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(71) Applicant *(for all designated States except US)*: **BIOGEN, INC.** [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GARBER, Ellen** [US/US]; 14 Donnell Street, Cambridge, MA 02138 (US). **LYNE, Paul** [US/US]; 72 Allston Street #3, Allston, MA 02139 (US). **SALDANHA, Jose, W.** [GB/GB]; 22A Lincoln Way, Enfield, Middlesex EN1 1TE (GB).

(74) Agent: **COX, Niki, D.**; Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142 (US).

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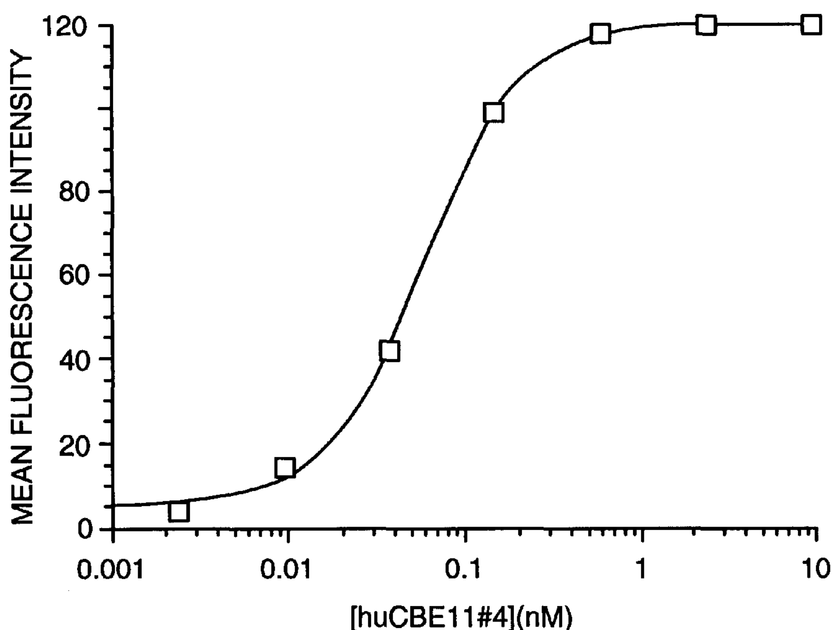
(54) Title: HUMANIZED ANTI-LT-β-R ANTIBODIES



(57) Abstract: Humanized antibodies to LT-β-R and method of use thereof are provided.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

HUMANIZED ANTI-LT-β-R ANTIBODIES

RELATED APPLICATIONS

This is a continuation-in-part of U.S.S.N. 60/299,987, filed on June 21, 2001 which is a continuation-in-part of U.S.S.N. 60/275,289, filed on March 13, 2001 which is a continuation-in-part of U.S.S.N. 60/240,285, filed on October 13, 2000. The entire disclosure of each of the aforesaid patent applications are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates generally to humanized antibodies specific for the lymphotoxin beta receptor (LT-β-R).

BACKGROUND OF THE INVENTION

Lymphotoxin beta receptor (referred to herein as LT-β-R) is a member of the tumor necrosis factor family which has a well-described role both in the development of the immune system and in the functional maintenance of a number of cells in the immune system including follicular dendritic cells and a number of stromal cell types (Matsumoto et al., *Immunol. Rev.* 156:137 (1997). Known ligands to the LT-β-R include LTα1/β2 and a second ligand called LIGHT (Mauri et al. *Immunity* 8:21 (1998)). Activation of LT-β-R has been shown to induce the apoptotic death of certain cancer cell lines *in vivo* (PCT/US96/01386). Treatment with agonist LT-β-R activating agents, such as specific humanized anti-LT-β-R antibodies, would thus be useful for treating or reducing the advancement, severity or effects of neoplasia in subjects (e.g., humans).

SUMMARY OF THE INVENTION

The present invention provides humanized anti-lymphotoxin beta receptor (LT-β-R) antibodies and methods of using these antibodies to treat or reduce the advancement, severity or effects of neoplasia in subjects (e.g., humans).

Specifically, the invention embraces a humanized antibody that specifically binds to LT-β-R (e.g., human LT-β-R). This antibody comprises light chain complementary determining regions defined by amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO: 1, and/or heavy chain complementary determining regions defined by amino acid residues 31 to 35, 50 to 66 and 99 to 109 of SEQ ID NO: 2 and in addition at least one (e.g., 1, 2, 3, 4, or 5) of the following residues in its light chain:

K3, W41, I46, Q69 and Y71; or at least one (e.g. 1, 2, 3, 4, or 5) of the following residues in its heavy chain: F37, T40, A49, M89 and V93 (Kabat numbering convention).

The humanized antibody of this invention may comprise a light chain variable domain sequence defined by amino acid residues 1 to 107 of SEQ ID NO:8 and/or a heavy chain variable domain sequence defined by amino acid residues 1 to 120 of SEQ ID NO:16. The humanized antibody may also comprise the same heavy and/or light chain polypeptide sequences as an antibody produced by cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765).

In another embodiment, the humanized antibody of this invention substantially retains the binding properties of the parent antibody. In one embodiment the humanized antibody of this invention binds to LT-β-R with a functional affinity, for instance, of about 1 pM to about 10 pM, alternatively, about 10 pM to about 20 pM, alternatively, about 20 pM to about 30 pM, alternatively, about 30 pM to about 40 pM alternatively, about 40 pM to about 50 pM, alternatively, about 50 pM to about 60 pM, alternatively, about 60 pM to about 70 pM, alternatively, about 70 pM to about 80 pM, and alternatively, about 80 pM to about 90 pM, wherein the functional affinity is measured by FACS in accordance with Example 8.

In another embodiment, the humanized antibody of this invention is linked to an immunotoxin (e.g., ricin A chain and *Pseudonomas toxin*). The humanized antibody of this invention can also be linked to a chemotherapeutic drug (e.g., Adriamycin, 5FU, Vinblastine, Actinomycin D, Etoposide, Cisplatin, Methotrexate and Doxorubicin) or to a radioisotype. The present invention also embraces a combination therapy in which for instance, the humanized antibody of the present invention which is linked to an immunotoxin is used in combination with a humanized antibody of the present invention which is linked to a chemotherapeutic drug. The present invention further embraces a composition suitable for administration to a mammal (ie human) having a tumor that overexpresses LTβR comprising a) a humanized anti- LTβR antibody either alone or linked to a immunotoxin or a chemotherapeutic drug and b) a cytotoxic factor, each present in amounts effective to reduce tumor volume upon administration to the mammal. The cytotoxic factor may include for instance, TNF-α, TNF-β, IL-1, INF-γ,

IL-2. Alternatively, the cytotoxic factor may by a chemotherapeutic drug. The chemotherapeutic drug may include for instance, Adriamycin, 5FU, Vinblastine, Actinomycin D, Etoposide, Cisplatin, Methotrexate and Doxorubicin.

The antibody of this invention can be, for instance, a whole antibody (i.e. with two full length light chains and two full length heavy chains) of any isotype and subtypes (e.g. IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgE, IgA1 and IgA2); alternatively, it can be an antigen-binding fragment (e.g., Fab, $F(ab')_2$, and Fv) of a whole antibody. Embraced in this invention are also a composition comprising a pharmaceutically acceptable carrier; an isolated nucleic acid comprising a coding sequence for SEQ ID NO:8; an isolated nucleic acid comprising a coding sequence for SEQ ID NO:16; an isolated nucleic acid comprising a coding sequence for the light chain of an antibody produced by cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765); an isolated nucleic acid comprising a coding sequence for the heavy chain of an antibody produced by cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765); an isolated nucleic acid comprising a coding sequence for residues 1-107 of SEQ ID NO:8; and an isolated nucleic acid comprising a coding sequence for residues 1-120 of SEQ ID NO:16.

Embraced within the present invention are also cells from cell lines that produce humanized anti-LTβR antibody, included, for instance, cell line E46.4 (ATCC patent deposit designation PTA-3357) and cell line E77.4 (ATCC patent deposit designation 3765). In one embodiment the cell line produces from about 250mg/L to about 300 mg/L of said antibody, alternatively, the cell line produces from about 300mg/L to about 350 mg/L of said antibody, alternatively, the cell line produces from about 350mg/L to about 400 mg/L of said antibody, alternatively, the cell line produces from about 400mg/L to about 450 mg/L of said antibody, alternatively, the cell line produces from about 450mg/L to about 500 mg/L of said antibody, alternatively, the cell line produces from about 500mg/L to about 550 mg/L of said antibody and alternatively, the cell line produces from about 550mg/L to about 600 mg/L of said antibody. The concentration of the antibody produced by the cell lines is measures as a harvest titer from a 10 day fed batch culture.

The present invention also provides a method of treating or reducing the advancement, severity or effects of neoplasia in a subject (e.g., human) comprising

administering to the subject an effective amount of an antibody of this invention. An effective amount of the composition can be administered in one or more dosages. In another embodiment the present invention provides a method of treating or reducing the advancement, severity or effects of neoplasia in a subject (e.g., human) comprising administering to the subject an effective amount of an antibody of this invention and a cytotoxic factor. The cytotoxic factor may include for instance, TNF-α, TNF-β, IL-1, INF-γ, IL-2. Alternatively, the cytotoxic factor may by a chemotherapeutic drug. The chemotherapeutic drug may include for instance, Adriamycin, 5FU, Vinblastine, Actinomycin D, Etoposide, Cisplatin, Methotrexate and Doxorubicin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a graph of cytotoxicity on WiDr cells. mCBE11 (murine) (diamond), huCBE11#2 (humanized anti-LT-β-R antibody comprising version 2 of the light chain (VL#2) and version 2 of the heavy chain(VH#2)) (circle), huCBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain (VL#3) and version 4 of the heavy chain (VH#4)) (star).

Figure 2 shows a graph of IL-8 agonism on A375 cells. mCBE11 (diamonds), huCBE11#2 (humanized anti-LT-β-R antibody comprising version 2 of the light chain (VL#2) and version 2 of the heavy chain(VH#2)) (circle), huCBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain (VL#3) and version 4 of the heavy chain (VH#4)) (stars).

Figure 3 shows a graph of tumor volume versus days of administration. mCBE11 (triangles), hu CBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain (VL#3) and version 4 of the heavy chain (VH#4)) (circles), no treatment (squares).

Figure 4 shows a graph of percent survival of animals versus days of post tumor injection. mCBE11 (triangles), huCBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain and version 4 of the heavy chain) (circles), no treatment (squares).

Figure 5 shows a graph of tumor volume versus days post-injection. HuCBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain (VL#3) and version 4 of the heavy chain (VH#4)) (circles) and control with no treatment (squares).

Figure 6 shows a graph of pre-grown tumor volume versus days of post-treatment. Control (square); huCBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain (VL#3) and version 4 of the heavy chain (VH#4)) at different dosages: 500ug (circles), 100ug (triangles), and 20ug (diamonds); mCBE11 (crosses).

Figure 7 shows a graph of percent survival of animals with pre-grown tumors versus days of post-treatment. Control (square); huCBE11#4 (humanized anti-LT-β-R antibody comprising version 3 of the light chain (VL#3) and version 4 of the heavy chain (VH#4)) at different dosages: 500ug (circles), 100ug (triangles), and 20ug (diamonds); mCBE11 (crosses).

Figure 8 shows a graph of mean fluorescence intensity versus huCBE11#4 concentrations on a log scale.

DETAILED DESCRIPTION

Sequence Identification Numbers

Nucleotide and amino acid sequences referred to in the specification have been given the following sequence identification numbers:

SEQ ID NO:1 – Amino acid sequence of mCBE11 heavy chain variable region.

SEQ ID NO:2 – Amino acid sequence of mCBE11 light chain variable domain.

SEQ ID NO:3 – Nucleic acid sequence of humanized CBE11 light chain variable region (version 1-VL#1).

SEQ ID NO:4 – Amino acid sequence of humanized CBE11 light chain variable region (version 1-VL#1).

SEQ ID NO:5 – Nucleic acid sequence of humanized CBE11 light chain variable region (version 2-VL#2).

SEQ ID NO:6 – Amino acid sequence of humanized CBE11 light chain variable region (version 2-VL#2)

SEQ ID NO:7 – Nucleic acid sequence of humanized CBE11 light chain variable region (version 3-VL#3).

SEQ ID NO:8 – Amino acid sequence of humanized CBE11 light chain variable region (version 3-VL#3)

SEQ ID NO:9 – Nucleic acid sequence of humanized CBE11 heavy chain variable region (version 1-VH#1)

SEQ ID NO:10 – Amino acid sequence of humanized CBE11 light chain variable region (version 1-VH#1)

SEQ ID NO:11 – Nucleic acid sequence of humanized CBE11 heavy chain variable region (version 2-VH#2)

5 SEQ ID NO:12 – Amino acid sequence of humanized CBE11 light chain variable region (version 2-VH#2)

SEQ ID NO:13 – Nucleic acid sequence of humanized CBE11 heavy chain variable region (version 3-VH#3)

SEQ ID NO:14 – Amino acid sequence of humanized CBE11 light chain variable

10 region (version 3-VH#3)

SEQ ID NO:15 – Nucleic acid sequence of humanized CBE11 heavy chain variable region (version 4-VH#4)

SEQ ID NO:16 – Amino acid sequence of humanized CBE11 light chain variable region (version 4-VH#4)

15 SEQ ID NO:17 - FR1 primer to introduce a Bsu36I site.

SEQ ID NO:18 - FR2 primer to introduce NciI and HpaII sites.

SEQ ID NO:19 - FR3 primer to introduce Bsu36I and PstI sites.

SEQ ID NO:20 FR2 primer to introduce SmaI site.

SEQ ID NO:21 - FR3 primer to introduce PvuI site.

20 SEQ ID NO:22 - FR2 primer to introduce SmaI and HhaI sites.

SEQ ID NO:23 - FR3 primer to introduce PvuII and FspI sites.

SEQ ID NO:24 - FR1 primer to introduce HinfI and NsiI sites.

SEQ ID NO:25 - FR2 primer to introduce HaeII and HhaI sites.

SEQ ID NO:26 - FR3 primer to introduce Bsu36I, DdeI and PstI sites.

25 SEQ ID NO:27 - FR1 primer to introduce EcoRV site.

SEQ ID NO:28 - FR3 primer to introduce RsaI site.

SEQ ID NO:29 - FR1 primer to introduce EcoRV site.

SEQ ID NO:30 - FR2 primer to introduce HindIII site.

SEQ ID NO:31 - FR3 primer to introduce RsaI site.

30 SEQ ID NO:32 - Full huCBE11 light chain (version 3) including constant domain.

SEQ ID NO:33 - Full huCBE11 heavy chain (version 4) including constant domain.

Definitions

The term humanized antibody, as used herein, refers to herein an antibody derived from a non-human antibody, typically murine, that retains or substantially retains the antigen-binding properties of the parent antibody but which is less immunogenic in humans.

5 The term complementarity determining region (CDR), as used herein, refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site as delineated by Kabat et al (1991).

The term framework region (FR), as used herein, refers to amino acid sequences 10 interposed between CDRs. These portions of the antibody serve to hold the CDRs in appropriate orientation (allows for CDRs to bind antigen).

The term constant region (CR) as used herein, refers to the portion of the antibody molecule which confers effector functions. In the present invention, murine constant regions are substituted by human constant regions. The constant regions of the 15 subject chimeric or humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu. Further, heavy chains of various subclasses (such as the IgG subclasses of heavy chains) are responsible for different effector functions and thus, by choosing the desired heavy chain constant region, antibodies with desired 20 effector function can be produced. Preferred constant regions are gamma 1 (IgGl), gamma 3 (IgG3) and gamma 4 (IgG4). More preferred is an Fc region of the gamma 1 (IgG1) isotype. The light chain constant region can be of the kappa or lambda type, preferably of the kappa type.

The term chimeric antibody as used herein refers to an antibody containing 25 sequences derived from two different antibodies, which typically are of different species. Most typically chimeric antibodies comprise human and murine antibody fragments, generally human constant and murine variable region.

The term immunogenicity as used herein refers to a measure of the ability of a targeting protein or therapeutic moiety to elicit an immune response (humoral or 30 cellular) when administered to a recipient. The present invention is concerned with the immunogenicity of the subject humanized antibodies.

Humanized antibody of reduced immunogenicity refers to a humanized antibody exhibiting reduced immunogenicity relative to the parent antibody, e.g., the murine antibody.

Humanized antibody substantially retaining the binding properties of the parent antibody refers to a humanized antibody which retains the ability to specifically bind the antigen recognized by the parent antibody used to produce such humanized antibody. Preferably the humanized antibody will exhibit the same or substantially the same antigen-binding affinity and avidity as the parent antibody. Ideally, the affinity of the antibody will not be less than 10% of the parent antibody affinity, more preferably not less than about 30%, and most preferably the affinity will not be less than 50% of the parent antibody. Methods for assaying antigen-binding affinity are well known in the art and include half-maximal binding assays, competition assays, and Scatchard analysis. Suitable antigen binding assays are described in this application.

The present invention is directed to humanized monoclonal antibodies which bind human LT-β-R and their use as therapeutic agents. The present invention is further directed toward nucleic acid sequences which encode said humanized antibodies, and their expression in recombinant host cells. More specifically, the present invention is directed toward humanized antibodies derived from murine CBE11 which specifically binds to human LT-β-R.

Murine CBE11 (mCBE11) is a murine IgG1, kappa antibody isolated from a mouse immunized with a human LT-β-R-Ig fusion protein (Browning et al., *J. Immunol.* 154: 33 (1995)). mCBE11 functionally activates LT-β-R both *in vitro* and *in vivo* (PCT/US96/01386) and its isolation and anti-tumor properties have been described (Browning et al. *J. Exp. Med.* 183:867 (1996). The hybridoma cell line which produces mCBE11 has been previously deposited with the American Type Culture Collection (ATCC) according to the provisions of the Budapest Treaty by the Applicants of the present invention and was assigned the ATCC accession number HB 11793. (PCT/US96/01386). Applicants have also shown that LT-β receptor cross-linking with various agonist anti-LT-β-R antibodies activate the LT-β receptor (i.e. can mimic the effects of the natural ligands). (PCT/US96/01386) Receptor activation in turn has been shown to inhibit tumor growth in a variety of *in vivo* tumor models for which LT-β receptor is expressed. LT-β receptor has been shown to be expressed on a number of

cancer cells including for example non small cell lung cancer cells (NSCLC), colorectal cancer cells (CRC), breast cancer cells, as well as on prostate, gastric, skin, stomach, esophagus and bladder cancer cells. Non-limiting examples of tumors that the agonist LT-β-R antibodies inhibit include the following solid tumors: HT29 colon adenocarcinoma, HT3 cervical carcinoma, A375 melanoma, MDA-231 breast carcinoma and primary colon tumors. Therefore, agonist LT-β-R antibodies possess properties which render it useful for treatment of diseases wherein LT-β-R activation and/or modulation of the LT-β-R / LT-β-R ligand interaction is desirable including for example the treating or reducing the advancement, severity or effects of neoplasia in a subject (e.g., human).

Humanizing the mCBE11 monoclonal antibody including the modeling analysis and back mutations required to substantially retain the binding properties of the mCBE11 monoclonal antibody is described herein.

Modeling Analysis Of The Mouse Variable Regions

The CDRs contain the residues most likely to bind antigen and must be retained in the reshaped antibody. CDRs are defined by sequence according to Kabat et al., Sequence of Proteins of Immunological Interest, 5th Edition, The United States Department of Health and Human Services, The United States Government Printing Office, 1991. CDRs fall into canonical classes (Chothia et al, 1989 Nature, 342, 877-883) where key residues determine to a large extent the structural conformation of the CDR loop. These residues are almost always retained in the reshaped antibody. The polypeptide sequence of the light chain variable domain of mCBE11 is shown below with the CDR's underlined and the residue position numbers are designated according with the Kabat numbering system:

```
1     DIKMTQSPSS MYASLGERVT ITCKAGQDIK SYLSWYQQKP
41    WKSPKILIYY ATRLADGVPS RFSGSGSGQD YSLTISSLES
81    DDTATYYCLQ HGESPWTFGG GTKLEIK
(SEQ ID NO:1)
```

The polypeptide sequence of the heavy chain variable domain of mCBE11 is shown below with the CDR's underlined and the residue position number are designated according with the Kabat numbering system:

```
1          EVQLVESGGG LVKPGGSLKL SCAASGFTFS DYYMYWFRQT
41         PEKRLEWVAT ISDGGSYTYY PDSVKGRFTI SRDNAKNNLY
```

```
81        LQMSSLKSED TAMYYCVREE NGNFYYFDYW GQGTTVTVSS
(SEQ ID NO:2)
```

The variable light and heavy chains of mCBE11 were compared with the consensus sequences for mouse and human subgroups (Johnson, G., Wu, T. T. Kabat Database and its applications: future directions Nucleic Acid Research, 29, 205-206, 2001; Wu and Kabat, J. Exp. Med. 132:211-250 (1970)) using the program FASTA. The mCBE11 variable light chain is a member of mouse subgroup V with a 74% identity in 110 amino acid overlap and the mCBE11 variable heavy chain is a member of mouse subgroup IIId with a 79% identity in 132 amino acid overlap. The variable light chain corresponds to human subgroup I with a 66% identity in 113 amino acid overlap. The variable heavy chain corresponds to human subgroup III with a 71% identity in 131 amino acid overlap.

The CDRs of the present invention were classified into canonical classes. The L1 loop fell into canonical class 2 (11 residue loop), L2 into class 1 (7 residues) and L3 into class 1 (9 residues). The H1 loop fell into class 1 (5 residues) and the H2 loop into class 3 (17) residues. The H3 loop did not belong to a canonical class.

The residues at the interface between the variable light and heavy chains have been defined (Chothia et al, 1985 J. Mol. Biol., 186, 651-663). These are usually retained in the reshaped antibody. In mCBE11 several of these residues are unusual at the interface, namely S34, I46, L89, H91 in VL and Y35, F37, V93, E95 in VH.

Unusual framework residues were determined by analysing all mouse and human variable chain sequences in the September 1999 version of the Kabat database [NCBI, NIH]. It is believed that mCBE11-specific differences might indicate somatic mutations that enhance binding activity if these differences were close to the binding site. Unusual mouse residues further away from the binding site and unusual human residues were removed in case they would create immunogenic epitopes in the reshaped antibody. Unusual framework residues found in the mCBE11 were K3, M11, Y12, W41, Q69, S72, D81, T83 in the light chain; and F37, T40, E42, A49, N77 in the heavy chain. While most of these residues were not retained in the humanized CBE11 antibodies some of these unusual framework residues were retained including for example F37 and A49 in the heavy chains.

Modeling The Structure Of The Variable Regions

The light and heavy chains of the present invention were aligned against the non-redundant database to determine structural frames to be used to construct three dimensional models of the mCBE11 light and heavy chains. Using BLAST the light chain was found to have 93% sequence identity to monoclonal murine antibody 5g9 (1AHW), and the heavy chain was found to have 81% sequence identity to murine IGGA2 Fab fragment (Fab 17/9) (1IFH). Using the molecular modeling package Sybyl (Tripos Inc.) the three dimensional structures of the light and heavy chains were built using the light chain of 5g9 and the heavy chain of IGGA2 Fab fragment, respectively. The structural integrity of the models was assessed at the console and were found to be reasonable.

Design Of The Reshaped Variable Regions

Homology matching was used to choose human acceptor frameworks to "accept" mCBE11 CDRs. Both the Kabat database and the non-redundant database from NCBI, ENTRZ (The National Institutes of Health) were searched using the software program BLAST. The choice of human acceptor frameworks was made based on sequence identity between mCBE11 frameworks and human frameworks (excluding frameworks from previously humanized antibodies).

The eventual choice of human frameworks was from antibody TNF-A1'CL (kabat ID 004770) against human tumor necrosis factor alpha (Griffiths et al, 1993 EMBO J. 12:725-734) for the variable light (VL) chain (human kappa subgroup I) and antibody FLA-IgG'CL (kabat id 040003) of unknown specificity (Malisan et al, 1996 Blood 87:717-724) for the variable heavy (VH) chain (human subgroup III). The human VL and VH frameworks have 15 and 11 residues differences compared to the murine sequences.

Back Mutations of the human frameworks

The most unpredictable procedure in the humanization of monoclonal antibodies is the identification of critical framework residues from the parent antibody (i.e. in the present case, the parent antibody is of mouse origin) that need to be retained in order to substantially retain the binding properties of the parent antibody while at the same time minimizing the potential immunogenicity of the resultant antibody. It is especially important to retain canonical residues, interface packing residues and unusual murine residues which are close to the binding site. In addition, residues in the 'Vernier Zone' (which forms a platform on which the CDRs rest) (Foote & Winter, 1992 J. Mol.

Biol. 224, 487-499) and those close to CDR H3 are considered. Mutations back to the parent antibody (i.e. back mutating from human framework residues to mouse) are referred to herein as back mutations.

Three versions of the variable light reshaped chain (hu-CBE11 VL) and four versions of the variable heavy reshaped chain (hu-CBE11 VH) have been made. In general, the first version contains the most back mutations and the third version contains the fewest (ie the most "humanized") except for the fourth version of the hu-CBE11 VH. The present invention contemplates humanized antibodies derived from mCBE11 which possess a variable light chain selected from the variable light chains described below (i.e. VL#1, VL#2 or VL#3) and a variable heavy chain selected from the variable heavy chains described below (i.e. VH#1, VH#2, VH#2, or VH#4) in any combination.

(A) Light chain:

3 Q (glutamine)->K (lysine) It is retained in the first version since previous reshaping experiments have shown (e.g. Kolbinger et al, 1993 Prot. Eng., 6, 971-980) it might be important for antigen binding or CDR conformation.

41 G (glycine)->W (tryptophan) It was retained in the first and second versions.

46 L (leucine)->I (isoleucine) It was retained in the first and second versions since it is both an interface residue and in the vernier zone. In addition, it is an unusual reside occurring 9 times in mouse sequences and once in human. It is likely to affect the packing of the variable chains and may contact CDRs.

69 T (threonine)->Q (glutamine) This residue is in the vernier zone and may influence CDR conformation. The change from a short T to a longer Q may also mean that it contacts antigen. The Q is unusual occurring 58 times in mouse and twice in human. It was retained in the first version.

71 F (phenylalanine)->Y (tyrosine) This residue is a canonical position and was retained in the all versions. It is also relatively unusual in human sequences only occurring 25 times.

(B) Heavy Chain

37 V (valine)->F (phenylalanine) It was retained in the first, second and fourth versions. The F at this position is unusual only occurring 15 times in mouse and 18 times in human. It is also an interface residue.

40 A (alanine)->T (threonine) It was retained in the first version. Mutation at this position has been tried in 5 previous humanization experiments although never the change from A to T. One example is the change from A to S in the veneering of BrE-3 (Couto et al, 1994 Hybridoma, 13, 215-219) in which binding affinity was increased, although the reason was never determined. In this case, the heavy chain was also human subgroup III.

49 S (serine)->A (alanine) This residue is under the CDRs and in the vernier zone and was retained in all versions.

89 V (valine)->M (methionine) It was retained the first version. This position has been back-mutated in several humanization experiments. It was retained the first version.

93 A (alanine)->V (valine) This position is both an interface residue and in the vernier zone. It was retained in the first and second versions.

The amino acid and nucleic acid sequences of each of the different versions of the variable light and heavy chains made are as follows:

Reshaped Variable Light Chains

Reshaped variable light chain of CBE11 - version 1 light chain (VL#1) (Plasmid pAND066)

```
  1  GATATTAAGATGACCCAGTCTCCATCATCCTTGTCTGCATCGGTGGGAGACAGGGTCACT  60
     D  I  K  M  T  Q  S  P  S  S  L  S  A  S  V  G  D  R  V  T
           aa3
 61  ATCACTTGCAAGGCGGGTCAGGACATTAAAAGCTATTTAAGCTGGTACCAGCAGAAACCA  120
     I  T  C  K  A  G  Q  D  I  K  S  Y  L  S  W  Y  Q  Q  K  P

121  TGGAAAGCGCCTAAGATCCTGATCTATTATGCAACAAGGTTGGCAGATGGGGTCCCATCA  180
     W  K  A  P  K  I  L  I  Y  Y  A  T  R  L  A  D  G  V  P  S
    aa41           aa46
181  AGATTCAGTGGCAGTGGATCTGGGCAAGATTATACTCTAACCATCAGCAGCCTGCAGCCT  240
     R  F  S  G  S  G  S  G  Q  D  Y  T  L  T  I  S  S  L  Q  P
                             aa69  aa71
241  GAGGATTTCGCAACTTATTACTGTCTACAGCATGGTGAGAGCCCGTGGACGTTCGGTGGA  300
     E  D  F  A  T  Y  Y  C  L  Q  H  G  E  S  P  W  T  F  G  G

301  GGCACCAAGCTGGAGATCAAA  321
     G  T  K  L  E  I  K
```

SEQ ID NO:3-represents the nucleic acid sequence of the reshaped VL#1 above.

SEQ ID NO:4-represents the amino acid sequence of the reshaped VL#1 above.

Reshaped variable light chain of CBE11 - version 2 light chain (VL#2) (Plasmid pAND070)

```
  1  GATATCCAGATGACCCAGTCTCCATCATCCTTGTCTGCATCGGTGGGAGACAGGGTCACT  60
     D  I  Q  M  T  Q  S  P  S  S  L  S  A  S  V  G  D  R  V  T

 61  ATCACTTGCAAGGCGGGTCAGGACATTAAAAGCTATTTAAGCTGGTACCAGCAGAAACCA  120
```

```
     I  T  C  K  A  G  Q  D  I  K  S  Y  L  S  W  Y  Q  Q  K  P

121  TGGAAAGCGCCTAAGATCCTGATCTATTATGCAACAAGGTTGGCAGATGGGGTCCCATCA 180
     W  K  A  P  K  I  L  I  Y  Y  A  T  R  L  A  D  G  V  P  S
    aa41           aa46
181  AGATTCAGTGGCAGTGGATCTGGTACAGATTATACTCTAACCATCAGCAGCCTGCAGCCT 240
     R  F  S  G  S  G  S  G  T  D  Y  T  L  T  I  S  S  L  Q  P
                                 aa71
241  GAGGATTTCGCAACTTATTACTGTCTACAGCATGGTGAGAGCCCGTGGACGTTCGGTGGA 300
     E  D  F  A  T  Y  Y  C  L  Q  H  G  E  S  P  W  T  F  G  G

301  GGCACCAAGCTGGAGATCAAA 321
     G  T  K  L  E  I  K
```

SEQ ID NO:5-represents the nucleic acid sequence of the reshaped VL#2 above.

SEQ ID NO:6-represents the amino acid sequence of the reshaped VL#2 above.

Reshaped variable light chain of CBE11 - version 3 light chain (VL#3) (Plasmid pAND074)

```
  1  GATATCCAGATGACCCAGTCTCCATCATCCTTGTCTGCATCGGTGGGAGACAGGGTCACT 60
     D  I  Q  M  T  Q  S  P  S  S  L  S  A  S  V  G  D  R  V  T

 61  ATCACTTGCAAGGCGGGTCAGGACATTAAAAGCTATTTAAGCTGGTACCAGCAGAAACCA 120
     I  T  C  K  A  G  Q  D  I  K  S  Y  L  S  W  Y  Q  Q  K  P

121  GGGAAAGCGCCTAAGCCTCTGATCTATTATGCAACAAGGTTGGCAGATGGGGTCCCATCA 180
     G  K  A  P  K  L  L  I  Y  Y  A  T  R  L  A  D  G  V  P  S

181  AGATTCAGTGGCAGTGGATCTGGTACAGATTATACTCTAACCATCAGCAGCCTGCAGCCT 240
     R  F  S  G  S  G  S  G  T  D  Y  T  L  T  I  S  S  L  Q  P
                                 aa71
241  GAGGATTTCGCAACTTATTACTGTCTACAGCATGGTGAGAGCCCGTGGACGTTCGGTGGA 300
     E  D  F  A  T  Y  Y  C  L  Q  H  G  E  S  P  W  T  F  G  G

301  GGCACCAAGCTGGAGATCAAA 321
     G  T  K  L  E  I  K
```

SEQ ID NO:7-represents the nucleic acid sequence of the reshaped VL#3 above.

SEQ ID NO:8-represents the amino acid sequence of the reshaped VL#3 above.

Reshaped Variable Heavy Chains:

Reshaped variable heavy chain of CBE11 - version 1 heavy chain (VH#1) (Plasmid pAND067)

```
  1  GAGGTACAACTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAGGCTC 60
     E  V  Q  L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  R  L

 61  TCCTGTGCAGCCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGTTTCGCCAGACT 120
     S  C  A  A  S  G  F  T  F  S  D  Y  Y  M  Y  W  F  R  Q  T
                                                   aa37     aa40
121  CCGGGAAAGGGGCTGGAGTGGGTCGCAACCATTAGTGATGGTGGTAGTTACACCTACTAT 180
     P  G  K  G  L  E  W  V  A  T  I  S  D  G  G  S  Y  T  Y  Y
                           aa49
181  CCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACAGCCTCTAC 240
     P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  S  L  Y

241  CTGCAGATGAGCAGCCTGAGGGCTGAGGACACAGCCATGTATTACTGTGTAAGAGAGGAG 300
     L  Q  M  S  S  L  R  A  E  D  T  A  M  Y  Y  C  V  R  E  E
                                          aa89        aa93
301  AATGGTAACTTTTACTACTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA 360
     N  G  N  F  Y  Y  F  D  Y  W  G  Q  G  T  T  V  T  V  S  S
```

SEQ ID NO:9-represents the nucleic acid sequence of the reshaped VH#1 above.

SEQ ID NO:10-represents the amino acid sequence of the reshaped VH#1 above.

<u>Reshaped variable heavy chain of CBE11 - version 2 heavy chain (VH#2)</u> (Plasmid pAND071)

```
  1  GAGGTACAACTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAGGCTC  60
     E  V  Q  L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  R  L

 61  TCCTGTGCAGCCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGTTTCGCCAGGCC  120
     S  C  A  A  S  G  F  T  F  S  D  Y  Y  M  Y  W  F  R  Q  A
                                                    aa37
121  CCGGGAAAGGGGCTGGAGTGGGTCGCAACCATTAGTGATGGTGGTAGTTACACCTACTAT  180
     P  G  K  G  L  E  W  V  A  T  I  S  D  G  G  S  Y  T  Y  Y
                             aa49
181  CCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACAGCCTCTAC  240
     P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  S  L  Y

241  CTGCAGATGAGCAGCCTGAGGGCTGAGGACACAGCTGTGTATTACTGTGTAAGAGAGGAG  300
     L  Q  M  S  S  L  R  A  E  D  T  A  V  Y  Y  C  V  R  E  E
                                                    aa93
301  AATGGTAACTTTTACTACTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA  360
     N  G  N  F  Y  Y  F  D  Y  W  G  Q  G  T  T  V  T  V  S  S
```

SEQ ID NO:11-represents the nucleic acid sequence of the reshaped VH#2 above.

SEQ ID NO:12-represents the amino acid sequence of the reshaped VH#2 above.

<u>Reshaped variable heavy chain of CBE11 - version 3 heavy chain (VH#3)</u> (Plasmid pAND075)

```
  1  GAGGTACAACTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAGGCTC  60
     E  V  Q  L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  R  L

 61  TCCTGTGCAGCCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTGCGCCAGGCC  120
     S  C  A  A  S  G  F  T  F  S  D  Y  Y  M  Y  W  V  R  Q  A

121  CCGGGAAAGGGGCTGGAGTGGGTCGCAACCATTAGTGATGGTGGTAGTTACACCTACTAT  180
     P  G  K  G  L  E  W  V  A  T  I  S  D  G  G  S  Y  T  Y  Y
                             aa49
181  CCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACAGCCTCTAC  240
     P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  S  L  Y

241  CTGCAGATGAGCAGCCTGAGGGCTGAGGACACAGCTGTGTATTACTGCGCAAGAGAGGAG  300
     L  Q  M  S  S  L  R  A  E  D  T  A  V  Y  Y  C  A  R  E  E

301  AATGGTAACTTTTACTACTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA  360
     N  G  N  F  Y  Y  F  D  Y  W  G  Q  G  T  T  V  T  V  S  S
```

SEQ ID NO:13-represents the nucleic acid sequence of the reshaped VH#3 above.

SEQ ID NO:14-represents the amino acid sequence of the reshaped VH#3 above.

<u>Reshaped variable heavy chain of CBE11 - version 4 heavy chain (VH#4)</u> (Plasmid pAND090)

```
  1  GAGGTACAACTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAGGCTC  60
     E  V  Q  L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  R  L
```

```
 61  TCCTGTGCAGCCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGTTTCGCCAGGCC 120
     S  C  A  A  S  G  F  T  F  S  D  Y  Y  M  Y  W  F  R  Q  A
                                                     aa37
121  CCGGGAAAGGGGCTGGAGTGGGTCGCAACCATTAGTGATGGTGGTAGTTACACCTACTAT 180
     P  G  K  G  L  E  W  V  A  T  I  S  D  G  G  S  Y  T  Y  Y
                             aa49
181  CCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACAGCCTCTAC 240
     P  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  S  L  Y

241  CTGCAGATGAGCAGCCTGAGGGCTGAGGACACAGCTGTGTATTACTGCGCAAGAGAGGAG 300
     L  Q  M  S  S  L  R  A  E  D  T  A  V  Y  Y  C  A  R  E  E

301  AATGGTAACTTTTACTACTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA 360
     N  G  N  F  Y  Y  F  D  Y  W  G  Q  G  T  T  V  T  V  S  S
```

SEQ ID NO:15-represents the nucleic acid sequence of the reshaped VH#4 above.

SEQ ID NO:16-represents the amino acid sequence of the reshaped VH#4 above.

Antibodies consisting of different versions of the light and heavy chains were made and used for further studies. For instance, the antibody consisting of reshaped huCBE11 version 3 light variable chain (VL#3) and reshaped huCBE11 version 4 heavy variable chain (VH#4), named huCBE11#4 or hCBE11, was made and cell lines, E46.4 and E77.4, producing the antibody were deposited with the A.T.C.C. depository (ATCC patent deposit designation PTA-3357 and 3765, respectively).

The invention further contemplates equivalents and variants of the reshaped VH and VL sequences, i.e. those containing one or more conservative amino acid substitution which do not substantially affect LT-β-R binding. Humanized LT-β-R antibodies containing these humanized variable heavy and light sequences may be obtained by recombinant methods as described in the Examples.

Uses

The humanized anti- LT-β-R antibodies of the present invention have use in treating disease conditions wherein LT-β-R activation is therapeutically beneficial. Such conditions include treating, preventing or reducing the advancement, severity or effects of neoplasia.

In one embodiment of the invention is a method of treating a mammal (i.e. human) for a condition associated with undesired cell proliferation by administering to the mammal a therapeutically effective amount of a composition comprising humanized LT-β-R antibodies of the present invention.

In another embodiment of the invention is a method of treating a mammal (i.e. human) having a solid tumor (i.e. a carcinoma) that overexpresses LT-β-R comprising administering to said mammal a humanized LT-β-R antibody that binds to LT-β-R in

an amount effective to reduce the tumor volume. Examples of cancers whose cell proliferation is modulated by LT-β-R may be screened by measuring in vitro the level of LT-β-R and/or LT-β-R ligand (ie LTα1β2 or LIGHT) message expressed in tumor tissue libraries. Tumor tissue libraries in which of LT-β-R and/or LT-β-R ligand (ie 5 LTα1β2 or LIGHT) message is highly expressed would be candidates. Tumor types contemplated in the present invention include solid tumors including but not limited to non small cell lung cancer (NSCLC), colorectal cancer (CRC), breast cancer, as well as on prostate, gastric, skin, stomach, esophagus and bladder cancer.

The humanized antibodies of the subject invention which are used in treating 10 conditions associated with undesired cell proliferation, in particular tumor therapy, advantageously inhibit tumor cell growth, as measured for example by a decrease in the tumor volume, greater than about 10%, 20%, 30% or 40% and most advantageously greater than about 50%. The humanized antibodies are obtained through screening (see, for example, the discussion in Example 3). For example, humanized antibodies 15 for use in the present invention can be selected on the basis of decreased tumor volume versus untreated cancer cells (e.g., greater than about 10%, 20%, 30%, 40% or 50%).

The present invention also provides pharmaceutical compositions comprising a humanized antibody of the present invention and a pharmaceutically acceptable excipient. Suitable carriers, for instance, and their formulations, are described in 20 Remington' Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically an appropriate amount of a pharmaceutically acceptable salt is used in the formulation to render the formulation isotonic. Examples of the carrier include buffers such as saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7.4 to 25 about 7.8. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers, which matrices are in the form of shaped articles, e.g. liposomes, films or microparticles. It will be apparent to those of skill in the art that certain carriers may be more preferable depending upon for instance the route of administration and concentration of the pharmaceutical 30 composition being administered.

Administration may be accomplished by injection (eg intravenous, intraperitoneal, subcutaneous, intramuscular) or by other methods such as infusion that ensure delivery to the bloodstream in an effective form.

The humanized antibodies of the present invention can be administered at an effective dose to treat the particular clinical condition addressed. Determination of a preferred pharmaceutical formulation and a therapeutically efficient dose regiment for a given application is well within the skill of the art taking into consideration, for example, the weight and condition of the patient, the extent of the desired treatment and the tolerance of the patient for the treatment. For example, an effective dosage will be in the range of about 0.05 to about 100 milligrams per kilogram of body weight per day. More particularly, about 0.05mg, 0.1 mg, 0.2 mg, 0.3 mg, 0.4mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1mg, 2 mg, 3 mg, 4mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 15 mg, 20 mg, or 25 mg, per kilogram body weight per day. Alternatively about 0.05 to about 100 milligrams, more particularly, about 0.05mg, 0.1 mg, 0.2 mg, 0.3 mg, 0.4mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1mg, 2 mg, 3 mg, 4mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 15 mg, 20 mg, or 25 mg, per kilogram body weight per week. Alternatively about 0.05 to about 100 milligrams, more particularly, about 0.05mg, 0.1 mg, 0.2 mg, 0.3 mg, 0.4mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1mg, 2 mg, 3 mg, 4mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 15 mg, 20 mg, or 25 mg, per kilogram body weight per two weeks. Alternatively about 0.05 to about 100 milligrams, more particularly, about 0.05mg, 0.1 mg, 0.2 mg, 0.3 mg, 0.4mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1mg, 2 mg, 3 mg, 4mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 15 mg, 20 mg, or 25 mg, per kilogram body weight per three weeks. Alternatively about 0.05 to about 100 milligrams, more particularly, about 0.05mg, 0.1 mg, 0.2 mg, 0.3 mg, 0.4mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1mg, 2 mg, 3 mg, 4mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 15 mg, 20 mg, or 25 mg, per kilogram body weight per four weeks.

Practice of the present invention will employ, unless indicated otherwise, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, protein chemistry, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, **Molecular Cloning: A Laboratory Manual,** 2nd edition. (Sambrook, Fritsch and Maniatis, eds.), Cold Spring Harbor Laboratory Press, 1989; **DNA Cloning**, Volumes I and II (D.N. Glover, ed), 1985; **Oligonucleotide Synthesis**, (M.J. Gait, ed.), 1984; U.S. Patent No.

4,683,195 (Mullis et al.,); **Nucleic Acid Hybridization** (B.D. Hames and S.J. Higgins, eds.), 1984; Transcription and Translation (B.D. Hames and S.J. Higgins, eds.), 1984; **Culture of Animal Cells** (R.I. Freshney, ed). Alan R. Liss, Inc., 1987; **Immobilized Cells and Enzymes,** IRL Press, 1986; **A Practical Guide to Molecular Cloning** (B. Perbal), 1984; **Methods in Enzymology,** Volumes 154 and 155 (Wu et al., eds), Academic Press, New York; **Gene Transfer Vectors for Mammalian Cells** (J.H. Miller and M.P. Calos, eds.), 1987, Cold Spring Harbor Laboratory; **Immunochemical Methods in Cell and Molecular Biology** (Mayer and Walker, eds.), Academic Press, London, 1987; **Handbook of Experiment Immunology,** Volumes I-IV (D.M. Weir and C.C. Blackwell, eds.), 1986; **Manipulating the Mouse Embryo,** Cold Spring Harbor Laboratory Press, 1986.

The following Examples are provided to illustrate the present invention, and should not be construed as limiting thereof.

EXAMPLES:

Example 1

Construction And Expression Of chCBE11

cDNAs encoding the murine CBE11 variable regions of the heavy and light chains were used to construct vectors for expression of murine-human chimeras (chCBE11) in which the muCBE11 variable regions were linked to human IgG1 and kappa constant regions. For construction of the heavy chain chimera, a 0.36 kb PstI-BstEII fragment from the CBE11 heavy chain subclone pEAG970 was subcloned into the phosphatased 2.82 kb PstI-BstEII vector fragment from the 5a8 heavy chain plasmid pLCB7 (5a8 is a molecularly cloned CD4-specific mAb previously characterized at Biogen), to add a murine heavy chain signal sequence and splice donor site to the muCBE11 heavy chain variable region. In this plasmid, called pEAG979, the heavy chain mature N-terminus differs by two residues from the N-terminal sequence of purified authentic CBE11 heavy chain derived from Edman degradation, since it was primer-determined during PCR. To correct the heavy chain N-terminus, pEAG979 was subjected to unique site elimination (USE) mutagenesis using an Amersham Pharmacia Biotech USE mutagenesis kit following the manufacturer's recommended protocol. Mutated plasmids were identified by screening for introduced AvaII, PstI, and RsaI changes. The heavy chain sequence in the resultant plasmid pEAG981 was

confirmed by DNA sequencing. The 0.44 kb NotI-HindIII heavy chain variable domain fragment from pEAG981 and the 1.21 kb HindIII-NotI fragment from the plasmid pEAG964, containing a human IgG1 constant region, were subcloned into the NotI site of the pCEP4 (Invitrogen) EBV expression vector-derived plasmid pCH269, producing plasmid pEAG983.

For construction of the light chain chimera, a 0.11 kb NotI-EcoRV fragment from the plasmid pMDR985 and a 0.37 kb EcoRV-BamHI fragment from the CBE11 light chain variable domain plasmid pEAG967 were subcloned into the phosphatased 2.94 kb NotI-BamHI vector fragment from Stratagene's pBluescriptIISK+ cloning vector, to add a murine light chain signal sequence and a 5' NotI site in the resulting plasmid pEAG978. This plasmid was subjected to USE mutagenesis using an Amersham Pharmacia Biotech USE mutagenesis kit following the manufacturer's recommended protocol, with mutagenic primers which encoded a V3K substitution to match the authentic CBE11 light chain N-terminus, and which introduced a BglII site at the 3' end of the light chain variable domain. Mutated plasmids were identified by screening for introduced BglII, EcoRV, and MseI site changes. The light chain sequence in the resultant plasmid pEAG980 was confirmed by DNA sequencing. The 0.41 kb NotI-BglII light chain variable domain fragment from pEAG980 and the 0.68 kb BclI-NotI fragment from the plasmid pEAG963, containing a human kappa light chain constant domain, were subcloned into the NotI site of the pCEP4 (Invitrogen) EBV expression vector-derived plasmid pCH269, producing plasmid pEAG982.

Expression vectors (chCBE11 heavy chain vector pEAG983 and chCBE11 light chain vector pEAG982) were co-transfected into 293-EBNA cells and transfected cells were tested for antibody secretion and specificity (empty vector- and ch5c8 (a molecularly cloned CD154-specific mAb previously characterized at Biogen)-transfected cells served as controls). Western blot analysis (developed with anti-human heavy and light chain antibodies) of protein A immunoprecipitates from whole cell lysates and conditioned medium indicated that chCBE11-transfected cells synthesized and efficiently secreted heavy and light chains at levels similar to ch5c8-transfected cells. FACS analysis of LT-β-R -expressing HT-29 cells stained with conditioned medium from transfected cells indicated that the chCBE11 antibody bound and produced staining patterns similar to those of muCBE11, while conditioned medium

from mock- and ch5c8-transfected cells failed to stain LT-β-R on HT-29 cells. Chimeric CBE11 produced from transient transfection was purified and demonstrated to induce IL-8 secretion by LT-β-R -expressing A375 melanoma cells and to inhibit growth of WiDRr adenocarcinoma cells in nude mice.

5 **Example 2**

Construction And Expression Of huCBE11

Design of the reshaped variable domains to produce humanized CBE11 (huCBE11) was carried out as described supra. The choice of the human acceptor frameworks was by homology matching: human kappa subgroup I mAb TNF-A1 for the
10 light chain (Griffiths et al., 1993), and human subgroup III mAb FLA-IgG for the heavy chain (Malisan et al., 1996). Three versions of each of the variable light and four versions of the variable heavy reshaped chains were designed. In general the first version contains the most back mutations to the murine donor sequences, while the last version contains the fewest (i.e., the most "humanized").

15 The huCBE11 variable regions were made by unique site elimination (USE) mutagenesis using an Amersham Pharmacia Biotech USE mutagenesis kit following the manufacturer's recommended protocol, using the chCBE11 variable domain plasmids as starting templates. The mutagenic primers for the framework (FR) changes are described below. The cDNA sequence of the human acceptor frameworks (Kabat
20 database #004770 for the light chain and Kabat #040003 for the heavy chain) were used, with silent mutations introduced to produce restriction site changes to facilitate identification of mutated plasmids. Mutated plasmids were identified by identified by screening for the introduced restriction site changes. The variable region cDNA sequences in the resultant plasmids were confirmed by DNA sequencing.

25 VH#1 used pEAG981 template with the following primers: FR1 primer 5' GCC TGG AGG GTC CCT GAG GCT CTC CTG TGC AGC CTC 3' (SEQ ID NO:17), which introduced a Bsu36I site; FR2 primer 5' GTT TCG CCA GAC TCC GGG AAA GGG GCT GGA GTG GGT CGC AAC 3' (SEQ ID NO:18), which introduced NciI and HpaII sites; and FR3 primer 5' CAG AGA CAA TGC CAA GAA CAG CCT CTA
30 CCT GCA GAT GAG CAG CCT GAG GGC TGA GGA CAC AGC CAT G 3' (SEQ ID NO:19), which introduced Bsu36I and PstI sites and removes an RsaI site. The resultant VH#1 plasmid was designated pAND067.

VH#2 used pAND067 template with the following primers: FR2 primer 5' CAT GTA TTG GTT TCG CCA GGC CCC GGG AAA GGG GCT GG 3' (SEQ ID NO:20), which introduced a SmaI site; and FR3 primer 5' GGG CTG AGG ACA CAG CTG TGT ATT ACT GTG TAA GAG 3' (SEQ ID NO:21), which introduced a PvuII site.
5 The resultant VH#2 plasmid was designated pAND071.

VH#3 used plasmid pAND067 template with the following primers: FR2 primer 5' GTG ACT ATT ACA TGT ATT GGG TGC GCC AGG CCC CGG GAA AGG GGC TGG AG 3' (SEQ ID NO:22), which introduced SmaI and HhaI sites; and FR3 primer 5' GAG GGC TGA GGA CAC AGC TGT GTA TTA CTG CGC AAG AGA
10 GGA GAA TGG TAA C 3' (SEQ ID NO:23), which introduced PvuII and FspI sites. The resultant VH#3 plasmid was designated pAND075.

Expression vectors for the huCBE11 heavy chains were made by subcloning the 0.44 kb NotI-HindIII heavy chain variable domain fragments from pAND067, pAND071, or pAND075, and the 1.21 kb HindIII-NotI fragment from the plasmid
15 pEAG964, containing a human IgG1 constant region, were subcloned into the NotI site of the pCEP4 EBV expression vector-derived plasmid pCH269, producing heavy chain expression vectors pAND069 (VH#1), pAND073 (VH#2), and pAND077 (VH#3).

VL#1 used plasmid pEAG980 template with the following primers: FR1 primer 5' CTT GCA AGT GAT AGT GAC CCT GTC TCC CAC CGA TGC AGA CAA
20 GGA TGA TGG AGA CTG GGT CAT C 3' (SEQ ID NO:24), which removed HinfI and NsiI sites; FR2 primer 5' CAT AAT AGA TCA GGA TCT TAG GCG CTT TCC ATG GTT TCT GCT G 3' (SEQ ID NO:25), which introduced HaeII and HhaI sites; and FR3 primer 5' GTA GAC AGT AAT AAG TTG CGA AAT CCT CAG GCT GCA GGC TGC TGA TGG TTA GAG TAT AAT CTT GCC CAG ATC 3' (SEQ ID
25 NO:26), which introduced Bsu36I, DdeI, and PstI sites. The resultant VL#1 plasmid was designated pAND066.

VL#2 used plasmid pAND066 template with the following primers: FR1 primer 5' GAT GGA GAC TGG GTC ATC TGG ATA TCA CCT CTG GCA CCT G 3' (SEQ ID NO:27), which introduced an EcoRV site; and FR3 primer 5' GAT GGT TAG AGT
30 ATA ATC TGT ACC AGA TCC ACT GCC ACT G 3' (SEQ ID NO:28), which introduced an RsaI site. The resultant VL#2 plasmid was designated pAND070.

VL#3 used plasmid pAND066 template with the following primers: FR1 primer 5' GAT GGA GAC TGG GTC ATC TGG ATA TCA CCT CTG GCA CCT G 3' (SEQ

ID NO:29), which introduced an EcoRV site; FR2 primer 5' CAA CCT TGT TGC ATA ATA GAT CAG AAG CTT AGG CGC TTT CCC TGG TTT CTG CTG GTA CC 3' (SEQ ID NO:30), which introduced a HindIII site and removed NcoI and StyI sites; and FR3 primer 5' GAT GGT TAG AGT ATA ATC TGT ACC AGA TCC ACT GCC ACT G 3' (SEQ ID NO:31), which introduced an RsaI site. The resultant VL#3 plasmid was designated pAND074.

Expression vectors for the huCBE11 light chains were made by subcloning the 0.41 kb NotI-BglII light chain variable domain fragments from pAND066, pAND070, or pAND074 and the 0.68 kb BclI-NotI fragment from the plasmid pEAG963, containing a human kappa light chain constant domain, were subcloned into the NotI site of the pCEP4 EBV expression vector-derived plasmid pCH269, producing light chain expression vectors pAND068 (VL#1), pAND072 (VL#2), and pAND076 (VL#3).

Expression vectors were co-transfected into 293-EBNA cells and transfected cells were tested for antibody secretion and specificity (empty vector-transfected cells served as negative control). Western blot analysis (developed with anti-human heavy and light chain antibodies) of protein A immunoprecipitates from whole cell lysates and conditioned medium indicated that huCBE11-transfected cells synthesized and efficiently secreted heavy and light chains at levels similar to chCBE11-transfected cells. FACS analysis of LT-β-R -expressing HT-29 cells stained with conditioned medium from transfected cells indicated that the huCBE11#3 mAb bound less well than huCBE11#1 and huCBE11#2 mAbs relative to chCBE11 (Table 1 below) where huCBE11#1 (VL#1 with VH#1); huCBE#2 (VL#2 with VH#2) and huCBE11#3 (VL#3 with VH#3). Mix and match co-transfections suggested that the reduction could be attributed to the VH#3, which differed from VH#2 at two framework residues: FR2 F37V and FR3 V93A. To examine the individual contributions of each of these changes, new heavy chain expression vectors were constructed. Plasmid pAND089, the F37V variant of VH#2, was made by subcloning the 311 bp NotI-PstI fragment from pAND075, the 126 bp PstI-HindIII fragment from pAND071, and the 1.21 kb HindIII-NotI fragment from the plasmid pEAG964 into the NotI site of the pCEP4 EBV expression vector-derived plasmid pCH269. Plasmid pAND090, the V93A variant of VH#2, was made by subcloning the 311 bp NotI-PstI fragment from pAND071, the 126 bp PstI-HindIII fragment from pAND075, and the 1.21 kb HindIII-NotI fragment from the plasmid pEAG964 into the NotI site of the pCEP4 EBV expression vector-derived

plasmid pCH269. These H2/H3 chimeric heavy chains were co-transfected into 293-EBNA cells with VL#2 or VL#3. FACS analysis indicated that the V93A H2 variant restored LT-β-R binding when paired with the VL#3 (Table 1 supra). The pAND076 and pAND090 pairing was designated huCBE11#4 (Table 1 supra).

5 Co-transfections of 293-EBNA cells with chCBE11 and huCBE11 versions #1-4 were scaled up and conditioned medium was harvested. Antibody was purified on Protein A-Sepharose. Purified mAbs were assayed for activity.

Table 1. FACS staining of HT-29 cells by chCBE11 and huCBE11

	Light chain	Heavy chain	Relacive MFI
ChCBE11	pEAG982	pEAG983	1.00
HuCBE11#1	pAND068	pAND069	1.00
HuCBE11#2	pAND072	pAND073	1.00
HuCBE11#3	pAND076	pAND077	0.62
L2/H3	pAND072	pAND077	0.42
L3/H2	pAND076	pAND073	1.00
L2/F37V H2	pAND072	pAND089	0.65
L2/V93A H2	pAND072	pAND090	0.75
L3/F37V H2	pAND076	pAND089	0.80
HuCBE11#4	pAND076	pAND090	1.00

10 Conditioned medium from transiently transfected cells was used to stain HT-29 cells by incubating for 30 min on ice, washing cells twice with FACS buffer (PBS with 5% FBS and 0.05% sodium azide), staining with PE-conjugated anti-human IgG (H + L), Jackson ImmunoResearch Laboratories, Inc., for 30 min on ice in FACS buffer, washing cells twice with FACS buffer, and resuspending in FACS buffer for analysis.

15 Relative MFI refers to mean MFI normalized to that observed for chCBE11. Data shown represents the average from two independent transfections.

Example 3

IL-8 Agonism on A375 cells

Purified mAbs were assayed for activity. Results of an IL-8 release assay on

20 A375 human melanoma cells are shown in **Figure 1** which measures the amount of IL-8 released upon the binding of anti-LT-β-R antibodies with an LT-β-R expressed on the surface of A375 human melanoma cells. A375 cells were plated at 10^5/ml into 96 well plates containing either soluble antibodies or antibodies captured onto goat anti-human

IgG Fc (Jackson ImmunoResearch Laboratories)-coated wells. The culture plates were incubated overnight. Conditioned medium was harvested and analyzed for IL-8 by ELISA.

Example 4

Cytotoxicity on WiDr cells

Results of a cytotoxicity assay using WiDr colon cancer cells with soluble anti-LT-β-R antibodies onto anti-human IgG Fc coated wells which demonstrate that anti-LT-β-R antibodies increases cytotoxicity in cancer cells as shown in **Figure 2**. WiDr cells were plated at $6x10^4$/ml in the presence of 80 units/ml huIFN-gamma into 96 well plates containing either soluble antibodies or antibodies captured onto goat anti-human IgG Fc (Jackson ImmunoResearch Laboratories)-coated wells. The culture plates were incubated for 5 days. MTT was added for 4 hrs and the resulting precipitate was dissolved by overnight incubation with 10% SDS in 10 mM HCl, and O.D.s were read on a microplate reader.

Example 5

The antibody consisting of reshaped huCBE11 version 3 light variable chain (VL#3) and reshaped huCBE11 version 4 heavy variable chain, named huCBE11#4 or hCBE11, was made and the cell line producing the antibody was deposited with the A.T.C.C. depository (ATCC patent deposit designation PTA-3357). The full polypeptide sequences of each of the light and heavy chains, including the constant domains are as follows:

Sequence of mature huCBE11 version 3 light chain (SEQ ID NO: 32):

1 DIQMTQSPSS LSASVGDRVT ITCKAGQDIK SYLSWYQQKP GKAPKLLIYY
51 ATRLADGVPS RFSGSGSGTD **Y**TLTISSLQP EDFATYYCLQ HGESPWTFGG
101 GTKLEIK [RTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201 LSSPVTKSFN RGEC]

CDRs are underlined; back mutation F71Y is bolded; constant domain is bracketed.

Sequence of mature huCBE11 version 4 heavy chain (SEQ ID NO: 33):

V37F S49A
1 EVQLVESGGG LVKPGGSLRL SCAASGFTFS DYYMYWFRQA PGKGLEWVAT

51 ISDGGSYTYY PDSVKGRFTI SRDNAKNSLY LQMSSLRAED TAVYYCAREE

101 NGNFYYFDYW GQGTTVTVSS [ASTKGPSVFP LAPSSKSTSG GTAALGCLVK

5 151 DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT

201 YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP

251 KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN

10 301 STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ

351 VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV

15 401 LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPG]

CDRs are underlined; back mutations V37F and S49A are bolded; constant domain is bracketed.

6 week old nude mice were injected intraperitoneally with 100 ug of anti-LFA3
20 antibody (1E6), 100 ug anti-LTBR antibody (huCBE11#4) or not injected (control). The animals were then injected subcutaneously with $1x10^6$ WIDR colon adenocarcinoma cells. The huCBE11#4-treated mice were retreated weekly with 100 ug of antibody and the mCBE11 animals were retreated on day 14 only. Tumor size was measured weekly and the volume of the tumor sphere calculated (**Figure 3**). Animals were sacrificed
25 when their tumors reached a volume of 2.0 cm^3 (16 mm diameter) and their death was noted on the survival chart (**Figure 4**).

Example 6

6 week old nude mice were either injected intraperitoneally with 100 ug anti-LTBR antibody (huCBE11#4) or were not injected (control). All animals were then injected
30 subcutaneously with $1x10^6$ WIDR colon adenocarcinama cells. The huCBE11#4-treated mice were retreated weekly with 100 ug of huCBE11#4. Tumor size was measured weekly and the volume of the tumor sphere calculated. Tumor volumes shown represent the average of 10 control animals and 8 huCBE11#4-treated animals (**Figure 5**). Weekly treatment with huCBE11#4 significantly inhibits the growth rate of WIDR
35 tumors implanted subcutaneously in nude mice. Animals treated with antibody through day 21 continue to show reduced tumor growth rates two week following the cessation of treatment.

Example 7

huCBE11#4 Slows Growth of Pregrown WIDR Tumors and Increases Survival in WIDR Tumor-bearing Nude Mice

10^6 WIDR cells were pregrown subcutaneously for 10 days in Nude mice. The mice received subcutaneous injections of either PBS or huCBE11#4 weekly or mCBE11 alternate weeks. Tumor weights were calculated from width and length measurements and animals with tumors over 2000 mg were sacrificed, their tumor weights at time of sacrifice continued into the statistical averaging. Error bars represent standard error. Tumor weights were calculated using the formula: (Width x Width x Length)/2= tumor weight in mg. The results are graphed in **Figure 6** and show that huCBE11#4 is able to slow pre-grown tumors in vivo.

In addition, tumors were grown and treated as described above and percent survival of the animals was measured. The results are graphed in **Figure 7** and show that huCBE11#4 is able to induce prolonged survival in vivo in mice with pregrown tumors.

Example 8

Antibody affinity measurement

HT-29 cells were grown in DMEM supplemented with L-glutamine, non-essential amino acids, sodium pyruvate and 10% fetal bovine serum. Cells were washed once with PBS and removed from the plate by incubating at room temperature for five minutes with PBS plus 20 mM EDTA. Cells were centrifuged at 1000 rpm (110 x g) for five minutes and resuspended to a density of 1 x 10^7 cells/mL in PBS.

HuCBE11#4 anti-LTβR antibody and humanized anti-CD40L as a negative control were diluted in PBS and a 12 point serial 1:4 dilution was made to a final concentration range of 2.37 pM – 10 μM. 100 μL cell suspension and 100 μL antibody dilution were added together to each well of a 96 well V-bottom microtiter plate. The antibody and the cells were incubated at 4°C for 2 hours. The plate was centrifuged at 1000 rpm (110 x g) for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was washed with cold PBS six times.

Goat-anti human IgG-phycoerythrin conjugate (Jackson Immunoresearch) was diluted 1:100 in PBS and 200 μL was added to each well. The cells were incubated with this secondary antibody for one hour at 4°C, centrifuged as described above, and washed once in cold PBS. The cells were then transferred to polystyrene test tubes.

Fluorescence intensity was measured on a FACS Calibur instrument (Beckton Dickinson).

The mean fluorescence intensity values of the staining for anti-CD40L non-specific binding control were plotted against the antibody concentration in Delta Graph.
5 The values were fit to a straight line and the theoretical non-specific binding values for each antibody concentration were subtracted from each data point for the huCBE11#4 dilution series.

These specific fluorescence intensity values were then plotted against huCBE11#4 concentrations on a log scale. The resulting curve is bell shaped and symmetrical, and
10 reflects self-inhibition of the antibody binding at high concentrations. The left half of this curve was fit to a four parameter equation to find the functional affinity of the antibody. The resulting curve fit gives an EC_{50} value of 60 pM for the huCBE11#4 binding to HT-29 cells.

It will be apparent to those skilled in the art that various modifications and
15 variations can be made in the polypeptides, compositions and methods of the invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided that they come within the scope of the appended claims and their equivalents.

What is claimed is:

1. A humanized anti-lymphotoxin-beta receptor (LT-β-R) antibody whose light chain complementary determining regions are defined by amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:1, and whose heavy chain complementary determining regions are defined by amino acid residues 31 to 35, 50 to 66 and 99 to 109 of SEQ ID NO: 2 and wherein the antibody comprises at least one of the following residues in its light chain: K3, W41, I46, Q69 and Y71; or at least one of the following residues in its heavy chain: F37, T40, A49, M89 and V93 (Kabat numbering convention).

2. A humanized anti-lymphotoxin-beta receptor (LT-β-R) antibody whose light chain complementary determining regions are defined by amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:1, and whose heavy chain complementary determining regions are defined by amino acid residues 31 to 35, 50 to 66 and 99 to 109 of SEQ ID NO:2 and wherein the antibody comprises residue Y71 in its light chain (Kabat numbering convention).

3. A humanized anti-lymphotoxin-beta receptor (LT-β-R) antibody whose light chain complementary determining regions are defined by amino acid residues 24 to 34, 50 to 56 and 89 to 97 of SEQ ID NO:1, and whose heavy chain complementary determining regions are defined by amino acid residues 31 to 35, 50 to 66 and 99 to 109 of SEQ ID NO:2 and wherein the antibody comprises residues F37 and A49 in its heavy chain (Kabat numbering convention).

4. The antibody of claim 1, wherein the antibody comprises a light chain variable domain sequence defined by amino acid residues 1 to 107 of SEQ ID NO:8.

5. The antibody of claim 1, wherein the antibody comprises a heavy chain variable domain sequence defined by amino acid residues 1 to 120 of SEQ ID NO:16.

6. The antibody of claim 4, wherein the antibody further comprises a heavy chain variable domain sequence defined by amino acid residues 1 to 120 of SEQ ID NO:16

7. An antibody comprising the same heavy and light chain polypeptide sequences as an antibody produced by cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765).

8. The antibody according to anyone of claims 1-7 wherein the antibody substantially retains the binding properties of the parent antibody.

9. The antibody according to anyone of claims 1-7 wherein the antibody is further linked to an immunotoxin.

5 10. The antibody according to anyone of claims 1-7 wherein the antibody is further linked to a chemotherapeutic drug.

11. A composition comprising an antibody of any one of claims 1-7 and a pharmaceutically acceptable carrier.

12. A method of treating or reducing the advancement, severity or effects of

10 neoplasia in a human comprising administering the composition of claim 11.

13. An isolated nucleic acid comprising a coding sequence for the light chain of an antibody produced by cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765).

14. An isolated nucleic acid comprising a coding sequence for the heavy chain

15 of an antibody produced by cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765).

15. An isolated nucleic acid comprising a coding sequence for residues 1 to 107 of SEQ ID NO:8.

16. An isolated nucleic acid comprising a coding sequence for residues 1 to 120

20 of SEQ ID NO:16.

17. A cell of cell line E46.4 (ATCC patent deposit designation PTA-3357) or cell line E77.4 (ATCC patent deposit designation 3765).

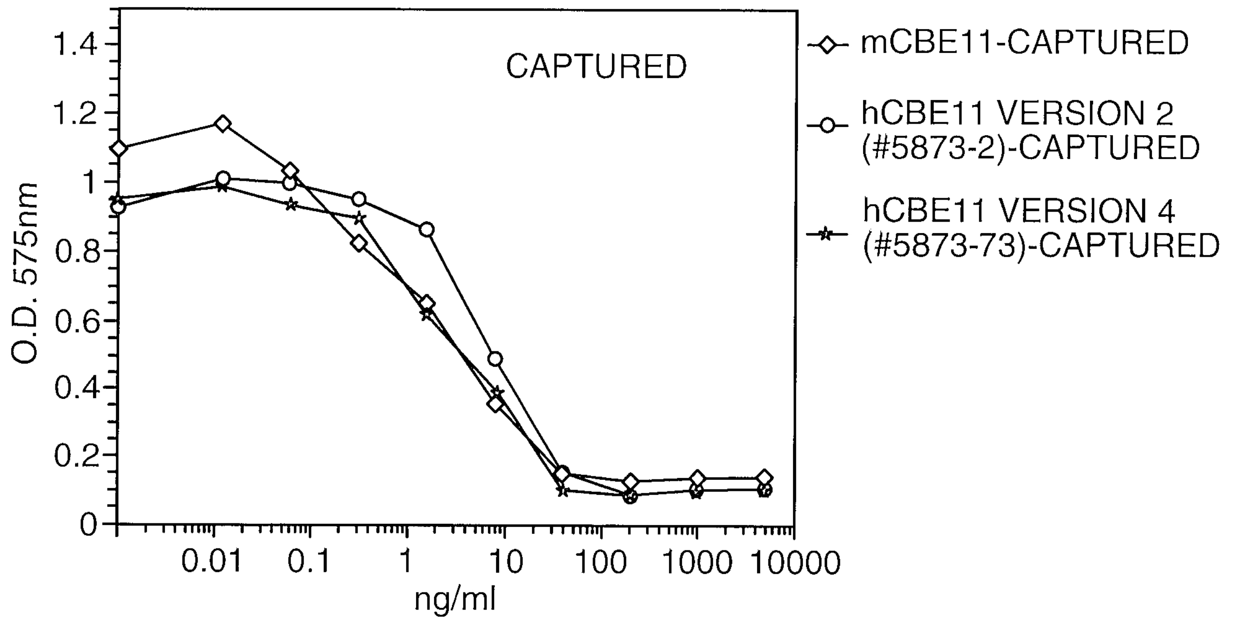


FIG. 1A

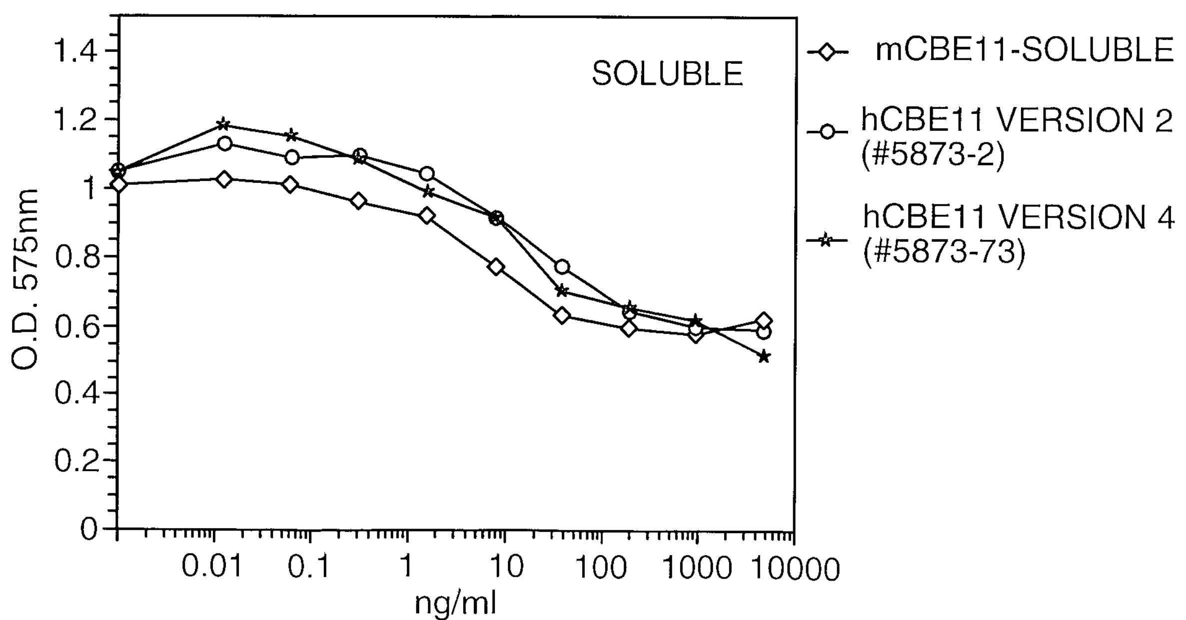


FIG. 1B

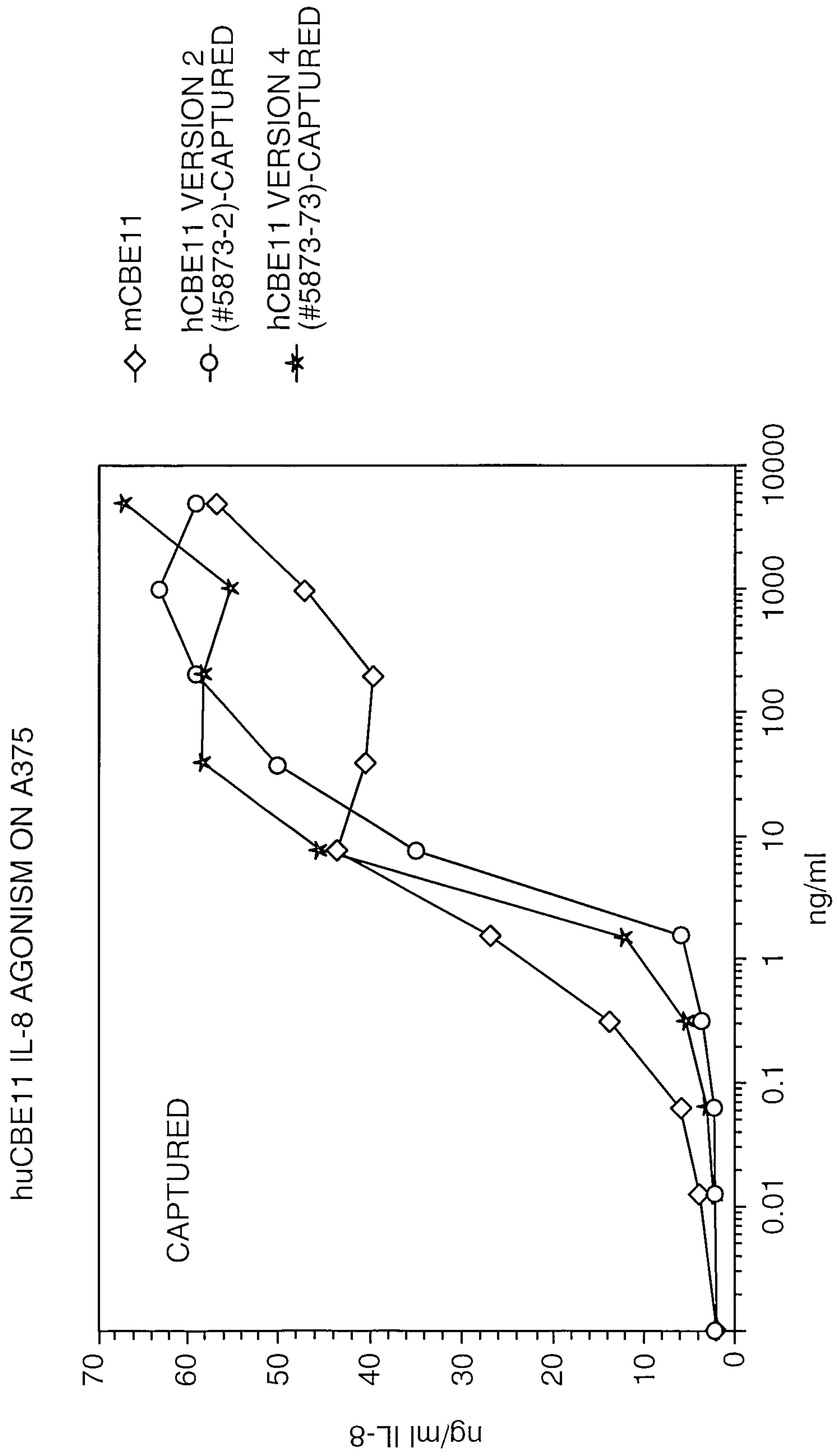


FIG. 2A

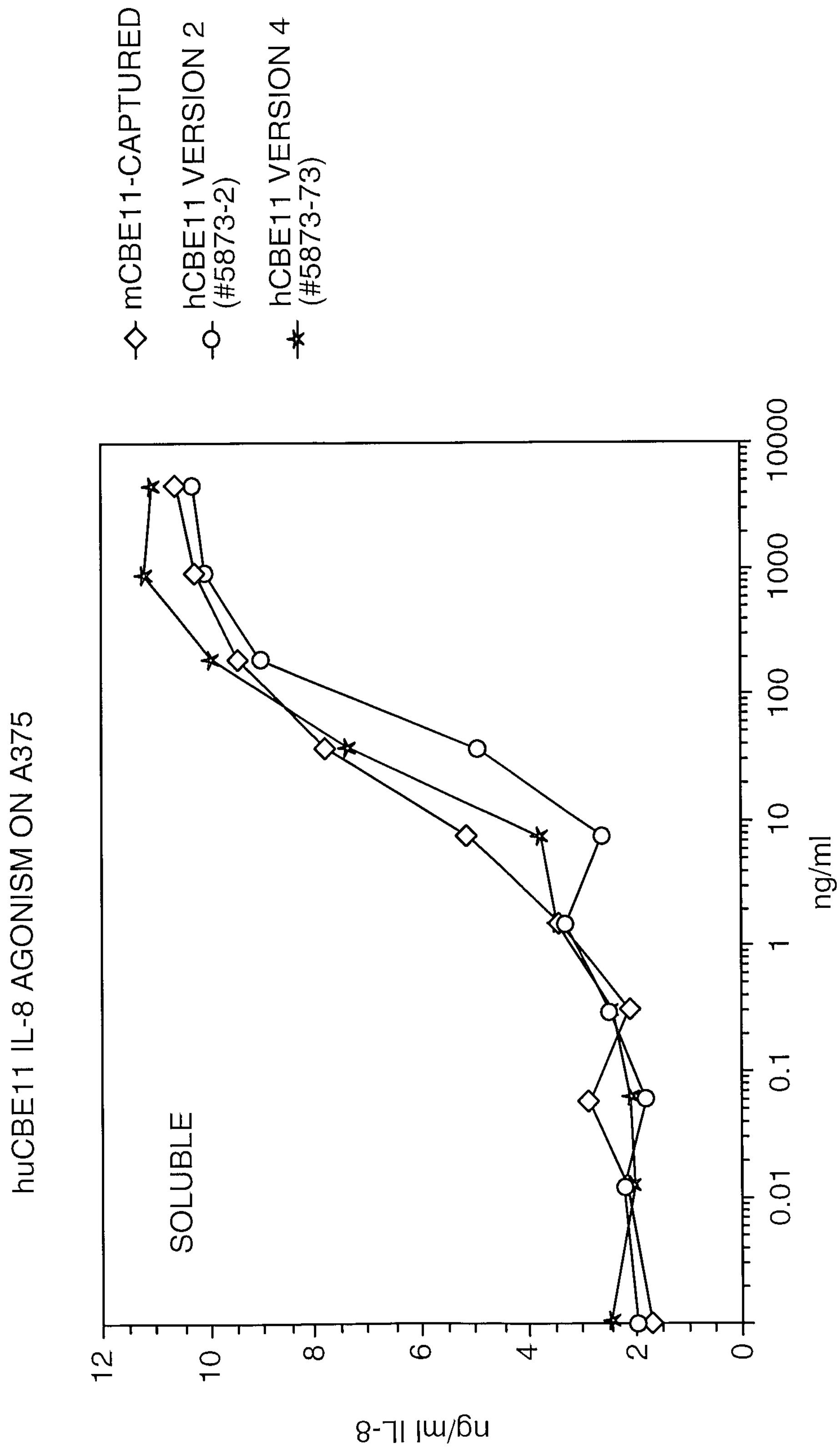


FIG. 2B

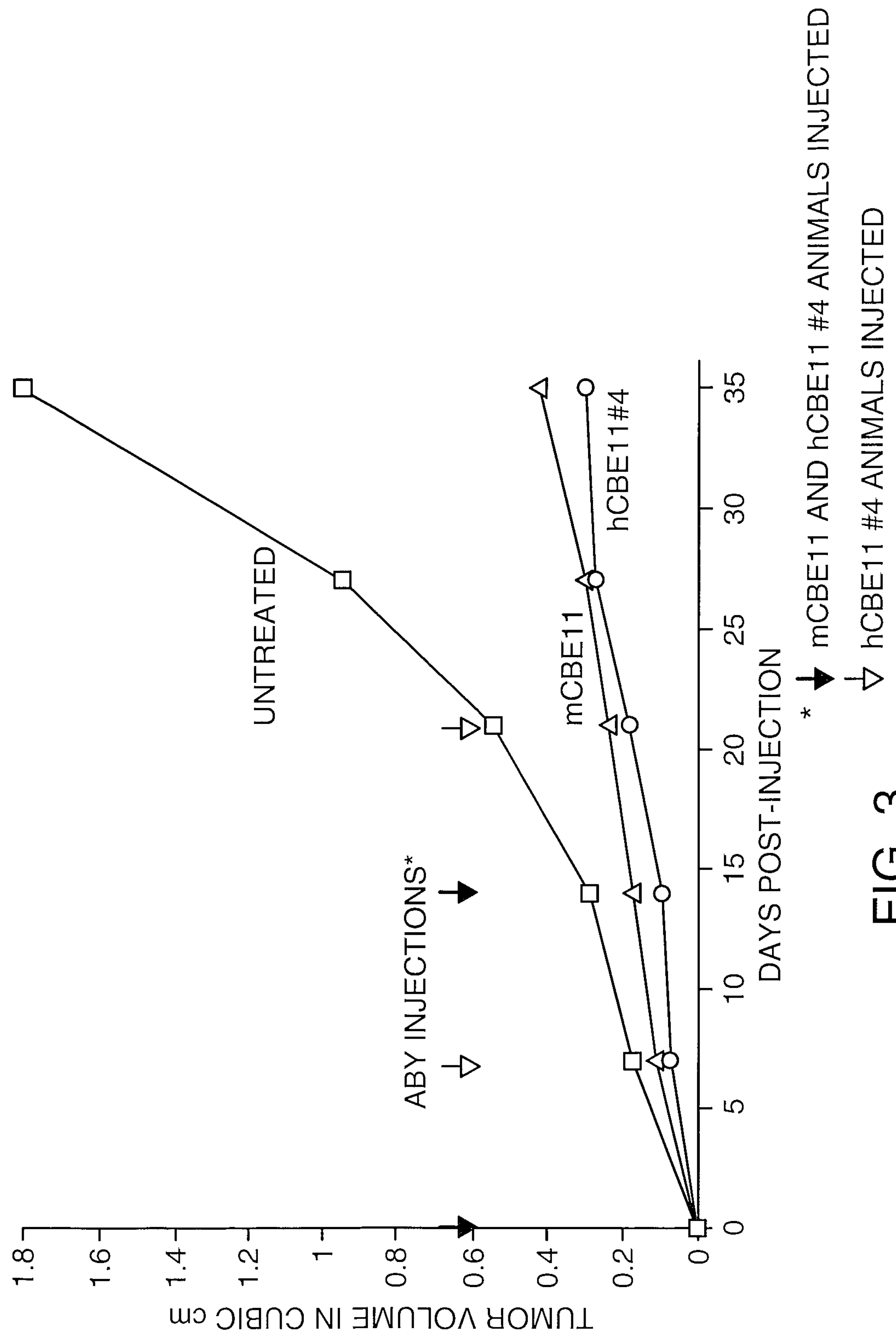


FIG. 3

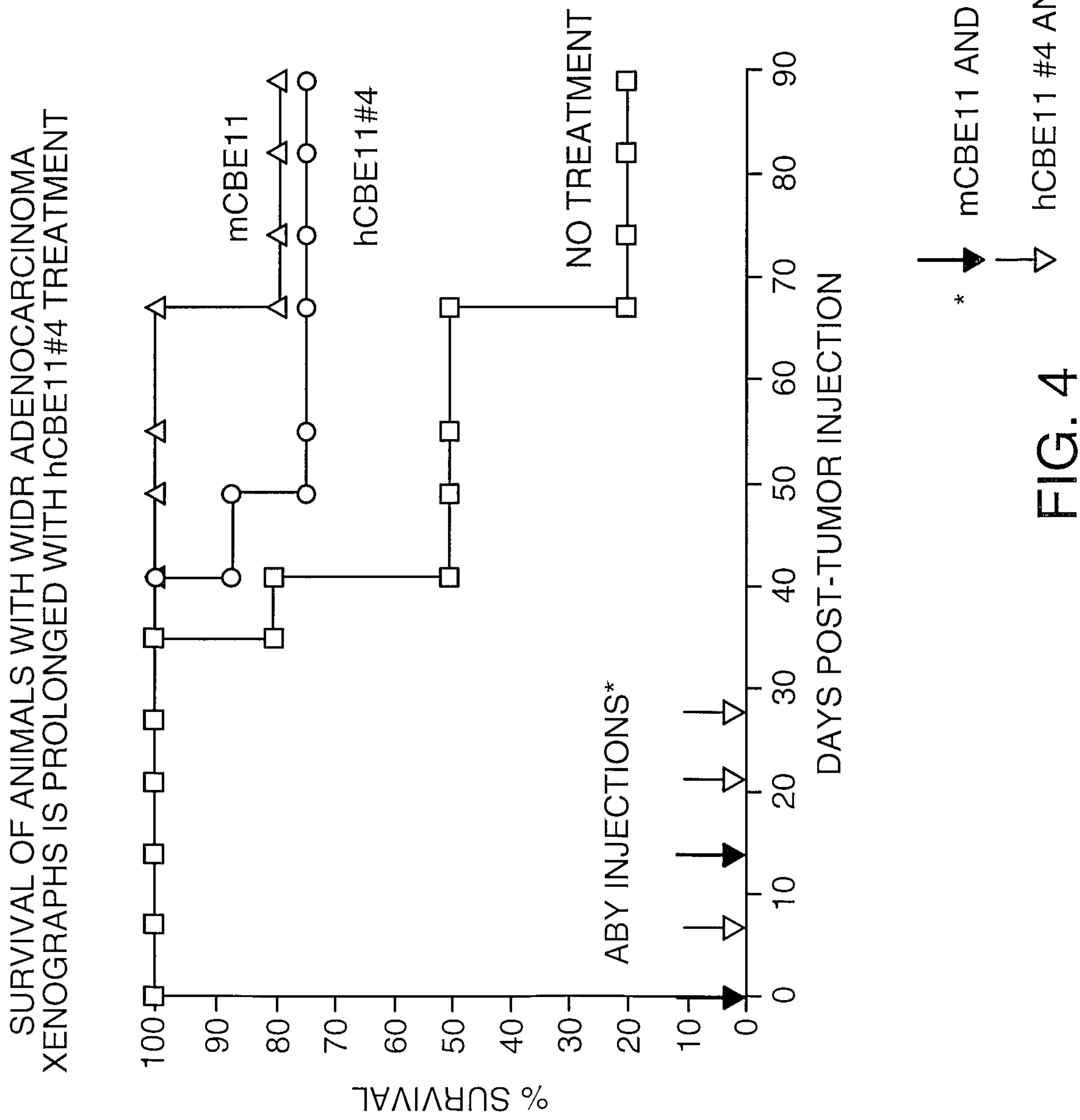
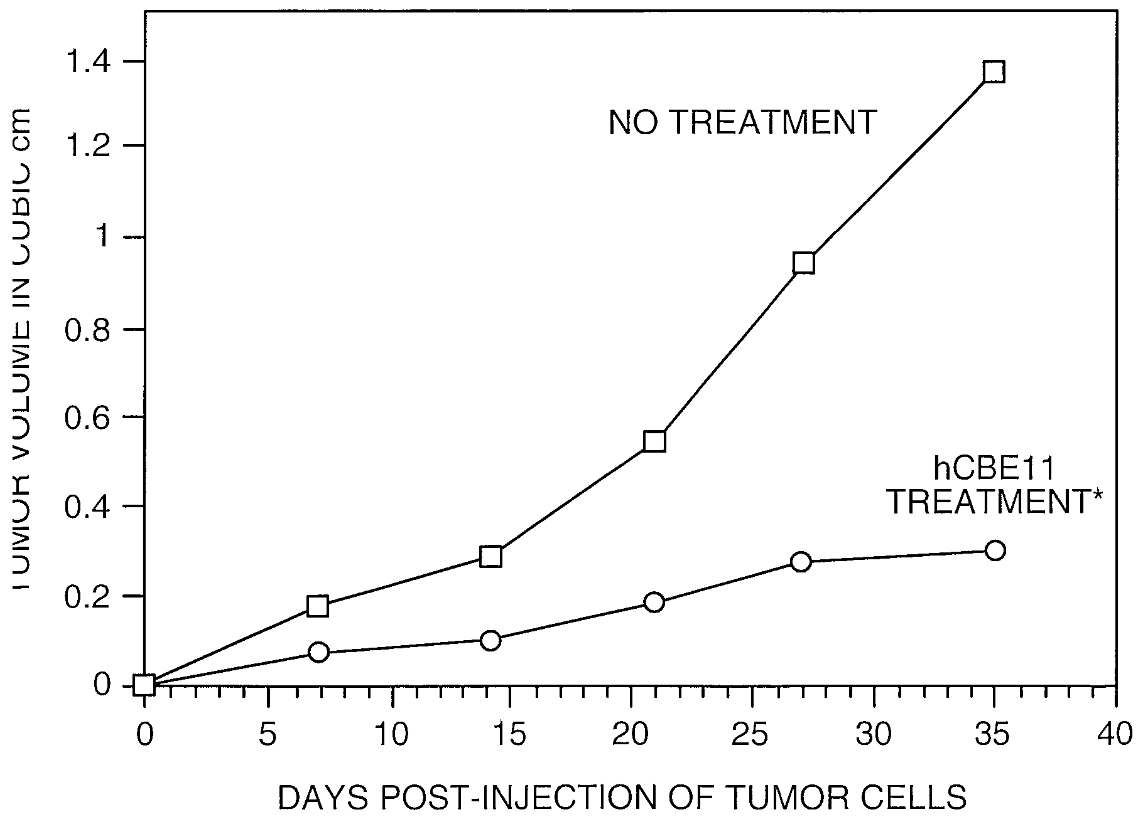


FIG. 4

HUMANIZED CBE11 INHIBITS GROWTH OF WIDR
TUMOR CELLS IN NUDE MICE



*hCBE11 (100 ug) INJECTED IP ON DAYS 0, 7, 14 & 21

FIG. 5

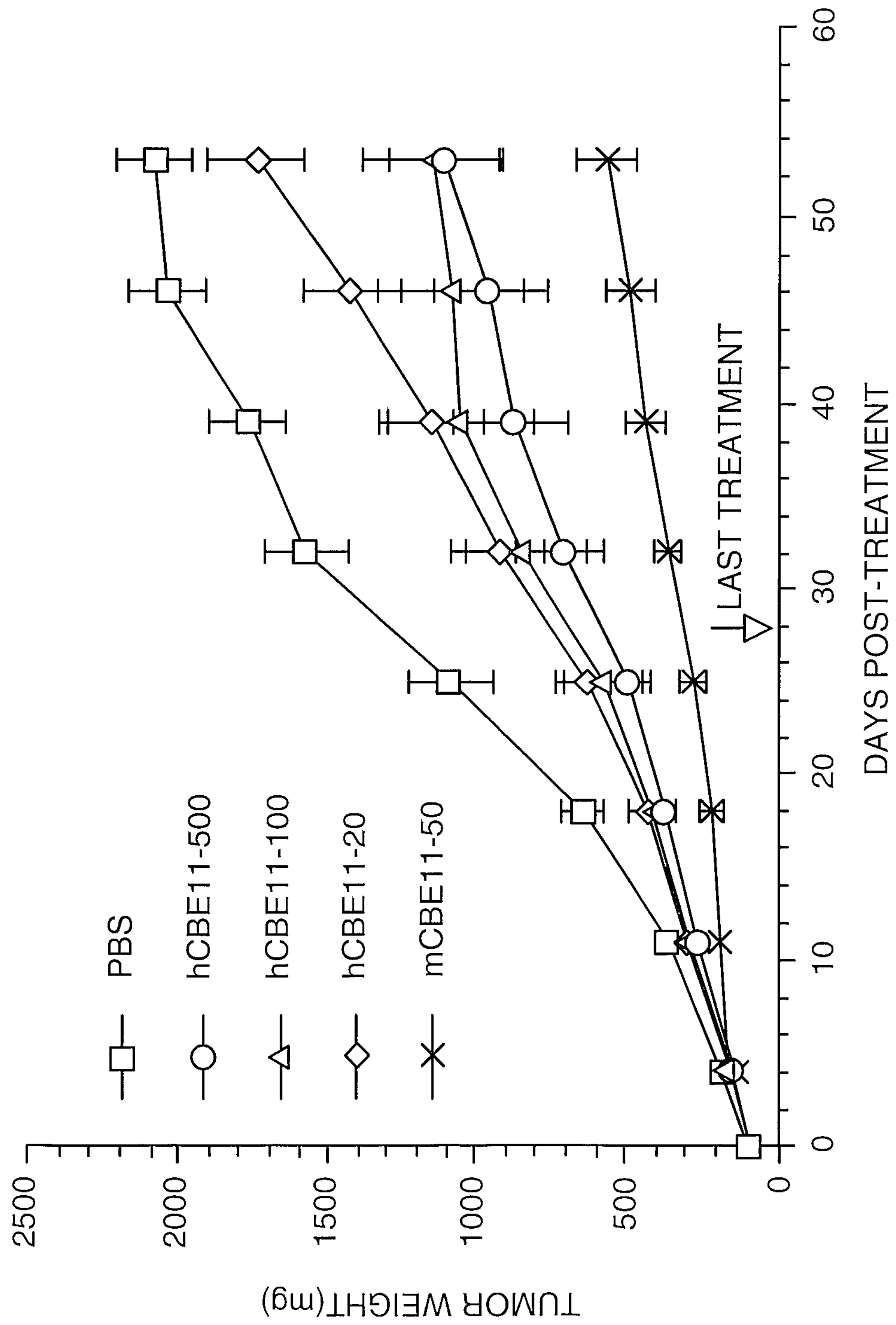


FIG. 6

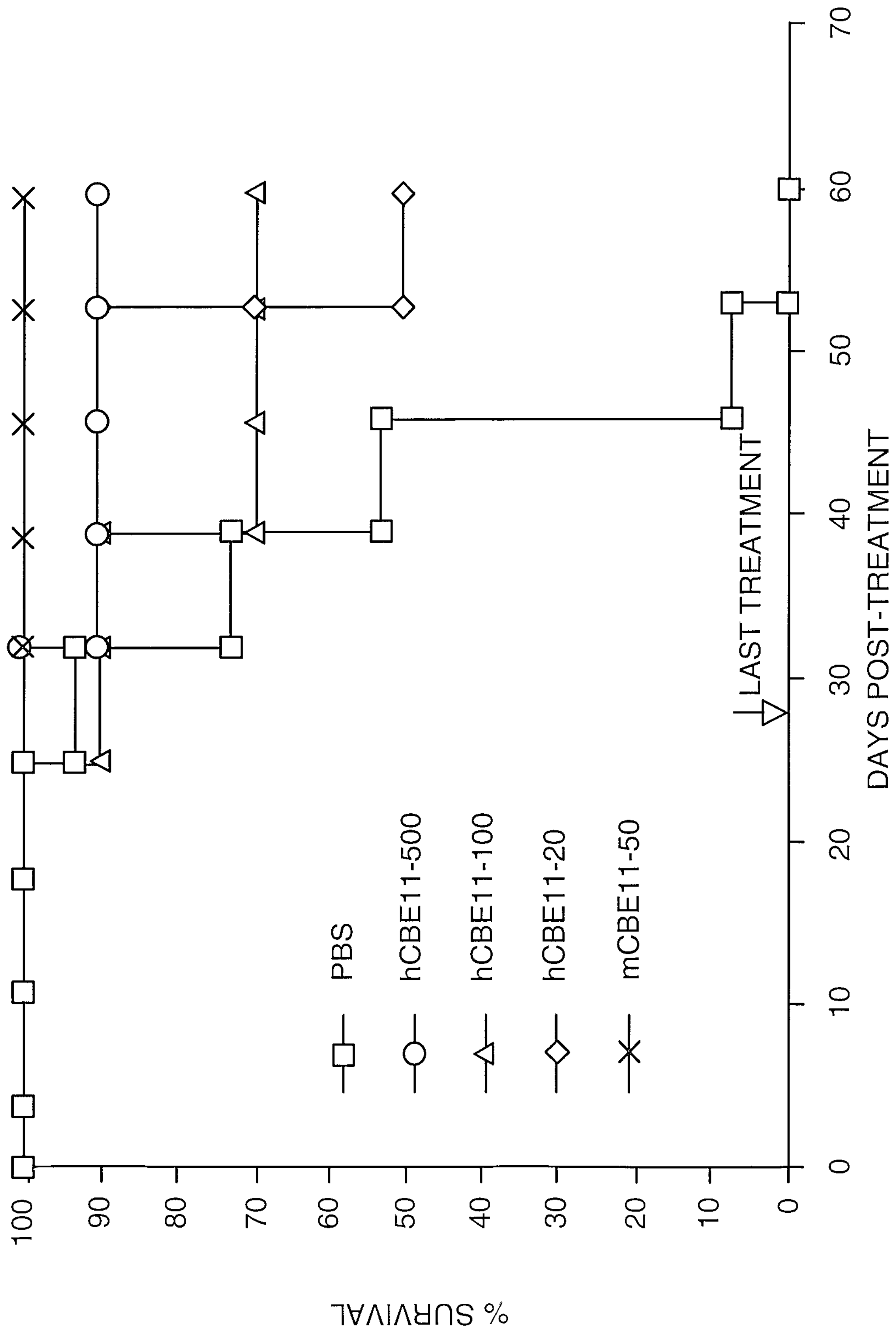


FIG. 7

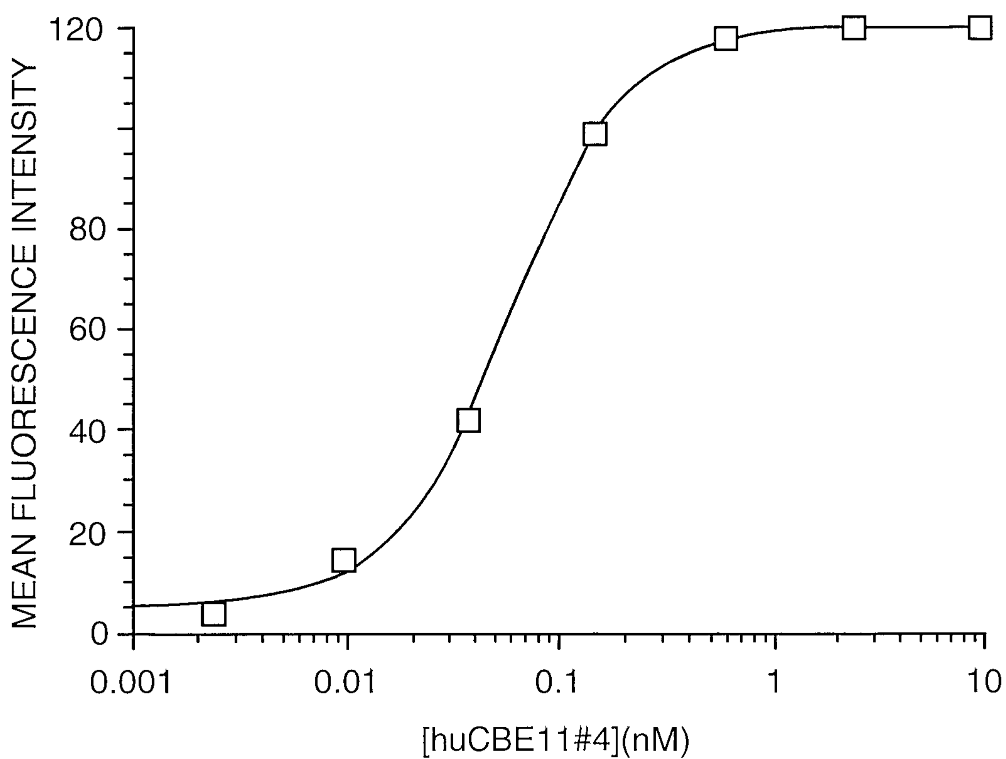


FIG. 8