Three New Monoclonal Antibodies That Define a Unique Antigen Associated With Prolymphocytic Leukemia/Non-Hodgkin's Lymphoma and Are Effectively Internalized After Binding to the Cell Surface Antigen

By Morihiro Okazaki, Yi Luo, Tin Han, Minoru Yoshida, and Ben K. Seon

Prolymphocytic leukemia (PLL) is closely related to chronic lymphocytic leukemia (CLL), but present with distinctive clinical/laboratory features and associated with much worse prognosis. In this study, we generated three new IgG1-κ monoclonal antibodies (MoAbs), termed SN8, SN8a, and SN8b, by use of an unconventional approach, ie, by using an isolated PLL antigen preparation to immunize mice. These MoAbs, particularly SN8, showed a highly selective reactivity to PLL and B non-Hodgkin's lymphoma (NHL) among various human leukemia/lymphoma specimens tested; eg, SN8 was capable of effectively distinguishing PLL from CLL as well as from hairy cell leukemia (HCL) cell specimens. The cell surface antigen defined by the three MoAbs was determined to be a covalently linked heterodimeric glycoprotein complex (gp49/40) consisting of a 49,000-dalton component (α-chain) and a 40,000-dalton component (β-chain). Epitope comparison showed that the epitope defined by SN8 (SN8 epitope) is in close proximity to SN8a epitope but in a distant position from SN8b epitope. Western blot analysis showed that both SN8 and SN8a epitopes are on the β-chain, but SN8b epitope was not detected on either the α- or the β-chain of the reduced antigen in the same analysis. Binding of either SN8 or SN8b to the cell surface gp49/40 did not cause significant downregulation of the antigen expression whereas binding of SN8a to the antigen caused small (approximately 20%) decrease in the antigen expression. Among the various normal peripheral blood cells, only a subpopulation (6.0% to 24.2% among different specimens derived from different donors) of B cells reacted with the SN8 series MoAbs; these MoAbs showed no significant reactivity against T cells, granulocytes, monocytes, erythrocytes, and platelets. Minimal or no significant reactivity (0 to 2.6% among different specimens) was detected against normal bone marrow cells. Ricin A-chain conjugates of the three MoAbs are all strongly effective for specific killing of SN8 antigen-expressing leukemia cells in the absence of any potentiation. Furthermore, the addition of 10 mmol/L NH₄Cl, a potentiator, enhanced strongly the cytotoxic activities of the SN8, SN8a, and SN8b conjugates. Thus, each of the three MoAbs was effectively internalized after binding to the cell surface antigen.

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SN3\(^{11}\) (anti-CD24), SN4\(^{12}\) (anti-CD9), SN5\(^{13}\) (anti-CD10), SN6\(^{10}\) (anti-GP160), G4-3A7\(^{12}\) (anti-HLA-DR), and B3-3D1\(^{9}\) (anti-HLA class I) were previously generated in our laboratory. Control murine IgG (MOPC 195variant; IgG1-k) was prepared in our laboratory.\(^9\)

MoAbs directed toward Leu1 (CD5), Leu4 (CD3), Leu12 (CD19), Leu16 (CD20), and HLA-DR (monomorphic) were purchased from Becton Dickinson (Mountain View, CA). Anti-human Ig A-chain MoAb was obtained from AMAC, Inc (Westbrook, ME).

**Fluorescein isothiocyanate (FITC)-labeled F(ab\(^{\prime}\))\(_{2}\) fragments of goat anti-human Ig chain (each of \(\kappa-, \lambda-, \gamma-, \alpha-, \) and \(\mu-\) chains) antibodies were purchased from TAGO, Inc (Burlingame, CA).

**Antigen preparation from leukemia cell membranes.** Antigen was prepared from the cell membranes of leukemia cells derived from a patient (NC) with B PLL.

The patient presented with clinical symptoms of typical B PLL, ie, massive splenomegaly, no lymphadenopathy, marked lymphocytosis with over 90% prolymphocytes, and a high white blood cell (WBC) count (2 \( \times 10^{9}\) /mm\(^{3}\)). The cell surface phenotype of the B PLL cells was Ig \(\kappa\), Ig \(\lambda\), Ig \(\mu\), Ig \(\gamma\), Ig \(\alpha\), CD3\(^{+}\), CD5\(^{+}\), CD9\(^{+}\), CD10\(^{-}\), CD19\(^{+}\), CD20\(^{+}\), CD24\(^{+}\), GP160\(^{-}\), and HLA-DR\(^{+}\). The results indicate that the malignant cells of the donor patient are relatively mature B cells derived from a monoclonal origin; the phenotype of the cells is consistent with that of B PLL cells.

**Generation of MoAbs.** MoAb was generated by immunizing two BALB/c mice with the isolated antigen preparation. Immunization of the mice was performed as previously described.\(^8\) Cell fusion, hybridoma screening, cloning, and MoAb class determination was performed as described before.\(^{9,16}\)

**Cellular radioimmunoassay (RIA) and fluorescence-activated cell sorter (FACS) analysis.** Details of the cellular RIA that was used for determining reactivity with MoAbs and various cultured and uncultured cells were described previously.\(^{9,16}\) 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 MoAbs with various cell specimens was also determined by FACS analysis. FACS analysis was performed as described previously.\(^{9}\)

**Radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** The PLL antigen preparation (see above) and an LcH-bound glycoprotein preparation of PLL (NC) cells were separately radiolabeled with \(^{125}\)I by an Iodo-gen method (see above). Titration experiments that were performed using a fixed amount (0.1 \(\mu\)g) of each \(^{125}\)I-labeled MoAb and twofold serial increments of BALL-1 cells showed that 88.4% and 20.6%, respectively, of the labeled SN8 and SN8b retained antigen-binding activity; however, the labeled SN8a lost nearly all of its binding activity presumably because of the presence of a tyrosine at the binding site of SN8a. Therefore, only SN8 and SN8b were used for the Scatchard plot analysis. In the analyses of the binding data for SN8 and SN8b, corrections were made for the above numbers. The radiolabeled SN8 and SN8b were determined to contain 1.93 and 2.14 iodine atoms per IgG molecule on the average, respectively. Scatchard analysis of the binding data was performed as described by Trucco et al.\(^{19}\) An equilibrium constant and an average maximal number of MoAb bound/cell were estimated by this analysis.

**Determination of antigen in the plasma of human leukemia-lymphoma (HLL) patients and healthy control donors.** A solid-phase RIA was used as described recently.\(^9\) MOPC 195variant and anti-human Ig A-chain MoAb (IgG1) were included in the test as an isotype-matching negative and a positive control, respectively.

A titration experiment showed that in the above solid-phase RIA, we can detect SN8 antigen contained in as little as 0.1 \(\mu\)g of cell membrane glycoproteins from B PLL cells.

**Antigenic modulation.** Regulation of antigen expression by SN8, SN8a, and SN8b was studied by incubating BALL-1 cells for varying times at 37°C with an excess of the individual MoAbs following a previously described procedure.\(^{20,21}\) Isotype-matching control IgG (MOPC 195variant) and control MoAb SN5 defining CD10\(^{10}\) were included in the assay as a negative and a positive control. The antigen on the cells was determined by a cellular RIA as described previously.\(^8\)

**Preparation of immunotoxin.** The purified MoAbs and an isotype-matching control mouse IgG (MOPC 195variant) were individually conjugated to RA as previously described.\(^{22}\) The conjugates were purified by gel filtration on a calibrated Sephacryl S-300 (Pharmacia) column followed by affinity chromatography on a Blue Sepharose (Pharmacia) column.\(^{23}\)

**Determination of cytotoxic activities of immunotoxins.** A protein synthesis inhibition assay was used to determine the in vitro cytotoxic activities of immunotoxins against HLL cells and control cells. Details of the procedures were described recently.\(^9\)
Table 1. Reactivity of SN8 Series MoAbs With Malignant or EBV-Transformed Human Hematopoietic Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin of Cell Line</th>
<th>SNB Cpm</th>
<th>SNBa Cpm</th>
<th>SNBb Cpm</th>
<th>Control* Cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLL pre-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NALM-1</td>
<td>CML-BC</td>
<td>444 ± 74</td>
<td>371 ± 36</td>
<td>351 ± 11</td>
<td>447 ± 80</td>
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<tr>
<td>NALM-6</td>
<td>ALL</td>
<td>694 ± 326</td>
<td>309 ± 18</td>
<td>423 ± 63</td>
<td>470 ± 145</td>
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<tr>
<td>HLL non-T/non-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>KM-3</td>
<td>ALL</td>
<td>284 ± 33</td>
<td>255 ± 31</td>
<td>414 ± 28</td>
<td>341 ± 75</td>
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<tr>
<td>NALM-16</td>
<td>ALL</td>
<td>341 ± 81</td>
<td>267 ± 18</td>
<td>304 ± 51</td>
<td>314 ± 32</td>
</tr>
<tr>
<td>REH</td>
<td>ALL</td>
<td>150 ± 27</td>
<td>233 ± 29</td>
<td>204 ± 24</td>
<td>165 ± 18</td>
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<tr>
<td>HLL B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALL-1</td>
<td>ALL</td>
<td>5,867 ± 519</td>
<td>4,946 ± 1,024</td>
<td>4,718 ± 252</td>
<td>279 ± 31</td>
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<tr>
<td>BALM-2</td>
<td>ALL</td>
<td>551 ± 104</td>
<td>566 ± 73</td>
<td>481 ± 99</td>
<td>574 ± 62</td>
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<td>Daudi</td>
<td>BL</td>
<td>3,077 ± 366</td>
<td>3,562 ± 519</td>
<td>3,250 ± 196</td>
<td>344 ± 55</td>
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<tr>
<td>SU-DHL-4</td>
<td>HL</td>
<td>8,030 ± 1,016</td>
<td>7,223 ± 121</td>
<td>6,378 ± 229</td>
<td>414 ± 69</td>
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<tr>
<td>U689-M</td>
<td>LS</td>
<td>2,703 ± 264</td>
<td>2,771 ± 485</td>
<td>2,271 ± 670</td>
<td>266 ± 1</td>
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<tr>
<td>Ramos</td>
<td>BL</td>
<td>4,432 ± 1,157</td>
<td>4,032 ± 377</td>
<td>2,814 ± 352</td>
<td>468 ± 16</td>
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<tr>
<td>Raji</td>
<td>BL</td>
<td>684 ± 127</td>
<td>730 ± 85</td>
<td>528 ± 9</td>
<td>454 ± 93</td>
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<tr>
<td>BALM-3</td>
<td>LY</td>
<td>5,382 ± 420</td>
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<td>470 ± 65</td>
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<tr>
<td>BALM-5</td>
<td>LY</td>
<td>1,578 ± 220</td>
<td>1,831 ± 326</td>
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<td>MO 1043</td>
<td>CLL</td>
<td>227 ± 18</td>
<td>255 ± 20</td>
<td>244 ± 19</td>
<td>269 ± 46</td>
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<td>Plasma</td>
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<td>ARH+77</td>
<td>MM</td>
<td>288 ± 48</td>
<td>451 ± 70</td>
<td>250 ± 42</td>
<td>280 ± 17</td>
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<td>RPMI 8226</td>
<td>MM</td>
<td>232 ± 19</td>
<td>415 ± 85</td>
<td>217 ± 30</td>
<td>242 ± 27</td>
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<tr>
<td>HS</td>
<td>MM</td>
<td>221 ± 46</td>
<td>581 ± 62</td>
<td>285 ± 56</td>
<td>389 ± 41</td>
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<td>HLL T</td>
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<tr>
<td>MOLT 4</td>
<td>ALL</td>
<td>242 ± 136</td>
<td>259 ± 74</td>
<td>221 ± 69</td>
<td>246 ± 57</td>
</tr>
<tr>
<td>JM</td>
<td>ALL</td>
<td>233 ± 60</td>
<td>242 ± 27</td>
<td>165 ± 28</td>
<td>233 ± 37</td>
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<tr>
<td>CCRF-HSB-2</td>
<td>ALL</td>
<td>200 ± 14</td>
<td>227 ± 47</td>
<td>217 ± 33</td>
<td>197 ± 14</td>
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<tr>
<td>Ichikawa</td>
<td>ALL</td>
<td>192 ± 5</td>
<td>304 ± 48</td>
<td>223 ± 46</td>
<td>251 ± 40</td>
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<td>HPB-MLT</td>
<td>LTL</td>
<td>283 ± 54</td>
<td>312 ± 56</td>
<td>278 ± 34</td>
<td>281 ± 26</td>
</tr>
<tr>
<td>HUT 78</td>
<td>SS</td>
<td>226 ± 23</td>
<td>366 ± 57</td>
<td>298 ± 87</td>
<td>344 ± 85</td>
</tr>
<tr>
<td>HLL myelo/monocytic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML-2</td>
<td>AML</td>
<td>339 ± 28</td>
<td>387 ± 63</td>
<td>342 ± 15</td>
<td>384 ± 69</td>
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<tr>
<td>HL-60</td>
<td>APL</td>
<td>166 ± 36</td>
<td>219 ± 24</td>
<td>601 ± 347</td>
<td>217 ± 31</td>
</tr>
<tr>
<td>U937</td>
<td>HL</td>
<td>346 ± 105</td>
<td>300 ± 67</td>
<td>246 ± 19</td>
<td>230 ± 44</td>
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<tr>
<td>HLL myeloid/erythroid</td>
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<td></td>
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<tr>
<td>K562</td>
<td>CML-BC</td>
<td>223 ± 10</td>
<td>261 ± 58</td>
<td>219 ± 32</td>
<td>205 ± 62</td>
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<tr>
<td>EBV-transformed B</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CCRF-SB</td>
<td></td>
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<tr>
<td>RPMI 8057</td>
<td></td>
<td>224 ± 94</td>
<td>321 ± 53</td>
<td>293 ± 62</td>
<td>321 ± 103</td>
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</tbody>
</table>

The reactivity was determined using 20 μL of a 10-fold dilution of culture fluids of individual hybridomas and 2 x 10⁶ cells in each test by means of a cellular RIA. Each test was performed in triplicate and the values given are the mean ± SD.

Abbreviations: CML-BC, chronic myelocytic leukemia in blast crisis; ALL, acute lymphoblastic leukemia; BL, Burkitt’s lymphoma; HL, histiocytic lymphoma; LS, lymphosarcoma; LY, lymphoma; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; LTL, leukemic phase of T-cell lymphoma; SS, Sézary syndrome; AML, acute myelocytic leukemia; APL, acute promyelocytic leukemia.

* An isotype-matching (IgG1) murine plasmacytoma IgG (10 μg/mL) dissolved in the hybridoma culture medium.

RESULTS

Initial characterization of MoAbs. In the present study, MoAbs were generated by immunizing two mice with an isolated leukemia antigen preparation (see Materials and Methods). Initial characterization of primary hybridoma cultures and cloned hybridomas was performed by testing against normal human peripheral blood lymphocytes (PBL), PLL cells, and selected human cell lines by means of a cellular RIA as described previously.⁶,⁶ Hybridoma clones 3A2-2E7-1F5 and 3B3-1D2-1A2 derived from mouse 1 and Q6-1D5-C6 from mouse 2 produced IgG1-κ MoAbs that showed a selective reactivity with PLL cells and some B HLL cell lines. These MoAbs were designated SN8, SN8a, and SN8b, respectively.

In Table 1, the test results of these MoAbs with 28 malignant and two Epstein-Barr virus (EBV)-transformed human cell lines are summarized. Among the 28 malignant HLL and myeloma cell lines tested, SN8 series MoAbs showed a significant reactivity with only seven relatively mature B HLL cell lines; ie, BALL-1, Daudi, SU-DHL-4, U689-M, Ramos, BALM-3, and BALM-5. Furthermore, these MoAbs did not react with two EBV-transformed nonmalignant cell lines tested. This restricted reactivity of SN8 series MoAbs with cultured cell lines is consistent with the reactivity of these
MoAbs with fresh (uncultured) HLL cell specimens as described below.

Reactivity with fresh (uncultured) HLL cells. Reactivity of SN8 series MoAbs with uncultured HLL cell specimens was determined by a cellular RIA and, in selected cases, also by FACS analysis.

The results of a cellular RIA with SN8 and uncultured HLL cell specimens from 80 different HLL patients are summarized in Fig 1. SN8 showed significant reactivity with all of the 8 B PLL, 9 of the 12 B non-Hodgkin's lymphomas (NHLs), the 1 B acute lymphoblastic leukemia (ALL), and 1 of the 5 hairy cell leukemia (HCL) specimens tested. SN8 did not show significant reactivity with any of the specimens of non-T/non-B ALL, T ALL, and myeloid/monocytic leukemias. Among the 23 B CLL specimens tested, only three showed weak but significant reactivity with SN8. Thus, SN8 shows a selective reactivity with PLL compared with CLL and hairy cell leukemia, which are both closely related to PLL in the differentiation pathway of B-cell ontogeny. The results of a cellular RIA were supported by FACS analysis, some of which are presented in Fig 2 where greater than 90% of a PLL cell specimen reacted with SN8 and virtually none of the cells of the two CLL specimens were reactive.

The reactivities of SN8a and SN8b with uncultured cell specimens of 60 and 44 HLL patients, respectively, were tested by a cellular RIA. The results are presented in Fig 1, B and C. SN8a and SN8b showed a selective reactivity with B PLL and B NHL samples as SN8 did. However, these MoAbs reacted with higher percentages (ie, 30.0% and 31.3%, respectively) of B-cell preparations derived from the three different donors reacted with SN8 (Fig 2, D through F). Two (nos. 1 and 3) of these B-cell preparations were tested for their reactivities with an anti–HLA-DR MoAb (monomorphic; Becton Dickinson) by FACS analysis. The test showed that 53.4% and 74.2%, respectively, of the B-cell preparations reacted with the anti–HLA-DR MoAb. These two B-cell preparations were also tested for their reactivities with SN8a and SN8b by FACS analysis.
These MoAbs reacted with 11.2% and 7.5%, respectively, of no. 1 sample as well as 23.0% and 13.5%, respectively, of no. 3 sample.

To further characterize the specificity of the MoAbs, SN8 series MoAbs were tested for their reactivities with normal (or nearly normal) BM specimens by a cellular RIA and FACS analysis; these BM specimens were obtained from four different ALL patients in remission. In the RIA, the reactivities of SN8 series MoAbs with each of the four BM samples (between 498 and 749 cpm) were slightly above the corresponding background values obtained by using an isotype-matching control IgG (28,130, 20,1, and 356 cpm, respectively, against the four samples), whereas anti-HLA-DR MoAb (Becton Dickinson) showed a strong reactivity with these BM samples (7225, 3221, 3581, and 5546 cpm, respectively). In an additional study, FACS analysis was performed with these BM samples. The results of SN8 with three of the four samples are shown in Fig 2 (A through C). Reactivity of SN8 with these four normal BM specimens was not detectable (<1% for two specimens) or marginal (approximately 1% and 2.6%, respectively, for the two other specimens).

Molecular nature of antigen. A leukemia antigen preparation from cell membrane glycoproteins of PLL cells was labeled with 125I and used for immunoprecipitation with SN8 series MoAbs and an isotype-matching control murine IgG (MOPC 195 variant). The immunoprecipitates were unreduced or reduced and analyzed by SDS-PAGE and autoradiographs were prepared. The results are shown in Fig 3. Under unreduced conditions, each of the SN8, SN8a, and SN8b immunoprecipitates showed a single component of approximately 81,000 daltons (lanes A through C), whereas no significant component was immunoprecipitated by the control IgG (lane D).

Under reduced conditions, each MoAb immunoprecipitate showed two components of approximately 49,000 (α-chain) and 40,000 (β-chain) daltons (lanes E through G), whereas the control IgG did not precipitate any significant component (lane H). Therefore, the antigen defined by the SN8 series MoAbs consists of two polypeptide chains covalently linked by disulfide bond(s). The antigen was designated gp49/40.

In a separate test, LcH-bound glycoproteins from cell membranes of PLL cells were labeled with 125I and used for immunoprecipitation. The results are consistent with the above-mentioned results obtained with a 125I-labeled leukemia antigen preparation (data not shown).

Epitope study by Western blot analysis. The assay was performed after reducing the antigen preparation to determine the component of the antigen with which the SN8 series MoAbs react (Fig 4). Both SN8 and SN8a were shown to bind to the 40-Kd component (β-chain) of gp49/40, whereas SN8b did not bind to either component. When the assay was performed with an unreduced sample, both SN8 and SN8a were found to bind strongly to the intact dimer antigen, whereas SN8b bound to the antigen only very weakly. The results show that epitopes defined by SN8 and SN8a reside on the β-chain of the antigen, but SN8b epitope was probably
Fig 3. SDS-PAGE of immunoprecipitates from a 125I-labeled PLL antigen preparation. In the immunoprecipitation, we used SN8 (lanes A and E), SN8a (lanes B and F), SN8b (lanes C and G), and control IgG (MOPC 195 variant; lanes D and H). Samples were analyzed after being unreduced (lanes A through D) or reduced with dithiothreitol (lanes E through H). BioRad Mr marker proteins of the heavy chain (52 K) of human IgG were used after reduction as references.

perturbed or damaged under the experimental conditions used for treating the sample (see Materials and Methods).

**Competitive antibody binding.** A competitive binding assay was performed to compare the epitopes defined by SN8 series MoAbs (Fig 5). Preincubation of gp49/40-expressing BALL-1 cells (see Table 1) with SN8 or SN8a completely blocked the subsequent binding of 125I-SN8 at the maximum. However, preincubation with SN8b or an isotype-matching control murine IgG did not inhibit the 125I-SN8 binding at all. These results indicate that the epitopes defined by SN8 and SN8a are in close proximity to each other but distant from the epitope defined by SN8b. These results are consistent with the above finding that both SN8 and SN8a bound to the same component (β-chain) in the Western immunoblotting.

**Antibody avidity and number of available epitopes on HLL cells.** Scatchard plot analyses of direct binding of radiolabeled SN8 and SN8b to BALL-1 cells were performed (Fig 6); the results showed equilibrium constants of 4.2 × 10^8 and 4.3 × 10^8 L/mol, respectively. In the same analysis, the average number of antibody molecules bound per cell was estimated to be 5.9 × 10^4 and 5.7 × 10^4, respectively, at antibody saturation. Because both SN8 (IgG1) and SN8b (IgG1) are bivalent antibodies, the average number of antigen on these cell specimens is probably onefold to twofold greater than the antibody number. In the present test, SN8a was not included because SN8a lost antigen-binding activity nearly completely after labeling with 125I (see Materials and Methods).

The present results show that both SN8 and SN8b possess a good binding avidity to BALL-1 cells and gp49/40 is expressed abundantly on BALL-1.

**Determination of circulating antigen in the plasma of HLL patients.** Circulating antigen in the plasma of patients may bind an administered MoAb and thereby inhibit the therapeutic efficacy of the administered MoAb and immunoconjugate. Therefore, we tested for circulating SN8 antigen in the plasma of HLL patients and healthy individuals (control) by using MoAbs SN8, SN8a, and SN8b and a solid-phase RIA. No significant amount of SN8 antigen was detected in any of the plasma samples derived from seven different B NHL patients and five different B CLL patients. Similarly, no significant SN8 antigen was detected in the plasma samples derived from five different healthy individuals.

**Regulation of antigen expression.** Binding of antibody to a cell surface antigen may induce antigenic modulation and downregulation of antigen expression, which may make antigen-targeting by antibody difficult. In the present study, we tested the effect of binding of SN8, SN8a, and SN8b to BALL-1 on the expression of gp49/40. Binding of either SN8 or SN8b to the cell surface gp49/40 did not cause significant downregulation of antigen expression whereas binding of SN8a to the antigen caused small (approximately 20%) decrease in the antigen expression (Fig 7).

Nevertheless, all the SN8 series MoAbs are effectively internalized into the HLL cells after binding as demonstrated by the effective killing of the HLL cells by the RA conjugates of these MoAbs (see below).

**Specific killing of HLL cells by RA conjugates of MoAbs.** Cytotoxic activities of RA conjugates of SN8, SN8a, and SN8b, and an isotype-matching control IgG were tested against gp49/40-expressing BALL-1 and gp49/40-negative control MOLT-4 cells in the absence or in the presence of
not inhibit the protein synthesis of control MOLT-4 cells at any of the concentrations tested (Fig 8B). NH₄Cl (10 mmol/L) enhanced the cytotoxic activities of immunotoxins against BALL-1 but not against MOLT-4. IC₅₀ of SN8-RA, SN8a-RA, and SN8b-RA against BALL-1 were 1.0 × 10⁻¹⁰, 1.2 × 10⁻¹⁰, and 4.8 × 10⁻¹⁰ mol/L, respectively, in the presence of 10 mmol/L NH₄Cl (broken lines in Fig 8A).

The control RA conjugate (MOPC-RA) did not show significant cytotoxicity against either BALL-1 or MOLT-4 in the absence or in the presence of NH₄Cl. In an additional test, RA conjugates of SN8, SN8a, and SN8b were tested for cytotoxicity against Daudi, a lymphoma cell line (see Table 1). IC₅₀ of SN8-RA, SN8a-RA, and SN8b-RA were 8.2 × 10⁻¹⁰, 6.7 × 10⁻¹⁰, and 2.4 × 10⁻⁹ mol/L, respectively. The cytotoxic activities of these conjugates against Daudi were potentiated by NH₄Cl; IC₅₀ in the presence of 10 mmol/L NH₄Cl were found to be 3.1 × 10⁻¹², 4.9 × 10⁻¹², and 1.3 × 10⁻¹¹ mol/L, respectively.

The present results show that each of the RA conjugates of the three MoAbs SN8, SN8a, and SN8b is effective in

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**Fig 4.** Western blot analysis of the epitopes defined by SN8 series MoAbs. A ¹²⁵I-labeled leukemia antigen preparation was reduced with 50 mmol/L dithiothreitol and subjected to SDS-PAGE. The separated proteins in the gels were transferred to nitrocellulose membranes. After blocking with normal goat serum, the membranes were incubated with SN8 (lane A), SN8a (lane B), SN8b (lane C), or control IgG (lane D). The membranes were further treated with ¹²⁵I-F(ab')₂ of affinity purified goat anti-mouse IgG antibodies. The marker proteins (shown in kilodaltons) are described in the legend to Fig 3 except for soybean trypsin inhibitor (21.5 K).

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10 mmol/L NH₄Cl, a potentiator. The results are shown in Fig 8. In the absence of any potentiators, SN8-RA could inhibit the protein synthesis of BALL-1 for 50% of the control at a concentration of 5.0 × 10⁻¹⁰ mol/L. The 50% inhibitory concentrations (IC₅₀) of SN8a-RA and SN8b-RA against BALL-1 were 2.6 × 10⁻⁹ and 3.0 × 10⁻⁹ mol/L, respectively (solid lines in Fig 8A). However, the three RA conjugates did
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Fig 6. Binding of $^{125}$I-labeled SN8 and SN8b to BALL-1. $r$ is the number of antibody molecules bound to one cell at a given dilution; $A$ is the molar concentration of total antibody; and $x$ is the molar concentration of bound antibody, so that $(A - x)$ is the molar concentration of the free antibody. The slope of the binding curve gives the equilibrium constant. $n$ is the maximum number of antibody molecules that can be bound to a single cell; and $K$ is the equilibrium constant for the reaction, expressed in liters per mole.

specific killing of gp49/40-expressing HLL cells. Furthermore, the results indicate that each of the three MoAbs is effectively internalized into target HLL cells after binding to the cell surface antigen.

DISCUSSION

The epitope defined by new MoAb SN8 is detected on malignant B cells with a restricted stage of maturation as well as on a small subpopulation of normal B cells in the peripheral blood of healthy individuals. SN8 reacted with all of the 8 B PLL specimens and 9 of the 12 B NHL specimens tested but not with most of B CLL, HCL, and non-T/non-B ALL (including pre-B ALL) specimens tested (Fig 1). In addition, SN8 reacted with the one uncultured B ALL cell specimen tested as well as one of the two B ALL cell lines tested (Fig 1 and Table 1). It is extremely interesting that SN8 is capable of effectively discriminating B PLL from B CLL and HCL as well as from non-T/non-B ALL. Among the previously reported MoAbs, MoAbs defining FMC-726 and CD22 appear to be able to discriminate B PLL from some cases of B CLL but not from HCL; it should be noted that molecular properties of the antigens defined by these MoAbs are different from gp49/40 defined by SN8.

It is believed that B CLL, B PLL, and HCL are closely related in the differentiation pathway of B-cell ontogeny while non-T/non-B ALL derives from normal counterparts at earlier stages of B-cell ontogeny by malignant transformation and clonal expansion. B ALL is closely related to B lymphomas and its phenotype corresponds to that of relatively mature B cells. B NHL consists of a heterogeneous group of malignant B cells with varying degrees of maturation but the normal counterparts in the majority of cases of B NHL appear to be relatively mature B cells. Phenotypic and genetic analyses suggest that B PLL derives from normal counterparts by malignant transformation at a later developmental stage than B CLL. For instance, B PLL cells express higher density of cell surface Ig than B CLL cells. Furthermore, it was reported that in most cases of B PLL, the malignant clone has both alleles of Ig heavy chain gene in a rearranged configuration, while in many cases of B CLL, only one allele of the gene is rearranged and the other is found in a germ line-like configuration. Similarly to B PLL, HCL also appears to derive from the clonal expansion of a B cell at a later developmental stage than B CLL. Between HCL and B PLL, the normal counterparts of HCL are probably more mature than those of B PLL in the differentiation pathway of B-cell ontogeny.

Fig 7. Regulation of gp49/40 expression on BALL-1 that has been incubated with SN8, SN8a, or SN8b. BALL-1 was incubated with an excess of purified MoAb SN8, SN8a, or SN8b (solid circles) or an isotype-matching purified control IgG (open circles) for varying periods of time. gp49/40 on the incubated cells was determined by a cellular RIA.
SN8 reacted well with B PLL but poorly with HCL, B CLL, and non-T/non-B ALL (Fig 1). Thus, the results presented in this report indicate that SN8 defines an epitope that is associated with a relatively narrow range of B-cell maturation. The epitopes defined by SN8a and SN8b appear to be associated with slightly wider ranges of B-cell maturation than SN8 epitope.

We would like to point out that the present MoAbs were generated using an unconventional approach, ie, by immunizing animals with an HLL antigen preparation rather than with intact HLL cells. Previously, we developed a novel system for isolating immunologically active HLL associated cell membrane antigen mixture. In the present study, this system was applied to isolating a B PLL-associated cell membrane antigen preparation that was used for generating MoAbs.

SN8 series MoAbs appear to be different from those previously reported MoAbs in the antibody specificity and/or in the molecular nature of the antigen defined. The antigen gp49/40 defined by the present MoAbs appears to be different from any of the reported CD series antigens. Among the CD series antigens, CD72 shows some similarity to gp49/40; CD72 is a heterodimer of 43,000- and 39,000-dalton components. However, besides the smaller molecular size of the heavier component of CD72 compared with the α-chain (gp49) of SN8 antigen, there are distinct differences in the specificity between anti-CD72 MoAb and SN8 series MoAbs. For instance, anti-CD72 MoAb reacted with 44% (7 of the 16 specimens tested) of non-T/non-B ALL specimens, all (3/3) of the HCL specimens, and NALM-6, a pre-B ALL cell line. In contrast, SN8 series MoAbs did not react with any of the non-T/non-B ALL specimens tested (ie, 0/13, 0/7, and 0/4, respectively, for SN8, SN8a, and SN8b), did not react with the majority of the HCL specimens (1/5, 1/4, and 0/3, respectively), and did not react with NALM-6 (see Fig 1 and Table 1).

As described above, anti-CD22 MoAbs show some similarity to SN8 series MoAbs in their specificity. However, CD22 is a single polypeptide chain antigen with a molecular weight of 135,000. Comparison of SN8 series MoAbs with previously reported MoAbs suggests that the present MoAbs may define a novel human B-cell-associated antigen or epitopes. However, it is prudent that any definite conclusions about the novelty of the present antigen be withheld until the chemical structure (eg, amino acid sequence) of the antigen defined by these MoAbs is determined.

The data presented here suggest the usefulness of SN8 series MoAbs, particularly SN8, for diagnosis of HLL and follow-up of B PLL and B NHL. The data also suggest the usefulness of these MoAbs for studying the pathogenesis of B PLL and B NHL. Another important application of these MoAbs may be their utilization as a specific delivery vehicle of a cytotoxic agent(s) to tumor targets.

As an initial test for the utility of SN8 series MoAbs for preparing immunoconjugates, these MoAbs were conjugated with RA, and the in vitro cytotoxic activities of the generated immunotoxins were determined. All of the three immunotoxins killed gp49/40-expressing HLL cells effectively while...
showing no significant cytotoxicity against control cells. Thus, these immunotoxins showed specific cytotoxicity and each of SN8 series MoAbs bound to the target antigen (gpl49/40) on HLL cells was effectively internalized into the cells. However, the binding of these MoAbs to HLL cells did not cause (SN8 and SN8b) or caused only a small degree (SN8a) of downregulation of antigen expression. These results may be explained by our recent finding that there is dynamic balance (or unbalance in some cases) between endocytosis and exocytosis-cell surface expression of an antigen on the cell surfaces. Therefore, an antibody-bound antigen can be effectively internalized even when no modulation of the overall cell surface expression of the antigen is observed. No significant amount of circulating antigen was detected in the plasma of HLL patients or healthy individuals. Although more studies are needed, the initial studies with these new MoAbs and their immunonoconjugates appear to suggest the potential of these MoAbs as delivery vehicles of cytotoxic agents to tumor targets.

ACKNOWLEDGMENT

We are grateful to Dr Maurice Barcos and David Chervinsky for providing us with specimens of patients. We thank Dr Soichiro Yokota, Hilda Tsai, and Barbara Dadey for their technical assistance and Dr Joseph Krasner, Suzanne Sabadaz, and Cheryl Zober for their help in the preparation of the manuscript.

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Three new monoclonal antibodies that define a unique antigen associated with prolymphocytic leukemia/non-Hodgkin’s lymphoma and are effectively internalized after binding to the cell surface antigen

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