(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/36005 A2

(51) International Patent Classification[7]: A61K 47/48, 49/00

(21) International Application Number: PCT/US00/31424

(22) International Filing Date:
15 November 2000 (15.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/165,563 15 November 1999 (15.11.1999) US

(71) Applicant: **UNIVERSITY OF SOUTHERN CALIFORNIA** [US/US]; 3716 South Hope Street, Suite 313, Los Angeles, CA 90007-4344 (US).

(72) Inventors: **PRESS, Michael**; USC/Norris Comprehensive Cancer Center and Hospital, University of Southern California, 1441 Eastlake Avenue, Los Angeles, CA 90033 (US). **PARK, Jinha**; 1218 Pine Street, #303, South Pasadena, CA 91030 (US).

(74) Agent: **YADAV, Rajiv**; McCutchen, Doyle, Brown & Enersen, LLP, Three Embarcadero Center, San Francisco, CA 94111 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

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.

WO 01/36005 A2

(54) Title: TARGETED DELIVERY OF THERAPEUTIC AND DIAGNOSTIC MOIETIES

(57) Abstract: Compositions and methods for improving cellular internalization of one or more compounds are disclosed. The invention provides a drug conjugate composition that can be delivered to a target cell, which comprises a carrier compound that has a binding specificity for a receptor molecule and is conjugated to a therapeutic or diagnostic moiety. When this composition is administered to a subject, the carrier compound binds to the receptor and is internalized by the target cell. Furthermore, monoclonal antibodies are disclosed that are internalized into target cells. The monoclonal antibodies of the invention are specific for target cells, particularly for cells expressing the surface antigen p185HER-2. The antibodies of the invention may be conjugated with a molecule for delivery into a target cell. Such molecules may be used for therapeutic treatment, including gene therapy, and for imaging. The invention also provides DNA sequences of the variable regions of particular monoclonal antibodies.

TARGETED DELIVERY OF THERAPEUTIC AND DIAGNOSTIC MOIETIES

RELATED APPLICATION DATA

This application claims the benefit under 35 U.S.C. 119(e) of United States Application Serial No. 60/165,563 filed November 15, 1999 which is herein incorporated by reference.

FIELD OF THE INVENTION

The compositions and methods of use described herein are in the area of materials and methods for enhancing cellular internalization.

BACKGROUND

It is well known in current chemotherapy protocols that administration of anti-mitotic drugs such as adriamycin, vincristine, cisplatin, doxorubicin, daunomycin and methotrexate, toxins such as diphtheria toxin, pseudomonas toxin and ricin, and anti-tumor drugs such as cyclophosphamide and isophosphamide in cancer chemotherapy has acute undesirable side effects on the normal cells of the patient, thus severely limiting the doses that can safely be administered. For a review, see DeVita, "Principles of Cancer Therapy", pages 765-788, in Petersdorf et al., Principles of Internal Medicine, 10th ed., McGraw-Hill, N.Y., 1983. Even in general therapeutic protocols it is often difficult to deliver compounds, such as proteins, peptides, genetic material, and other drugs and diagnostic compounds intracellularly because cell membranes often resist the passage of these compounds.

Many approaches have been explored to improve the effectiveness and specificity of cancer chemotherapy and drug delivery. One approach has been to attempt to specifically direct anticancer drugs to malignant cells, so that their effect on normal cells would be minimal. This approach is generally referred to as "drug targeting." In one example of this approach, the drug is conjugated to a biodegradable polyamino acid macromolecular base or to such a polyamino acid base that is also linked to a carrier agent. Theoretically, degradation of the polyamino acid base in the target cells releases the cytotoxic drug. Unfortunately, the use of a polyamino acid

base may reduce the ability of the conjugate to penetrate many tumors efficiently. Further, there is a dearth of appropriate carrier agents. Although, several compounds have been considered as carrier agents, they suffer from disadvantages including unpredictability of their internalization into cells.

Thus, it would be advantageous to have new methods for delivering agents intracellularly. In particular, it would be highly desirable to have available a drug delivery system that delivers a chemotherapeutic drug predictably to tumor cells. To achieve this goal several groups have tested $p185^{HER-2}$ overexpressing cells as a target for gene therapy (12-15). Targeted delivery of genes has been attempted by altering cognate receptors on virions with cellular receptor ligands and single-chain antibodies (16-19). Although these vectors have successfully bound $p185^{HER-2}$ overexpressing cells, none have resulted in significant rates of gene transduction. A humanized monoclonal antibody, Herceptin™, has been used to successfully treat some women with $^{HER-2}$/neu overexpressing breast and ovarian cancers, demonstrating that $p185^{HER-2}$ can be used to selectively target human cancers (10, 11). While outcomes have improved for patients treated with Herceptin™, more effective classes of antibody-based therapies need to be developed to capitalize on cancer specific antigen expression. Moreover, because relatively little is known about cellular responses after binding of a vector construct there is a need to assess the efficiency of post-binding receptor-mediated endocytosis, which will help to improve the efficacy of novel therapies.

SUMMARY

The present invention relates to compositions and methods for enhancing intracellular delivery of bioactive and/or diagnostic agents and to provide less invasive methods for delivering high molecular weight and labile drugs, such as proteins and nucleic acid molecules, and diagnostic agents.

More specifically, the invention relates to a carrier compound, such as a monoclonal antibody, to deliver other molecules or gene therapy vectors specifically to cells that can be identified by a specific surface marker protein and which, once

bound, would be internalized by a normal cellular process called receptor-mediated endocytosis.

Compositions and methods for improving cellular internalization of one or more compounds are disclosed. The invention provides a drug conjugate composition that can be delivered to a target cell, which comprises a carrier compound that has a binding specificity for a receptor molecule and is conjugated to a therapeutic or diagnostic moiety. When this composition is administered to a subject, the carrier compound binds to the receptor and is internalized by the target cell.

Further, the invention provides methods for treating disease states and inhibiting tumors using the compositions of the invention. Also described are methods of using the compositions and compounds of the invention to detect disease states and tumors, and to screen for putative compositions of the invention.

The invention also provides four novel monoclonal antibodies that recognize the extracellular domain of $p185^{HER-2}$, a membrane receptor protein, for use in internalization-dependent therapies. All four antibodies bind to $p185^{HER-2}$ and two of them recognize accessible epitopes of $p185^{HER-2}$ on viable cells. Because the successful gene therapy vector will require not only cell targeting, but internalization of the vector and expression of a therapeutic gene, it is important to evaluate as many of these steps as possible prior to construction of the vector. To this end, the invention discloses characterization of the internalization potential of the four anti-$p185^{HER-2}$ monoclonal antibodies using a rapid, quantifiable radioimmunoassay. The results of the invention show that two $p185^{HER-2}$ monoclonal antibodies, 8H11 and 10H8, will bind to viable, intact target cells, and will be internalized and trafficked through an endosomal pathway and, thus, establish that the antibodies will be useful in targeting treatment vectors that require internalization for therapeutic effect.

Additionally, the DNA sequences of the genes which encode the unique variable heavy (VH) and variable light (VL) regions of the monoclonal antibodies, Mabs, 8H11 and 10H8 are disclosed.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Purification of recombinant $^{HER-2}$/*neu* extracellular domain (ECD) protein with poly-His tag. The cDNA of $^{HER-2}$/*neu* was cut between the NcoI and SphI sites and subcloned into an expression vector for production of a 70 kDa epitope-tagged protein. TM=transmembrane domain. Purified protein was resolved by SDS-PAGE, transferred to nitrocellulose and detected by an antibody to the poly-His tag.

Figure 2. Western Blots of the total protein lysates from human and mouse cell lines. Monoclonal antibodies 5A7, 11F11, 8H11, and 10H8 were tested for binding to $p185^{HER-2}$ in total protein lysates from normal human mammary epithelial cells (HMEC), human breast carcinoma cells overexpressing $^{HER-2}$/*neu* (SKBR-3), human epidermoid carcinoma cells overexpressing EGF receptor (A431), NIH3T3 cells which lack $^{HER-2}$/*neu* expression, and NIH/189 cells engineered to overexpress $^{HER-2}$/*neu*.

Figure 3. Immunoprecipitation of $p185^{HER-2}$ by monoclonal antibody 8H11. Total protein lysates were incubated with the 8H11 monoclonal antibody. Immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. $p185^{HER-2}$ was detected by a rabbit polyclonal antibody recognizing a carboxy-terminal epitope of $p185^{HER-2}$.

Figure 4. Immunohistochemical localization of $p185^{HER-2}$. Tissue sections from a single archival paraffin-embedded breast tumor were analyzed for monoclonal antibody localization to membranes of carcinoma cells. Membrane localization of anti-$p185^{HER-2}$ monoclonal antibodies were detected by the peroxidase-anti-peroxidase method.

Figure 5. Flow cytometry using novel monoclonal antibodies to detect $p185^{HER-2}$ on SKBR-3 cells. SKBR-3 cells overexpressing $p185^{HER-2}$ were incubated with indicated monoclonal antibody and then tagged with secondary FITC-labeled antibodies. 8H11 and 10H8 monoclonal antibodies detected $p185^{HER-2}$ on the surface of viable SKBR-3 cells. IgG isotype control, 5A7 and 11F11 show no fluorescence.

Figure 6. Receptor-mediated endocytosis assays showing internalization and catabolism of monoclonal antibodies 8H11 and 10H8 by NIH/189 cells. (Top panels) Monoclonal antibodies labeled with ^{125}I were incubated on ice with NIH/189 cells. % total cpm indicate fractions of label on the surface, within the cells, and in the supernatant at various incubation times at 37 °C. Mean cpm were calculated for triplicate wells. (Bottom panels) Supernatant cpm was treated with 25% TCA and divided between TCA precipitatable cpm (representing intact antibodies) and TCA soluble cpm (representing small MW metabolites of antibodies). Increased TCA soluble cpm fractions shows degradation and exocytosis of internalized monoclonal antibodies. (A and B) 8H111 (C and D) 10H8.

Figure 7 shows the sequences of the variable regions of monoclonal antibody 8H11 both for the light chain (upper) and for the heavy chain (lower).

Figure 8 shows the sequences of the variable regions of monoclonal antibody 10H8 both for the light chain (upper) and for the heavy chain (lower).

DEFINITIONS

The term "antibody or antibody molecule" in the various grammatical forms is used herein as a collective noun that refers to a population of immunoglobulin molecules and/or immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

The phrase "monoclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody

combining sites, each immunospecific for a different epitope, e.g., a bispecific monoclonal antibody.

Use of the term "having the binding specificity of" indicates that equivalent monoclonal antibodies compete for binding to a preselected target epitope.

The terms "conjugate composition," "conjugate" and "composition" are used interchangeably throughout the specification.

The term "nucleotide sequence" refers to a heteropolymer of nucleotides or the sequence of nucleotides. One of skill in the art will readily discern from contextual cues which of the two definitions is appropriate. The terms "nucleic acid" and "polynucleotide" are also used interchangeably herein to refer to a heteropolymer of nucleotides. Generally, nucleic acid segments provided by this invention may be assembled from fragments of the genome and short oligonucleotide linkers, or from a series of oligonucleotides, or from individual nucleotides, to provide a synthetic nucleic acid which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon, or a eukaryotic gene.

The terms "oligonucleotide fragment" or a "polynucleotide fragment," "portion," or "segment" refer to a stretch of nucleotide residues which is long enough to use in polymerase chain reaction (PCR) or various hybridization procedures to identify or amplify identical or related parts of mRNA or DNA molecules.

"Oligonucleotides" or "nucleic acid probes" are prepared based on the polynucleotide sequences provided herein. Oligonucleotides comprise portions of such a polynucleotide sequence having at least about 15 nucleotides and usually at least about 20 nucleotides. Nucleic acid probes comprise portions of such a polynucleotide sequence having fewer nucleotides than about 6 kb, usually fewer than 1 kb. After appropriate testing to eliminate false positives, these probes may, for example, be used to determine whether specific mRNA molecules are present in a cell or tissue.

The term "probes" includes naturally occurring or recombinant or chemically synthesized single- or double-stranded nucleic acids. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Probes of the present invention, their preparation and/or labeling are elaborated in Sambrook, J. *et al.*, 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York; or Ausubel, F. *et al.*, 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York, both of which are incorporated herein by reference in their entirety.

The term "recombinant," when used herein to refer to a polypeptide or protein, means that a polypeptide or protein is derived from recombinant (*e.g.*, microbial, mammalian, or insect-based) expression systems. "Microbial" refers to recombinant polypeptides or proteins made in bacterial or fungal (*e.g.*, yeast) expression systems. As a product, "recombinant microbial" defines a polypeptide or protein essentially free of native endogenous substances and unaccompanied by associated native glycosylation. Polypeptides or proteins expressed in most bacterial cultures, *e.g.*, *E. coli*, will be free of glycosylation modifications; polypeptides or proteins expressed in yeast will have a glycosylation pattern in general different from those expressed in mammalian cells.

The term "recombinant expression vehicle or vector" refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. An expression vehicle can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be

subsequently cleaved from the expressed recombinant protein to provide a final product.

The term "recombinant expression system" means host cells which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit extrachromosomally. Recombinant expression systems as defined herein will express heterologous polypeptides or proteins upon induction of the regulatory elements linked to the DNA segment or synthetic gene to be expressed. This term also means host cells which have stably integrated a recombinant genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers. Recombinant expression systems as defined herein will express polypeptides or proteins endogenous to the cell upon induction of the regulatory elements linked to the endogenous DNA segment or gene to be expressed. The cells can be prokaryotic or eukaryotic.

The term "active" refers to those forms of the polypeptide which retain the biologic and/or immunologic activities of any naturally occurring polypeptide.

The term "naturally occurring polypeptide" refers to polypeptides produced by cells that have not been genetically engineered and specifically contemplates various polypeptides arising from post-translational modifications of the polypeptide including, but not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

The term "derivative" refers to polypeptides chemically modified by such techniques as ubiquitination, labeling (*e.g.*, with radionuclides or various enzymes), pegylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins.

The term "recombinant variant" refers to any polypeptide differing from naturally occurring polypeptides by amino acid insertions, deletions, and substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing

activities of interest, such as cellular trafficking, may be found by comparing the sequence of the particular polypeptide with that of homologous peptides and minimizing the number of amino acid sequence changes made in regions of high homology.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, *i.e.*, conservative amino acid replacements. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

Alternatively, where alteration of function is desired, insertions, deletions or non-conservative alterations can be engineered to produce altered polypeptides. Such alterations can, for example, alter one or more of the biological functions or biochemical characteristics of the polypeptides of the invention. For example, such alterations may change polypeptide characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate. Further, such alterations can be selected so as to generate polypeptides that are better suited for expression, scale up and the like in the host cells chosen for expression. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges.

As used herein, "substantially equivalent" can refer both to nucleotide and amino acid sequences, for example a mutant sequence, that varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and subject sequences. Typically, such a substantially equivalent sequence varies from one of those listed herein by no more than about 2% (*i.e.*, the number of individual residue substitutions, additions, and/or deletions in a substantially equivalent sequence, as compared to the corresponding reference sequence, divided by the total number of residues in the substantially equivalent sequence is about 0.02 or less). Such a sequence is said to have 98% sequence identity to the listed sequence. In one embodiment, a substantially equivalent, *e.g.*, mutant, sequence of the invention varies from a listed sequence by no more than 2% (98% sequence identity); in a variation of this embodiment, by no more than 0.5% (99.5% sequence identity); and in a further variation of this embodiment, by no more than 0.1% (99.9% sequence identity). Substantially equivalent, *e.g.*, mutant, amino acid sequences according to the invention generally have at least 98% sequence identity with a listed amino acid sequence, whereas substantially equivalent nucleotide sequence of the invention can have lower percent sequence identities, taking into account, for example, the redundancy or degeneracy of the genetic code. For the purposes of determining equivalence, truncation of the mature sequence (*e.g.*, via a mutation which creates a spurious stop codon) should be disregarded.

Where desired, an expression vector may be designed to contain a "signal or leader sequence" which will direct the polypeptide through the membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from heterologous protein sources by recombinant DNA techniques.

A polypeptide "fragment," "portion," or "segment" is a stretch of amino acid residues of at least about 5 amino acids, often at least about 7 amino acids, typically at least about 9 to 13 amino acids, and, in various embodiments, at least

about 17 or more amino acids. To be active, any polypeptide must have sufficient length to display biologic and/or immunologic activity.

Alternatively, recombinant variants encoding these same or similar polypeptides may be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic system. Mutations in the polynucleotide sequence may be reflected in the polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide, to change characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate.

The term "activated" cells as used in this application are those which are engaged in extracellular or intracellular membrane trafficking, including the export of neurosecretory or enzymatic molecules as part of a normal or disease process.

The term "purified" as used herein denotes that the indicated nucleic acid or polypeptide is present in the substantial absence of other biological macromolecules, *e.g.*, polynucleotides, proteins, and the like. In one embodiment, the polynucleotide or polypeptide is purified such that it constitutes at least 95% by weight, more preferably at least 99.8% by weight, of the indicated biological macromolecules present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000 daltons, can be present).

The term "isolated" as used herein refers to a nucleic acid or polypeptide separated from at least one other component (*e.g.*, nucleic acid or polypeptide) present with the nucleic acid or polypeptide in its natural source. In one embodiment, the nucleic acid or polypeptide is found in the presence of (if anything) only a solvent, buffer, ion, or other component normally present in a solution of the same. The terms "isolated" and "purified" do not encompass nucleic acids or polypeptides present in their natural source.

By "pharmaceutically acceptable salt" it is meant those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge, et al. describe pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 1977, 66: 1-19. The salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or separately by reacting the free base function with a suitable organic acid. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphersulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like.

As used herein, the term "pharmaceutically acceptable ester" refers to esters which hydrolyze in vivo and include those that break down readily in the human body to leave the parent compound or a salt thereof. Suitable ester groups include, for example, those derived from pharmaceutically acceptable aliphatic carboxylic acids, particularly alkanoic, alkenoic, cycloalkanoic and alkanedioic acids, in which each alkyl or alkenyl moiety advantageously has not more than 6 carbon atoms. Examples of particular esters includes formates, acetates, propionates, butyrates, acrylates and ethylsuccinates.

The term "pharmaceutically acceptable prodrugs" as used herein refers to those prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals with undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention. The term "prodrug" refers to compounds that are rapidly transformed in vivo to yield the parent compound of the above formula, for example by hydrolysis in blood. A thorough discussion is provided in T. Higuchi and V. Stella, Pro-drugs as Novel Delivery Systems, Vol. 14 of the A. C. S. Symposium Series, and in Edward B. Roche, ed., Bioreversible Carriers in Drug Design, American Pharmaceutical Association and Pergamon Press, 1987, both of which are incorporated herein by reference.

It is well known in the art that modifications and changes can be made in the structure of a polypeptide without substantially altering the biological function of that peptide. For example, certain amino acids can be substituted for other amino acids in a given polypeptide without any appreciable loss of function. In making such changes, substitutions of like amino acid residues can be made on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity, and the like.

The term "open reading frame," ORF, means a series of nucleotide triplets coding for amino acids without any termination codons and is a sequence translatable into protein.

The term "expression modulating fragment," EMF, means a series of nucleotides which modulates the expression of an operably linked ORF or another EMF.

The term "infection" refers to the introduction of nucleic acids into a suitable host cell by use of a virus or viral vector.

The term "transformation" means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration.

The term "transfection" refers to the taking up of an expression vector by a suitable host cell, whether or not any coding sequences are in fact expressed.

Each of the above terms is meant to encompasses all that is described for each, unless the context dictates otherwise.

DETAILED DESCRIPTION OF THE INVENTION

Successful targeted drug delivery requires cellular internalization and cell recognition. The binding of ligands or assembly proteins to surface receptors of eucaryotic cell membranes initiates or accompanies a cascade of nonequilibrium phenomena culminating in the cellular invagination of membrane complexes within clathrin-coated vesicles. This process is known as receptor-mediated endocytosis (RME). RME is the primary means by which several types of bioactive molecules, particularly macromolecules, enter eukaryotic cells and can be exploited to develop carrier agents for targeted delivery of drugs.

The second prong of a successful drug delivery regimen requires the use of well-characterized reagents that specifically recognize tumor cells in the host background and preferentially bind to such cells rather than to normal cells. Recent identification of differences in the genotype and molecular stucture of cancer cells has provided an opportunity to develop such targetable reagents for the specific therapy of selected human cancers (1-4). For example, amplification of the $^{HER-2}$/neu oncogene in human breast cancers, endometrial cancers, ovarian cancers, gastric cancers, and salivary gland carcinomas provides a potential molecular target for these cancers (5-9). $^{HER-2}$/neu gene amplification is associated with overexpression of the $p185^{HER-2}$ protein.

As can be seen from the Examples below, two monoclonal antibodies were isolated from mouse immune cells and characterized to bind specifically to the

HER-2/neu oncogene's protein receptor in its extracellular domain. The Examples establish that both monoclonal antibodies bind to the protein on live cells overexpressing this protein and trigger internalization of monoclonal antibodies in a pathway that includes the endosome. Following routing to endosomes, the monoclonal antibody and bound HER-2/neu protein are degraded in lysosomes and extruded from the cell (41).

Both monoclonal antibodies have been characterized extensively for binding to the protein product of the HER-2/neu oncogene. They have also been shown to be internalized specifically in HER-2/neu overexpressing cells and show evidence of endosomal location followed by degradation and extrusion into extracellular spaces as protein fragments found floating outside the cells. The genes encoding each VH and VL region of monoclonal antibodies 8H11 and 10H8 have been isolated, cloned, and sequenced. Predicted amino acids were cross-referenced to a database of amino acid sequences of monoclonal antibodies and confirmed as genuine monoclonal antibody variable regions. Amino acid sequence analysis of a partial tryptic digest of one of the expressed genes confirmed that the predicted amino acid sequence was correct.

Since the process of ligand-induced receptor-mediated endocytosis is a common one for cellular down-regulation of surface proteins, monoclonal antibodies of the invention may be exploited for the ability to trigger internalization of "piggybacked" gene therapy vectors, therapeutic toxins, or imaging molecules. Toxin proteins, chemicals, or isotopic labels may also be conjugated to the monoclonal antibodies to deliver these effector molecules into a cell in order to enhance targeted therapies or create imaging agents which rely on endosome localization. Gene therapy and immunotoxin therapy may be effectively enhanced when the effector vector or protein can be predictably delivered into an endosome of a targeted cell. Similarly, radiographic imaging may be established by such targeted moieties.

Conjugates Compositions

In the general case, the carrier component of the conjugate has a cell-specific binding component (or site) and a moiety-binding component (or site). The cell-specific binding component specifically binds a cellular surface structure which

mediates its internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding component can be a natural or synthetic ligand which binds the receptor. The ligand can be an antibody, a protein, polypeptide, carbohydrate, lipid or a combination thereof which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the conjugate composition to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages (lymphoma), mannose-6-phosphate glycoproteins for fibroblasts (fibrosarcoma), intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures as discussed below.

The moiety conjugated to the carrier for internalization is an oligonucleotide, an antibody, monoclonal or polyclonal, an antibody fragment, a therapeutic toxin, or an imaging molecule, such as an isotope label.

In one embodiment, the invention is a conjugate composition for targeted delivery to a target cell that comprises a carrier compound that has a binding specificity for a receptor molecule and a moiety conjugated to the carrier compound. This composition is internalized by the target cells when the carrier compound binds to the receptor molecule. In a preferred embodiment, the carrier compound is an antibody, either monoclonal or polyclonal, or a fragment of such antibodies. In various embodiments, the invention provides for monoclonal antibodies Mab 8H11, 10H8, 5A7 and 11F11 as carrier compounds.

The conjugates of the invention are useful for delivering different moieties to various kinds of cells including, but not limited to, cancer cells. Thus, the receptor molecule, in certain embodiments, is a polypeptide overexpressed by cancer cells, for example, the $p185^{HER-2}$ protein.

In a preferred embodiment, the conjugate of the invention targets delivery of a moiety to cancer cells that overexpress the $^{HER-2}$/neu oncogene. This composition comprises a monoclonal antibody that is anti-$p185^{HER-2}$ protein and a moiety conjugated to the antibody. The antibody has a binding specificity for the $p185^{HER-2}$ protein and it is internalized when it binds to the protein. The monoclonal antibody, in certain embodiments of the invention, is one of Mab 8H11, Mab 10H8, Mab 5A7 and Mab 11F11. The moiety conjugated to the antibody is a therapeutic moiety or an imaging compound.

The moiety-binding component conjugates the moiety to be delivered. Conjugation with the moiety must be sufficiently stable *in vivo* to prevent significant uncoupling of the moiety extracellularly prior to internalization by the target cell. However, the conjugate is cleavable under appropriate conditions within the cell so that the moiety is released in functional form. For example, the conjugate can be labile in the acidic and enzyme rich environment of lysosomes. A non-covalent bond based on electrostatic attraction between the binding component and the moiety provides extracellular stability and is releasable under intracellular conditions.

By way of example preparation, to form the conjugate, the moiety and carrier are mixed and incubated under conditions conducive to complexation. For example, the moiety and carrier can be mixed at the appropriate ratio in 2 M NaCl and the solution can be diluted to 0.15 M and filtered to provide an administrable composition.

ANTIBODIES

In preferred embodiments, the carrier compounds of the present invention are antibodies, monoclonal or polyclonal, or fragments thereof. Example monoclonal antibodies are Mab 8H11, 10H8, 5A7 and 11F11. Moreover, moieties of the conjugate, therapeutic or imaging, can also be antibodies.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (Campbell, A.M., Monoclonal Antibodies Technology: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth *et al., J. Immunol.* **35**:1-21 (1990); Kohler and Milstein, *Nature 256*:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al., Immunology Today* **4**:72 (1983); Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. (1985), pp. 77-96).

Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with a peptide, e.g., the receptor molecule, $p185^{HER-2}$ protein. Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the peptide. One skilled in the art will recognize that the amount of the peptide used for immunization will vary based on the animal which is immunized, the antigenicity of the peptide and the site of injection. The peptide that is used as an immunogen may be modified or administered with an adjuvant to increase the peptide's antigenicity. Methods of increasing the antigenicity of a peptide are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as globulin or β-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells. Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz *et al., Exp. Cell Research,* **175**:109-124 (1988)).

Hybridomas secreting the desired antibodies are cloned and the class and subclass is determined using procedures known in the art (Campbell, A.M., Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1984)). Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to the targeted peptides, e.g., $p185^{HER-2}$ protein.

Techniques described for the production of minibodies and other modified antibodies can also be adapted to produce various types of antibody modifications containing 10H8 or 8H11 variable heavy and variable light chain sequences. (42,43).

Other methods of producing a monoclonal antibody, a hybridoma cell, or a hybridoma cell culture also are well known. See, for example, the method of isolating monoclonal antibodies from an immunological repertoire as described by Sastry *et al.* (1989) *Proc. Natl. Acad. Sci. USA*, 86:5728-5732; and Huse *et al.* (1989) *Science*, 246:1275-1281.

For polyclonal antibodies, antibody containing antiserum is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

The present invention further provides the above-described antibodies in detectably labeled form. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as

horseradish peroxidase, alkaline phosphatase, *etc.*) fluorescent labels (such as FITC or rhodamine, *etc.*), paramagnetic atoms, *etc.* Procedures for accomplishing such labeling are well-known in the art, for example, see (Sternberger, L.A. *et al., J. Histochem. Cytochem.* **18**:315 (1970); Bayer, E.A. *et al., Meth. Enzym.* **62**:308 (1979); Engval, E. *et al., Immunol.* **109**:129 (1972); Goding, J.W. *J. Immunol. Meth.* **13**:215 (1976)).

If desired, the antibodies can also be used to make anti-idiotype atibodies which in turn can be humanized as is known in the art to prevent immunological responses. Humanized monoclonal antibodies offer particular advantages over murine monoclonal antibodies, particularly insofar as they can be used therapeutically in humans. Specifically, human antibodies are not cleared from the circulation as rapidly as "foreign" antigens, and do not activate the immune system in the same manner as foreign antigens and foreign antibodies.

The antibody of the invention can also be a fully human antibody such as those generated, for example, by selection from an antibody phage display library displaying human single chain or double chain antibodies such as those described in de Haard, H.J. *et al.* (1999) *J. Biol. Chem.* 274:18218-30 and in Winter, G. *et al.* (1994) *Annu. Rev. Immunol.* 12:433-55.

The labeled antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays to identify cells or tissues in which a fragment of the receptor molecule of interest is expressed. The antibodies also may be used directly in therapies or other diagnostics. The present invention further provides the above-described antibodies immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir, D.M. *et al.,* Handbook of Experimental Immunology 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986); Jacoby, W.D. *et al., Meth. Enzym. 34* Academic Press, N.Y. (1974)). The immobilized antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays as well as for immuno-

affinity purification of receptors for which the antibodies have a binding affinity. The antibodies can also be used in research to further elucidate the functioning of the signaling pathways in activation of cells by growth factors, hormones, cytokines or the like.

Antisera titer may be established through several means known in the art, such as by dot blot and density analysis, and also by precipitation of radiolabeled peptide-antibody complexes using a protein, secondary antisera, cold ethanol or charcoal-dextran followed by activity measurement with a gamma counter. If desired, the highest titer antisera may be purified on affinity columns. For example, a peptide such as the receptor molecules $p185^{HER-2}$ may be coupled to a commercially available resin and used to form an affinity column. Antiserum samples may then be passed through the column so that antibodies to the peptide bind (via the peptide) to the column. These bound antibodies are subsequently eluted, collected and evaluated for determination of titer and specificity.

An additional way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to determine the amino acid residue sequence of the CDR regions of the antibodies in question. Antibody molecules having identical, or functionally equivalent, amino acid residue sequences in their CDR regions have the same binding specificity. Methods for sequencing polypeptides are well known in the art. This does not suggest that antibodies with distinct CDR regions cannot bind to the same epitope.

Exemplary antibodies for use in the present invention include intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), and also referred to as antibody fragments. The Fab fragment, lacking Fc receptor, is soluble, and affords therapeutic advantages in serum half life, and diagnostic advantages in modes of using the soluble Fab fragment. The preparation of a soluble Fab fragment is generally known in the immunological arts and can be accomplished by a variety of methods.

For example, Fab and F(ab')$_2$ portions (fragments) of antibodies are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well known. See for example, U.S. Pat. No. 4,342,566 to Theofilopolous and Dixon. Fab' antibody portions also are well known and are produced from F(ab') .sub.2 portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide.

Polynucleotides and Nucleic Acids of the Invention

Nucleotide sequences of the invention are reported below. The present invention also provides genes corresponding to the cDNA sequences disclosed herein. The corresponding genes can be isolated in accordance with known methods using the sequence information disclosed herein. Such methods include the preparation of probes or primers from the disclosed sequence information for identification and/or amplification of genes in appropriate genomic libraries or other sources of genomic materials.

The compositions of the present invention include,but are not limited to, isolated polynucleotides, including recombinant DNA molecules, and cloned genes or degenerate variants thereof, especially naturally occurring variants such as allelic variants.

Nucleic Acids of the Invention

SEQ ID NO: 1 encodes the variable light region of Mab 8H11, SEQ ID NO:2 encodes the variable heavy region of Mab 8H11, SEQ ID NO:3 encodes the variable light region of Mab 10H8, and SEQ ID NO:4 encodes the variable heavy region of Mab 10H8. In particular embodiments, the isolated polynucleotides of the invention include, but are not limited to, polynucleotides comprising the nucleotide sequence of SEQ ID NO: 1, or polynucleotides comprising the nucleotide sequence of SEQ ID NO: 2, or polynucleotides comprising the nucleotide sequence of SEQ ID NO: 3, or polynucleotides comprising the nucleotide sequence of SEQ ID NO: 4. The

polynucleotides of the invention additionally include the complement of any of the polynucleotides recited above.

The invention also provides for polynucleotides as moieties to be used in the conjugate compositions of the invention. For example, the invention provides for targeted delivery of gene therapy vectors.

The polynucleotides of the invention also provide polynucleotides including nucleotide sequences that are substantially equivalent to the polynucleotides recited above. The invention also provides the complement of the polynucleotides including a nucleotide sequence that has at least about 95%, more typically at least about 99%, and even more typically at least about 99.5%, sequence identity to a polynucleotide encoding a polypeptide recited above. The polynucleotide can be DNA (genomic, cDNA, amplified, or synthetic) or RNA. Methods and algorithms for obtaining such polynucleotides are well known to those of skill in the art and can include, for example, methods for determining hybridization conditions which can routinely isolate polynucleotides of the desired sequence identities.

A polynucleotide according to the invention can be joined to any of a variety of other nucleotide sequences by well-established recombinant DNA techniques (*see* Sambrook J *et al.* (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY). Useful nucleotide sequences for joining to polynucleotides include an assortment of vectors, *e.g.*, plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art. Accordingly, the invention also provides a vector including a polynucleotide of the invention and a host cell containing the polynucleotide. In general, the vector contains an origin of replication functional in at least one organism, convenient restriction endonuclease sites, and a selectable marker for the host cell. Vectors according to the invention include expression vectors, replication vectors, probe generation vectors, and sequencing vectors. A host cell according to the invention can be a prokaryotic or eukaryotic cell and can be a unicellular organism or part of a multicellular organism.

The sequences falling within the scope of the present invention are not limited to the specific sequences herein described, but also include allelic variations thereof. For example, allelic variations can be routinely determined by comparing the sequence provided in SEQ ID NO: 1, a representative fragment thereof, or a nucleotide sequence at least 98 % identical to SEQ ID NO: 1 or nucleotides 226-2836 of SEQ ID NO: 1, with a sequence from another human isolate. An allelic variation is more typically at least 99% identical to SEQ ID NO: 1 and even more typically 99.8% identical to SEQ ID NO: 1. Furthermore, to accommodate codon variability, the invention includes nucleic acid molecules coding for the same amino acid sequences as do the specific ORFs disclosed herein. In other words, in the coding region of an ORF, substitution of one codon for another which encodes the same amino acid is expressly contemplated. Any specific sequence disclosed herein can be readily screened for errors by resequencing a particular fragment, such as an ORF, in both directions (*i.e.*, sequence both strands).

The present invention further provides recombinant constructs comprising a nucleic acid having the sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or a fragment thereof. The recombinant constructs of the present invention comprise a vector, such as a plasmid or viral vector, into which a nucleic acid having the sequence of one of the above-noted sequences or a fragment thereof is inserted, in a forward or reverse orientation. In the case of a vector comprising one of the ORFs of the present invention, the vector may further comprise regulatory sequences, including for example, a promoter, operably linked to the ORF.

Large numbers of suitable vectors and promoters are known to those of skill in the art and are commercially available for generating the recombinant constructs of the present invention. The following vectors are provided by way of example. Bacterial: pBs, phagescript, PsiX174, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLneo, pSV2cat, pOG44, PXTI, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia).

The nucleic acid sequences of the invention are further directed to sequences which encode variants of the described nucleic acids. These amino acid sequence variants may be prepared by methods known in the art by introducing appropriate nucleotide changes into a native or variant polynucleotide. There are two variables in the construction of amino acid sequence variants: the location of the mutation and the nature of the mutation. The amino acid sequence variants of the nucleic acids are preferably constructed by mutating the polynucleotide to give an amino acid sequence that does not occur in nature. These amino acid alterations can be made at sites that differ in the nucleic acids from different species (variable positions) or in highly conserved regions (constant regions). Sites at such locations will typically be modified in series, *e.g.*, by substituting first with conservative choices (*e.g.*, hydrophobic amino acid to a different hydrophobic amino acid) and then with more distant choices (*e.g.*, hydrophobic amino acid to a charged amino acid), and then deletions or insertions may be made at the target site. Amino acid sequence deletions generally range from about 1 to 30 residues, preferably about 1 to 10 residues, and are typically contiguous. Amino acid insertions include amino- and/or carboxy-terminal fusions ranging in length from one to one hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions may range generally from about 1 to 10 amino residues, preferably from 1 to 5 residues. Examples of terminal insertions include the heterologous signal sequences necessary for secretion or for intracellular targeting in different host cells.

In a preferred method, polynucleotides encoding the novel nucleic acids are changed via site-directed mutagenesis. This method uses oligonucleotide sequences that encode the polynucleotide sequence of the desired amino acid variant, as well as a sufficient adjacent nucleotide on both sides of the changed amino acid to form a stable duplex on either side of the site of being changed. In general, the techniques of site-directed mutagenesis are well known to those of skill in the art and this technique is exemplified by publications such as, Edelman *et al.*, DNA **2**:183 (1983). A versatile and efficient method for producing site-specific changes in a polynucleotide sequence was published by Zoller and Smith, Nucleic Acids Res.

10:6487-6500 (1982). PCR may also be used to create amino acid sequence variants of the novel nucleic acids. When small amounts of template DNA are used as starting material, primer(s) that differs slightly in sequence from the corresponding region in the template DNA can generate the desired amino acid variant. PCR amplification results in a population of product DNA fragments that differ from the polynucleotide template encoding the polypeptide at the position specified by the primer. The product DNA fragments replace the corresponding region in the plasmid and this gives the desired amino acid variant.

A further technique for generating amino acid variants is the cassette mutagenesis technique described in Wells *et al.*, Gene **34**:315 (1985); and other mutagenesis techniques well known in the art, such as, for example, the techniques in Sambrook *et al.*, *supra*, and Current Protocols in Molecular Biology, Ausubel *et al.*, *supra* Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the invention for the cloning and expression of these novel nucleic acids. Such DNA sequences include those which are capable of hybridizing to the appropriate novel nucleic acid sequence under stringent conditions.

Therapeutic Compositions

The conjugates of the invention may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with a conjugate of the present invention. Additionally, the conjugates of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid-base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. A sustained-release matrix desirably is chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (copolymers of

lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxcylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid).

When used in the above or other treatments, a therapeutically effective amount of one of the conjugates of the present invention may be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt form. By a "therapeutically effective amount" of the compound of the invention is meant a sufficient amount of the compound to treat an angiogenic disease, (for example, to limit tumor growth or to slow or block tumor metastasis) at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the conjugate of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the conjugate at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

The conjugate compositions of the present invention can be used in the form of salts derived from inorganic or organic acids. These salts include but are not limited to the following: acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsufonate, digluconate,

glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxy-ethansulfonate (isothionate), lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, pcrate, pivalate, propionate, succinate, tartrate, thiocyanate, phosphate, glutamate, bicarbonate, p-toluenesulfonate and undecanoate. Water or oil-soluble or dispersible products are thereby obtained.

Examples of acids which may be employed to form pharmaceutically acceptable addition salts include such inorganic acids as hydrochloric acid, sulphuric acid and phosphoric acid and such organic acids as maleic acid, succinic acid and citric acid. Other salts include salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium or magnesium or with organic basis. Preferred salts of the conjugate compositions of the invention include phosphate, tris and acetate.

The total daily dose of the conjugate compositions of this invention administered to a human or lower animal may range from about 0.001 to about 1 mg/kg of patients body mass/day. If desired, the effective daily dose may be divided into multiple doses for purposes of administration; consequently, single dose compositions may contain such amounts or submultiples thereof to make up the daily dose.

Alternatively, a conjugate of the present invention may be administered as pharmaceutical compositions containing the conjugate of interest in combination with one or more pharmaceutically acceptable excipients. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The compositions may be administered parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), rectally, or bucally. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions for parenteral injection comprise pharmaceutically-acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservative, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide, poly(orthoesters) and poly(anhydrides). Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

The injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Topical administration includes administration to the skin or mucosa, including surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, may be prepared as a dry powder which may be pressurized or non-pressurized. In non-pressurized powder compositions, the active ingredient in finely divided form may be used in admixture with a larger-sized pharmaceutically-acceptable inert carrier comprising particles having a size, for example, of up to 100 micrometers in diameter. Suitable inert carriers include sugars such as lactose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10 micrometers.

Alternatively, the composition may be pressurized and contain a compressed gas, such as nitrogen or a liquified gas propellant. The liquified propellant medium and indeed the total composition is preferably such that the active ingredient does not dissolve therein to any substantial extent. The pressurized composition may also contain a surface active agent, such as a liquid or solid non-ionic surface active agent or may be a solid anionic surface active agent It is preferred to use the solid anionic surface active agent in the form of a sodium salt.

A further form of topical administration is to the eye. A conjugate of the invention is delivered in a pharmaceutically acceptable ophthalmic vehicle, such that the conjugate is maintained in contact with the ocular surface for a sufficient time period to allow the conjugate to penetrate the corneal and internal regions of the eye, as for example the anterior chamber, posterior chamber, vitreous body, aqueous humor, vitreous humor, cornea, iris/ciliary, lens, choroid/retina and sclera. The pharmaceutically-acceptable ophthalmic vehicle may, for example, be an ointment, vegetable oil or an encapsulating material. Alternatively, the conjugates of the invention may be injected directly into the vitreous and aqueous humour.

Compositions for rectal or vaginal administration are preferably suppositories which may be prepared by mixing the conjugates of this invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at room temperature but liquid at body

temperature and therefore melt in the rectum or vaginal cavity and release the active conjugate.

Conjugates of the present invention may also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically-acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to a conjugate of the present invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 et seq.

While the conjugates of the invention can be administered as the sole active pharmaceutical agent, they may also be used in combination with one or more agents which are conventionally administered to patients for treating angiogenic diseases. For example, the conjugates of the invention are effective over the short term to make tumors more sensitive to traditional cytotoxic therapies such as chemicals and radiation. The conjugates of the invention also enhance the effectiveness of existing cytotoxic adjuvant anti-cancer therapies. The conjugates of the invention may also be combined with other antiangiogenic agents to enhance their effectiveness, or combined with other antiangiogenic agents and administered together with other cytotoxic agents. In particular, when used in the treatment of solid tumors, conjugates of the invention may be administered with IL-12, retinoids, interferons, angiostatin, endostatin, thalidomide, thrombospondin-1, thrombospondin-2, captopryl, anti-neoplastic agents such as alpha inteferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methortrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PRO-MACE/MOPP (prednisone, methotrexate (w/leucovin rescue), doxorubicin, cyclophosphamide, taxol, etoposide/mechlorethamine, vincristine,

prednisone and procarbazine), vincristine, vinblastine, angioinhibins, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like as well as with rediation.

Total daily dose of the compositions of the invention to be administered to a human or other mammal host in single or divided doses may be in amounts, for example, from 0.0001 to 300 mg/kg body weight daily and more usually 1 to 300 mg/kg body weight.

It will be understood that agents which can be combined with the conjugate of the present invention for the inhibition, treatment or prophylaxis of angiogenic diseases are not limited to those listed above, but include in principle any agents useful for the treatment or prophylaxis of angiogenic diseases.

Methods for Treating Diseases

The invention provides for methods for treating various disease states. Generally, the methods comprise administering to a subject the conjugates of the invention. In preferred embodiments, as a person of ordinary skill in the art would recognize, the conjugates are administered in a therapeutically effective amount and with a pharmaceutically acceptable carrier as discussed above. The disease state to be treated is, *inter alia*, cancer, psoriasis, macular degeneration, a neurological disease or restenosis in a tissue. The cancers to be treated include, but are not limited to, colorectal cancer, breast cancer, gastric cancer, esophageal cancer, small cell lung carcinoma, and different types of lymphoma such as Burkitt's lymphoma and B follicular cell lymphoma.

In some embodiments, the conjugates of the invention are administered to inhibit tumor growth or metastasis in a tissue. The tumor may be a melanoma, carcinoma, sarcoma, fibrosarcoma, glioma or astrocytoma.

The conjugates of the invention can also be used to inhibit angiogenesis, which plays an important role in a variety of disease processes. As angiogenesis inhibitors, conjugates of the invention are useful in the treatment of both primary and metastatic solid tumors, including carcinomas of breast, colon, rectum,

lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gallbladder and bile ducts, small intestine, urinary tract (including kidney, bladder and urothelium), female genital tract, (including cervix, uterus, and ovaries as well as choriocarcinoma and gestational trophoblastic disease), male genital tract (including prostate, seminal vesicles, testes and germ cell tumors), endocrine glands (including the thyroid, adrenal, and pituitary glands), and skin, as well as hemangiomas, melanomas, sarcomas (including those arising from bone and soft tissues as well as Kaposi's sarcoma) and tumors of the brain, nerves, eyes, and meninges (including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas, and meningiomas). Such conjugates may also be useful in treating solid tumors arising from hematopoietic malignancies such as leukemias (i.e. chloromas, plasmacytomas and the plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia) as well as in the treatment of lymphomas (both Hodgkin's and non-Hodgkin's lymphomas). In addition, these conjugates or genes which encode their expression may be useful in the prevention of metastases from the tumors described above either when used alone or in combination with radiotherapy and/or other chemotherapeutic agents.

The patient treated in the present invention in its many embodiments is desirably a human patient, although it is to be understood that the principles of the invention indicate that the invention is effective with respect to all mammals, which are intended to be included in the term "patient". Such a patient can be, for example, a pig, a cow, a horse, a goat, a sheep, a mule, a donkey, a dog, a cat, a rabbit, a mouse and a rat.

In an additional related embodiment, a tissue to be treated is a tumor tissue of a patient with a solid tumor, a metastases, a skin cancer, a breast cancer, a hemangioma or angiofibroma and the like cancer. Typical solid tumor tissues treatable by the present methods include lung, pancreas, breast, uterus, salivary gland, stomach, colon, laryngeal, ovarian, Kaposi's Sarcoma and the like tissues.

In a related embodiment, the invention contemplates the practice of the method in conjunction with other therapies such as conventional chemotherapy or

radiation therapy directed against solid tumors and for control of establishment of metastases. The administration of the invention's conjugates is typically conducted during or after chemotherapy. In addition, it is preferred to administer the conjugates after surgery where solid tumors have been removed as a prophylaxis against metastases.

The dosage ranges for the administration of a conjugate of the invention depend upon the form of the conjugate, and its potency, and are amounts large enough to produce the desired effect in which the disease symptoms are ameliorated. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage also can be adjusted by the individual physician in the event of any complication.

The conjugates of the invention can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule. Thus, conjugates of the invention including monoclonal antibodies, polypeptides, and derivatives thereof can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, topically, intraocularly, orally, intranasally and can be delivered by peristaltic means.

The compositions of this invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the patient to be treated, capacity of the patient's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration also are variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

Detection Methods

The conjugates of the invention also are suitable for detection of disease in tissues. In preferred embodiments, the tissue is *ex vivo*. For example, where the conjugate is an antibody, it can be used in immunohistochemical techniques to stain tissues *ex vivo*. Immunological techniques such as immunostaining and ELISA are described in, for example, Receptor Binding Techniques, Methods in Molecular Biology. 106. ed. M. Keen. Humana Press, 1999; Brooks *et al.* (1998) *Cell* 92:391-400; Brooks *et al.* (1996) *Cell* 85:683-693; and Brooks *et al.* (1993) *J. Cell. Biol.* 122:1351-1359.

The conjugates of the invention, once bound to the target tissue can be detected either directly or indirectly. Direct detection can be performed on conjugates that comprise a moiety, which is detectable label such as a fluorochrome, a radioactive tag, paramagnetic heavy metal or diagnostic dye.

For *in vivo* detection, it is preferable to use a detectably labeled conjugate. The labeled conjugate is administered to a patient intravenously, intramuscularly, transdermally, intrasynovially, intratumorally, intraocularly, intranasally, intrathecally, topically or orally. Labels suitable for detection within a patient are particularly preferred. For example, paramagnetically labeled conjugates

can be detected by magnetic resonance imaging. Radioactively tagged conjugates also can be detected.

Other applications

The invention also provides for a method for screening for the carrier compounds of the invention. In a preferred embodiment, such methods comprise providing a putative carrier, measuring the (first) affinity of the putative carrier compound to bind to $p185^{HER-2}$, and measuring the (second) affinity of an antibody for binding with $p185^{HER-2}$. The putative carrier is selected as a carrier of the invention, if the second affinity is less than the first affinity. In various embodiments, the second affinity is measured for antibodies that include Mab 8H11, Mab 10H8, Mab 5A7 and Mab 11F11.

Further, the invention provides for determining the internalization potential of putative carrier compounds. In these methods, the fraction of Mab 8H11 that is internalized by NIH/189 cells is compared to the fraction of the putative carrier that is internalized by NIH/189 cells. The internalization potential of the putative carrier is high, if the fraction of the putative carrier internalized by NIH/189 cells is higher than the fraction of Mab 8H11 internalized by NIH/189 cells. In an alternative embodiment, Mab 10H8 is used to determine the internalization potential of the putative carrier.

The carrier compounds of the present invention may be chemically coupled to isotopes, enzymes, carrier proteins, cytotoxic agents, fluorescent molecules, chemiluminescent, bioluminescent and other compounds for a variety of applications. For example, a carrier compound may be labeled to facilitate testing of its ability to bind antisera or to detect cell types which possess a relevant receptor. The coupling technique is generally chosen on the basis of the functional groups available on the amino acids of the peptide including, but not limited to amino, sulfhydral, carboxyl, amide, phenol, and imidazole groups. Various reagents used to effect such couplings include among others, glutaraldehyde, dizodized benzidine, carbodiimide, and p-benzoquinone.

The efficiency of the coupling reaction is determined using different techniques appropriate for the specific reaction. For example, radiolabeling of the carrier compound with I^{125} may be accomplished using chloramine T and NaI^{125} of high specific activity. The reaction is terminated with sodium metabisulfite and the mixture is desalted on disposable columns. The labeled peptide is eluted from the column and fractions are collected. Aliquots are removed from each fraction and radioactivity measured in a gamma counter. In this manner, a labeled carrier compound may be obtained which is free from unreacted NaI^{125}.

Kits for measurement of receptors that facilitate cellular internalization are also contemplated as part of the present invention. Antisera that possess the highest titer and specificity and can detect receptor molecules in extracts of plasma, tissues, and in cell culture media may be used to establish assay kits for rapid, reliable, sensitive, and specific measurement and detection of receptors. These assay kits may employ (but are not limited to) the following techniques: competitive and non-competitive assays, radioimmunoassay (RIA), bioluminescence and chemilurninescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood, and immunocytochemistry. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established by means well known to those skilled in the art.

Another kit may be used to visualize or localize receptor moleculse in tissues and cells. Immunohistochemistry techniques and kits, for example, which employ such techniques are well known to those of ordinary skill in the art. Such a kit provides antisera to the receptor, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualize the primary antiserum. Using this methodology, biopsied tumors may be examined for the presence of the peptide receptor.

EXAMPLES

The following examples serve to illustrate the present invention. Selection of analytical methods as well as the concentration of reagents, temperatures, and the values of other variables are only to exemplify application of the present invention and are not to be considered limitations thereof.

In particular the examples illustrate that targeted gene therapy and immunotherapy capitalize on the strategies of viruses and biological toxins for cellular internalization after docking to surface receptors. Receptor-mediated endocytosis is the most common mode of vector and toxin internalization (31, 32). Rational cell-specific vector design for gene therapy and immunotherapy thus requires development of monoclonal antibodies directed to cell surface antigens, such as $p185^{HER-2}$, expressed by target cells (16, 18, 33). Furthermore, the internalization potential of these monoclonal antibodies should be assessed prior to retargeting of gene therapy or immunotherapy vectors to ensure efficient delivery of genes or toxins to subcellular sites of action. Here a radioimmunoassay was used to identify two novel monoclonal antibodies which were internalized after binding a cell surface receptor and, therefore, may be useful for delivery of genes and/or toxins into target cells via an endolysosomal pathway.

Some monoclonal antibodies mimic the effects of ligands in experimental systems. Monoclonal antibodies directed against the rat p185 receptor trigger receptor dimerization, phosphorylation, and downregulation of rat p185, whereas monovalent Fab' have not (34). Another $p185^{HER-2}$ monoclonal antibody was shown to undergo receptor-mediated endocytosis by electron microscopy following capping at membranes of $p185^{HER-2}$ overexpressing NIH 3T3 cells (30). Intracellular trafficking was previously documented by immunogold detection of other monoclonal antibodies in membrane-bound organelles (29). Although this assay has been used to determine the subcellular fate of the murine precursor to the therapeutic monoclonal antibody Herceptin™, only a few other monoclonal antibodies in experimental protocols for immunotoxin therapies or targeted gene therapies have been tested for internalization (30, 35, 36). Furthermore, the radioimmunoassays used did not

identify evidence of endolysosomal trafficking. Here, a rapid radioimmunoassay has been tested for its utility in determining the post-binding subcellular fates of novel monoclonal antibodies to the $p185^{HER-2}$ receptor.

Evidence of 8H11 and 10H8 antibody trafficking through receptor-mediated endocytosis and degradation by lysosomal sorting was demonstrated. TCA soluble fractions of supernatant counts were found in 8H11 and 10H8 assays. No TCA soluble counts were found in the supernatants of NIH 3T3 pulsed control cells. The radioimmunoassay therefore delineated the fates of antibodies to be assessed for receptor-mediated endocytosis.

Future retargeting of vectors and toxins can be improved by characterizing the post-binding fates of the engineered constructs. In particular, non-viral antibody vectors which lack viral endosome escape domains have been fused with viral or toxin polypeptides known to disrupt endosomal membrane integrity (15, 32, 37-39). Endosomal routing is critical for these added domains to mediate cytoplasmic entry of molecular conjugates. Since divalency is required for antibodies to trigger receptor dimerization and internalization, divalent binding sites may be needed in at least some engineered vectors to ensure receptor-mediated endocytosis (34, 40). Therefore, the radioimmunoassay described may be useful in predetermining antibodies and antibody-based vectors for dimerization and subsequent receptor-mediated endocytosis.

To develop effective monoclonal antibody-based therapies, determining the internalization potential and, particularly, the trafficking of monoclonal antibody-antigen to an endolysosomal pathway is important. After altering targeting specificities, prokaryotic and plant toxin-monoclonal antibody conjugates and targeted viral and non-viral vector conjugates should be assessed for retention of internalization potential. Hence, the radioimmunoassay which determined the internalization of monoclonal antibodies 8H11 and 10H8 could be used to assess the subcellular fates of newly developed monoclonal antibodies against cancer specific surface antigens to improve internalization-dependent therapies.

GENERAL METHODOLOGY

Production and isolation of monoclonal antibodies to the extracellular domain (ECD) of $p185^{HER-2}$ involved the use of recombinant ECD^{HER-2} protein or viable $^{HER-2}$/neu overexpressing cell lines for immunization of BALB/c mice, and screening of hybridomas with ELISA and immunocytochemical assays. The monoclonal antibodies were purified by affinity chromatography. The specificity of the antibodies was determined with Western immunoblot analyses, immunoprecipitation assays, and immunohistochemistry. The ability of monoclonal antibodies to specifically recognize viable $^{HER-2}$/neu overexpressing cell lines was evaluated with fluorescence microscopy and flow cytometry. Cellular trafficking of monoclonal antibodies was characterized with radioimmunoassay methods.

Protein production and purification

A truncated $p185^{HER-2}$ protein was synthesized and injected into mice as the initial immunogen for generating anti-$p185^{HER-2}$ monoclonal antibodies. The cDNA of the $^{HER-2}$/neu gene was cut between the NcoI and SphI sites, yielding a portion of the open reading frame which includes the initiation codon extending to the transmembrane domain (20). The 2-kilobase DNA insert was ligated into the multiple cloning site immediately downstream of an inducible trp-lac promoter of a pTrcHisA expression vector (Invitrogen, San Diego, CA). Successful in-frame subcloning of the $^{HER-2}$/neu gene was confirmed by restriction fragment analysis and direct DNA sequencing (Amersham Pharmacia Biotech, Piscataway, NJ). TOP10 E. coli (Invitrogen) were transformed using the $^{HER-2}$/neu expression vector and induced to produce a fusion protein containing a leader peptide with six contiguous histidine residues followed by the ECD^{HER-2}. Proteins were purified by conditions using metal chelate affinity chromatography on Ni2+-NTA-agarose as suggested by the manufacturer (QIAGEN, Valencia, CA).

Cell culture

In addition to recombinant ECD^{HER-2} protein, $p185^{HER-2}$ overexpressing intact cells (NIH/189 and SKBR-3 cells) were used to immunize BALB/c mice. NIH/189 and NIH 3T3 cells were used as sources of intact or solubilized $p185^{HER-2}$

protein for immunologic assays. The NIH/189 cell line, which has been described previously as a $p185^{HER-2}$ overexpressor, was a generous gift from C. Richter King (21).

NIH 3T3, NIH/189, and A431 human epidermoid carcinoma cell lines were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies). The SKBR-3 human breast adenocarcinoma cell line was grown in McCoy's 5A media supplemented with 10% FBS and penicillin/streptomycin. Normal HMEC (human mammary epithelial cell) cells were grown in media formulated by the supplier of the cells (Clonetics, San Diego, CA). These cells were used as non-$p185^{HER-2}$ expressing controls for Western blots and immunoprecipitations. Sp2/0-Ag14 mouse myeloma cells were grown in RPMI-1640 media supplemented with sodium pyruvate, L-glutamine, penicillin/streptomycin, and 15% FBS prior to cell fusion.

Immuization

Two sets of 8-9 week-old female BALB/c mice were immunized with either protein or live cells as immunogens. Three female BALB/c mice received three sequential immunizations of 100 mg, 50 mg, and 50 mg each of the truncated $^{HER-2}$/neu protein. The first immunization of ECD^{HER-2} was an intraperitoneal injection of protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). At weeks 3 and 5, mice were given two immunizations of 50 mg of protein mixed with Freund's incomplete adjuvant (Sigma). The mouse that yielded two monoclonal antibodies (5A7 and 11F11) in this work was given three further boosts of 50 mg ECD^{HER-2}.

Mice were also inoculated with live $p185^{HER-2}$-overexpressing cells in order to ensure the widest range of monoclonal antibodies against all potential ECD^{HER-2} epitopes, including glycosylated regions. Initial immunizations were intraperitoneal injections of 2-5 x106 SKBR-3 human breast cancer cells in serum-free RPMI-1640 media over a 7 month period. The mouse that yielded monoclonal antibodies 8H11 and 10H8 had another boost of 5x106 NIH/189 cells in Freund's incomplete adjuvant injected intraperitoneally and 1x106 cells injected

subcutaneously. The final immunizations before fusion were an intraperitoneal boost of 10x106 NIH/189 cells in PBS and an intravenous injection of 50 mg of ECD^{HER-2} protein in a buffer containing 0.1M NaH2PO4 and 10 mM TrisHCl, pH 8.0.

Hybridoma production and monoclonal antibody screening

Spleen cells from BALB/c mice immunized with either protein or cells were fused to Sp2/0-Ag14 mouse myeloma cells to generate hybridoma cells (22). Cells were fused with polyethylene glycol in a method modified from Köhler and Milstein (23). Hybridoma cells were selected for anti-$p185^{HER-2}$ antibody production by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry.

Protein and cell ELISA

ELISA was used as the primary method to screen hybridoma cell clone supernatants for secretion of antibodies. ECD^{HER-2} (250 ng/well) protein was coated onto 96-well plates in PBS. After blocking in 3% bovine serum albumin (BSA), 50 mL of supernatant were incubated from 1-2 hours at room temperature. After washing 3 times with PBS, a 1:2000 dilution (in 3% BSA) of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) was incubated for 30 minutes at room temperature. Positive wells were visualized by o-phenylene diamine (OPD) substrate (Sigma) and read at O.D. 490 nm on an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT).

To ensure detection of antibodies recognizing conformational and polysaccharide epitopes, cell ELISA using NIH3T3 and NIH/189 cells was employed to detect such clones. Cells (1x104/well) were fixed using 100% methanol onto 96-well Linbro Titertek (ICN, Irvine, CA) plates after overnight attachment onto 1.5% gelatin (Difco, Detroit, MI). The protocol for protein ELISA was used for cell ELISA.

Immunohistochemistry

Frozen and paraffin-embedded surgical biopsies of both known low and high $p185^{HER-2}$ expressor breast cancers were tested for binding of $p185^{HER-2}$ by

these four monoclonal antibodies. The use of human tissue was approved prior to this work by the Institutional Review Board of the USC School of Medicine.

Frozen tissue section immunohistochemistry was used as a secondary screening method to detect positive hybridomas that secreted antibodies to intact $p185^{HER-2}$ from human tissue. $p185^{HER-2}$ in tissue sections were detected by the peroxidase-anti-peroxidase technique as previously described (24).

Breast cancer cases embedded in paraffin as a single multi-tumor block were also tested for $p185^{HER-2}$ recognition. Formalin-fixed breast cancer biopsies with known low and high expression of $p185^{HER-2}$ protein were embedded in a specified matrix in a single paraffin block (25). Monoclonal antibodies purified from ascites were used at equal concentrations to compare localization in a single specimen.

Western blots and immunoprecipitation

Monoclonal antibodies were used to probe for full-length $p185^{HER-2}$ in total protein lysates of both mouse and human cell lines loaded equally in SDS-PAGE. The method used has been previously described (26).

Immunoprecipitations demonstrated the ability of monoclonal antibodies to bind to full length $^{HER-2}$/neu in soluble detergent lysates of mouse and human cell lines. The immunoprecipitation method used has been previously described (27). A rabbit polyclonal antibody (R60) previously reported for use in $p185^{HER-2}$ Western blots was used (9). A 1:2000 dilution of R60 polyclonal sera in 10% goat serum was used to detect the C-terminus of immunoprecipitated $p185^{HER-2}$ protein in the blot.

Flow cytometry

A prerequisite for the determination of cellular trafficking of monoclonal antibody-antigen complexes is the binding of monoclonal antibody to its target on the surface of $p185^{HER-2}$ overexpressing cells. Fluorescence-activated cell sorting (FACS) was used to determine ligation of monoclonal antibodies to native $p185^{HER-2}$ on the surface of SKBR-3 cells. The flow cytometry method has been previously described (28). Cells were analyzed on a Coulter Elite ESP Cell Sorter

(Beckman Coulter, Miami Lakes, FL). Mouse IgG isotype control was used as a negative control for FACS (Zymed).

Radioimmunoassay

A method to detect internalization and intracellular trafficking of monoclonal antibodies directed against cell-surface antigens was used as previously described to characterize anti-p185^{HER-2} monoclonal antibodies with this potential (29, 30). Monoclonal antibodies 8H11 and 10H8 were labeled with 125I (Amersham Pharmacia) using the Iodo-Gen method (Pierce). NIH 3T3 and NIH/189 cells were pulsed with labeled monoclonal antibodies and chased with 1% BSA/RPMI-1640. Supernatant, acid-releasable surface-bound, and intracellular label were collected and counted on a Cobra Automated Gamma Counter (Packard, Meriden, CT). The supernatant fraction was further divided between 25% TCA precipitable and 25% TCA soluble components representing shed, intact monoclonal antibodies and degraded monoclonal antibodies, respectively. These fractions were collected at times of 0, 1, 4, 19, and 24 hours. All timepoint determinations were corrected for non-specific initial binding (T=0 hours) by subtracting counts in fractions after surface blocking by unlabeled monoclonal antibodies.

RESULTS

Four monoclonal antibodies have been isolated and characterized for their individual characteristics in standard immunoassays and in an internalization assay to determine their potential for use with internalization-dependent therapies.

Production and characterization of monoclonal antibodies. A 70 kDa, truncated ECD^{HER-2} protein was isolated from inclusion bodies of bacteria which had been transformed by an inducible expression plasmid (Fig. 1). This ECD^{HER-2} protein solution was used as the immunogen in mice producing hybridoma clones expressing 5A7 and 11F11 monoclonal antibodies recognizing p185^{HER-2} in ELISA assays, Western immunoblot and immunohistochemistry of tissue sections but not in viable cells (see below). BALB/c mice were also immunized with viable cells overexpressing p185^{HER-2} as well as the recombinant ECD^{HER-2} protein in order to isolate hybridoma clones 8H11 and 10H8.

Both protein and cell ELISA were used as the primary screening methods for detection of antibodies to $p185^{HER-2}$ from hybridoma supernatants. Supernatants positive in either of these screens were further tested for the presence of antibodies which could detect full-length endogenous $p185^{HER-2}$ in frozen breast cancer tissue from a patient with $^{HER-2}$/neu gene overexpression. The monoclonal antibodies from hybridomas which bound $p185^{HER-2}$ in these screens were 5A7, 11F11, 8H11, and 10H8. The hybridoma cells were isolated as monoclonally derived cell lines after cloning twice by limiting dilution. 5A7, 11F11, and 8H11 were of the IgG1/k class and isotype. 10H8 was of the IgG2a/k class and isotype.

The specificity of the monoclonal antibodies was tested in Western blots, immunoprecipitation assays, immunohistochemistry, and immunofluorescence. By ELISA, all four monoclonal antibodies bound to ECD^{HER-2} purified from bacterial cell lysates. Cell ELISA performed with whole cells either overexpressing or not expressing $^{HER-2}$/neu showed that all four antibodies bound $p185^{HER-2}$ on NIH/189 cells but did not bind to antigens on the surface of wild-type NIH 3T3 cells (Table 1). In Western blots, three of the monoclonal antibodies, 5A7, 11F11, and 10H8, recognized human $p185^{HER-2}$ from either SKBR-3 cells (endogenous) or NIH/189 cells (transfected) (Fig. 2). There was no cross-reactivity identified with other proteins in these cell lines or with EGF-R expressed in A431 cells. The 8H11 monoclonal antibody did not detect denatured $p185^{HER-2}$ on Western blots. Both 10H8 and 8H11 were able to bind to soluble $p185^{HER-2}$ in immunoprecipitation reactions while 5A7 and 11F11 were not able to bind to soluble $p185^{HER-2}$ (Fig. 3). The presence of additional bands in the immunoblots reflects either alternatively spliced variants or proteolytically degraded fragments of $p185^{HER-2}$.

Recognition of endogenous $p185^{HER-2}$ in membranes of human tissue.

The ability to bind endogenous forms of $p185^{HER-2}$ in breast cancer tissue was tested with known low- and high-expression tissue samples by immunohistochemistry. All four monoclonal antibodies recognized endogenous $p185^{HER-2}$ on the cell membranes of tumor cells from a frozen human breast cancer biopsy (Table 1). As a control, the monoclonal antibodies were tested on a known

low-expressor of $p185^{HER-2}$ and did not show localization in the membranes (data not shown). The utility of these monoclonal antibodies for measuring $p185^{HER-2}$ status in archival paraffin-embedded biopsies was also tested. Similar to the findings in Western blots, 8H11 was unable to recognize $p185^{HER-2}$ while 5A7, 11F11, and 10H8 were able to recognize $p185^{HER-2}$ in the formalin-fixed tissue sections (Fig. 4).

Monoclonal antibody binding to viable intact $p185^{HER-2}$ overexpressing cells.

While the utility of these monoclonal antibodies in standard immunoassays was demonstrated, the potential therapeutic utility of these monoclonal antibodies needed to be assessed on viable cells. The binding of monoclonal antibodies to $p185^{HER-2}$ was assessed in viable, intact cells by flow cytometric assays using SKBR-3 human breast cancer cells. Only two monoclonal antibodies, 8H11 and 10H8, were able to bind to $p185^{HER-2}$ expressed on the surface of these cells (Fig. 5). Both 8H11 and 10H8 shifted the population of cells indicating a high level of fluorescence labeling. 5A7 and 11F11 did not bind to SKBR-3 cells and exhibited a fluorescence equivalent to that of control IgG antibody. Similar observations were made by fluorescence microscopy (data not shown).

Internalization and cellular trafficking of anti-$p185^{HER-2}$ antibodies in p185HER 2 overexpressing cells.

Most targeted therapies require antibodies to be able to target cell-specific antigens and trigger internalization. To determine the subcellular distribution of bound monoclonal antibodies, radioimmunoassays were performed to determine the relative amounts of labeled antibodies in each compartment of the cultured NIH/189 cells. The relative percentage of 125I labeled 8H11 and 10H8 in different cellular fractions was found to be similar for both 8H11 and 10H8 (Fig. 6). In these pulse-chase experiments, 8H11 and 10H8 were found to be almost exclusively surface-bound at the beginning of the experiment (T=0 hour), and to be internalized subsequently. Surface label decreased to 35% and 25% of total cpm for 8H11 and 10H8, respectively, over a 24 hour period when maintained at 37°C (Fig. 6A and 6C). At 1 hour of incubation, intracellular 125I rose to a peak and then leveled off throughout the assay. The net loss of surface-bound labeled monoclonal antibody was offset by a net increase in supernatant radioactivity. This supernatant fraction was

further divided between TCA precipitable and TCA soluble fractions representing intact monoclonal antibody and degraded monoclonal antibody fragments. The net increase of TCA soluble counts rose faster than the TCA precipitable fractions reflecting a higher proportion of internalized, lysosomally degraded, and exocytosed monoclonal antibody fragments compared with passively shed monoclonal antibody (Fig. 6B and 6D). After 24 hours, 45% of monoclonal antibody 8H11 was internalized, degraded, and exocytosed (Fig. 6A). A similar amount of monoclonal antibody 10H8 was internalized but a higher fraction of the monoclonal antibody was shed (Fig. 6C). As a control to test non-specific internalization, labeled 8H11 and 10H8 were both found exclusively in the supernatant fraction of NIH 3T3 cells. Separation of TCA precipitable from TCA soluble fractions showed that both 8H11 and 10H8 were found exclusively as intact proteins (data not shown). Thus, both 8H11 and 10H8 neither bound to proteins on the surface of NIH 3T3 cells nor were internalized by endocytosis, trafficked to lysosomes, and exocytosed.

Table 1. Characteristics of anti-p185^{HER-2} Monoclonal Antibodies

Name of mAb	Antigen	Mouse mAb isotype and subclass	ECD protein ELISA[a]	NIH 3T3 wild-type cell ELISA[b]	NIH/189 cell ELISA[c]	Western blot detection of p185^{HER-2}	IP of p185^{HER-2}	IHC on frozen biopsies	IHC on paraffin biopsies	Indirect immuno-fluorescence detection of SKBR-3 cells
5A7	ECD protein	IgG_1/P	+	-	+	+	-	+	+	-
11F11	ECD protein	IgG_1/P	+	-	+	+	-	+	+	-
8H11	Live cells	IgG_1/P	+	-	+	-	+	+	-	+
10H8	Live cells	IgG_1/P	+	-	+	+	+	+	+	+

[a] HER-2/*neu* receptor extracellular domain (ECD) polypeptide; ~70 kDa.
[b] NIH 3T3 cells lack p185 HER-2 expression.
[c] NIH/189 cells have p185 HER-2 overexpression.
Abbreviations used: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; IHC, immunohistochemistry.

BIBLIOGRAPHY

All of the following publications which are cited in the body of the instant specification are hereby incorporated by reference in their entirety.

1. Benz, C. C., Brandt, B. H., and Zanker, K. S. Gene diagnostics provide new insights into breast cancer prognosis and therapy, Gene. 159: 3-7, 1995.

2. Dhingra, K., Hittelman, W. N., and Hortobagyi, G. N. Genetic changes in breast cancer - consequences for therapy?, Gene. 159: 59-63, 1995.

3. Hung, M.-C., Matin, A., Zhang, Y., Xing, X., Sorgi, F., Huang, L., and Yu, D. HER-2/neu-targeting gene therapy - a review, Gene. 159: 65-71, 1995.

4. Sledge, G. W. Implications of the new biology for therapy in breast cancer, Sem. Oncol. 23: 76-81, 1996.

5. Albino, A. P., Jaehne, J., Altorki, N., Blundell, M., Urmacher, C., Lauwers, G., Niedzwiecki, D., and Kelsen, D. P. Amplification of HER-2/neu gene in human gastric adenocarcinomas: Correlation with primary site., European J. Surg. Oncol. 21: 56-60, 1995.

6. Press, M. F., Hung, G., Pike, M. C., George, J., Dietz-Band, J., James, W., Slamon, D. J., Batsakis, J. G., and El-Naggar, A. K. Amplification and overexpression of HER-2/neu in carcinomas of the salivary gland: Correlation with poor prognosis, Cancer Res. 54: 5675-5682, 1994.

7. Saffari, B., Jones, L. A., El-Naggar, A., Felix, J. C., George, J., and Press, M. F. Amplification and overexpression of HER-2/neu (c-erbB2) in endometrial cancers: correlation with overall survival, Cancer Res. 55: 5693-5698, 1995.

8. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science. 235: 177-182, 1987.

9. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer, Science. 244: 707-712, 1989.

10. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous recombinant

humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer, J. Clin. Oncol. 14: 737-744, 1996.

11. Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., Paton, V., Shak, S., Lieberman, G., and Slamon, D. Efficacy and safety of Herceptin (Humanized anti-HER2 antibody) as a single agent in 222 women with HER2 overexpression who relapsed following chemotherapy for metastatic breast cancer, Proc. Am. Soc. Clin. Oncol. 17: 97a, 1998.

12. Foster, B. J. and Kern, J. A. HER2-targeted gene transfer, Hum. Gene Ther. 8: 719-727, 1997.

13. Schnierle, B. S., Moritz, D., Jeschke, M., and Groner, B. Expression of chimeric envelope proteins in helper cell lines and integration into Moloney murine leukemia virus particles, Gene Therapy. 3: 334-342, 1996.

14. Han, X., Kasahara, N., and Kan, Y. W. Ligand-directed retroviral targeting of human breast cancer cells, Proc. Natl. Acad. Sci. USA. 92: 9747-9751, 1995.

15. Fominaya, J. and Wels, W. Target cell-specific DNA transfer mediated by a chimeric multidomain protein, J. Biol. Chem. 271: 10560-10568, 1996.

16. Cosset, F.-L. and Russell, S. J. Targeting retrovirus entry, Gene Therapy. 3: 946-956, 1996.

17. Kasahara, N., Dozy, A. M., and Kan, Y. W. Tissue-specific targeting of retroviral vectors through ligand-receptor interactions, Science. 266: 1373-1376, 1994.

18. Schnierle, B. S. and Groner, B. Retroviral targeted delivery, Gene Therapy. 3: 1069-1073, 1996.

19. Somia, N. V., Zoppe, M., and Verma, I. M. Generation of targeted retroviral vectors by using single-chain variable fragment: an approach to in vivo gene delivery, Proc. Natl. Acad. Sci. USA. 92: 7570-7574, 1995.

20. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene, Science. 230: 1132-1139, 1985.

21. DiFiore, P. P., Pierce, J. H., Krause, M. H., Segatto, O., King, C. R., and Aaronson, S. A. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells, Science. 237: 178-182, 1987.

22. Shulman, M., Wilde, C. D., and Köhler, G. A better cell line for making hybridomas secreting specific antibodies, Nature. 276: 269-270, 1978.

23. Köhler, G. and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity, Nature. 256: 495-497, 1975.

24. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the $^{HER-2}$/neu proto-oncogene in normal human adult and fetal tissues., Oncogene. 5: 953-952, 1990.

25. Petrosyan, K. and Press, M. F. Multispecimen tissue blocks in pathology: an improved technique of preparation, Laboratory Investigation. 77: 541-542, 1997.

26. Zhou, Y. and Lee, A. S. Mechanism for the suppression of the mammalian stress response by genistein, an anticancer phytoestrogen from soy, J. Natl. Cancer Inst. 90: 381-388, 1998.

27. Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T. E., Mann, M., and Lieber, M. R. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells, Nature. 388: 492-495, 1997.

28. Yang, X. and Page, M. An Mr 7-kDa membrane protein overexpressed in human multidrug-resistant ovarian cancer cells, Cancer Lett. 88: 171-178, 1995.

29. Press, O. W., Hansen, J. A., Farr, A., and Martin, P. J. Endocytosis and degradation of murine anti-human CD-3 monoclonal antibodies by normal and malignant T-lymphocytes, Cancer Res. 48: 2249-2257, 1988.

30. De Santes, K., Slamon, D., Anderson, S. K., Shepard, M., Fendly, B., Maneval, D., and Press, O. Radiolabeled antibody targeting of the $^{HER-2}$/neu oncoprotein, Cancer Res. 52: 1916-1923, 1992.

31. Hunter, E. and Swanstorm, R. Retrovirus envelope glycoproteins, Curr. Topics Micro. Immuno. 157: 188-253, 1990.

32. Michael, S. I. and Curiel, D. T. Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway, Gene Therapy. 1: 223-232, 1994.

33. Frankel, A. E., FitzGerald, D., Siegall, C., and Press, O. W. Advances in immunotoxin biology and therapy: a summary of the Fourth International Symposium on Immunotoxins, Cancer Res. 56: 926-932, 1996.

34. Yarden, Y. Agonistic antibodies stimulate the kinase encoded by the neu protooncogene in living cells but the oncogenic mutant is constituitively active, Proc. Natl. Acad. Sci. USA. 87: 2569-2573, 1990.

35. Tagliabue, E., Centis, F., Campiglio, M., Mastroianni, A., Martignone, S., Pellegrini, R., Casalini, P., Lanzi, C., Menard, S., and Colnaghi, M. I. Selection of monoclonal antibodies which induce internalization and phosphorylation of p185HER2 and growth inhibition of cells with HER2/neu gene amplification, Int. J. Cancer. 47: 933-937, 1991.

36. Maier, L. A., Xu, F. J., Hester, S., Boyer, C. M., McKenzie, S., Bruskin, A. M., Argon, Y., and Bast, R. C. Requirements for the internalization of a murine monoclonal antibody directed against the $^{HER-2}$/neu gene product c-erbB-2, Cancer Res. 51: 5361-5369, 1991.

37. Fisher, K. J. and Wilson, J. M. The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer, Biochem. J. 321: 49-58, 1997.

38. Fisher, K. J. and Wilson, J. M. Biochemical and functional analysis of an adenovirus-based ligand complex for gene transfer, Biochem. J. 299: 49-58, 1994.

39. Uherek, C., Fominaya, J., and Wels, W. A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery, J. Biol. Chem. 273: 8835-8841, 1998.

40. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A., and Greene, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies, Cell. 41: 695-706, 1985.

41. Park, J.M., Yang, X, Park, J.J., Press, O.W., Press, M.S. Assessment of Novel Anti-p185HER-2 Monoclonal Antibodies for Internalization Dependent Therapies, Hybridoma, 18: 487-495 (1999).

42. Hu S., Shively, L., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y., Shively, J.E., Wu A.M. Minibody: A Novel Engineered Anti-Carcinoembryonic Antigen Antibody Fragment (Single-Chain Fv-CH3) Which Exhibits Rapid, High-Level Targeting of Xenografts. Cancer Research. 56(13):3055-61 (1996)

43. Wu, A.M., Yazaki, P.J., Tsai, S.W. Nguyen, K., Anderson, A.L., McCarthy, D.W., Welch, M.J., Shively, J.E., Williams, L.E., Raubitschek, A.A., Wong, J.Y., Toyokumi, T., Phelps, M.E., Gambhir, S.S. High-Resolution MicroPET Imaging of Carcinoembryonic Antigen-Positive Xenografts by Using a Copper-64-Labeled Engineered Antibody Fragment. Proceedings of the National Academy of Sciences of the United States of America. 97(15):8495-500 (2000).

CLAIMS

What is claimed is:

1. A cohnjugate composition for targeted delivery to a target cell, the composition comprising:
   a carrier compound that has a binding specificity for a receptor molecule; and
   a moiety conjugated to the carrier compound,
   wherein the composition is internalized by said target cell when the carrier compound binds to the receptor molecule.

2. The composition of claim 1 wherein the carrier compound is an antibody.

3. The composition of claim 2 wherein said antibody is monoclonal.

4. The composition of claim3 wherein the antibody is Mab 8H11.

5. The composition of claim3 wherein the antibody is Mab 10H8.

6. The composition of claim3 wherein the antibody is Mab 5A7.

7. The composition of claim3 wherein the antibody is Mab 11F11.

8. The composition of claim 2 wherein the antibody is a fragment of a monoclonal antibody.

9. The composition of claim 2 wherein said antibody is polyclonal.

10. The composition of claim 1 wherein said target cell is a cancer cell.

11. The composition of claim 10 wherein said receptor is a polypeptide overexpressed by the cancer cell.

12. The composition of claim 11 wherein said polypeptide is $p185^{HER-2}$ protein.

13. The composition of claim 1 wherein the moiety is an oligonucleotide segment.

14. The composition of claim 1 wherein the moiety is an antibody.

15. The composition of claim 14 wherein the moiety is a monoclonal antibody.

16. The composition of claim 14 wherein the moiety is a fragment of a monoclonal antibody.

17. The composition of claim 14 wherein the moiety is a polyclonal antibody.

18. The composition of claim 1 wherein the t moiety is a toxin.

19. The composition of claim 1 wherein the t moiety is an imaging molecule.

20. The composition of claim 19 wherein the imaging molecule is an isotope label.

21. A method of treating a disease state comprising administering the composition of claim 1.

22. The method of claim 21 wherein said composition is administered intravenously, transdermally, intrasynovially, intramuscularly, intratummorally, intraocularly, intranasally, intrathecally, topically or orally.

23. The method of claim 21 wherein said composition is administered as part of chemotherapy.

24. The method of claim 21 wherein said composition is administered in conjunction with radiation.

25. The method of claim 21 wherein the disease is a cancer, psoriasis, macular degeneration, a neurological disease, or restenosis in a tissue.

26. A method of inhibiting tumor growth or metastasis in a tissue comprising administering the composition of claim 1.

27. The method of claim 26 wherein said composition is administered intravenously, transdermally, intrasynovially, intramuscularly, intratumorally, intraocularly, intranasally, topically or orally.

28. The method of claim 26 wherein said composition is administered as part of chemotherapy.

29. The method of claim 26 wherein said composition is administered in conjunction with radiation.

30. The method of claim 26 wherein the tumor or metastasis is a melanoma, carcinoma, sarcoma, fibrosarcoma, glioma or astrocytoma.

31. A method of inhibiting psoriasis, macular degeneration, or restenosis in a tissue by administering the composition of claim 1.

32. The method of claim 31 wherein said composition is administered intravenously, transdermally, intrasynovially, intramuscularly, intratummorally, intraocularly, intranasally, intrathecally, topically or orally.

33. The method of claim 31 wherein administering the composition is part of chemotherapy.

34. The method of claim 31 wherein administering the composition is in conjunction with radiation.

35. A method of detecting angiogenesis in a tissue by contacting the composition of claim 1 with said tissue.

36. The method of claim 35 wherein said tissue is *ex vivo*.

37. The method of claim 35 wherein said tissue is *in vivo* and said composition is administered intravenously, transdermally, intrasynovially, intramuscularly, intratummorally, intraocularly, intranasally, intrathecally, topically or orally.

38. The method of claim 35 wherein said moiety is a fluorochrome, radioactive tag, paramagnetic heavy metal, diagnostic dye or enzyme.

39. A method of detecting tumors or tumor invasion in a tissue by administering the composition of claim 1.

40. The method of claim 39 wherein said tissue is *ex vivo*.

41. The method of claim 39 wherein said tissue is *in vivo* and said composition is administered intravenously, transdermally, intrasynovially, intramuscularly, intratummorally, intraocularly, intranasally, intrathecally, topically or orally.

42. The method of claims 39 wherein said moiety is a fluorochrome, radioactive tag, paramagnetic heavy metal or diagnostic dye.

43. An antibody directed towards $p185^{HER-2}$ protein.

44. The antibody of claim 43 wherein the antibody is Mab 8H11.

45. The antibody of claim 43 wherein the antibody is Mab 10H8.

46. The antibody of claim 43 wherein the antibody is Mab 5A7.

47. The antibody of claim 43 wherein the antibody is Mab 11F11.

48. A method for screening for carrier compounds of claim 1, the method comprising:
providing a putative carrier compound;
measuring a first affinity of said putative carrier compound to bind to $p185^{HER-2}$;
measuring a second affinity of an antibody for binding with $p185^{HER-2}$; and
selecting said putative carrier compound as a carrier compound of claim 1 if said second affinity is less than said first affinity.

49. The method of claim 48 wherein the antibody is Mab 8H11.

50. The method of claim 48 wherein the antibody is Mab 10H8.

51. The method of claim 48 wherein the antibody is Mab 5A7.

52. The method of claim 48 wherein the antibody is Mab 11F11.

53. A method to determine the internalization potential of a putative carrier compound, the method comprising:
determining a first fraction of Mab 8H11 that is internalized by NIH/189 cells; and
determining a second fraction of the putative carrier compound that is internalized by NIH/189 cells
whereby the internalization potential of the putative carrier is high if the second fraction is substantially equal to or greater than the first fraction.

54. A conjugate composition for targeted delivery to a cancer cell that overexpresses the $^{HER-2}$/neu oncogene, the composition comprising:
a monoclonal antibody that is anti-$p185^{HER-2}$ protein; and
a moiety conjugated to the monoclonal antibody,
wherein the composition is internalized by said cancer cell when the antibody binds to the $p185^{HER-2}$ protein.

55. The composition of claim 54 wherein the antibody is 8H11.

56. The composition of claim 54 wherein the antibody is 10H8.

57. The composition of claim 54 wherein the antibody is 5A7.

58. The composition of claim 54 wherein the antibody is 11F11.

59. The composition of claim 54 wherein the moiety is a therapeutic moiety.

60. The composition of claim 54 wherein the moiety is an imaging molecule.

61. An isolated polynucleotide encoding the variable light region of Mab 8H11 wherein said polynucleotide is substantially equivalent to SEQ ID NO: 1.

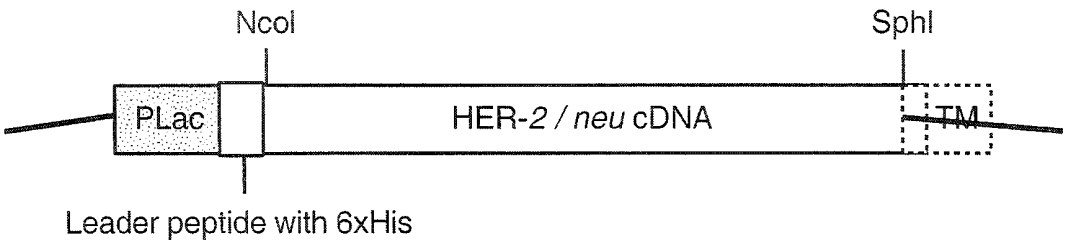
62. An isolated polynucleotide which comprises the complement of the polynucleotide of claim 61.

63. A vector comprising the isolated polynucleotide of claim 61.

64. An isolated polynucleotide encoding the variable heavy region of Mab 8H11 wherein said polynucleotide is substantially equivalent to SEQ ID NO: 2.

65. An isolated polynucleotide encoding the variable light region of Mab 10H8 wherein said polynucleotide is substantially equivalent to SEQ ID NO: 3.

66. An isolated polynucleotide encoding the variable heavy region of Mab 10H8 wherein said polynucleotide is substantially equivalent to SEQ ID NO: 4.


NcoI
SphI
PLac
HER-2 / neu cDNA
TM
Leader peptide with 6xHis

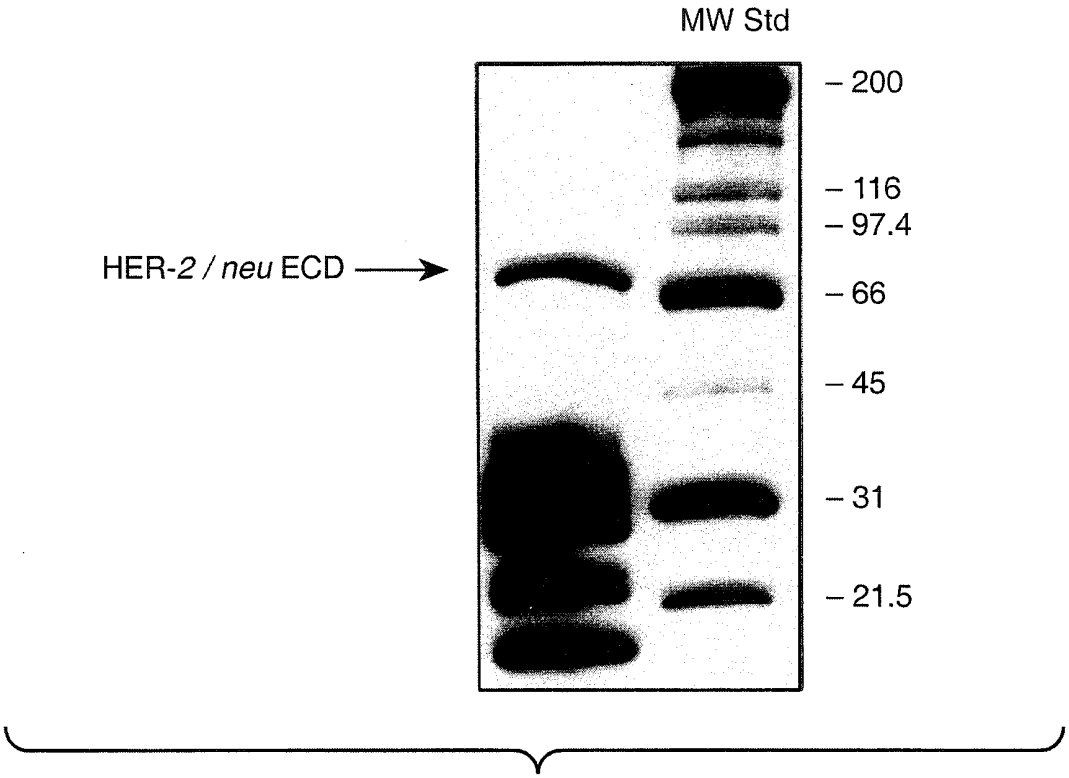

MW Std
– 200
– 116
– 97.4
– 66
– 45
– 31
– 21.5
HER-2 / neu ECD

FIG._1

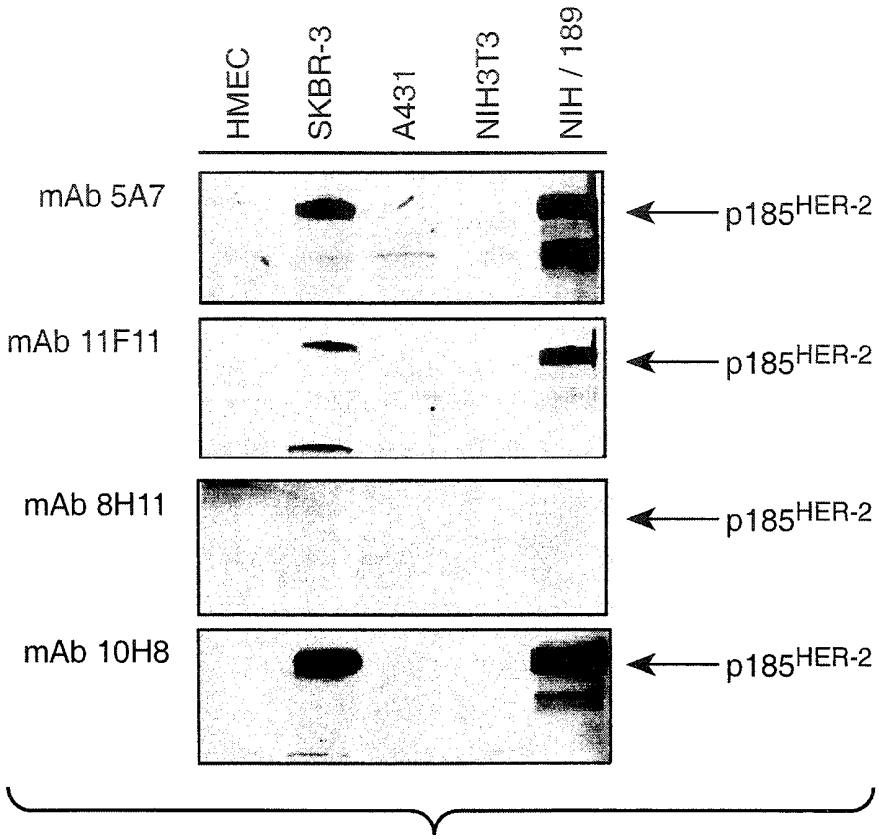

HMEC
SKBR-3
A431
NIH3T3
NIH / 189
mAb 5A7
mAb 11F11
mAb 8H11
mAb 10H8
p185HER-2
p185HER-2
p185HER-2
p185HER-2

FIG._2

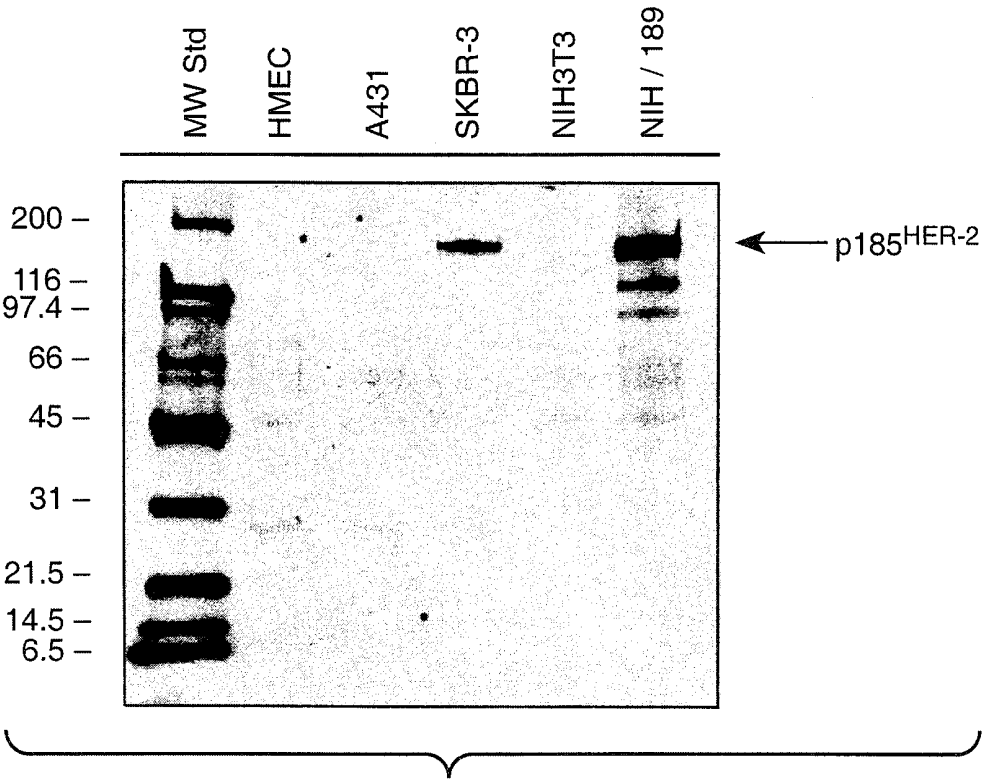
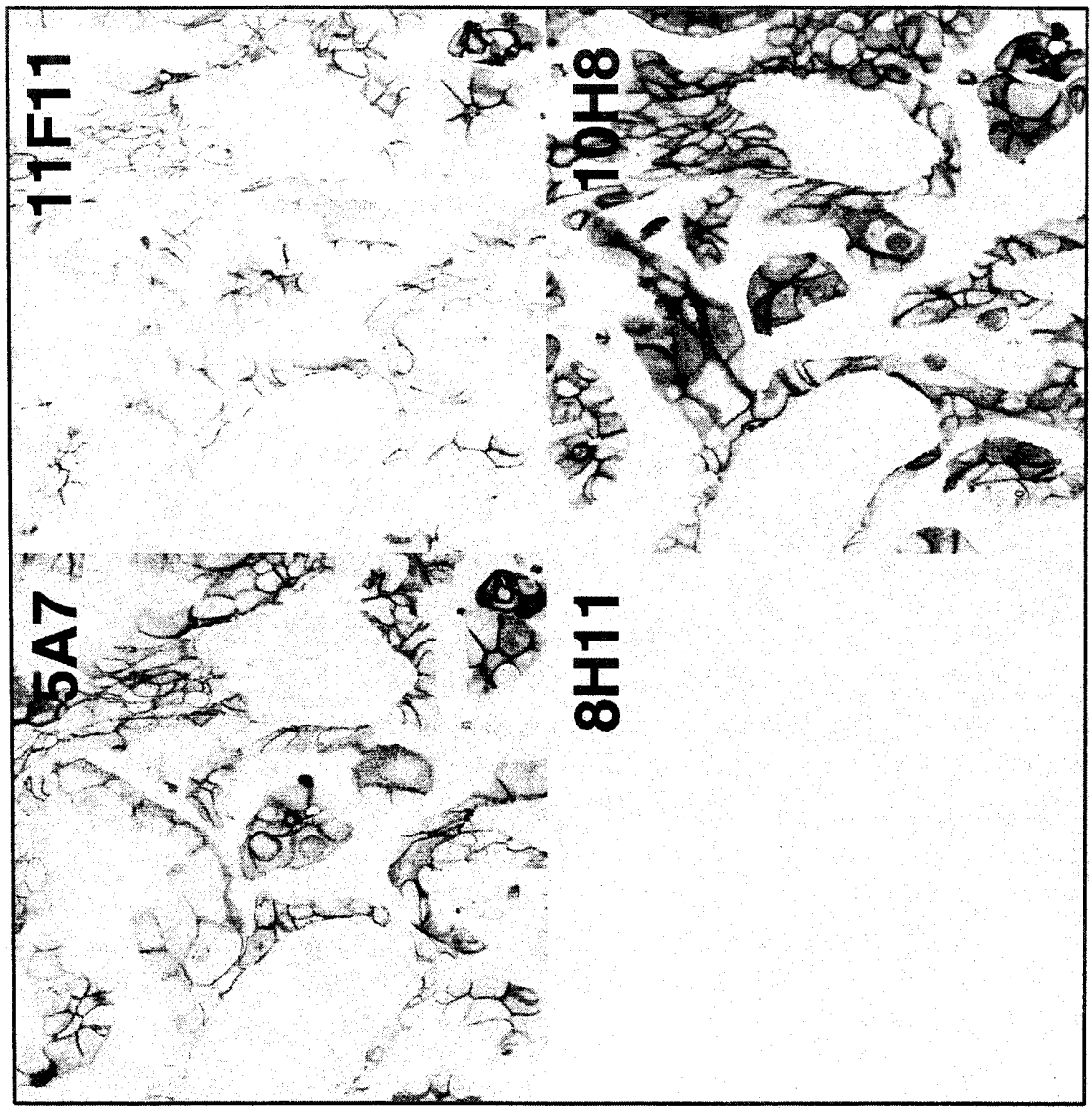

MW Std
HMEC
A431
SKBR-3
NIH3T3
NIH / 189
200 –
116 –
97.4 –
66 –
45 –
31 –
21.5 –
14.5 –
6.5 –
p185HER-2

FIG._3

FIG._4



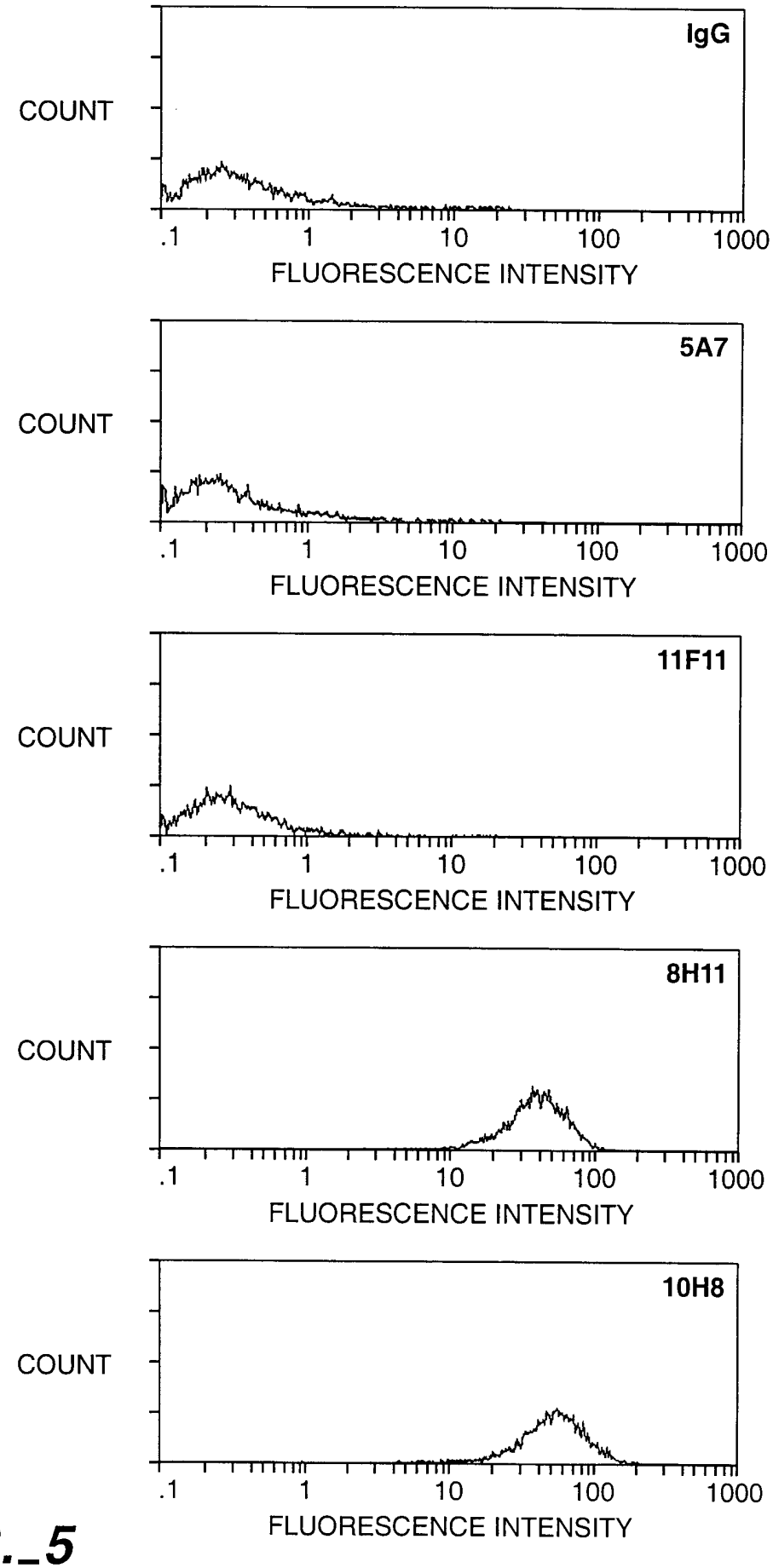

IgG
COUNT
.1
1
10
100
1000
FLUORESCENCE INTENSITY
5A7
COUNT
.1
1
10
100
1000
FLUORESCENCE INTENSITY
11F11
COUNT
.1
1
10
100
1000
FLUORESCENCE INTENSITY
8H11
COUNT
.1
1
10
100
1000
FLUORESCENCE INTENSITY
10H8
COUNT
.1
1
10
100
1000
FLUORESCENCE INTENSITY

FIG._5

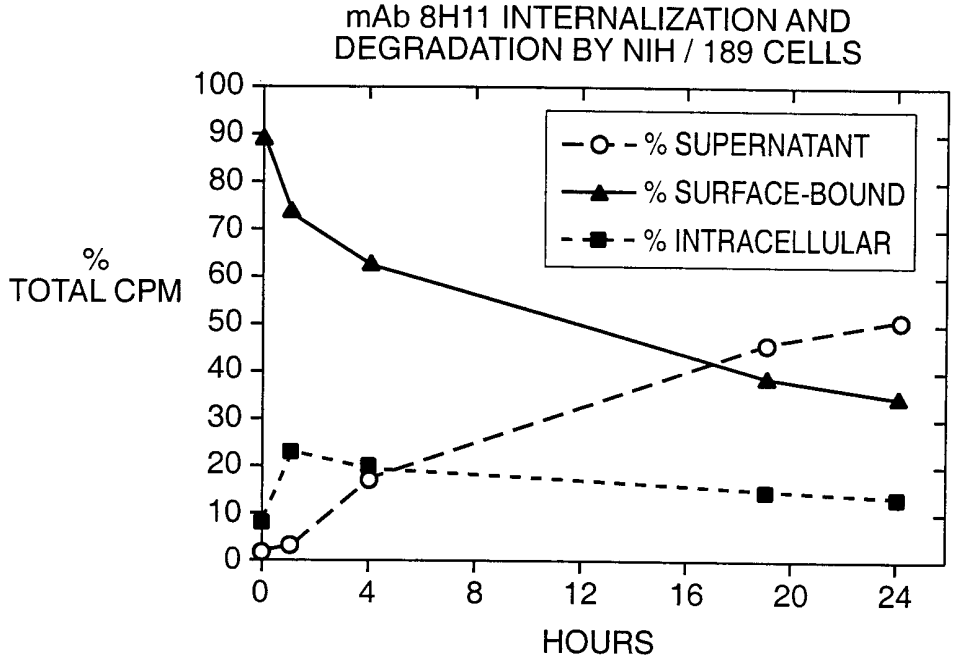

mAb 8H11 INTERNALIZATION AND
DEGRADATION BY NIH / 189 CELLS
% SUPERNATANT
% SURFACE-BOUND
% INTRACELLULAR
% TOTAL CPM
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS

FIG._6A

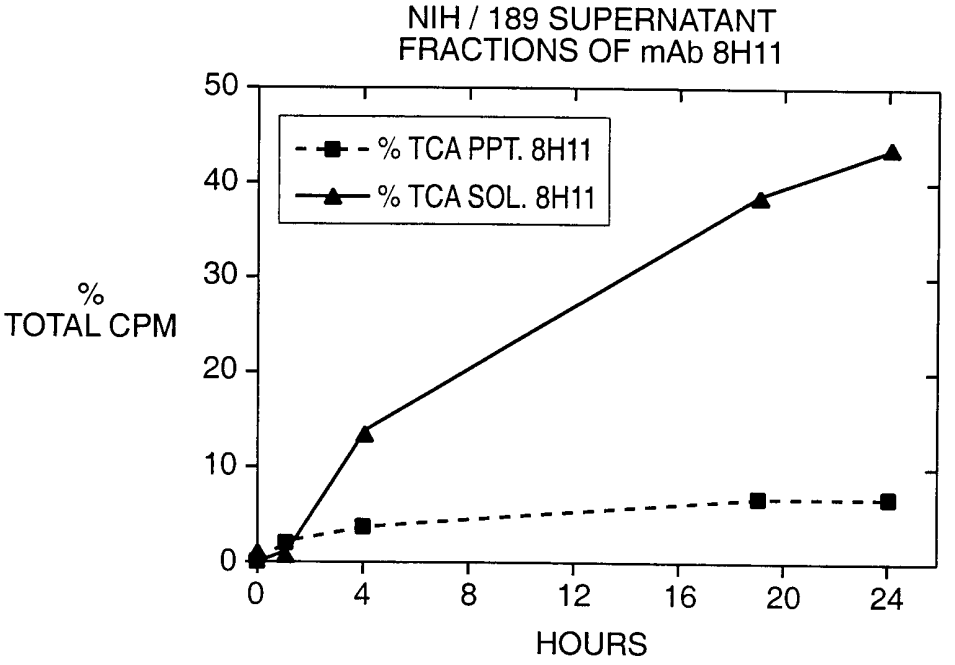

NIH / 189 SUPERNATANT
FRACTIONS OF mAb 8H11
% TCA PPT. 8H11
% TCA SOL. 8H11
% TOTAL CPM
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS

FIG._6B

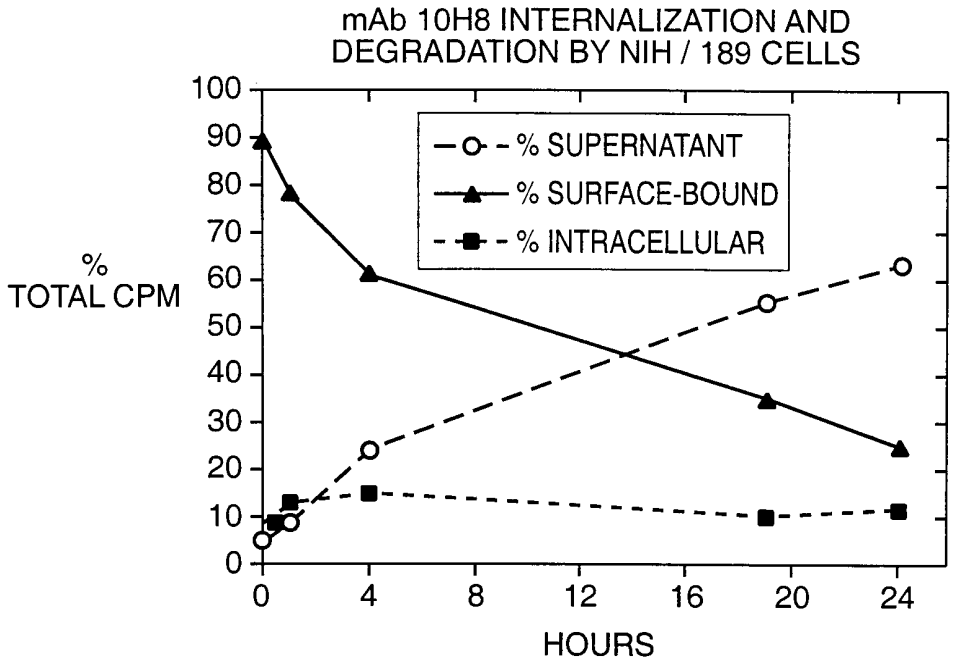

mAb 10H8 INTERNALIZATION AND
DEGRADATION BY NIH / 189 CELLS
% SUPERNATANT
% SURFACE-BOUND
% INTRACELLULAR
%
TOTAL CPM
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS

FIG._6C

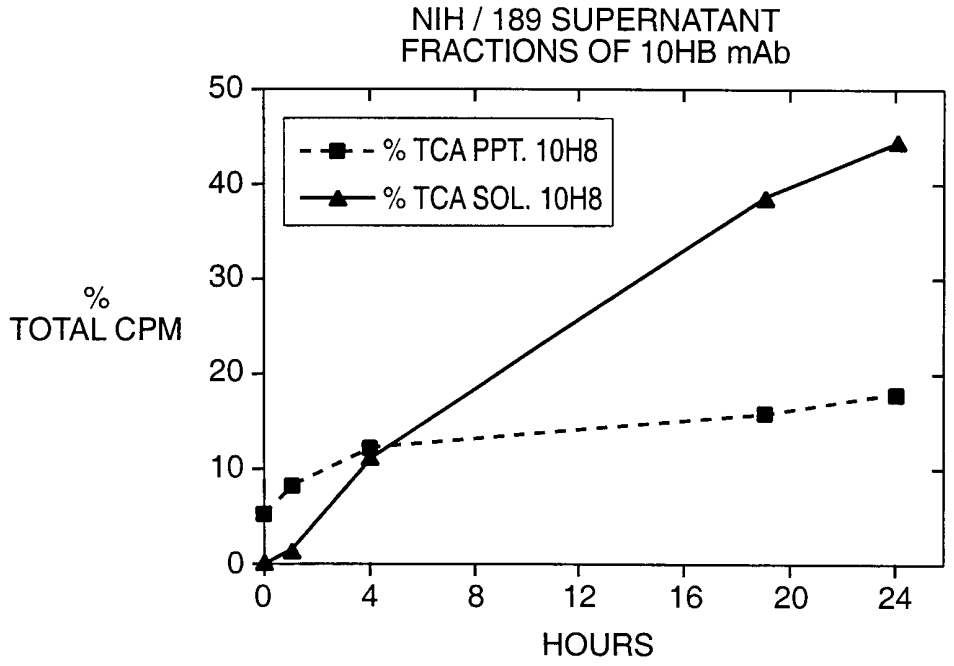

NIH / 189 SUPERNATANT
FRACTIONS OF 10HB mAb
% TCA PPT. 10H8
% TCA SOL. 10H8
%
TOTAL CPM
50
40
30
20
10
0
0
4
8
12
16
20
24
HOURS

FIG._6D

Variable Light 8H11

TTGGTGATATCTTGATGACCCAATCTCCACTCTCCATGTATACATCTCTAGGAGAGAGAGTCA
CTATCACTTGCAAGGCGAGTCAGGACATTAATAGCTATTTAAGCTGGTTCCAGCAGAAACCAG
GGAAATCTCCTAAGACCCTGATCTATCGTGCAAACAGATTGGTAGATGGGGTCCCATCAAGGT
TCAGTGGCAGTGGATCTGGGCAAGATTATTCTCTCACCATCAGCAGCCTGGAGTATGAAGATA
TGGGAATTTATTATTGTCTACAGCATGATGAGTTTCCGTGGACGTTCGGTGGAGGCACCAAGT
TGGAAATCAAACGGGCTGATGCTGCACCAACTGTATCCATCTTCCCACCA

Variable Heavy 8H11

TTAGGTGCAGCTGCAGGAGTCTGGACCTGAGCTGAAGAAGCCTGGAGAGACAGTCAAGATCTC
CTGCAAGGCTTCTGGGTATACCTTCACAAACTATGGAATGAACTGGGTGAAGCAGGCTCCAGG
AAAGGGTTTAAAGTGGATGGGCTGGATAAACACCAATATTGGAGAGCCAACATATACTGAAGA
GTTCAAGGGACGGTTTGCCTTTTCTTTGGGAACCTCTGCCAGCACTGCCTTTTTGCAGATCAA
CAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCAAGAGATGATGGTTACGGGAATCG
TGTTAGTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATC
TGTCTATCCACTGGCCCCTGAA

FIG._7

Variable Light 10H8

TTGGTGATATCTTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCT
CCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTATTTACATTGGT
ACCTGCAGAGGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAATCGATTTTCTG
GGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAG
TGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCTCCGTGGACGT
TCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCACCAACTGTATCCATCTTCC
CACCATCCGAATTCCGAA

Variable Heavy 10H8

TTAGGTCCAGCTGCAGGAGTCTGGGGGAGACCTAGTGAGTCCTGGAGGGTCCCTGAAGCTCTC
CTGTGCAGCCTCTGGATTCACTTTCAGTAGTAATGGCATGTCTTGGGTTCGCCAGACTCCAGA
CAAGAGGCTGGAGTGGATCGCAACCATTAGTGGTGGTGGTTATTACATCTACTATCCAGACAG
TGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTATACCTGCAAATGAG
AAGTCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAGACATGGGGACGATAATAGCTC
CTACCTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACAACAGCCCC
ATCGGTCTATCCACTGGC

FIG._8