Monoclonal Antibody SN10 Which Shows a Highly Selective Reactivity with Human B Leukemia-Lymphoma and Is Effectively Internalized into Cells

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ABSTRACT

The monoclonal antibody termed SN10 (IgG1-κ) which was generated and characterized in the present study shows a highly selective reactivity with fresh (uncultured) human leukemia-lymphoma cells. The antigen defined by SN10 is a cell surface glycoprotein composed of a single polypeptide chain of M, 36,000 and designated as gp36.

The primary reactivity of SN10 is against mature B-lineage leukemia-lymphoma cells. For instance, SN10 reacted with all of the 17 B non-Hodgkin's lymphoma specimens, all of the 15 B chronic lymphocytic leukemia specimens, both of the 2 B prolymphocytic leukemia specimens, all of the 3 B hairy cell leukemia specimens, and 2 of the 3 B acute lymphoblastic leukemia specimens tested.

Of normal peripheral blood cells, only a marginal reactivity of SN10 was detected with a minor subpopulation (<1-4% among different specimens) of isolated B-cells from healthy donors. No significant reactivity of SN10 was detected against any other isolated normal peripheral blood cells which include T-cells, granulocytes, monocytes, erythrocytes, and platelets. Furthermore, no significant reactivity of SN10 was detected against normal bone marrow specimens.

In immunohistological studies using frozen tissue sections, SN10 reacted well with malignant lymphomas and showed varying patterns of reaction with hyperplastic reactive lymph nodes. Various normal human tissues tested were unreactive with SN10.

In general, glycoprotein 36 was more abundantly expressed on fresh (uncultured) leukemia-lymphoma cells than on cultured leukemia-lymphoma cell lines.

No significant amount of circulating SN10 antigen was detected in the plasma of leukemia-lymphoma patients or normal healthy donors.

Scatchard plot analysis of direct binding of radiolabeled SN10 to a fresh (uncultured) B non-Hodgkin's lymphoma cell specimen, a fresh B chronic lymphocytic leukemia cell specimen, and DND-39 (an American Burkitt's lymphoma cell line) showed equilibrium constants of 5.2, 5.8, and 6.8 × 10^3 liters/mmol, respectively. Thus, SN10 shows a high binding avidity to each of the 3 B leukemia-lymphoma cell specimens tested.

Ricin A chain conjugate of SN10 killed leukemia-lymphoma cells effectively, whereas the same conjugate showed no cytotoxicity against control cells. Thus, SN10 bound to target antigen on the cell surface was effectively internalized into the cell.

The present results suggest the potential of SN10 for therapy as well as for diagnosis of various forms of leukemia-lymphoma, particularly mature B-lineage leukemia-lymphoma.

INTRODUCTION

During the last few decades, considerable improvement in the therapy of various forms of human LL has been achieved primarily because of the successful application of chemio-therapy (e.g., reviewed in Refs. 1 and 2). However, many forms of LL are still associated with poor prognosis. These LL include low-grade NHL, prolymphocytic leukemia, B ALL, acute myelogenous leukemia, and chronic myelogenous leukemia (2-6). In this regard, there still exists a strong need for the development of a new modality for treating LL.

Recently, several mAbs directed toward LL associated antigens have been shown to be useful for diagnosis and follow-up of LL (7). However, few, if any, anti-LL mAbs were clearly shown to be effective in the in vivo therapy (serotherapy) of LL. Therefore, the generation and characterization of new effective anti-LL mAbs are valuable.

A promising approach to the serotherapeutic utilization of anti-LL mAbs is to target LL cells by conjugating a mAb with an appropriate cytotoxic agent such as chemotherapeutic drugs, toxin subunits, or radioisotopes (reviewed in Refs. 8-12). The tumor specificity of the mAb is of paramount importance in the preparation of an effective immunocomjugate of an anti-LL mAb. In addition, a high binding avidity of the mAb to target antigen and a relatively abundant target antigen on the cells are important. Furthermore, in most cases, the mAb needs to be effectively internalized into the target cells after binding to the cell surface antigen. However, antibody binding to the target antigen should not induce a strong down-regulation of antigen expression (13).

A mAb termed SN10 which was generated and characterized in the present study meets these five important requirements for preparing a potentially effective immunocomjugate. Furthermore, SN10 meets another important requirement for preparing an effective immunocomjugate, i.e., no significant amount of circulating SN10 antigen was detected in the plasma of LL patients and healthy individuals. The lack of circulating antigen will facilitate targeting the tumor cells in vivo by a mAb. In addition, mAb SN10 reacts with a relatively wide range of LL. The availability of a sufficiently large candidate patient population for clinical trials will be important for developing a mAb and an immunocomjugate.

Thus, SN10 appears to have good potential for serotherapy as well as for diagnosis of LL. This mAb was generated by using an antigen preparation which was isolated by our established novel procedure (14, 15).

MATERIALS AND METHODS

Cells. Various established human cell lines were cultured in RPMI 1640 medium supplemented with 4-8% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 μg/ml) as described previously (16, 17). Fresh (uncultured) cell and tissue specimens from patients with various LLs were kindly provided by Drs. Tin Han, Edward S. Henderson, and Cameron K. Tebbi and David Chervinsky of our institute. Normal (or nearly normal) bone marrow specimens were from patients who were in remission and had a morphologically normal bone marrow. Mononuclear cells were isolated from the bone marrow aspirates by Ficoll-Hypaque gradient centrifugation.

B-cells, T-cells, granulocytes, monocytes, erythrocytes, and platelets of normal peripheral blood from healthy volunteers were isolated as previously described (16, 18).
Control Murine MAbS, Control Murine IgG, and Reagents. Leu 16
(anti-CD20 mAb; IgG1) and anti-HLA-DR mAb (IgG2a) were
purchased from Becton Dickinson (Mountain View, CA). Anti-human
immunoglobulin λ chain mAb (IgG1) was obtained from AMAC, Inc.
(Westbrook, ME). Control murine IgG (MOPC 195 variant; IgG1) was
prepared in our laboratory.

L-[3H]Thymidine and leucine-free medium were purchased from ICN
Biomedicals, Inc. (Irvine, CA) and GIBCO Laboratories (Grand Island,
NY), respectively. Ricin A chain was obtained from Inland Laboratories
(Austin, TX).

Antigen Preparation Used for Generating MAbS. Antigen preparation
was isolated from the cell membranes of an approximately equal
mixture of NHL cells derived from two patients with B-cell type NHLs,
i.e., nodular poorly differentiated lymphocytic lymphoma and small
lymphocytic lymphoma. The procedures involved are based on our
earlier methods for isolating T and non-T ALL antigen preparations
(16, 18). A brief description of the present isolation system is given
below. Cell membranes were isolated from the NHL cells, and the
membrane antigens were solubilized by deoxycholate treatment.
The solubilized antigens were fractionated by affinity chromatography on
serially connected columns of LcH and Ricinus communis lectin. The
LcH-bound and Ricinus communis lectin-bound glycoconjugates
(mostly glycoproteins) were individually eluted. The LcH-bound and
elated glycoproteins were further subjected to passive immunoaffinity
chromatography (14, 15) by passing the glycoproteins through three
serially connected immunoadsorbent columns. These immunoadsor-
bents (anti-IgG, anti-IgM, and anti-IgA) were linked to solid
immobilized 1 mg/ml of Protein A-Sepharose CL-4B, and rabbit anti-human peripheral blood lympho-
cyte antibodies coupled to Sepharose CL-4B. Materials in these pass
through fractions were pooled and concentrated.

Generation of MAbS. Monoclonal antibody was generated by immu-
nizing a BALB/c mouse with the isolated antigen preparation. Immu-
nization of the mouse was carried out as previously described (16). Cell
fusion, hybridoma screening, cloning, and mAb class determination
was carried out as described before (16, 19).

Cellular RIA and FACS Analysis. Details of the cellular RIA which
was used for determining reactivity with mAbs and various cultured
and uncultured cells were described previously (16, 19). It should be
noted that Fc receptors on the target cells are blocked with human IgG
during the assay. In selected cases, the reactivity of mAbs with various
cell specimens was also determined by FACS analysis. FACS analysis
was carried out as described previously (20). Briefly, one million cells
were suspended in 10–50 μl of RPMI 1640 medium containing 25 mM
4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 0.1% human IgG,
0.5% bovine serum albumin, 2 mM EDTA, Trasylol (50 kallikrein
units/ml), and 0.1% NaN3 and were allowed to stand for 30 min at
4°C. Then, the cells were incubated with 100 μl of hybridoma culture
supernatant or an isotype matching control mouse IgG solution (10
μg/ml) for 90 min at 4°C. After three washes with cold phosphate
buffered saline, the cells were incubated with fluorescein-conjugated
F(ab')2 fragment of sheep anti-mouse immunoglobulin (Sigma Chemi-
cal Co., St. Louis, MO) for 90 min at 4°C. The incubated cells were
washed three times with cold phosphate buffered saline and fixed with
formaldehyde. The fixed cells were analyzed by FACS using a Becton
Dickinson FACS 440.

Immunohistochemical Staining. Malignant NHL lymph nodes and
various normal human tissues were frozen, sectioned, and fixed in
acetone at 4°C and for 5 min as described by Barcos et al. (21). The
fixed tissue sections were stained using Sigma ExtrAvidin staining kits
(EXTRA-2) by the avidin-biotin-peroxidase complex procedure follow-
ing the manufacturer’s directions.

Radioimmuno precipitation and SDS-PAGE. Three LcH-bound gly-
coprotein preparations were isolated from the cell membranes of fresh
(uncultured) malignant cells from a B CLL patient, a B prolymphocytic
leukemia patient, and two B NHL patients, as described above.

The three glycoprotein preparations (a CLL, a prolymphocytic leu-
kemia, and an NHL preparation) were separately radiolabeled with
125I using an IODO-GEN coated Minisorp tube as described previously (14,
18). The three radiolabeled preparations were used separately for immu-
noprecipitation as described previously (18, 19). The immunoprecip-
itation in the present experiment was carried out using Pansorbin
coated with affinity purified rabbit anti-mouse IgG antibodies and mAb
SN10 (IgG1), isotype matching control mouse IgG (MOPC 195 variant)
or control mAbs. The specific and control immunoprecipitates were
washed as previously described (18). The radiolabeled antigens in
the washed immunoprecipitates were released from the Pansorbin by
boiling for 3 min in the presence of 2.5% SDS and in the presence or
absence of 0.1 M dithiothreitol. The released antigens were analyzed by
SDS-PAGE as described before (18, 19) and an autoradiogram was
prepared by using a Kodak X-OMAT AR film and X-Omatic intensi-
fying screen as previously described (22).

Enzyme Treatments of Cell Surface Antigens. SN10 antigen (gp36)
expressing lymphoma cells were treated with various enzymes as de-
scribed previously (23). gp36 remaining on the treated cells was deter-
mined using a cellular RIA (16).

Determination of Antigen in the Plasma of LL Patients. A previously
reported solid phase RIA (24) was used with a modification. Briefly,
50 μl, in triplicate, of serial dilutions of plasma from healthy donors
(control), B NHL patients, and B CLL patients was incubated at 4°C
overnight in the individual wells of 96 well microtiter plates. Then, 50
μl of a 1.6% bovine serum albumen solution in Tris buffer was added
and the incubation continued for 1 h at room temperature. The Tris
buffer consisted of 20 mM Tris-HCl, pH 7.0, containing 0.13 M NaCl,
2 mM EDTA, 0.03% NaN3, and Trasylol (20 kallikrein units/ml). After
the incubation, the wells were washed 3 times with Tris buffer contain-
ing 0.2% bovine serum albumin. The wash was followed by adding 100
μl of hybridoma culture fluid, or an appropriate dilution of control
mAb or control IgG, and then incubating at 4°C for 1 h. After the wells
were washed, 125I labeled F(ab')2 of affinity purified goat anti-mouse
IgG antibodies which had been adsorbed with human IgG coupled to
Sepharose CL-4B was added and incubated for 1 h at 4°C. The wells
were washed and cut out, and the radioactivity was counted in a γ-ray
spectrometer.

MOPC 195 variant (IgG1) and anti-human immunoglobulin λ chain
mAb (IgG1) were included in the test as an isotype matching negative
and a positive control, respectively.

A titration experiment showed that in the above described solid
phase RIA, we can detect SN10 antigen (gp36) contained in as little as
1 μg of total crude cell membrane protein preparation from B NHL
cells. However, if SN10 antigen in the plasma is substantially different
from the membrane SN10 antigen in their physicochemical properties,
the sensitivity of this assay may change significantly.

Direct Binding of 125I Labeled MAb to LL Cells and Scatchard Plot
Analysis. The equilibrium constant between 125I labeled SN10 and a fresh
(uncultured) B CLL cell specimen, a fresh B NHL cell specimen, or
DND-39 (an American Burkit's lymphoma cell line) cells was
determined as described by Trucco et al. (25). The radiolabeling of
the purified mAb SN10 was carried out by a method using IODO-GEN
(see above). Scatchard analysis of the binding data was carried out as
previously described (25, 26). An equilibrium constant and an average
maximal number of mAbs bound/cell were estimated by this analysis.

Regulation of Antigen Expression. Antigenic modulation and down-
regulation of gp36 was studied by incubating gp36 expressing DND-39
cells for various times at 37°C with an excess of SN10 or an isotype
matching control IgG (MOPC 195 variant; IgG1-x) following a previ-
ously described procedure (13, 23). A preliminary titration experiment
showed that 10 μg of purified SN10 mAb/1 x 106 DND-39 cells
represents antibody excess. The gp36 on the incubated cells was deter-
mined by an indirect cellular RIA after appropriate washings of the
cells as described previously (13, 23). In the indirect RIA, SN10 was
freshly added to the SN10 or control IgG treated cells and incubated
for 1 h at 4°C, followed by washings and the addition of 125I labeled
F(ab')2 of affinity purified goat anti-mouse IgG antibodies (16). The
addition and incubation with fresh SN10 was necessary for determining
gp36 on the control IgG treated DND-39. Therefore, the same addition
and incubation with fresh SN10 was carried out with the SN10 treated
DND-39. Also, we carried out a control experiment in which fresh

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SN10 was not added. In this experiment, the $^{125}$I labeled F(ab')$_2$ of affinity purified goat anti-mouse IgG antibodies were added to the DND-39 cells which had been previously incubated with an excess of SN10 and washed. The result of this control test was almost identical to that of the above test in which fresh SN10 was added.

As a positive control, an excess of mAb SN5 defining CD10 (CALLA) was incubated with NALM-6 cells (13). Preparation of Immunotoxin. The purified mAb and an isotype match control mouse IgG (IgGl) were individually conjugated to ricin A chain as previously described (27). The conjugates were purified by gel filtration on a calibrated Sephacryl S-300 column (27) followed by affinity chromatography on a Blue Sepharose column (28).

Determination of Activity of Immunotoxin. The in vitro cytotoxic activity of immunotoxin was determined by two different methods, i.e., a protein synthesis inhibition assay (29) and a direct test of cytotoxicity against LL cells and control cells (29). The present procedure of the protein synthesis inhibition assay is briefly described below.

DND-39, a gp36 expressing lymphoma cell line, or MOLT-4, a gp36 negative T ALL cell line, was incubated in triplicate, with varying concentrations of SN10-RA or control IgG-RA for 24 h. The incubated cells were centrifuged, washed, and resuspended in leucine-free medium containing 1 uCi of $[^3H]$leucine. The cell suspension was incubated for 4 h and centrifuged, and the pelleted cells were washed. The cells were harvested on glass fiber filters using a multiple semiautomatic cell harvester (type 7010; Skatron Inc., Sterling, VA), and the $[^3H]$ radioactivity was determined in a liquid scintillation spectrometer. Protein synthesis in the conjugate treated cells is expressed as the percentage of $[^3H]$leucine incorporated into control cells not exposed to conjugate.

RESULTS

Initial Characterization of mAb. Reactivity of culture supernatants of 48 hybridoma primary cultures and hybridoma clones derived from the selected primary cultures were initially characterized using a cellular RIA with various cultured and uncultured cells.

Based on these test results, the mAb termed SN10, which was produced by hybridoma clone 2B-4G9-E7, was chosen for further studies. The properties of SN10 are described in this report. SN10 was found to be an IgG1-k antibody. The reactivity of SN10 with various human LL cell lines and EB virus transformed nonmalignant cell lines is summarized in Table 1. SN10 reacted with all of the 10 mature B LL cell lines tested. In addition, SN10 reacted with 2 (i.e., REH and KM-3) of the 3 immature non-T/non-B (B-cell lineage) LL cell lines, one (i.e., NALM-1) of the 2 immature pre-B LL cell lines, and two (i.e., ARH-77 and HS) of the 3 plasma cell lines tested. However, SN10 did not react significantly with T, myelo-monocytic, and myeloidoerythroid LL cell lines (Table 1).

SN10 showed a weak but definite reactivity against 3 EB virus transformed nonmalignant B-cell lines. It should be noted, however, that EB virus is absent in many of the SN10 reactive LL cell lines, e.g., REH, KM-3, NALM-1, and SU-DHL-4 (30).

Reactivity of SN10 with Fresh (Uncultured) LL Cells. Reactivity of SN10 with uncultured LL cells was routinely determined by a cellular RIA and, in selected cases, also by FACS analysis. The results of a cellular RIA with uncultured LL specimens from 68 different LL patients are summarized in Fig. 1. The test results are consistent with those obtained using cultured LL cell lines (Table 1). SN10 reacted with all but one of mature B LL cell specimens tested. SN10 reacted with all of the 17 B NHL, all of the 15 B CLL, both of the 2 B prolymphocytic lymphoma; APL, acute promyelocytic leukemia.

![](https://example.com/figure1.png)

Fig. 1. Reactivity of SN10 with fresh (uncultured) LL cells as determined by a cellular RIA. Individual cell specimens were derived from peripheral blood, bone marrow aspirates, and lymph nodes of 68 different LL patients. In the cellular RIA, three different controls were included with each test sample (19). One of these was control mouse IgG1 (10 µg/ml) in the hybridoma culture medium in place of the culture fluid of hybridoma SN10. Radioactivity counts of this control were subtracted from those of individual test samples in each test. The other two controls were a positive cell line and a negative cell line in place of the target cell specimen. Pre-B ALL was included in the group of non-T/non-B ALL in the figure. Abbreviations: PLL, prolymphocytic leukemia; HCL, hairy cell leukemia; AML, acute myeloid leukemia; AMOL, acute monocytic leukemia; AMMOL, acute monomyelomonocytic leukemia.
phocytic leukemia, all of the 3 B hairy cell leukemia, and 2 of the 3 B ALL specimens tested.

However, it should be noted that generally the reactivity of SN10 with uncultured B LL cell specimens is stronger than with cultured B LL cell lines.

SN10 did not react significantly with T NHL, T CLL, or T ALL specimens. With a few exceptions, non-T/non-B (including pre-B) ALL specimens did not react or reacted only marginally with SN10. SN10 reacted with 3 of the 6 acute myelocytic, monocytic, or myelomonocytic leukemia specimens tested.

An example of FACS analysis with a B NHL and a B CLL specimen is shown in Fig. 2. The majority (77 and 72%, respectively) of the B NHL and B CLL specimens reacted with SN10.

Reactivity with Uncultured Normal Cells. B-cells, T-cells, monocytes, granulocytes, erythrocytes, and platelets were isolated from peripheral blood of three healthy donors and tested for reactivity with SN10 by a cellular RIA. SN10 showed a weak marginal reactivity with B-cell specimens but no significant reactivity with other cell specimens. Therefore, the reactivity of SN10 with B-cells was further tested by FACS analysis. The FACS analysis results of 2 of the 3 specimens tested are shown in Fig. 2. A weak marginal reactivity of SN10 was detected with a minor subpopulation (<1, 2.7, and 3.9%) of the B-cell preparations derived from the three different donors. One of these B-cell preparations was tested for its reactivity with an anti-HLA-DR mAb (monomorphic; Becton Dickinson) by FACS analysis. The test showed that 77.5% of the B-cell preparation reacted with the anti-HLA-DR mAb.

To further characterize the specificity of the mAb, SN10 was tested for its reactivity with normal (or nearly normal) bone marrow specimens by FACS analysis; these bone marrow specimens were obtained from 5 different LL (4 ALL and one acute myelocytic leukemia) patients in remission. FACS analysis results of 2 of the 5 bone marrow specimens are shown in Fig. 2. Reactivity of SN10 with these 5 normal bone marrow specimens was not detectable (<1% for 2 specimens) or marginal (approximately 1% for the 3 other specimens). These bone marrow samples were tested for their reactivity with an anti-HLA-DR mAb (monomorphic) by FACS analysis. The tests showed that 20.8, 25.7, 27.9, 11.3, and 18.6% of these samples reacted with the anti-HLA-DR mAb.

Reactivity with Solid Tissues as Determined by Immunohistochemical Staining. Immunohistochemical assays performed on frozen sections showed good reactivity of SN10 with malignant lymph nodes from four B NHL patients tested. Reactivity of SN10 with two of these NHL lymph node samples are illustrated in Fig. 3; these two are lymph nodes involved by follicular small cleaved cell lymphoma (Fig. 3c) and diffuse small cleaved cell lymphoma (Fig. 3c). An isotype matching control murine IgG (IgG1) did not show significant reaction with the NHL samples except for a focal reaction due to endogeneous peroxidase; an example is shown in Fig. 3b. SN10 showed varying patterns of reactivity with hyperplastic reactive lymph nodes from three donors. An example is shown in Fig. 3d; sparse to moderate numbers of the germinal center and interfollicular cells are SN10 positive, while the majority of follicular mantle zone cells are SN10 positive. G4–3A7 (anti-HLA-DR; Ref. 20), an isotype matching positive control mAb, reacted strongly with both germinal center and mantle zone cells and moderately with the interfollicular cells of the same reactive lymph node.

No significant reactivity with SN10 was noted in normal epidermis, lung, kidney, or small intestinal mucosa.

Determination of Circulating Antigen in the Plasma of LL Patients. Circulating antigen in the plasma of patients may bind an administered mAb and thereby inhibit the therapeutic efficacy of the administered mAb and immunonjugate. Therefore, we tested for circulating SN10 antigen in the plasma of LL patients and healthy individuals (control) by using a solid phase RIA. A titration experiment showed that this RIA allows us to detect SN10 antigen contained in as little as 1 μg of total crude cell membrane protein preparation from B NHL cells. No significant amount of SN10 antigen was detected in any of the plasma samples derived from 7 different B NHL patients and 5 different healthy individuals (Fig. 4). Similarly, no significant SN10 antigen was detected in the plasma samples derived from 7 different B CLL patients.

Molecular Nature of Antigen. Cell membranes were prepared from a mixture of malignant cells from two B NHL patients. Cell membranes were also prepared from malignant cells of a B CLL patient as well as of a B prolymphocytic leukemia patient. Glycoproteins were isolated separately from each of the three cell membrane preparations and radiolabeled individually with 125I.

The immunoprecipitates obtained by using the radiolabeled samples and SN10 (lgG1-α), an isotype matching control mouse IgG, or control mAbs were analyzed by SDS-PAGE and autoradiographs were prepared. Some of the results are shown in Fig. 5.

The results of the NHL sample are shown in Fig. 5, lanes A–E. Under reduced conditions, the SN10 immunoprecipitate showed a single component of approximately M, 36,000 (lane A), whereas no significant component was immunoprecipitated by an isotype matching mAb Leu 16 (anti-CD20 mAb; lane B) or by an isotype matching control mouse IgG (MOPC 195 variant; lane C). Under unreduced conditions, the SN10 immunoprecipitate showed a single component with a molecular size similar to that of the reduced antigen (lane D), whereas
Fig. 3. Reactivity of SN10 with lymphomatous and reactive lymph nodes as determined by immunohistochemical staining. a, follicular small cleaved cell lymphoma: SN10 reactivity is confined to the majority of the lymphocytes within the neoplastic follicles. The interfollicular areas are essentially negative. b, negative isotype matching control IgG with the same lymph node (as a) focal reaction due to endogenous peroxidase in the interfollicular histiocytes is noted. c, diffuse small cleaved cell lymphoma: the majority of the lymphocytes are SN10 positive. d, reactive lymphoid hyperplasia: the majority of follicular mantle zone lymphocytes are SN10 positive. Sparse to moderate numbers of SN10 positive cells are also noted within the germinal center and interfollicular areas (frozen sections; × 240).

Fig. 4. Absence of gp36 in the plasma of LL patients and healthy individuals. A solid phase RIA was used to determine circulating gp36 in the plasma of 7 B NHL patients, 7 B CLL patients, and 5 healthy individuals. An isotypic matching control IgG (MOPC 195 variant) and anti-human immunoglobulin A chain mAb (IgGl) were used along with SN10 as a negative and a positive control. gp36 was not detectable in any of the tested specimens. Therefore, only the results of NHL and healthy control specimens are shown. A titration experiment was carried out separately to determine the sensitivity of the RIA for detecting gp36 (see “Materials and Methods”).

This conclusion was supported by the following results obtained with a B CLL sample (lanes F–K) and a B prolymphocytic leukemia sample (lanes L and M). Under reduced conditions with the CLL sample, SN10 immunoprecipitated a single component of M, 36,000 (lane F), whereas anti-HLA DR mAb, a control mAb, immunoprecipitated two components of 33,000 (α chain) and 28,000 daltons (β chain) (lane G). The control IgG immunoprecipitated no significant component (lane H). Essentially, the same results were obtained with the CLL sample under unreduced conditions (lanes I–K).

With a B prolymphocytic leukemia sample, SN10 immunoprecipitated an M, 36,000 component (lane L), whereas the control IgG precipitated only a minor nonspecific component (lane M).

Thus, an M, 36,000 antigen was specifically immunoprecipitated by SN10 from each of the three samples tested, i.e., a B NHL, a B CLL, and a B prolymphocytic leukemia sample. The SN10 antigen was designated gp36.

It should be noted that the reduced SN10 antigen and unreduced SN10 antigen are in slightly different positions relative to the molecular weight markers; the marker proteins used were reduced before the gel analysis. Such small differences between reduced and unreduced proteins were previously observed for other proteins (31). The molecular weight of the SN10 antigen was estimated using the relative mobility of the reduced antigen

the control IgG immunoprecipitate did not show any significant component (lane E).

Therefore, the SN10 antigen is a glycoprotein with an approximate M, of 36,000 and consists of a single polypeptide chain.
because it was compared to molecular weight marker proteins that had been reduced before the gel analysis.

CD20 antigen has a molecular weight similar to that of the SN10 antigen, although its specificity is substantially different from that of SN10 (32, 33). Therefore, we compared the SN10 antigen with the CD20 antigen (Fig. 5). No significant component was immunoprecipitated from our glycoprotein mixtures by Leu 16, an anti-CD20 mAb from Becton Dickinson, whereas an M, 36,000 component was specifically immunoprecipitated from the same sample by SN10. This finding is consistent with the fact that the CD20 antigen is not a glycoprotein (33), whereas the SN10 antigen is a glycoprotein. To further compare the SN10 antigen with the CD20 antigen, we carried out a sequential immunoprecipitation experiment using the glycoprotein mixtures, Leu 16 and SN10. Leu 16 did not preclear SN10 antigen at all. Taken together, the above results indicate that the SN10 antigen is different from the CD20 antigen.

Biochemical Nature of Epitope. Daudi, a Burkitt’s lymphoma cell line (Table 1), which expresses gp36 on the cell surface, was treated with trypsin and mixed glycosidases, and the antigenic determinant remaining on the treated cells was tested for by means of a cellular RIA. As a control, a mAb L1-1E5-2C8 which defines an epitope containing sialic acid(s) of the CD24 antigen (34) was used. The results are shown in Table 2. The epitope defined by SN10 was strongly reduced by treatment with trypsin but not effected by treatment with mixed glycosidases. Treatment with the same glycosidases as well as trypsin substantially reduced the epitope defined by the control mAb L1-1E5-2C8. It should be noted that, on the cells, carbohydrates are attached to protein moieties which interact with the lipid bilayers of cell membranes. Therefore, carbohydrate as well as the protein moieties of a glycoprotein on cell surfaces may be eliminated by proteolytic digestion as in the case for the sialic acid residue defined by L1-1E5-2C8.

In conclusion, the present data suggest that SN10 epitope is primarily composed of the protein moiety of the glycoprotein antigen on Daudi cells.

Antibody Avidity and Number of Available Epitopes on LL Cells. Scatchard plot analysis of direct binding of radiolabeled SN10 to an uncultured B NHL cell specimen, an uncultured B CLL cell specimen, and cultured DND-39 cells showed equilibrium constants of 5.2, 5.8, and 6.8 x 10⁶ liters/mol, respectively. In the same analyses, the average number of antibody molecules bound/cell was estimated to be 7.4, 2.9, and 2.1 x 10⁵, respectively, at the antibody saturation for the NHL, CLL, and DND-39 cells. Since SN10 (IgG1) is a bivalent antibody, the average number of antigens on these cell specimens is probably 1- to 2-fold of the above number of antibodies.

Thus, SN10 shows a high binding avidity to each of the three B LL cell specimens tested, and the SN10 antigen is expressed relatively abundantly on these cell specimens.

Regulation of Antigen Expression. Binding of antibody to a cell surface antigen may induce antigenic modulation (35, 36) and down-regulation of antigen biosynthesis and expression (13). However, binding of SN10 to gp36 on DND-39 cells did not cause significant down-regulation of antigen expression as determined by a cellular RIA (Fig. 6; “Materials and Methods”). Nevertheless, mAb SN10 is effectively internalized into the LL cells after binding as demonstrated by the effective killing of the LL cells by the RA conjugate of SN10 (see below).

Specific Killing of LL Cells by RA Conjugate of SN10. Specific cytotoxic activity of the RA conjugate of SN10 was tested against gp36 expressing LL cells and gp36 negative control cells using two different in vitro assays, i.e., a protein synthesis inhibition assay and a direct cytotoxicity test (see “Materials and Methods”). The results obtained by using the first assay procedure are shown in Fig. 7. The SN10-RA conjugate showed significant inhibition of protein synthesis against gp36 expressing DND-39 at concentrations >5 nm but not against gp36 negative MOLT-4. The control IgG-RA conjugate showed no significant inhibitory activity against either DND-39 or MOLT-4. In the above test, no immunotoxin potentiatior such as NH₄Cl

Table 2 Binding of mAbs to enzyme treated LL cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reduction in antibody binding by enzyme treatment (%)</th>
<th>SN10</th>
<th>L1-1E5-2C8</th>
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<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Trypsin</td>
<td>0.1</td>
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<td>88.2</td>
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<td></td>
<td>1.0</td>
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<td>90.6</td>
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<td>Mixed glycosidases</td>
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<td></td>
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</tr>
</tbody>
</table>

* The amount of enzymes shown is mg/ml.

* As a reference, the results of mAb L1-1E5-2C8 directed to neuraminic acid residue(s) of the CD24 antigen (34) are included. For L1-1E5-2C8, K/M-3 cells (see Table 1) were incubated as described above. L1-1E5-2C8 reacts poorly with Daudi cells.
was added. Addition of 10 mM NH₄Cl potentiated the specific cytotoxicity of SN10-RA by approximately 10-fold (data not shown). Results consistent with the above were obtained for the specific cytotoxic activity of SN10-RA by using the second assay procedure, i.e., a direct cytotoxicity test (data not shown).

Thus, SN10-RA is effective in specific killing of gp36 expressing LL cells. Furthermore, the present results indicate that mAb SN10 is effectively internalized into target LL cells after binding to the cell surface antigen.

DISCUSSION

In the past several years, a number of investigators reported many mAbs which reacted with human normal and malignant B-cells (reviewed in Refs. 37 and 38). However, only a small number of these mAbs show a highly selective reactivity with malignant B-cells over normal B-cells (39–44).

A new mAb SN10 which was generated and characterized in this study shows a highly selective reactivity with malignant B-cells. Furthermore, SN10 appears to be different from those previously reported mAbs in the antibody specificity and/or in the molecular nature of the antigen defined. The antigen defined by SN10 appears to be different from any of the reported CD series (1–78) antigens (45). However, it is prudent that any definite conclusions about the novelty of SN10 antigen be withheld until either SN10 is compared with other mAbs in an International Workshop or the chemical structure (e.g., amino acid sequence) of the antigens defined by these mAbs is determined.

The antigen defined by SN10 is a cell surface glycoprotein composed of a single polypeptide chain of Mr, 36,000 and designated gp36. The epitope defined by SN10 appears to be primarily composed of the protein moiety of gp36 on Daudi lymphoma cells. In general, gp36 is expressed to a greater degree on fresh (uncultured) LL cells than on cultured LL cell lines. At present, we do not know the origin of gp36. However, it may be a transformation associated antigen because (a) SN10 strongly reacts with B ALL cells, (b) SN10 shows a weak marginal reaction with a few percent (e.g., < 1, 2.7, and 3.9%) of normal peripheral blood B-cells, (c) SN10 does not react significantly with normal bone marrow cells, (d) SN10 reacts with EB virus transformed nonmalignant B-cell lines as well as EB virus negative LL cell lines, and (e) SN10 shows varying patterns of reactivity with the hyperplastic reactive lymph nodes.

Recently, SN10 was further tested for reactivity with concanavalin A activated T-cells. In this test, we included an anti-transferrin receptor mAb (OKT9) as a positive control as previously described (23). SN10 did not react significantly with either activated or preactivated T-cells, while OKT9 showed a strongly enhanced reactivity with the activated T-cells as compared to its reactivity with preactivated T-cells.

Characterization of the molecular properties, particularly amino acid sequence, of gp36 will be valuable for understanding the origin of the antigen. Currently, we are contemplating a study to isolate sufficient quantities of purified gp36 for determining the partial amino acid sequence of the antigen.

We would like to point out that mAb SN10 was generated using an unconventional approach, i.e., immunizing animals (mice) with an LL antigen preparation rather than intact LL cells. Previously, we developed a novel system for isolating immunologically active LL associated cell membrane antigen mixtures (14, 15). Using such antigen preparations isolated from T and non-T/non-B (immature B lineage including pre-B) LL cells to immunize mice, we previously generated several mAbs directed to T or non-T LL associated cell surface antigens (16, 18, 19, 23, 46). In the present study, we extended these studies and prepared a B LL associated cell membrane antigen preparation from mononuclear cells derived from malignant spleens of two patients with B NHL (see “Materials and Methods”). SN10 was generated by immunizing mice with this antigen preparation.

Recently, several mAbs reactive with normal and malignant B-cells have been widely used for diagnosis and follow-up of LL (reviewed in Ref. 7). SN10 will be particularly useful for such purposes because it reacts strongly with many malignant cells and tissues of B-lineage but shows little reactivity with normal counterparts, i.e., normal peripheral blood B-cells and normal bone marrow B-cells. SN10 showed no significant reactivity with a variety of other normal cells and normal solid tissues tested. The normal cells include T-cells, granulocytes, monocytes, erythrocytes, and platelets. The normal solid tissues include epidermis, kidney, lung, and small intestine. Thus, SN10 shows a remarkably high tumor specificity.

SN10 may be useful for studying the tumorigenic mechanisms in LL. Another important application of antitumor mAbs will be utilization of mAbs as a specific delivery vehicle of a
cytotoxic agent(s) to tumor targets.

However, only those mAbs which meet several fundamental criteria (see "Introduction" and below) may have potential for an effective delivery vehicle. These criteria include relatively high tumor specificity, high antigen binding affinity, high antigen density, effective internalization into the target cells, noninduction of down-regulation of antigen expression, and lack of circulating antigen in the plasma of patients (see "Introduction"). SN10 appears to meet these fundamental requirements to serve as an effective delivery vehicle.

As an initial test for the usefulness of SN10 for preparing immunoconjugates, SN10 was conjugated with RA, and the in vitro cytotoxic activity of SN10-RA was determined. SN10-RA killed LL cells effectively, whereas the same conjugate showed no cytotoxicity against control cells (Fig. 7). Thus, SN10 bound to the target antigen (gp36) on LL cells was effectively internalized into the cells. However, the binding of mAb SN10 to LL cells did not cause significant down-regulation of gp36 expression as assayed by our test procedure (Fig. 6; "Materials and Methods").

Although RA conjugate of SN10 is effective in the in vitro killing of cultured LL cells, its activity is not particularly strong. However, it is likely that SN10-RA conjugates show stronger cytotoxic activity against fresh (uncultured) LL cells compared to cultured LL cells since, in general, the former expresses more gp36 than the latter. The in vivo cytotoxic activity of SN10-RA remains to be determined. It should be noted that no clear correlation has been observed between the in vitro cytotoxic activity of an immunotoxin and its in vivo cytotoxic efficacy (13, 47).

Another important use of SN10 will be radioimmunodetection and radioimmunotherapy of NHL after SN10 is conjugated to an appropriate radioisotope because SN10 displays a remarkably high tumor specificity and is an IgG1 antibody. IgG1 antibodies show less nonspecific binding (e.g., binding to Fc receptors) than IgG2a, IgG3, and IgM antibodies.

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Monoclonal Antibody SN10 Which Shows a Highly Selective Reactivity with Human B Leukemia-Lymphoma and Is Effectively Internalized into Cells

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