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212,545 ANTIBODY-GOLD CLUSTER CONJUGATES James F. Hainfeld

TITLE:

ANTIBODY-GOLD CLUSTER CONJUGATES

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RELATED APPLICATION

The instant application is a continuation-in-part application of co-pending application S.N. 041,054 filed April 22, 1987.

BACKGROUND OF THE INVENTION

Effective detection, diagnosis, and therapy of certain disorders such as cancer have long been an object of intense research. The development of monoclonal antibodies has significantly improved the practitioners' ability to target and deliver diagnostic and therapeutic agents to specific target cells. The use of antibodies, both polyclonal and monoclonal, to mark tissues or molecules has been an important tool in cancer imaging and therapy, and electron microscopy (EM).

It has been shown that whole antibodies can be labeled with radioisotopes and used for the detection of cancer in humans (Burchiel et al., Pat. No. 4,311,688 and Carlsson et al., Pat. No. 4,232,119).

It has also been shown that the Fab portion of an immunoglobulin can be labeled and used in diagnostic and

detection regimens [Sjoquist et al., Pat. No. 3,966,898; Ling, Pat. No. 4,298,593; Aebi et al., PNAS, USA, 74:5514 (1977)].

It has also been shown that metal chelates can be conjugated to monoclonal antibodies and used in diagnostic and therapeutic techniques (Gansow et al., Pat. Nos. 4,454,106 and 4,472,509).

It has also been shown that electron dense moieties such as ferritin [Singer, Nature, 183:1523 (1959)] or colloidal gold [De Mey et al., Pat. No. 4,446,238 and Faulk et al., Immunochemistry, 8:1081 (1971)] can be used as a marker for visualization of antibody labels in the electron microscope.

It has also been shown that immunodiffusion assay techniques can be developed using antibody-metal compound conjugates in which the metal or metal compound has a particle size of at least 5 nm (Leuvering et al., Pat. No. 4,313,734).

In the present invention, antibodies or active antibody fragments are covalently combined with stable arrangements of multiple copies of gold, called clusters below. The gold clusters may be radioactive when such activity provides diagnostic or therapeutic capabilities. These antibody-gold cluster conjugates retain the antibody specificity and activity, and are useful in any electron microscopy, diagnostic techniques, antigen localization studies and therapies that rely upon the delivery of an effective dose to a targeted site.

Although each of the above-noted disclosures are adequate for some uses, each suffers from a deficiency now overcome by the present invention. Some of the labels noted

above are generally too large for submolecular mapping in electron microscope studies. For example, an antibody molecule is about 15 nm in diameter and a colloidal gold complex is about 12-35 nm in diameter or larger (4,446,238 discloses a diameter range of 20-40 nm). A conjugated label of these components is therefore about 27-50 nm in diameter, too large for meaningful electron microscopy studies in many fields where electron microscopy could be of value. In contrast, the antibody-gold cluster conjugates of the present invention can possess diameters of about 5.0 nm, ideal for microscopy studies.

Some of the references noted above are also deficient in their inability to couple more than a few radioactive elements onto an antibody without losing the antibody activity, thus limiting the radioactive dose that can be delivered to a specific site (for imaging, diagnosis, or therapy). It is an object of the present invention to utilize a cluster system whereby enough radioactive dose can be delivered to a specific site. This application is of critical importance in the use of radiation to tumor therapy for certain forms of cancer. The 3.1 day half-life of the radioactive gold (^{199}Au) conjugates herein described is compatible with the use of these antibody-gold cluster conjugates in cancer therapy because it limits extended doses of radiation to the patient.

It is also an object of the present invention to take advantage of the small size of the antibody-gold cluster conjugates. For example, it is possible to more effectively and efficiently treat or detect cancer by using the advantages

inherent in the small size of a Fab'-Au_{11} conjugate which permits penetration of tissues and tumors much more rapidly than previous attempts with whole antibodies coupled to radioisotopes. The small size of the conjugate also greatly improves resolution in electron microscopy studies in which specific sites or molecules or biological structures are labeled.

It is also an object of the present invention to provide a cluster-conjugate in which many different clusters may be coupled to an antibody or antibody fragment. The present invention shows that clusters containing 55 gold atoms can be targeted to a tumor via the anti-tumor antibody to which the clusters are attached, thus enhancing the ability to deliver the required radiation dose for therapy. Additionally, multiple detectable atoms via a cluster enables improved signal-to-noise ratios, and therefore earlier detection of small tumors.

Although tumor imaging, diagnosis, and therapy are applications of the antibody-gold cluster conjugates of the present invention, use of antibodies to a variety of other antigens broaden the usefulness of such gold cluster conjugates. For example, antibodies to blood clots, fibrin, viruses, and specific tissues can be conjugated to gold clusters and used to image and detect phlebitis, embolisms, viral infections and organ abnormalities.

SUMMARY OF THE INVENTION

The gold conjugates of the present invention are a significant improvement on known methods and compositions for

covalently attaching antibodies to a stable cluster of multiple atoms or chemical groups. The small size of the conjugate overcomes previous problems with size or with the inability to load more than a few atoms onto an antibody. In the preferred embodiment, a Fab' fragment is covalently attached to an undecagold cluster, which may be the radioactive Au_{11} cluster, such that the antigenic specificity and capacity of the antibody are preserved, while at the same time, the distance of the gold cluster to the target antigen is no more than 5.0 nanometers (nm).

DESCRIPTION OF THE FIGURES

Figure 1 shows a reaction scheme for labeling a Fab' fragment with the Au_{11} cluster.

Figure 2 shows the size comparison of antibody labels used in electron microscopy. A commonly used conventional label (A) relies on staphylococcal protein A binding to the Fc region of an immunoglobulin; the overall dimension is 25 nm or greater. The label of the present invention (B), an undecagold cluster bound to a Fab' fragment, is no more than 5.0 nm.

SPECIFIC DESCRIPTION OF THE INVENTION

A general method and compositions have been developed for covalently linking an antibody or antibody fragment to a stable cluster of multiple atoms of gold in such a manner that the antigenic specificity and capacity of the antibody is preserved. These antibody-gold cluster conjugates exhibit a broad range of applications. "Cluster" as used throughout this specification, is defined as a stable group of gold atoms. These include, but are not limited to, clusters containing 11

or 55 gold atoms, or multiple radioactive atoms, such as ^{198}Au, and ^{199}Au. The undecagold complex (Au_{11}) was described in Safer et al., Science, 218:290-291 (1982) and McPartlin et al., Chem. Comm., p. 334 (1969); monofunctional forms of this complex have been described in Safer et al., J. Inorg. Biochem., 26:77-91 (1986) and Reardon et al., Biochemistry, 23:3849 (1984). These complexes are water soluble and capable of being covalently linked through the external organic shell to specific residues or molecules. As is shown below, a maleimide group on the organic shell reacts directly with free sulfhydryls on the antibody or antibody fragment.

The antibody or antibody fragment used in this invention may be any polyclonal or monoclonal antibody or antibody fragment which forms an immunochemical reaction with an antigen. "Antigen" therefore is a term of art denoting any substance or molecule which induces the formation of an antibody (i.e., that can trigger an immune response), and can be a virus, a bacterium, a fungus, a parasite, tissues or cells not naturally a member of a hosts family of tissues or cells, or even a portion or product of any of these organisms. "Antigenic" or immunogenic" are used to describe the capacity of a given substance to stimulate the production of antibodies. "Antibody" is a term of art denoting the soluble substance or molecule secreted or produced by an animal in response to an antigen, and which has the particular property of combining specifically with the antigen which induced its formation. Antibodies, also known as immunoglobulins, are classified into five distinct classes--IgG, IgA, IgM, IgD, and

IgE. The basic IgG immunoglobulin structure consists of two identical light polypeptide chains and two identical heavy polypeptide chains (linked together by disulfide bonds). When IgG is treated with the enzyme papain, an antigen binding fragment can be isolated, termed Fab. When IgG is treated with pepsin (another proteolytic enzyme), a larger fragment is produced, $F(ab')_2$. This fragment can be split in half by reduction to Fab'. The Fab' fragment is slightly larger than the Fab and contains one or more free sulfhydryls from the hinge region (which are not found in the smaller Fab fragment). The term "antibody fragment" is used herein to define the portion known as Fab'. It is well known in the art to treat antibody molecules with pepsin in order to produce antibody fragments [Gorevic et al., Methods of Enzymol., 116:3 (1985)].

The term "immunochemical reaction" is used to denote the specific interaction which occurs between an antigen and its corresponding antibody. Methods for obtaining antibodies have been extensively discussed and are well-known in the art. A useful reference for obtaining monoclonal antibodies is Koprowski et al., U.S. Patent No. 4,196,265. The selection of the antibody for the practice of this invention will depend upon the end use for which the antibody-gold cluster conjugate will be employed. Such selection is within the skill in the art.

Of particular importance to the present invention is the size of the antibody or antibody fragment molecules. The antibody molecule is about 15nm in diameter and the Fab'

fragment is about 5.0 by 4.0 by 3.0 nm (about 50 kD). This size makes the antibody label five to ten times smaller than the gold antibody labels synthesized according to the prior art. This smaller size also permits the use of the label for discerning specific sites on single protein molecules or nucleic acid chains, and also permits the labeling of secluded sites previously inaccessible by larger labels, a quality particularly useful in electron microscopy,

The rabbit IgG described in the examples has only one disulfide in the hinge region of the antibody (see figure 1). Human antibodies have between 2 and 15 disulfides in the hinge region, most of which remain after pepsin digestion (see Gorevic et al., cited above). These human antibody fragments drastically increase the capability of loading clusters onto the antibody label, which should make the fragments easily visible in thin sections or conventional electron micrographs without the sacrifice of the high resolution of this technique.

Other antibodies are useful in the present invention, including but not limited to, rabbit anti-flagella antibodies, rabbit anti-22s and anti-14s dynein antibodies, mouse monoclonal antibodies to bovine factor V. The labeling procedure of the present invention is generally applicable to a broad range of antibodies and antibody fragments.

The antibodies are generally maintained in an aqueous solution that contains an ionic compound. A physiologic normal saline is preferred. 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 used in the

practice of this invention.

One aspect of the present invention is covalently linking the cluster or clusters to the antibody or antibody fragment. For example, the undecagold complex is water soluble and can be covalently linked through its organic shell to specific residues or molecules. In this case one maleimide group in the organic shell reacts directly with free sulfhydryl(s) located on the antibody fragment.

It is known in the art that Fab' fragments can be prepared that have one free sulfhydryl group at a location distant from the antibody-antigen binding region. See Martin et al., Biochemistry, 20:4229 (1981). Such antibody fragments can be coupled to a gold cluster and still retain full antigenic specificity and capacity.

The synthesis of the antibody-cluster conjugates of the present invention is illustrated by the formation of the antibody fragment (Fab')-undecagold (Au_{11}) cluster shown in Figure 1. First, IgG molecules are digested with the enzyme pepsin to form $F(ab')_2$. This fragment is reduced with a suitable reducing agent such as dithiothreitol (DTT) to form Fab' fragments. Treatment with DTT in this manner exposes a specific thiol (-SH) to which the activated gold cluster is bound.

Another method of forming antibody-gold cluster conjugates involves oxidation of the carbohydrates on the IgG molecules with periodate to form aldehyde groups, adding clusters containing free amines, and finally, stabilizing the Schiff's bases thus formed using borohydride. The result is a

cluster cross-linked through the carbohydrate moiety on the IgG molecule.

Another method of forming whole antibody-cluster conjugates involves reduction of IgG with DTT, forming relatively short-lived free sulfhydryls. DTT is removed by column chromatography and the free sulfhydryls reacted quickly with appropriate gold clusters.

To preserve the maximum biological activity of the antibody, the use of strong acids or bases to adjust pH should be avoided (strong acid or base causes localized denaturation in the solution). The pH is preferably controlled by including a suitable buffer in the aqueous solution containing the antibody. For example, $NaHCO_3$ is known in the art and may be employed in the practice of this invention. The choice of the appropriate buffer is within the skill of the art.

As has been described above, this invention may take many forms, all dependent upon the end use of the antibody-cluster conjugate. For example, Fab'-Au_{11} conjugate is useful for diagnosing and treating cancer--using an anti-tumor antibody and a radioactive gold cluster, the conjugate selectively binds to the tumor cells reactive with the Fab' fragment. Concurrently, the radioactive gold atoms decay with emission of a gamma ray, thus permitting imaging of the tumor. The 11-gold cluster conjugated to 17-1A monoclonal antibody is useful for the detection of human colon carcinoma.

Also, decay produces beta particles in an energy range suitable for killing surrounding tumor cells within a small, several cell radius. Using these clusters, therefore, enough

radioactive dose may be delivered to treat cancers. The 3.1 day half-life of the radioactive gold (^{199}Au) is also compatible with limiting extended doses of radiation to the patient.

When the antibody-gold cluster conjugate is being used for microscopy purposes or nuclear medicine, the gold cluster labeled antibodies can be enhanced for easier detection by a silver reaction. This produces a controlled size silver particle around each cluster that can be seen, for example, in the electron microscope and light microscope, depending on the size and number of particles. The antibody-gold cluster conjugates enhanced by a silver reaction have advantages over antibodies labeled with conventional labels. The gold cluster and its attachment to antibodies is very stable and not affected by extremes in pH or ionic strength as are many other labels. It has a longer shelf life and is useable under more conditions. Equally important, its preparation and use are simple and reliable.

The antibody-gold cluster conjugates enhanced by a silver reaction have a variety of applications, including diagnostic medicine, electron microscope studies, and light microscope studies. Any antibody testing could be accomplished with this improved product, such as testing for AIDS or any other viral or bacterial presence. A simple "dipstick" test based on this technology could be used. Here the antigen to be detected is bound to a solid phase material on a stick, which is then inserted into the antibody-cluster solution. If antigen is present, antibody and cluster will bind to it.

After rinsing, a color is developed from the gold. The conjugates are also useful in electron microscopy because of the small size of the Fab'-Au_{11} (5-10 times smaller than other antibody labels), which permits this reagent to diffuse into tissues and samples better and to label at a higher resolution. Subsequent enlargement of the gold by silver makes visualization straightforward. Also the poor stability of conventional antibody labels makes them unuseable in some cases. The antibody-gold cluster labels have superior stability properties.

Silver exists in an oxidation state in many compounds. Gold can, via redox, reduce the silver to silver metal, Ag°, which precipitates out around the other metal. In the present invention, the gold cluster serves as a reaction site for silver deposition. The initial grains grow with time until they can be seen easily with an electron or light microscope. The gold cluster serves as a nucleation site for specific silver deposition.

Another application of the present invention is the use of the antibody-gold cluster conjugates in electron microscopy (EM). For example, the 11-gold cluster bound to Fab' antibody fragments is 5-10 times smaller than any other EM antibody labels currently available. The significantly smaller size of the conjugates of the present invention greatly improve the resolution of EM studies in which specific sites or molecules or biological structures are labeled, thus permitting or enhancing domainal mapping of single molecules or pinpointing specific molecules, sites or sequences in supramolecular

complexes.

The antibody-cluster conjugates of this invention may be administered in vivo in any pharmaceutically suitable carrier. As noted above, a physiologic normal saline solution can be used, and may optionally include an appropriate amount of carrier protein such as human serum albumin (for antibody stabilization). The appropriate concentration of any biologically active material in a carrier is routinely determined by practitioners in the art.

EXAMPLE 1

The reaction scheme shown in Figure 1 is used for labeling Fab' with undecagold. Rabbit IgG (from Cooper Biomedical, Inc.) was digested with pepsin to yield $F(ab')_2$ fragments joined by a disulfide (A,B). The constant fragment (F_c) regions were removed by a Staph A-Sepharose column; other digested fragments were removed on a high-pressure liquid chromatography (HPLC) Fractogel TSK HW-55 (S) column. Next, 20 mM dithiothreitol (DTT) was used to reduce the disulfide link (B,C), which produced the Fab' fragment (2 hour reaction time under N_2). DTT was removed with the HPLC column. Between runs through the column, protein was concentrated to 0.5 to 1.0 mg/ml with Amicon Centricon 10 microconcentrators. Undecagold was activated immediately prior to use by reaction of monoaminopropyl-Au_{11} and N-methoxycarboxyl maleimide to form monomaleimidopropyl-Au_{11} (E). The product was purified on a carboxylic CM Sepahrose column. The Fab' and activated Au_{11} were incubated overnight at 4°C (D-F). Unreacted Au_{11} was removed with an HPLC Fractogel TSK HW-40(S) column.

EXAMPLE 2

In the undecagold-labeled Fab' fragments produced according to Example 1, the Fab molecules show in scanning transmission electron microscope images quantitative labeling--the undecagold spots (0.8 nm diameter) are at one end of these 50 kD fragments. Some Fab' fragments show more than one Au_{11} cluster attached. Each Fab' fragment binds usually one undecagold cluster, but sometimes two or three (and rarely four). Gold clusters on 724 Fab' molecules were counted to determine a more exact stoichiometry. The mean was found to be 1.5 ± 0.7 (S.D.) clusters per Fab'. A spectral assay of the Fab'-Au_{11} complex in solution indicated a stoichiometric ratio of 1.4 Au_{11} to 1 Fab'. The undecagold cluster on the Fab' was located in the same place on each Fab' molecule (at one end), as expected from the position of the free sulfhydryl group. When two undecagold clusters were found on a Fab' fragment, the second site was always 1.8 to 2.8 nm from the first, which again indicated specific attachment. Similarly, when a third undecagold cluster was present, it was 1.8 to 2.8 nm from the first; the three clusters usually formed a triangular cap (sometimes they formed a linear array) on one end of the Fab' molecule. The undecagold clusters were always localized at one end of the Fab' molecule. Some of these multiply labeled Fab' fragments can be identified when they are bound to the antigen and therefore do not appear to effect antibody activity.

EXAMPLE 3

8 mg of pure gold was irradiated at the Brookhaven National Laboratory (BNL) High Flux Beam Reactor (HFBR) for 2

hours. While this does not produce carrier free ^{199}Au, it is a convenient method to yield the levels of specific radioactivity necessary for preliminary tests. Irradiation of enriched ^{198}Pt is one method to yield highly pure ^{199}Au (after separation). The double neutron capture on natural gold employed in fact yielded both ^{199}Au and ^{198}Au. The irradiated gold was then dissolved in 5 drops of aqua regia. It was stirred with mild heating (80°C) until dissolved. Next it was taken to dryness with heat. 5 drops of concentrated HCl was added dropwise and the solution again heated to dryness in order to drive off the nitric acid. The material was dissolved in 5 ml of water to make chloroauric acid ($HAuCl_4$). This was then converted by reduction to AuCN.

The gold cyanide recovered was suspended in 3 ml of absolute ethanol. Two substituted triphenyl phosphines ($P\emptyset R_3$, $\emptyset$ = phenyl) were synthesized. One of the organic groups, R', contained a propylamine (R' = $-CO-NH(CH_2)_3-NH_2$), whereas the other was R = $CO-NH-CH_3$. A 20 to 1 proportion of R to R' yields a predominantly monofunctional eleven gold atom cluster, Au_{11} $(P\emptyset R_3)_6 P\emptyset R_2 R'$.

These phosphines were prepared as follows: First tolyl magnesium bromide was synthesized by reacting p-bromotoluene with magnesium metal in tetrahydrofuran. This Grignard reagent was reacted with phosphorous trichloride in tetrahydrofuran with refluxing. The product was tris-p-tolyphosphine. After crystallization, this material was treated with sodium carbonate and potassium permanganate in water to yield tris-p-carboxyphenylphosphine oxide. Treatment of this with

methanolic hydrochloric acid yielded tris-p-carbomethoxy-phenylphosphine oxide. Trichlorosilane in benzene converted this to tris-p-carbomethoxyphenylphosphine. A solution of potassium hydroxide in 1:1 methanol water was added. After refluxing, tris-p-carboxyphenylphosphine was isolated. This product was reacted with 1,1-carboxyl diimidazole. Methylamine and 1,3-diaminopropane were mixed in a 20:1 proportion in dimethylformamide. This was added to the above material and a mixture of mostly 6 parts tris-p-N-methyl-carboxamidophenyl-phosphine and one part 4-N-(-aminoalkyl)carboxamidophenyl-di(4-N-methylcarboxamidophenyl) phosphine was produced.

A stoichiometric amount of this 6:1 mixture of phosphines, 16 mg, was dissolved in 2 ml of methanol, which was added to the AuCN suspension. With stirring, 0.4 ml of a 0.1 M ethanolic solution of sodium borohydride was slowly added. Afterwards, a few drops of acetone were used to quench any excess borohydride.

The reaction mixture was next taken to dryness <u>in vacuo</u> and redissolved in 2.5 ml of water and 2.5 ml of methanol. 1.5 g of Dowex I (chloride form) was added and allowed to react overnight. After filtering, the Au_{11} clusters were separated from other reactants by passage through a Sephadex G25 column equilibrated in 0.6 M triethylammonium bicarbonate buffer containing 5% methanol. The appropriate peak fraction was dried <u>in vacuo</u>. At this point a CM-Sepharose ion exchange column was sometimes employed to purify further the clusters to obtain only the monofunctionalized specie.

The single primary amine on the Au_{11} cluster was next

converted into an N-alkylmaleimide which would react specifically with free sulfhydryl groups. This was done by reacting the above described cluster with N-methoxycarboxyl maleimide and isolating the product on a CM Sepharose column. The resultant monomaleimidopropyl gold clusters were then ready for labeling the appropriately prepared antibodies.

17-1A monoclonal antibodies, which were reactive toward human colon carcinoma, were treated in two ways for labeling. One method was used to label whole IgG molecules; the other was to label the smaller Fab' antibody fragments. These two species have different molecular weights and have the gold label at possibly different sites. Consequently, their biodistribution in an animal after injection, blood clearance times, and tumor/non-tumor localization ratio will be different. This latitude in properties will provide some optimization when choosing a preparation for diagnosis or therapy.

The IgG labeling was accomplished as follows: An aliquot of 17-1A IgG was treated with dithiothreitol (DTT, final concentration 20 mM) for an hour at room temperature, to transiently reduce some disulfides. Next, DTT was removed with a Sephadex G25 column equilibrated in 10 mM sodium phosphate buffer, pH 7.0. A 5-fold molar excess of monomaleimidopropyl gold cluster was added and allowed to react at 4°C overnight. The excess unreacted gold cluster was removed by multiple washings on an Amicon Centricon-30 filter or passage down a DuPont GF 250 gel exclusion column equilibrated with 0.2 M sodium phosphate buffer, pH 7.0.

The Fab' labeling was done similarly: 17-1A IgG was digested with pepsin overnight to yield $F(ab')_2$ fragments which were purified on a DuPont GF250 HPLC column. The $F(ab')_2$ material was reduced to Fab' with 20 mM DTT for 1 hour at room temperature. Successive steps in gold labeling were identical to the above IgG labeling description.

A typical stoichiometry of the labeling was 1:1 for the Fab' (one Au_{11} cluster on each Fab' molecule) and 2 Au_{11} clusters per whole IgG molecule.

EXAMPLE 4

The IgG used was rabbit anti-horse ferritin. To demonstrate that the gold-labeled Fab' fragments retained their antibody activity, they were mixed in 20-fold molar excess with horse ferritin; each ferritin molecule has a shell of 24 similar subunits that encapsulate the 8.0-nm diameter core of iron oxides. After a 1-hour incubation, any unbound material was eliminated by passage through a TSK-HW55(S) HPLC column.

Scanning transmission electron microscope images of ferritin molecules with Fab'-Au_{11} molecules bound were taken. In these images, the maximum distance of the gold clusters from the edge of the ferritin molecules is 5.0 nm; some clusters are much closer. The number of Au_{11} clusters that could be counted per ferritin molecule was 7.3 ± 3.8 S.D. (207 molecules examined) and varied from 0 to 21. Note that ferritin is normally used as an EM label, but with this technique it is labeled with a marker whose electron dense core is 1/10 the size of the ferritin core.

Other antibodies labeled by this technique include rabbit anti-flagella antibodies, rabbit anti-22S and anti-14S dynein antibodies and two mouse monoclonal antibodies to bovine factor V. This indicates that the labeling procedure described should be generally applicable to many antibodies.

EXAMPLE 5

Preliminary *in vitro* testing of a monoclonal antibody to human colon carcinoma coupled to undecagold clusters shows that the Fab'-Au_{11} conjugate retains its specificity for the tumor and does not bind to a control melanoma. The antibody used is "17-1A", commercially available from Centocor, Inc. These antibody-cluster conjugates were also assayed by electron microscopy, high pressure liquid chromatography, and spectroscopy and appeared to follow the normal course of results indicated in Examples 1-4. The number of gold clusters bound to each Fab' in this example was 0.7-1.0.

The *in vitro* test involved incubating the Fab'-Au_{11} with either colorectal carcinoma cells or a control melanoma for one hour, centrifugation to isolate the cell-bound antibody, and assay of the bound antibody with a second antibody [to $F(ab')_2$] which was itself radiolabeled with iodine. A titer curve was produced. Compared to previous attempts at radiolabeling the 17-1A antibody by other methods, the present invention's results are qualitatively better since antibody activity and specificity was preserved. It is predicted that the use of radioactive gold will not affect the results, since chemically the radioactive gold will behave

identically to Au_{11}.

Kinetic, or time course studies of the reaction of the 11-gold cluster with 17-1A Fab' fragments (incubations at 37°C) show that after 30 minutes, 56% labeling occurs; after one hour, 79%; and after two hours, the reactions were complete. This is important since radioactive gold has a half life of 3.1 days, and therefore, short coupling reaction times are advantageous.

The amount of gold cluster necessary to label the 17-1A Fab' fragments was also studied. It was found that one gold cluster to one Fab' fragment resulted in more than 85% coupling. This high yield means that when high specific activity radiogold is used, there will be minimal amounts of waste generated, and approximately only enough radioactive gold need be produced for the dose being injected. This makes the radioisotope production more economical than presently available techniques.

EXAMPLE 6

The 17-1A Fab' fragment-(radioactive gold)-undecagold cluster conjugate was incubated *in vitro* with tumor cells (LS180- a cell culture of human colon carcinoma) and non tumor cells (WM164 - a cell culture of melanoma as control) and the radioactivity of the gold was used to assay binding. The radioactive gold was localized completely on the tumor cells with none (<0.4%, or background) on the non tumor cells. This demonstrates the fact that gold cluster attachment to the anti-tumor antibody does not diminish its specificity for tumor cells.

This provides direct evidence for radioactive gold localization on tumor cells. In Example 5, an indirect cell binding assay was reported where non-radioactive gold cluster labeled antibodies were bound to tumors. Their attachment was determined by a second antibody standard radioimmunoassay procedure. Those results showed no loss of activity or specificity as a result of gold labeling.

EXAMPLE 7

Several *in vivo* tests in mice have been completed to assess both biodistribution with time and toxicity.

I. Toxicity and Biodistribution of Radioactive Gold Clusters (No Antibody).

Radioactive gold cluster was prepared as described that had a specific activity of 40 mCi/mg of gold metal. Various doses of gold cluster were injected into the tail vein of healthy mice: cluster containing 7.8 μg of gold metal into one group, 12.6 μg into another and 24 μg into the third. Each group consisted of 6 animals, for a total of 18 mice. Three mice from each group were sacrificed after 2 hours and the remaining 3 were terminated after 24 hours in order to determine the biodistribution in different organs as a function of time.

Results

One result was that no mice died or reacted unusually to the gold cluster during or following injection. The biodistribution studies showed that 44% of the clusters were excreted after 2 hours and 56% after 24 hours. Typically the blood had 2.8%, the spleen 0.4%, the stomach 0.4%, kidney 5.1%,

bone 2.5%, gut and feces 3.3%, liver 19.3% and, carcass 13.7% of the injected amount after 24 hours. These numbers are % counts per organ normalized to 25 gm mice.

Conclusions

The highest dose of cluster injected, which contained 24 μg of gold metal, is 6,000 times higher than what would be injected in a diagnostic or therapeutic dose. The fact that no noticeable side effects were observed indicates that the gold cluster by itself is probably not toxic. The biodistribution study indicated that more than half of the cluster (56%) was excreted by 24 hours. This is not unexpected because of its low molecular weight, about 5000 daltons.

II. Biodistribution of Gold Clusters Conjugated to Anti-Tumor Antibodies in Animals Bearing Human Tumors

Nude mice bearing human colon carcinoma or melanoma tumors were injected with radioactive gold clusters conjugated to anti-tumor antibodies. Two antibodies were gold labeled, one (17-1A) against the human colon carcinoma and another non-relevant antibody, $H_2$4B5 anti-influenza. The non-relevant tumor (melanoma) and non-relevant gold conjugated antibody ($H_2$4B5) served as controls. A total of 53 mice were used. In order to assess the efficacy of using gold labeled Fab' versus whole IgG, both types of antibody were labeled and tested. In addition, two crosslinking arms between the gold cluster and antibody were used, one, as described in the preparation example, was N-methoxycarbonyl maleimide (NMCM), whereas the other was n-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), This was to test if a different covalent crosslinker between

the gold cluster and antibody or antibody fragment yielded conjugates with different stabilities or body distributions *in vivo*.

Results

In one animal, the 17-1A Fab' labeled with gold showed that 16% of the injected radioactive gold was localized on the relevant human colon carcinoma tumor after 24 hours, whereas the same antibody injected into mice with the non-relevant tumor showed only 2.5% accumulation on that tumor. Lower accumulation had occured after 2 hours (5.2% on the tumor). This was for 17-1A Fab' using the MBS crosslinker to gold cluster and was the best case in the study. The other animals and variables in this study gave similar results: The 17-1A IgG with MBS gave 10.7% on the relevant tumor and 3.3% on the nonrelevant tumor after 24 hours. IgG with NMCM gave 10.0% and 3.1% respectively, whereas the Fab' with NMCM gave 10.1% and 2.8%. The nonrelevant gold labeled antibody gave 2.6% on the tumor. Typically, the liver accumulated 10% of the counts injected after 24 hours. As expected from the known better clearance of Fab' from the blood through the kidneys as opposed to IgG, the kidneys showed more counts with the Fab' labeled samples with comparably reduced blood levels. For example, the kidneys had 26.7% for Fab'-Au_{11} (using MBS) and the blood 11.8% after 24 hours, whereas the IgG-Au_{11} (using MBS) had values of 8.6% and 16.9% respectively.

Conclusion

The labeled antibody against a human tumor concentrates on this tumor, whereas there is little accumulation in

control tumors or with control antibodies. There does not appear to be a breakdown of the antibody and gold cluster which would be evidenced by a significant accumulation in the liver. This is a problem with many other radiolabeled antibody systems.

ABSTRACT

ANTIBODY-GOLD CLUSTER CONJUGATES

Antibody- or antibody fragment-gold cluster conjugates are shown wherein the conjugate size can be about 5.0 nm. Methods and reagents are disclosed in which antibodies or Fab' fragments thereof are covalently bound to a stable cluster of gold atoms.

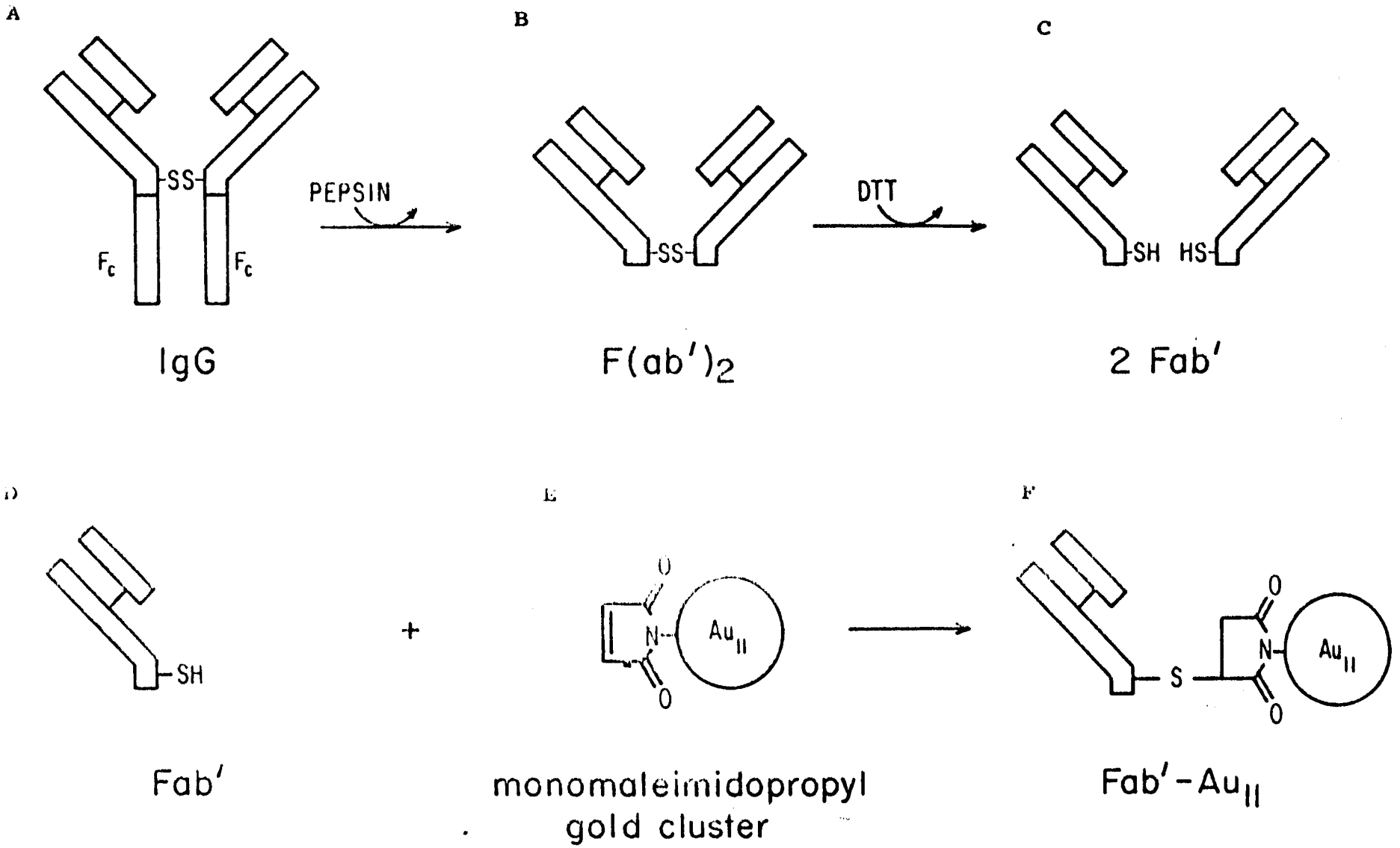
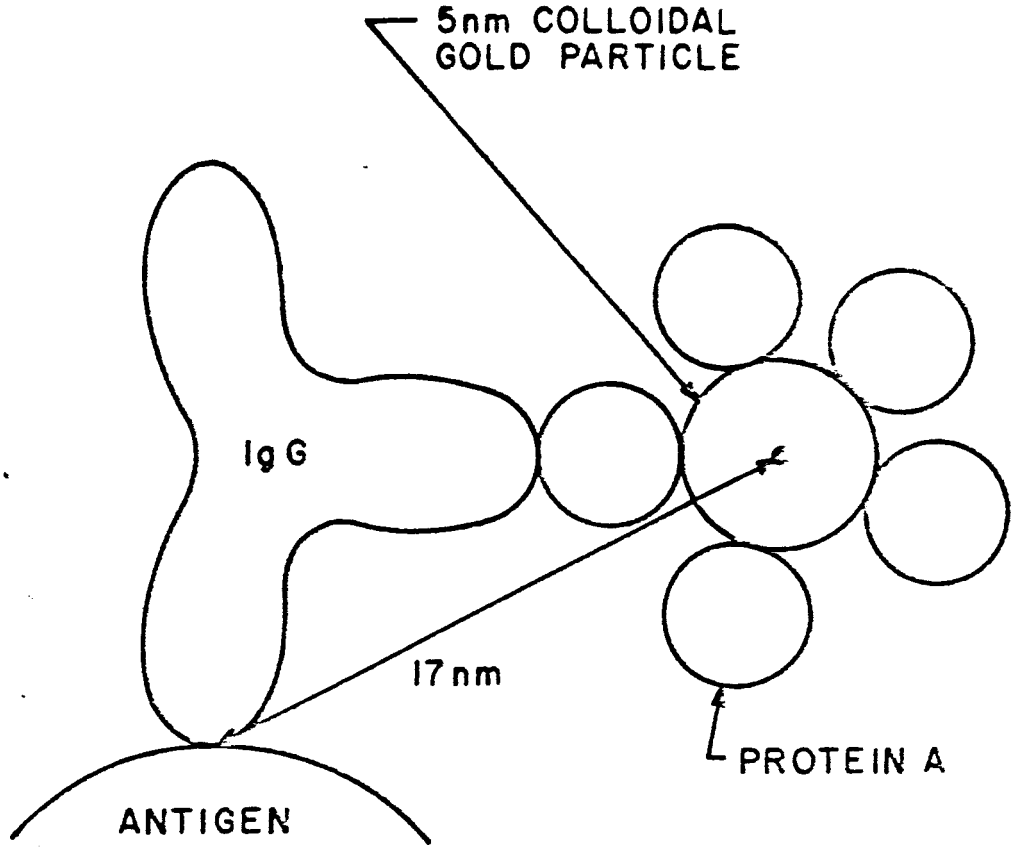

A
SS
Fc
Fc
IgG
PEPSIN
B
SS
F(ab')2
DTT
C
SH
HS
2 Fab'
D
SH
Fab'
+
E
O
N
O
Au11
monomaleimidopropyl
gold cluster
F
S
O
N
O
Au11
Fab'-Au11

FIG. 1

(a)


5nm COLLOIDAL
GOLD PARTICLE
IgG
17nm
PROTEIN A
ANTIGEN

(b)

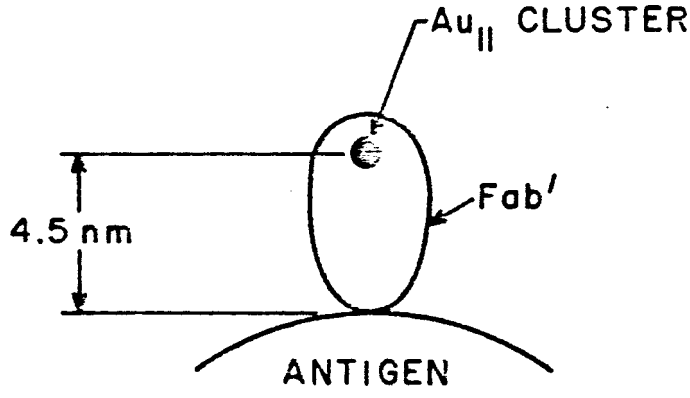

Au11 CLUSTER
Fab'
4.5 nm
ANTIGEN

FIG.2