

Señor
DIRECTOR DIVISIÓN DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
E. S. D.

REF: Expediente No. NC2017/0006740, solicitud de patente de invención a favor de **GENENTECH INC.** Título: **"ANTICUERPOS ANTI-CD79B"**

RESPUESTA A EXAMEN DE FONDO

JUAN PABLO CADENA SARMIENTO, portador de la tarjeta profesional No. 57.492 del C.S.J., obrando como apoderado de la sociedad solicitante me permito dar respuesta al Oficio No. 14442 relacionado con la patentabilidad de la presente invención y notificado el 5 de diciembre de 2018.

1. Título

A este respecto, me permito resaltar que, siguiendo la amable sugerencia del Examinador, y con el ánimo de facilitar el trámite, el título de la invención se modifica de la siguiente manera:

" Anticuerpos anti-CD79b y Anticuerpos anti-CD79b/CD3 biespecíficos "

Considerando lo anterior, de manera respetuosa solicito que la objeción a este respecto sea retirada.

2. Claridad de las Reivindicaciones

i) En primer lugar, con relación a la reivindicación 1, me permito resaltar que contrario a lo mencionado por el señor Examinador en el Oficio Técnico No. 14442, la reivindicación 1 es clara, concisa y además cuenta con pleno soporte en el Capítulo Descriptivo de la solicitud, ya que el anticuerpo anti-CD79b aislado allí reclamado se caracteriza principalmente por comprender (a) un dominio de unión CD79b y (b) seis regiones hipervariables que a su vez se encuentran definidas por secuencias de aminoácidos específicas.

Así, es claro que la materia consignada en la actual reivindicación 1 cumple con una definición precisa del anticuerpo, es más, tal y como el mismo Examinador lo sugiere en el Oficio Técnico No. 14442, un anticuerpo **debe ser definido exclusivamente** a partir de sus características técnicas esenciales, tales como:

- Mediante las secuencias completas que lo conforman en la cadena ligera y cadena pesada; o

- Mediante las secuencias variables de la cadena ligera y pesada; o
- Mediante las secuencias de los 6 CDR's que conforman las cadenas.

con el único objetivo de distinguir inequívocamente la invención del estado de la técnica. De esta manera, **es evidente que las seis regiones hipervariables de la cadena pesada y ligera que a su vez se encuentran definidas por secuencias de aminoácidos específicas, del anticuerpo de la reivindicación 1 cumplen a cabalidad con lo anteriormente mencionado.** Es más, esta misma información de igual forma se encuentra estipulada dentro del numeral 5.2.1.2.5. de la Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad. Por lo tanto, me permito manifestar que la materia reclamada de ninguna manera puede ser afectada por falta de claridad o concisión.

Adicionalmente, y a modo de aclaración me permito resaltar que las secuencias de aminoácidos mencionadas dentro de esta reivindicación se encuentran definidas en el Listado de secuencias originalmente presentado, al igual que dentro del Capítulo Descriptivo Modificado. Así las cosas, no es correcto afirmar que las secuencias aquí presentadas carecen de sustento, ya que la descripción de la solicitud divulga por completo las cadenas de aminoácidos recitadas, y más aún los residuos de aminoácidos en posiciones específicas que pueden ser sustituidos.

En este sentido, de manera atenta solicito la consideración de los anteriores argumentos y el retiro de la objeción a este respecto.

ii) Por otro lado, en cuanto a la reivindicación 11 de la presentes solicitud, de manera respetuosa me permito resaltar que el anticuerpo anti-CD79b allí reclamado encuentra pleno soporte en el Capítulo Descriptivo de la solicitud, ya que las mutaciones del mismo a los residuos de aminoácidos N297, L234, L235 y/o D265 de acuerdo con la numeración EU se describen a lo largo de la descripción, por ejemplo, en las páginas 6, 11, 90, 122. Así, me permito manifestar que la materia recitada en esta reivindicación corresponde a **una realización preferida que indica claramente la mutación realizada.**

Adicionalmente, me permito resaltar que las reivindicaciones dependientes de esta, de igual forma mencionan características técnicas esenciales y adicionales que permiten una caracterización completa de la invención, por lo que de igual forma deberían ser tenidas en cuenta al momento de sugerir una modificación, ya que, por ejemplo tal y como lo mencionan las reivindicaciones 12 y 13, estas de igual manera especifican mutaciones de sustitución en los residuos de aminoácidos N297G, N297A, L234A, L235A, y D265A, así como el residuo de aminoácido 297 para N297G.

Así las cosas, me permito manifestar que seguir el consejo sugerido por el Examinador, en cuanto a restringir aún más la materia reclamada, correspondería a una limitación injusta del concepto inventivo desarrollado por el Solicitante. Por lo tanto, de forma respetuosa solicito a ese Despacho que las consideraciones anteriormente expuestas

sean tenidas en cuenta al momento de evaluar el presente memorial de respuesta, y por ende las objeciones a este respecto sean retiradas.

3. Nivel Inventivo

i) Con respecto al nivel inventivo de las reivindicaciones 1, 2-16, 28-33 me permito resaltar que contrario a los señalamientos establecidos por el Examinador, la presente solicitud, tal y como se encuentra actualmente reclamada difiere en diferentes aspectos con los documentos del arte anterior citados en el Oficio Técnico No. 14442.

Particularmente, D1 describe anticuerpos anti-CD79b humanizados y sus inmunoconjugados útiles para el tratamiento de tumores hematopoyéticos (reivindicaciones 1-258 de D1), en donde principalmente este documento enseña formas biespecíficas anti-CD79b con un enlace a un dominio de unión CD3, y en donde dichos anticuerpos usan los dominios variables de cadena pesada (VH) y de cadena liviana (VL) de los anticuerpos monoclonales de ratón SN8 descritos en el documento **Okazaki *et al.*, Blood, 81: 84-94 (1993)**, del cual se adjunta una copia al presente memorial de respuesta.

ii) Por su parte, D2 se dirige a describir moléculas diacuerpo y sus usos en el tratamiento de una variedad de enfermedades y trastornos, incluidos trastornos inmunológicos, enfermedades infecciosas, intoxicación y cáncer. Las moléculas divulgadas por este documento comprenden dos cadenas polipeptídicas que se asocian para formar al menos dos sitios de unión a epítopos, que pueden reconocer los mismos o diferentes epítopos en antígenos.

iii) Sin embargo, y teniendo en cuenta la materia actualmente reclamada en el capítulo Reivindicatorio de la invención, me permito resaltar que el objeto de las reivindicaciones de la presente solicitud, **difiere de estos documentos en características como: las secuencias de aminoácidos de las CDR del anticuerpo anti-CD79b.**

Pese a lo anterior, y más aún reconociendo que la presente solicitud es diferente de los documentos del arte citados, el señor Examinador afirma que el problema a resolver por la presente invención puede considerarse simplemente como una modificación de los anticuerpos anti-CD79b de D1 para generar más anticuerpos anti-CD79b capaces de inducir la muerte del trastorno proliferativo de las células B o un trastorno autoinmune, además, que D2 enseña el desarrollo de DARTs caracterizados porque contienen un dominio de unión a antígeno CD79b capaces de inducir la muerte de células B, por lo que es útil para el tratamiento de un trastorno proliferativo de células B o un trastorno autoinmune. Sin embargo, el señor Examinador **no considera a fondo realmente cada una de las diferencias que existen entre la presente solicitud y los documentos referenciados.**

En este sentido, con el propósito de demostrar que la presente solicitud además de ser diferente de las divulgaciones referenciadas, presenta un efecto técnico sorprendente, me permito resaltar que el **Ejemplo 1.1**, del Capítulo Descriptivo de la presente invención compara la idoneidad de los anticuerpos anti-CD79b como ramas o brazos objetivo para un anticuerpo biespecífico dependiente de células T (TDB), en donde, tal y como se menciona en la página 125 de la descripción de la invención, concretamente en el aparte titulado **1. Selección de rama de antígeno CD79b TDB-anti-CD79b**, el anticuerpo SN8 humanizado fue escogido como un objetivo para un anticuerpo biespecífico dependiente de células T (TDB).

Particularmente, la presente invención menciona que se han generado conjugados de anticuerpo-fármaco (ADC) (tales como el anticuerpo humanizado anti-CD79b (SN8 humanizado) conjugado con monometilauristatina E (MMAE) por un conector disociable por proteasa), que ha demostrado, en la clínica, ser eficaz para el tratamiento de NHL (Patente de los Estados Unidos Nro. 8.088.378, la cual corresponde a la Solicitud PCT del documento D1), siendo esta la referencia principal de la fuente del anticuerpo SN8.

Así las cosas, sobre la base del éxito clínico del ADC anti-CD79b, el anticuerpo humanizado SN8 se presentó en un formato de anticuerpo biespecífico dependiente de células T (TDB) a fin de utilizar el alto potencial citotóxico de las células T en la erradicación de células tumorales. Sin embargo, dicho anticuerpo **exhibió una muerte de células B deficiente** cuando se emparejó con el brazo/rama CD3 UCHT1.v9. De igual manera, se observaron resultados deficientes de destrucción celular para un segundo anticuerpo TDB, el cual se generó usando el anticuerpo anti-CD79b 2F2.

Sin embargo, y a pesar de estos resultados, se generaron anticuerpos monoclonales anti-CD79b adicionales, en donde dos de estos anticuerpos anti-CD79b (CD79b.F6 y CD79b.A7) se ensayaron también como anticuerpos de formato bisfab biespecíficos anti-CD79b/CD3, en donde particularmente el anticuerpo monoclonal CD79b.A7, descrito en el Capítulo Descriptivo de la presente solicitud, **mostró una muerte eficiente de células B** cuando se emparejó con el brazo/rama CD3 UHT1.v9, tal y como se describe en las páginas 126 y 127 del Capítulo Descriptivo de la solicitud a continuación en la Figura 1:

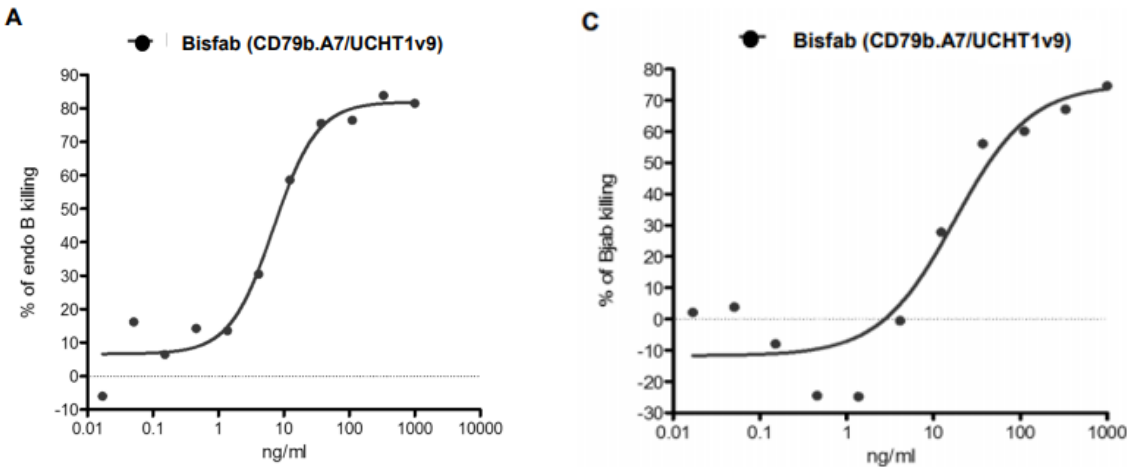
Figura 1. Aparte tomado de las páginas 126 y 127 del Capítulo Descriptivo de la presente invención.

Se generaron anticuerpos monoclonales anti-CD79b como se describe anteriormente. Dos de estos anticuerpos anti-CD79b (CD79b.F6 y CD79b.A7) se ensayaron también como anticuerpos de formato bisfab biespecíficos anti-CD79b/CD3. Como se expone en la Figura 1C, en un ensayo de muerte de célula B endógena, el CD79b.F6/UHT1.v9 bisfab biespecífico exhibió mejorada muerte de células B en comparación con K&H SN8.v28/UHT1.v9 biespecífico (EC₅₀ de 33 ng/ml en comparación con 189 ng/ml). Adicionalmente, como se muestra en la Figura 1C, en el ensayo de muerte de célula B endógena, el bisfab biespecífico CD79b.A7/UHT1.v9 mejoró drásticamente la muerte de células B en comparación o bien con bisfab biespecífico CD79b.F6/UHT1.v9 o K&H biespecífico SN8.v28/UHT1.v9 (EC₅₀ 12 ng/ml en comparación con 33 ng/ml y 189 ng/ml). El CD79b.A7/UHT1.v9 bisfab biespecífico fue adicionalmente ensayado para determinar la muerte de célula B endógena y la activación de células T CD8+ usando donantes adicionales. Como se muestra en las Figuras 2A y C, en el ensayo de muerte de célula B endógena que se describe anteriormente, el bisfab biespecífico CD79b.A7/UHT1.v9 usando dos diferentes donantes produjo la

eficiente muerte de células B, con un EC₅₀ de 7.0 ng/ml y 18 ng/ml, respectivamente. Al mismo tiempo, como se muestra en las Figuras 2B y D, en el ensayo de activación de células T CD8+ que se describe anteriormente, el bisfab biespecífico CD79b.A7/UHT1.v9 logró la eficiente activación de células T, según lo evidenciado por el % de células T CD69+CD25+ en células T CD8+ con un valor EC₅₀ de 17 ng/ml y 17 ng/ml, respectivamente.

Así, y como prueba fehaciente de lo anteriormente mencionado, las Figuras 2A y C, reafirman que el bisfab biespecífico CD79b.A7/UHT1.v9, usando dos diferentes donantes **produjo una muerte eficiente de células B**, tal y como se observa a continuación:

Figura 2. Figuras 2A y C del Capítulo Descriptivo de la presente invención.



Esta mejora de la eficacia sobre los anticuerpos TDB SN8 y 2F2 también se observó en el CD79b.A7v14b optimizado cuando se combina con brazos/ramas CD3 adicionales, como se demuestra en el Ejemplo 1.3 de la presente descripción.

iv) De conformidad con lo anterior, me permito resaltar que en adición a demostrar **la superioridad de la presente solicitud con respecto a los anticuerpos de D1**, como brazo objetivo para los anticuerpos TDB, los anticuerpos CD79b de la presente invención también muestran **una afinidad mejorada por la diana CD79b como un anticuerpo monoespecífico**, en donde inicialmente, si bien el injerto humanizado del CD79b.A7 no mostró afinidad de unión por el objetivo (páginas 129 y 139 del Capítulo Descriptivo de la solicitud), posterior a diferentes esfuerzos de humanización, la afinidad de las variantes humanizadas mejoró dramáticamente y sorprendentemente por una sustitución de una serina por la cisteína en la posición 46 de la cadena ligera.

Así, de lo anterior, me permito resaltar que esta selección puntual para la sustitución única, resulta en **un impacto significativo en la afinidad de unión y por ende, de ninguna manera podría ser un desarrollo predecible**.

Con base en los resultados mencionados anteriormente, en adición a esto, los inventores de la presente solicitud de igual forma optaron por realizar optimizaciones adicionales **para aumentar la afinidad de los anticuerpos humanizados y, finalmente, para eliminar las responsabilidades de fabricación para llegar a la variante optimizada CD79b.A7.v14b** (páginas 130-134 del Capítulo Descriptivo de la invención). Por lo anterior, queda en evidencia que la invención actualmente reclamada cumple con el requerimiento de nivel inventivo.

v) En este sentido, teniendo en cuenta los datos resaltados y la argumentación expuesta anteriormente, me permito manifestar que para determinar si una invención tiene o no nivel inventivo, siempre es necesario considerar cada uno de los elementos, y características establecidas en la materia reclamada como un **todo y no como porciones separadas de una misma invención**. Así las cosas, no basta con la existencia y divulgación de los elementos y características de una invención de manera aislada o individualmente para concluir la falta de nivel inventivo, sino por el contrario, en su totalidad, estos deben integrar y enseñar de manera evidente la manera de llegar a la solución al problema técnico, por lo tanto, es claro que la presente invención, goza de nivel inventivo.

Además de lo anterior, es relevante considerar que tal y como lo expone el Manual para el Examen de Solicitudes de Patentes de Invención en las Oficinas de Propiedad Industrial de los países de la Comunidad Andina *“Para juzgar si la invención definida por las reivindicaciones realmente se deriva de manera evidente del estado de la técnica, hay que determinar si carece de nivel inventivo cuando se consideran las diferencias entre ésta y el estado de la técnica más cercano. El examinador tiene la carga de probar que la invención carece de nivel inventivo y no sólo limitarse a establecer las diferencias entre la solicitud y dicho estado de la técnica”*¹, por lo anterior, me permito llamar la atención del Examinador en el hecho que cada invención es de cierta medida una combinación de elementos relacionados entre sí, por lo que no es suficiente afirmar que dichos elementos, características, etapas, en el presente caso, ya habían sido previamente

¹ Manual para el Examen de Solicitudes de Patentes de Invención en las Oficinas de Propiedad Industrial de los países de la Comunidad Andina, Sección 10.

divulgados, partiendo de combinaciones de enseñanzas convenientes, y que por ende, la combinación de fracciones de diferentes documentos permiten la deducción obvia de la invención reclamada.

Por el contrario, en primera instancia es importante entender que el nivel inventivo es **un proceso creativo, innovador y único** para cada invención, en donde la cuestión para el Examinador se enfoca en si la invención reivindicada es o no evidente para un técnico en la materia.² En sí, para evaluar el nivel inventivo de una invención, el Examinador debería determinar si los elementos y/o características ya conocidas podrían haberse combinado para dar como resultado una invención particular y más aun sabiendo que existen diferencias claras entre la invención y los documentos del arte citados, las cuales conllevan a obtener un efecto técnico particular.

Así, considerando lo anterior, me permito resaltar que el análisis de nivel inventivo expuesto en el Oficio 14442 es errado y de ninguna manera podría afirmarse que la presente solicitud es evidente u obvia a la luz de las enseñanzas de D1 y D2.

Adicionalmente, me permito resaltar que a partir de la lectura del arte anterior citado, no es posible deducir los anticuerpos y modalidades preferidas de la presente invención, ya que las invenciones de patente tienen nivel inventivo cuando el producto o procedimiento no es **fácilmente derivable** por la persona medianamente versada en la materia o cuando presentan un **efecto inesperado o mejorado impredecible de los documentos citados**, como en la presente invención. Por lo anterior, me permito manifestar que **nada en el arte previo sugiere ni enseña** la invención particular de esta solicitud, ni mucho menos, el efecto de la misma.

Así las cosas, y en vista de lo anteriormente expuesto, respetuosamente solicito el retiro de las objeciones de la presente solicitud y la concesión del privilegio solicitado, ya que en opinión del solicitante cumple con todos los requisitos establecidos en la Decisión 486.

ANEXOS

- Documento Okazaki *et al.*, Blood, 81: 84-94 (1993).

Atentamente,

JUAN PABLO CADENA SARMIENTO

T.P. No. 57.492 del C.S.J.

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² Íbidem.

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 B PLL antigen preparation to immunize mice. These MoAbs, particularly SN8, showed a highly selective reactivity to B PLL and B non-Hodgkin's lymphoma (NHL) among various human leukemia-lymphoma specimens tested; eg, SN8 was capable of effectively distinguishing B PLL from B 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 (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 potentiators; furthermore, the addition of 10 mmol/L NH_4Cl , 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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PROLYMPHOCYTIC leukemia (PLL) was initially reported in 1974 by Galton et al¹ as a variant of chronic lymphocytic leukemia (CLL) but with distinct clinical and laboratory features. The distinction between PLL and CLL is clinically important because PLL is a relentlessly progressive disease with a worse prognosis and usually resistant to the chemotherapy that is often effective in CLL.¹⁻³ The majority (approximately 75%) of PLL cases are of B-cell origin^{2,5} as are most cases (approximately 98%) of CLL.^{2,6,7} Furthermore, B PLL appears to be closely related to HCL in the differentiation pathway of B-cell ontogeny.²

In this article, we report the generation and characterization of three monoclonal antibodies (MoAbs) termed SN8, SN8a, and SN8b that were generated by immunizing two mice with an isolated PLL antigen preparation. These MoAbs, particularly SN8, showed a highly selective reactivity with B PLL and B NHL. The surface antigen defined by the SN8 series MoAbs was identified as a covalently linked heterodimer (gp49/40). Comparison of SN8 series MoAbs and SN8 antigen with the reported MoAbs and their antigens suggests that

SN8 series MoAbs may define a novel leukemia/lymphoma associated antigen (see Discussion).

Because the present MoAbs show a highly selective reactivity as well as high binding avidity (SN8 and SN8b) and are of IgG1 isotype, they may be useful for the diagnosis of leukemia/lymphoma, for studying the pathogenesis of PLL and perhaps for studying normal human B-cell differentiation.

In addition, ricin A-chain (RA) conjugates of the three MoAbs are all effective for the specific killing of SN8 antigen-expressing leukemia cells. Furthermore, binding of these MoAbs to the cell surface antigen induced no significant (SN8 and SN8b) or only a small (SN8a) downregulation of antigen expression. The results suggest the potential of these MoAbs as a specific delivery vehicle of cytotoxic agents to tumor targets.

MATERIALS AND METHODS

Cells. Various established human cell lines were cultured in RPMI 1640 medium supplemented with 4% to 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 $\mu\text{g}/\text{mL}$) as described previously.⁸ Peripheral blood, bone marrow (BM) aspirates, and lymph node specimens from cancer patients were obtained at the Roswell Park Cancer Institute clinics as described previously.⁹ Mononuclear and blast cells were isolated from the cell suspensions of these specimens by centrifugation on a Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient. Normal (or nearly normal) BM specimens were from patients who were in remission and had a morphologically normal BM. Mononuclear cells were isolated from the BM aspirates by Ficoll-Paque gradient centrifugation.

B cells, T cells, granulocytes, monocytes, erythrocytes, and platelets of normal peripheral blood were isolated from buffy coat preparations of healthy volunteers as previously described.^{8,10}

Reagents, control MoAb, and control IgG. L-[³H]leucine and leucine-free medium were purchased from ICN Biomedicals, Inc (Irvine, CA) and GIBCO Laboratories (Grand Island, NY), respectively. RA was obtained from Inland Laboratories (Austin, TX). MoAbs

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ANTI-LEUKEMIA/LYMPHOMA MONOCLONAL ANTIBODIES

SN3¹¹ (anti-CD24), SN4¹² (anti-CD9), SN5¹³ (anti-CD10), SN6¹⁰ (anti-GP160), G4-3A7¹² (anti-HLA-DR), and B3-3D1⁹ (anti-HLA class I) were previously generated in our laboratory. Control murine IgG (MOPC 195variant; IgG1- κ) 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 λ -chain MoAb was obtained from AMAC, Inc (Westbrook, ME).

Fluorescein isothiocyanate (FITC)-labeled F(ab')₂ fragments of goat anti-human Ig chain (each of κ -, λ -, γ -, α -, and μ -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 ($2 \times 10^5/\text{mm}^3$). The cell surface phenotype of the B PLL cells was Ig κ^+ , Ig λ^- , Ig μ^+ , Ig γ^- , Ig α^- , 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.

The procedures for isolating the cell membrane antigen are based on a modification of our previously reported novel isolation system.^{14,15} A brief description of the present isolation system is given as follows: Cell membranes were prepared from the leukemia cells and cell membrane antigens were solubilized by deoxycholate treatment. The solubilized antigens were fractionated by affinity chromatography on serially connected columns of *Lens culinaris* lectin (LcH) and *Ricinus communis* lectin (RCA). The LcH-bound and RCA-bound glycoconjugates (mostly glycoproteins) were individually eluted, combined, and subjected to passive immunoaffinity chromatography. To this end, the combined glycoproteins were passed through three serially connected immunoabsorbent columns. These immunoabsorbents consisted of anti-HLA class I MoAb (B3-3D1), anti-HLA-DR MoAb (G4-3A7), and rabbit anti-normal human peripheral blood lymphocyte antibodies, all coupled to Sepharose CL-4B (Pharmacia). Materials in the pass-through fractions were pooled and concentrated.

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.^{8,16}

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.^{8,16} 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 ¹²⁵I using an Iodogen-coated (Pierce, Rockford, IL) Minisorp tube (Vanguard International, Neptune, NJ) as described previously.¹⁰ The two radiolabeled preparations were used separately for immunoprecipitation and SDS-PAGE as previously described.^{10,16} Autoradiograph was prepared by using Kodak X-OMAT AR film and X-Omatic intensifying screen (Eastman Kodak, Rochester, NY) as previously described.¹⁷

Western blot analysis. This analysis was performed as described by Inman et al.¹⁸ The PLL antigen preparation was unreduced or

reduced for 1 hour with 50 mmol/L dithiothreitol. The reducing agent was removed from the reduced sample by a cycle of dilution and concentration using a Centricon-10 microconcentrator (Amicon, Danvers, MA). Then SDS was added to the sample at the final concentration of 0.05% and the sample was subjected to SDS-PAGE. The separated proteins in SDS-PAGE were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) with 25 mmol/L Tris-192 mmol/L glycine buffer (pH 8.3) containing 10% methanol. After preincubation with 10% normal goat serum, the membranes were incubated with hybridoma ascites or control ascites diluted in 10 mmol/L Tris-HCl buffer (pH 7.4) containing 0.9% NaCl and 5% bovine serum albumin (Tris-saline-BSA), or Tris-saline-BSA containing an isotype-matching control murine IgG (MOPC 195variant). The membranes were washed with 10 mmol/L Tris buffer (pH 7.4) containing 0.9% NaCl and 0.05% NP-40 (Tris-saline-NP40), treated with ¹²⁵I-labeled F(ab')₂ of affinity-purified goat anti-mouse IgG antibodies, and washed with Tris-saline-NP40. The membranes were dried and autoradiograph was prepared as described above.

Competitive antibody binding. This assay was performed to make a comparison among epitopes defined by SN8, SN8a, and SN8b. The assay was performed following our previously described procedure.¹³

Direct binding of ¹²⁵I-labeled antibodies to human leukemia cells and Scatchard plot analysis. The purified MoAbs SN8, SN8a, and SN8b were individually radiolabeled with ¹²⁵I by an Iodo-gen method (see above). Titration experiments that were performed using a fixed amount (0.1 μg) of each ¹²⁵I-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 λ 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 μg 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 CD10¹³ 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) column²² 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

		Reactivity (cpm)			
Cell Line	Origin of Cell Line	SN8	SN8a	SN8b	Control*
HLL pre-B					
NALM-1	CML-BC	444 ± 74	371 ± 36	351 ± 11	447 ± 80
NALM-6	ALL	694 ± 325	309 ± 18	423 ± 63	470 ± 145
HLL non-T/non-B					
KM-3	ALL	284 ± 33	255 ± 31	414 ± 28	341 ± 75
NALM-16	ALL	341 ± 81	267 ± 18	304 ± 51	314 ± 32
REH	ALL	158 ± 27	233 ± 29	204 ± 24	165 ± 18
HLL B					
BALL-1	ALL	5,867 ± 519	4,946 ± 1,024	4,718 ± 252	279 ± 31
BALM-2	ALL	551 ± 104	566 ± 73	481 ± 99	574 ± 62
Daudi	BL	3,077 ± 366	3,562 ± 519	3,250 ± 196	344 ± 55
SU-DHL-4	HL	8,030 ± 1,015	7,223 ± 121	6,378 ± 229	414 ± 69
U689-M	LS	2,703 ± 264	2,771 ± 485	2,271 ± 670	266 ± 1
Ramos	BL	4,432 ± 1,157	4,032 ± 377	2,814 ± 352	468 ± 16
Raji	BL	584 ± 127	730 ± 85	528 ± 9	454 ± 93
BALM-3	LY	5,382 ± 420	5,330 ± 598	4,839 ± 199	470 ± 65
BALM-5	LY	1,578 ± 220	1,831 ± 326	1,532 ± 115	282 ± 49
MO 1043	CLL	227 ± 18	255 ± 20	244 ± 19	269 ± 46
Plasma					
ARH-77	MM	288 ± 48	451 ± 70	250 ± 42	280 ± 17
RPMI 8226	MM	232 ± 19	415 ± 85	217 ± 30	242 ± 27
HS	MM	221 ± 46	581 ± 62	285 ± 55	389 ± 41
HLL T					
MOLT 4	ALL	242 ± 136	259 ± 74	221 ± 69	246 ± 57
JM	ALL	233 ± 60	242 ± 27	155 ± 28	233 ± 37
CCRF-HSB-2	ALL	200 ± 14	227 ± 47	217 ± 33	197 ± 14
Ichikawa	ALL	192 ± 5	304 ± 48	223 ± 46	251 ± 40
HPB-MLT	LTL	283 ± 54	312 ± 56	278 ± 34	281 ± 26
HUT 78	SS	226 ± 23	366 ± 57	298 ± 87	344 ± 85
HLL myelo/monocytic					
ML-2	AML	339 ± 28	387 ± 63	342 ± 15	384 ± 69
HL-60	APL	166 ± 36	219 ± 24	601 ± 347	217 ± 31
U937	HL	346 ± 105	300 ± 67	246 ± 19	230 ± 44
HLL myeloerythroid					
K562	CML-BC	223 ± 10	261 ± 58	219 ± 32	205 ± 62
EBV-transformed B					
CCRF-SB		165 ± 14	246 ± 50	254 ± 50	216 ± 36
RPMI 8057		224 ± 94	321 ± 53	293 ± 62	321 ± 103

The reactivity was determined using 20 μ L of a 10-fold dilution of culture fluids of individual hybridomas and 2×10^5 cells in each test by means of a cellular RIA. Each test was performed in triplicate and the values given are the mean $\pm$ 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, Sezary syndrome; AML, acute myelocytic leukemia, APL, acute promyelocytic leukemia.

* An isotype-matching (IgG1) murine plasmacytoma IgG (10 μ g/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.^{8,16} 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 CLL samples compared to SN8 (13.6%). Thus, the degree of PLL selectivity of these MoAbs is less than that of SN8.

Reactivity with uncultured normal cells. B cells, T cells, monocytes, granulocytes, erythrocytes, and platelets were isolated from the peripheral blood of three healthy donors and tested for reactivity with SN8 series MoAbs by a cellular RIA. These MoAbs showed a moderate reactivity with B-cell specimens but no significant reactivity with other cell specimens. Therefore, the reactivities of SN8 series MoAbs with B cells were further tested by FACS analysis. The FACS analysis results of SN8 are shown in Fig 2. A subpopulation (6.0%, 17.4%, and 24.2%, respectively) of the 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.

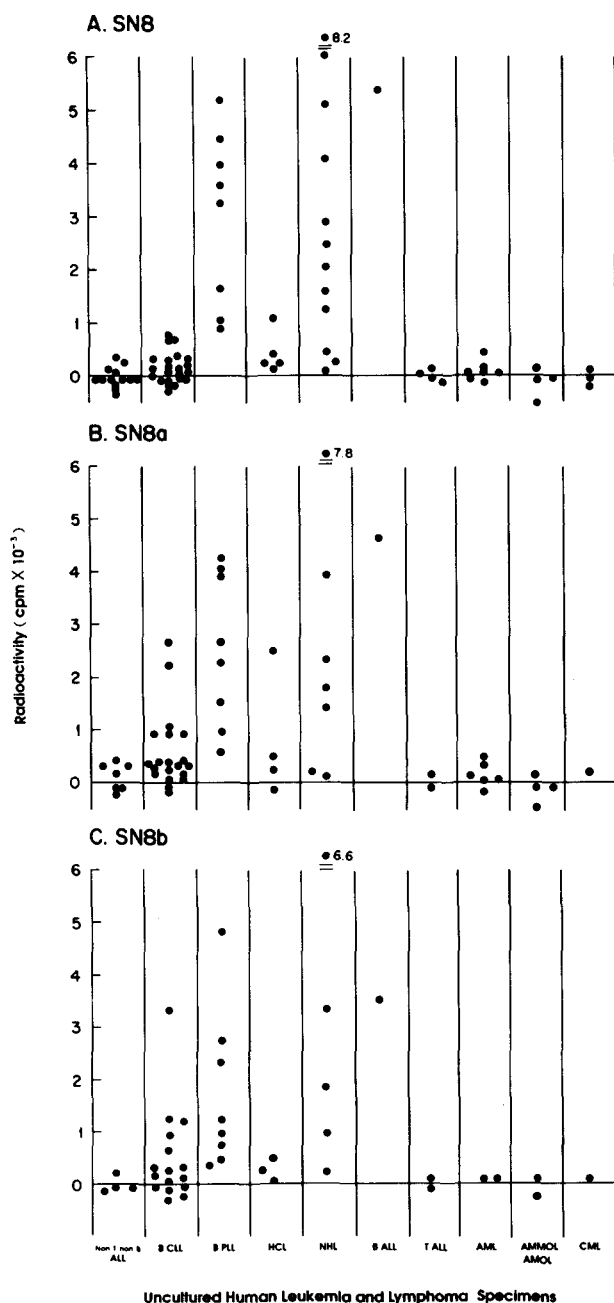


Fig 1. Reactivity of SN8 with fresh (uncultured) HLL cells as determined by a cellular RIA. Individual cell specimens in panels A through C were derived from peripheral blood, BM aspirates, or lymph nodes of 80, 60, and 44 different HLL patients, respectively. In the cellular RIA, three different controls were included with each test sample.⁸ One of these was control mouse IgG1 (10 µg/mL) in the hybridoma culture medium in place of the culture fluid of hybridoma SN8. 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 used in the figure: ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; HCL, hairy cell leukemia; NHL, non-Hodgkin's lymphoma; AML, acute myelocytic leukemia; AMOL, acute monocytic leukemia; AMMOL, acute myelo-monocytic leukemia; CML, chronic myelocytic leukemia. The types of NHL specimens used are as follows: 2 diffuse small cleaved cell (SN8⁺, SN8a⁺, SN8b⁺; SN8⁺), 1 diffuse large cell (SN8⁺, SN8a⁺, SN8b⁺), 2 diffuse mixed cell (SN8⁺, SN8a⁺, SN8b⁺; SN8⁺; SN8⁻), 3 follicular small cleaved cell (SN8⁺, SN8a⁺, SN8b⁺; SN8⁺; SN8⁻), 3 small lymphocytic (SN8⁻, SN8a⁻, SN8b⁻; SN8⁺, SN8a⁺; SN8⁻, SN8a⁻), and 1 follicular mixed cell (SN8⁺) lymphoma.

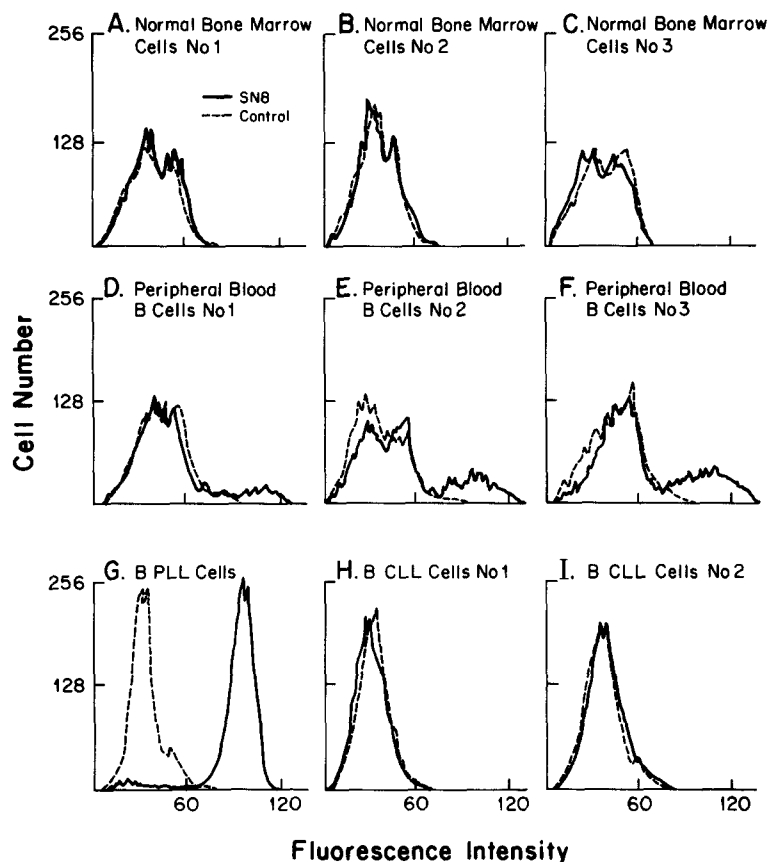


Fig 2. FACS analysis of SN8 reactivity with various human cells. Target cells were allowed to react with SN8 or an isotype-matching control mouse IgG (MOPC 195variant; IgG1- κ) and stained with fluorescence-conjugated F(ab')₂ of sheep anti-mouse Ig antibodies. The three BM specimens shown were obtained from three different ALL patients in remission and mononuclear cells were isolated for use in the test. The three B-cell specimens were individually isolated from the peripheral blood of three healthy donors. The B PLL and B CLL specimens were uncultured cell specimens. The fluorescence intensity is on a log scale.

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 (281, 300, 201, 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).

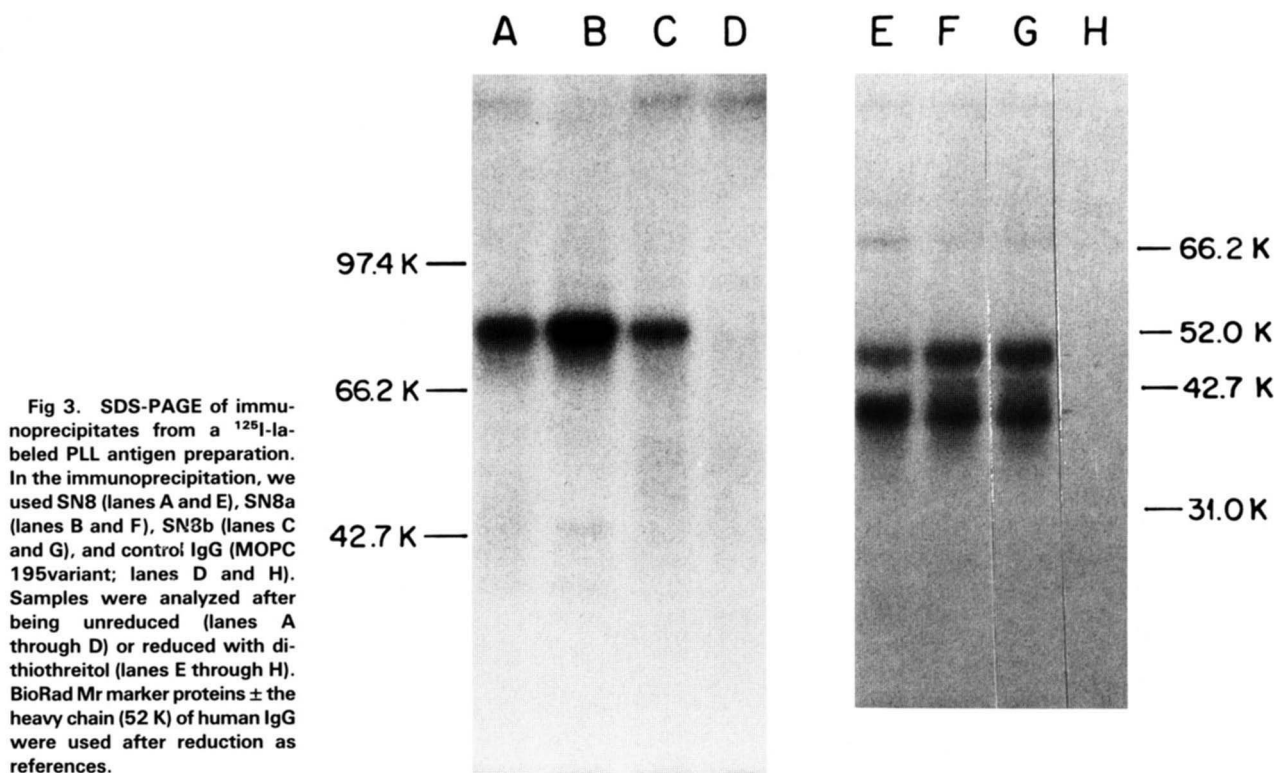
Molecular nature of antigen. A leukemia antigen preparation from cell membrane glycoproteins of PLL cells (see Materials and Methods) was labeled with ¹²⁵I and used for immunoprecipitation with SN8 series MoAbs and an isotype-matching control murine IgG (MOPC 195variant). The immunoprecipitates were unreduced or reduced and analyzed by SDS-PAGE and autoradiographs were prepared. The re-

sults 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



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 ^{125}I -SN8 at the maximum. However, preincubation with SN8b or an isotype-matching control murine IgG did not inhibit the ^{125}I -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 ^{125}I (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 relatively 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^{24,25} and downregulation of antigen expression,²¹ 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, 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

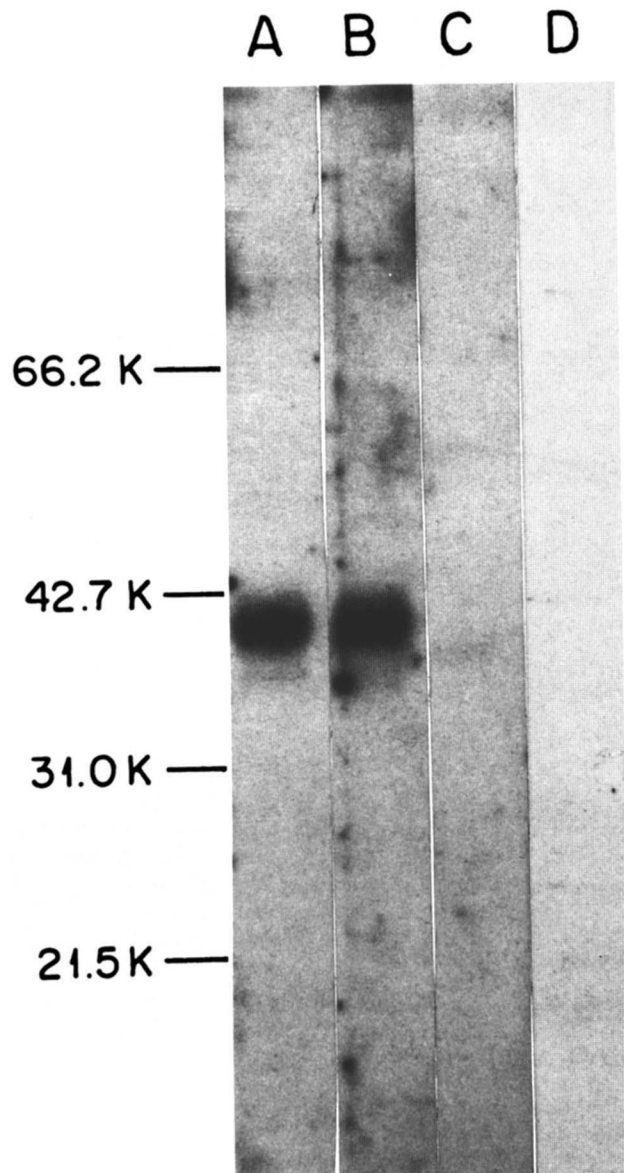


Fig 4. Western blot analysis of the epitopes defined by SN8 series MoAbs. A ^{125}I -labeled leukemia antigen preparation was reduced with 50 mmol/L dithiothreitol and subjected to SDS-PAGE. The separated proteins in the gels were transferred to nitrocellulose membranes. After blocking with normal goat 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 ^{125}I -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).

10 mmol/L NH_4Cl , 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 (IC_{50}) 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

not inhibit the protein synthesis of control MOLT-4 cells at any of the concentrations tested (Fig 8B). NH_4Cl (10 mmol/L) enhanced the cytotoxic activities of immunotoxins against BALL-1 but not against MOLT-4. IC_{50} 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 NH_4Cl (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 NH_4Cl . In an additional test, RA conjugates of SN8, SN8a, and SN8b were tested for cytotoxicity against Daudi, a lymphoma cell line (see Table 1). IC_{50} 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 NH_4Cl ; IC_{50} in the presence of 10 mmol/L NH_4Cl 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

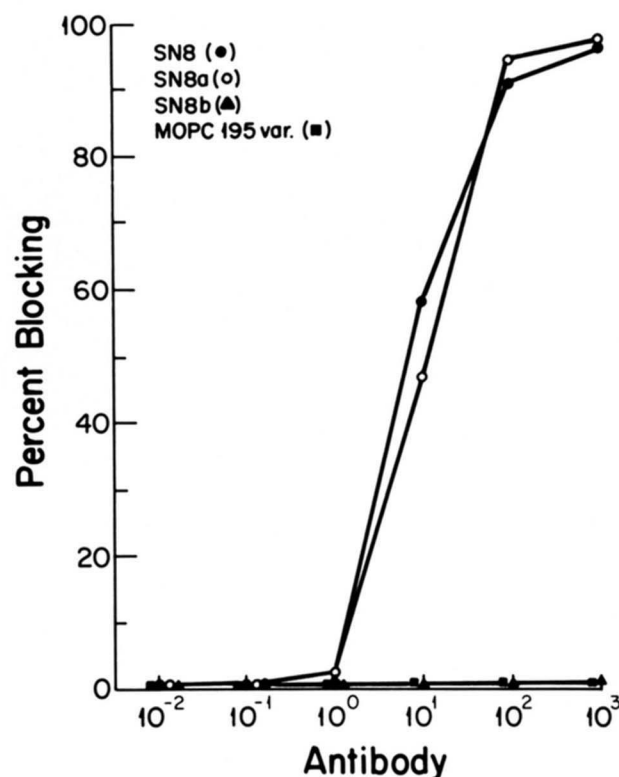


Fig 5. Competitive binding between MoAbs to SN8 antigen on BALL-1 cells as measured by a cellular RIA. BALL-1 cells were incubated with serial dilutions of individual SN8 series MoAbs or an isotype-matching control IgG for 1 hour at 4°C. ^{125}I -SN8 was then added, and the incubation was continued for an additional 1 hour. Abscissa is nanograms of the purified SN8 series MoAbs and control IgG. Radioactivity of ^{125}I -SN8 bound to BALL-1 was in the range between 5.7 and 6.1×10^3 cpm when BALL-1 was preincubated with 10^{-2} to 10^3 ng of the control IgG or SN8b. The radioactivity was 331 and 190 cpm, respectively, when BALL-1 was preincubated with 10^3 ng of SN8 or SN8a.

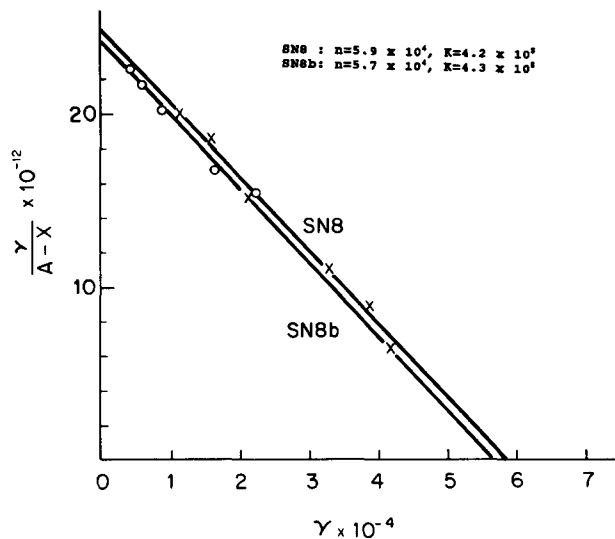


Fig 6. Binding of ^{125}I -labeled SN8 and SN8b to BALL-1. r is the number of antibody molecules bound to one cell at a given dilution; A is the molar concentration of total antibody; and x is the molar concentration of bound antibody, so that $(A - x)$ is the molar concentration of the free antibody. 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 specimen tested as well as with 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 CD22²⁷ appear to be able to discriminate B PLL from some cases of B CLL but not from HCL²; 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.^{2,6,7} B ALL is closely related to B lymphomas^{6,28,29} 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.³⁰ 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.⁵ 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.³¹ 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.² 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.²

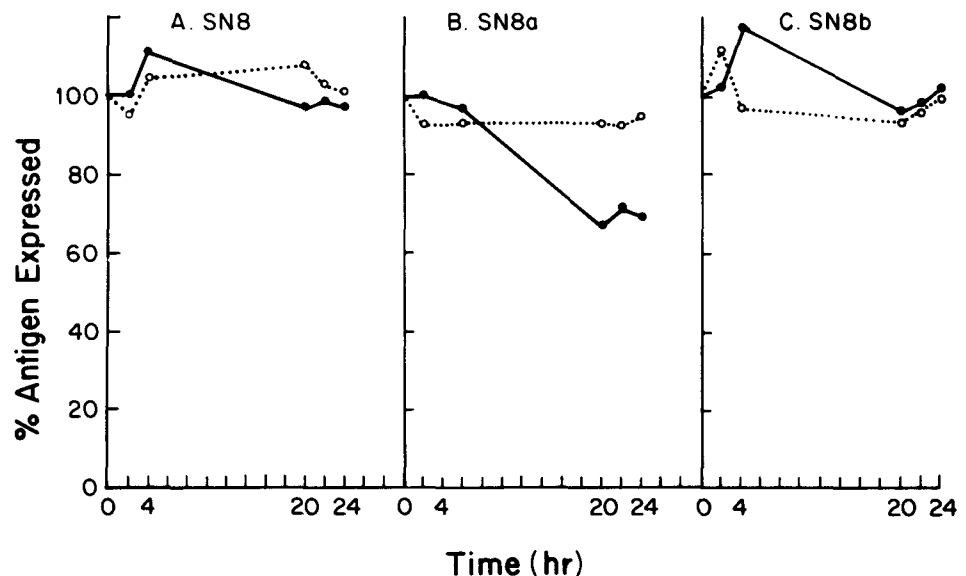


Fig 7. Regulation of gp49/40 expression on BALL-1 that has been incubated with SN8, SN8a, or SN8b. BALL-1 was incubated with an excess of purified MoAb SN8, SN8a, or SN8b (solid circles) or an isotype-matching purified control IgG (open circles) for varying periods of time. gp49/40 on the incubated cells was determined by a cellular RIA.

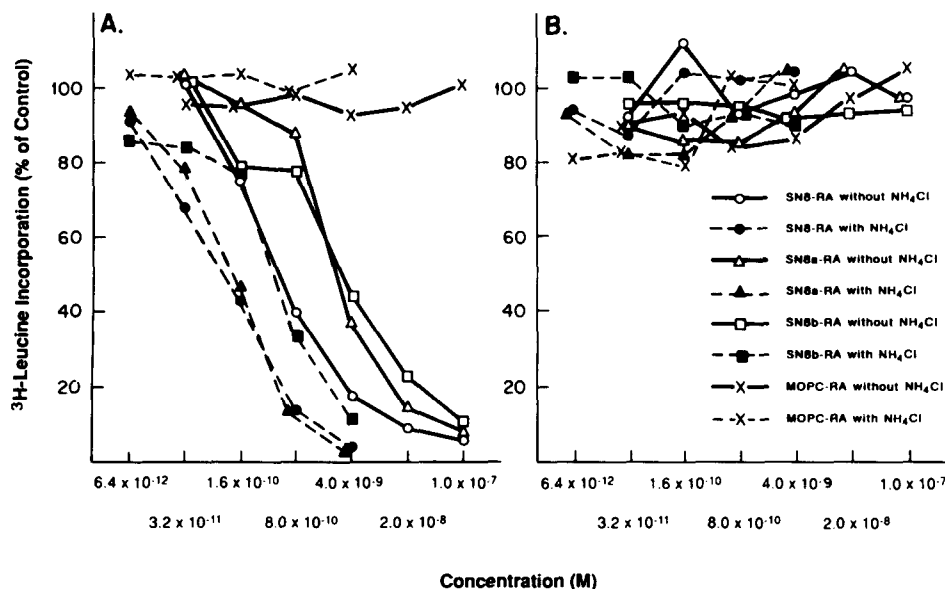


Fig 8. Cytotoxic activities of RA conjugates of SN8 series MoAbs as measured by a protein synthesis inhibition assay. BALL-1 (panel A) and gp49/40-negative MOLT-4 (control; panel B) were incubated with varying concentrations of individual SN8 series conjugates and a control conjugate in leucine-free medium for 24 hours in the absence (solid lines) or presence (broken lines) of 10 mmol/L NH_4Cl , a potentiator. The cells were pulsed with ^3H -leucine and incubated for 4 hours. The pulsed 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 percent of [^3H]leucine incorporated into control cells not exposed to conjugate. SN8-RA, SN8a-RA, SN8b-RA, and control conjugate are indicated by circles, triangles, squares, and X, respectively, in the figure.

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 mixtures.^{14,15} 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.²⁷ 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.²⁷ 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 135,000.²⁷ 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.³²⁻³⁵

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 (gp49/40) 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.²¹ Therefore, an antibody-bound antigen can be effectively internalized even when no modulation of the overall cell surface expression of the antigen is observed.^{9,21} 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 immunoconjugates appear to suggest the potential of these MoAbs as delivery vehicles of cytotoxic agents to tumor targets.

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