SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 379 FASE NACIONAL INCLUIDO CAPITULO II
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 5
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 13-90540- -7
Trámite: 11
Evento: 379
Actuación: 519
Oficio: 10823
Folios: 5

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I		Capítulo II	**X**
No. Solicitud Internacional	PCT/EP2011/067339				
Fecha de Presentación Internacional	04/10/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No 13 090540 00000 0000	08/Abril/2013
Reivindicaciones:	Radicado No 13 090540 00000 0000	08/Abril/2013
	Radicado No 13 090540 00005 0000	08/Julio/2013
Secuencias:	Radicado No 13 090540 00000 0000	08/Abril/2013
Dibujos:	Radicado No 13 090540 00000 0000	08/Abril/2013
Prioridad:	EP 101 86468 4	04/Octubre/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

En el presente caso se estudiaran las reivindicaciones desde la 1 hasta la 26 (Radicado No 13 090540 00005 0000).

Título de la solicitud: “Agentes de unión a CD 33”

El problema planteado en esta solicitud consiste en la necesidad de disminuir el número de células mieloides con el fin de tratar enfermedades como la leucemia mieloide aguda.

Para resolver este problema técnico la solicitud en estudio proporciona anticuerpos de unión a CD33.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K39/395; C07K16/28

4. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

1- La reivindicaciones 1 y 3 carecen de claridad al definir un anticuerpo a partir de la caracterización del epítope al cual se une. Se sugiere definir el anticuerpo indicando la secuencia aminoácida de las regiones variables de la cadena ligera y pesada ó las secuencias de las 6 CDRs de las cadenas pesada y ligera.
2- Las reivindicaciones 2, 5, 6, 16 -18 mencionan las CDRs y las cadenas pesadas y ligeras de los anticuerpos, características técnicas esenciales que deben estar incluidas en la reivindicación independiente. Se sugiere entonces definir de manera clara y concisa los anticuerpos indicando la secuencia aminoácida de las regiones variables de la cadena ligera y pesada ó las secuencias de las 6 CDRs de las cadenas pesada y ligera en la reivindicación 1.
3– La reivindicación 3 define un anticuerpo de unión a CD33 a partir de la técnica e espectrofotometría con la que se reconoce el epítope, lo cual no es admisible. Se sugiere definirlo indicando la secuencia aminoácida de las

regiones variables de la cadena ligera y pesada ó las secuencias de las 6 CDRs de las cadenas pesada y ligera.

4– Las reivindicaciones 4, 7 se refieren a la KD de un agente de unión, que es un resultado a alcanzar y no definen al anticuerpo, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, que contribuyen a la obtención de ese efecto.

5– Las reivindicaciones 10 - 12 mencionan una función efectora, que es un resultado a alcanzar y no definen al anticuerpo, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, que contribuyen a la obtención de ese efecto.

6– La reivindicación 13 menciona un porcentaje de modulación, que es un resultado a alcanzar y no definen al anticuerpo, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, que contribuyen a la obtención de ese efecto.

7- La molécula de ADN de las reivindicaciones 19 y 20 está caracterizada por codificar la región variable de cadena pesada y ligera, no corresponde a las características técnicas esenciales de un ácido nucleico. Se sugiere definirlo indicando la secuencia nucleótida que contiene e indicar el número de la misma (SEQ ID NO).

8- El vector de la reivindicación 21 debe estar caracterizado indicando tipo de vector y la secuencia recombinante que contiene.

9- En la reivindicación 22 la célula debe estar definida indicando a qué tipo de célula corresponde.

10- El método de la reivindicación 23 carece de claridad porque no está definido a través de una secuencia lógica de etapas en que debiera realizarse como lo son las etapas para producir anticuerpos de unión a CD33, no se define el tipo de vector para transfectar la célula huésped. Se sugiere, entonces, definir la invención por sus características esenciales.

11- La composición farmacéutica de las reivindicaciones 24 y 25 debe estar definida a partir de sus ingredientes y proporciones de los mismos que tenga sustento experimental en el capítulo descriptivo. La mención general del ingrediente activo y del vehículo farmacéuticamente activo no corresponde a una característica técnica esencial.

12– La reivindicación 26 se refiere a la disminución del número de células que expresan células CD33, que es un resultado a alcanzar y no definen a la composición, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, que contribuyen a la obtención de ese efecto.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: octubre 04 de 2010 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	Cancer supplement. Philp C. Caron Vol 73, No 3 (Pág. 1049-1056)	"Murine and humanized constructs of monoclonal antibody M195 (Anti-CD33) for the therapy of acute myelogenous leukemia"	01/febrero/1994
D2	Cáncer research Philp C. Caron (Pág. 6761-6767)	"Biological and inmunological features of humanized M195 (Anti CD33) Monoclonal antibodies"	15/Diciembre/1992
D3	WO2008058021	"Methods of treating neoplastic, autoinmune and inflammatory diseases"	15/Mayo/2008
D4	US 8124731	"Optimized Fc variants and methods for their generation"	28/Febrero/2012

El documento D1[1] divulga inmunoglobulinas de unión a CD33 humanizadas (Pág. 1049)

El documento D2[2] divulga inmunoglobulinas quiméricas y humanizadas que comprenden injertos de regiones de terminantes de complementariedad (Pág. 6761)

El documento D3[3] divulga anticuerpos de unión a CD33 en el tratamiento de enfermedades neoplásicas (Resumen).

El documento D4[4] divulga variantes Fc optimizadas de anticuerpos anti-CD33 (Resumen).

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en *"un agente de unión a CD33 que se une al CD33 humano, definiéndose el agente de unión a CD33 por unir un epitopo dentro de la secuencia…"*

1 1 http://www.ncbi.nlm.nih.gov/pubmed/8306247

2 2 http://www.ncbi.nlm.nih.gov/pubmed/1458463

3 3http://worldwide.espacenet.com/publicationDetails/description;jsessionid=942C59E817EDDE2A7E6130171423A9F3.espacenet_levelx_prod_1?CC=WO&NR=2008058021A3&KC=A3&FT=D&ND=&date=20080703&DB=&&locale=en_EP

4 4http://worldwide.espacenet.com/searchResults?compact=false&ST=singleline&query=US8124731&locale=en_EP&DB=worldwide.espacenet.com

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Agente de unión a CD33.	Anticuerpo anti-CD33 (Pág. 1049))

Las características esenciales del anticuerpo de la reivindicación 1 habían sido divulgadas previamente en el documento D1, el cual enseña, anticuerpo de unión anti-CD33 M195 para el tratamiento de leucemia mieloide (Pág. 1049). El documento también divulga anticuerpos humanizados y células huésped.

En consecuencia, la reivindicación 1 no es nueva o carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 3, 4, 7-13, 19-22 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel inventivo (Art18 D486)

La reivindicación 2 consiste en *un agente de unión a CD33 según la reivindicación 1, caracterizado porque tiene una región variable de cadena pesada que comprende CDR1, CDR2 y CDR3 y una región variable de cadena ligera que comprende CDR4, CDR5, CDR6…"*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Agente de unión a CD33 que comprende:	Anticuerpo de unión a CD33 que comprende: (Pág. 1049)	Anticuerpo de unión a CD33 (Pág. 6761)
CDR1 SEQ ID NO 1-14, CDR2 SEQ ID NO 15-28, CDR3 SEQ ID NO 29-42, CDR4 SEQ ID NO 43-56, CDR 5 SEQ ID NO 57-70, CDR6 SEQ ID NO 71-84.	Injertos de CDRs (Pág. 1050)	Injertos de CDRs (Pág. 6761)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga un anticuerpo de unión a CD33 que comprende (Pág. 1049) injertos de CDRs (Pág. 1050).

El documento D2 divulga un anticuerpo de unión a CD33 que comprende injertos de regiones determinantes de complementariedad CDRs (Pág. 6761).

La diferencia consiste en que la invención divulga que el agente de unión a CD33 comprende: CDR1 SEQ ID NO 1-14, CDR2 SEQ ID NO 15-28, CDR3 SEQ ID NO 29-42, CDR4 SEQ ID NO 43-56, CDR 5 SEQ ID NO 57-70, CDR6 SEQ ID NO 71-84.

El efecto que logra el incluir regiones determinantes de complementariedad CDRs alternativas es la afinidad por células CD33.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo proveer anticuerpos de unión a células CD33 alternativos.

Sin embargo, anticuerpos de unión a células CD33, ya está divulgado en el documento D2 que se refiere a que se encuentran disponibles las técnicas para injertar regiones de CDRs de IgG sobre regiones marco de IgG humana (Pág.6765).

En consecuencia la persona del oficio normalmente versada en la materia, incluiría CDRs del documento D2 en el anticuerpo divulgado en el documento D1 para llegar así al objeto de la reivindicación 2 de la solicitud. Por lo cual se considera obvia.

Las reivindicaciones 6, 8, 9, 16 – 18 no mencionan características técnicas adicionales por lo cual tampoco tienen nivel inventivo.

Conclusión:
La reivindicación 2 cumple el requisito de novedad (Art. 16) pero no cumple con el requisito de nivel inventivo (Art. 18).

La reivindicación 14 consiste en *"un agente de unión según cualquiera de las reivindicaciones 12-13 en el que las mutaciones en el dominio Fc se localizan en una o más posiciones seleccionadas de los aminoácidos en las posiciones 332 y/o 239 y/o 236 según el índice de EU de Kabat"*

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	**D3**	**D4**
Agente de unión a CD33 que comprende:	Anticuerpo anti-CD33 (Párr. [0187]).	
Mutaciones en el dominio Fc en las posiciones 332 y/o 239 y/o 236	Modificaciones en la región Fc (Párr. [0187]).	Modificación aminoácida en la posición 332 de región Fc en anticuerpo de unión a CD33 (Col. 9 ln 4-12).

El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 14 porque divulga un anticuerpo de unión a CD33 y la introducción de sustituciones de uno o más aminoácidos en la región Fc de anticuerpos de unión a CD33 (Párr.[0187]). El documento también divulga la producción de anticuerpos de unión a CD33 que comprende: transfectar la célula

huésped con la secuencia nucleótida del vector, cultivar célula huésped y obtener el anticuerpo (Párr. [0202-0222]) y una composición farmacéutica que comprende anticuerpo de unión a CD33 y un vehículo farmacéuticamente aceptable (Párr. [0237, 0240]).

El documento D4 divulga la posición 332 de la región FC en anticuerpo anti-CD33 (Col. 9 ln 4-12).

La diferencia consiste en que la invención divulga mutaciones en el dominio Fc en las posiciones 239 y/o 236.

No se evidencia un efecto técnico inesperado, respecto a la función efectora divulgada en el documento D3.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo proveer anticuerpos de unión a células CD33 con mutaciones en la región Fc.

Sin embargo, sustitución en la posición 332 de la región Fc para anticuerpo anti-CD33 ya está divulgada en el documento D4 que se refiere a las posiciones de los aminoácidos en la región Fc susceptibles de sustitución, además también divulga los métodos para obtener dichas modificaciones en la región Fc (Col. 9 ln 4-12).

En consecuencia la persona del oficio normalmente versada en la materia, incluiría una sustitución aminoácida del documento D4 en la región Fc del anticuerpo divulgado en el documento D3 para llegar así al objeto de la reivindicación 2 de la solicitud. Por lo cual se considera obvia.

Las reivindicaciones 15, 23 - 26 no mencionan características técnicas adicionales por lo cual tampoco tiene nivel inventivo.

Conclusión:
La reivindicación 14 cumple el requisito de novedad (Art. 16) pero no cumple con el requisito de nivel inventivo (Art. 18).

7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Cancer supplement. Philp C. Caron Vol 73, No 3 (Pág. 1049-1056)	1, 3, 4, 7-13,19-22	Novedad
D1	Cancer supplement. Philp	2, 6, 8, 9, 16-18,	Nivel inventivo

	C. Caron Vol 73, No 3 (Pág. 1049-1056)		
D2	Cáncer research Philp C. Caron (Pág. 6761-6767)	2, 6, 8, 9, 16-18,	Nivel inventivo
D3	WO 2008058021	14 y 15.	Nivel inventivo
D3	WO 2008058021	23-26	Nivel inventivo
D4	US 8124731	14 y 15.	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-09-02

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Murine and Humanized Constructs of Monoclonal Antibody M195 (Anti-CD33) for the Therapy of Acute Myelogenous Leukemia

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Long-term survival rates of patients with acute myelogenous leukemia treated with intensive chemotherapy are 15–20%, despite efforts to develop new treatment strategies. Murine M195 (^{131}I-M195), an anti-CD33, immunoglobulin (Ig) G2a monoclonal antibody has reactivity restricted to early myeloid cells and myeloid leukemic blasts but not hematopoietic progenitors. Previous trials in patients with relapsed or refractory myeloid leukemia showed that ^{131}I-M195 rapidly targeted to the bone marrow and internalized into target cells.

This article describes a therapeutic dose escalation study in which 24 patients received from 50 mCi/m^2 to 210 mCi/m^2 of ^{131}I-M195 in divided doses. Cytoreduction of peripheral cell counts and bone marrow blasts occurred without nonhematopoietic toxicity. Doses of ^{131}I-M195 greater than 135 mCi/m^2 were associated with marrow cytoreduction sufficient to necessitate bone marrow transplant. However, 37% of the patients developed human anti-mouse antibody, preventing retreatment.

To decrease immunogenicity and improve effector function, chimeric IgG1 and IgG3, and complementarity-determining region-grafted, humanized IgG1 and IgG3 versions of mouse M195 were developed by genetic engineering techniques. The new versions maintained specificity and biologic function, and they were superior to the mouse M195 in their ability to perform antibody-dependent cellular cytotoxicity against leukemia cells. Humanized M195, but not chimeric M195, showed a 4–8.6 times higher avidity than its mouse counterpart. Because effector function of IgG depends to a large extent on Fc clustering, a homodimeric HuG1 also was developed. Homodimeric HuG1 showed an ability to cause additional dramatic improvements in effector functions, as well as an ability to internalize and retain radioisotope in target leukemia cells. Monomeric and dimeric forms of humanized M195 may be advantageous in the therapy of acute myelogenous leukemia. *Cancer* 1994; 73:1049–56.

Key words: monoclonal antibody, M195, anti-CD33, acute myelogenous leukemia, chimeric and humanized antibodies, antibody-dependent cellular cytotoxicity.

Mouse monoclonal IgG2a antibody M195 binds to CD33, a 67-kilodalton (KD) glycoprotein, and is rapidly internalized into target cells.[1–3] It is specifically reactive with acute myelogenous leukemia (AML) cells and early myeloid progenitors, but not hematopoietic stem cells.[1,2] This sparing of normal stem cells, as well as its rapid accessibility to malignant cells in the blood, makes it an ideal candidate for leukemia therapy. Because M195 kills target cells with guinea pig and rabbit complement,[1] M195 has been used for ex vivo bone marrow purging of AML.[4]

Although M195 is not intrinsically cytotoxic in vitro, an initial pilot trial of 10 patients with myeloid leukemias showed rapid and specific bone marrow targeting and efficient internalization of ^{131}I-M195 into blasts.[5] Using an optimal biologic dose of antibody for radioimmunotherapy from this original trial, a dose escalation trial of ^{131}I-M195 for relapsed or refractory

Presented at the Fourth Conference on Radioimmunodetection and Radioimmunotherapy of Cancer, Princeton, New Jersey, September 17–19, 1992.

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myeloid leukemias was conducted and the results are described in this report. Safe, leukemic cytoreduction was seen with ^{131}I-M195.

In the hope of improving effector function and to avoid neutralizing human anti-mouse antibodies (HAMA) responses, genetic engineering techniques have allowed for the production of chimeric IgG1 and IgG3 (ChG1, ChG3), combining mouse variable regions and human constant regions; and more fully humanized, "CDR-grafted" IgG1 and IgG3 (HuG1, HuG3), so that only the original mouse antigen binding sites are retained.[6] Avoidance of HAMA may allow for longer half-lives in vivo and the ability for repeated treatments.[7] In addition, humanized monoclonal antibodies (MoAb) may obviate the need for toxins or radiolabels. Several humanized MoAb, including CAMPATH-1H,[8] anti-Tac-H,[9] and humanized versions of two murine MoAb against herpes simplex virus gB and gD glycoproteins,[10] offer selective advantages over their murine counterparts.

Hence, this report also describes new humanized constructs of M195. These humanized versions showed superiority over their murine parent MoAb in immune effector function and higher avidity than the original MoAb.[11] To further attempt to improve on biologic and immunologic effector functions of HuG1, a mutation in the heavy chain gene allowed disulfide bonds to form a homodimeric HuG1 (HdIgG). HdIgG showed dramatic improvements over the monomer,[12] in part, similar to that seen previously for a chimeric IgG dimer.[13] The potential use of humanized M195 constructs in the therapy of AML is discussed.

Materials and Methods

Cells and Cell Lines

Hematopoietic cell lines, including SKLy16, RAJI, HL60, and U937 cells, were obtained from the human tumor banks of the Cancer Immunology Laboratory at Sloan-Kettering Institute. Hematopoietic cell lines were grown in RPMI1640/5% newborn calf serum/10% serum-plus (Hazelton Biologics, Lenexa, KS). Heparinized peripheral blood samples were obtained from healthy volunteers and patients on the Leukemia Service at Memorial Hospital on IRB-approved protocols. Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation.

Monocloncal Antibodies

Monoclonal antibody M195 (anti-CD33) originated in Balb/c mice immunized with leukemia cells from a patient with AML, and was produced from hybridomas and purified as described previously.[1,2] Sp2/0 hybridoma cell lines secreting the chimeric and humanized M195 were grown in vitro, and the MoAb were purified on PA-Sepharose (Pharmacia, Piscataway, NJ) by affinity chromatography using sequential pH step elutions.[1] Purity was determined on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie brilliant blue. Purification of clinical grade M195 was done from mouse ascites at Sloan-Kettering Institute essentially as described.[14] The product was tested for the presence of murine viruses, endotoxin, pyrogen, bacteria, fungal contamination, mycoplasma, and DNA. General safety testing was done on mice and guinea pigs. Human-mouse chimeric IgG, with human-derived constant regions for IgG1 and IgG3, and "CDR-grafted" humanized M195, retaining only the CDRs and other sterically important amino acids from the mouse IgG, were constructed as described.[6] The construction of homodimeric IgG (HdIgG) was recently described[12] by introducing a mutation at the γ1 chain CH3 region gene to change a serine to a cysteine, allowing interchain disulfide bond formation at the COOH terminal of the IgG.

Radioiodination and Radioimmunoassay

For the clinical trial, 2 mg M195 was labeled with an appropriate amount of iodine-131 (New England Nuclear, Wilmington, DE) using chloramine-T and aseptic pyrogen-free technique.[15] In the initial M195 trial, optimal bone marrow targeting and minimal blood pooling occurred when less than 5 mg/M^2 antibody was infused. In addition, pharmacokinetics correlated with tumor burden.[5] Hence, in this trial, to maximize ^{131}I delivery to the marrow, M195 doses were adjusted for estimated leukemia burden: unlabeled M195 was added to bring the total antibody dose to 3 mg/M^2 plus an additional 0.1 mg/M^2 for every 10,000 peripheral white blood cells per microliter at the time of infusion.[15] This escalation was based on the known number of target sites per leukemia cell.[1] Doses for injection were greater than 98% free of ^{131}I and showed immunoreactivities of about 60% in one-step binding assay in antigen excess.[15]

Immunoreactivity of the new constructs was determined by incubating 3–5 ng of radiolabeled MoAb with an excess of antigen (5×10^6) HL60 cells for 1 hour at 4°C. Specific binding in these radiobinding assays was determined by subtracting the amount of radiolabeled MoAb bound in the presence of an excess of unlabeled MoAb as previously described.[1]

Trial Design

Details of the trial and its design have been published.[15] Patients with relapsed or refractory acute nonlymphocytic leukemia, blastic chronic myelogenous leukemia or myelodysplastic syndrome (MDS), or chronic myelomonocytic leukemia were eligible if their blasts expressed CD33 antigen in greater than 25% of cells by flow cytometry. All patients were not undergoing chemotherapy (except for hydroxyurea) for at least 3 weeks before entering this study. Hydroxyurea was permitted to control peripheral blood counts up to 2 days before beginning treatment. Entry criteria included serum creatinine and bilirubin less than 2 times normal values, no positive blood cultures for bacteria within the preceding 10 days and Karnofsky Performance Status (KPS $\geq$ 50%).

Seven ^{131}I dose levels were employed: 50, 70, 90, 105, 135, 160, and 210 mCi/M^2. A minimum of three patients were treated at each dose level. Because the possibility of irreversible marrow ablation became apparent at higher dose levels, patients were treated with 160 mCi/M^2 or greater only if they were eligible for allogeneic or autologous (prior stored remission marrow) transplantation.

^{131}I-M195 was administered by 20-minute intravenous infusion in 2–4 divided doses given at least 48 hours apart to allow for reexpression of the CD33 antigen on the surface of target cells between infusions. Physical examination, complete blood cell count, coagulation indices, and biochemical and electrolyte values were measured daily. Before the second or third antibody infusions, patients underwent whole-body anterior and posterior gamma camera imaging.[15]

Toxicity was assessed according to the common criteria established by the National Cancer Institute. This trial was conducted under IRB approval, with informed consent or assent obtained from all participants.

Flow Cytometry

Peripheral blood cells from selected patients were examined for expression of M195 antigen (CD33) and other myeloid cell surface markers before, 1 hour, and 24 hours after treatment with M195, using an EPIC Profile flow cytometer.[1] Goat anti-mouse Ig-fluorescein isothiocyanate conjugate was used without primary antibody to coat and detect M195 bound cells in vivo. Specificity studies of the humanized constructs were done by direct and indirect flow cytometry using goat anti-human Ig-fluoroscein isothiocyanate conjugate as described.[11]

Quantitation of Killing of Bone Marrow Blasts

Bone marrow biopsies taken before therapy and at least 7 days after completion of therapy were assessed for absolute mononuclear cell count per millimeter of marrow sample, using the technique of Blumenreich et al.[16] The values were multiplied by the percentage of blasts present on a simultaneously obtained marrow aspirate smear to calculate the absolute number of blasts present per millimeter of sample. Values before and after treatment were compared to determine the resulting change in absolute blast concentration.

Human Anti-Mouse Antibody

Plasma samples were tested for HAMA beginning 8 days after the last infusion and then every 2–3 weeks thereafter using an enzyme-linked immunosorbent assay against purified mouse M195 and human-mouse chimeric M195[6] essentially as described.[14]

Avidity Determinations

Scatchard analyses were done as previously described.[9] Comparison of antigen–antibody avidity between different M195 constructs was done in competition with ^{125}I-M195 and ^{125}I-HuG1. Increasing amounts of cold MoAb were incubated with 2×10^5 HL60 cells and 50 ng ^{125}I-MoAb in 200 μl RPMI for 1 hour at 0°C.[11] Cells were washed 3 times in RPMI and counted. To avoid nonspecific and Fc-receptor binding, competition assays were done in the presence of human serum. Samples were run in duplicate and the avidity of binding was measured by comparing the concentrations of unlabeled competitor where 50% reduction of binding was achieved as described.[9] Experiments were repeated 2–3 times each, and arithmetic means of the data are presented.

Internalization Studies

Internalization of the HuG1 or HdIgG was measured from 0 to 48 hours by incubating 0.01 μg/ml to 2 μg/ml radiolabeled MoAb with 1–2 million HL60 cells/ml in RPMI 1640/2% human AB serum.[11,12] Cells then were washed twice in RPMI, and the surface-bound M195 was stripped with 1 ml 50 mM glycine/150 mM NaCl, pH 2.8 at 24°C for 10 minutes. The amount (ng) of MoAb per million cells remaining after the acid wash (i.e., internalized), or in the supernatant (i.e., cell surface) was determined.

Complement-Mediated Cytotoxicity

Twenty-five microliters of HL60 cells (5×10^6 cells/ml) or fresh leukemia samples were incubated with 25 μl diluted rabbit or human complement and 25 μl serial dilutions of MoAb at 37°C for 60 minutes.[11] Monoclonal antibody M31 (IgM anti-CD15) was used as a positive control. Live and dead cells were enumerated using trypan blue or propidium iodide exclusion.

Rabbit serum was purchased from PelFreeze (Rogers, AK); human sera were obtained from volunteers. All complement sources were stored at −70°C until use and were not reused. Complement was used at the maximum concentrations not showing nonspecific lysis of the target cells, usually from 1:6 to 1:10 final dilution.

Antibody-Dependent Cellular Cytotoxicity

Chromium release assays to determine if chimeric, humanized, or homodimeric M195 MoAb were capable of mediating antibody-dependent cellular cytotoxicity (ADCC) were conducted as described previously.[1] Peripheral blood mononuclear cells from human volunteers were used as effector cells, and HL60 cells were used as positive targets. Assays were conducted at 37°C for 5 hours and harvested using a Skatron press, and released ^{51}Cr was counted in a Packard gamma counter. Detergent lysed cells were used as a 100% control. Effector cell only and MoAb only treated target cells were used as negative controls. CD33-negative RAJI cells and a control HuG1 MoAb (Fd 79)[10] were used as controls. Samples were done in quadruplicate and the mean was presented. Experiments were repeated 3–5 times. Standard deviations were always less than 10% of the mean value.

Results

Patient Studies

As described previously,[15] the 24 patients on study included 16 with refractory or relapsed AML, 5 with blastic MDS, 2 with chemotherapy-related secondary AML, and 1 with myeloblastic CML. Patients were heavily pretreated with chemotherapy, and 7 of these 24 patients had relapsed after allogeneic (n = 6) or autologous (n = 7) bone marrow transplantation. Patient median age was 40 years.

Marked uptake of ^{131}I-M195 into all areas of bone marrow as well as variable blood pooling could be seen by whole-body imaging at 48 or 72 hours. Flow cytometric studies using peripheral blood from 11 patients with adequate numbers of circulating leukemia cells were done to demonstrate the presence of surface bound M195 after infusion. Using direct immunofluorescence with MY9 (CD33), mean peak channel changes showed that 29% (range, 18–47%) of available CD33 sites were occupied at 1 hour, with concomitant binding of FITC-goat anti-mouse Ig (GAM) on a mean of 67% (range, 42–89%) of gated cells.

Table 1. Cytoreduction of Leukemia Cells in Blood and Bone Marrow After ^{131}I-M195 Therapy

	Level	Dose*	Decreased blood counts	Bone marrow depletion†
I	3 Patients	50	2/3	2/3
II	4 Patients	70	4/4	2/3
	1 Retreated			
III	3 Patients	90	3/3	2/2
	1 Retreated			
IV	3 Patients	105	3/3	3/3
V	2 Patients	135	2/2	2/2
VI	6 Patients	160	6/6	6/6
VII	3 Patients	210	3/3	3/3

* ^{131}I-M195 was administered by 20-minute intravenous infusion in two to four divided doses.

† Biopsies were taken prior to therapy and were assessed for absolute mononuclear cell count per millimeter of marrow at least 7 days after completion of therapy.

The cytoreductive effect of ^{131}I-M195 on peripheral blood and bone marrow is seen in Table 1. Modest to significant drops in peripheral white and leukemia cell counts were seen in all but one patient (96%). Although the duration of nadir was variable (6–43 days) and not directly dose dependent at lower dose levels (up to and including 105 mCi/m^2), pancytopenia was profound and long in duration (12 days or greater) at dose levels greater than 105 mCi/m^2. These included eight patients who underwent bone marrow transplantation, who previously had been ineligible for BMT due to refractory or relapsed leukemia. Bone marrow examination showed a direct correlation between ^{131}I dose level and mean blasts killed, with 17 of 19 (89%) of patients tested demonstrating decreases in the absolute number of blasts per millimeter of bone marrow biopsy.

Despite large cell kills, up to 1 kg (10^{12} cells), no laboratory evidence of tumor lysis or disseminated intravascular coagulation (DIC) was demonstrated. More than 99% of blasts were killed in some patients. No complete responses were seen at nontransplant dose levels. Clinical parameters were improved, however. A patient with acute promyelocytic leukemia who was in fulminant DIC before treatment showed clinical and laboratory resolution of DIC after treatment. Another patient who had transformed into AML from MDS, and had been refractory to chemotherapy, returned to her baseline MDS for a duration of 3 months.

The presence of HAMA in sera was examined. Thirty-seven percent of the patients developed a positive titer. In two patients that showed positive titers, retreatment with ^{131}I-M195 was unsuccessful, with poor bone marrow targeting and absence of cytoreduction. The only acute toxicities included bone pain and low-grade fever in each of four patients. One patient who developed hyperbilirubinemia with venoocclusive disease during her initial transplant developed hyperbilirubinemia on this study. Hematologic toxicity was difficult to evaluate because of baseline pancytopenia seen in these patients.

Humanized Antibodies

Specificity, Immunoreactivity, and Avidity of Humanized Antibodies

Because of the encouraging results of these clinical trials, and to reduce the likelihood of HAMA formation, humanized constructs were produced[6] and their biologic and immunologic characteristics were examined.[11,12] Chimeric and humanized IgG1 and IgG3, as well as homodimeric humanized IgG1, were analyzed for their specificity relative to the parental mouse M195. When tested against a panel of $CD33^+$ and $CD33^-$ cell lines by radioimmunoassay, all constructs maintained specificity. Specificity of the chimeric and humanized M195 was tested against fresh hematopoietic samples from 47 patients using a direct fluorescein conjugate of HuG1-M195, and confirmed for myeloid leukemia cells.[11]

The ability of the genetically engineered monomeric and dimeric constructs to bind to CD33 expressing HL60 cells was determined by radiobinding assays by incubating 3–5 ng of MoAb in the presence of excess antigen. Total immunoreactivity of the four radiolabeled constructs was 50%, similar to that of the original M195. This value nearly doubled to 85% for the HdIgG.

Direct radioimmunoassay of the constructs showed that binding was both specific and saturable. Scatchard analysis has shown that although the chimeric M195 showed similar or slightly lower avidity than the mouse M195, to our surprise both HuG1 and HuG3 showed a higher avidity than the parent Ig.[6] Not unexpectedly, HdIgG showed similar avidity to HuG1-M195.[12]

Because radiolabeling of the MoAb may cause damage during the radioiodination of the Ig, avidities were compared between different constructs and ^{125}I-M195 or ^{125}I-HuG1 using competition radiobinding assays.[11] The higher avidities of the humanized M195 determined by Scatchard analysis were confirmed by these assays. HuG1 and HuG3 showed an 8.6- and 4-fold higher avidity, respectively, than the mouse M195. The avidities of the ChG1 and ChG3 were confirmed by competition assays to be similar to that of the mouse.

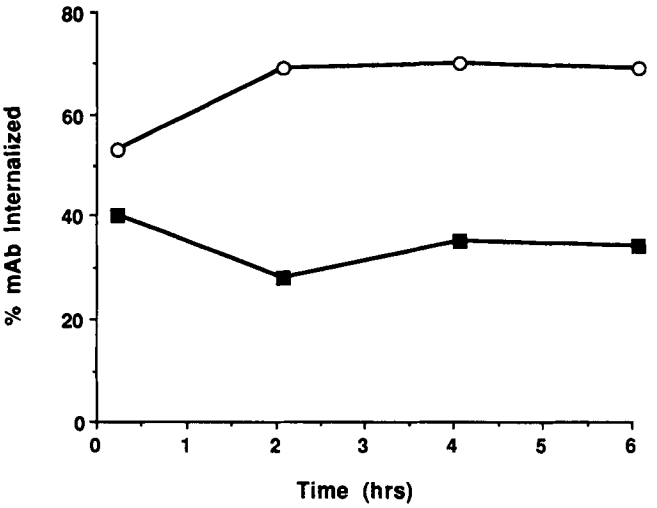

Figure 1. Percentage of HuG1 (■-■) and HdIgG (O-O) internalized over 6 hours at 37°C. Radiolabeled monoclonal antibody at 1 μg/ml was incubated with 2×10^6 HL60 cells in a final volume of 200 μl, and internalization was measured as described in the text. Each time point was done in triplicate and SD less than 10%.

Internalization

It was known from previous studies that M195 rapidly internalized into target cells, which was important for the therapeutic delivery of radioisotope into cells.[5] Both radiolabeled HuG1 and HuG3 became associated over time in an acid-resistant compartment (i.e., not on the cell surface) at 37°C. By 4 hours, 30% of the radiolabel was internalized into the cell. In contrast, HdIgG internalized much more rapidly than HuG1, with 70% of the radiolabeled MoAb being retained inside the cell by 4 hours (Fig. 1). Experiments performed at 0°C did not show internalization of any of the constructs. An extended analysis over 48 hours showed persistence of HdIgG within cells, demonstrating that dimerization extended the length of time that M195 could be retained inside the cell after binding to the surface.[12]

Effector Function

One of the main purposes of studying humanized MoAb is to determine whether they are more effective at cell killing by immune effector functions, i.e., complement-mediated cytotoxicity (CMC) or ADCC. All of the constructs showed HL60 leukemia cell killing using rabbit complement, but not human serum, as a source of complement.[11] For rabbit CMC, the murine and new monomeric constructs yielded 50% cell lysis at a MoAb

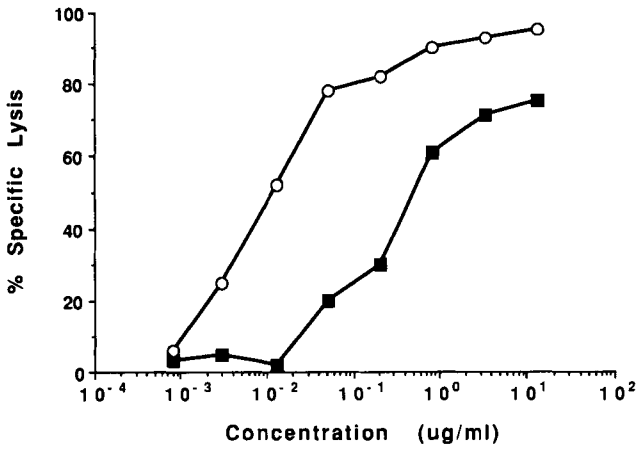

Figure 2. Complement-mediated cytotoxicity of HuG1 (■-■) and HdIgG (O-O). Dilutions of monoclonal antibodies were added to 10^5 HL60 cells with rabbit complement at final dilutions of 1:6 at 37°C for 1 hour. Cell kill was determined by trypan blue exclusion.

concentration of 0.1–0.5 μg/ml. All constructs showed a prozone effect, with decreases in activity at higher concentrations. HdIgG was at least 100-fold more potent on a molar basis at rabbit complement-mediated cytotoxicity than was HuG1-M195 (Fig. 2). Although no cytotoxicity for HuG1 was seen below a concentration of 0.01 μg/ml, HdIgG showed a linear increase in cell lysis starting at a concentration of 0.001 μg/ml.[12]

Unlike the mouse version, both chimeric and humanized M195 possessed the new capability of ADCC.[11] Cell killing in the range of 20–30% for ChG1 and ChG3, and 10–20% for HuG1 and HuG3, was seen in 5-hour chromium release assays at high E:T ratios (above 50:1) and MoAb concentrations 1 μg/ml and above. Killing by ADCC was linearly correlated with increasing E:T ratios (Fig. 3). Monoclonal antibody levels greater than 10 μg/ml did not demonstrate any improved level of killing, and in some cases, reduced levels of killing at higher antibody concentrations were seen. No killing was seen with a control HuG1 MoAb (Fd79)[10] and HL60 cells, or with HuG1-M195 and $CD33^-$ RAJI cell lines. Similar to CMC, HdIgG was also 100-fold more potent on a molar basis at ADCC than HuG1.[12] At each E:T ratio, HdIgG was 2- to 5-fold more effective over a 50-fold concentration range.

Discussion

This report summarizes clinical work with ^{131}I-M195 and the development of newly designed humanized versions with their improved cytotoxicity and biologic functions. This was the first trial of a monoclonal antibody with significant single agent activity against AML in vivo.[15] This therapeutic trial of radiolabeled M195 in patients with relapsed and refractory myeloid leukemia has shown M195 to be an effective agent for cytoreduction without nonhematologic toxicity. Peripheral blood and bone marrow involvement was decreased in more than 90% of patients. M195 is capable of destroying up to nearly 1 kg of leukemia in patients without tumor lysis. The amount of blasts killed (up to 2.5 logs) compared favorably with that reported for anthracycline/cytosine-arabinoside therapy.[16] Despite dose escalation from 50 to 210 mCi/m^2, the maximum tolerated dose of ^{131}I-M195 was not reached, based on dose-limiting nonhematologic toxicity. Hematologic toxicity appeared to be based not only on the radiation dose level, but also on the previous treatment affecting the patient's bone marrow reserve.

The amount of cell kill from ^{131}I beta-emission is highly dependent on the concentration of CD33 target, in addition to the "field of kill effect" due to the range of emission of 50 cell diameters for beta emitters. This predicts that ^{131}I-M195 is useful for marrow ablation for patients in relapse. Doses greater than 135 mCi/m^2 ^{131}I caused prolonged pancytopenia of longer than 14 days. In eight patients who were previously not candidates for bone marrow transplantation, leukemia blast reduction with M195 was sufficient to proceed to transplantation.

It was previously shown that M195 is capable of rapidly targeting to areas of bone marrow with leukemic involvement while sparing normal tissues.[5] In this study, successful bone marrow imaging was demonstrated as late as 72 hours and at least 168 hours into a course of MoAb with relatively low doses (3 mg/m^2) of MoAb administration. Cellular targeting was confirmed

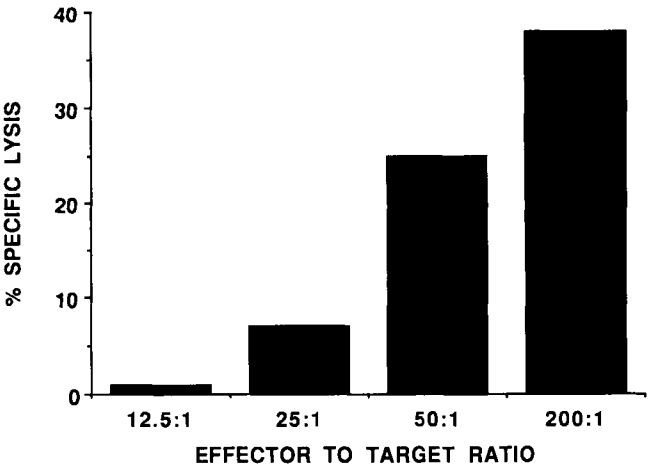

Figure 3. Antibody-dependent cellular cytotoxicity of HuG1 as a function of E:T ratio. Target HL60 cells were incubated at 37°C with monoclonal antibody at 1 μg/ml and peripheral blood mononuclear cells as effectors. ^{51}Cr-release was measured 5 hours later as described in the text.

by flow cytometry, and this dose was more than sufficient to target all cells with minimal excess. The combination of low nontarget doses and high specific activity of labeled antibody make it an exceptionally effective agent for massive cell kill.

Retreatment in two patients with HAMA resulted in rapid clearance of antibody and reduced effectiveness of the mouse MoAb.[15] To avoid neutralizing HAMA responses, and to take advantage of natural immune effector functions, obviating the need for radioisotopes and immunotoxins, humanized versions of M195 were constructed. These newly genetically engineered chimeric and humanized M195 constructs show improvements over the murine MoAb in both avidity and recruitment of host effector cells.

To our surprise, the humanized, but not chimeric versions, show a markedly higher avidity than that of the original mouse MoAb.[11] Competition experiments using either ^{125}I-HuG1 or ^{125}I-M195 corroborated the suggested higher avidities of HuG1 and HuG3, as calculated from the Scatchard plots.[6] These results are probably more accurate than the Scatchard plots because radioiodination may affect binding characteristics. Other humanized MoAb, including anti-TAC-H[9] and anti-Herpes MoAb[10] showed slightly lower, but not improved, avidities compared with the original parental murine MoAb. Comparisons of the variable region sequences of M195[6] showed a loss of a carbohydrate binding site in the variable region from the CDR-grafted M195 MoAb. This change in the carbohydrate region may be responsible for the dramatic difference in avidities of HuG1 and HuG3. This hypothesis is being investigated.

The ability of M195 to internalize and carry radioactivity into target cells is crucial to the in vivo therapeutic effects seen in this study, or for the use of toxins to purge bone marrows of AML cells in vitro.[17] The ability of M195 to persist inside cells as shown here by flow cytometry is in contrast to studies of ^{131}I-P67, which was poorly retained by cells, because of rapid endocytosis and expulsion of degraded metabolites.[18] HdIgG was more than 2 times more efficient than any monomeric M195 at staying inside the cell, in light of a similar binding avidity. This more efficient internalization held true over 48 hours. Thus, radiation doses to target cells would be twice as high for the same added dose and same toxicity. Although others have found that cross-linked IgG bound to Fc receptors more avidly than monomeric Ig,[19–21] our system is unique in that binding and internalization occur through specific Fab binding to CD33 receptors.

Patients with AML who received infusion of autologous marrow treated with anti-MY9 and rabbit complement after myeloablative chemotherapy showed trilineage engraftment and remission.[22] Although all of the monomeric versions performed CMC with rabbit complement,[11] HdIgG was at least 100-fold more potent at cell killing when compared with HuG1.[12] This was most likely due to the proximity of Fc receptors, because binding and activation of C1q requires the formation of doublet IgG on the cell surface.[23] The ability of HdIgG to allow spatial arrangements to occur is consistent with the mechanism of a cluster of IgGs needed for the attachment of C1q,[24–26] or for the critical spacing of epitopes on the cell surface necessary for CMC to occur.[27] The additional evidence that bispecific antibodies that are capable of binding to two antigens,[28] and a chimeric antibody with dual Fc regions[29] were superior to monomeric MoAb in CMC, attests to the improvement seen with HdIgG.

Although HuG1[11] and HdIgG[12] were capable of fixing human complement, neither MoAb lysed HL60 cells in the presence of human complement. The ability of M195 to perform CMC using human complement was a function of CD33 antigen density. Effective killing of CD33$^+$ AL67 fibroblasts, but not HL60 cells with a 27-fold lower antigen density, was seen with human serum as a source of complement.[12]

One of the advantages of chimeric and humanized M195 is their ability to perform ADCC with human PBMC. This new effector function over the original murine MoAb has been reported for other chimeric[9,30] and humanized[9] MoAb. The much improved level of ADCC with HdIgG can be attributed to dual Fc receptors that would allow effectors and targets to be in close proximity. The spacing of IgG on the target cell surface is crucial for the enhanced susceptibility of K lymphocytes to bind and lyse antibody-coated red blood cells.[31]

The improved biologic properties and effector functions of humanized and homodimeric IgG1 M195 show promise for future in vivo therapies of AML. This clinical trial validates the use of radiolabeled mouse M195 for cytoreduction and as a preconditioning regimen at higher doses of radioactivity for allogeneic BMT in refractory leukemia. But it is our hope that radiolabels and immunotoxins will be replaced by the use of humanized monomeric or dimeric M195, alone or in combination with cytokines, for the treatment of myeloid leukemias. The most likely use of humanized versions of M195 will be for the elimination of minimal residual disease after chemotherapy, with the aim of preventing relapse and sustaining durable remissions in patients with myeloid leukemia.

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Biological and Immunological Features of Humanized M195 (Anti-CD33) Monoclonal Antibodies

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Biological and Immunological Features of Humanized M195 (Anti-CD33) Monoclonal Antibodies[1]

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ABSTRACT

Human-mouse chimeric immunoglobulins G1 and G3 (IgG1 and IgG3) (ChG1, ChG3) and "complementarity-determining region"-grafted, humanized IgG1 and IgG3 (HuG1, HuG3) constructs of the mouse monoclonal antibody (mAb) M195 were characterized. M195 is a murine immunoglobulin G2a (IgG2a), anti-CD33 mAb, specifically reactive with acute myelogenous leukemia cells, that is active as an antileukemia agent in humans. The new mAb constructs maintained specificity and biological function, including rapid internalization after binding to the cell surface, which has been important for delivery of therapeutic isotopes in patients. Although previously reported complementarity-determining region-grafted mAbs had reduced avidities, the HuG1 and HuG3 M195 showed up to an 8.6- and 4-fold higher binding avidity, respectively, than the original murine mAb. All constructs were effective at mediating rabbit complement-mediated cytotoxicity against HL60 targets. Fibroblasts transfected with *CD33* genes and expressing high levels of CD33 antigen were also lysed in the presence of human complement, but HL60 cells or fibroblasts with lower CD33 levels were not killed. Thus, the inability of M195 and constructs to kill HL60 targets with human complement is due to the much lower antigen density on HL60 cells compared to CD33$^+$ fibroblasts. Unlike the murine M195, the chimeric and humanized M195 demonstrated antibody-dependent cell-mediated cytotoxicity using human peripheral blood mononuclear cells as effectors. Because the chimeric and humanized M195 have improved avidities as compared to the original M195 and have, in addition, the potential to avoid human anti-mouse antibody responses and to recruit human effector functions, these new constructs may be useful therapeutically, either alone or conjugated to toxins or isotopes, in the treatment of acute myelogenous leukemia.

INTRODUCTION

Mouse monoclonal IgG2a[3] antibody M195 (mAb M195) is specifically reactive with the CD33 antigen on AML cells and early myeloid progenitor cells, but not with normal tissues or hematopoietic stem cells (1–5). This sparing of normal stem cells makes it an ideal candidate for leukemia therapy and for *ex vivo* bone marrow purging of AML cells (6–8). M195 reacts with the CD33 antigen, a M_r 67,000 glycoprotein, and is rapidly internalized into target cells upon binding. M195 is not intrinsically cytotoxic *in vitro*. Although M195 kills target cells with guinea pig and rabbit complement, it is not effective with human complement or effector cells. However, the efficient binding and internalization of M195 have allowed the use of radiolabeled mAb in trials for AML therapy (9, 10). These trials have demonstrated that specific targeting of ^{131}I-M195 can result in marked leukemia cytoreduction, therapeutic responses, or marrow ablation with no toxicity.

Genetic engineering techniques are now available to produce new human-mouse chimeric mAbs that combine mouse variable regions with human constant regions (Fig. 1) or more fully humanized mAbs that have human framework and constant regions. These humanized mAbs have been "CDR-grafted" so that only the original mouse antigen binding sites are retained. The new constructs offer advantages over murine mAbs in being more effective in recruiting human effector functions and in avoiding neutralizing HAMA responses. Avoidance of HAMA may allow for repeated treatments without loss of effectiveness and a longer circulating time for the mAb. The added features may allow us to avoid the use of radiolabels and toxins in clinical therapeutic trials (11–16).

One humanized IgG1 mAb, CAMPATH-1H, derived from CDRs from a rat mAb together with human framework regions, has been used in a clinical situation (15). CAMPATH-1H produced remissions in two patients with refractory non-Hodgkin's lymphoma. Mathieson *et al.* (16) have also used CAMPATH-1H in one patient with systemic vasculitis.

A second humanized mAb, anti-Tac-H, reactive with the IL-2 receptor, has recently been constructed using computer-aided three-dimensional structural design to maintain avidity at its binding site (17). Humanized anti-Tac, as well as the chimeric version, blocks T-cell activation and has the added advantage over the original murine mAb of performing ADCC against human tumor targets (18). More recently, humanized versions of two murine mAbs against herpes simplex virus gB and gD glycoproteins have resulted in similar binding, virus neutralization, and cell protection as seen with the original murine mAb (19).

This paper describes four new reconstructed M195 mAbs in comparison to the original mouse M195. The four new constructs compare favorably to the parental M195 with respect to their specificity, immunoreactivity, and internalization, and they showed increased avidity of binding and improved immunological functions, such as ADCC. Therefore, these constructs hold potential promise for a new approach to the treatment of AML.

MATERIALS AND METHODS

MAb. Mab M195 (anti-CD33) originated in BALB/c mice immunized with leukemia cells from a patient with AML and was produced from hybridomas and purified as described previously (4, 5). Human-mouse chimeric IgG, with human-derived constant regions for IgG1 and IgG3 (ChG1, ChG3), and CDR-grafted humanized M195, retaining only the CDRs and other sterically important amino acids from the mouse IgG (HuG1, HuG3), were constructed as described (20). Sp2/0 hybridoma cell lines secreting the chimeric and humanized M195 were grown *in vitro*, and the mAbs were purified on PA-Sepharose (Pharmacia, Piscataway, NJ) by affinity chromatography using sequential pH step elutions (4). Purity was determined on SDS-polyacrylamide gels stained with Coomassie brilliant blue.

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[3] The abbreviations used are: IgG, immunoglobulin G; IgM, immunoglobulin M; mAb, monoclonal antibody; AML, acute myelogenous leukemia; HAMA, human anti-mouse antibody; IL-2, interleukin 2; SDS, sodium dodecyl sulfate; PBMC, peripheral blood mononuclear cells; CMC, complement-mediated cytotoxicity; MTT, dimethylthiazol diphenyltetrazolium bromide: thiazolyl blue; ADCC, antibody-dependent cellular cytotoxicity; CDR, complementarity-determining region.

Cells and Cell Lines. Hematopoietic cell lines, including SKLy16, RAJI, HL60, and U937 cells, were obtained from the human tumor banks of the Cancer Immunology Laboratory at Sloan-Kettering Institute. Hematopoietic cell lines were grown in RPMI 1640/5% newborn calf serum/10% serum-plus (Hazelton Biologics, Inc., Lenexa, KS). Mouse NIH-3T3 fibroblasts transfected with the *ras* gene to enhance growth rate and AL67 cells, which were NIH3T3 cells transfected with the *ras* and *CD33* genes and expressing cell surface CD33 (21), were the generous gift of Dr. Look, Dr. Ashman, and Dr. Peiper (St. Jude's Children Research Hospital, Memphis, TN). Fibroblasts were grown in RPMI 1640/10% serum-plus/10% fetal calf serum with 2 mM glutamine.

Heparinized peripheral blood samples were obtained from healthy volunteers and patients on the Leukemia Service at Memorial Hospital on institutional review board-approved protocols. PBMC were separated on Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation.

Direct and indirect flow cytometry was conducted as described (5).

Radioiodination and Radioimmunoassay. Purified mAbs were labeled with Na-^{125}I (New England Nuclear, Boston, MA) using chloramine-T to start and sodium metabisulfite to stop the reaction. MAbs were labeled to 2 to 10 μCi/μg of protein. Immunoreactivity was determined by incubating 3 to 5 ng of radiolabeled mAb with an excess of antigen (5×10^6) HL60 cells for 1 h at 4°C. Immunoreactivity of the four radiolabeled constructs and the original M195 mAb was determined to be about 50%. Specific binding in these radiobinding assays was determined by subtracting the amount of radiolabeled mAb bound in the presence of an excess of unlabeled mAb as previously described (4).

Competition Radioimmunoassay. Comparison of antigen-antibody avidity between different M195 constructs was done in competition with ^{125}I-M195 and ^{125}I-HuG1. Increasing amounts of cold mAb were incubated with 2×10^5 HL60 cells and 50 ng of ^{125}I-mAb in 200 μl of RPMI for 1 h at 0°C. Cells were washed 3 times in RPMI and counted. To avoid nonspecific and Fc-receptor binding, competition assays were done in the presence of human serum. Samples were run in duplicate, and the avidity of binding was measured by comparing the concentrations of unlabeled competitor where 50% reduction of binding was achieved as described (18). Experiments were repeated 2 to 3 times each, and arithmetic means of the data are presented.

Modulation of Cell Surface Antigen. Internalization of the cell surface antigen was measured by incubating 0.5 μg/ml of radiolabeled mAb with 5×10^5 HL60 cells over time at 0° and 37°C. Cell pellets were washed twice in RPMI, and then surface-bound M195 was stripped with 1 ml of 50 mM glycine/150 mM NaCl, pH 2.8, at 24°C for 10 min. Total cell-associated radioactivity and acid-resistant (internalized) radioactivity were determined.

CMC. Twenty-five μl of HL60 cells (5×10^6 cells/ml) or fresh leukemia samples were incubated with 25 μl of diluted rabbit or human complement and 25 μl of serial dilutions of mAb at 37°C for 60 min. MAb M31 (IgM anti-CD15) was used as a positive control. Live and dead cells were enumerated using trypan blue or propidium iodide exclusion.

Rabbit serum was purchased from PelFreeze (Rogers, AK); human sera were obtained from volunteers. All complement sources were stored at −70°C until use and not reused. Complement was used at the maximum concentrations not showing nonspecific lysis of the target cells, usually from 1:6 to 1:10 final dilution.

Alternately, CMC was determined using the MTT (Sigma Co., St. Louis, MO) assay as described previously (22). MTT measures cell viability and correlates well with the trypan blue method. AL67 fibroblasts are incubated with MTT together with mAb and human complement for 4 h, and the metabolized MTT (blue formazon precipitate) is solubilized with 0.04 M HCl in 2-propanol. The assay is then quantified using an enzyme-linked immunosorbent assay at 570 nm (BT2000 Microkinetics Reader, Fisher Biotech, Springfield, NJ).

Measurement of CD33 Density on AL67 Cells. The antigen density of CD33 on HL60 cells *versus* AL67 fibroblasts was estimated by determining antigen levels and dividing by cell surface area. Cell diameter was measured both by forward light scatter on flow cytometry and by direct microscopy. The surface area of nonadherent AL67 fibroblast cells was calculated to be 1.6 times that of HL60 cells. Radiobinding experiments showed that the number of M195 antigen sites was 42 times greater for AL67 fibroblasts than HL60 cells (420,000 *versus* 10,000). The relative increase in the number of sites was confirmed by measuring mean peak fluorescence using a flow cytometer. Assuming a spherical shape for AL67 cells when in suspension, a calculation of the surface area yielded an average antigen density of CD33 epitopes that was 27-fold greater for AL67 fibroblasts than HL60 cells.

Enzymatic Cleavage of CD33 from the Cell Surface. CD33+ AL67 fibroblasts were incubated with 0.35 units of proteinase K (Bethesda Research Labs, Gaithersburg, MD) at 37°C. At various time intervals, an aliquot was washed twice with medium and split in half, and cells were tested as a target for CMC with human complement and HuG1 as described above. The number of antigen binding sites was quantified using direct radioimmunobinding and also by indirect flow cytometry on the second aliquot.

ADCC. Chromium release assays to determine if chimeric or humanized M195 mAbs were capable of mediating ADCC were conducted as described previously (4). PBMCs from human volunteers were used as effector cells, and either HL60 cells or CD33+ AL67 fibroblasts were used as positive targets. Assays were conducted at 37°C for 5 h and harvested using a Skatron press, and released ^{51}Cr was counted in a Packard gamma counter. Detergent lysed cells were used as a 100% control. Effector cell only and mAb only treated target cells were used as negative controls. CD33-negative RAJI and NIH-3T3 cells and a control HuG1 mAb (Fd 79) (19) were used as controls. Samples were done in quadruplicate, and the mean was presented. Experiments were repeated 3 to 5 times. Standard deviations were always less than 10% of the mean value.

RESULTS

Specificity. Four new M195 mAb constructs were made (Fig. 1) as described (20). Specificity was first confirmed against a panel of CD33$^+$ and CD33$^-$ cell lines by radioimmunoassay and then against fresh hematopoietic samples from 47 patients using a direct fluorescein conjugate of HuG1 M195. The specificity for myeloid leukemia cells and monocytes in direct comparison with other anti-CD33 antibodies and the absence of

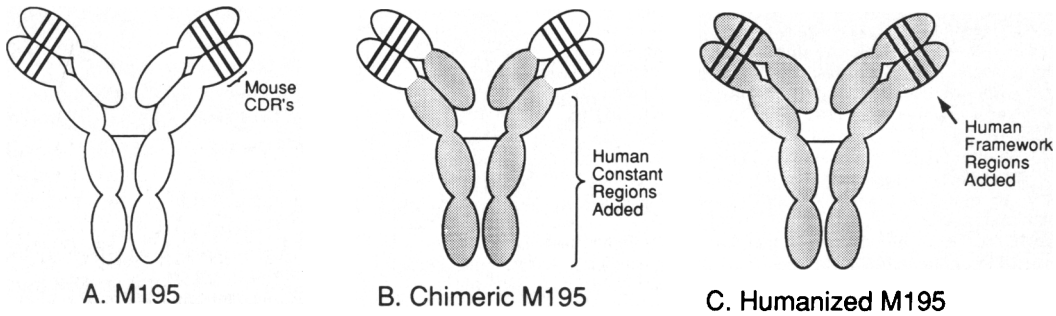

Fig. 1. Diagram of chimeric and humanized M195 constructs. *A*, parental IgG2a murine mAb. In *B*, chimeric IgG1 (ChG1) and IgG3 (ChG3) were made by combining mouse M195 variable regions with human constant regions. In *C*, humanized IgG1 (HuG1) and IgG3 (HuG3) are entirely human except for CDR-grafted murine-derived antigen binding sites and other selected amino acids. ○, sequences of mouse origin; ●, sequences of human origin.

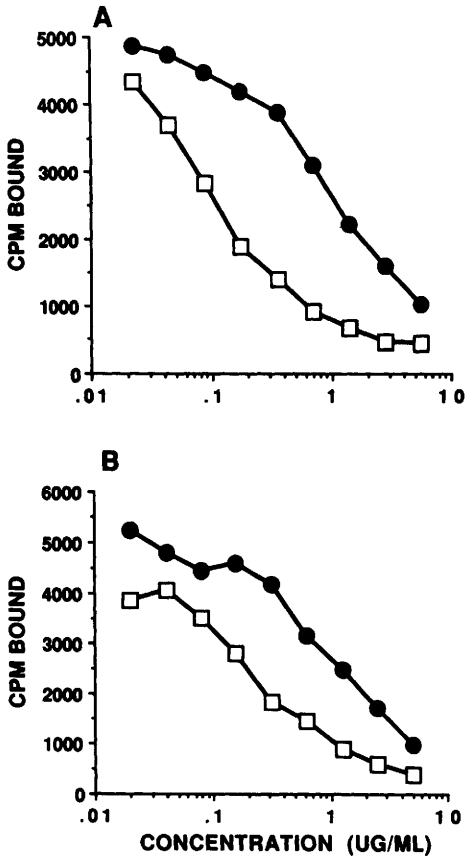

Fig. 2. Competition binding assays with murine M195 and HuG1. HL60 cells (2×10^5) were incubated with increasing amounts of cold murine M195 (●) and HuG1 (□) and 50 ng of mouse ^{125}I-M195 (*A*) or ^{125}I-HuG1 (*B*) for 1 h at 4°C in the presence of 2% human AB serum. Cells were then washed, and cell-associated radioactivity was measured. The HuG1 showed an increased binding avidity to HL60 cells compared to the parental murine mAb. Regression analysis showed the linear portions of the two curves to be significantly different ($P < 0.0001$ for both figures).

reactivity with cells of other lineages were confirmed in these assays. The analysis included blasts, lymphocytes, monocytes, and myeloid cells, separately gated. Immunohistochemistry confirmed lack of reactivity with normal tissues as well.

Competition Radiobinding Assays. Initial binding curves had suggested that the CDR-grafted constructs had similar or perhaps improved binding avidity as compared to the mouse M195 (20). To accurately compare the avidity of binding of the human constructs to mouse M195, unlabeled mouse M195 and constructs were allowed to compete with radiolabeled murine M195 for antigen sites on HL60 cells. Human serum was added to prevent potential artifacts attributable to nonspecific and human Fc receptor binding. Murine M195 has an avidity of 2.2×10^9 M^{-1} (20). This value was fixed, and the avidity of the new constructs was computed relative to it. These experiments showed that the binding avidity of ChG1 was comparable to that of the mouse M195 ($K_a = 2.2 \times 10^9$ M^{-1}), while ChG3 showed slightly less binding avidity than the original mAb ($K_a = 1.5 \times 10^9$ M^{-1}). Similar competition experiments using unlabeled constructs in competition with radioiodinated mouse M195 or unlabeled constructs in competition with radioiodinated HuG1 showed a statistically significant 8.6- ($K_a = 1.9 \times 10^{10}$ M^{-1}) and 5- ($K_a = 1.1 \times 10^{10}$ M^{-1}) fold, respectively, greater calculated avidity of binding for the HuG1 as compared to the mouse M195 (Fig. 2). HuG3 also showed a 4-fold greater avidity than the murine M195 ($K_a = 8.8 \times 10^9$ M^{-1}). The increased avidities of the humanized mAbs as measured by these more accurate competition experiments were consistent with, but greater in magnitude than, the increased avidities suggested by Scatchard analysis (20).

Internalization of Bound M195. Studies *in vitro* and in humans *in vivo* have shown that M195 is capable of rapidly internalizing into target cells upon binding to antigen (4, 9). Direct radioimmunobinding studies at 37°C showed that the radiolabeled mouse mAb became associated with HL60 cells over time in an acid-resistant compartment, *i.e.*, not on the cell surface (Fig. 3*A*). Both HuG1 (Fig. 3*B*) and HuG3 (not shown) behaved with similar kinetics. At 0°C, no radioactivity entered the cell above a baseline level less than 10% of the total cpm. However, at 37°C the amount of radiolabel inside the cell steadily increased. By 4 h, 25% of the cell-associated radiolabel was internalized into the cell.

CMC. Assays were performed to determine whether M195 constructs were capable of killing cells in the presence of rabbit and human complement. All four mAbs were capable of CMC of HL60 cells with rabbit complement (Fig. 4). Murine M195,

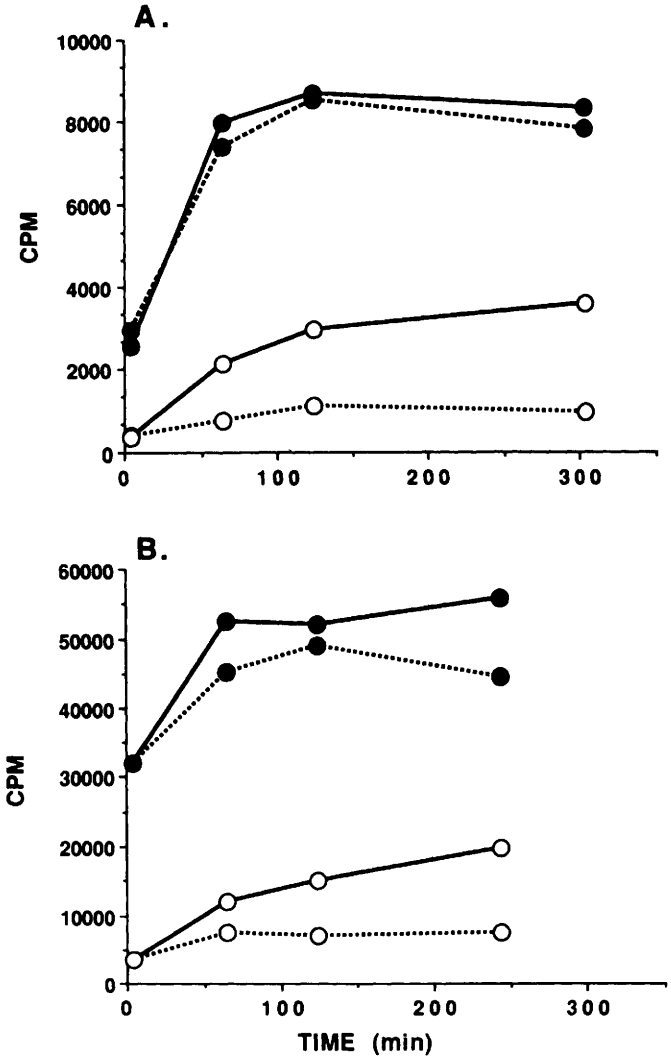

Fig. 3. Internalization of mouse or humanized ^{125}I-M195 over time. HL60 cells were incubated with murine ^{125}I-M195 (specific activity, 1.2×10^3 cpm/ng) (*A*) or ^{125}I-HuG1 M195 (specific activity, 8×10^3 cpm/ng) (*B*) for the times shown along the *abscissa*. Total cell-associated cpm were determined at 0°C (●- - -●) and 37°C (●——●). Cell surface-bound IgG was then stripped using 50 mM glycine-HCl, 150 mM NaCl, pH 2.8, and internalized cpm were measured at 0°C (O- - -O) and 37°C (O——O).At 37°C, both the mouse and humanized mAb showed that 25% of the label entered the cell by 4 h.

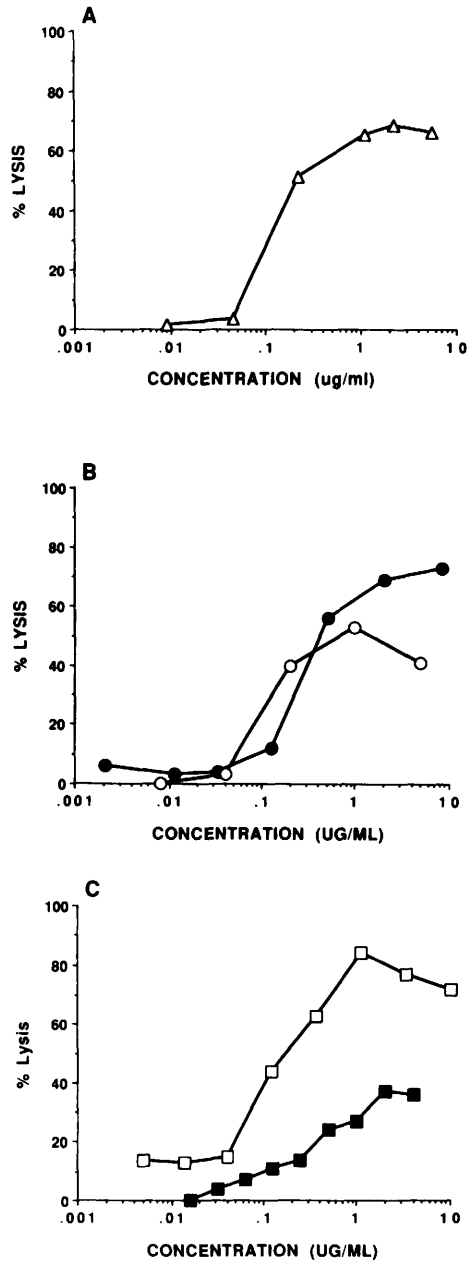

Fig. 4. CMC on HL60 cells with M195 constructs using rabbit complement. Twenty five μl of HL60 cells (5×10^5 cells/ml) were incubated for 1 h at 37°C with equal volumes of diluted rabbit complement and serial dilutions of mAb. Trypan blue exclusion or propidium iodide was used as an indicator. Murine M195 (△) (*A*), ChG1 (○—○) and ChG3 (●—●) (*B*), and HuG1 (□) and HuG3 (■) (*C*) were all effective at lysing the HL60 cells with rabbit complement. All constructs showed a prozone effect at the highest concentrations of mAb.

ChG1, ChG3, and HuG1 yielded 50% cell lysis at a concentration of 0.1 to 0.5 μg/ml. However, HuG3 was consistently less efficient at CMC with rabbit complement, achieving less than 50% maximum cell killing. All four constructs showed a prozone effect, with a decline in activity at the highest concentrations of mAb. None of the mAbs was effective in lysing HL60 cells when human serum from several normal individuals at varying concentrations was used as a source of complement.

AL67 fibroblasts, which are mouse NIH3T3 cells that express high levels of human CD33$^+$ antigen, were studied to determine whether an increased antigen density would permit M195 and its constructs to kill by activation of the complement cascade using human complement. Assayed by trypan blue exclusion, M195 and all four constructs were cytotoxic to the AL67 fibroblasts with both rabbit and human complement. Control NIH3T3 fibroblasts did not bind mAb and were not killed by M195 or the constructs in the presence of either complement. To quantitate the titer of the constructs for human CMC against the adherent AL67 fibroblasts, an MTT assay, which measures cellular metabolism, was used (Fig. 5). Human CMC was antibody concentration dependent. Murine M195, ChG1, and HuG1 demonstrated cytotoxicity curves with 50% cell lysis seen at 1 to 3 μg/ml (Fig. 5). HuG3 was also similar to murine M195, while ChG3 was less effective at cell killing with human complement, even at high concentrations of antibody (results not shown).

Correlation of CD33 Antigen Density and CMC. To determine a relationship between the number of antigen sites and CMC activity using the HuG1 and human complement, AL67 fibroblasts were incubated with proteinase K at varying time intervals to cleave CD33 sites. The number of remaining CD33 sites correlated with the ability of HuG1 to cause cell killing using human complement. As shown in Fig. 6, a critical number of CD33 sites was needed for CMC to occur. When fewer than 150,000 sites were present, CMC was minimal. Above this critical level, the amount of CMC linearly increased with the number of CD33 antigen sites.

Antibody-dependent Cellular Cytotoxicity. The capabilities of M195 constructs at mediating ADCC were determined in chromium release assays. Mouse M195 did not show evidence of ADCC using PBMCs as effector cells. In contrast, experiments with ChG1 and ChG3, as well as HuG1 and HuG3, demonstrated a dose and effector cell-dependent activity against HL60 targets. ChG1 and ChG3 showed a maximum killing of 20 to 30% in 5-h assays at high effector:target ratios (100:1) and increasing antibody concentrations (1 to 10 μg/ml) (results not shown). The CDR-grafted HuG1 showed slightly lower levels of ADCC, with a maximum killing of 10 to 20% at 5 h of incubation with HL60 target cells as shown in a representative experiment (Fig. 7*A*). HuG3 also showed similar levels of ADCC. No additional killing was seen with any construct at concentrations above 10 μg/ml and, in some cases, reduced effects were seen with increasing antibody, possibly due to increased modulation. When CD33+ AL67 fibroblasts were used as target cells, HuG1 showed high levels of killing (40 to 70%)

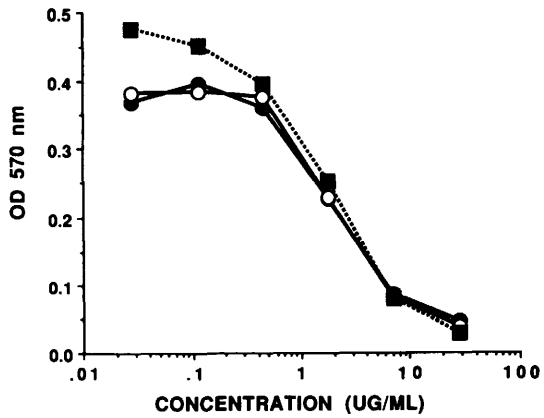

Fig. 5. CMC on CD33$^+$ AL67 fibroblasts with M195 constructs using human complement. An MTT assay (see text) was used to quantitate the titer of mAb for human CMC on AL67 fibroblasts that have been transfected with the *ras* gene and express high levels of human CD33 antigen. Cells are incubated with human complement and then MTT for 4 h. The assay is then quantified using an ELISA at 570 nm. M195 (●), ChG1 (■), and HuG1 (○), as well as HuG3 (results not shown), all demonstrated a similar dose-dependent cytotoxicity curve. The ability of M195 mAbs to perform human CMC with CD33$^+$ fibroblasts may be explained by their higher antigen density compared to HL60 cells.

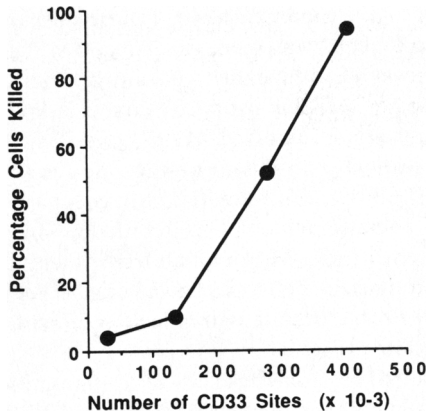

Fig. 6. Correlation of CD33 antigen density and CMC using HuG1 and human complement. CD33+ AL67 fibroblasts were incubated with 0.35 units of proteinase K at varying time intervals. The number of sites was determined by direct radioimmunoassay and was plotted against the percentage of cells killed after CMC in the same aliquot. Above a critical number of CD33 sites, the ability of HuG1 to mediate CMC using human complement linearly increased with the antigen density.

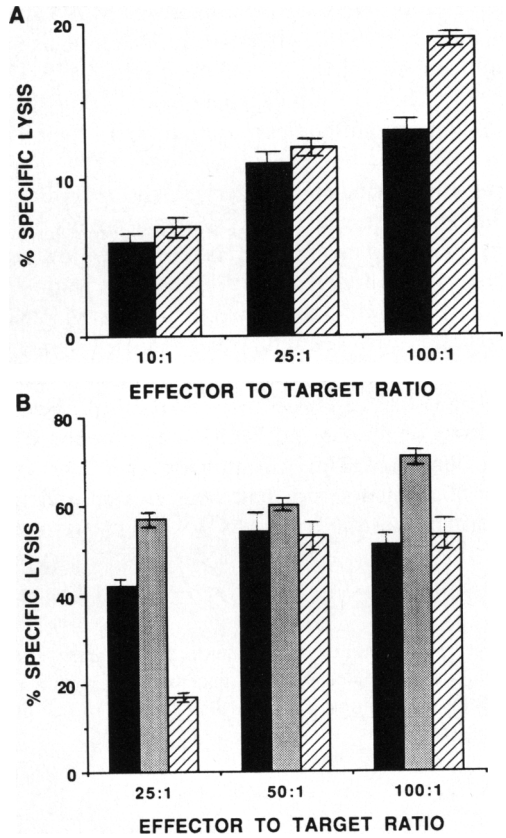

Fig. 7. ADCC of humanized M195 as a function of concentration and effector:target ratio at 5 h of incubation. Chromium release assays were conducted as described in "Materials and Methods" using either HL60 or CD33+ AL67 fibroblasts as target cells and PBMCs as effector cells. *A*, HuG1 at 1 μg/ml (■) and 10 μg/ml (▨) against HL60 cells; *B*, HuG1 at 0.1 μg/ml (■), 0.33 μg/ml (□), and 3.3 μg/ml (▨) against AL67 fibroblasts. HuG1 demonstrated dose-dependent and effector cell-dependent increases in ADCC against HL60 cells. When tested against CD33+ AL67 fibroblasts, HuG1 showed higher levels of killing than against HL60 cells at a log lower concentration. *Columns*, mean; *base*, SD.

at a log lower concentration. This high level of killing was independent of effector:target ratio (Fig. 7*B*).

These results for ADCC varied, depending on the source of effector cells, largely because of variations in nonspecific effector cell killing of targets in the absence of mAb. No killing was seen with a control HuG1 mAb (FD79) (19) and HL60 cells or with HuG1 M195 and CD33-negative RAJI cells or NIH3T3 cells. In other experiments (not shown), killing was seen at mAb concentrations ranging from 1 to 100 μg/ml and at effector:target ratios of 10 to 100:1, but not at 5:1.

DISCUSSION

Techniques are now available to genetically graft the mouse IgG CDR regions onto human IgG framework regions, resulting in new humanized IgG that are entirely human except for the hypervariable regions involved in antigen binding (12–14). The two major aims in developing a humanized M195 mAb are to take advantage of natural immune effector functions to destroy leukemic cells and to avoid neutralizing HAMA responses. In this way, an unmodified M195 may be used rather than relying on conjugation to radiolabels or toxins. Newly genetically engineered chimeric and humanized versions of M195, a mouse IgG2a mAb reactive with the CD33 antigen of early myeloid cells, show improvements over the murine mAb in both avidity and recruitment of host effector cells.

ChG1 and ChG3 bound to HL60 cells with similar or slightly less avidity than that of the original murine mAb. Other chimeric mAbs have shown similar avidities to the mouse immunoglobulin from which they were derived (23–25). In contrast, the complete reengineering of the humanized IgG did modify the avidity, in this case toward increased avidity. The HuG1 and HuG3 showed a markedly higher avidity, respectively, than the original mouse M195, in competition with radiolabeled M195 for binding to HL60 cells. Since competition experiments for HuG1 were done using either ^{125}I-HuG1 or ^{125}I-M195, it is unclear which result is more accurate and may relate to radioiodination damage. Since radioiodination would decrease avidity, the higher avidity is the more accurate description. These results corroborated the suggested higher binding avidities of the human mAb, compared to the murine and chimeric M195, as calculated from the Scatchard plots (20).

Direct radiobinding experiments to determine avidities as are done for Scatchard analysis may be affected by artifacts generated in the radioiodination of the immunoglobulin either due to radioiodination in the binding site or denaturation during the process. There are five tyrosine residues in the heavy chain CDR and one tyrosine residue in the light chain CDR of M195 that could be attacked in the radioiodination procedure.

This is the first reported instance of a newly constructed humanized mAb having a higher avidity than the parental murine mAb. Other humanized mAbs, anti-Tac-H (18) and mAbs against herpes simplex virus gB and gD glycoproteins (19), showed comparable to slightly lower, but not improved, avidities compared to the original mouse mAb in competition binding experiments. Comparisons of the variable region sequences of M195 (20) have revealed a loss of a carbohydrate binding site in the variable region from the CDR-grafted M195 antibodies. Thus, the mechanism for the increased avidity involves a change in a carbohydrate content in the humanized V region relative to the mouse V region. This hypothesis has now been confirmed by construction and analysis of new variable regions with and without the carbohydrate site.[4]

The ability to internalize and carry radioactivity into target cells has been important in the *in vivo* therapeutic effects of M195 (9, 10), or for the use of toxins to purge bone marrows of

[4] Manuscript in preparation.

AML cells *in vitro* (26). Here we show that the HuG1 and HuG3 were also capable of internalizing into HL60 cells over time at 37°C. Antigenic modulation may pose specific problems for future mAb trials. Although a rapid decrease of mAb from the cell surface allows for radiolabel and toxins to enter the cell and cause killing, a disadvantage of this process would be limited access of the mAb for CMC and ADCC (14, 27).

One of the advantages of chimeric and human mAbs over murine mAbs is their ability to mediate ADCC (18, 28, 29) with human PBMC. It is surprising that the humanized M195 mAbs were slightly less effective at ADCC than the chimeric mAbs. One possible explanation is the change in a carbohydrate moiety attachment site on the IgG framework region that occurred during reconstruction increased avidity and rates of modulation (not shown). This alteration may be responsible for the decreased activity.

Isotype is of importance in determining the level of ADCC. Junghans *et al.* (18) reported that only the human G1 isotype (chimeric and humanized variants) of anti-Tac-H promoted ADCC. Brüggemann *et al.* (30) also found that chimeric IgG1 was the most effective isotype in mediating ADCC against a hapten-derivatized human T-cell line. Among the CAM-PATH-1 family of mAb, only rat IgG2b was active in ADCC (31). Others have found higher levels of macrophage ADCC when IgG mAbs of different isotypes and recognizing different antigens were used in combination against lymphoma targets (32).

Cytokines may be necessary to optimize ADCC activity by increasing various effector cell functions and numbers. IL-2 has been shown to potentiate ADCC of human effector cells with mouse anti-melanoma mAb (33, 34), against malignant B-cell lines using the murine Lym-1 mAb (35), and against the IL-2 receptor using chimeric and humanized anti-Tac mAb (18). In addition, γ- interferon (36) and granulocyte-macrophage (37) and macrophage colony-stimulating factor (38) have also been shown to augment ADCC against human tumor cells using human effector cells. Combining IL-2 and PBMCs as effector cells to augment chimeric and humanized M195-mediated ADCC showed that similar enhancement occurred against HL60 target cells (data not shown).

Murine, ChG1, ChG3, HuG1, and HuG3 all killed HL60 cells at low concentrations in the presence of rabbit complement. This shows that M195 is capable of binding rabbit C1q and initiating the complement cascade. Although HuG3 was the least effective mAb in this regard, this cannot be explained by differences in isotype alone, since ChG3 was as efficient at rabbit CMC. The ChG3 showed decreased avidity, as well as increased instability in solution over time (months), as evidenced by progressive precipitation and breakdown on SDS-gels. This subtle instability may result from the long hinge region of the IgG3 and may account for the reduced activities seen (30).

None of the M195 constructs showed any killing of HL60 cells with human complement. The ability to kill AL67 cells with M195 constructs was a function of CD33 antigen density, since effective killing of AL67 fibroblasts, but not HL60 cells with a 27-fold lower antigen density, was seen with dilute human serum as a complement source. The lack of killing of HL60 cells could not be solely attributed to homologous restriction factors on HL60 cells (39), since reduction of the number of CD33 sites on AL67 cells by enzymatic cleavage also rendered them insusceptible to CMC. Others have found that the tumoricidal effects of an IgG2a mAb correlated with antibody density on the tumor cells (40, 41). Because more efficient killing of AL67 cells was seen, this suggested, as with CMC, that ADCC was also dependent on antigen density. Marked killing (80%) was seen against AL67 targets at low effector:target ratios and low humanized M195 concentrations. We are currently investigating drugs to up-regulate the CD33 antigen density on HL60 cells. This might permit adequate C1q binding and, hence, enhancement of CMC with human complement using M195 constructs. MAbs which have potent CMC *in vitro* have shown tumoricidal effects *in vivo*, but whether lack of these effects *in vitro* will correlate with a lack of effect in humans *in vivo* is not known (14).

The mouse M195 alone has shown no immunological effector functions in humans in human trials (9). Thus, the evidence that the engineered M195 constructs demonstrate ADCC and the ability to fix human complement *in vitro* is encouraging. Although not directly cytoxic via human CMC with the expected numbers of CD33 sites for a typical leukemia cell, opsonization via complement receptors may be possible. It is therefore possible that immunological functions *in vivo* will also be enhanced. Clinical trials using humanized M195 are planned to see whether they will be effective antileukemic agents.

Although chimeric mAbs are the most effective mediators of ADCC *in vitro*, they are still capable of generating HAMA and human antichimeric antibodies that might limit their usefulness *in vivo* (14). Hence, a humanized version of M195 may still be favored over the murine and chimeric mAbs, regardless of their biological functions, especially as carriers of toxins or radioisotopes. The increased avidity of HuG1 may allow us to use significantly smaller doses *in vivo* to achieve saturation of target cells. We have already shown therapeutic activity with ^{131}I-M195 in patients with refractory and relapsed leukemias, but retreatments have been limited by the development of HAMA (10). ^{131}I-HuG1 or ^{131}I-HuG3 may avoid this problem. Indeed, repeated doses of humanized M195 may potentially be given with the avoidance of HAMA responses, which have previously resulted in shortened serum half lives *in vivo* and in limited treatment efficacy of the mouse M195 mAb (10).

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