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① SOLICITUD DE:

☐ Corrección de Error Material☒

Modificación a una solicitud

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④ Número de expediente:

Título

05-048.853

"ANTICUERPOS ANTI-CD33 Y METODO PARA TRATAMIENTO
 DE LEUCEMIA MIELOIDE AGUDA UTILIZANDO LOS MISMOS"

⑤ Descripción de la corrección o modificación solicitada

De conformidad con el Artículo 34 de la Decision 486 de la Comisión de la Comunidad

Andina, presento nuevas páginas 14,57,58,59,68,69 y 72 del capítulo descriptivo con

algunas modificaciones efectuadas. Estas modificaciones no implican ningún cambio

sustancial, ni material de la solicitud presentada inicialmente. Anexamos nuevo listado

de secuencias.

⑥ Comprobante de pago No. 08-60,610 Fecha: 29 de julio de 2008

⑦ Anexos:

☒ Comprobante de pago de la tasa☐ Poderes, si fuere el caso.☐ Certificado de Existencia y Representación legal de las partes, cuando sean personas jurídicas.

⑧

NOMBRE **HUMBERTO RUBIO CAMACHO**

FIRMA

Cpim.-

C.C. 17'192.062 de Bogotá T.P. 50.007



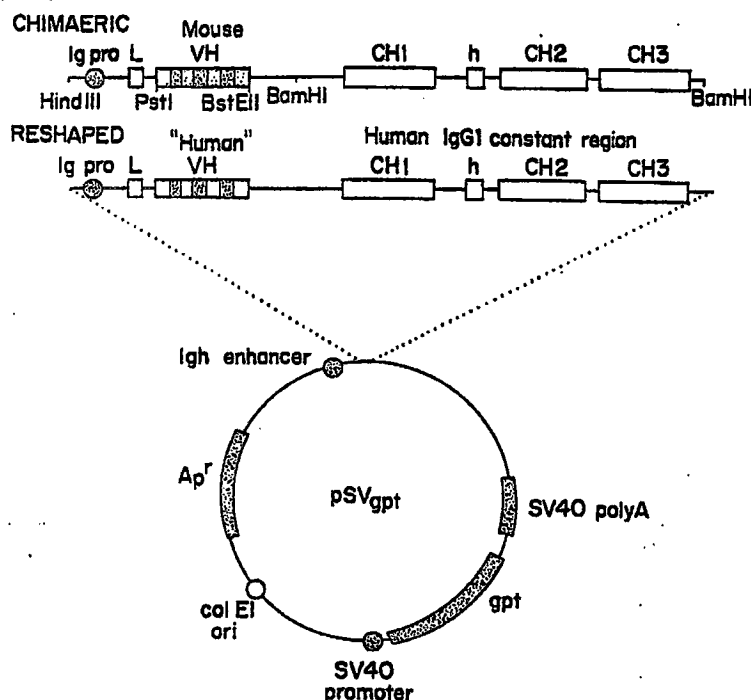
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(21) International Application Number: PCT/US93/10384 (22) International Filing Date: 4 November 1993 (04.11.93) (30) Priority data: 9223377.4 4 November 1992 (04.11.92) GB (71) Applicant (for all designated States except US): MEDAREX, INC. [US/US]; 22 Chambers Street, Princeton, NJ 08542 (US). (72) Inventors; and (75) Inventors/Applicants (for US only) : TEMPEST, Philip, R. [GB/GB]; 63 Brighton Place, Aberdeen AB1 6RT (GB). HARRIS, William, J. [GB/GB]; 3 Caesar Avenue, Carnoustie DD7 6DR (GB). CARR, Frank, J. [GB/GB]; Birchlea, The Holdings, Balmedie, Aberdeenshire AB23 8XU (GB).	(74) Agents: MANDRAGOURAS, Amy, E. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US). (81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.	

(54) Title: HUMANIZED ANTIBODIES TO Fc RECEPTORS FOR IMMUNOGLOBULIN G ON HUMAN MONONUCLEAR PHAGOCYTES



(57) Abstract

Humanized antibodies are described which are specific to an Fc receptor (FcR). The humanized antibodies have at least a portion of a complementarity determining region (CDR) derived from a non-human antibody, e.g. murine, with the remaining portions being human in origin. The use of humanized antibodies rather than murine antibodies in human therapy should alleviate some of the problems associated with the use of some murine monoclonal antibodies because only the substituted CDRs will be foreign to a human's immune system. The humanized antibodies can be used in the same manner as their murine counterparts.

HUMANIZED ANTIBODIES TO Fc RECEPTORS FOR IMMUNOGLOBULIN G ON HUMAN MONONUCLEAR PHAGOCYTES

5 Background

Human Fc γ receptors (Fc γ R) (reviewed in Fanger, M.W., et al. (1989) Immunology Today 10:92-99), of which there are three structurally and functionally distinct types (i.e., Fc γ RI, Fc γ RII and Fc γ RIII), are well-characterized cell surface glycoproteins that mediate phagocytosis or antibody-dependent cell cytotoxicity (ADCC) of immunoglobulin G (IgG) opsonized targets. Antibodies have been made which are directed towards Fc γ R for various purposes, e.g., targeting of immunotoxins to a particular target cell type, or radioimaging a particular target cell type. The antibodies typically have been murine antibodies.

Murine monoclonal antibodies are sometimes desirable for human therapeutic applications because the antibodies can be purified in large quantities and are free of contamination by human pathogens such as the hepatitis or human immunodeficiency virus. Murine monoclonal antibodies have been used in some human therapies, however, results have not always been desirable due to the development of an immune response to the "foreign" murine proteins. The immune response has been termed a human anti-mouse antibody or HAMA response (Schroff, R., et al. (1985), Cancer Res., 45, 879-885) and is a condition which causes serum sickness in humans and results in rapid clearance of the murine antibodies from an individual's circulation. The immune response in humans has been shown to be against both the variable and the constant regions of the murine immunoglobulin.

Recombinant DNA technology has provided the ability to alter antibodies by substituting specific immunoglobulin regions from one species with immunoglobulin regions from another species. Neuberger et al. (Patent Cooperation Treaty Patent Application No. PCT/GB85/00392) describes a process whereby the complementary heavy and light chain variable domains of an Ig molecule from one species may be combined with the complementary heavy and light chain Ig constant domains from another species. This process may be used to substitute the murine constant region domains to create a "chimeric" antibody which may be used for human therapy. A chimeric antibody produced as described by Neuberger et al. would have the advantage of having the human Fc region for efficient stimulation of antibody mediated effector functions, such as complement fixation, but would still have the potential to elicit an immune response in humans against the murine ("foreign") variable regions.

Winter (British Patent Application Number GB2188538A) describes a process for altering antibodies by substituting the complementarity determining regions (CDRs) with those from another species. This process may be used to substitute the CDRs from the murine variable region domains of a monoclonal antibody with desirable binding properties

(for instance to a human pathogen) into human heavy and light chain Ig variable region domains. These altered Ig variable regions may then be combined with human Ig constant regions to create antibodies which are totally human in composition except for the substituted murine CDRs. The "reshaped" or "humanized" antibodies described by Winter elicit a
5 considerably reduced immune response in humans compared to chimeric antibodies because of the considerably less murine components. Further, the half life of the altered antibodies in circulation should approach that of natural human antibodies. However, as stated by Winter, merely replacing the CDRs with complementary CDRs from another antibody which is
10 specific for an antigen such as a viral or bacterial protein, does not always result in an altered antibody which retains the desired binding capacity. In practice, some amino acids in the framework of the antibody variable region interact with the amino acid residues that make up the CDRs so that amino acid substitutions into the human Ig variable regions are likely to be required to restore antigen binding.

15 Summary of the Invention

The present invention pertains to humanized antibodies specific to an Fc receptor (FcR). The humanized antibodies have at least a portion of a complementarity determining region (CDR) derived from a non-human antibody, e.g., murine, with the remaining portions being human in origin. The use of humanized antibodies rather than
20 murine antibodies in human therapy should alleviate some of the problems associated with the use of some murine monoclonal antibodies because only the substituted CDRs will be foreign to a human host's immune system.

The present invention further pertains to the use of humanized antibodies specific to an FcR as components in heteroantibodies, bifunctional antibodies, or
25 immunotoxins. The humanized antibody specific to an FcR may be used in the same manner and for the same purpose as its corresponding murine counterpart. For example, the humanized anti-Fc receptor antibody of this invention can be used to treat cancer, allergies, and infectious and autoimmune diseases. Diagnostic applications of the antibodies include their use in assays for FcRI levels and assays for substances that influence FcR levels.

30

Brief Description of the Drawings

Figure 1 compares the amino acid sequences of murine 022 VH (SEQ ID NO:3) with the amino acid sequences of humanized NEWM-based VH (022 NMVH) (SEQ ID NO:1) and humanized KOL-based VH (022 KLVH) (SEQ ID NO:2). The CDRs are
35 boxed. Murine residues retained in the human portion are indicated by the inverted black triangles.

Figure 2 compares the amino acid sequences of murine 022 VK (SEQ ID NO:28) with humanized REI-based VK (022 HuVK) (SEQ ID NO:4). The CDRs are boxed. Murine residues retained in the human portion are indicated by the inverted black triangles.

Figure 3 depicts the vector used for expression of the humanized or chimeric 022 heavy chain gene.

Figure 4 depicts the vector used for expression of the humanized or chimeric 022 kappa chain gene.

Figure 5 depicts the binding of the test antibodies in the enzyme linked immunoassay described in the example.

Detailed Description

The present invention pertains to a humanized antibody specific for an Fc receptor. The humanized antibody is made up of a human antibody having at least a portion of a complementarity determining region (CDR) derived from a non-human antibody. The portion is selected to provide specificity of the humanized antibody for a human Fc receptor. The humanized antibody has CDR's derived from a non-human antibody and the remaining portions of the antibody molecule are human.

The antibody may be a complete antibody molecule having full length heavy and light chains or any fragment thereof, e.g., Fab or (Fab')₂ fragment. The antibody further may be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner et al. (U.S. Patent No. 4,946,778, issued August 7, 1990), the contents of which is expressly incorporated by reference.

The human antibody of the present invention may be any human antibody capable of retaining non-human CDRs. The preferred human antibody is derived from known proteins NEWM and KOL for heavy chain variable regions (VHs) and REI for Ig kappa chain, variable regions (VKs). These proteins are described in detail in the examples below.

"Complementarity determining region" (CDR) is an art recognized terminology and the technique used for locating the CDRs within the described sequences also is conventional.

The portion of the non-human CDR inserted into the human antibody is selected to be sufficient for allowing binding of the humanized antibody to the Fc receptor. A sufficient portion may be selected by inserting a portion of the CDR into the human antibody and testing the binding capacity of the created humanized antibody using the enzyme linked immunosorbent assay (ELISA) described in the examples below.

All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the

humanized antibody to the Fc receptor. The exemplified non-human CDR is derived from a murine antibody, particularly the CDR is derived from a monoclonal antibody (mab), mab 22. The mab 22 antibody is specific to the Fc receptor and further is described in U.S. patent application Serial No. 07/151,450, filed on February 2, 1988, and in Fanger et al. (U.S. Patent
5 No. 4,954,617, issued September 4, 1988). The entire contents of the aforementioned pending application and issued patent are expressly incorporated by reference.

The CDRs are derived from a non-human antibody specific for a human Fc receptor. The CDRs can be derived from known Fc receptor antibodies such as those discussed in the Fanger et al. patent application and issued patent cited above (hereinafter
10 Fanger et al.). The CDR may be derived from an antibody which binds to the Fc receptor at a site which is not blocked by human immunoglobulin G. The antibody also may be specific for the high affinity Fc receptor for human immunoglobulin G. Examples of antibodies from which the non-human CDRs may be derived are mab 32, mab 22, mab 44, mab 62, mab 197 and anti-FcRI antibody 62. The humanized mab 22 antibody producing cell line has been
15 deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession no. CRL 11177.

The present invention also pertains to bifunctional antibodies or heteroantibodies having at least one humanized antigen binding region derived from a humanized anti-Fc receptor antibody and at least one antigen binding region specific for a
20 target epitope. The humanized antigen binding region may be derived from a humanized anti-Fc receptor antibody as described above. Bifunctional and heteroantibodies having an antibody portion specific for an Fc receptor are described in detail by Fanger et al.

It should be understood that the humanized antibodies of the present invention may be used in the same manner, e.g., as components of immunotoxins or heteroantibodies,
25 as their corresponding non-humanized counterparts described by Fanger et al. The humanized antibodies further share the same utilities as their non-humanized counterparts. All aspects of the teachings of the Fanger et al. application and patent are incorporated by reference.

The humanized antibody of the present invention may be made by any method
30 capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on March 26, 1987), the contents of which is expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed
35 mutagenesis as described in the examples below.

The humanized antibody of the present invention may be made as described in the brief explanation below. A detailed method for production is set forth in the examples. It should be understood that one of ordinary skill in the art may be able to substitute known

conventional techniques for those described below for the purpose of achieving the same result. The humanized antibodies of the present invention may be produced by the following process:

- 5 (a) constructing, by conventional techniques, an expression vector containing an operon with a DNA sequence encoding an antibody heavy chain in which the CDRs and such minimal portions of the variable domain framework region that are required to retain antibody binding specificity are derived from a non-human immunoglobulin, and the remaining parts of the antibody chain are derived from a human immunoglobulin, thereby producing the vector of the invention;
 - 10 (b) constructing, by conventional techniques, an expression vector containing an operon with a DNA sequence encoding a complementary antibody light chain in which the CDRs and such minimal portions of the variable domain framework region that are required to retain donor antibody binding specificity are derived from a non-human immunoglobulin, and the remaining parts of the antibody chain are derived from a human immunoglobulin,
 - 15 thereby producing the vector of the invention;
 - (c) transfecting the expression vectors into a host cell by conventional techniques to produce the transfected host cell of the invention; and
 - (d) culturing the transfected cell by conventional techniques to produce the altered antibody of the invention.
- 20 The host cell may be cotransfected with the two vectors of the invention, the first vector containing an operon encoding a light chain derived polypeptide and the second vector containing an operon encoding a heavy chain derived polypeptide. The two vectors contain different selectable markers, but otherwise, apart from the antibody heavy and light chain coding sequences, are preferably identical, to ensure, as far as possible, equal
- 25 expression of the heavy and light chain polypeptides. Alternatively, a single vector may be used, the vector including the sequences encoding both the light and the heavy chain polypeptides. The coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both.
- The host cell used to express the altered antibody of the invention may be
- 30 either a bacterial cell such as Escherichia coli, or a eukaryotic cell. In particular a mammalian cell of a well defined type for this purpose, such as a myeloma cell or a Chinese hamster ovary cell may be used.
- The general methods by which the vectors of the invention may be constructed, transfection methods required to produce the host cell of the invention and
- 35 culture methods required to produce the antibody of the invention from such host cells are all conventional techniques. Likewise, once produced, the humanized antibodies of the invention may be purified according to standard procedures of the art, including cross-flow

filtration, ammonium sulphate precipitation, affinity column chromatography, gel electrophoresis and the like.

It should be understood that the humanized antibodies of this invention perform in a manner which is the same or similar to that of the non-humanized versions of the same antibodies. It also is noted that the humanized antibodies of this invention may be used for the design and synthesis of either peptide or non-peptide compounds (mimetics) which would be useful for the same therapy as the antibody (Saragobi et al., Science 253:792-795 (1991)), the contents of which is expressly incorporated by reference.

The following examples are provided as a further illustration of the present invention and should in no way be construed as being limiting.

EXAMPLES

In the following examples all necessary restriction and modification enzymes, plasmids and other reagents and materials were obtained from commercial sources unless otherwise indicated.

In the following examples, unless otherwise indicated, all general recombinant DNA methodology was performed as described in "Molecular Cloning, A Laboratory Manual" (1982) Eds T. Maniatis et al., published by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, the contents of which is expressly incorporated by reference.

In the following examples the following abbreviations were employed:

	dCTP	deoxycytidine triphosphate
	dATP	deoxyadenosine triphosphate
	dGTP	deoxyguanosine triphosphate
25	dTTP	deoxythymidine triphosphate
	DTT	dithiothreitol
	C	cytosine
	A	adenine
	G	guanine
30	T	thymine
	PBS	phosphate buffered saline
	PBST	phosphate buffered saline containing 0.05% Tween 20 (pH 7.5)

Example 1 - Production of Humanized Antibodies Specific for an Fc Receptor

The source of the donor CDRs used to prepare the humanized antibody was a murine monoclonal antibody, mab 22, which is specific for the Fc receptor. A mab 22

hybridoma cell line (022WCL-1) was established. Cytoplasmic RNA was prepared from the mab 22 cell line using the method described by Favoloro et al. (Methods in Enzymology 65, 718-749 (1980)), the contents of which is expressly incorporated by reference. The cDNA was synthesized using IgG1 and kappa constant region primers. The primer CG1FOR was used for the heavy chain variable (VH) region and the primer CK2FOR was used for the Ig kappa chain variable region (VK). The cDNA synthesis reactions mixtures consisted of 1 µg RNA, 0.5µM CG1FOR or CK2FOR, 250 µM each of dATP, dCTP, dGTP, and dTTP, 50 mM Tris HCl (pH 7.5), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂ and 20µ RNAguard (sold by Pharmacia, Milton Keynes, U.K.) in a total volume of 50 µl. The samples were heated at 72°C for two minutes and slowly cooled to 37°C. Murine moloney leukemia virus reverse transcriptase (100 µl - sold by Life Technologies, Paisley, U.K.) was added to the samples and the transcriptase containing samples were incubated at 42°C for sixty minutes.

VH and VK cDNAs were then amplified using the polymerase chain reaction (PCR) as described by Saiki et al. (Science 239, 487-491 (1988)), the contents of which is expressly incorporated by reference. The primers used in the above steps were as follows:

CG1FOR (SEQ ID NO:5) 5' GGAAGCTTAGACAGATGGGGGTGTCGTTTTG 3'
 VH1FOR (SEQ ID NO:6) 5' TGAGGAGACCGGTGACCGTGGTCCCTTGGCCCCAG 3'
 20 VH1BACK (SEQ ID NO: 7) 5' AGGTSMARCTGCAGSAGTCWGG 3'
 SH1BACK (SEQ ID NO:8) 5' TGGAATTCATGGRATGGAGCTGGRTCWTBHTCTT 3'
 SH2BACK (SEQ ID NO:9) 5' TGGAATTCATGRACCTCDGGYTCAACTKRRTTT 3'
 CK2FOR (SEQ ID NO:10) 5' GGAAGCTTGAAGATGGATACAGTTGGTGCAGC 3'
 25 VK1BACK (SEQ ID NO:11) 5' GACATTCAGCTGACCCAGTCTCCA 3'
 VK5BACK (SEQ ID NO:12) 5' TTGAATTCGGTGCCAGAKCWSAHATYGTKATG 3'
 VK6BACK (SEQ ID NO:13) 5' TTGAATTCGGTGGCAGAKCWSAHATYGTKCTC 3'
 VK7BACK (SEQ ID NO:14) 5' TTGAATTCGGAGCTGATGGGAACATTGTAATG 3'

30 Restriction sites incorporated in primers to facilitate cloning are underlined.

The PCR amplification of murine Ig DNA was conducted using the methodology described by Orlandi et al. (Proc. Natl. Acad. Sci USA 86, 3833-3838 (1989)), the contents of which is expressly incorporated by reference. The DNA/primer mixtures consisted of RNA/cDNA hybrid (10 µl) and 25pmol each of CG1FOR and SH1BACK or SH2BACK for PCR amplification of VH. The DNA/primer mixtures consisted of RNA/cDNA hybrid (10 µl) and 25pmol each of CK2FOR and VK1BACK, VK5BACK, VK6BACK, VK7BACK for PCR amplification of VK. dATP, dCTP, dGTP and dTTP (250

5 μM each), 10mM Tris HCl (pH 8.3), 60mM KCl, 1.5mM MgCl₂, 0.01% (w/v) gelatin, 0.01% (v/v) Tween 20, 0.01% (v/v) NP40 and 2.5μ Amplitaq (sold by Cetus, Beaconsfield, U.K.) were added to the samples in a final volume of 50 μl. The samples were subjected to 25-30 thermal cycles of PCR at 94°C for thirty seconds, 55°C for thirty seconds, 72°C for one minute and a final cycle at 72°C for five minutes.

10 The amplified VH and VK DNAs were run on a low melting point agarose gel and purified by Elutip-d column chromatography (sold by Schleicher and Schueell, Anderman, Walton, U.K.) for cloning and sequencing. The purified VH DNAs were cut with Eco I or Pst I and Hind III and cloned into M13mp18 and mp19 (sold by Pharmacia, Milton Keynes, U.K.). The purified VK DNAs were cut with Pvu II or Eco I and Hind III and cloned into M13mp18 and mp19. For general cloning methodologies see Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)), the contents of which are expressly incorporated by reference. The resulting collection of clones were sequenced by the dideoxy method using T7 DNA polymerase (sold by Pharmacia, Milton Keynes, U.K.) as described by Sanger et al. 15 (Proc. Natl. Acad. Sci. USA 74, 5463-5467, (1979)), the contents of which are expressly incorporated by reference.

20 From the sequences of the O22 VH and VR domains the CDR sequences were determined with reference to the database of Kabat et al. ("Sequences of Proteins of Immunological Interest" US Department of Health and Human Services, US Government Printing Office), the contents of which is expressly incorporated by reference, and utilizing computer assisted alignment with other VH and VK sequences.

25 Transfer of the murine O22 CDRs to human frameworks was achieved by oligonucleotide site-directed mutagenesis as described by Nakamye et al. (Nucleic Acids Res 14, 9679-9687 (1986)), the contents of which is expressly incorporated by reference. The primers used were as follows:

30 KLVHCDR1 (SEQ ID NO:15): 5' TGCCTGTCTCACCCAATACATGTAA
TTGTCACTGAAATGAAGCCAGACGMGGAGCGGACAG

 KLVHCDR2 (SEQ ID NO:16): 5' TGTAATCTTCCCTTCACACTGTCTGGATAGTA
GGTGTAACCTACCACTCACTAATGGTTGCAACCCACTCAGG

35 KLVHCDR3 (SEQ ID NO:17): 5' GGGGTCCCTTGGCCCCAGTAGTCCATAGC
CCCCTCGTACCTATAGTAGCCTCTTGCAAAAAATAGA

 NMVHCDR1 (SEQ ID NO:18): 5' TGGCTGTCTCACCCAATACATGTAATTGT
CGCTGAAAATGAAGCCAGACACGGTGCAGGTCAGGCTCA

NMVHCDR2 (SEQ ID NO:19): 5' TTGCTGGTGTCTCTCAGCATTGTCACTCTC
CCCTTCACACTGTCTGGATAGTAGGTGTAACCTACCACCA
TCACTAATGGTTCCAATCCACTCAA

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NMVHCDR3 (SEQ ID NO:20): 5' AGACGGTGACCAAGGACCCTTGGCCCCAG
TAGTCCATAGCCCCCTCGTACCTATAGTAGCCTCTTGACAATAATAG

HuVKCDR1 (SEQ ID NO:21): 5' CTTCTGCTGGTACCAGGCCAAGTAGTTCTTC
10 TGATTTGAACTGTATAAAACACTTTGACTGGACTTACAGGTGATGGTCAC

HuVKCDR2 (SEQ ID NO:22): 5' GCTTGGCACACCAGATTCCCTAGTGGATG
CCCAGTAGATCAGCAG

15 HuVKCDR3 (SEQ ID NO:23): 5' CCTTGGCCGAACGTCCACGAGGAGAGGTAT
TGATGGCAGTAGTAGGTGG

The primer for NMVHCDR1 was extended to include a change of NEWM residues Ser 27
20 Thr 28 to Phe 27 Ile 28. The primer for NMVHCDR2 was extended to include a change of
NEWM residue Val 71 to Arg 71.

The DNA templates used for mutagenesis of VHs comprised human
framework regions from the crystallographically solved protein NEW described by Saul et al.
(J. Biol. Chem. 53, 585-597 (1978)) or KOL described by Schmidt et al. (Z. Physical Chem.
25 364, 713-747 (1983)). The DNA templates used for mutagenesis of VKs comprised human
framework regions from the crystallographically solved protein REI described by Epp et al.
(Eur. J. Biochem. 45, 513-524 (1974)). The contents of each of the forementioned references
are expressly incorporated by reference.

M13 based templated M13VHPCR1 (for NEWMVH), M13VHPCR2 (for
30 KOLVH) and M13VKPCR2 (for REIVK) comprising human frameworks with irrelevant
CDRs were prepared as described by Riechmann et al. (Nature 332, 323-327 (1988)), the
contents of which are expressly incorporated by reference. Oligonucleotide site-directed
mutagenesis was carried out using the following protocol. A 5-fold molar excess of each
phosphorylated mutagenic oligonucleotide was added along with the universal M13
35 sequencing primer (5'- GTAAAACGACGGCCAGT) (SEQ ID NO:24). All of the primers
were annealed in 20ul 0.1M TrisHCl (pH8.0) and 10mM MgCl₂ by heating to 70-85°C for
two minutes and slowly cooling to room temperature. 10 mM DTT, 1 mM ATP, 40 µM each
of dATP, dCTP, dGTP and dTTP, 2.5µ T7 DNA polymerase (sold by United States

Biochemicals) and 0.5 μ T4DNA ligase (sold by Life Technologies, Paisley, U.K.) was added to the annealed DNA in a reaction volume of 30 μ l and incubated at 22° - 37°C for one to two hours. The newly extended and ligated strand was preferentially amplified over the parental strand in a thermostable DNA polymerase directed reaction using the M13 reverse sequencing primer (5' AACAGCTATGACCATG) (SEQ ID NO:25). The reverse sequencing primer is not complementary to the parental strand. The reaction mixture of 50 μ l contained 1 μ l extension/ligation product, 25 pmol M13 reverse sequencing primer, 250 μ M each of dATP, dCTP, dGTP and dTTP, 1 μ Vent DNA polymerase (sold by New England Biolabs, Bishop's Stortford, U.K.) or 2.5 μ Amplitaq (sold by Cetus, Beaconsfield, U.K.) in the appropriate buffer supplied by the enzyme manufacturer and was subjected to thirty thermal cycles of 94°C, 30s, 55°C, 30s, 75° or 72°C, 90s; ending with 5 min at 72°C. A 4 μ l aliquot of this sample was then amplified by PCR using both M13 universal and reverse sequencing primers in a reaction mixture of 50 μ l containing 25 pmol of each primer, 250 μ M each of dATP, dCTP, dGTP and dTTP, 2.5 μ Amplitaq (Cetus) in the buffer supplied by the enzyme manufacturer. Amplified DNAs were digested with HindIII and BamHI and cloned into M13mp19 and sequenced.

Mutagenesis of M13VHPCR2 KOL VH residue Leu71 to Arg71 was by the overlap/extension PCR method of Ho *et al.* (*Gene*, 77, 51-55 (1989)), the contents of which is expressly incorporated by reference. The overlapping oligonucleotides used were 5' - TTTACAATATCGAGACAACAGCAA (SEQ ID NO:26) and 5' - TTGCTGTTGTCTCTCGATTGTAAA (SEQ ID NO:27).

The amino acid sequences of the humanized antibodies were compared to the known murine antibodies as shown in Figures 1 and 2. The CDR replaced VH and VK genes were cloned into expression vectors pSVgpt and pSVhyg as shown in Figures 3 and 4 as described by Orlandi *et al.* (cited *supra*). The CDR replaced NEWMVH and KOLVH genes together with the Ig heavy chain promoter, appropriate splice sites and signal peptide sequences were excised from M13 by digestion with HindIII and BamHI and cloned into the pSVgpt expression vector containing the murine Ig heavy chain enhancer, the gpt gene for selection in mammalian cells and genes for replication and selection in *E. coli*. The plasmid also contains a human IgG1 constant region as described by Takahashi *et al.* (*Cell* 29, 671-675 (1982)). The construction of the kappa chain expression vector was essentially the same except that the gpt gene was replaced by the hygromycin resistance gene and contains a human kappa constant region (Hieter *et al.*, *Cell* 22, 197-207 (1980)). The contents of each of the forementioned references are expressly incorporated by reference.

Approximately 5 μ g of each heavy chain expression vector and 10 μ g of the kappa chain expression vector were digested with PvuI. The DNAs were mixed together, ethanol precipitated and dissolved in 25 μ l water. Approximately 5-10 x 10⁶ NSO cells (from European Collection of Animal Cell Cultures, Porton Down, U.K.) were grown to semi-

confluency in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (Myoclon plus, Gibco, Paisley, Scotland), harvested by centrifugation and resuspended in 0.5ml DMEM together with the digested DNA in a cuvette. After five minutes in ice, the cells were given a single pulse of 170V at 960 μ F (Gene-Pulser, Bio-Rad, Richmond, CA) and left in ice for a further twenty minutes. The cells were then put into 20ml DMEM + supplemented with 10% FCS and allowed to recover for twenty-four to forty-eight hours. After this time, the cells were distributed into a 24-well plate and selective medium was applied (DMEM, 10% FCS, 0.8 μ g/ml mycophenolic acid and 250 μ g/ml xanthine). After three to four days, the medium and dead cells were removed and replaced with fresh selective medium. Transfected clones were visible with the naked eye ten days later.

The presence of human antibody in the medium of wells containing gpt+ transfectants was measured using conventional enzyme linked immunosorbent assay (ELISA) techniques. Wells of a microtitre plate (Immolon, Dynatech, Chantilly, VA) were coated with 100ng goat anti-human IgG antibodies (SeraLab, Crawley Down, U.K.) in 100 μ l 50mM carbonate buffer pH9.6. After washing with PBST (Phosphate buffered saline pH 7.2 containing 0.05% Tween 20) culture medium in 100 μ l PBST (5-50 μ l) was added to each well for one hour at 37°C. The wells were then emptied, washed with PBST and 100 μ l of 1:1000 dilution peroxidase conjugated goat anti-human kappa constant region antibodies (SeraLab, Crawley Down, U.K.) were added for one hour at 37°C. The wells were emptied, washed with PBST and 100 μ l OPD substrate buffer (400 μ g/ml Q-phenylenediamine in 24mM citrate/42mM sodium phosphate pH 5. and 0.0003% (v/v) H₂O₂) was added. The reaction was stopped after a few minutes by the addition of 12.5% H₂SO₄ (25 μ l) and the absorbance at 492nm was measured.

The antibody secreting cells were expanded and antibody was purified from the culture medium by protein A affinity chromatography as described by Harlow and Lane (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), the contents of which is expressly incorporated by reference.

The binding of the antibodies to antigen was measured by ELISA. Wells of a microtitre plate (Immulon 1, Dynatech, Chantilly, VA) were coated with 200ng goat anti-human IgM antibodies (Sera-lab, Crawley Down, U.K.) in 100 μ l 50mM carbonate buffer pH 9.6 at 37°C for at least one hour. Wells were emptied and washed once with PBST and blocked with 1% BSA in PBS at room temperature for thirty minutes. The wells were emptied and washed with PBST and Cos supernatant containing FcRI/IgM fusion protein was added and incubated for one hour at room temperature. Wells were then emptied and washed three times with PBST and test antibodies diluted in 1% BSA/PBS were added and incubated for one hour at room temperature. In addition, each well contained 2 μ g human IgG1, lambda antibody (Sigma, Poole, U.K.) The wells were then emptied, washed three times with PBST and 40ng peroxidase goat anti-human kappa constant region antibodies (Sera-Lab, Crawley

A Tetravalent Single-chain Antibody-Streptavidin Fusion Protein for Pretargeted Lymphoma Therapy¹

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ABSTRACT

Single-chain Fv antibody fragments from the CD20-specific murine monoclonal antibody B9E9 were genetically engineered as streptavidin fusions [single-chain Fv-streptavidin (scFvSA) fusion protein] for use in pretargeted radioimmunotherapy. The scFvSA constructs were expressed as soluble, tetrameric species in the periplasm of *Escherichia coli*. Expression levels were affected by the order of the variable regions and the length and composition of the single-chain Fv linker. The best expressor was obtained with the variable regions in the heavy chain-light chain configuration separated by a 25-mer Gly₄Ser linker. This construct produced 250–300 mg of soluble, tetrameric fusion protein per liter of fermentor culture. The fusion protein (*M_r* 173,600) was purified from crude lysates by iminobiotin affinity chromatography with an overall yield of about 50% and was analyzed for functionality both *in vitro* and *in vivo*. Immunoreactivity of the scFvSA fusion protein and its nanomolar affinity to CD20-positive Ramos cells were comparable with the B9E9 monoclonal antibody. The fusion protein had a biotin dissociation rate identical to recombinant streptavidin and bound an average of 3.6 biotins/molecule of a possible 4 biotins/molecule. Labeled fusion protein cleared from the blood of BALB/c mice with a β half-life of about 16 h. In nude mice bearing Ramos xenografts, the fusion protein demonstrated sufficient tumor localization of functional streptavidin to enable efficient, tumor-specific targeting of a subsequently administered radionuclide-chelate/biotin molecule. These results suggest that large quantities of functional scFvSA can be produced for clinical testing as a therapy for non-Hodgkin's lymphoma.

INTRODUCTION

mAbs³ directed against the CD20 antigen are finding wide application as a means to target radiation to B-cell tumors of NHL. The CD20 antigen is a nonglycosylated phosphoprotein present on the surface of normal peripheral B-cell lymphocytes, malignant cells of pre-B-cell acute lymphoblastic leukemia, malignant cells of B-type chronic leukemia, and B-cell lymphomas. Compared with carcinomas, lymphomas and leukemias generally provide better access to target antigen, and the circulating disease cells can experience direct cytotoxic effects via the binding of whole antibodies. These qualities, along with the inherent radiosensitivity of hematological malignancies, have made RIT of lymphomas an application with established clinical efficacy (1–3). The clinical studies of Press *et al.* (4) and Liu *et al.* (5) have documented both the steep dose-response curve for radiation in NHL and the benefit of increased radiation dose in prolonging disease-free survival. Pretarget[®] RIT offers the potential to achieve the benefits of high-dose radiation without the need for stem

cell rescue. This is accomplished by injecting the antibody and radiation source separately, thereby decoupling the pharmacokinetics of the antibody from the radionuclide (6).

Pretargeted RIT is a multistep process that exploits the high-affinity interaction between streptavidin and biotin ($K_a \approx 10^{-15}$ M). In the first step of the process, an antibody linked to streptavidin is administered systemically. After sufficient time has elapsed to enable peak tumor uptake, the excess antibody/streptavidin is removed from the circulation by *in vivo* complexation with a biotinylated poly(GalNAc) "clearing" agent. The resultant complexes are excreted via the liver. The clearing step is essential to achieve the highest absolute concentration of antibody receptor at tumor sites but still maintain low absolute blood and whole body concentrations. Radiation is delivered to the tumor in a third step by administration of radiolabeled DOTA-biotin. This low molecular weight cytotoxic molecule readily penetrates into the tumor, where it is captured by prelocalized antibody-streptavidin conjugate. Unbound radioactivity is eliminated from the body via the urine. Rapid uptake of the therapeutic isotope at tumor sites (before significant loss of potency attributable to radioactive decay) and efficient renal elimination of excess radioactivity represent the fundamental advantages of the pretargeted approach as compared with conventional RIT, in which the radioisotope is directly conjugated to the antibody. This approach has been tested preclinically in mice, producing cures of human small cell lung, colon, and breast cancer xenografts (6), and clinically in patients with adenocarcinoma (7–9) and NHL (10).

In contrast to antibody-streptavidin chemical conjugates, a genetically fused targeting agent has a well-defined, homogeneous composition and is simpler and less expensive to manufacture. Here, we report the genetic engineering and *in vitro* and *in vivo* characterization of *Escherichia coli*-produced B9E9 scFvSA fusion protein for use as a first component in pretargeted RIT.

MATERIALS AND METHODS

Genetic Constructions. Total RNA (SNAP kit; Invitrogen) was prepared from B9E9 hybridoma cells expressing murine IgG2a anti-CD20 mAb (Bioprobe BV, Amstelveen, the Netherlands). The cDNAs for the V_L (κ) and V_H of B9E9 were obtained by a reverse transcription reaction using oligonucleotides for the antibody constant regions (5'-TAGCTGGCGGCCCGCTTGAAGCTCTTGACAAT for V_L and 5'-TAGCTGGCGGCCCGCTTCTGTCCACCTTGCTGC for V_H). The variable regions were PCR-amplified from the cDNA using the constant region primers and degenerate variable region primers (5'-TGCCGTGAATTCATTGCTGACCTGCTC for V_L and 5'-TGCCGTGAATTCGTSMACTGACGAGSARTCWGG for V_H). The DNA sequences of the variable regions have been deposited in the GenBank database (accession numbers AF277091 for V_H and AF277092 for V_L). The PCR fragments were cloned into an *EcoRI*/*NotI*-digested vector and sequenced using a BigDye kit (PE Applied Biosystems). The NH₂-terminal amino acids of the variable regions were reconstituted based on the consensus sequence (11), including introduction of a serine residue at position 5 in the V_L gene. Primers used were 5'-TTACGGCCATGCTGACATCGTGTGCTGCGAGTCTCCAGCAATCTGTCT and 5'-CACGAGATCTTCAGCTCCAGCTTGGTCCCA for V_L and 5'-CGGAGGCTCAGACCAAGTTTCAGCTGGTCCAGTCAGGGGCTGAGCTGGTGAAG and 5'-GAGCCAGAGCTCACGGTGACCGTGGTCCCTGCGCCCCA for V_H.

The variable regions were ordered in the V_H-V_L or V_L-V_H configuration and

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³ The abbreviations used are: mAb, monoclonal antibody; NHL, non-Hodgkin's lymphoma; RIT, radioimmunotherapy; scFv, single-chain Fv; scFvSA, scFv-streptavidin; V_L, variable light chain; V_H, variable heavy chain; IPTG, isopropyl- β -D-galactopyranoside; HPLC, high performance liquid chromatography; GalNAc, N-acetyl galactosamine; DOTA, 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid; % ID/g, percentage of injected dose per gram; AUC, area under the curve.

separated by linkers of various lengths or compositions (Table 1). The streptavidin coding region, leader sequence, and approximately 310 bp of the upstream region were PCR-amplified from *Streptomyces avidinii* (ATCC 27419; Ref. 12). The streptavidin gene was separated from the scFv gene by a linker encoding amino acids GSGSA. Plasmids contained a *lac* promoter, a pBR origin of replication, and a gene encoding either β -lactamase (*bla*; ampicillin resistant) or aminoglycoside 3'-phosphotransferase (*neo* from Tn5; kanamycin resistant).

Expression. Transformants of *E. coli* strain XL1Blue (Stratagene, La Jolla, CA) were grown overnight at 30°C in Terrific broth (20 ml; Sigma) containing carbenicillin (50 μ g/ml). The culture was diluted 100-fold into fresh medium and grown in a shaking incubator at 30°C. When the culture attained an $A_{600\text{ nm}}$ of 0.3–0.5, IPTG (Amersham Pharmacia Biotech, Piscataway, NJ) was added to a final concentration of 0.2 mM, and incubation was continued overnight. Periplasmic extracts were prepared for analysis of the scFvSA expression level. Cells were resuspended in an ice-cold solution of 20% sucrose, 2 mM EDTA, 30 mM Tris (pH 8.0), and 2.0 mg/ml lysozyme and incubated on ice for 30 min. Supernatants were analyzed on 4–20% Tris-glycine SDS-PAGE gels (Novex) under nonreducing, nonboiled conditions, and gels were stained with Coomassie Blue.

For fermentation cultures, the primary inoculum (50 ml) was grown overnight at 30°C in a shake flask containing Terrific broth plus kanamycin or carbenicillin (50 μ g/ml), depending on the selectable marker of the plasmid. The culture was then diluted 100-fold into the same medium and grown at 30°C for an additional 4–5 h. This secondary inoculum (0.5 liter) was transferred to a 14 liter BioFlo 3000 fermentor (New Brunswick Scientific) containing 8 liters of complete *E. coli* medium [per liter: 6 grams of Na_2HPO_4 , 3 grams of KH_2PO_4 , 0.5 gram of NaCl, 3 grams of $(\text{NH}_4)_2\text{SO}_4$, 48 grams of yeast extract (Difco), 0.25 ml of Mazu DF204 antifoam (PPG Industries Inc., Pittsburgh, PA), 0.79 gram of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.044 gram of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 3 ml of trace elements (per liter: 0.23 gram of CoCl_2 , 0.57 gram of H_3BO_3 , 0.2 gram of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 3.5 grams of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 4.0 grams of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 gram of ZnCl_2 , 1.35 grams of thiamine, and 0.5 gram of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$]. The medium contained galactose at an initial concentration of 5 grams/liter as a carbon source plus 50 μ g/ml kanamycin or carbenicillin for plasmid retention. The culture was grown at 30°C and induced with IPTG (0.2 mM) at 6 h postinoculation. The pH was maintained at 7.0 by the automatic addition of either phosphoric acid or NaOH. Dissolved oxygen concentration was maintained at $\geq 30\%$ throughout the run, using agitation speeds of 400–800 rpm and oxygen supplementation as necessary. A galactose solution (50%) was fed to a total of 20–25 grams/liter over a 9-h period after exhaustion of the initial galactose present in the medium. Cells were harvested at 24–26 h postinoculation in a continuous flow centrifuge (Pilot Powerfuge; Carr Separations, Franklin, MA), washed with PBS [10 mM sodium phosphate and 150 mM NaCl (pH 7.2)], and pelleted by centrifugation. A typical fermentation produced 80–90 grams of cells (wet weight) per liter of culture medium.

A rhodamine-biotin assay was used for quantifying the fusion protein in fermentor-grown cells. Cells were washed twice in PBS, resuspended in ice-cold 30 mM Tris and 1 mM EDTA (pH 8.0) to 20% (w/v), and disrupted through two cycles of microfluidization (Microfluidics International, Newton, MA). The fusion protein in centrifuged lysates was complexed with excess rhodamine-derivatized biotin, which was prepared as follows: 5 (and 6)-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene

OR) was coupled to biocytin (Pierce, Rockford IL) through the formation of a stable amide bond. The reaction mixture was purified by HPLC using a Dynamax semipreparative C-18 column (Rainin Instrument Co., Woburn, MA). The effluent was monitored at 547 nm, and peak fractions were collected and analyzed by mass spectrometry. Fractions corresponding in molecular weight to biocytin-rhodamine conjugate were pooled and concentrated by roto-evaporation (Büchi, Flawil, Switzerland). An excess of purified biocytin-rhodamine conjugate was added to the supernatant of a centrifuged sample of crude lysate and analyzed by size exclusion chromatography using a Zorbax GF-250 column (MAC-MOD, Chadds Ford, PA) equilibrated in 20 mM sodium phosphate containing 15% DMSO at a 1.0 ml/min flow rate. The effluent was monitored at 547 nm using a Varian Dynamax PDA-2 detector, and the peak area corresponding to fusion protein elution was determined using a Varian Dynamax HPLC Data System (Walnut Creek, CA). The concentration of fusion protein in the crude lysate was calculated by comparison with a standard analyzed under the same conditions. The molar extinction coefficient for the fusion protein standard was calculated using a previously described method summing the relative contributions of amino acids absorbing at 280 nm (13).

Purification. The iminobiotin affinity matrix used for the isolation of fusion protein was prepared by reacting epoxide-activated Macro-prep matrix (Bio-Rad, Hercules, CA) with 112 μ mol of *N*-(3-amino-propyl)-1,3 propane diamine (Sigma) per gram of matrix in 0.2 M carbonate buffer. The reaction was stopped after 8 h by filtering the slurry through a sintered glass funnel and rinsing the matrix with distilled water. Residual epoxides were inactivated by reacting the matrix with 0.1 M sulfuric acid for 4 h at 80°C, and the matrix was rinsed again. The amine-derivatized matrix was suspended in PBS, and the pH was increased to 8.5 by the addition of a 10% volume of 0.5 M sodium borate (pH 8.5). *N*-hydroxy-succinimide-iminobiotin (Pierce) was dissolved in DMSO and added to the suspended matrix at a ratio of 2.6 mg/gram of matrix. After a 4-h reaction, the matrix was rinsed with distilled water, followed by several alternating washes with sodium carbonate buffer (pH 11) and sodium acetate buffer (pH 4) and a final rinse with distilled water. The matrix was stored as a slurry in 20% ethanol.

Cells (650–750 grams, wet weight) from the fermentation culture were washed and lysed at a concentration of 20% (w/v) by microfluidization as described above. A 5% solution of polyethylenimine (M_n 1,200; Aldrich, Milwaukee, WI), adjusted to pH 8.0 with concentrated HCl, was added to the lysate (0.3 ml/g cells) and precipitated overnight at 4°C. The lysate solution was centrifuged (10,000 $\times g$ for 90 min) to pellet the cellular debris. The supernatant was decanted and filtered through a 0.2 μ m filter. Glycine was added to the clarified lysate solution to a final concentration of 50 mM, and NaCl was added to a final concentration of 0.3 M. The lysate solution was then applied over an iminobiotin affinity column equilibrated in 50 mM glycine (pH 9.2) containing 0.5 M NaCl. The column was washed with 20 column volumes of equilibration buffer and then eluted with 0.2 M sodium acetate buffer (pH 5.0) containing 0.1 M NaCl. The pH of the eluted product was neutralized with 0.5 M Tris buffer (pH 8.0), and then the product was exhaustively dialyzed in PBS at 4°C.

To reduce protein aggregation, the iminobiotin-purified scFvSA fusion protein was treated with 30% DMSO in PBS for 5–7 h at room temperature and dialyzed in PBS at 4°C. Preparations were concentrated to 2–3 mg/ml using a YM30 membrane (Millipore) and filter sterilized for aseptic storage at 4°C.

Biochemical Characterization. Purified fusion proteins were analyzed on 4–20% Tris-glycine SDS-PAGE gels under nonreducing conditions. Before electrophoresis, samples were mixed with SDS loading buffer and incubated at either room temperature or 100°C for 5 min. Gels were stained with Coomassie Blue, or the protein bands were transferred to polyvinylidene difluoride membranes (Novex). Immunoblot analysis was performed essentially as described previously (14). Peroxidase-conjugated goat anti-streptavidin polyclonal antibody (Zymed, San Francisco, CA) and TMB substrate (Vector Laboratories, Burlingame, CA) were used for detection.

Size exclusion HPLC (Beckman) was performed on a Zorbax GF-250 column with a 20 mM sodium phosphate/0.5 M NaCl mobile phase and a Varian Dynamax detector set at $\lambda_{280\text{ nm}}$. This system, connected in series with a Varian Star 9040 refractive index detector and a MiniDawn light-scattering instrument (Wyatt Technologies, Santa Barbara, CA), was also used to determine the molecular weight of the intact tetramer and aggregate. A dn/dc value of 0.185 for a protein in an aqueous buffer solution was used in the calculations

Table 1 Summary of expression levels of B9E9 scFvSA genetic variants

V_L - V_H -SA		V_H - V_L -SA	
Linker type ^a	Expression (mg/liter) ^b	Linker type	Expression (mg/liter)
15-mer G4S	+	15-mer G4S	60
18-mer G4S	++	18-mer G4S	195
25-mer G4S	++	25-mer G4S	300
35-mer G4S	++		
18-mer pKOD	40	18-mer pKOD	60
18-mer pKOD2	++	18-mer pKOD2	+++

^a G4S linkers encode repeating units of amino acids GGGGS; pKOD and pKOD2 linkers encode amino acids GLEGSPEAGLSPDAGSGS and GLEGSPEAGLSPD-AGSDS, respectively.

^b The expression levels of the fusion proteins were based on 8 liter fermentor runs except for those qualitative data designated as + symbols, which are based on SDS-PAGE analysis of shake flask cultures.

(15). Automated amino acid sequencing was performed using a Procise 494 sequenator (Applied Biosystems, Inc., Foster City, CA).

For molecular weight determination, liquid chromatographic separation was conducted with a Hewlett Packard series 1100 system fitted with a Jupiter C-18 column (300 Å; 3.2 × 50 mm; 5 µm) and a C-18 "SafeGuard" column (Phenomenex, Torrance, CA) at a flow rate of 500 µl/min. The mobile phase was composed of water/1% formic acid (buffer A) and acetonitrile/1% formic acid (buffer B). The gradient applied was 2% buffer B for 3 min, rising to 99% buffer B within 7 min. B9E9 scFvSA was eluted at a retention time of 8.7 min. The analytical column was interfaced with an electrospray ionization ion trap mass spectrometer (LSQ, Thermoquest, San Jose, CA). The instrument was calibrated with myoglobin and operated in the positive ion mode with the heated capillary set to 200°C and 5.1 kV applied to the electrospray needle. The data were acquired in a full scan mass spectrometry mode [m/z (500–2000 Da/z)]. The molecular weights and amino acid sequences of tryptic peptides generated from B9E9 scFvSA were determined by liquid chromatography-mass spectrometry/mass spectrometry as described by Covey *et al.* (16).

Relative immunoreactivity was assessed in a competitive binding assay using flow cytometry that measured the binding of fluorescein-labeled B9E9 mAb to the CD20-positive Ramos cell line (Burkitt lymphoma; ATCC CRL-1596) in the presence of various concentrations of unlabeled antibody or fusion. B9E9 mAb was labeled using fluorescein *N*-hydroxysuccinimidate, and an optimized amount of this conjugate was mixed with serial dilutions (3–200 µg/ml) of B9E9 mAb standard or molar equivalents of B9E9 scFvSA and incubated with 1×10^6 cells at 4°C for 30 min. Samples were washed and then analyzed on a single laser FACSCalibur (Becton Dickinson). After gating on single cells, the geometric mean fluorescence intensity was determined from a histogram plot of fluorescence. The concentration of competitor antibody required for IC_{50} of fluorescein-B9E9 binding was calculated using nonlinear regression analysis for one-site binding. The percentage of immunoreactivity was calculated according to the following formula: $(IC_{50} \text{ mAb} / IC_{50} \text{ scFvSA}) \times 100$.

Immunoreactivity of radiolabeled antibodies was determined by a cell binding assay. The B9E9 scFvSA fusion protein was stably radioiodinated using [^{125} I]-*N*-succinimidyl para-iodobenzoyl ester (NEN Research Products) according to the method of Wilbur *et al.* (17). ^{125} I-labeled B9E9 mAb or fusion protein was incubated with a fixed concentration of radiolabeled protein (50 ng/ml) and varying amounts of antigen (0.25 – 10×10^6 Ramos cells). Nonspecific binding was determined in the presence of excess cold mAb or fusion protein (50 µg/ml). After a 2-h incubation at ambient temperature, bound and free antibodies were separated by centrifugation through oil (1:1, dinonyl phthalate:dibutyl phthalate) and counted on a gamma counter. Immunoreactivity was calculated from nonlinear regression analysis of a plot of bound (specific + nonspecific binding)/total (bound + free) versus cell number (18).

Avidity was assessed using saturation binding experiments that measure specific binding of radiolabeled mAb or fusion protein (0.025–50 ng/ml) at equilibrium in the presence of excess antigen (10^7 cells). Nonspecific binding was determined in the presence of excess cold mAb or fusion protein (50 µg/ml). Mixtures were incubated and centrifuged as described above. The equilibrium dissociation constant (K_d) was calculated from nonlinear regression analysis of nanomolar bound versus nanomolar total radioligand using immunoreactivity-adjusted antibody concentrations (19). The calculated immunoreactivities for ^{125} I-labeled scFvSA and mAb were 79% and 67%, respectively.

The rate of biotin dissociation was determined at 37°C in 0.25 M sodium phosphate, 0.15 M NaCl, and 0.25% BSA (pH 7.0) containing 10 µM fusion protein or recombinant streptavidin (control), 0.06 µM [^{3} H]biotin (58 mCi/µmol; NEN Research Products) and 30 mM ascorbate as [^{3} H]biotin stabilizer. After incubation to reach equilibrium, biocytin (4 mM; Sigma) was added to initiate irreversible dissociation of [^{3} H]biotin. Aliquots were withdrawn periodically and diluted 20-fold in PBS containing 0.5% BSA. The samples were split for assessment of total and free [^{3} H]biotin, the latter determined after protein precipitation using sequential additions of ZnSO₄/NaOH (60 µM each). Radioactivity was assessed in a fluoroscintillate using a Hewlett Packard beta counter. Linear regression analysis of a plot of the natural logarithm (fraction bound) versus time yielded a dissociation rate constant.

Biotin binding capacity was determined by incubation of the fusion protein with a 9-fold molar excess of [^{3} H]biotin. The amount of [^{3} H]biotin associated

with the fusion protein was determined by liquid scintillation after the removal of uncomplexed biotin using a PD-10 size exclusion column (Pharmacia).

Immunohistology was conducted by PhenoPath Laboratories (Seattle, WA). Fluoresceinated B9E9 scFvSA, C2B8/SA chemical conjugate, B9E9 mAb, or a murine IgG2a isotype-matched control (Sigma F6522) was reacted with cryosections of normal and tumor human tissues, which were peroxidase blocked with 0.3% H₂O₂ and biotin blocked with Biotin Blocking System (DAKO X0590). Binding was detected with horseradish peroxidase-conjugated rabbit anti-FITC mAb (DAKO P0404).

Preclinical Pretargeting Studies. The B9E9 scFvSA fusion protein was labeled with ^{125} I as described above. Methods have been described previously for preparation of the synthetic clearing agent, designated as biotin-LC-NM-(GalNAc)₁₆ (20), radiolabeled DOTA-biotin (6), and the anti-CD20 C2B8/SA chemical conjugate (10). The murine CC49 scFvSA (V_H-V_L 25-mer) fusion protein, which was immunoreactive to TAG-72 antigen and used as a negative control, was prepared in house.⁴

All animal studies were conducted under the supervision of the NeoRx Animal Care and Use Committee. Disappearance of the fusion protein from the blood was assessed by measuring the amount of radioactivity in serially collected blood samples after i.v. injection of ^{125} I-labeled scFvSA (600 µg) in normal female BALB/cBkl mice (B&K Universal, Kent, WA). At 18 h postinjection, some mice received a single i.v. injection of the synthetic clearing agent (100 µg). Blood samples were obtained by retro-orbital bleeding.

Pretargeted RIT studies were conducted in female nude mice bearing well-established Ramos xenografts (100–400 mm³). Tumor-bearing Bkl: BALB/c/*nu/nu* nude mice were obtained by implanting 5 – 25×10^6 cultured cells s.c. in the flank 10–25 days before study initiation. Mice were injected i.v. at $t = -24$ h with ^{125} I-labeled B9E9 scFvSA (600 µg; 3.488 nmol; 5 µCi) or with the positive control C2B8/SA chemical conjugate (400 µg; 1.905 nmol; 5 µCi), followed 20 h later ($t = -4$) by 100 µg (11.558 nmol) of the synthetic clearing agent. ^{111} In-labeled DOTA-biotin (1.0 µg; 1.239 nmol; 10 µCi) was injected into each mouse 4 h after the clearing agent ($t = 0$).

In the ^{90} Y-DOTA-biotin studies, mice received i.v. injections of the unlabeled B9E9 scFvSA (600 µg; 3.488 nmol) or anti-TAG-72 murine CC49 scFvSA (600 µg; 3.488 nmol) and received an injection of 100 µg (11.558 nmol) of the synthetic clearing agent 20 h later. ^{90} Y-DOTA-biotin (1.0 µg; 1.239 nmol; 100 µCi) was injected into each mouse 4 h after the clearing agent. Groups of four mice per time point were bled and sacrificed at 2, 24, 48, and 120 h after the injection of radiolabeled DOTA-biotin. Whole organs and tissue were isolated and weighed, and radioactivity was assessed in a Packard Cobra-II gamma counter as described previously (6).

RESULTS

B9E9 scFvSA Design. The V_H and V_L DNA fragments were amplified from the B9E9 hybridoma cell line expressing the murine IgG2a anti-CD20 antibody. Comparison of the amino acid sequence of the B9E9 variable regions showed high homology with other well-characterized anti-CD20 antibodies, e.g., C2B8 and 1F5 (Fig. 1; Refs. 21 and 22). The V_H and V_L cDNAs were constructed as a scFv, which was fused to the full-length streptavidin gene of *Streptomyces avidinii* (Fig. 2). The scFv-streptavidin gene was designed for expression of soluble, tetrameric fusion protein in the *E. coli* periplasm, which has an oxidizing environment enabling disulfide bond formation. The tetrameric structure of the protein is derived from using streptavidin, which spontaneously forms a stable homotetramer resistant to proteolysis. The scFvSA gene encodes a mature protein of 421 amino acids with a calculated monomeric weight of M_r 43,400 (most abundant mass), whereas the soluble tetramer that forms in the periplasm is M_r 173,600.

***E. coli* Expression and Purification.** A number of genetic variants were constructed that contained scFv linkers of different lengths and composition or the variable regions in different order (Table 1). These constructs were analyzed first in shake flask cultures for expression,

⁴ J. Schultz, Y. Lin, J. Sanderson, and Y. Zuo, unpublished data.

A

B9E9 **d**IVLSQSPAILASAPGKVTMT**CRASSSVsy**mhwyQKPGSSPKFWIYATS
 C2B8 **q**IVLSQSPAILASAPGKVTMT**CRASSSVsy**lhwfQKPGSSPKFWIYATS
 1F5 **q**IVLSQSPAILASAPGKVTMT**CRASSSVsy**lhwfQKPGSSPKFWIYATS

B9E9 **N**LASGVParFSGSGSGTSYSLTISRVEAEDAATYyCqGwISNpPTFGaGTK
 C2B8 **N**LASGVParFSGSGSGTSYSLTISRVEAEDAATYyCqGwISNpPTFGaGTK
 1F5 **N**LASGVParFSGSGSGTSYSLTISRVEAEDAATYyCqGwISNpPTFGaGTK

B

B9E9 **L**ELKQVQLyQsGAELVKPGASVKMSCKASGYTFTSYNMEWVKQTPGGGLE
 C2B8 **L**ELKQVQLyQsGAELVKPGASVKMSCKASGYTFTSYNMEWVKQTPGGGLE
 1F5 **L**ELKQVQLyQsGAELVKPGASVKMSCKASGYTFTSYNMEWVKQTPGGGLE

B9E9 **W**IGATYFGNGDTSYNQRFGKATLTADKSSSTAYMQLSSITSED
 C2B8 **W**IGATYFGNGDTSYNQRFGKATLTADKSSSTAYMQLSSITSED
 1F5 **W**IGATYFGNGDTSYNQRFGKATLTADKSSSTAYMQLSSITSED

B9E9 **S**AVYYCARaqlrpnwyYFdvWGAGTTvTVS
 C2B8 **S**AVYYCARetyyggdwYFdvWGAGTTvTVS
 1F5 **S**AVYYCARehygnwyYFdvWGAGTTvTVS

Fig. 1. Amino acid homology of the V_L (A) and V_H (B) regions of B9E9 and the anti-CD20 antibodies C2B8 (21) and 1F5 (22). The complementarity-determining regions are shown in **bold type**, and amino acids that differ between the constructs are indicated in **lowercase letters**.

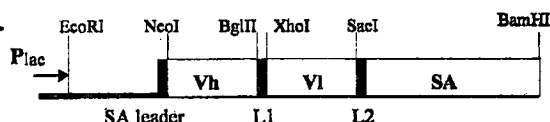


Fig. 2. Schematic of a B9E9 scFvSA genetic construct showing restriction sites. The scFvSA gene is composed of the scFv of the murine IgG2a anti-CD20 B9E9 antibody fused to the full-length, genomic streptavidin of *S. avidinii*. The leader sequence is from streptavidin, and L1 and L2 are linkers separating the components. The construct is expressed from the IPTG-inducible *lac* promoter.

as visualized on Coomassie Blue-stained SDS-PAGE gels. The fusion protein migrated as a tetramer in nonboiled, nonreduced samples and as a monomer in samples boiled for 5 min. The linker size, composition, and order of the variable regions significantly affected the expression level (Table 1). Several clones that showed good expression in shake flask cultures were grown further in an 8-liter fermentor and analyzed for expression level and purification recovery. One scFvSA with a 25-mer Gly₄Ser scFv linker (Fig. 2, L1) in the V_H-V_L orientation expressed at 250–300 mg/liter and was purified from homogenized cell extracts with a 50–60% yield on a single iminobiotin affinity column. This purified fusion protein was analyzed further for protein characterization, immunoreactivity, biotin binding ability, immunohistology, blood clearance rate, and *in vivo* tumor targeting.

Biochemical Characterization. SDS-PAGE demonstrated that the fusion protein was purified to >95% homogeneity after iminobiotin chromatography (Fig. 3A). The major band migrated at the expected molecular weight of ~174,000, and minor isoforms were evident. These isoforms were also detected with polyclonal anti-streptavidin antibody on Western gel analysis (Fig. 3B). However, all bands resolved into a single species of *M_r* ~ 43,000 when the protein was boiled before electrophoresis, consistent with a single protein entity dissociable into its homogeneous subunit.

On size exclusion HPLC, the purified protein exhibited a major peak with a retention time appropriate for the tetramer and a minor (~5%) peak representing a higher molecular weight, aggregated species (Fig. 4). Light-scattering HPLC indicated that the aggregate peak had a molecular weight of ~350,000, suggesting it was a dimer of the tetramer. The deconvoluted mass spectrum of the monomer showed a molecular weight of 43,402, which is in agreement with the calculated most abundant mass of 43,400. Liquid chromatography-mass spectrometry/mass spectrometry analysis of the tryptic peptides of B9E9

scFvSA identified 15 of 21 predicted peptide fragments with the correct molecular weight and amino acid sequences yielding near complete sequence information. In addition, N₂-terminal sequencing of the purified protein revealed that the leader sequence was cleaved at the expected signal peptidase site adjacent to the first amino acid of the V_H region.

Immunoreactivity was assessed by flow cytometry in a competitive assay with fluorescein-labeled B9E9 mAb for binding to CD20-positive human lymphoma Ramos cells. The tetravalent scFvSA was about twice as immunoreactive (~185%) as the divalent B9E9 mAb on a molar basis and nearly equivalent (~93%) to B9E9 mAb when adjusted for its tetravalency (Fig. 5). Scatchard analysis indicated that the fusion protein retained the same relative nanomolar avidity as the B9E9 mAb. *K_d* values were 9.75 and 12.44 nM, and *K_a* values were 1.02×10^8 and 0.80×10^8 M⁻¹ for B9E9 mAb and scFvSA, respectively.

B9E9 scFvSA was further characterized for its biotin binding ability. The biotin dissociation rate was identical to that of recombinant streptavidin (*t*_{1/2} = 379 and 364 min, respectively), indicating fully functional biotin binding. Determination of the biotin binding capacity revealed that B9E9 scFvSA had an average of 3.6 of 4 possible sites available for binding.

The binding of B9E9 scFvSA to cryosections of normal and human tumor tissues was compared with that of B9E9 mAb, C2B8/SA chemical conjugate (10), and a murine IgG2a isotype control mAb. All of the CD20 antibodies showed positive signals in a cell membrane pattern on B-cell aggregates, as expected from the known distribution of these cells. Thus, appropriate foci in sections of the

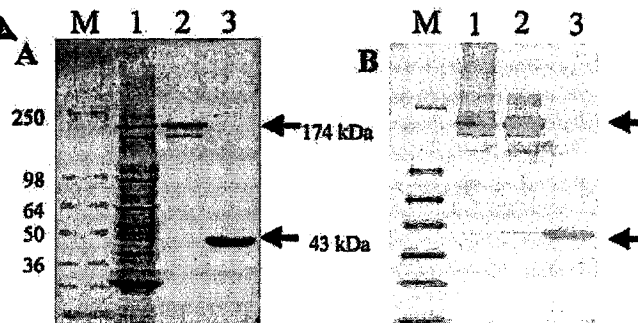


Fig. 3. SDS-PAGE (A) and Western blot analyses (B) of unpurified and purified B9E9 scFvSA. Lane M is the molecular weight markers (SeeBlue; Novex). Lane 1 shows the *E. coli* crude lysate containing the B9E9 scFvSA. Lanes 2 and 3 show the iminobiotin-purified protein either not boiled (Lane 2) or boiled for 5 min (Lane 3). SDS-PAGE gels were stained with Coomassie Blue, and Western blots were detected with horseradish peroxidase-conjugated goat anti-streptavidin polyclonal antibody.

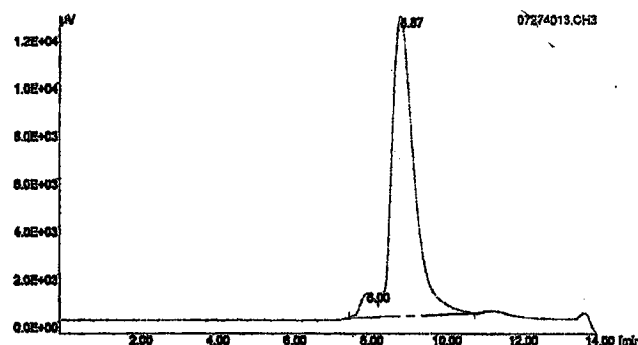


Fig. 4. HPLC size exclusion profile of iminobiotin-purified B9E9 scFvSA. The major peak at 8.87 min (94%) is the tetramer; the minor peak at 8.00 min (6%) is an aggregate of the tetramer.

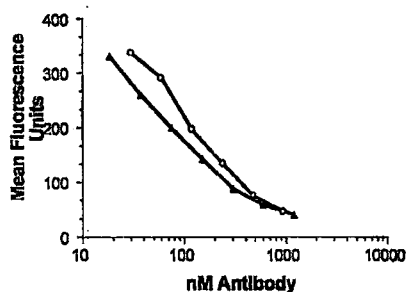


Fig. 5. Competitive immunoreactivity assay. Serial dilutions of B9E9 scFvSA ($\blacktriangle$) or mAb ($\circ$) were competed with fluorescein-labeled B9E9 mAb for binding to CD20-positive human lymphoma Ramos cells.

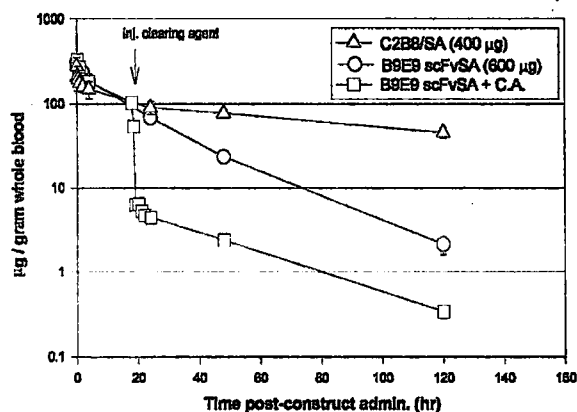


Fig. 6. Whole blood clearance of ^{125}I -labeled C2B8/SA chemical conjugate (400 μg) or ^{125}I -labeled B9E9 scFvSA (600 μg) with or without biotinylated poly(GalNAc) clearing agent treatment (100 μg) in normal BALB/c mice ($n = 3$ mice/group). The clearing agent was injected 18 h after the labeled fusion protein.

intestine (large and small), lymph node, spleen, thymus, and tonsil and in malignant B-cell lymphoma showed strong signals with the B9E9 scFvSA, B9E9 mAb, and C2B8/SA conjugate.

Clearance Rate and Biodistributions. Blood clearance studies were conducted in normal mice to examine the potential of the fusion protein in pretargeted RIT and to compare it with the C2B8/SA chemical conjugate. ^{125}I -labeled B9E9 scFvSA had a blood clearance half-life ($t_{1/2\beta}$) of 16 h, which was faster than the 46-h half-life of the C2B8/SA chemical conjugate (Fig. 6). The fusion protein was rapidly removed from the blood by a single i.v. injection of biotinylated poly(GalNAc) clearing agent. The scFvSA concentration dropped from 102 to 6.3 $\mu\text{g}/\text{gram}$ of whole blood at 1 h after administration of the clearing agent.

The B9E9 fusion protein was examined in the full pretargeting protocol in female nude mice bearing human lymphoma Ramos xenografts. The dose of fusion protein used in this study was determined empirically by comparison with the blood AUC achieved with the C2B8/SA conjugate. ^{125}I -labeled B9E9 scFvSA was injected i.v. at $t = -24$ h, the synthetic clearing agent was injected at $t = -4$ h, and ^{111}In -labeled DOTA-biotin was injected at $t = 0$ h. As expected on the basis of the blood clearance results, the ^{125}I -labeled scFvSA concentration in blood and in most well-perfused soft tissues was very low (Fig. 7). Liver uptake and retention are functions of complexation with the clearing agent, which binds to asialoglycoprotein receptors in the liver. The kidney and intestinal concentrations are believed to be the result of hepatic metabolism of the fusion protein and subsequent renal or biliary excretion of radiolabel. A relatively high concentration of the labeled scFvSA targeted the tumor and was retained there. The

apparent concentration decrease is attributable to the tumor volume doubling time of approximately 24 h in this xenograft model.

Stable delivery and retention of ^{111}In -DOTA-biotin at the tumor were also observed (Fig. 8). The highest concentration was at the tumor (both stoichiometrically and relative to blood pool concentration) at all time points. Peak concentrations of ^{111}In -DOTA-biotin at the tumor were 17–24% ID/g (mean, $21.66 \pm 3.17\%$ ID/g) and occurred by 2 h postadministration. This was a significantly higher peak uptake than that observed with the pretargeted C2B8/SA conjugate at the comparable time point (mean, $8.40 \pm 0.62\%$ ID/g; $P < 0.05$; data not shown). Tumor:blood ratios of pretargeted B9E9 scFvSA increased from ~ 90 at 2 h after DOTA-biotin injection to >700 by 24 h after DOTA-biotin injection. ^{111}In -DOTA-biotin concentration in the blood and in most well-perfused soft tissues was very low, generally $<2\%$ of the injected dose in any of the assayed tissues at all time points. Significant liver uptake of ^{111}In -DOTA-biotin was not observed, indicating that the ^{125}I -labeled scFvSA had been efficiently internalized by the action of the clearing agent, making it unable to bind subsequently administered radiobiotin. In these experiments, no effort was made to optimize the dose of the fusion protein, clearing agent, or DOTA-biotin, nor was any effort made to optimize the schedule of administration of these components.

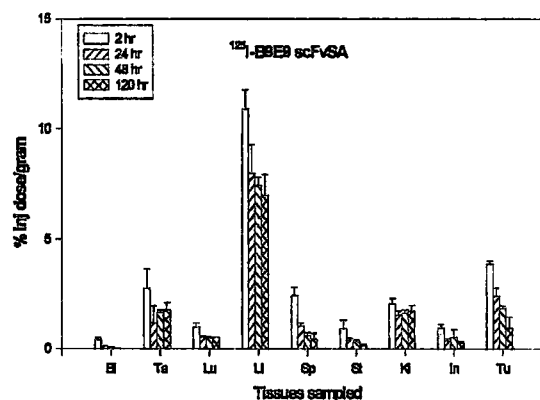


Fig. 7. The biodistribution of ^{125}I -labeled B9E9 scFvSA in a pretargeted RIT study using Ramos human lymphoma xenografts in female nude mice ($n = 3$ mice/group). ^{125}I -labeled B9E9 scFvSA (600 μg) was injected i.v. at $t = -24$ h, clearing agent (100 μg) was injected at $t = -4$ h, and ^{111}In -DOTA-biotin (1.0 μg) was injected at $t = 0$ h. Mice were sacrificed at 2, 24, 48, or 120 h after injection of DOTA-biotin. Bl, blood; Ta, tail; Lu, lung; Li, liver; Sp, spleen; St, stomach; Ki, kidney; In, intestine; Tu, tumor.

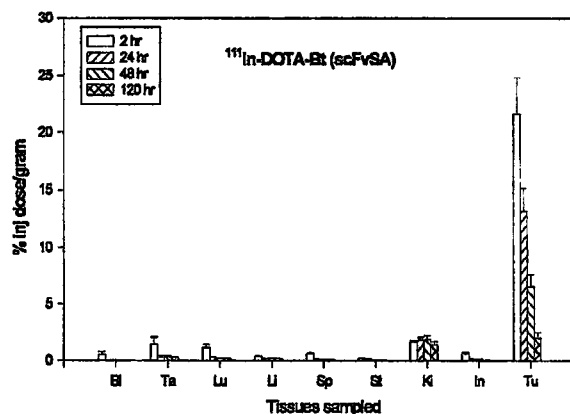


Fig. 8. The biodistribution of ^{111}In -DOTA-biotin in a pretargeted RIT study using Ramos xenografted mice receiving B9E9 scFvSA (600 μg). Mice were sacrificed at 2, 24, 48, or 120 h after injection of DOTA-biotin. Bl, blood; Ta, tail; Lu, lung; Li, liver; Sp, spleen; St, stomach; Ki, kidney; In, intestine; Tu, tumor.

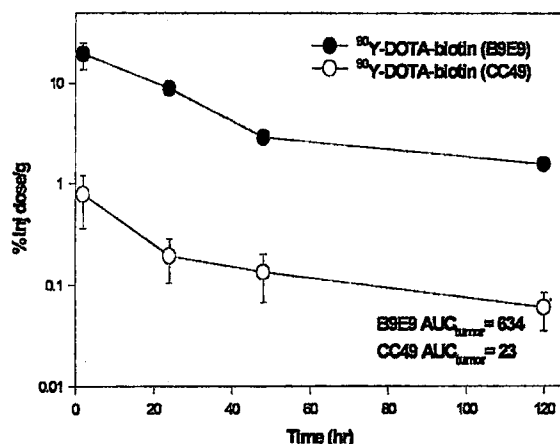


Fig. 9. Radioactivity concentrations in tumor resulting from pretargeted ^{90}Y -DOTA-biotin with either B9E9 scFvSA or nonspecific CC49 scFvSA as the targeting vehicle. Data are presented as the % ID/g of tissue at 2, 24, 48, and 120 h after injection of DOTA-biotin. AUC values were calculated by using trapezoidal integration from 0 to 120 h after injection.

The CC49 scFvSA fusion protein, which is immunoreactive to an antigen (TAG-72) not expressed by Ramos cells, was used as a negative control to assess antigen-specific tumor localization. Consistent with our prior studies, targeting with ^{90}Y -DOTA-biotin (mean, $19.55 \pm 5.86\%$ ID/g) was equivalent to targeting with ^{111}In -DOTA-biotin (mean, $21.66 \pm 3.17\%$ ID/g). By contrast, there was no targeting of ^{90}Y -DOTA-biotin to the tumor by the negative control fusion protein ($0.78 \pm 0.42\%$ ID/g). Nontarget tissue concentrations of CC49 pretargeted ^{90}Y -DOTA-biotin were nearly identical to the B9E9 values in all tissues at all time points, demonstrating the absence of antigen-mediated uptake in normal mouse tissues of either antibody fusion construct. The rapid tumor uptake achieved with B9E9 scFvSA resulted in a 2700% greater tumor dose as compared with the CC49 scFvSA (Fig. 9), whereas the % ID/g-h AUCs of blood showed similar total values (9% ID/g-h versus 18% ID/g-h for B9E9 and CC49 fusion proteins, respectively). Ratios of tumor AUC: blood AUC were >60 for the B9E9 group versus 1.2 for the CC49 group.

DISCUSSION

The B9E9 scFvSA fusion protein was designed to be a homogeneous and cost-effective bifunctional vehicle to mediate biotin-streptavidin pretargeted RIT of NHL. *E. coli* was chosen as the expression host because the periplasm allows disulfide formation and contains low levels of free biotin that would otherwise occlude the binding sites of streptavidin. In addition, fermentation conditions for *E. coli* are readily scaleable, enabling production of the gram quantities needed for clinical trials. Initial efforts to produce high levels of a V_L - V_H B9E9 scFvSA with a 15-mer Gly₄Ser linker were unsuccessful, leading us to generate numerous genetic variants with different scFv linkers and different orders of the variable regions. Two constructs, both V_H - V_L with either a 18-mer or a 25-mer Gly₄Ser linker, were highly expressed (>100 mg/liter) in *E. coli* as soluble, tetrameric fusion proteins. Both purified proteins had apparently identical functional characteristics *in vitro* and *in vivo* and could only be differentiated by their expression level.

Although high level *E. coli* expression of antibody fragments has been achieved, most scFvSA fusion proteins are poorly expressed or insoluble in the periplasmic space (23–27). Despite the fact that insoluble proteins in the cytoplasm or periplasm can occasionally be refolded from inclusion bodies, this approach is not practical for large

scale or high molecular weight proteins. Multiple factors, such as the leader sequence, the linker used, the order of variable regions, the host strain, and the growth conditions, affect the periplasmic expression of antibody fragments and the degree of aggregation (28–31). In this study, the scFvSA expression levels were affected dramatically by the order of the variable regions and the length and/or composition of the scFv linker. No variations were made in the leader sequence or the linker between the scFv and streptavidin, and these also may be amenable to further optimization.

The *in vitro* and *in vivo* functionality demonstrated by the B9E9 scFvSA is consistent and reproducible. *In vitro*, the competitive immunoreactivity with the B9E9 mAb showed evidence of increased avidity of the tetramer, a phenomenon already noted with trivalent and tetravalent Fab' constructs (32). Also exhibited is the intact immunoreactivity of the variable regions of the whole antibody when incorporated into the scFv motif. The biotin binding capacity of the scFvSA was nearly equivalent to that of commercially available recombinant streptavidin, whereas the biotin dissociation rate of the B9E9 scFvSA at physiological temperature was unperturbed. These data indicate that the fusion of B9E9 scFv to the NH₂ terminus of each subunit of streptavidin had a negligible effect on the tertiary and quaternary structural requirements of streptavidin to mediate high-affinity biotin binding. The biochemical uniformity of the purified B9E9 scFvSA alone makes it a superior test agent compared with our first-generation mAb-streptavidin covalent conjugates. In addition, the fusion protein exhibits increased antigen-binding avidity, which should decrease streptavidin dissociation from tumor, a key factor in the mathematical modeling of multistep delivery protocols (33, 34). This could be particularly critical in our pretargeting protocol, which rapidly and dramatically disrupts the equilibrium between tumor-bound and circulating immunoconjugate by administration of a clearing agent.

In vivo, the B9E9 scFvSA exhibited more rapid systemic clearance than our mAb-streptavidin conjugates, which is consistent with its lack of the Fc region of the antibody. However, the greater molecular weight of the scFvSA tetramer (~174,000) compared with conventional antibody fragments (Fab, Fab', and scFv) provides the fusion protein with sufficient hydrodynamic radius to avoid direct renal elimination via glomerular filtration. In fact, no intact fusion protein or monomeric subunits can be detected in animals' urine after systemic administration (data not shown). The B9E9 scFvSA half-life of ~16 h appears to be sufficient to allow extravasation from the blood and efficient binding to the tumor-associated antigen. Dual-label coinjection studies of equimolar amounts of C2B8 mAb and B9E9 scFvSA in mice bearing lymphoma xenografts have shown that peak tumor concentrations of both proteins are equivalent, despite the prolonged, elevated blood concentration of the C2B8 molecule. In general, the absence of the whole antibody Fc region should minimize uptake of B9E9 scFvSA in specialized antibody reservoirs, such as the Brambell receptor (35). In our full pretargeting protocol, B9E9 scFvSA is directed quantitatively from the blood to the liver for hepatic processing in a manner similar to that described for other conjugates and tumor models (6).

In recent years, immunotherapy of relapsed or refractory low-grade NHL with CD20 mAbs has been shown to be effective in inducing an objective tumor response. Clinical studies using unlabeled antibody (C2B8, rituximab) and radiolabeled antibodies (Y2B8 and Bexxar) have been recently reviewed by others (1–3). The work of Press *et al.* (4) and Liu *et al.* (5) using myeloablative doses of ^{131}I -labeled B1 has proven that dose intensification of I-131 by a factor of ~8 results in a higher complete response rate and a longer duration of response, albeit at the cost of stem cell rescue. Whether given in nonmyeloablative or myeloablative protocols, RIT continues to show promise for replacing total body irradiation in the treatment of NHL. Our own

pilot clinical pretargeting efforts in this indication using C2B8/SA conjugate as a model targeting vehicle yielded promising results in terms of clinical responses, absolute tumor-targeting efficiency, and minimal myelotoxicity, even at high ^{90}Y doses (10).

ACKNOWLEDGMENTS

We thank Gina Desfachelles for technical assistance in the mouse studies, Santosh Kumar (University of Washington, Seattle, WA) for NH_2 -terminal sequencing, Mark Hylarides for the deaggregation protocol, and Steve Goshorn for cloning of the streptavidin gene and construction of the initial fusion protein cassette.

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2. Objeto de la invención

- Título de la solicitud (folio 1): "Anticuerpos anti-CD33 y método para tratamiento e leucemia mieloide aguda utilizando los mismos".
- La presente solicitud se refiere a anticuerpos que enlazan CD33.
- El problema planteado en esta solicitud es la necesidad de inmunocóncugados mas seguros y efectivos que enlacen de manera específica a CD33 y que puedan ser utilizados en el tratamiento de leucemia mieloide aguda (AML).
- Para solucionar este problema técnico la solicitud en estudio proporciona un anticuerpo aislado o un fragmento de enlace de epitope del mismo, que comprende por lo menos una región de determinación complementaria, y que tiene habilidad para enlazar CD33.
- La Clasificación Internacional de Patentes (IPC) es: A61K 39/395.

3. No es una invención (artículo 15 D 486 literal a)-f))

El artículo 15 en su literal b) señala que:

No se consideran invenciones el todo o parte de los seres vivos tal como se encuentran en la naturaleza, los procesos biológicos naturales, el material biológico existente en la naturaleza o aquel que pueda ser aislado inclusive genoma o germoplasma de cualquier ser vivo natural.

- La reivindicación 1 y dependientes enseñan una anticuerpo aislado o fragmento de enlace de epítope del mismo que comprende una región de determinación complementaria y que tiene habilidad para enlazar CD33.

Este despacho considera que dichas reivindicaciones no son una invención debido a que cubren anticuerpos aislados que contengan alguna de las secuencias de aminoácidos reivindicadas y a los cuales no se les realizó ningún procedimiento no natural.

Por tal razón las reivindicaciones 1 y sus dependientes no pueden ser consideradas invenciones.

4. Excepción a la patentabilidad (artículo 20 D486 literal a)-d))

El literal c del artículo 20 de la decisión 486 establece que "no serán patentables:

- d) Los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales".*

- Las reivindicaciones 37 a 56 implican un método para tratar a un sujeto con una enfermedad en el que se expresa CD33.

De lo anterior la(s) reivindicaciones 37 a 56 no pueden ser consideradas patentables.

5. Reivindicaciones relativas a un uso o a un segundo uso (artículos 14 y 21 D 486)

- Las reivindicaciones 33 a 36 obedecen al uso de los compuestos reivindicados para inhibir el crecimiento de la célula que expresa CD33.

Por dicha razón, las reivindicaciones analizadas no se pueden considerar invenciones y que por lo tanto no pueden obtener patente.

6. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria: 11 de julio de 2002

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

Nº	Documento	Título	Fecha de publicación*
D1	WO 94/10332	Humanized antibodies to Fc receptors for immunoglobulin G on human mononuclear phagocytes	11 mayo 1994
D2	Shcultz et al.	A tetravalent Single- chain antibody-streptavidin fusion protein for pretargeted lymphoma therapy	1 diciembre 2000

7. Análisis de patentabilidad (artículo 14 D486)

Nivel inventivo (artículo 18 D486)

- La reivindicación 3 y sus dependientes divulgan un fragmento de enlace de epítoto caracterizado porque la región variable de cadena pesada tiene por lo menos 90% de identidad de secuencia a una secuencia de aminoácidos representada por la secuencia No.ID. SEC.: 7.

El documento del Estado de la Técnica más próximo es D2 (ver folios 258 a 261) que enseña un epítoto que presenta una secuencia de anticuerpo humanizado que posee una similaridad cercana al 90% con la reivindicada en la secuencia ID NO. 7 de la solicitud. Como se puede observar en la figura dos de D2.

La única diferencia con D2 es que las secuencias no son exactamente iguales, pero para cualquier técnico medio en la materia presenta obviedad la construcción de moléculas de anticuerpo con la misma función de enlace a CD33 variando aminoácidos que no impliquen cambios funcionales a nivel de actividad.

- La reivindicación 6 y sus dependientes divulgan un fragmento de enlace de epitoto caracterizado porque la región variable de cadena ligera que tiene por lo menos 90% de identidad de secuencia a una secuencia de aminoácidos representada por la secuencia No.ID. SEC.: 8.

El documento del Estado de la Técnica más próximo es D1 (ver folios 250 a 257) que enseña un epítipo que presenta una secuencia de anticuerpo humanizado que posee una similaridad cercana al 90% con la reivindicada en la secuencia ID NO. 8 de la solicitud. Como se puede observar en la figura dos de D1.

La única diferencia con D1 es que las secuencias no son exactamente iguales, pero para cualquier técnico medio en la materia presenta obviedad la construcción de moléculas de anticuerpo con la misma función de enlace a CD33 variando aminoácidos que no impliquen cambios funcionales a nivel de actividad

El problema técnico planteado en esta solicitud que era de proporcionar un anticuerpo aislado o un fragmento de enlace de epítipo del mismo, que comprende por lo menos una región de determinación complementaria, se había resuelto previamente mediante el mismo tipo de compuestos ya conocidos. Por esta razón se considera que las variaciones que se realicen en la secuencia o el desarrollo de anticuerpos provenientes de diferentes murinos y humanizados son evidentes para una persona medianamente versada en la materia.

De esta forma se establece que las reivindicaciones 3 a 16 no cumplen con el requisito de Nivel Inventivo.

8. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

Nº	Documento Nº	Reivindicaciones afectadas	Requisito que afecta
D1	WO 94/10332	6 – 8, 9 – 16	Nivel Inventivo
D2	Shcultz et al.	3 – 5, 9 – 16	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 60 días contados a partir 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.

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.


ALIX CARMENZA CÉSPEDES DE VERGEL
Directora de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 19.11.2009

CONCEPTO TÉCNICO No. 4059	EXAMINADOR TÉCNICO Código No. 202089
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