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(54) Title: IMPROVED DELIVERY OF DIAGNOSTIC AND THERAPEUTIC AGENTS TO A TARGET SITE

(57) Abstract

An *in vivo* method for delivering a diagnostic or therapeutic agent to a target site in a mammal, wherein a targeting species including a targeting moiety and a diagnostic or therapeutic agent or a binding site for a subsequently administered diagnostic or therapeutic agent conjugate, the targeting moiety having a primary binding site whereby it specifically binds to the target, is administered and allowed to accrete at the target site, is improved by injecting into the circulatory system of the mammal a clearing agent that binds to the primary binding site of the targeting species, whereby non-localized primary targeting species is cleared from circulation. The method is especially useful in pretargeting methods because the clearing agent does not remove the primary targeting species or block secondary binding sites of the primary targeting species.

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IMPROVED DELIVERY OF DIAGNOSTIC AND THERAPEUTIC AGENTS TO A TARGET SITE

Background of the Invention

1 - Field of the Invention

The present invention relates to improved clearing agents for use in targeting diagnostic or therapeutic agents to a target site in a mammal. The present invention also relates to improved methods for diagnosing or treating patients using improved clearing agents.

2 - Description of Related Art

The detection of a target site benefits from a high signal-to-background ratio of detection agent. Therapy benefits from as high an absolute accretion of therapeutic agent at the target site as possible, as well as a reasonably long duration of uptake and binding. In order to improve the targeting ratio and amount of agent delivered to a target site, the use of targeting vectors comprising diagnostic or therapeutic agents conjugated to a targeting moiety for preferential localization has long been known.

Examples of targeting vectors include diagnostic or therapeutic agent conjugates of targeting moieties such as antibody or antibody fragments, cell- or tissue-specific peptides, and hormones and other receptor-binding molecules. For example, antibodies against different determinants associated with pathological and normal cells, as well as associated with pathogenic microorganisms, have been used for the detection and treatment of a wide variety of pathological conditions or lesions. In these methods, the targeting antibody is directly conjugated to an appropriate detecting or therapeutic agent as described, for example, in Hansen et al., U.S. Patent No. 3,927,193 and Goldenberg, U.S. Pat. Nos. 4,331,647, 4,348,376, 4,361,544, 4,468,457, 4,444,744, 4,460,459, 4,460,561, 4,624,846 and 4,818,709, the disclosures of all of which are incorporated herein by reference.

One problem encountered in direct targeting methods, *i.e.*, in methods wherein the diagnostic or therapeutic agent (the "active agent") is conjugated directly to the targeting moiety, is that a relatively small fraction of the conjugate actually binds to the target site, while the majority of conjugate remains in circulation and compromises in one way or another the function of the targeted conjugate. In the case of a diagnostic conjugate, for example, a radioimmunoscintigraphic or magnetic resonance imaging conjugate, non-targeted conjugate which remains in circulation can increase background and decrease resolution. In the case of a therapeutic conjugate having a very toxic therapeutic agent, *e.g.*, a radioisotope, drug or toxin, attached to a long-circulating targeting moiety such as an antibody, circulating conjugate can result in unacceptable toxicity to the host, such as marrow toxicity or systemic side effects.

Pretargeting methods have been developed to increase the target:background ratios of the detection or therapeutic agents. Examples of pre-targeting and biotin/avidin approaches are described, for example, in Goodwin et al., U.S. Patent No. 4,863,713; Goodwin et al., J. Nucl. Med. 29:226, 1988; Hnatowich et al., J. Nucl. Med. 28:1294, 1987; Oehr et al., J. Nucl. Med. 29:728, 1988; Klibanov et al., J. Nucl. Med. 29:1951, 1988; Sinitsyn et al., J. Nucl. Med. 30:66, 1989; Kalofonos et al., J. Nucl. Med. 31:1791, 1990; Schechter et al., Int. J. Cancer 48:167, 1991; Paganelli et al., Cancer Res. 51:5960, 1991; Paganelli et al., Nucl. Med. Commun. 12:211, 1991; Stickney et al., Cancer Res. 51:6650, 1991; and Yuan et al., Cancer Res. 51:3119, 1991; all of which are incorporated by reference herein in their entirety.

In pretargeting methods, a primary targeting species (which is not bound to a diagnostic or therapeutic agent) comprising a first targeting moiety which binds to the target site and a binding site that is available for

binding by a subsequently administered second targeting species is targeted to an *in vivo* target site. Once sufficient accretion of the primary targeting species is achieved, a second targeting species comprising a diagnostic or therapeutic agent and a second targeting moiety, which recognizes the available binding site of the primary targeting species, is administered.

An illustrative example of pretargeting methodology is the use of the biotin-avidin system to administer a cytotoxic radioantibody to a tumor. In a typical procedure, a monoclonal antibody targeted against a tumor-associated antigen is conjugated to avidin (or biotin) and administered to a patient who has a tumor recognized by the antibody. Then the therapeutic agent, *e.g.*, a chelated radionuclide covalently bound to biotin (or avidin), is administered. The radionuclide, via its attached biotin (or avidin), is taken up by the antibody-avidin (or -biotin) conjugate pretargeted to the tumor.

Pretargeting is an approach which offers certain advantages over the use of direct targeting methods. For example, use of the pretargeting approach for the *in vivo* delivery of radionuclides to a target for therapy, e.g., radioimmunotherapy, reduces the marrow toxicity caused by prolonged circulation of a radioimmunoconjugate. This is because the radioisotope is delivered as a rapidly clearing, low molecular weight chelate rather than directly conjugated to a primary targeting molecule, which is often a long-circulating species.

One problem encountered with two-step pretargeting methods is that circulating primary targeting species (antibody-avidin or -biotin conjugate, for example) interferes with the targeting of active agent species (biotin- or avidin-active agent conjugate) at the target site by competing for the binding sites on the active agent-conjugate. This problem typically is avoided or minimized by the use of a three-step approach wherein a clearing agent is administered as an intermediate step of the above two-step approach. The clearing agent binds

and removes circulating primary conjugate which is not bound at the target site.

Paganelli *et al.* (J. Nucl. Med. 31:735, 1990 and Cancer Res. 51:5960, 1991) disclose a 3-step approach wherein a biotinylated antibody is administered, followed by cold, *i.e.*, non-labeled and non-conjugated, avidin to clear nontargeted antibody. Radiolabeled biotin is then administered which binds to the avidin retained in the body, presumably where the avidin has complexed to the biotinylated antibody.

When antibody-avidin is used as the primary targeting moiety, excess circulating conjugate is cleared by injecting a biotinylated polymer such as biotinylated human serum albumin. This type of agent forms a high molecular weight species with the circulating avidin-antibody conjugate which is quickly recognized by the hepatobiliary system and deposited primarily in the liver.

To speed up this hepatobiliary recognition process, the clearing agent may be substituted with sugar residues, primarily galactose, such that the galactosylated complex is recognized by the asialoglycoprotein receptors in the liver. By using a galactosylated biotin-protein, substantially all circulating streptavidin-antibody and galactosylated biotin-protein is deposited into the liver on the first pass through, making the clearing process very fast and efficient. With circulating avidin conjugate removed, excess biotin-chelate-radionuclide is rapidly eliminated, preferably renally. Because the radionuclide spends a very short time in circulation, considerably less marrow toxicity to the patient is seen compared to when the radionuclide is attached directly to the antibody.

Examples of this methodology are disclosed, *e.g.*, in Axworthy *et al.*, PCT Application No. WO 93/25240; Paganelli *et al.*, "Monoclonal Antibody Pretargeting Techniques For Tumour Localization: The Avidin-Biotin System", *Nucl. Med. Comm.*, Vol. 12:211-234, (1991); Oehr

et al., "Streptavidin And Biotin As Potential Tumor Imaging Agents", *J. Nucl. Med.*, Vol. 29:728-729, (1988); Kalofonos *et al.*, "Imaging Of Tumor In Patients With Indium-111-Labeled Biotin And Streptavidin-Conjugated Antibodies: Preliminary Communication", *J. Nucl. Med.*, Vol 31:1791-1796, (1990); Goodwin *et al.*, "Pre-Targeted Immunoscintigraphy Of Murine Tumors With Indium-111-Labeled Bifunctional Haptens", *J. Nucl. Med.*, Vol. 29:226-234, (1988). Improved pretargeting methods using the biotin-avidin system are disclosed, e.g., in co-pending U.S. Patent Applications Serial Nos. 07/933,982, 08/051,144 and 08/062,662, the disclosures of which are incorporated by reference herein in their entirety.

Pretargeting as it has been practiced to date suffers from certain drawbacks. First among these is the very low amount of radionuclide delivered to the target site compared to when the radionuclide is directly attached to an antibody. Using the above example, the problem of low target accretion is exacerbated by the choice of the clearing agent used for the avidin-antibody conjugate. In that approach, it has been found that the clearing agent also removes antibody-avidin conjugate previously bound to the target site. This substantially reduces the amount of antibody-avidin at the target, typically by 50% or more. Further, the use of a biotinylated clearing agent tends to partially block remaining antibody-avidin sites at the target, thereby reducing the number of sites available for binding by the biotinylated diagnostic or therapeutic agent.

A need exists, therefore, for improved clearing agents which work efficiently and rapidly, but do not block the binding sites at the target site, and which do not remove primary conjugate localized at the target site.

Summary of the Invention

One object of the present invention is to provide improved clearing agents for clearing non-targeted

primary targeting species efficiently and quickly from circulation.

Another object of the present invention is to provide improved methods of *in vivo* diagnosis or therapy using clearing agents which efficiently clear non-localized targeting species from circulation.

These and other objects are realized by providing, in an *in vivo* pretargeting method for delivering a diagnostic or therapeutic agent to a target site in a mammal, wherein:

a primary targeting species is administered to the mammal which binds via a primary, target-specific binding site to the target site or to a substance produced by or associated with the target site and which comprises a second binding site which binds an active agent conjugate or an intermediate which in turn binds an active agent conjugate, sufficient time being allowed for said primary targeting species to localize at the target site;

a clearing agent is then administered that binds to the primary targeting species, sufficient time being allowed for said clearing agent to clear said primary targeting species from circulation; and

an active agent conjugate, comprising a moiety that binds to the second binding site of the primary targeting species conjugated to a diagnostic or therapeutic agent, is then administered to said mammal, sufficient time being allowed for the conjugate to localize at the target site,

the improvement wherein the clearing agent binds to the primary binding site of the primary targeting species, whereby substantially only non-localized primary targeting species is cleared and targeted primary targeting species is not removed from the target site, nor is the second binding site of the primary targeting species blocked by the clearing agent.

In another aspect of the present invention, the clearing agent is substituted with sugar residues, such as galactose residues, that selectively bind to the

hepatic asialoglycoprotein receptor, whereby hepatic clearance is effected substantially in a single pass.

Additional objects and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages may be realized and obtained by means of the processes and compositions particularly pointed out in the appended claims.

Detailed Description

The following terms are used in this application:

Target site: A specific site to which a diagnostic or therapeutic agent is to be delivered, such as a cell or group of cells, tissue, organ, tumor or lesion.

Primary targeting moiety: A moiety that binds to the target site or to a substance produced by or associated with the target site via a primary binding site. For example, proteins, peptides, polypeptides, glycoproteins, lipoproteins, phospholipids, oligonucleotides, steroids, alkaloids or the like, *e.g.*, hormones, lymphokines, growth factors, albumin, cytokines, enzymes, immune modulators, receptor proteins, antisense oligonucleotides, antibodies and antibody fragments, which preferentially bind marker substances that are produced by or associated with the target site.

Direct targeting species: A species comprising a primary targeting moiety and an active agent.

Primary targeting species: A species comprising a primary targeting moiety which binds via a primary binding site to the target site and a second binding site that is available for binding by a subsequently administered second targeting moiety. The second binding site may be a region that is a natural part of the primary targeting moiety, a modified region of the targeting moiety, or a region that is conjugated to the targeting moiety, such as a biotin or avidin molecule conjugated to the primary targeting moiety.

Second targeting moiety: A moiety that binds to the second binding site of the primary targeting species, such as an avidin or biotin moiety.

Second targeting species: Species comprising a second targeting moiety and an active agent.

Clearing agent: Agent which clears non-localized primary targeting species from circulation.

Active agent: A diagnostic or therapeutic agent.

Avidin: A family of proteins functionally defined by their ability to bind biotin with high affinity and specificity. Avidins are fairly small oligomeric proteins, made up of four identical subunits, each bearing a single binding site for biotin. Avidins can therefore bind up to four moles of biotin per mole of avidin. Avidins include proteins (a) produced by amphibians, reptiles and avians, which is present in their eggs and known as avidin, and (b) produced by a streptomyces, *Streptomyces avidinii*, and known as streptavidin. As used herein "avidin" includes all of the above proteins.

Biotin: As used herein, "biotin" includes biotin, commercial biotin products in which the biotin has been modified by the addition of alkyl groups, and biotin derivatives such as active esters, amines, hydrazides and thiol groups with the complimentary reactive groups on polymers being amines, acyl and alkyl leaving groups, carbonyl groups and alkyl halides or Michael-type acceptors.

The clearing agents of the present invention comprise a moiety which binds the primary binding site of the primary targeting species. That is, the clearing agents of the present invention bind the region of the primary targeting moiety which binds to the target site. This is in sharp contrast to previously used clearing agents which bind to other regions of the primary targeting species, such as biotin or avidin moieties conjugated to the primary targeting moiety.

The clearing agent of the present invention may comprise any molecule which is a specific binding complement to the primary binding site of the primary targeting moiety. In one embodiment of the present invention, the primary targeting species is an antibody, and the clearing agent comprises an antibody which recognizes the primary binding site (the antigen-binding region) of the targeting antibody, *i.e.*, the clearing agent comprises an anti-idiotypic second antibody. The clearing agents also may be non-antibody species that bind to the primary binding site of the targeting antibody.

When non-antibody primary targeting species are used, such as small peptides, steroids, hormones, cytokines, neurotransmitters, or other targeting species which preferentially bind marker substances that are produced by or associated with the target site, the clearing agent may comprise an antibody that specifically binds to the receptor-binding site (the primary binding site) of the targeting species. A non-antibody clearing agent also may used which binds to the primary binding site of the non-antibody primary targeting species.

Because the clearing agents of the present invention bind to the primary binding site of the primary targeting species, they can only bind circulating primary binding species, and cannot bind species already bound to the target site. The clearing agents of the present invention therefore offer distinct advantages over clearing agents currently used, and avoid the problems discussed above. That is, the present clearing agents do not block the second binding sites of the primary targeting species and do not remove primary targeting species from the target site.

In a preferred embodiment of the invention, the clearing agent is conjugated to sugar residues such as galactose which bind to the hepatic asialoglycoprotein receptor, whereby the clearing agent and clearing agent-primary targeting species complexes are rapidly

recognized by liver hepatocytes. Use of galactosylated clearing agents, therefore, ensures near-total hepatocytic recognition and sequestration within minutes post-injection, generally substantially in a single pass through the liver.

The clearing agents of the present invention may be used in any method in which a targeting species is used to deliver an active agent to a target site. For example they may be used in the direct targeting methods and pretargeting methods discussed above. The clearing agents of the present invention also may be used in the three- and four-step methods disclosed in U.S. Serial Nos. 08/051,144, 07/933,922 and Axworthy PCT Application No. WO 93/25240, the contents of which are herein incorporated by reference in their entirety.

In a direct targeting method, a species comprising the primary targeting moiety and the active agent is administered and allowed to localize. Then, a clearing agent according to the present invention is administered to remove non-localized circulating targeting species.

In a three-step pretargeting method, the primary targeting species is administered and allowed to localize. Then, the clearing agent is administered to remove non-localized primary targeting species. Then, the second targeting species is administered. For example, an antibody-biotin (or antibody-avidin) primary targeting species is administered and allowed to localized, followed by the administration of an anti-idiotype-antibody clearing agent according to the present invention. Then an avidin-active agent (or biotin-active agent) second targeting species is administered.

Using an anti-idiotype clearing agent in a biotin-avidin pretargeting system avoids the aforementioned disadvantages of known clearing agents. For example, since the clearing agent does not contain biotin or avidin, none of the avidin-antibody conjugate localized at the target site is compromised by unwanted blocking of binding sites. Moreover, the anti-idiotypic second

antibody clearing agent recognizes only the antigen-binding region (paratope) of the first antibody, hence the second antibody is capable of rapidly binding to circulating antibody, but is unable to bind to target-bound avidin-antibody conjugate and therefore is unable to remove avidin-antibody from the target.

In a preferred embodiment of the present invention, the antibody-streptavidin targeting step and the anti-idiotype clearing step are followed by the injection of a monobiotinylated-dextran-$(boron)_x$ conjugate. Boron, as the B-10 isotope, is used for neutron capture reactions for therapy. Recently Holmberg and Meurling disclosed a method of attaching up to 1500 boron atoms per 70 kD dextran unit. Holmberg *et al.*, *Bioconjugate Chem.* 4: 570-573 (1993). Such a unit attached directly to an antibody would significantly alter antibody properties. However, in itself it displays very attractive properties since it retains its water solubility. After limited biotinylation, such a monobiotinylated-dextran-$(boron)_{1500}$ species retains the solubility properties, and is capable of delivering up to 6000 boron atoms to the target, by virtue of the tetravalency of the streptavidin-IgG conjugate previously localized at the tumor. This is significantly more boron-10 than can be delivered by methods using IgG-polymer-boron-10 conjugates, which typically produce conjugates containing in the 100s of boron atoms per antibody.

U.S. Serial Nos. 08/051,144 and 07/933,922, discussed above, describe targeting species, primary and second targeting moieties, and active agents which may be used in accordance with the present invention. For example, 08/051,144 sets forth antibodies, antibody fragments and other proteins which are useful as primary targeting moieties.

08/051,144 also discloses radionuclides, paramagnetic ions and fluorescence-emitters which may be used as detection or therapeutic agents, as well as beta- and alpha-emitters and neutron-capturing agents, such as

Boron and Uranium, which can be used as therapeutic agents.

Other therapeutic agents useful in accordance with the present invention include, drugs, fluorescent dyes activated by non-ionizing radiation, hormones, hormone antagonists, receptor antagonists, enzymes or proenzymes activated by another agent, autocrines or cytokines. Toxins also can be used in the methods of the present invention, such as plant and bacterial toxins, such as, abrin, alpha toxin, diphtheria toxin, exotoxin, gelonin, pokeweed antiviral protein, ricin, and saporin. Other therapeutic agents useful in the present invention include anti-DNA, anti-RNA, radiolabeled oligonucleotides, such as anti-sense oligodeoxy ribonucleotides, anti-protein and anti-chromatin cytotoxic or antimicrobial agents.

As set forth in 08/051,144, the targeting moiety may be conjugated to the active agent by methods known to those skilled in the art. US Patent 5,057,313, Shih *et al.*, hereby incorporated by reference, teaches one method for conjugating biotin or avidin to an active agent.

Other examples of methods of conjugating avidin to a detection or therapeutic agent include the following: (a) the chloramine-T or Bolton-Hunter procedures for conjugating iodine, (b) the procedures described by Griffiths et al. (*Cancer Res.* 51: 4594, 1991) or Fritzberg et al. (U.S. Pat. No. 5,120,526) to conjugate technetium or rhenium (c) through bifunctional chelating agents as described by Meares et al. (*Br. J. Cancer* 62: 21, 1990) to conjugate metallic nuclides. Additionally, avidin or biotin can be bound to dendrimers by procedures described for amino-containing proteins as described by Hnatowich et al. (*J. Nucl. Med.* 28: 1294, 1987). Biotin can be readily conjugated to proteins (including antibodies and their fragments) via the proteins' lysine and cysteine residues, and, if available, their oxidized carbohydrate groups.

The present invention also may be used with other antibody-based pretargeting systems (i.e., those not utilizing biotin and avidin). For example, the specific hybridization of complementary DNA fragments may be used as the recognition mechanism of a pretargeting system. In such a method, one strand of DNA is bound to an antibody and the complementary strand is bound to a therapeutic radioisotope which is administered later. Bos *et al.*, *Cancer Res.* 54: 3479-3486 (1994). A major advantage of this system over biotin/avidin systems could be the presumed lower immunogenicity of a relatively short piece of DNA compared to the highly immunogenic 60,000 Dalton avidin species.

Another approach to pretargeting involves administering an enzyme linked to an antibody, followed by administering a high-affinity enzyme inhibitor (specific for the enzyme) bound to a chelate-isotope complex. This method has the advantage over previous bispecific methods of retaining both antigen binding sites of the antibody, and the further advantage of utilizing a high affinity (K_d, dihyrofolate reductase:methotrexate = 10^{-10}) secondary targeting mechanism. As with the DNA method discussed above, antigenicity may be less of a problem than in the avidin/biotin system.

A physiological solution of the targeting species is advantageously metered into sterile vials, e.g., at a unit dosage of about 1.0 - 500 mg targeting species/vial, and the vials are either stoppered, sealed and stored at low temperature, or lyophilized, stoppered, sealed and stored.

Variations and modifications of these formulations will be readily apparent to the ordinary skilled artisan, as a function of the individual needs of the patient or treatment regimen, as well as of variations in the form in which the radioisotopes may be provided or may become available.

Routes of administration for the composition include intravenous, intraarterial, intrapleural, intraperitoneal, intrathecal, subcutaneous or by perfusion.

Methods useful for internal detection and/or treatment of tumors and/or other lesions, such as cardiovascular lesions (clots, emboli, infarcts, *etc.*), infectious diseases, inflammatory diseases, and autoimmune diseases are disclosed in U.S. Patent 4,782,840; U.S.Patent 4,932,412; and co-pending U.S. application Serial No. 07/879,857, the disclosures of which are incorporated herein by reference. The methods of the present invention can be used to enhance the methods disclosed in these references. The present invention also may be practiced in conjunction with intraoperative probes, endoscopic and laparascopic uses, and in methods for imaging normal organs. The methods of the present invention can be used in other methods that will be apparent to those skilled in the art.

The methods of the present invention also can be practiced either with scintigraphic or magnetic resonance imaging agents, as described, for example, in 08/051,144.

The embodiments of the invention may be further illustrated through examples which show aspects of the invention in detail. These examples illustrate specific elements of the invention and are not to be construed as limiting the scope thereof.

EXAMPLES

Example 1.

PREPARATION OF ANTIBODY-STREPTAVIDIN IMMUNOCONJUGATES

In this procedure, strepatavidin (SA) is substituted with a limited number of maleimido groups, and an IgG moiety is thiolated, after which the two components are mixed to effect protein conjugation.

The present invention also may be used with other antibody-based pretargeting systems (i.e., those not utilizing biotin and avidin). For example, the specific hybridization of complementary DNA fragments may be used as the recognition mechanism of a pretargeting system. In such a method, one strand of DNA is bound to an antibody and the complementary strand is bound to a therapeutic radioisotope which is administered later. Bos *et al.*, *Cancer Res.* 54: 3479-3486 (1994). A major advantage of this system over biotin/avidin systems could be the presumed lower immunogenicity of a relatively short piece of DNA compared to the highly immunogenic 60,000 Dalton avidin species.

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Routes of administration for the composition include intravenous, intraarterial, intrapleural, intraperitoneal, intrathecal, subcutaneous or by perfusion.

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In this procedure, strepatavidin (SA) is substituted with a limited number of maleimido groups, and an IgG moiety is thiolated, after which the two components are mixed to effect protein conjugation.

A. SA-SMCC

A thiol-activated streptavidin is prepared by reacting streptavidin lysine residues with a limited amount of the S-N cross-linker, sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce Chemical Co., Rockford IL). Briefly, a solution of streptavidin (950 μl), containing 10 mg of streptavidin (1.67×10^{-7} mol) dissolved in 0.1 M borate buffer, pH 8.3, containing 10 mM EDTA, is treated with 35 μL of a freshly prepared solution of 10 mg/ml of sulfo-SMCC (FW 436.4; 350 μg; 8.03×10^{-7} mol; 4.8 molar excess to SA). After 1 hour stirring at room temperature, the protein is purified from low MW materials on two G-50-80 size-exclusion spin-columns equilibrated in 0.1 M PBS, pH 6.4.

B. IMMU-14-2-IT

IMMU-14 (anti-carcinoembryonic antigen antibody) (3.3 ml of 7.23 mg/ml material; 23.8 mg, 1.54×10^{-7} mol) in 40 mM phosphate buffered 0.9% sodium chloride (PBS), pH 7.4, is treated with 300 μl 0.5 M borate buffer, pH 8.5, to give an antibody solution at a final pH of 8.1. The mixture is stirred at room temperature and treated with 8 μl of a solution of 100 mmolar 2-iminothiolane (FW 137.6; 8×10^{-7} mol) in water [5.2:1 2-IT:mab ratio]. The mixture is incubated for 50 min at room temperature and then purified on 4 x G-50-80 size-exclusion spin-columns equilibrated in 0.1 M phosphate buffer, pH 6.4, containing 10 mM EDTA, to give IMMU-14-SH in approximately 4.0 ml of buffer (5.9 mg/ml assuming 100% mab recovery).

C. SA-IMMU-14 IgG

The SA-SMCC product (1.1 ml) from Example 1A above is diluted to 15 ml with 0.1 M phosphate buffer, pH 6.4, containing 10 mM EDTA, and reacted with the IMMU-14-IT (4.0 ml), added in one portion, to give a final reaction volume of approximately 20 ml (molar reaction ratio 0.92:1, IMMU-14:SA), with rapid stirring at room temperature. Stirring is continued for a further 1 hour. The reaction mixture is concentrated to under 5 ml in two

Centriplus-100 (Amicon, Beverly MA) concentrators and the mixture is then applied to a preparative HPLC column (Altex Spherogel™, TSK-G-3000, 2.15 x 60 cm, Beckman, Fullerton CA) equilibrated in 0.2 M PBS, pH 6.8, and developed at 1 ml/min. The desired product eluted as the second peak, after a smaller peak consisting of higher molecular weight species.

Example 2.

PREPARATION OF ANTIBODY FRAGMENT-STREPTAVIDIN IMMUNOCONJUGATES

In this procedure, strepatavidin (SA) is substituted with a limited number of maleimido groups, and reacted with Fab-SH, after which the products, consisting of Fab-SA, $(Fab)_2$-SA, $(Fab)_3$-SA and $(Fab)_4$-SA, are separated on the basis of their size.

A. Streptavidin-SMCC

Streptavidin is activated with SMCC, using sulfo-SMCC. A streptavidin solution (0.5 ml), containing 1.75 mg of streptavidin dissolved in 100 mM Tris.HCl buffer, pH 8.6, containing 10 mM EDTA and 10 mM magnesium chloride is treated with 5 μl of a freshly prepared solution of 10 mg/ml of sulfo-SMCC.(0.05 mg). After 30 minutes stirring at room temperature, the protein is purified from low MW materials on a G-50-80 size-exclusion spin-column in 0.1 M PBS, pH 7.4. The recovered protein concentration is calculated from measurement of the λ_{280} absorbance of a small diluted aliquot of the product.

B. Fab-SH

IMMU-14-$F(ab)_2$ at 15 mg/ml (5 mg) is reduced with 20 mM cysteine, for 1 hour at 37°C, to give IMMU-14-Fab-SH. The progress of the reduction is followed by analytical size-exclusion HPLC. The IMMU-14-Fab-SH is separated from unreacted cysteine using a G-50-80 size-exclusion spin-column equilibrated with 100 mM PBS pH 7.4, containing 2mM EDTA. The recovered protein concentration is calculated from measurement of the λ_{280} absorbance of a small diluted aliquot of the product.

c. Fab-SA, $(Fab)_2$-SA, $(Fab)_3$-SA and $(Fab)_4$-SA

The two reaction products from Examples 2A and 2B above are mixed together and stirred 30 minutes at room temperature. The approximate molar ratios involved in this reaction are 1.75 mg streptavidin (2.9×10^{-8}Mol) and 5mg IMMU-14 Fab (1.0×10^{-7} Mol), which is a 1:3.4 molar ratio of SA:Fab-SH. At the end of the 30 minute reaction, 5μl of a 10 mg/ml freshly-prepared solution of cysteine.HCl (50μg; 2.85×10^{-7} Mol; 10-fold theoretical excess to SA-SMCC; approximate concentration of 0.285 molar in cysteine, which is not enough to further reduce MAb) in water is added, and the stirring is continued at room temperature for a further 15 minutes in order to block any remaining unreacted maleimide residues. At the end of this 15 minute blocking reaction, the reaction mixture is purified by preparative size-exclusion HPLC, as described in Example 1. Samples are collected from the column in the order of decreasing molecular weight.

Example 3.

GALACTOSYLATION OF THE ANTI-IDIOTYPIC WI2 ANTIBODY

The WI2 antibody is described in Losman et al., Int. J. Cancer 56:580-584 (1994), the contents of which are incorporated by reference. It is an anti-idiotypic antibody to the murine anti-CEA mab, MN-14.

Proteins are glycosylated according to the method outlined by Ong *et al.*, *Cancer Res.* 51: 1619-1626, (1991). Briefly, cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-β-D-galactopyranoside (Sigma Chemical Co., St. Louis MO) is dissolved in dry methanol to give a 0.1 M solution and then mixed with a 1/10th volume of sodium methoxide (J.T. Baker Chemical Co., Phillipsburg NJ). The flask contents are stoppered to prevent absorption of water, and then left to stand 48 hours at room temperature. The solution may be stored for a period of months in the refrigerator.

For conjugation to each mg of WI2 antibody, 500 μl of the imido ester solution is evaporated to dryness and then redissolved in a 1 mg/ml solution of WI2 in 0.25M

sodium borate buffer, pH 8.5. After standing for 2 hours, the galactosylated WI2 is dialyzed into 0.1M phosphate buffered saline, pH 7.4.

Example 4.

PREPARATION OF BIOTIN-(CARBORANE)$_n$-DEXTRAN CONJUGATE

A. Thiolated biotin

A solution of 5-(biotinamido)pentylamine (32.85 mg, 0.1mM) in 100 μl 0.05 M borate buffer, pH 8.5, is stirred at room temperature and treated with a solution of 2-iminothiolane (13.76 mg, 0.1mM) in 100 μl water. The mixture is incubated 30 minutes at room temperature and used immediately in reaction 4C below, or is aliquoted and frozen for future use.

B. (Allyl)$_n$-dextran

The boronation aspect of this procedure is the same as that described by Holmberg *et al.*, *Bioconjugate Chem.* 4: 570-573 (1993). Dextran (20g, 70,000 MW) is dissolved in 150 ml of distilled water containing 5 g of sodium hydroxide and 0.2 g of sodium borohydride, and treated with allyl bromide (35g, 0.3 mol) at 40 °C. The mixture is stirred for 3 hours at 60 °C, neutralized with acetic acid, and the product is purified by repeat precipitation in ethanol prior to drying to constant weight.

C. Biotin-(carborane)$_n$-dextran

To 20 mg of this (allyl)$_n$-dextran (2.5×10^{-7} mol, approximate FW 80,000, approximately 220 allyl groups per dextran unit or 5.5×10^{-5} mol allyl moiety) in 200 μl of water is added 30 mg sodium barocoptate, $Na_2B_{12}H_{11}SH$ (Boron Biologics, BSH, 1.4×10^{-4}mol), 2 μl (2×10^{-6} mol) of the above solution of 5-[(biotinamido)-4-pentylamidino]butanethiol prepared in Example 4A, and ammonium persulfate (20 mg). The reaction is stirred for 2 hours at 50 °C, and the product, biotin-(carborane)$_n$-dextran, is purified from low molecular weight materials on G-50-80 size-exclusion spin-columns. The product is quantitatively analyzed for boron content by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The ratio of biotin per dextran unit is determined by the

agent's ability to bind to known diminishing amounts of radiolabeled streptavidin. Binding is detected by a shift in the radioactivity peak of the radioiodinated streptavidin to higher molecular weight, by size-exclusion HPLC utilizing an in-line radioactivity detector.

Example 5

PREPARATION OF P-[5(-BIOTINAMIDO)PENTYL(AMINO) THIOUREAYL]-2-BENZYL-DIETHYLENETRIAMINEPENTAACETIC ACID (BPD)

5-(Biotinamido)pentylamine (50.7 mg, 1.54 x 10^{-4} mol) is dissolved in 700 μl of 0.1 M sodium phosphate, pH 8.5, and treated with p-(isothiocyanato)-2-benzyldiethylene triaminepentaacetic acid (43 mg, 3.23 x 10^{-5} mol). The pH is raised to 9 with approximately 300 μl of saturated sodium phosphate, and the reaction is incubated for 30 minutes at 37°C, while maintaining the pH at 9. The reaction mixture is filtered and the product (BPD) obtained in pure form using preparative reverse-phase HPLC, consisting of 3 tandemly coupled 40 x 100 mm Waters Prep-Pak RCM base columns. The columns are equilibrated for 10 minutes in 0.1% trifluoroacetic acid in water, and the desired product is eluted with a 10-30% gradient of 90% acetonitrile containing 0.1% trifluoroacetic acid, over 30 minutes, at a flow rate of 75 ml/minute.

Example 6

HIGH SPECIFIC ACTIVITY YTTRIUM-90 RADIOLABELING OF BPD

A 5 mCi sample of Y-90 (supplied in 10 μl of 0.05 M hydrochloric acid) is treated with 100 μl of 0.5 M sodium acetate buffer, pH 6, followed by 250 μl of a solution of BPD in 0.05 M acetate buffer, pH 8.1. The radiolabeling is allowed to proceed for 30 minutes, prior to radioanalysis using size-exclusion HPLC. A quantitative incorporation of yttrium-90 into the BPD is obtained. The specific activity of this labeling is approximately 2000 mCi of yttrium-90 per mg of BPD.

Example 7

***IN VIVO* LOCALIZATION OF YTTRIUM-90-BPD TO PRETARGETED STREPTAVIDIN-IMMU-14**

Athymic nude mice are injected subcutaneously with sufficient GW-39 tumor cells (expressing carcinoembryonic antigen) to produce a 200-300 μg solid tumor xenograft at 10 days post-implantation. At this time, a 250 μg dose of streptavidin-IMMU-14 is administered per animal. As with the IMMU-14 antibody itself, the maximum amount of streptavidin-IMMU-14 is localized onto the target tumor at 3 days post-administration. Then a streptavidin-IMMU-14 blood-clearing dose of galactose-WI2 is given to each animal, causing essentially all remaining circulating streptavidin-IMMU-14 to be immediately bound to the galactose-WI2 and deposited into the liver. Two hours after this, Y-90-BPD is given to the animals. A saturating dose of the Y-90-BPD binds to the localized streptavidin-IMMU-14 at the tumor, while the bulk of the non-tumor-bound Y-90-BPD is excreted through the urine within a 2-4 hour period. A similar amount of Y-90 is localized to the tumor with this method as with using a directly labeled Y-90-IMMU-14 conjugate but, without the latter's extended blood circulation, the current approach is much less toxic to the animal.

Example 8

LOCALIZATION OF BIOTIN-(CARBORANE)$_n$-DEXTRAN TO PRETARGETED STREPTAVIDIN-IGG

Athymic nude mice are injected subcutaneously with sufficient GW-39 tumor cells (expressing carcinoembryonic antigen) to produce a 200-300 μg solid tumor xenograft at 10 days post-implantation. At this time a 250 μg dose of streptavidin-IMMU-14 is administered per animal. As with the IMMU-14 antibody itself, the maximum amount of streptavidin-IMMU-14 is localized onto the target tumor at 3 days post-administration. Then a streptavidin-IMMU-14 blood-clearing dose of galactose-WI2 is given to each animal, causing essentially all remaining circulating streptavidin-IMMU-14 to be immediately bound to the galactose-WI2 and deposited into the liver. Two hours after this, a tumor-saturating dose of biotin-(carborane)$_n$-dextran is given to the animals, with the excess non-tumor-targeted biotin-(carborane)$_n$-dextran cleared from the circulation via the hepatobiliary and renal systems.

Example 9

PREPARATION OF BIOTINYLATING AGENTS

A. 2-AMINO-6-N(t-BUTOXYCARBONYL)AMINO-1-HEXANOL (Formula Ia of Table 1, R'=t-BOC)

N(t-butoxycarbonyl)lysine was reacted with borane-tetrahydrofuran complex in tetrahydrofuran (THF), at temperatures not exceeding 10°C, in an argon atmosphere for a period of 1-2 hours. Excess borane is decomposed by careful addition of aqueous THF. The product is then refluxed with 5 M aqueous sodium hydroxide solution for a period of 18h, cooled to room temperature, concentrated on a rotary evaporator to remove THF, and thoroughly extracted with chloroform. The chloroform extract is dried over anhydrous sodium sulfate, and evaporated to obtain the product (colorless liquid; 70% yield) in which the terminal N-protecting group is intact.

B. 2-(Biotinamido)-6-amino-1-hexanol
(Formula IIa in Table 1, R'=H)

N-Hydroxysuccinimidylbiotin (Sigma Chemical Co., St. Louis, MO) is mixed with the amine of formula Ia (described above) in equimolar ratio in dimethylformamide containing one equivalent of triethylamine. The reaction mixture is stirred at room temperature for 18h in an argon atmosphere. The reaction mixture is concentrated to a small volume under high vacuum, diluted with ether, and the precipitated product is washed with ether and 2-propanol, and dried to obtain a product of formula IIa wherein R'=t-BOC. Amine-deprotection is carried out by stirring with neat trifluoroacetic acid for 30-60 min. at room temperature, followed by isolation of the product of formula IIa wherein R'=H.

EXAMPLE 10

PREPARATION OF BIOTINYLATED DEXTRAN (STEPWISE APPROACH)

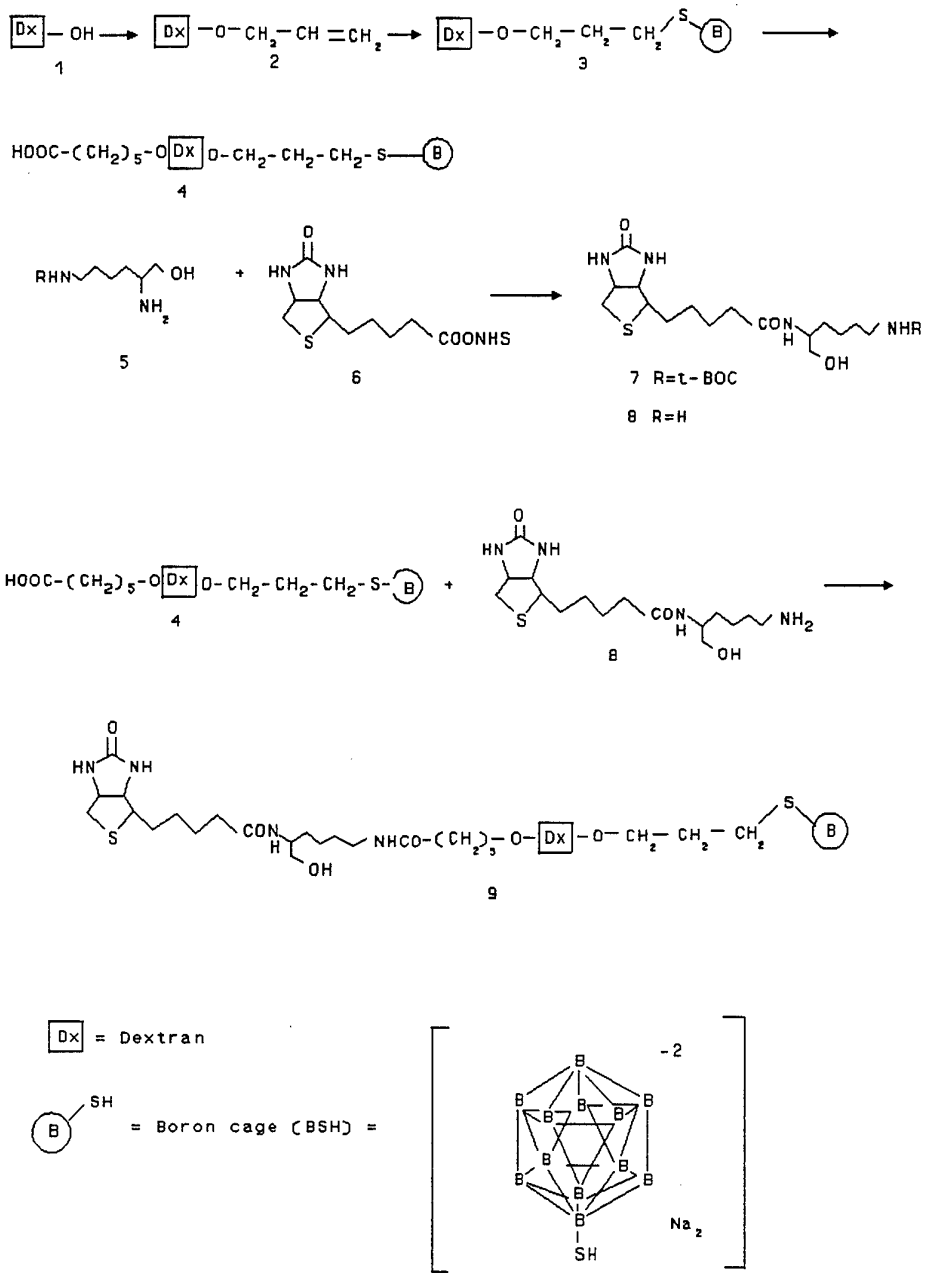
Carboxypentyl-$(allyl)_n$-dextran is prepared by dissolving 1 g of $(allyl)_n$-dextranExample 4B) in 10 ml of 4M aqueous sodium hydroxide, heating with 3.0 g of 6-bromohexanoic acid at 60-80°C for 3h, and purifying the product by dialysis against water. The extent of carboxyalkylation is determined by titration with a standard solution of sodium hydroxide. 20 mg of carboxypentyl-$(allyl)_n$-dextran (dissolved in 0.2 ml of water) is boronated with 30 mg of sodium borocaptate at 50°C for 2h, as described in Example 4C, to obtain (carboxypentyl)m-dextran-$(sulfhydrylborane)_n$ where m and n refer to the number of carboxyalkyl and borocaptate groups introduced onto the dextran.

Using any of the biotinylating agents of the general formula II of Table 1, $(carboxypentyl)_m$-dextran-$(sulfhydrylborane)_n$ is derivatized using water soluble peptide coupling agent EDC [1-ethyl 3-(3-dimethylaminopropyl)carbodiimide] and N-hydroxysulfosuccinimide. The number of biotin residues (most preferably one residue) introduced per dextran chain is controlled by the amount of biotinylating agent

used in the reaction. Final molar substitution ratios are derived from determinations of boron-dextran ratio (from boron determinations), and biotin-dextran ratio (from determination of the extent of binding to known concentrations of streptavidin).

Table 2 illustrates the steps involved using one biotinylating agent. (The compounds in Table 2 are numbered independently of those of Table 1. The biotinylating agent 8 in Table 2 is the same as formula IIa (R'=H) of Table 1.)

TABLE 2



EXAMPLE 11

COUPLING A BIOTINYLATING AGENT OF GENERAL FORMULA II WITH THE METAL-CHELATING AGENT DOTA

1,4,7,10-Tetraazacyclododecane N,N',N",N'"-tetraacetic acid (DOTA) forms kinetically stable chelates with metal ions of lanthanide series (such as yttrium and gadolinium) of the periodic table. DOTA N-

hydroxysulfosuccinimide ester is prepared following a known procedure (Lewis M.R., *et al.*, *Bioconjugate Chem.*, 5: 565-576, 1994), by mixing 60 mg (128 μmol) of trisodium DOTA and 27.7 mg (28 μmol) of N-hydroxysulfosuccinimide, in 0.96 ml of water, and incubating this solution with 49 μl of a freshly prepared solution of 'EDC' (50 mg/ml) at 4°C for 30 min. 1 ml of this solution contains 12.68 μmol (theoretical) of the monoactivated DOTA sulfosuccinimide.

An excess of this reagent is reacted with any of the amine-deprotected biotinylating reagent shown in Table 1, and stirred at 4°C for a period of 18-24 hours. The biotinylated DOTA product is purified on a reverse phase preparative HPLC column using acetonitrile-water gradient-elution at a flow rate of 1ml/min and monitoring the eluent with a refractive index detector. The purified material is analyzed by NMR spectroscopy and mass spectrometry.

Example 12.

TARGETING OF STREPTAVIDIN-MAB [SA-IMMU-14] CONJUGATES TO TUMOR XENOGRAFTS

This experiment is performed in order to determine the protein dose of SA-IMMU-14 which is necessary to saturate all available antigen binding sites in a tumor xenograft. Swiss nu/nu mice are injected subcutaneously in the back with sufficient cells of the GW-39 human tumor cell line to produce a solid tumor nodule of 100-200 mg at approximately ten days post-injection of cells. After tumors have grown to a suitable size, animals are split into groups of four to five per group and injected with increasing amounts of I-125-labeled-SA-IMMU-14 corresponding to 10 μg, 50 μg, 100 μg, 250 μg and 500 μg per [approximately] 20 g animal. Animals are anesthetized and euthanized at 24 hours post-injection of the I-125-SA-IMMU-14, and the major internal organs (liver, spleen, lung, kidney, blood and bone) as well as the GW-39 tumor xenograft are excised and counted for radioactivity content. From one experiment, the dose of SA-IgG required

to saturate all available antigen tumor sites, for a tumor of this size, was determined to be about 250 μg of SA-IMMU-14 per animal.

Example 13.

INVESTIGATION OF ANTI-IDIOTYPIC CLEARANCE OF CIRCULATING SA-IMMU-14.

Groups of animals, consisting of four to six nu/nu mice per group bearing GW-39 tumor xenografts produced as described in Example 12, are injected with 250 μg each of I-125-SA-IMMU-14. After 24 hours, groups of animals are given five-fold and ten-fold excesses of the radioidinated (I-131) anti-idiotype antibody WI2 or the radioiodinated (I-131) anti-idiotype antibody WI2 multiply substituted with residues of the monosaccharide D-galactose. A control group of animals is not given either WI2 moiety.

Groups of animals given the WI2 and the galactose-derivatized WI2, at both molar excesses, are taken and sacrificed at 1, 4, 24 and 48 hours post-injection of the anti-idiotype antibody. Organs are obtained as described in Example 12, and counted for radioactivity on a suitable counter through two separate channels, one set for the low energy emitter I-125, and the second channel set to read the high energy emitter I-131. Both antibodies exhibit a rapid clearance from the bloodstream of the I-125-SA-IMMU-14, with I-125-SA-IMMU-14 retained at the tumor xenograft. Other than at the tumor, most I-125 radioactivity is located in the liver, indicating that both species of WI2 at both dose levels are effective at clearing the circulating I-125-SA-IMMU-14. Animals not given clearing mab exhibit normal long-term retention of I-125-SA-IMMU-14 in the blood stream. Significant amounts of non-galactosylated WI2 remain in circulation out to 48 hours post-injection whereas essentially all galactosylated WI2 is cleared into the liver within 1 hour post-injection.

From this work, it is established that a five-fold excess of anti-idiotype antibody is sufficient to clear

all circulating I-125-SA-IMMU-14, and that this amount of clearing mab does not substantially remove I-125-SA-IMMU-14 from the tumor. It is further established that the clearance of the targeting first antibody occurs very rapidly, within 1 hour. Finally, it is established that the galactose-WI2 is cleared from circulation very rapidly whereas the non-galactosylated WI2 circulated for the extended periods typical of a non-derivatized immunoglobulin.

Example 14.

DELIVERY OF INDIUM-111 TO TUMOR XENOGRAFTS USING A PRETARGETING PROTOCOL.

Swiss nu/nu mice bearing GW-39 tumor xenografts, produced as described in Example 12, are injected with I-125-SA-IMMU-14 followed 24 hours later by injection with a five-fold molar excess of galactosylated WI2 anti-idiotype mab. At 3 hours after the injection of the second antibody, three groups of 5-8 mice per group are given an injection of 10 μCi of 2 mCi/mg indium-111-labeled p-5-(biotinamido-pentyl(amino)thioureayl-2-benzyldiethylenetriaminepentaacetic acid (In-111-BPD). At 24 hours after the injection of the second antibody, two groups of eight animals each are given the same dose of In-111-BPD. A further three groups of animals are given In-111-BPD with no prior antibody injections. Animals given each of the three protocols are sacrificed at times corresponding to 1 hour (each protocol), 3 hours (In-111-BPD only protocol), 24 hours (each protocol), and 72 hours (first protocol, animals injected with gal-WI2 three hours after I-125-SA-IMMU-14) post-administration of the In-111-BPD. Collected tissues are again counted through dual windows on a suitable counter set for I-125 and In-111.

Animals given In-111-BPD without prior antibody injections exhibit very rapid clearance of indium from all of the above organs, and within 1 hour post-injection less than 1.8% of the injected dose remain in all organs combined. No tumor localization is seen in this group.

Animals in protocol 1, injected with In-111-BPD three hours after injection of the gal-WI2 show greater tumor accumulation of both I-125-SA-IMMU-14 and In-111-BPD at both common sacrifice times (1 and 24 hour post-administration of In-111-BPD). Although absolute amounts of In-111-BPD in tumors is greater in the animals given the agent 3h after administration of the gal-WI2 clearing mab, tumor/non-tumor ratios in both groups are comparable, with high tumor/blood ratios of In-111 of 5-6:1 and 12-25:1 seen at 1 hour and 24 hours post-injection of the In-111-BPD, respectively.

Example 15

CURE OF TUMOR-BEARING NUDE MICE USING A PRETARGETING PROTOCOL.

Swiss nude (nu/nu) mice are injected sub-cutaneously in the back with sufficient cells of the GW-39 human tumor cell line to produce a solid tumor xenograft of 100-200 mg in size at approximately ten days post-injection of the cells. Animals bearing tumors of suitable size are split into five groups of 15-20 each and two of the groups are treated by intravenous injection with a 250 μg (a tumor-saturating) dose of SA-IMMU-14. A third group is given Y-90-IMMU-14, labeled in the normal manner by direct attachment of chelate to the antibody. After 24 hours, the two groups given the SA-IMMU-14 injection are given a five-fold molar excess of galactose-derivatized anti-idiotypic mab to IMMU-14 (gal.-WI2). After a further two hours, the animals in one of the groups of antibody-treated animals and the animals in one of the untreated groups of animals are given 50% of the maximum tolerated dose (determined empirically) of Yttrium-90-BPD.

The five groups of animals thus obtained are then as follows:

Group 1 - Untreated with any reagents.

Group 2 - Animals treated with the two cold antibodies SA-IMMU-14 and gal.WI2.

Group 3 - Animals treated with Y-90-BPD only.

Group 4 - Animals treated with the directly labeled Y-90-IMMU-14 reagent.

Group 5 - Animals treated with all three reagents; SA-IMMU-14, gal.-WI2 and Y-90-BPD.

Animals are tagged and returned to appropriate cages, fed and watered *ad libitum*, and measured for disease status biweekly. Animal weights are recorded and the sizes of the tumor xenografts measured using calipers. Within 6 weeks of implantation all animals in the untreated group (group 1) die due to their rapidly growing tumors. Animals in group 2, given just the cold antibodies, also die within 8 weeks post-injection. Animals in the group given just the Y-90-BPD reagent (group 3) also die this quickly since the isotope is not in the circulation for a long enough period of time to exert a non-specific radiation effect against the tumors. Animals in group 4 show a considerable improvement in overall survival time, out to 18-26 weeks post-implantation. This life extension is obtained at the cost of a considerable loss of weight (15-20% of body weight) in the first two to three weeks post-treatment with the Y-90-IMMU-14. The tumors regress after the treatment but resume their growth patterns after 15-20 weeks post-treatment, and the animals in this group eventually succumb to the tumors. Animals in group 5 are still alive at over 1 year post-treatment with full regression of tumors, no evidence of recurring tumor, and due to the specific nature of the therapeutic radiation delivered without the toxic effects typical of a long-circulating isotope, the animals in this group exhibit minimum weight loss.

Example 16

CURE OF TUMOR-BEARING NUDE MICE USING AN ALTERNATE PROTOCOL.

Swiss nude (nu/nu) mice are injected subcutaneously in the back with sufficient cells of the GW-39 human tumor cell line to produce a solid tumor xenograft of 100-200 mg in size at approximately ten days post-injection of the cells. Animals bearing tumors of suitable size are split into five groups of 15-20 each and two of the groups are treated by intravenous injection with a 250 μg(a tumor-saturating) dose of SA-IMMU-14. A third group is given Y-90-IMMU-14, labeled in the normal manner by direct attachment of chelate to the antibody. After 24 hours, the two groups given the SA-IMMU-14 injection are given a five-fold molar excess of anti-idiotypic mab to IMMU-14 (WI2). After a further two hours, the animals in one of the groups of antibody-treated animals and the animals in one of the untreated groups of animals are given 50% of the maximum tolerated dose (determined empirically) of Yttrium-90-BPD.

The five groups of animals thus obtained are then as follows:

Group 1 - Untreated with any reagents.

Group 2 - Animals treated with the two cold antibodies SA-IMMU-14 and WI2.

Group 3 - Animals treated with Y-90-BPD only.

Group 4 - Animals treated with the directly labeled Y-90-IMMU-14 reagent.

Group 5 - Animals treated with all three reagents: SA-IMMU-14, WI2 and Y-90-BPD.

Animals are tagged and returned to appropriate cages, fed and watered *ad libitum*, and measured for disease status biweekly. Animal weights are recorded and the sizes of the tumor xenografts measured using calipers.

Within 6 weeks of implantation all animals in the untreated group (group 1) die due to their rapidly growing tumors. A few animals in group 2, given just the cold antibodies survive longer than the group 2 animals

from Example 15, due to the induction of an immune response by the long-circulating WI2 mab, but most die within 8 weeks post-injection. Animals in the group given just the Y-90-BPD reagent (group 3) also die quickly since the isotope is not in the circulation for a long enough period of time to exert a non-specific radiation effect against the tumors. Animals in group 4 show a considerable improvement in overall survival time, out to 18-26 weeks post-implantation. This life extension is obtained at the cost of a considerable loss of weight (15-20% of body weight) in the first two to three weeks post-treatment with the Y-90-IMMU-14. The tumors regress after the treatment but will resume their growth patterns after 15-20 weeks post-treatment, and the animals in this group eventually succumb to the tumors. Animals in group 5 are still alive at over 1 year post-treatment with full regression of tumors, no evidence of recurring tumor, and due to the specific nature of the therapeutic radiation delivered without the toxic effects typical of a long-circulating isotope, the animals in this group exhibit minimum weight loss.

Example 17

TREATMENT OF HUMAN CANCER USING A PRETARGETING PROCEDURE.

A patient presenting with a carcinoembryonic antigen (CEA)- producing cancer is treated with a 1 mg dose of I-131-SA-hIMMU-14 [SA-hIMMU14 is a conjugate corresponding to streptavidin-humanized (complementarity determining region-) cdr-grafted version of the IMMU-14 mab], labeled with 5 mCi of I-131 radionuclide (prepared by the iodogen method), in order to determine the localization qualities of the SA-hIMMU-14 conjugate.

With strong positive localization indicated from this initial injection, a 0.5 g dose of the SA-IMMU-14 is then infused over a 1 hour period using a sterile, non-pyrogenic, isotonic solution of the conjugate. After 24 hours post-injection of the targeting antibody conjugate, a 0.5 g first antibody-clearing dose of the galactosylated anti-idiotypic mab, gal.-hWI2, is infused

in a similar manner. Two hours after administration of the hWI2, an infusion of 200 mCi of the therapy agent, Y-90-BPD, dissolved in 200 ml of phosphate buffered saline containing 1 % v/v human serum albumin is begun. The patient is monitored for adverse reactions during the infusion of the isotope. After the infusion is complete, the patient's blood and urine are analyzed and quantified for the presence of radioyttrium out to 48 hours post-injection to determine the amount of isotope in circulation and the amount eliminated via the urine. The patient is observed periodically for the next two years, during which time is seen a complete response to the treatment.

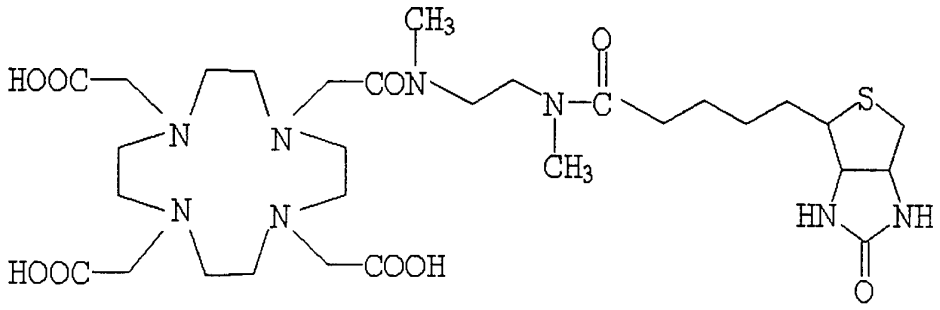
Example 18

TREATMENT OF HUMAN CANCER USING AN ALTERNATE PROCEDURE.

A patient presenting with a carcinoembryonic antigen (CEA)-producing cancer is treated with a 1 mg dose of I-131-SA-hIMMU-14 [SA-hIMMU14 is a conjugate corresponding to streptavidin-humanized (complementarity determining region-) cdr-grafted version of the IMMU-14 mab], labeled with 5 mCi of I-131 radionuclide (prepared by the iodogen method), in order to determine the localization qualities of the SA-hIMMU-14 conjugate. With strong positive localization indicated from this initial injection, a 0.5 g dose of the SA-IMMU-14 is then infused over a 1 hour period using a sterile, non-pyrogenic, isotonic solution of the conjugate. After 48 hours post-injection of the targeting antibody conjugate, a 0.5 g first antibody-clearing dose of the anti-idiotypic mab, hWI2, is infused in a similar manner. Four hours after administration of the hWI2, an infusion of 200 mCi of the therapy agent, Y-90-(2-biotinylmethylamidoethyl) methylamido-DOTA, dissolved in 200 ml of phosphate buffered saline containing 1 % v/v human serum albumin is begun. The patient is monitored for adverse reactions during the infusion of the isotope. After the infusion is complete the patient's blood and urine are analyzed and quantified for the presence of radioyttrium out to 48

hours post-injection, to determine the amount of isotope in circulation and the amount eliminated via the urine. The patient is observed periodically for the next two years, during which time the cancer exhibits a full response to the treatment over this extended period of time.

(2-biotinylmethylamidoethyl)methylamido-DOTA:



It will be apparent to those skilled in the art that various modifications and variations can be made to the processes and compositions of this invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

What is claimed is:

1. Use of a composition comprising a clearing agent which binds to a primary binding site of a primary targeting species, in the preparation of an agent for use in an *in vivo* pretargeting method for delivering a diagnostic or therapeutic agent to a target site in a mammal,

wherein, in said method, a primary targeting species is administered to a mammal, which binds via a primary, target-specific binding site to a target site or to a substance produced by or associated with a target site and which comprises a second binding site which binds an active agent conjugate or to an intermediate which in turn binds an active agent conjugate, sufficient time being allowed for the primary targeting species to localize at the target site;

said composition is then administered, sufficient time being allowed for the clearing agent to clear the primary targeting species from circulation, wherein the clearing agent binds to the primary binding site of the primary targeting species, whereby substantially only non-localized primary targeting species is cleared and targeted primary targeting species is not removed from the target site, nor is the second binding site of the primary targeting species blocked by the clearing agent; and

an active agent conjugate, comprising a moiety that binds to the second binding site of the primary targeting species conjugated to a diagnostic or therapeutic agent, is then administered to the mammal, sufficient time being allowed for the conjugate to localize at the target site.

2. The use of claim 1, wherein said clearing agent comprises pendant terminal sugar residues that selectively bind to the hepatic asialoglycoprotein receptor, whereby hepatic clearance is effected substantially in a single pass.

3. The use of claim 2, wherein said sugar residues are galactose residues.

4. The use of claim 1, wherein said primary targeting species is a conjugate of an antibody or antibody fragment and avidin, and said active agent conjugate is a conjugate of biotin and said diagnostic or therapeutic agent.

5. The use of claim 1, wherein said primary targeting species is a conjugate of an antibody or antibody fragment and biotin, and said active agent conjugate is a conjugate of avidin and said diagnostic or therapeutic agent.

6. The use of claim 1, wherein said primary targeting species is a conjugate of an antibody or antibody fragment and biotin, and said active agent conjugate is a conjugate of biotin and said diagnostic or therapeutic agent; and wherein, in said method, avidin is administered to said mammal after localization of said primary targeting species.

7. The use of any one of claims 4-6, wherein said clearing agent is an anti-idiotypic antibody.

8. The use of claim 1, wherein said active agent conjugate comprises a diagnostic agent.

9. The use of claim 8, wherein said diagnostic agent is selected from the group consisting of radionuclides, paramagnetic ions and fluorescence-emitters.

10. The use of claim 1, wherein said active agent conjugate comprises a therapeutic agent.

11. The use of claim 10, wherein said therapeutic agent is a radionuclide or neutron-capturing boron addend.

12. The use of claim 10, wherein said therapeutic agent is selected from the group consisting of drugs, toxins, fluorescent dyes, hormones, hormone antagonists, receptor antagonists, enzymes, proenzymes, autocrines and cytokines.

13. The use of claim 10, wherein said therapeutic agent is selected from the group consisting of anti-DNA, anti-RNA, radiolabeled oligonucleotides, anti-protein, anti-chromatin, cytotoxic agents and antimicrobial agents.

14. The use of claim 1, wherein said primary targeting species is a non-antibody primary targeting species which preferentially binds marker substances that are produced by or associated with said target site.

15. The use of claim 1, wherein said primary targeting species is selected from the group consisting of small peptides, steroids, hormones, cytokines, and neurotransmitters.

16. The use of claim 15, wherein said clearing agent comprises an antibody.

17. In an *in vivo* pretargeting method for delivering a diagnostic or therapeutic agent to a target site in a mammal, wherein in said method,

a primary targeting species is administered to said mammal which binds via a primary, target-specific binding site to the target site or to a substance produced by or associated with the target site and which comprises a second binding site which binds an active agent conjugate or to an intermediate which in turn binds an active agent conjugate, sufficient time being allowed for said primary targeting species to localize at the target site;

a clearing agent is then administered that binds to said primary targeting species, sufficient time being allowed for said clearing agent to clear said primary targeting species from circulation; and

an active agent conjugate, comprising a moiety that binds to said second binding site of said primary targeting species conjugated to a diagnostic or therapeutic agent, is then administered to said mammal, sufficient time being allowed for said conjugate to localize at said target site,

the improvement wherein said clearing agent binds to said primary binding site of said primary targeting

species, whereby substantially only non-localized primary targeting species is cleared and targeted primary targeting species is not removed from said target site, nor is said second binding site of said primary targeting species blocked by said clearing agent.

INTERNATIONAL SEARCH REPORT

Int. tional application No.
PCT/US96/08696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395; G01N 33/53
US CL :424/131.100, 178.1; 435/7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/131.100, 178.1; 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO, A1, 89/10140 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED) 02 November 1989, pages 33-38.	1-3, 12, 14, 16, 17 --- 1-17
X, P --- Y, P	US, A, 5482698 (GRIFFITHS) 09 January 1996, see entire document.	1, 4-6, 11-13, 15-17 --- 1-9, 11-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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