(12) UK Patent Application (19) GB (11) 2 122 641 A

(21) Application No 8315483
(22) Date of filing 6 Jun 1983
(30) Priority data
(31) 386110
386109
(32) 7 Jun 1982
7 Jun 1982
(33) United States of America (US)
(43) Application published 18 Jan 1984
(51) INT CL³
C12P 1/00 A61K 43/00
(52) Domestic classification
C6F HA2
A5B 180 28X 28Y 317 31X 31Y 38Y 394 39X H
G6R 2
(56) Documents cited
GBA 2109407
(58) Field of search
C6F

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(54) Metal chelate conjugated monoclonal antibodies, wherein the metal is an α emitter

(57) Methods of manufacturing and purifying metal chelate conjugated monoclonal antibodies are described, wherein the chelated metal emits alpha radiation. The conjugates are suited for therapeutic uses being substantially free of nonchelated radiometal.

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SPECIFICATION

Metal chelate conjugated monoclonal antibodies

TECHNICAL FIELD

This invention relates generally to metal chelate conjugated monoclonal antibodies. This invention also relates to a method for treating cellular disorders, particularly cancer, which employs a radiometal chelate conjugated monoclonal antibody.

BACKGROUND OF THE INVENTION

Effective therapeutic methods for the treatment of cellular disorders such as cancer have been the object of intensive research. Conventional therapy employs surgery, radiation and chemotherapy. Each of these methods suffers a serious drawback in that it is not highly selective between healthy and cancerous cells. In order to be effective, these methods kill or remove large amounts of healthy tissue. Furthermore, chemotherapy adversely affects the immune system so that death or serious illness often arises from fungal, bacterial or viral infections.

The development of monoclonal antibodies has opened the possibility of selectively delivering therapeutic agents or diagnostic agents to specific target cells. Monoclonal antibodies are immunoglobulins of well-defined chemical structure. A characteristic feature of monoclonal antibodies is reproducibility of function and high specificity.

Radioiodine bound directly to monoclonal antibodies has been used for diagnosis and therapy. Iodine-131 has had some therapeutic success for large tumours, but radioiodine labeled antibodies have been ineffective in the treatment of small tumor foci or metastases. In addition, specifically bonded antibodies are relatively rapidly catabolized by the target cell. Catabolism, therefore, leads to the incorporation of metabolized iodine in the excretory organs, i.e., kidney, bladder and stomach. In addition, attempts to transport toxins via monoclonal antibodies to tumor cells have not resulted in a successful therapeutic method.

It has been suggested in the literature that diethylenetriaminepentaacetic acid (DTPA) can form stable metal complexes when attached to protein. Krejcarek et al., *Biochem. & Biophys. Res. Commun.* 77:581 (1977). Imaging of target sites *in vivo* with radiometal-DTPA conjugated polyclonal antibodies prepared according to the method of Krejcarek have been reported by Khaw et al., *Science 209*:295 (1980). Despite separation, by gel chromatography and dialysis, of free and chelated metal from metal chelate conjugated polyclonal antibodies the gamma images included in the article show that a high proportion of the radiometal localized in the liver.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide improved therapeutic methods.

It is another object of the present invention to provide an effective method of treating cellular disorders employing monoclonal antibodies.

It is a further object of the present invention to provide a method of selectively targeting lethal doeses of radiation to diseased cells which causes little or no destruction of healthy cells.

It is also an object of the present invention to provide a method of selectively treating small tumor foci and metastases.

It is another object of the present invention is to provide a method of introducing selectively targeted radiometals *in vivo* which avoids appreciable radiometal incorporation into healthy organs of the body.

In one of its aspects, the present invention provides a method of treating cellular disorders comprising contacting a target cell with radiometal chelate conjugated monoclonal antibodies wherein said radiometal is an alpha particle emitting metal nuclide. In another embodiment, the present invention contemplates a method comprising introducing into body fluid metal chelate conjugated monoclonal antibodies wherein said conjugated chelate is a derivative of diethylenetriaminepentaacetic acid, said conjugate being substantially free of adventitiously bound ions of said metal and retaining substantially all of the activity and selectivity of the antibody. Such a technique is suitable for both diagnostic and therapeutic purposes. The present invention also provides a method for producing a metal chelate conjugated monoclonal antibody of an alpha emitting radiometal wherein the metal chelate conjugated antibody is passed through a chromotography column having one or more layers selected from the group of ion retardation resins, anion exchange resins, cation exchange resins and a chelating ion exchange resin, and a final layer comprising a sizing matrix.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employes metal chelate conjugated monoclonal antibodies for therapeutic techniques, particularly *in vivo*.

This invention also provides metal chelated conjugated antibodies which retain their biological activity and specificity, and which are substantially free of adventitiously bonded metals. Adventitiously bonded metals are not stable and result in free metal entering the blood. Metals which are released in the blood can be bound by transferrin or other metal binding protein (e.g., ferritin) which are present in

blood. Such bound metals are retained in the circulatory system for considerable periods of time and are cleared by the reticuloendothelial system (RES). Such clearance results in a concentration of the metal in the liver and spleen. It is apparent that random, long term circulation of radioactive metals in the body or concentration of radioactive metals in the liver and spleen are highly undesirable. The practice of this invention can alleviate these serious problems.

Monoclonal antibodies are immunoglobulins of well-defined chemical structure, in contrast to polyclonal antibodies which are heterogeneous mixtures of immunoglobulins. A characteristic feature of monoclonal antibodies is reproducibility of function and specificity, and such antibodies can be and have been developed for a wide variety of target antigens, including tumor cells. Methods for obtaining monoclonal antibodies have been extensively discussed and are well-known in the art. A useful text is *Monoclonal Antibodies* (R. H. Kennett, T. J. McKearn & K. B. Bechtol eds. 1980). *See also* Koprowski et al. U.S. Patent 4,196,265. The selection of a monoclonal antibody for the practice of this invention will depend upon the end use for which the metal chelate conjugated monoclonal antibody will be employed. Such selection is within the skill of the art.

The antibodies are generally maintained in an aqueous solution that contains an ionic compound. A physiologic normal saline solution is very often employed and is widely available. Other ionic solutions, such as those containing sodium or potassium phosphate, sodium carbonate and the like, are known in the art and may also be employed.

A wide variety of organic chelating agents or ligands can be conjugated to monoclonal antibodies. Organic ligands to be conjugated to monoclonal antibodies may be chosen from among either the natural or synthetic amines, porphyrins, aminocarboxylic acids, iminocarboxylic acids, ethers, thiols, phenols glycols and alcohols or the polyamines, polyaminocarboxylic acids, polyiminocarboxylic acids, aminopolycarboxylic acids, iminopolycarboxylic acids, nitrilocarboxylic acids, dinitrilopolycarboxylic acid, polynitrilopolycarboxylic acids, ethylenediaminetetracetates, diethylenetriaminepenta or tetracetates, polyethers, polythiols, cryptands, polyetherphenolates, polyetherthiols, ethers of thioglycols or alcohols, polyaminephenols, all either acyclic, macrocyclic, cyclic, macrobicyclic or polycyclic, or other similar ligands which produce highly stable metal chelates or cryptates. Obviously, the choice of the ligand depends upon the metal to be chelated and is within the skill of the art.

The ligand used in certain embodiments of this invention possesses a nonmetal bonded organic functional group suitable for bonding to the monoclonal antibody. Functional groups may be chosen from among the carboxylic acid groups, diazotiazable amine groups, succinimide esters, anhydrides, mixed anhydrides, benzimidates, nitrenes, isothiocyanates, azides, sulfonamides, bromoacetamides, iodoacetamides, carbodiimides, sulfonylchlorides, hydrazides, thioglycols, or any reactive functional group known in the art as a biomolecular conjugating or coupling agent.

The present invention preferably employs a derivative of diethylenetriaminepentaacetic acid (DTPA). It has been found that DTPA ligands tightly bind metal ions and that the DTPA derivative (hereinafter referred to as chelate) forms a chelate conjugated monoclonal antibody that is highly stable, both with respect to the metal chelate binding and with respect to chelate-antibody conjugate. These properties are of great importance, particularly for *in vivo* applications. For example, if the chelate releases the metal ion after introduction into the blood, these ions will tend to be bound by transferrin, or the like, and be distributed generally in the circulatory system of the body. Moreover, the ions will ultimately tend to collect and remain in organs such as the liver and spleen. These effects can have serious consequences depending on the toxicity of the metal and its radioactivity. Furthermore, if the chelate does not form a highly stable conjugate with the antibody, there is a significant reduction in the amount of metal delivered to the target site and a corresponding decrease in efficacy.

In the preparation of the metal chelate conjugated monoclonal antibodies of the present invention, it is important to avoid metal contamination from outside sources. Labware should be plastic or glass cleansed of exogenous metal. All stock solutions should be metal depleted by, for example, column chromotography with a suitable resin.

For ease of presentation, the present invention will be described with respect to the DTPA chelate. The preferred chelate is prepared from an amine salt of DTPA. Amine is used broadly and includes primary, secondary and teriary amines that will completely deprotonate the DTPA. Selection of an appropriate amine is within the skill of the art and the efficacy of any amine (including ammonia) can readily by determined. A particularly preferred amine is triethylamine. At least about 5 equivalents of the amine is added to an aqueous solution of DTPA and warmed to complete the reaction. The reaction produces a pentakis(amine)DTPA salt according to the following equation wherein triethylamine is the amine:

$$5\,(CH_3CH_2)_3N + DTPA \rightarrow [(CH_3CH_2)_3N]_5DTPA$$

Solid DTPA-amine salt can be recovered by evaporating or freeze-drying the solution to remove the water and excess amine.

The actual chelate is a DTPA derivative. A functional group is added to the DTPA and the DTPA is bonded through it to amine groups on the monoclonal antibody. Esters of a haloformic acid are reacted with the DTPA-amine salt to make the chelate employed by the present invention. By ester of a

haloformic acid is meant an ester of the general formula XC(O)—O—R wherein X is a halogen, preferably a chloride, and R is any suitable functional group, preferably containing not more than about 6 carbon atoms. The selection of R and X is within the skill of the art taking into consideration the stability of the chelate and steric hinderance when the chelate is reacted with the monoclonal antibody. A preferred ester is isobutylchloroformate.

In an exemplary preparation, approximately equimolar amounts of haloformic acid ester and DTPA-amine salt are dissolved in a polar organic solvent such as pure, dry acetonitrile. Excess of the halomformic acid ester should be avoided because it will block a metal chelation site on the modified DTPA ligand. The temperature of the reaction is generally not critical and can be chosen to provide a salt that is either partially or substantially precipitated. The reaction preferrably is carried out at a temperature low enough to precipitate substantially all of the haloamine salt by-product of the reaction. When the amine employed is triethylamine and the ester is an ester of chloroformic acid, the temperature should be in the range of from about −20°C to about −70°C. Maintaining the temperature in this range drives the equilibrium reaction to the right, producing a high yield of a mixed carboxycarbonic anhydride of DPTA according to the following equation:

$$X—C(O)—O—R + [(CH_3CH_2)_3N]_5DTPA \rightleftharpoons$$

$$[(CH_3CH_2)_3N]_4DTPA—C(=O)—O—C(=O)—O—R + [(CH_3CH_2)_3NH]\,X$$

By carrying out the above reaction in the temperature range specified, a high concentration of the chelate can be produced substantially free of the haloamine salt by-product. For example, approximately 0.25 mM of the pentamkis(triethylamine)DTPA salt can be dissolved in 0.5 ml acetonitrile and reacted with 35 microliters of isobutylchloroformate. After approximately 45 minutes at −70°C, the solution can be centrifuged to remove the precipitate leaving a supernatant liquid containing the desired chelate at a concentration of about 0.5 M. The chelate is desirably introduced into the chelate-antibody conjugation reaction at a concentration of at least about 0.25 M in the organic solvent. Such concentrations of chelate permit the use of relatively small amounts of organic solvents in the conjugation reaction mixture. Excessive amounts of organic solvent in the reaction mixture should be avoided because the solvent can produce adverse effects with respect to the biological activity and specificity of the antibody.

The chelate conjugated monoclonal antibody is formed by adding the chelate in the organic solvent to an aqueous saline antibody solution. It is important to carry out the reaction of the modified DTPA and antibody at a pH not higher than about 7.2. The chelate-antibody reaction competes with the decomposition of the chelate caused by its reaction with water. If the pH is too low, however, the chelate undergoes acid catalysed decomposition and the biological activity and specificity of the antibody is diminished. The pH is desirably in the range of from about 6.0 to about 7.2, preferably as close to 7.0 as practicable. In this range, the reaction of the DTPA chelate with water is less detrimental to the chelate-antibody reaction.

While the above discussion has focused on DTPA, it is within the skill of the art to form conjugates employing other ligands. *See, e.g.,* 73 *Proc. Natl. Acad. Sci. USA* 3803 (Nov. 1976).

To preserve the maximum biological activity of the antibody, the use of strong acids or bases to adjust pH should be avoided for any chelate-antibody preparation. Use of a strong acid or base can cause localized denaturation int he solution. The pH can be controlled in the aqueous solution of monoclonal antibody by including a suitable buffer. For example, $NaHCO_3$ at a concentration of approximately 0.1 M can be used. Other buffers such as MES (2-(N-morpholino)ethane sulfonic acid) are known in the art and may also be employed. The choice of an appropriate buffer is within the skill of the art.

When the chelate solution is added to the aqueous antibody solution, both should be at about 0°C. The temperature of the solution generally should not be allowed to rise above about 4°C during the course of the reaction. Use of temperatures in the range of about 0 to about 4°C tends to avoid decomposing the antibody and also reduce chelate decomposition. Duration of the reaction is not critical so long as the reaction is permitted to go to completion and the solution may be left in the cold overnight.

The chelate-to-antibody mole ratio may vary widely depending upon the use for which the conjugate is intended. The mole ratio of chelate-to-antibody can broadly range from about 0.1 to about 10 or higher and preferably from about 0.25 to about 5. In many instances the mole ratio of chelate to antibody will range from about 0.5 to about 3.

In general an excess of chelate is employed in the reaction because the chelate will decompose to some extent in the aqueous solution. The number of chelates bound per molecule of antibody will be a function of both the concentration of the chelate and the concentration of the antibody in the reaction

mixture, with high concentrations tending to provide more chelates per antibody. If the amount of antibody employed is relatively small and a relatively dilute solution is employed, a substantial excess of chelate may be required. For example, a molar excess of approximately 600:1 of chelate may be required to react with an antibody solution having a protein concentration of about 5 to 10 mg per ml in order to provide approximately 1.5 chelates bonded per molecule of antibody. Molar excesses as low as 100:1 can be employed, however, and still produce an average of about 0.5 chelates bonded per molecule of antibody. Adding too many chelate molecules per antibody molecule can reduce the biological activity and specificity of the antibody.

When the addition of the chelate to the antibody has gone to completion, substantial amounts of decomposed chelate may be present in the solution. This can occur for any chelate-antibody conjugate. The decomposed chelate should be removed while retaining the biological activity and specificity of the antibody. Dialysis or chromatography can, for example, be employed. If desired, a first dialysis against dilute ascorbic acid and EDTA solution to remove any residual iron which may be present in the chelate or the protein. A purified chelate conjugated antibody can be produced by dialysis of the reaction mixture over a 48 hour period against three 1 liter changes of an aqueous solution at about 4°C and a pH of about 6 containing 50 mM citrate and 200 mM sodium chloride with 1 ml Chelex 100 resin (Bio-Rad) in the dialysis vessel. A final dialysis into 1 liter of solution containing 10 mM MES and 200 mM sodium chloride at 4°C and pH 6 completes purification of the protein. Variations of dialysis procedures are known and are within the skill of the art.

Metal chelation is carried out in an aqueous solution and, once again, desirably avoids the use of strong acids or bases. Metal chelation for any chelate-antibody conjugate is carried out at a pH which does not significantly reduce the biological activity or specificity of the antibody. Generally, the acceptable range is from about pH 3.2 to about pH 9, however, particular antibodies may have to be restricted to a narrower range. At a pH below about 3.5, adventitious binding of metal ions to antibodies is substantially impaired for many metals. A preferred range, therefore, is often from about pH 3.2 to about pH 3.5. Factors peculiar to solutions of the metal employed, however, may permit a pH above 3.5. The selection of the appropriate pH within the range is within the skill of the art.

In the present invention, a weakly chelating acid or base is desirably employed as a buffer. Citric acid or glycine are useful buffers. Still other buffers are, of course, known in the art. The present invention contemplates a solution of chelate conjugated antibodies adjusted to the desired pH with a weakly chelating acid or base buffer and without the addition of a strong acid or base. To this solution is added a metal salt. If the metal salt is in solution, that solution also has its pH adjusted with a chelating buffer. The pH of the metal solution, however, can be adjusted with strong acids or bases prior to its addition to the chelate conjugated antibody solution.

Any acceptable metal salt can be employed to make the metal chelate conjugated monoclonal antibodies. Typical salts may include halides (e.g., chlorides), nitrates, perchlorates, or the like. The metal salt is employed in as high a concentration as is practicable. Radiation exposure of individuals handling the preparation will generally set a limit below one equivalent metal per chelate binding site.

The duration of the reaction is not critical unless the pH is near the outside limits of pH acceptable to the antibody. At or near such pH limits, the reaction times generally should be under about 1 hour and preferably about 30 minutes or less. Indeed, from the standpoint of economy of time, reaction times generally within these periods are desired. It is also preferred to carry out the chelation in the presence of a water soluble, nonchelatable, biologically innocuous reducing agent, such as ascorbic acid, to prevent trace iron from being chelated. The reaction is usually completed by adding trisodium citrate in sufficient quantity so that the solution pH is raised to a point that the metal conjugate is no longer labile. It has been determined that most DTPA complexes are at especially stable at a pH of about 6. Other weak bases, or acids when the reaction is above pH 6, may be used so long as they do not adversely affect the antibodies. Their selection is within the skill of the art.

The reaction solution will, generally require purification prior to its use *in vivo*, and may also require purification prior to *in vitro* use. Nonbonded metal and adventitiously bonded metal should be removed. The discussion herein refers to adventitiously bound metal ions. Some of the metal, however, may be insecurely held by the chelates and acts in the same manner as adventitiously bound metal ions.

When a radioactive metal is employed which has a short half life, it is especially important that the purification step be as expeditious as possible. The present invention contemplates a relatively fast purification by use of chromatography and this facet of the invention is applicable to chelate-antibody conjugates in general. By employing one or more ion exchange, retardation or chelating resins in conjunction with a sizing matrix (e.g., gel) the metal chelate conjugated monoclonal antibodies of the present invention can be quickly and thoroughly purified.

Different ion exchange resins can be employed singly, or any combination of an ion retardation resin, a cation exchange resin, an anion exchange resin or a chelating ion exchange resin can be employed. The selection of an appropriate resin or resins, their extent of class-linkage, chemical form and mesh size is within the skill of the art.

Cation exchange resins employed in the present invention frequently are strongly acidic polystyrene gel-type resins (e.g., Dowex 50WX8) or other non-polystyrene strongly acidic resins such as Zeocarb 215 (Permutit Co.). Additional suitable acidic resins can include weakly acidic gel polystyrene

resins, macroporous gel polystyrene resins, or macroreticular carboxylic acid cation exchange resins. Anion exchange resins can include strongly basic polystyrene gel-type resins (e.g., Dowex 1X8) or other less basic resins such as pyridinium polymer-type and phenolic polyamine-type resins. Chelating resins may be Chelex 100, or any resin of the type which is a styrene divinyl benzene copolymer containing paired imminodiacetate ions (e.g., Dowex A-1). Useful retardation resins include those containing paired anion and cation exchange sites (e.g., Bio-Rad AG 11-A8). These resins are usually made by polymerizing acrylic acid inside a strongly basic resin such as one having quaternary ammonium groups in a styrene-divinyl benzene copolymer lattice. The above discussion includes only representative examples of each resin; still other resins are also known in the art. A compendium of commercially available resins with brief descriptions of their properties and applications is contained, *inter alia*, in Bio-Rad Laboratories, 1982 Price List H. The choice and combination of resins will depend upon the particular separation problem encountered and is within the skill of the art in view of the disclosure herein. A useful reference is J. Khym, *Analytical Ion-Exchange Procedures in Chemistry and Biology* (1974).

Sizing matrices are also well known in the art. These include polyacrilamides, agraroses, polysaccharides or the like. A particularly useful sizing matrix is a polysaccharide gel (e.g., Sephadex G-50 gel). Examples of polyacrilamide gels are the Bio-Gel P Series (Bio-Rad Labs). The choice of the sizing matrix will depend upon the protein to be purified and is within the skill of the art.

In the practice of this invention the various resins can be established as layers within a column and the solution to be purified can be fed either downwardly through the column or upwardly through the column. Downward feed is a preferred laboratory technique when radioactive compounds are employed because gravity flow requires little or no auxiliary equipment or instrumentation. The choice of bed heights, flow rates, and the like are easily within the skill of the art.

It appears that highly charged metals are adventitiously bound, at times, by the antibody at ionic sites along the surface of the protein. At other times adventitiously bound metals appear to be included with the folds of the antibody protein. These metals can be released into solution but also can be reabsorbed from the solution in an equilibrium-type process. The retardation or ion exchange resins employed in the purification of this invention are used in order to shift the equilibrium and permit metals to be removed from the antibody. For example, as the antibody passes through an ion retardation resin, the passage of metal ions in the solution is slowed, but the protein is not. Adventitiously bonded, highly charged (+3 or higher), metal ions are then released into the solution to reestablish equilibrium. As those ions are released into solution, however, they in turn are retarded by the resin to cause a continuing metal ion release by the antibody.

A level of ion exchange resin may be employed below the ion retardation resin to tightly bind the separated, highly charged ions and to continue the separation process. As the resin depletes the protein solution of free, highly charged ions, equilibrium is again reestablished between free and adventitious metal ions. However, throughout this process, metal ions inside the chelate are retained with the antibody.

It has been determined, however, that mere use of an ion retardation or ion exchange resin is not satisfactory to provide an effective removal of substantially all adventitiously bound metals. In order to complete the purification a sizing matrix is employed. The antibody solution which enters the matrix is already partially depleted in free, highly charged metal ion content. In the sizing matrix, further depletion occurs. As the solution moves through the matrix, the antibodies are not retarded while the ions are. The resulting solution taken off from the sizing matrix is substantially free of adventitiously bound metals. Such loosely bound metals can be reduced to not more than about six percent of the total metal content of conjugate so that at least about 94% of the metal carried by the conjugate is bound by the chelate stably. Desirably, at least about 97% of the total metals bound by the chelate. It is possible to obtain metal levels in which 98% or more of the metal is bound by the chelate. Dialysis can be employed to determine stably bound metal content.

A preferred method of purification is an ion retardation resin (Bio-Rad AG 11-A8) over a cation exchange resin (Bio-Rad AG 50WX8) and a gel (Pharmacia Sephadex G-50). In the purification of technicium chelate conjugated monocloncal antibodies for the present invention, the preferred column contains an ion retardation resin (Bio-Rad AG 11-A8) over a cation exchange resin (Bio-Rad 50WX8) over an anion exchange resin (Bio-Rad AG 1X8) and a sizing matrix gel (Pharmacia Sephadex G-50).

In the process of this invention, the antibody is retained in nonaggregated form. Aggregation of antibodies, whether by clumping or by cross-linking, results in a loss of antibody specificity which, of course, is undesirable. Aggregation can be caused by excessively high concentrations of antibodies in a carrier, or by contact with chemicals that cause protein cross-linking such as, for example, carbodiimdes. Standard sedimentation tests, size matrixing, or the like, can be employed to determine if the antibodies have aggregated. Indeed, the antibody specificity tests discussed herein will reflect aggregation as a loss of specificity.

The activity and specificity of the conjugated antibody products of this invention are maintained at a level of at least about 80%, and preferably at least about 90% of the activity and specificity of the antibody that was employed to produce the conjugate. Particularly preferred solutions are characterized by antibody activity and specificity of at least about 95% and, indeed, products have been produced

which retain the activity and specificity of the original antibody virtually unchanged. The activity and specificity of antibodies are routinely measured in the art by binding of antibodies, *in vitro*, to an epitope. The degree of activity and specificity of the final antibody product can readily be determined simply by repeating the initial test with the final conjugated product.

The invention contemplates an *in vivo* therapeutic procedure in which radiometal chelate conjugated monoclonal antibodies are introduced into the body and allowed to concentrate in the target region. There are a variety of radiometal isotopes which form stable DTPA complexes and emit cytotoxic alpha particles. The therapeutic effect occurs when the conjugates are near or in contact with and bind to the targeted cells. Cell death, it is believed, is a direct or indirect result of the radiation event of the radiometal which is positioned in close proximity to the cell.

The benefits of this aspect of the invention are several. First, the high specificity of the conjugated monoclonal antibody minimizes the total radiation dosage. Only enough radiation for the target cells need be employed. In addition, radiometal chelates generally are cleared rapidly from the body should the conjugated antibody be disrupted. The isotope can be short-lived and the affinity constant by which the isotope is retained in the DTPA chelate is very high resulting in a stably bound metal. Finally, since the the amount of radiometal employed is minimized, the radiation hazard to persons preparing and administering the radiometal chelate conjugated antibody is significantly reduced.

Because of the properties of the DTPA radiometal chelate conjugated monoclonal antibody employed by the present invention, tissue damage or whole body dose during therapy are markedly reduced as compared to that from presently employed methods of radiation therapy such as isotope implants, external radiation therapy, and immunoradiotherapy employing iodine-131 labeled polyclonal or autologus antibodies. Additionally, both biological and physical half-lives of the targeting radiobiological may now be controlled, minimizing whole body radiation effects. Since radiation is targeted specifically to cell types (e.g., neoplastic cells) a therapeutic dose is delivered specifically to malignant cells, either localized or metastasized. The ability of radiometal chelate conjugated monoclonal antibody to provide an effective dose of therapeutic radiation specifically to metastasized cells is also unique and singularly useful for cancer therapy.

The present invention employs the metal chelate conjugated monoclonal antibody containing an alpha emitting radiometal to treat cellular disorders. it is desirable in most applications that the radiometal have a half-life of less than about 4 days and decay rapidly to a stable isotope once the alpha particle is emitted. The preferred isotopes employed in the present invention are bismuth-211, bismuth-212, bismuth-213 and bismuth-214. Bismuth-212, with a half-life of 60.6 minutes, is particularly preferred.

The monoclonal antibody employed is specific for the diseased cell which is to be killed. Cell death is caused by decay of the radiometal and can occur in one of two ways. First, if the alpha particle is emitted in the direction of the diseased cell, a single hit in the cell nucleus can be cytotoxic. The isotope to which the radiometal decays after emitting the alpha particle is ejected from the chelate on a trajectory opposite that of the alpha particle. The bound cell, therefore, can still be hit even when the alpha particle is emitted on a trajectory away from the cell. A single hit in the cell membrane by the decayed isotope can cause irreparable cell injury leading to cell death. The relatively high effectiveness of the alpha particle means that less radioactive material can be employed. Selectivity of the monoclonal antibody and the short range (a few cell diameters) of the alpha particles minimizes the destruction of healthy tissue on a cellular level.

Bismuth-212 decays by one of two different pathways. Approximately 64% of the bismuth-212 decays via beta emission to polonium-212 which has a half-life of 0.3 microseconds. The polonium-212 decays to stable lead-208 after emitting an alpha particle with a range of approximately 90 microns. The other 36% of the bismuth-212 decays to thallium-208 by emitting an alpha particle with a range of approximately 35 to 50 microns. The thallium-208, with a half-life of 3 minutes, then decays via beta emission to stable lead-208.

Generators for Bi-212 have been described in the literature by Gleu, et al., *Z. Anorg. Alleg. Chem. 290*:270 (1957), and by Zucchini, et al., *Int. J. Nucl. Med. & Biol.* (June, 1982), (the abstract of the manuscript was distributed at the August, 1981, ACS meeting in New York). A useful generator consists of Th-228 in the tetravalent state absorbed on a 3 x 5 mm bed of sodium titanate contained in a quartz column above a coarse fritted glass disc sealed in the column. The titanate tightly retains both Th-228 and its Ra-224 daughter. When water is passed through the titanate, the Rn-220 daughter of the Ra-224 isotope dissolves into the water and passes through the fritted disc and is collected in a 10 cc glass reservoir filled with water. The aqueous Rn-220 solution flows from the reservoir into a 10 mm diameter column containing approximately 1 ml of a strongly acidic ion exchange resin such as Bio-Rad AG-50 WX8 cation exchange resin. Rn-220 decays substantially within 5 minutes in the reservoir to Pb-212 which is absorbed upon passage through the resin. At flow rate of approximately 1.5 ml/min through the resin, about 85% of the Pb-212 produced is collected in the column where it decays to its Bi-212 daughter.

When the desired amount of Bi-212 has been formed on the resin, it may be eluted by acid according to procedures entirely familar to those skilled in the art. A useful method of elution for both Pb-212 and Bi-212 is to pass 5 ml of 2 N HCl through the resin. Alternatively, if only Bi-212 is desired,

1.5 ml of 0.5 M HCl can be passed through the resin.

While metals that emit beta particles or Auger electrons can be employed for therapy, alpha emitting radiometals are preferred for several reasons. First, alpha nucleotide radiation characteristically has a short range in tissue and a very high linear energy transfer vis-a-vis beta or Auger radiation. Alpha radiation can kill a cell with only one hit to the nucleus and will kill substantially any cell with 10 hits or less. In addition, the decay also emits an isotope (e.g., T1-208 or Pb-208 in the case of Bi-212) which can also cause cell death. The range of alpha particles is usually less than about 150 microns in tissue. In contrast, beta and Auger particles require hundreds of hits in the nucleus before causing cell death and have ranges in tissue on the order of tenths of millimeters to centimeters. When employing beta particles, a higher dose is required and the decay of substantially more radiolabeled antibodies will be needed to achieve cell death. Thus, specifically bound antibodies will be catabolized releasing beta emitting radiometals into the blood. Alpha-emitting radiometals kill relatively quickly so that fewer antibodies are catabolized.

The metal chelate conjugated antibodies of this invention can be administered *in vivo* in any suitable pharmaceutical carrier. As noted earlier, a physiologic normal saline solution can appropriately be employed. Often the carrier will include a minor amount of carrier protein such as human serum albumin to stabilize the antibody. The concentration of metal chelate conjugated antibodies within the solution will be a matter of choice. Levels of 0.5 mg per ml are readily attainable but the concentrations may vary considerably depending upon the specifics of any given application. Appropriate concentrations of biologically active materials in a carrier are routinely determined in the art.

The effective dose of radiation or metal content to be utilized for any application will also depend upon the particulars of that application. In treating tumors, for example, the dose will depend, *inter alia*, upon tumor burden, accessability and the like. Somewhat similarly, the use of metal chelate conjugated antibodies for diagnostic purposes will depend, *inter alia*, upon the sensing apparatus employed, the location of the site to be examined and the like. In the event that the patient has circulating antigen in addition to those located at the site, the circulating antigens can be removed prior to treatment. Such removal of antigens can be accomplished, for example, by the use of unlabeled antibodies, or by plasmaphoresis in which the patient's serum is treated to remove antigens.

The following examples are included to better illustrate the practice of this invention. These examples are included for illustrative purposes only and are not intended in any way to limit the scope of the invention.

EXAMPLE I

One hundred miligrams of DTPA was weighed into a flask and to this was added 1 ml of water. This solution was reacted with 0.125 g redistilled triethylamine. The reaction solution was warmed to complete the reaction and a solid product was collected by freeze drying.

The freeze dried solid was dissolved in 0.5 ml of pure, dry acetonitrile and 35 ul isobutylchloroformate added at a temperature of approximately −20°C and brought down to about −70°C. After about 45 minutes, the solution was centrifuged in an Eppendorf vial. The supernatant liquid was collected which contained the desired mixed carboxycarbonic anhydride of DTPA at a concentration of approximately 0.5 M.

The monoclonal antibody employed was designated 103A5 and was obtained by fusing P3X63Ag8 mouse myeloma cells with the isolated spleen cells of C56B1/6 mice which had been immunized with purified retrovirus glycoprotein of 70,000 daltons (gp 70) obtained as described by M. Strand and J. T. August, 251 *J. Biol. Chem.* 559 (1976). The fusion was carried out as described by M. Strand, 77 *Proc. Natl. Acad. Sci. USA* 3234 (1980).

A 114 ul solution containing 2 mg of monoclonal antibody 103A5 in 0.1 M $NaHCO_3$ at a pH of approximately 7.2 and 150 mM sodium chloride was prepared and pipetted into a Nunc vial. Then, 33 ul of a 0.1 M $NaHCO_3$ solution at a pH of 7.0 was added to the vial. Finally, 26.4 ul of the mixed carboxycarbonic anhydride of DTPA (0.5 M in acetonitrile) was added after cooling the chelate and antibody solutions to approximately 0°C. The reaction was allowed to proceed overnight.

The product was first dialyzed at 4°C, against one liter of 30 mM ascorbic acid, 5 mM EDTA, 200 mM NaCl and 20 mM of sodium citrate (pH 7.0). The resulting solution was dialyzed at 4°C against three one liter changes of 50 mM citrate, 200 mM sodium chloride at pH 6.0, and 1 ml Chelex 100 resin (Bio-Rad) over a 48 hour period. Finally, the resulting solution was dialyzed for 8 hours against one liter of a solution that had a concentration of 10 mM MES and 200 mM sodium chloride at pH 6.0. Approximately 1.7 mg of chelate conjugated monoclonal antibody was recovered. Analogous experiments employing C-14 labeled DTPA were analyzed by scintillation counting and shown to contain approximately 1.5 chelates per antibody molecule.

Forty microliters of Indium-111 chloride solution (New England Nuclear Corp.) was adjusted to pH 3.0 by the addition of 11.4 ul of 0.4 M citric acid at pH 5.0. A separate solution was prepared containing 250 micrograms of chelate conjugated monoclonal antibody in a total volume of 21.6 microliters. The solution had a concentration of 200 mM sodium chloride and 10 mM MES at a pH of 6.0. The solution was adjusted to pH 4.6 by the addition of 6 ul of 0.25 M citric acid at a pH of 3.0.

The metal chelate conjugated monoclonal antibody was prepared by combining the indium

chloride and chelate conjugated antibody solutions and allowing them to react for approximately 30 minutes at ambient temperature. The reaction was terminated by adding 25 ul of a saturated solution of trisodium citrate to adjust the pH to about 6.

The chelate conjugated antibody was purified by chromatography on 9 cm long column containing 1.0 ml of an ion retardation resin (AG 11-A8) available from Bio-Rad) above 1.0 ml of a cation exchange resin (AG-50-WX8, H^+ form, 200—400 mesh available from Bio-Rad) above 7 ml of Sephadex G-50 gel (Pharmacia). A solution with concentrations of 200 mM sodium chloride and 10 mM MES at a pH of 6.0 was used as the eluant and was used pre-equilibrate the column.

The eluate was collected in 0.5 ml fractions. The two fractions with most of the protein were shown to contain 150 ug of monoclonal antibody labeled with 157.1 microcuries Indium-111. Dialysis at 4°C against one liter of an aqueous solution of 20 mM MES and 200 mM sodium chloride at pH 6.0 showed less than 6% loss of Indium. The antibody was shown to retain substantially 100% of its biological activity and specificity by *in vitro* tests. *In vivo* imaging in leukemic mice highlighted the tumor site in the spleen. When administered to normal mice there was no uptake by the spleen.

EXAMPLE II

A hybridoma was obtained by fusing P3 653 mouse myeloma cells with the isolated spleen cells of C56B1/6 mice which had been immunized with purified tumor-associated ferritin isolated from the human spleen. A hybridoma was isolated that produced an anti-ferritin antibody designated 263D5. The antibody was specific for human ferritin and did not react with ferritin of other mammalian species.

The procedure of Example 2 was repeated to provide an indium-111 containing DTPA conjugated monoclonal antibody. A physiologic normal saline solution containing the metal chelate conjugated monoclonal antibody was injected into normal and leukemic mice. In both the leukemic and normal mice, radio imaging showed that there was no concentration of radio labeled metal. These tests demonstrated that the chelate was stable *in vivo* both with respect to the chelate-antibody conjugation and with respect to the retention of the radioactive metal. Neither the spleen nor the liver was highlighted in the images.

EXAMPLE III

The following example employs a gamma particle emitting isotope to demonstrate the selective localization of radiometals (including alpha particle emitting isotopes) achieved by the present invention.

Indium-111 chelate conjugated monoclonal antibodies were prepared from an antibody specific for human breast tumor. The hybridoma that produced the antibody was prepared from a fusion of mouse myeloma and mouse spleen cells. The hybridoma and antibody are described in 78 *Proc. Natl. Acad. Sci.* 3199 (1981).

The procedure employed was substantially the same as the procedures of Examples I and II, except for the following. First, the step of dialyzing the chelate conjugated monoclonal antibody against ascorbate-EDTA was omitted. Second, 10 microliters of 0.1 M ascorbate at pH 4 was added to the indium-111 solution prior its addition to the aqueous saline solution of the chelate conjugated monoclonal antibody.

The labeling efficiency exhibited a three-fold increase over the methods of Example I and II. The final product was labeled with approximately 2.1 microcuries per microgram.

Ten micrograms of the indium-111 chelated conjugated monoclonal antibody collected from the purification column was diluted to 100 microliters with an aqueous solution of phosphate buffered saline. The diluted indium-111 conjugated antibody was injected into the tail vein of a nude, athymic mouse in which a human breast tumor had been grown. The human breast tumor cells expressed an antigen for the antibody. Seventy-two hours after injection, a clear and well-defined gamma camera image demonstrated high localization of indium-111 in the tumor tissue. No similar localization of the indium-111 in the liver or spleen was observed.

EXAMPLE IV

The following tables demonstrate that a radiometal-DTPA chelate, as opposed to free radiometal, does not localize in the liver and spleen and is rapidly excreted through the kidneys and stomach. The uptake of radiometal into the organs of normal and leukemic mice was determined by the following procedure. Six-week old normal mice and mice made leukemic eight days previously by the injection of Rauscher leukemia virus were injected intraperitoneally with 5 micrograms (5 microcurie per microgram) of free Bi-207, DTPA chelated Bi-207 and DTPA chelated Sc-46. Eighteen and forty-two hours later, mice were sacrificed, their organs weighed, and the amount of radioactivity associated with the organs determined. In order to normalize for differences in the injections, in body weights, and in times of excising the organs, the amount of radioactivity per gram of tissue was divided by the amount the radioactivity per gram of blood, and results are expressed as this ratio. The results are shown in Tables 1, 2 and 3.

TABLE 1
Means and standard errors of the mean of ratio/blood of 5 organs of 14-day leukemic and normal mice at 18 and 42 hours after injection of free ^{207}Bi[a].

	18 hours		42 hours
Tissue	Leukemic	Normal	Leukemic
Heart	2.35 ± 0.65[b]	4.0 ± 0.30	1.48 ± 0.12
Liver	31.3 ± 7.50[c]	29.0 ± 0.35	43.3 ± 2.65
Spleen	7.18 ± 2.03[d]	21.1 ± 0.65	8.70 ± 0.66
Kidney	69.4 ± 43.7	612.6 ± 47.8	89.3 ± 9.18
Stomach	1.35 ± 0.35	6.95 ± 0.65	5.01 ± 0.43

a = injected dose = 7×10^6 cpm per mouse
b = approximately 0.13% of total cpm in heart
c = approximately 40% of total cpm in liver
d = approximately 12% of total cpm in spleen

TABLE 2
Means and standard errors of the mean of ratio/blood of 5 organs of leukemic and normal mice at 18 hours after injection with ^{207}Bi-DTPA chelate

	^{207}Bi-DTPA[a]	
Tissue	Leukemic	Normal
Heart	2.60 ± 0.33[b]	2.45 ± 0.21
Liver	19.12 ± 6.32[c]	10.93 ± 1.24
Spleen	13.1 ± 2.6[d]	12.4 ± 2.0
Kidney	294.0 ± 37.0	281.0 ± 26.0
Stomach	7.52 ± 2.44	7.20 ± 2.90

a = injected dose 3.8×10^6 cpm per mouse
b = approximately 0.003% of total cpm in the heart
c = approximately 0.21% of total cpm in the liver
d = approximately 0.06% of total cpm in the spleen

TABLE 3
Means and standard errors of the mean of ratio/blood of 5 organs of leukemic and normal mice at 18 hours after injection with ^{46}SC-DTPA chelate

	^{46}SC-DTPA[a]	
Tissue	Leukemic	Normal
Heart	3.66 ± 0.21[b]	4.49 ± 0.84
Liver	9.96 ± 0.57[c]	7.23 ± 1.04
Spleen	3.17 ± 0.39[d]	5.69 ± 0.28
Kidney	42.1 ± 0.9	29.5 ± 5.5
Stomach	12.45 ± 5.43	7.76 ± 2.06

a = injected dose 5.4×10^6 cpm per mouse
b = approximately 0.006% of total cpm in the heart
c = approximately 0.14% of total cpm in the liver
d = approximately 0.08% of total cpm in the spleen

The data in the above tables demonstrates that DTPA chelated bismuth and scandium do not concentrate in the liver or spleen of mice as opposed to free bismuth. High concentrations of chelated metal in the kidneys demonstrates that it is being voided through the urine. The variation in kidney concentrations between leukemic and normal mice is attributable to frequent voiding by the leukemic mice due to stress.

EXAMPLE V

Tests were conducted to determine the effect of bismuth alpha radiation on mammalian cells. F-46 leukemic cells were grown *in vitro* in Dulbacco's Modified Eagle medium containing 10% heat inactivated fetal calf serum to provide a cell population of 1×10^5 in each well. The cell populations were exposed to bismuth-212 by adding serial dilutions (as indicated in Table 4) in the growth medium. The cells were then grown for 96 hours and the number of surviving cells was determined. The results are shown in Table 4 below.

TABLE 4

Dose (Rads)	Mean Number of Surviving cells (10^5)	Standard Deviation	% Survival
0	6.9	1.4	100
0.2	8.0	0.5	116
0.4	6.1	1.7	88
0.8	6.7	1.4	97
1.5	6.0	2.8	87
3.1	6.0	0.6	87
6.2	5.0	0.6	72
12.3	2.9	0.4	42
24.6	2.2	0.4	32
49.2	1.1	0.4	16
98.4	0.6	0.1	9

From the above data of Table 4, employing standard calculation methods, D_0 (37% survival) is 38.5 rads. This demonstrates that bismuth-212 emits highly cytotoxic, densely ionizing radiation. By comparison, 900 rads of sparsely ionizing radiation from a cobalt-60 source was required to achieve the same results. For a discussion of radiation doses see: nm/mird pamphlets No's 1 (revised) and 10.

Since modifications will be apparent to those skilled in the art, it is intended that this invention be limited only by the scope of the appended claims.

CLAIMS

1. A method for preparing metal chelate conjugated monoclonal antibodies comprising:

(a) providing chelate conjugated monoclonal antibodies;

(b) adding a metal salt to an aqueous solution of said recovered chelate conjugated monoclonal antibodies and a buffer to form metal chelate conjugated antibodies, said metal being an alpha emitting radiometal;

(c) passing the aqueous solution containing said metal chelate conjugated monoclonal antibodies through a chromatography column, said column having one or more layers selected from the group consisting of an ion retardation resin, an anion exchange resin, a cation exchange resin and a chelating ion exchange resin, and a final layer comprising a sizing matrix; and

(d) recovering from said column a solution of metal chelate conjugated antibodies having at least about 80% of said metal contained in said solution complexed by said chelate conjugated to said monoclonal antibodies.

2. The method of claim 1 wherein said chelate conjugated monoclonal antibody is prepared by contacting a carboxycarbonic anhydride of diethylenetriaminepentaacetic acid with an aqueous solution of monoclonal antibodies.

3. The method of claim 2 wherein said carboxycarbonic anhydride of diethylenetriaminepentaacetic acid is the reaction product of an amine diethylenetriaminepentaacetic acid salt with an ester of a haloformic acid in an organic solvent at a temperature less than about −20°C.

4. The method of claim 3 wherein said ester of a haloformic acid is isobutylchloroformate.

5. The method of claim 1 wherein said alpha emitting radiometal is selected from the group consisting of Bi-211, Bi-212 and Bi-213.

6. The method of claim 5 wherein said alpha emitting radiometal is Bi-211.

7. An aqueous solution of metal chelate conjugated monoclonal antibodies wherein at least about 94% of said metal complexed by the chelate portion of said conjugate and said metal is an alpha emitting radiometal.

8. The solution of claim 7 wherein at least about 97% of said metal is complexed by the chelate portion of said conjugate.

9. The solution of claim 7 wherein said conjugated antibodies retain at least about 80% of their activity and specificity.

10. The solution of claim 7 wherein said conjugated antibodies retain at least about 95% of their activity and specificity.

11. The solution of claim 8 wherein said conjugated antibodies retain at least about 97% of their activity and specificity.

12. The method of claim 7 wherein said alpha emitting radiometal is selected from the group consisting of Bi-211, Bi-212, Bi-213.

13. The method of claim 12 wherein said alpha emitting radiometal is Bi-212.

New claims or amendments to claims filed on 28th June '83

Superseded claims 12—13

New or amended claims:—

12. The solution of claim 7 wherein said alpha emitting radiometal is selected from the group consisting of Bi-211, Bi-212, Bi-213.

13. The solution of claim 12 wherein said alpha emitting radiometal is Bi-212.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1984. Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.