I-labeled McAb SN1 solution were then added and the incubation continued for an additional 1 h. Cells were washed three times and the radioactivity in the washed cell pellet determined in a γ-ray spectrometer. The percentage of inhibition of binding was calculated from the means of triplicate determinations according to the formula:

\[ \text{Percentage of blocking} = \left( 1 - \frac{B}{A} \right) × 100 \]

where A is radiolabeled antibody bound to cells preincubated with buffer, and B is radiolabeled antibody bound to cells preincubated with test antibody or control mouse IgG.

Neuraminidase (Sialidase) Treatment. TALLA-expressing T ALL cells and control cells were treated with neuraminidase following a previously reported procedure (10, 11). The cells were treated with neuraminidase (Vibrio cholerae; Calbiochem-Behring, San Diego) at 0.17 I.U. enzyme/ml and at pH 6.7. The enzyme action was stopped by the addition of excess volume of cold RPMI 1640-HEPES and by washing the cells.

RESULTS

Radioimmunoprecipitation and SDS-PAGE of TALLA. A leukemia antigen preparation was radiolabeled with 125I by the Bolton-Hunter method or the IODO-GEN method (see “Materials and Methods” for details). The first method radiolabels NH2-groups of the proteins whereas the second method primarily radiolabels tyrosine residues of the proteins. In initial experiments, no antigen band was detected either from the radiolabeled antigen preparations by following the conventional method of immunoprecipitation and SDS-PAGE (e.g., see Ref. 3 and “Materials and Methods”). Therefore, we investigated various experimental parameters which might cause the lack of the antigen band being seen. Fig. 1A shows an example of these experiments. When the control precipitate and the specific immunoprecipitate were treated in a conventional way before analysis by SDS-PAGE, no antigen band was detected for either precipitate (Fig. 1A, lanes 1 and 2). However, a distinct M, 150,000 component was detected for the specific immunoprecipitate (Fig. 1A, lane 1) when the heating of the sample at 100°C was replaced with the incubation at 37°C. Under the same conditions, this M, 150,000 component was not detected for the control precipitate (Fig. 1A, lane 3). The results shown in Fig. 1A were reproducible in several experiments using several different radiolabeled antigen preparations labeled by either the Bolton-Hunter method or the IODO-GEN method. Also, the results show that TALLA has an approximate molecular weight of 150,000. To investigate the subunit structure of TALLA, the control precipitate and the specific immunoprecipitate were reduced before analysis by SDS-PAGE and the results are shown in Fig. 1B, lanes 1 and 2. A component with a molecular weight of 42,000 and a widely diffuse component of approximately M, 30,000 were detected only for the specific immunoprecipitate (lane 2); the fact that two well-defined bands of the heavy and light chains of the reduced McAb SN1 were detected in a parallel lane on the same slab gel (lane 3) indicates that the band diffusion was not caused by an inappropriate gel analysis.

Antigenic Modulation. Modulation of TALLA on human T leukemia cells was studied by incubating JM cells with an excess amount (6 μg IgG per 1.5 × 10^8 cells) of purified SN1 and control mouse IgG (MOPC 195 variant) for various periods of time. As a positive control, NALM-6 cells which express CALLA were incubated with McAb J5 (IgG2a) and a control mouse IgG (RPC5; IgG2a). It was previously reported that J5 induces antigenic modulation of CALLA on the cells (13). TALLA and CALLA on the incubated cells were quantitated by a cellular radioimmunoassay (1). No significant effect of the incubation with SN1 on the TALLA expression was observed in these experiments.
(Fig. 2A), whereas a significant decrease in the expression of CALLA was observed (Fig. 2B). Therefore, it was concluded that SN1 does not induce antigenic modulation of TALLA.

Test for Reactivity of SN1 with Normal Tissues. Reactivity of SN1 with detergent extracts of various human normal solid tissues was determined by our recently developed solid phase RIA (6). McAb SN5 (IgG1) and mouse plasmacytoma MOPC 21 (IgG2a) were included in the assay as a positive and a negative control, respectively. SN5 was recently generated in our laboratory (12); it was shown to be directed to CALLA and reactive with kidney (12). Two specimens derived from two different donors were used for each tissue in the assay. Furthermore, a 4-fold and a 20-fold dilution of the tissue extract were used for individual specimens in the assay. The assay results obtained using the two different dilutions were consistent; therefore, only the results of the 4-fold dilutions are shown (Table 1). As expected, SN5 showed a strong reaction with both kidney specimens tested. However, SN1 did not show a significant reaction with any of kidney, spleen, liver, lung, heart, pancreas, and lymph node.

Generation and Initial Characterization of SN1a and SN1b. Initial characterization of the primary cultures and the cloned hybridomas was carried out by a cellular RIA using various cells as the targets, as previously described (1, 3). The results indicated that hybridoma clones M3-3D9 and G5-3D9-2D1 produced McAbs with the same specificity as that of McAb SN1. Therefore, these hybridomas were designated SN1a and SN1b, respectively. McAbs SN1a and SN1b reacted with three of three T ALL cell lines tested (MOLT-4, JM and CCRF-HSB2) but did not react with any of the following human cell specimens: normal peripheral blood lymphocytes, normal thymocytes, a Japanese T lymphoma cell line (HPB-MLT), 2 non-T/non-B ALL cell lines (KM-3 and REH), a pre-B ALL cell line (NALM-6), a pre-B chronic myelocytic leukemia in blast crisis cell line (NALM-1), and 2 nonmalignant B cell lines (RPMI 8057 and CCRF-SB). This pattern of reactivity agrees completely with that of SN1 (1). Both SN1a and SN1b were found to be IgG1 in a double diffusion agarose plate test (3).

Competitive Antibody Binding. To gain information about the epitopes on the TALLA molecule recognized by SN1, SN1a, and SN1b, we carried out a competitive binding assay (Fig. 3). In this assay, preincubation of TALLA-expressing JM cells with SN1, SN1a, and SN1b completely blocked the subsequent binding of $^{125}$I-labeled SN1 at the maximum. Preincubation with a control McAb SN2 or a control mouse IgG (MOPC 195 variant) did not or only marginally blocked the subsequent binding. The results indicate that the epitopes recognized by these three McAbs are in close proximity to one another on the TALLA molecule on the cells.

Increased Expression of TALLA by Neuraminidase Treatment. TALLA-expressing JM (a T ALL cell line) cells were treated with neuraminidase and tested for TALLA with a cellular RIA using SN1, SN1a, and SN1b. As a control, NALM-6 cells were treated in parallel and the treated cells were tested using McAb L1-1E5-2B3. This McAb was recently generated in our laboratory and the antigenic determinant defined by this McAb contains a neuraminic acid residue on human non-T leukemia cells (11). Treatment of JM cells with neuraminidase resulted in increase of TALLA expression by 31, 35, and 29%, respectively, as determined using SN1, SN1a, and SN1b. On the other hand, treatment of NALM-6 cells with the neuramin-
idase eliminated almost completely (i.e., 95%) the antigenic determinant defined by L1-1E5-2B3.

DISCUSSION

In the clinical utilization of an anti-tumor McAb, specificity of the McAb is of paramount importance. In this regard, anti-human T ALL McAb SN1 demonstrates an extremely high specificity (1, 2). Furthermore, SN1 conjugate with ricin A-chain shows highly specific killing of T ALL cells both in vitro (14, 15) and in vivo (16, 17). However, the molecular nature of TALLA defined by SN1 has been unknown. In the present work, we show that TALLA is a glycoprotein with an approximate molecular weight of 150,000. This molecule appears to be composed of a M, 42,000 and a diffuse M, 30,000 subunit (Fig. 1). On this basis, TALLA may be a dimer of a complex of the two subunits similar to the IgG molecule. Further studies are needed to confirm this postulation. TALLA is peculiar in that heating at 100°C for 2 min renders it undetectable by SDS-PAGE. Such heating of immunoprecipitates containing antigens is a universally used routine procedure before analysis of the antigens by SDS-PAGE (reviewed in Ref. 18). There are a number of cases where researchers were unable to detect antigens by immunoprecipitation and SDS-PAGE. The reasons for this failure can be various; in some cases it could be due to degradation of the antigen by brief heating at 100°C as we found for TALLA in the present work. Heating at 37°C can easily be substituted for heating at 100°C (see “Materials and Methods” for details). It was previously reported that a major obstacle in using McAbs for therapy of leukemia patients can be the antigenic modulation of target tumor cells which occurs on the binding of McAbs; thus, tumor cells may escape binding of additional antibody molecules (19). Therefore, we tested if SN1 binding to TALLA on T leukemia cells causes antigenic modulation of TALLA; no antigenic modulation of TALLA was found (Fig. 2). In another set of experiments, we investigated the reactivity of SN1 with various normal tissues (Table 1). SN1 did not show any significant reactivity with these tissues.

In the present study we generated two new hybridomas that produce individual McAbs with the same specificity as that of SN1; these McAbs were designated SN1a and SN1b. These McAbs together with SN1 were used to compare the epitopes of the TALLA molecule on the T leukemia cells. In the first test, a competitive binding assay was used. The results show that the three epitopes recognized by SN1, SN1a, and SN1b are sufficiently close to one another to allow complete reciprocal inhibition of binding to TALLA present on leukemia cells. In another test, we investigated the effect of neuraminidase treatment on the expression of the epitopes recognized by the three McAbs. Interestingly, the neuraminidase treatment of T leukemia cells significantly (approximately 30%) increased the expression of all three epitopes. This enhanced expression of the epitopes may be due to the elimination of a negative charge(s) of the neuraminic acid residue(s) in the vicinity of the epitopes; in this instance, the negative charges around the epitopes effect the antigen-antibody interaction unfavorably. Alternatively, the enhanced expression may be due to the elimination of the neuraminic acid residues which block sterically the accessibility of the epitopes to SN1, SN1a, and SN1b. These first and second possibilities are not mutually exclusive. In any event, the present finding will be useful for practical purposes. For instance, pretreatment of leukemia cells with the enzyme will increase the susceptibility of the leukemia cells to in vitro killing by the SN1 series McAbs or immunotoxins containing these McAbs as well as increase the sensitivity of detection of the leukemia cells in the diagnosis.

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