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Immunology

Distinct human leukemia-associated cell surface glycoprotein GP160 defined by monoclonal antibody SN6

(human non-T-cell and myelo-monocytic leukemia antigen/cell surface glycoprotein/immunodiagnosis)

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ABSTRACT **In this study, a monoclonal antibody (mAb) termed SN6 was generated by immunizing a mouse with a non-T-cell leukemia antigen preparation isolated from cell membranes of leukemia cells derived from a patient (FJ) with non-T/non-B-cell-type acute lymphoblastic leukemia (ALL). SN6 was tested against a variety of cultured and uncultured human cell specimens by using a sensitive cellular radioimmunoassay. Among the 26 cultured malignant and nonmalignant cell lines tested, SN6 reacted with all of the 6 leukemic non-T/non-B (including pre-B)-cell lines tested—i.e., KM-3, NALM-16, REH, NALL-1, NALM-1, and NALM-6. Of these cell lines, 5 were derived from individual patients with ALL; the remaining 1 was from a patient with chronic myelocytic leukemia in blast crisis. In addition, SN6 reacted with 3 of 3 leukemic myelo-monocytic cell lines tested—i.e., ML-2, HL-60, and U937. SN6 did not react with any other cell lines. A consistent result was obtained with 42 fresh (uncultured) cell specimens derived from individual patients with several different types of leukemias. SN6 reacted with 11 of 16 non-T/non-B (including pre-B)-cell ALL specimens. In addition, it reacted with various myelo-monocytic leukemia cell specimens to various degrees. SN6 did not show a significant reaction with normal peripheral blood cells tested, which included B cells, T cells, granulocytes, monocytes, and erythrocytes. However, it reacted with a small population (≈1% as determined by immunofluorescence staining) of normal bone marrow cells. The approximate molecular mass of the glycoprotein antigen defined by SN6 was determined to be 160,000 by radioimmunoprecipitation followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Only one component of 80,000 daltons was formed upon reduction of the 160,000 molecular mass antigen. Therefore, this antigen is apparently a homodimer of a 80,000-dalton subunit. This conclusion was further corroborated by two-dimensional gel analysis, which showed a single well-defined spot for the reduced antigen. We designate this distinct human leukemia-associated cell surface antigen "GP160."**

During the past decade substantial progress has been made in the classification and characterization of human leukemia. In particular, the somatic-cell hybridization technology developed by Kohler and Milstein (1) has contributed greatly to this progress. Monoclonal antibodies (mAbs) have proven to be a powerful tool in studying cell surface antigens, including tumor-associated antigens (2–5). Furthermore, they have good potential for tumor therapy.

In this paper, we report the generation and characterization of a mAb termed SN6 that was found to define a distinct human leukemia-associated cell surface glycoprotein. SN6 reacts with non-T-cell-type (non-T) acute lymphoblastic leukemia (ALL) cells and myelo-monocytic leukemia cells but does not react with various normal cells from healthy donors. Therefore, SN6 may be useful in studying target cells for human leukemogenesis as well as for diagnosis.

MATERIALS AND METHODS

Cells. Established human cell lines were cultured in RPMI 1640 medium supplemented with 4–10% fetal calf serum as described (6). Peripheral blood and bone marrow aspirate specimens from cancer patients were provided to us by D. J. Higby, C. K. Tebbi, T. Han, E. S. Henderson, A. I. Freeman, and D. Chervinsky of the Clinics of Roswell Park Memorial Institute (RPMI). Mononuclear and blast cells were isolated from these specimens by centrifugation on a Ficoll–Paque gradient (7). Various types of cells of normal peripheral blood were isolated from buffy-coat preparations of healthy volunteers as described (8, 9). Normal peripheral blood B cells were isolated from the buffy-coat preparations as described by Werner *et al.* (10).

Antigen Preparation from Leukemia Cell Membranes. Antigen was prepared from cell membrane of leukemia cells derived from a patient (FJ) with non-T/non-B-cell-type (non-T/non-B) ALL. The procedures involved are based on modifications of our earlier method for isolating human T-cell leukemia antigen preparations (6). A brief description of the present isolation system is given as follows. Cell membranes were prepared from the leukemia cells, and the cell membrane antigens were solubilized by sodium deoxycholate treatment. The solubilized antigens were fractionated by affinity chromatography on serially connected columns of *Lens culinaris* lectin and *Ricinus communis* lectin. The *L. culinaris* lectin-bound and *R. communis* lectin-bound glycoconjugates (mostly glycoproteins) were individually eluted, combined, and subjected to passive immunoaffinity chromatography (6, 11). To this end, the combined glycoproteins were passed through three serially connected immunoadsorbent columns. These immunoadsorbents consist of affinity-purified rabbit anti-human β_2-microglobulin antibodies coupled to Sepharose CL-4B, rabbit anti-human cultured normal B-cell antibodies coupled to Sepharose CL-4B, and rabbit anti-human uncultured normal peripheral blood lymphocyte antibodies coupled to Sepharose CL-4B. Materials in the pass-through fractions were pooled and concentrated, and the passive immunoaffinity chromatography was repeated once.

Generation of mAb. mAb was generated by immunizing a BALB/c mouse with the isolated antigen preparation. Immunization of the mouse was carried out as described (8). Cell fusion, hybridoma screening, cloning, and mAb class determination was carried out as described (8, 9).

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Abbreviations: non-T and non-T/non-B, non-T-cell type and non-T/non-B-cell type; mAb, monoclonal antibody; ALL, acute lymphoblastic leukemia.

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Cellular Radioimmunoassay. Cellular radioimmunoassay was used to determine the reactivity of murine mAb with various cultured and uncultured human target cells. Details of the radioimmunoassay were as described (8). It should be noted that Fc receptors on the target cells are blocked with human IgG during the assay.

Immunofluorescence Staining. An indirect procedure was carried out as described (9).

Radioimmunoprecipitation and NaDodSO$_4$/PAGE. These experiments were carried out as described (9) and are briefly described below. The leukemia antigen preparation isolated from leukemia cell membrane glycoconjugates was radiolabeled with ^{125}I by using an Iodo-Gen (Pierce)-coated Minisorp tube (Vangard International, Neptune, NJ). To reduce the background radioactivity during the radioimmunoprecipitation, the ^{125}I-labeled leukemia antigen preparation was pretreated by incubating for 1 hr at 0°C with Pansorbin (Calbiochem), which had been coated with affinity-purified rabbit anti-mouse IgG antibodies and rabbit anti-*L. culinaris* lectin antibodies. For the specific immunoprecipitation, the pretreated radiolabeled sample was incubated, in duplicate, for 1 hr at 0°C with Pansorbin coated with rabbit anti-mouse IgG and mAb SN6 (IgG1). Control immunoprecipitates were prepared by using Pansorbin coated with rabbit anti-mouse IgG and control mouse IgG1 (MOPC 195 variant). The specific and control immunoprecipitates were washed twice with Tris·HCl buffer (pH 7.2) containing 0.5% taurocholate (a detergent; see refs. 12 and 13), 0.15 M NaCl, 2 mM EDTA, 0.1% bovine serum albumin, Trasylol (100 kallikrein units/ml), and 0.05% NaN$_3$ (Tris/taurocholate buffer). The immunoprecipitates were further washed twice with Tris/Renex 30 buffer (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) containing 0.01% cytochrome *c*. The radiolabeled antigens of the washed immunoprecipitates were released from the Pansorbin by boiling for 3 min in the presence of 2.5% NaDodSO$_4$ and in the presence or absence of 0.1 M dithiothreitol. The released antigens were analyzed by NaDodSO$_4$/PAGE as described (9).

Two-Dimensional Gel Electrophoresis. The radiolabeled antigen obtained by specific radioimmunoprecipitation (see above) was further analyzed by the method of O'Farrell (14). Briefly, the sample was first subjected to isoelectric focusing with Pharmalyte 3-10 (Pharmacia) in the first dimension. Control gels were run in parallel to measure the pH gradient in the gel. The pH gradient was determined as described by O'Farrell (14). In the second dimension, the sample was subjected to NaDodSO$_4$/PAGE using BioRad Protean dual slab cell equipment.

RESULTS

Generation of Hybridoma and Initial Characterization. Hybridoma SN6 was obtained from hybridoma primary culture N1 by using a limiting-dilution procedure (15). Culture supernatant of N1 already showed good specificity for non-T leukemia cells in a sensitive cellular radioimmunoassay; it reacted with three of three non-T/non-B leukemia cell lines tested but did not react with any of five T-cell leukemia lines, two B-cell leukemia-lymphoma lines or two normal B-cell lines (see below). The high specificity of the primary culture N1 is probably due to our using an isolated leukemia antigen preparation rather than whole leukemia cells to immunize the mouse that provided spleen cells for the cell fusion (see also refs. 8 and 9).

More than 30 good hybridoma clones were obtained from N1 by a limiting dilution, and 1 (i.e., N1-3A1, designated "SN6") of these was chosen for further study. Serial dilutions of culture fluid and ascites of hybridoma SN6 were tested against various cell lines. The results are shown in Fig. 1. The culture fluid of SN6 showed a strong reaction against two non-T/non-B leukemia cell lines (NALM-16 and KM-3) and a pre-B-cell leukemia line (NALM-6) but a weaker reaction against ML-2, a myelo-monocytic leukemia cell line. However, SN6 culture fluid showed no reactivity against CCRF-SB, a normal B-cell line, at any dilution tested (Fig. 1 *Upper*). The reactivity of SN6 ascites was consistent with that of SN6 culture fluid, although only NALM-6, KM-3, and CCRF-SB were tested (Fig. 1 *Lower*). The culture fluid of hybridoma SN6 was tested further against various (total of 26) cultured cell lines, and the results are summarized in Table 1. SN6 reacted with 4 of 4 leukemic non-T/non-B-cell lines tested, and 2 of 2 leukemic pre-B-cell lines. In addition, it reacted with 3 of 3 leukemic myelo-monocytic cell lines tested. However, SN6 did not react with any of the other human leukemia–lymphoma cell lines or nonmalignant cell lines tested (Table 1). Antibody SN6 was found to be IgG1 in a double-diffusion agarose plate test (9).

Reactivity of SN6 with Uncultured Normal Cells. To further define the specificity, SN6 was tested by a cellular radioimmunoassay against various normal peripheral blood cells isolated from buffy coat of healthy donors, and the results are summarized in Table 2. SN6 did not show a significant reaction with the normal cells tested—i.e., mononuclear cells, lymphocytes, B cells, T cells, granulocytes, mono-

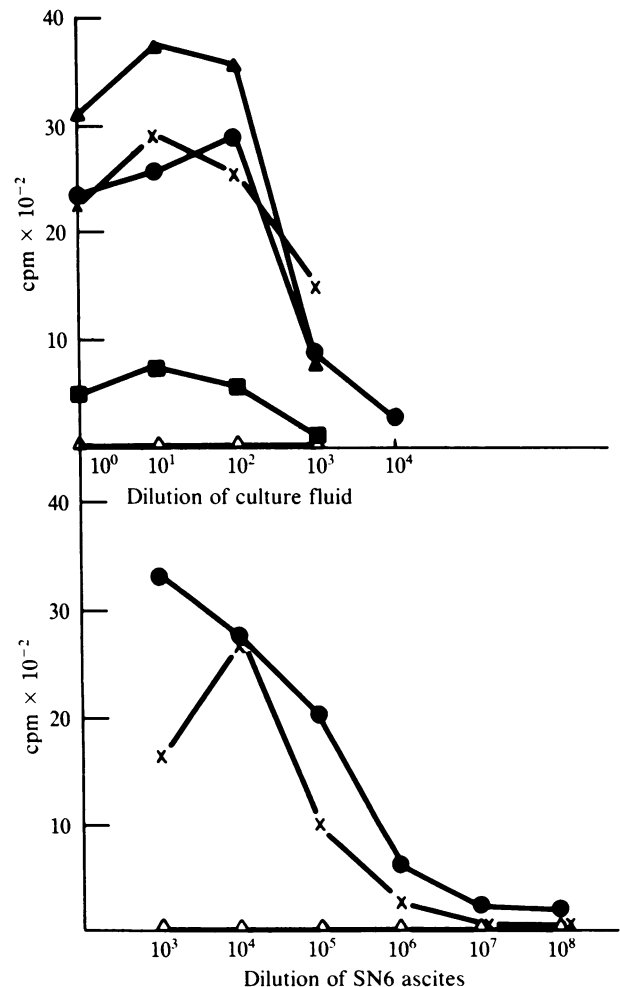

FIG. 1. Reactivity of culture fluid and ascites of hybridoma SN6. Serial dilutions of culture fluid (*Upper*) and ascites (*Lower*) were tested against target cells by a cellular radioimmunoassay. Control tests and subtraction of control values were carried out as described (see ref. 8). ▲, NALM-16; X, KM-3; ●, NALM-6; ■, ML-2; △, CCRF-SB.

Table 1. Reactivity of culture fluid of hybridoma SN6 with human leukemia–lymphoma (HLL) and normal cell lines

Cell line	Origin of cell line	Reactivity, cpm	
		SN6	Control*
HLL non-T/non-B-cell			
KM-3	ALL	3246 ± 245	210 ± 10
NALM-16	ALL	3497 ± 302	406 ± 96
REH	ALL	2478 ± 570	183 ± 31
NALL-1	ALL	1813 ± 78	223 ± 16
HLL pre-B-cell			
NALM-1	CML-BC	1916 ± 168	270 ± 17
NALM-6	ALL	3053 ± 474	308 ± 30
HLL B-cell			
BALL-1	ALL	246 ± 63	277 ± 29
BALM-2	ALL	264 ± 88	213 ± 12
Daudi	BL	279 ± 32	337 ± 13
HLL T-cell			
MOLT-4	ALL	159 ± 17	188 ± 28
JM	ALL	127 ± 13	167 ± 13
CCRF-CEM	ALL	373 ± 43	225 ± 20
CCRF-HSB-2	ALL	186 ± 6	195 ± 10
PEER	ALL	197 ± 24	301 ± 56
DND-41	ALL	179 ± 36	194 ± 3
Ichikawa	ALL	222 ± 8	237 ± 35
HPB-MLT	LTL	245 ± 97	210 ± 33
HuT 78	SS	337 ± 34	322 ± 92
HLL myelo-monocytic			
ML-2	AML	909 ± 16	145 ± 23
HL-60	APL	1693 ± 68	110 ± 26
U937	HL	2634 ± 295	171 ± 29
HLL myeloerythroid			
K-562	CML-BC	200 ± 28	168 ± 11
HLL undefined			
SU-DHL-1	HL	195 ± 33	197 ± 97
Normal B cell			
CCRF-SB		221 ± 31	232 ± 5
RPMI 1788		148 ± 12	215 ± 28
RPMI 8057		365 ± 27	250 ± 58

Properties of the HLL cell lines listed are reviewed in ref. 16. The reactivity was determined by using 20 μl of a 1:9 dilution of culture fluid of hybridoma SN6 (IgG1) and 2×10^5 cells in each test by means of a cellular radioimmunoassay. Each test was carried out in triplicate, and the values given (in cpm) are the mean of triplicates ± the standard deviation. CML-BC, chronic myelocytic leukemia in blast crisis; BL, Burkitt lymphoma; LTL, leukemic phase of T-cell lymphoma; SS, Sezary syndrome; AML, acute myelocytic leukemia; APL, acute promyelocytic leukemia; HL, histiocytic lymphoma.
*Mouse plasmacytoma IgG1 (10 μg/ml) dissolved in the hybridoma culture media.

cytes, and erythrocytes. SN6 was tested further against normal bone marrow specimens derived from three individuals. A weak reaction with SN6 was observed for all three specimens. One of these specimens was further tested by an indirect immunofluorescence staining, and ≈1% of the cells was found to react with SN6.

Reactivity with Uncultured Cancer Cells. The reactivity of SN6 with fresh (uncultured) cell specimens derived from 42 patients with various leukemias was determined by a cellular radioimmunoassay, and the results are summarized in Table 3. SN6 reacted with 11 of 16 non-T/non-B (including pre-B) ALL specimens. Among the 16 specimens, 3 were common ALL antigen-negative, but 13 were common ALL antigen-positive. Note that SN6 reacted with two of the three common ALL antigen-negative ALL specimens as well as with 9 of the 13 common ALL antigen-positive ALL specimens. In contrast to mAbs defining common ALL antigen [e.g., J5 (17) and SN5 (H. Matsuzaki, Y. H., T. Fukukawa, M. P. Barcos, and B.K.S., unpublished data)], SN6 also

Table 2. Reactivity of uncultured normal human peripheral blood cells with SN6

Cells	Reactivity*
Mononuclear cells	0/4
Lymphocytes	0/4
B cells	0/4
T cells	0/4
Granulocytes	0/3
Monocytes	0/4
Erythrocytes	0/2

Individual cell specimens were obtained from different donors and tested separately. In a typical experiment, the cell number used in each test was as follows: 1×10^6 for mononuclear cells, lymphocytes, B cells, T cells, and erythrocytes; 3×10^5 cells for granulocytes and monocytes. Each test using individual cell specimens was carried out in triplicate by means of a cellular radioimmunoassay.
*Number of reactive specimens per total number of specimens tested.

reacted with acute myelomonocytic leukemia cells (3 of 3 specimens tested), acute myelocytic leukemia cells (2 of 5 specimens tested), and acute monocytic leukemia cells (1 of 4 specimens tested). In addition, SN6 reacted with chronic myelocytic leukemia cells (1 of 3 specimens tested) and a specimen of chronic myelocytic leukemia in blast crisis. However, SN6 did not react with any of T-cell ALL specimens, a B-cell ALL specimen, and B-cell chronic lymphocytic leukemia specimens. These test results with fresh (uncultured) leukemia specimens are consistent with the test results obtained with cultured established cell lines (see Table 1). SN6 appears to be useful in identifying a subgroup of non-T/non-B (including pre-B) ALL cells and myelo-monocytic leukemia cells (see also the *Discussion*).

Radioimmunoprecipitation and NaDodSO$_4$/PAGE. The cell membrane leukemia antigen preparation was labeled with ^{125}I and used for immunoprecipitation. Under reduction of the immunoprecipitates, SN6 immunoprecipitate showed a single major component with an approximate size of 80,000 daltons and two minor components with smaller sizes (Fig. 2, lane A). The minor components are apparently of nonspecific nature because the control precipitate also showed these minor components in a parallel experiment (Fig. 2, lane B). This conclusion was supported by the results from the

Table 3. Reactivity of SN6 with uncultured human leukemia cells

Disease of patient	Reactivity*
Non-T/non-B ALL†	11/16
CALLA-negative ALL	2/3
CALLA-positive ALL	9/13
T-cell ALL	0/4
B-cell ALL	0/1
B-cell chronic lymphocytic leukemia	0/5
Acute myelocytic leukemia	2/5
Acute myelo-monocytic leukemia	3/3
Acute monocytic leukemia	1/4
Chronic myelocytic leukemia (CML)	1/3
CML in blast crisis	1/1

Individual cell specimens were derived from peripheral blood or bone marrow of different patients. In the radioimmunoassay, three different controls were included with each cell specimen. These were mouse IgG1 (10 μg/ml) in the hybridoma culture medium in place of the culture fluid of hybridoma (see Table 1). The other two controls were NALM-6 cells (a positive control) and CCRF-SB (a negative control) in place of the target cell specimen. CALLA, common ALL antigen.
*Number of reactive specimens per total number of specimens tested.
†Pre-B-cell ALL is included in this group.

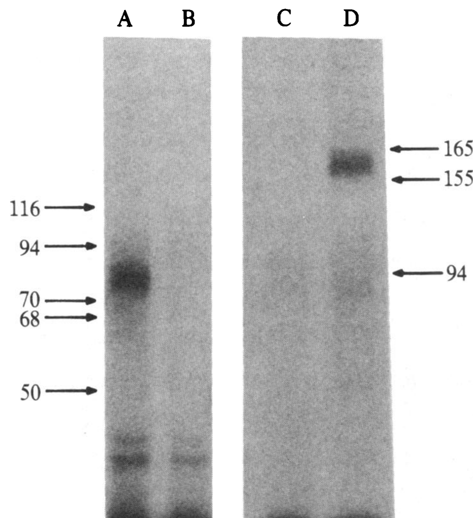

FIG. 2. $NaDodSO_4$/PAGE of SN6 immunoprecipitates from a ^{125}I-labeled leukemia antigen preparation and control precipitates (lanes B and C). The samples in lanes A and B were reduced with dithiothreitol and analyzed by using 7% gels. The samples in lanes C and D were unreduced and analyzed by using 6% gels. The marker proteins (shown in kDa) are: β' subunit (165,000 daltons) and β-subunit (155,000) of RNA polymerase, β-galactosidase (116,000), phosphorylase B (94,000), IgM μ chain (70,000), bovine serum albumin (68,000), and IgG γ chain (50,000).

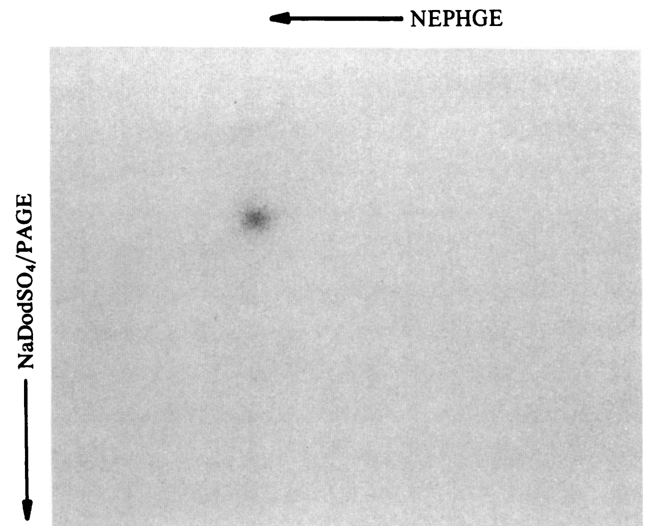

FIG. 3. Two-dimensional gel analysis of the ^{125}I-labeled SN6 immunoprecipitate. The immunoprecipitate prepared as in Fig. 1 was reduced and subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension for 2000 V·hr on a gradient of pH 3–10. The material in the gel was then electrophoresed in the second dimension on an $NaDodSO_4$/8% polyacrylamide gel. The autoradiogram shows a single discrete ^{125}I-labeled spot.

unreduced immunoprecipitates. The unreduced SN6 immunoprecipitate showed only a single component with an approximate molecular mass of 160,000 (Fig. 2, lane D), whereas the unreduced control precipitate showed no significant component (Fig. 2, lane C). Therefore, SN6 antigen termed "GP160" is apparently a homodimer of a 80,000-dalton subunit. This conclusion was corroborated by two-dimensional gel analysis of the reduced immunoprecipitates as described below.

Two-Dimensional Gel Analysis. Two-dimensional gel analysis of the reduced SN6 immunoprecipitate was carried out to investigate whether GP160 is a homodimer composed of a single 80,000-dalton subunit or a heterodimer composed of two different 80,000-dalton subunits. Furthermore, such an analysis will provide us with information about the charge homogeneity (or heterogeneity) and isoelectric point of the antigen. The results obtained with the reduced SN6 immunoprecipitate are shown in Fig. 3. A single well-defined spot with an isoelectric point of 5.3 was observed; the result strongly supports our notion that GP160 is a homodimer of a single 80,000-dalton subunit. A parallel two-dimensional gel analysis of the reduced control precipitate showed no detectable spot.

DISCUSSION

Among different types of human leukemia–lymphoma, ALL has been best characterized with respect to the cell surface marker profiles. One of the reasons for this has been that many malignant cell lines with different phenotypes (e.g., T cell, non-T/non-B-cell, B cell, and pre-B-cell) have been successfully established from peripheral blood lymphoblasts of patients with ALL, and these cell lines have been extensively utilized for studies of cell surface markers (reviewed in refs. 16 and 18). Furthermore, recent development of hybridoma technology and availability of mAbs greatly enhanced the characterization, classification, and diagnosis of ALL. It is important to note that different prognoses are associated with different types of ALL (19–26). Therefore, the determination of phenotypes of ALL cells is valuable for developing appropriate protocols for the treatment of individual ALL patients.

Despite recent significant advances in understanding and diagnosis of ALL, its origin and pathogenesis remain elusive. Until now, no particular virus has been identified as being specifically associated with ALL. Three known human T lymphotropic retroviruses (HTLV-I, HTLV-II, and HTLV-III/LAV, now called HIV for human immunodeficiency virus) appear not to be associated with ALL (including T-cell ALL). Recent studies by several investigators failed to reveal any specific association of known oncogenes with ALL (27–29). Furthermore, extensive epidemiological studies by many investigators [e.g., a collaborative study by Greaves *et al.* involving 20 medical centers in 18 countries (30)] failed to reveal any clear etiologic agent associated with ALL with a probable exception of the atomic bomb in Japan (31). Thus, little is known about the pathogenesis of ALL.

In this regard, new high-specificity mAbs defining unique leukemia-associated antigens may be valuable in investigating pathogenesis of ALL as well as in diagnosis and perhaps therapy. Previously, we had generated and characterized two groups of anti-human T-cell leukemia mAbs—i.e., SN1 series mAbs defining a T-cell ALL-specific antigen termed "TALLA" (8, 32) and SN2 series mAbs defining a T-cell leukemia-associated antigen termed GP37 (9, 33). These mAbs were generated by immunizing mice with a T-cell leukemia antigen preparation isolated by utilizing a novel system of purification (6). In the present work, we extended the above studies and utilized a non-T leukemia antigen preparation isolated in a way similar to that for the T-cell leukemia antigen preparation (see *Materials and Methods*). Recently we generated SN3 series mAbs (34, 35), SN4 series mAbs (unpublished data), and SN5 series mAbs (unpublished data) by using non-T leukemia antigen preparations obtained in a similar way. SN6 mAb generated in the present work by using a non-T leukemia antigen preparation apparently defines a unique leukemia-associated cell surface glycoprotein, termed "GP160," based on the comparison of the reported mAbs with SN6 with regard to the specificity of mAbs and the molecular nature of the antigens defined by the mAbs. SN6 reacts with non-T ALL cells and myelo-monocytic leukemia cells but did not show any significant reaction against a

number of normal peripheral blood cells (Table 2). However, it reacts with ≈1% of normal bone marrow cells. One interesting postulation would be that GP160 is a marker of pluripotent stem cells or a marker of stem cells common to lymphoid and myeloid cells, and these stem cells bearing GP160 are the targets for leukemogenesis of non-T ALL and myelo-monocytic leukemia. The presence of a common progenitor of myeloid and lymphoid cells in normal and disturbed hemopoiesis has been reported (36–38). However, little is known about the target cells for leukemogenesis of human lymphoid and myeloid leukemias.

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