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GOLD, ELECTRON MICROSCOPY, AND CANCER THERAPY

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Abstract

Radioactive gold has properties suitable for radiotherapy and can provide lethal irradiation to cells. If the gold is conjugated to a targeting molecule, such as an antibody, it may be possible to specifically deliver the dose to tumor cells. Various gold particles are possible candidates and include gold colloids with adsorption of antibodies or gold clusters with covalent attachment. Different sizes of gold particles are available and some may be preferred for certain situations. Problems with intravenous injection and *in vivo* delivery are numerous, and a more tractable application is the direct instillation into the urinary bladder of radiogold immunoconjugates to treat superficial bladder carcinoma. Preliminary studies indicate the feasibility of this approach.

Key Words: Gold, radioactive gold, Au-198/199, undecagold, Nanogold, colloidal gold, cancer, bladder carcinoma, immunotherapy, radioimmunotherapy.

Introduction

Gold technology

Gold is used as a label and has become the preferred marker for electron microscopy due to its high electron scattering and the development of methods to produce uniform sizes and conjugate them to a variety of molecules. Antibodies are adsorbed to colloidal gold particles in low ionic strength solutions. A further advance was the use of large multiple gold atom compounds (or clusters), such as the undecagold cluster (Fig. 1). The organic shell permits covalent attachment to an Fab' antibody fragment (or other molecule) as shown in Figure 2 (Hainfeld, 1987, 1989). This adds stability to the conjugate and serves also to orient the antibody with the hypervariable region facing outward, since attachment is via the hinge sulfhydryl. Also, the

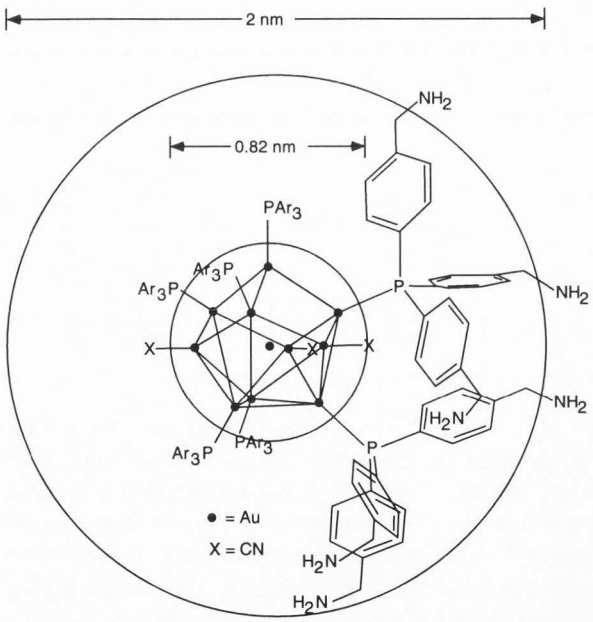

Figure 1. The undecagold cluster.

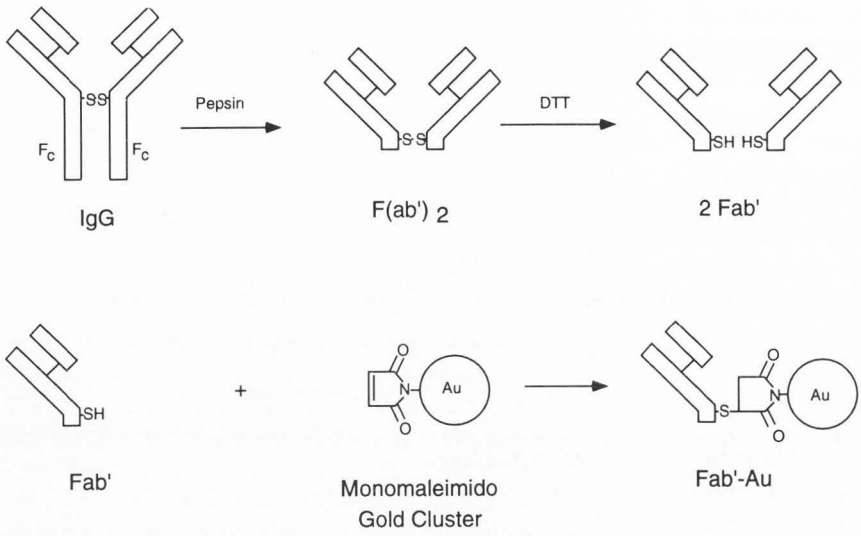

Figure 2. Covalent coupling of gold to Fab' fragment at the hinge sulfhydryl.

gold clusters may be made monofunctional (Yang *et al.*, 1984; Safer *et al.*, 1986) so that just one antibody (IgG or Fab') is attached per gold particle (Fig. 3). In addition, the reactive groups on the gold clusters may be made to couple to various moieties: a maleimide group to react with free sulfhydryls, a N-hydroxysuccinimide ester to couple to amines, or an amino group to link to aldehydes, which may be formed by oxidizing carbohydrate moieties. Other chemistries are also possible.

Gold technology has developed to the point where fairly uniform sizes of gold spheres may be prepared. Colloidal golds may be made that are ~ 1 to 50 nm in size; the ones > 5 nm can be quite uniform, but the smaller sizes show much more variation in size, frequently ≥ 100% for 1-2 nm. In contrast, the gold clusters, e.g., Au_{11} and $Au_{1.4}$ nm (Nanogold), are gold compounds and contain a definite number of gold atoms (11 or 67, respectively), so their size is extremely uniform (gold cores of 0.82 nm or 1.4 nm, respectively).

All of these gold particles can be silver enhanced, leading to higher sensitivity and better visibility. The small gold particles can then not only be seen in the electron microscope, but in the light microscope or with the unaided eye, e.g., on blots developed with silver.

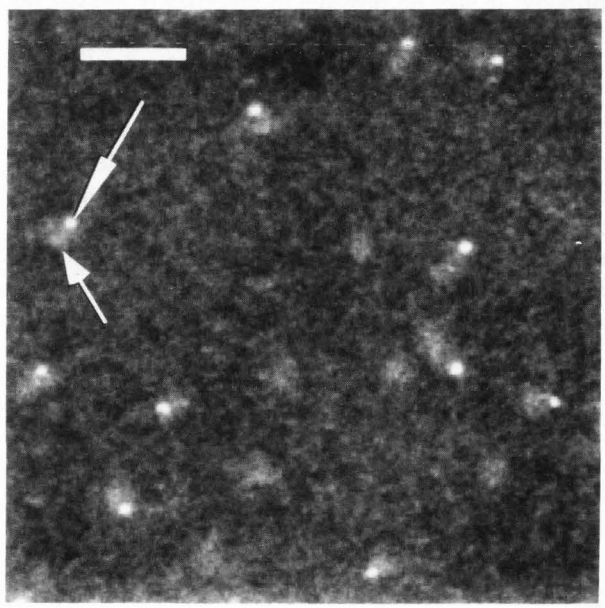

Figure 3. 1.4 nm gold clusters (Nanogold) covalently attached to Fab' fragments. Thin arrow points to a gold cluster (white dot), attached to the hinge sulfhydryl of a Fab' antibody fragment (thicker arrow, grey density mass). Bar = 20 nm.

Use of gold in diagnosis and therapy

With all of this technology involving gold and immunolabeling, or targeting using lectins or other molecules, one might ask if this could be applied to medical applications such as cancer diagnosis or therapy. For diagnosis, gold immunoconjugates could be used to detect tumor antigens from the blood, or to do immunocytochemistry of biopsied material. Serum detection schemes usually employ microtiter plate assays, Westerns, or types of dot blots. Although enzyme linked assays are the most common (using alkaline phosphatase or horseradish peroxidase to produce colored products), gold conjugates have also been used. Another example is the home pregnancy test kit that shows a pink positive color, the pink being ~20 nm colloidal gold. Gold of this size has the unique property of having an extinction coefficient of $\sim 10^8$, or about 1000 times more than most other colored compounds, including fluorescent ones. This makes a few picomoles of gold directly visible with the unaided eye. Gold particles may also be silver enhanced to improve the sensitivity even further.

Table 1. Properties of radioactive gold-198 and 199 (Lederer and Shirley, 1986).

	$t_{1/2}$ (days)	β^- (keV)	γ, keV (%)	β^- range in water
^{198}Au	2.7	312 (avg.); 961 (max.)	412 (95)	460 μm avg.; 4.0 mm max.
^{199}Au	3.1	86 (avg.); 453 (max.)	159 (37); 208 (22)	100 μm avg.; 1.0 mm max.

In vivo imaging using gold may someday be possible, since gold may be sensitively detected using X-ray absorption or X-ray induced X-ray fluorescence. This would require adequate targeting to tumors and the proper instrumentation. Gold is also sensitively detected by mass spectrometry (to ~1 ppb) or neutron activation, so that other forms of probe measurements and diagnostic procedures could be envisioned. Recently, 16 nm colloidal gold stabilized with bovine serum albumin (BSA) was injected into test animals to test leakage into alveolar lung mucous which would be used to diagnose pulmonary leakage (Darien *et al.*, 1995). The lung mucous samples were analyzed for gold by neutron activation.

For therapy, gold materials might also be useful. Various gold compounds have been used for some time to treat arthritis. However, no gold formulations have yet been found for effective cancer therapy in the same way as some other organometallics such as cisplatin.

Radioactive gold

Radioactive gold presents an interesting extension to the usefulness of the rest of gold technology. Several isotopes are possible, but perhaps the two most useful ones are ^{198}Au and ^{199}Au. Their properties are shown in Table 1.

^{198}Au may be produced from neutron irradiation of natural gold in a research nuclear reactor. However, the specific activity is limited, i.e., only about 1 in 3,000 gold atoms becomes radioactive with typical irradiation times and fluxes. No carrier added ^{199}Au (where all gold atoms are radioactive) may be produced from an enriched ^{198}Pt target, and later purified from the platinum (Anderson *et al.*, 1988; Kolsky and Mausner, 1993). The 3 day half life is acceptable since it gives time for preparation and administration, but would not reside long-term in a patient (avoiding the so-called "night light" effect). Gold is a low to intermediate range β emitter, with a range of several cells. This may be preferable to the use of ^{90}Y (avg. β^- = 934.8 keV, max. = 2283.9 keV), for example, which has an average range of 4 mm (1.0 cm max.) and may damage adjacent tissue. One also has the choice of using ^{198}Au or ^{199}Au to optimize the range desired, that of ^{199}Au being ~1/5 of that of ^{198}Au (100 μm versus 460 μm). ^{198}Au and ^{199}Au also emit γ's suitable for imaging, although the 412 keV of ^{198}Au is higher than normally used for imaging studies. Imaging is an important clinical requirement since different patients respond variably to antibodies. This means that a low dose could be used to determine tumor localization and exact dosimetry before a therapeutic (and potentially hazardous) dose was administered. Only a few elements meet the requirement of appropriate (low to intermediate) β with a γ suitable for imaging: ^{47}Sc, ^{67}Cu, ^{105}Rh, ^{131}I, ^{153}Sm, and ^{198}Au and ^{199}Au (Mausner *et al.*, 1988) and ^{177}Lu. Therefore, radioactive gold turns out to be one of the few best isotopes for radionuclide therapy. The use of radiogold solely for diagnostic imaging is not apparently advisable, since the β emission would induce tissue damage. Other isotopes, that emit mainly γ rays, are more suitable, such as ^{111}In, ^{99m}Tc, ^{201}Tl, and ^{125}I, although ^{125}I does not have a high enough γ energy for efficient imaging.

Radiogold should therefore be further considered for therapeutic applications. The basic idea is to target the gold to tumor cells, which would then be locally irradiated and die. This modality seems best suited to tracking down small metastases or cells that escape surgery, and is probably not suited to treating large tumor masses that can be effectively removed by surgery. Since most patients with cancer die due to metastatic growths, where surgery is not effective or possible, this area of application is important. Many anti-tumor antibodies have been developed worldwide to pursue immunotherapies. These antibodies may be used either alone, in hopes of stimulating antibody-dependent cellular cytotoxicity (ADCC) or the complement system, or as conjugates to target a cytotoxic agent (e.g., ricin), or a radioisotope; boron (or uranium) neutron capture therapy is yet another variation. Drugs, prodrugs, and coagulant-stimulating tissue factors have also been used. Other targeting molecules besides antibodies are possible and include peptides, hormones, and growth factors (e.g., epidermal growth factor). The avidin-biotin method has been proposed as a two step targeting procedure, and gold may be made with either of these molecules attached.

The usual sequence for testing the effectiveness of a radioimmunoconjugate is to first test it *in vitro* (binding to tumor and non-tumor cells grown in cell culture), check serum stability, then inject it into nude mice

which have human tumor xenografts (formed by injecting human tumor cells subcutaneously which then grow into a tumor mass). Further animal testing should include toxicity and antigenicity studies. Favorable products may go on to human clinical trials.

Immunotherapy: general problems

Most of the clinical trials in this area to date have been done with ^{90}Y or ^{131}I due to their availability and ease of antibody coupling. Although there have been some occasional remissions and favorable responses {e.g., 4 patients out of 9 with B-cell lymphoma had complete remissions using ^{131}I anti-CD20 antibody, (Kaminski *et al.*, 1993)}, the overall success of antibody therapy (including ADCC, toxin and radioconjugates) has been disappointing. The main reason for this is the low tumor to non-tumor localization of the antibody (for a review of the problems with radioimmunoconjugates, see: Sands, 1988). Generally, there is high uptake in the liver, as well as spleen, bone, and frequently kidney. Although specific tumor uptake is evident, there is frequently a higher amount in other tissues (Reilly, 1991). In human trials, typically only ~0.007% of the injected materials localizes in the tumor (per gram, Sands, 1988), meaning that most of the antibody, radioactivity or toxins are in other tissues. It takes ~10,000 rad to kill tumor cells (Bigler *et al.*, 1988), but only 500-1,000 rad to lethally damage the bone marrow {serious bone marrow depression was observed in 21% of patients receiving bone marrow doses exceeding 200 rad (Bigler *et al.*, 1988)}. One cannot simply raise the amount injected to kill the tumor, since the lethal dose to other organs would be exceeded. There are several explanations: for this lack of good tumor to non-tumor localization: one is the instability of the radioisotope-antibody conjugate. Typically, diethylenetriaminepentaacetic acid (DTPA) chelators have been covalently attached to IgG's which then chelate the radiometal. Each chelator has a dissociation constant for the metal-chelator complex, and there is a finite release rate of the metal. Once released, a metal ion can bind to other serum components or may have natural affinity for particular organs; e.g., yttrium goes to bone, indium goes to the liver, and iodine to the thyroid. Chelation chemistry has improved and highly stable conjugates are now available which virtually eliminate this problem (Li and Meares, 1993). A second effect is that the conjugates are not native molecules and may be removed by the liver; this is particularly true with IgG aggregates, colloids, liposomes (Allen, 1994), and perhaps other more native looking constructs. Aggregated antibodies lead to increased liver uptake, but more careful chromatographic separation permits only monomers to be selected for use. Another important factor in liver and spleen uptake is due to the antibody processing of monoclonal antibodies (MAbs) by these organs.

Another barrier to effective delivery is the poor penetration of antibodies into tumor masses, due to their large size (Langmuir *et al.*, 1991). They must pass through the endothelium of the blood vessels to reach the first tumor cells, then pass between cells to go to the next layer. This is not a favorable situation, and explains why the better results with radioimmunotherapy have been obtained with blood borne lymphomas which are readily accessible, being already in the blood compartment, than with solid carcinomas. The endothelium is an interesting barrier, and different tissues have varying porosity, leading to different tissue specific macromolecular leakage rates. Fortunately, higher endothelial leakage is frequently observed in tumor vasculature (Jain and Gerlowski, 1986). Histological examination of radioimmunoconjugate localization in tumors indicates it to be primarily along the blood vessels and not highly correlated with tumor cell antigen expression (Esteban *et al.*, 1994). One might propose smaller targeting molecules, such as $F(ab')_2$, Fab fragments, single chain antibodies, or peptides to improve tumor penetration. However, a disadvantage of small molecules has been that for $\leq$ 60,000 daltons (D), they are rapidly cleared by the kidney and do not allow adequate tumor accumulation. For imaging, this is an advantage, since background blood concentration is reduced quickly, and less delivery to the tumor is acceptable. For therapy, $F(ab')_2$ fragments are considered best (Yorke *et al.*, 1991), since tumor accumulation rises for about 22 hours (Andrew *et al.*, 1990), and the retention time of $F(ab')_2$ in the blood is consistent with this (whole IgG often shows better tumor uptake at 48 hours). There are also Fc receptors on some cells, and the use of $F(ab')_2$ fragments eliminates this non-specific binding. Even with all these factors, fairly similar results have been obtained for IgG and $F(ab')_2$.

A further explanation of why so little antibody accumulates on the tumor is the dilution effect. Dye injected into a river with a dye-binding site downstream on the shore will only accumulate a small fraction of the material. The mouse to human weight ratio is ~2800, so that, e.g., a 1 g tumor in a mouse with a localization of 20% injected dose per gram (id/g), might be expected in a human to achieve 1/2800 of this, or 0.007% id/g, due to antibody dilution; this is in the range found by clinical trials (Sands, 1988). The injected immunoconjugates are spread far and wide and once passing through tissue endothelium, may reside there during the course of study. Another factor is the antibody itself, its binding constant, the heterogeneity of tumors, and cross reactivity with other tissues. Since mouse monoclonals are typically used, there is the problem of human anti-mouse antibody

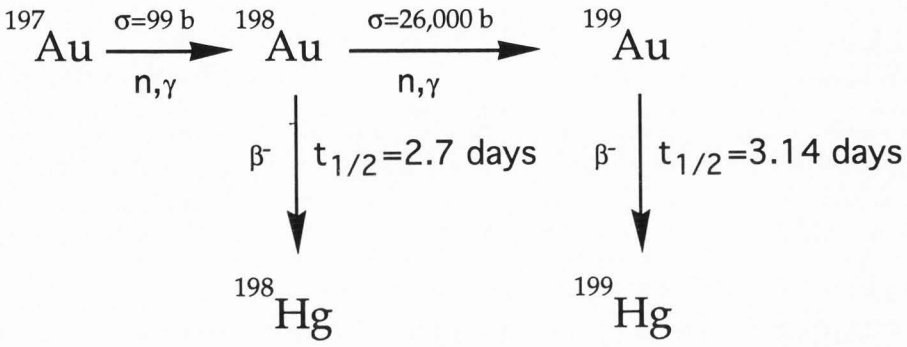

Figure 4. Production of ^{198}Au and ^{199}Au from neutron irradiation of natural gold.

forming (the HAMA response), which may hinder multiple treatments. The search for better antibodies, their genetic manipulation and humanization has improved this aspect to some extent (Buist *et al.*, 1995). Unfortunately, with all of these negative factors, adequate tumor to non-tumor ratios for effective therapy in humans has generally not been achieved.

Radiogold immunotherapy of bladder carcinoma, first proposed by de Harven *et al.* (1992), avoids most of the above difficulties and is discussed later.

Results Using Radioactive Gold

Preparation of low-specific activity Au-198

We have studied the use of gold as the radioisotope for potential therapy (Hainfeld *et al.*, 1990). The first aspect is the production of the radioactive gold. Neutron irradiation of clusters or antibody-gold conjugates directly was avoided, since samples must be dried, sealed in a vacuum, and beam heating may approach 400°C. Gold metal was therefore irradiated, and synthesis of gold clusters or gold colloids was done after activation. The following activation reaction (Fig. 4) describes the preparation of ^{198}Au from natural Au-197:

Irradiation times used were typically 2 to 8 hours at a neutron flux of 8 x 10^{14} neutrons/cm^2·s; the sample size was 2 mg, and the product was low-specific activity gold, ~80 mCi/mg, which is equivalent to ~1 in 3,000 of the gold atoms being radioactive. About 90% of the radiogold produced is ^{198}Au, and about 10% ^{199}Au. Although the radioactive gold eventually decays to mercury, the amount of mercury produced *in vivo* for therapeutic doses of gold is far below chemical toxicity levels.

In vitro tumor cell binding of gold immunoconjugates

Since much of our work was directed toward intravenous administration of the immunoconjugate for targeting of carcinomas, the use of gold colloids was avoided, since other colloids are known to be rapidly cleared from the blood by the liver. Gold atoms do not bind to the standard chelates (such as DTPA) used in this type of work with other radiometals, but covalent linking of gold clusters has been demonstrated to be feasible and stable, and they can be prepared in high yield.

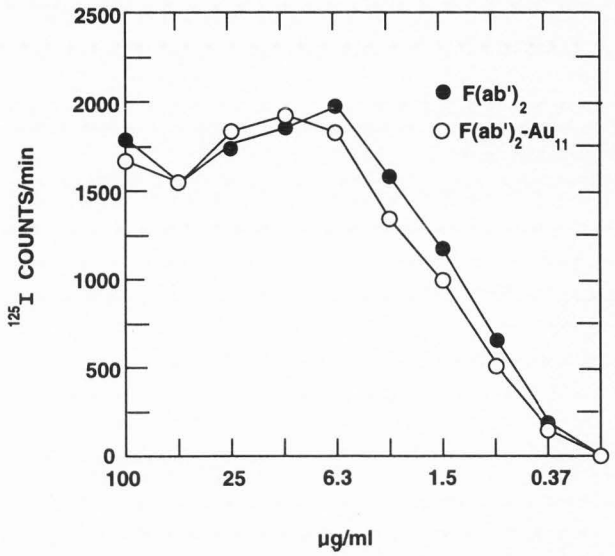

Figure 5. Cell binding assay using 17-1A $F(ab')_2$-Au_{11} compared to native $F(ab')_2$.

Undecagold clusters covalently bound to IgG, $F(ab')_2$, or Fab' fragments were tested. The monoclonal antibody 17-1A, reactive with human colon carcinomas was used and the target cells were LS180; control cells were a melanoma line, WM164. The gold labeled antibodies showed high retention of immunoreactivity on cell binding assays: 76% for Fab'-Au_{11}, 80% for $F(ab')_2$-Au_{11}, and 83% for IgG-Au_{11}, using non-radioactive gold and a second antibody (goat anti-mouse) labeled with ^{125}I. An example is shown in Figure 5. In these second antibody assays, the labeling with gold cluster was close to 100% (one gold cluster per antibody), so that the results should, in fact, be measuring activity of the conjugate and not of the native antibody.

Table 2. Cell binding of radiogold antibody conjugates to tumor and control cells. Results are expressed as the percent of applied radioactivity remaining with the cells after washing.

Sample	% bound to tumor cells	% bound to control cells
Fab'-Au_{11}	7.8 ± 0.5	1.9 ± 0.2
$F(ab')_2$-Au_{11}	16.1 ± 0.1	0.3 ± 0.1
IgG-Au_{11}	14.3 ± 0.9	0.8 ± 0.1
Au_{11}	0.5 ± 0.1	0.5 ± 0.1

In vitro cell binding of radioactive undecagold immunoconjugates

Next, radioactive gold clusters were used, and the binding to tumor and control cells was measured by γ-counting the gold directly (no second antibody was used). Microtiter plates were used that contained equal numbers of cells with various dilutions of undecagold immunoconjugates. After incubation with the cells for 1 hour, the plates were centrifuged, the supernatant removed, and fresh buffer applied (radioimmunoassay buffer, or "RIA", which is phosphate buffered saline plus 5% horse serum). Cells were resuspended, then spun again. Three such washes were done so as to remove any unbound antibody/gold. Since the total amount of gold applied was known, the remaining bound gold may be quantified. Typical results are shown in Table 2.

Good specificity is shown, and was maximal for the $F(ab')_2$-Au_{11}. The Au_{11} by itself showed only a background level, and did not appear to have any significant cellular uptake. The reasons that the specific uptake is not 100% are: the antibody-antigen binding is in equilibrium, immunoreactivity being < 100%, incomplete labeling and competition by native antibody, or other factors. Cell binding of 15-20% is not uncommon for many radiolabeled antibodies in this form of assay.

In vivo targeting of radioactive gold cluster (Au_{11}) immunoconjugates in nude mice with human tumor xenografts

A second level of testing for tumor targeting is to inject the preparation into mice, usually intravenously, through a tail vein. After various times, the mice are euthanized (killed) and dissected, and the different tissues and organs are placed in separate containers, weighed and counted. This then gives the biodistribution *in vivo* of the radioisotope. Time points are usually 24 and 96 hours, with additional points as necessary. Three or four mice are used per data point for statistical accuracy. Nude mice (which are immunodeficient) with the target human tumor implanted (a xenograft) are used to determine tumor localization. Human tumor cells grown in culture are injected subcutaneously, usually in the shoulder area, and after 2 to 3 weeks develop into a solid tumor mass about 0.5 g in size, complete with vascularization. While not a perfect model, it does permit human tumors to be used *in vivo* with the appropriate anti-human tumor antibody. Radiolabeled antibodies generally reach a maximal tumor uptake at ~20 hours (Andrew *et al.*, 1990), with slow washout after several days, whereas other tissues generally show quicker depletion, although liver and bone can show stable uptake; because of these pharmacokinetics, time points a few minutes or hours after injection are not usually taken.

Radioactive undecagold clusters covalently conjugated to monoclonal antibody 17-1A injected into nude mice with human tumor xenografts gave the biodistributions shown in Table 3. ^{111}Indium-DTPA conjugate results are also shown for comparison using the same antibody.

From these data, several features are evident: the localization of undecagold (^{198}Au)-antibodies generally mimicked the distribution of the ^{111}indium conjugate, but bone uptake was significantly lower with the gold (2.9 times less for the IgG conjugate). Also, the tumor localization and the tumor to non-tumor ratios were better for the $F(ab)'_2$-Au_{11} than the $F(ab)'_2$-In. Both conjugates showed high kidney accumulation, especially for the antibody fragments, partially because of their small size; however, the gold showed higher kidney uptake in all cases. Effective therapy could be achieved with tumor to non-tumor ratios of 10-50; here values of 0.5 to 11.6 were obtained with the IgG-Au_{11}, thus falling short of the required distribution. Generally speaking, this has been the experience with immunotherapy, both with radioisotopes and toxin conjugates: biodistributions are not favorable enough.

Use of multiple gold atom clusters and high-specific activity ^{199}Au

One aspect of the use of gold clusters is that more than one radioactive atom could be delivered per antibody. This could be very important since most radioimmunotherapy trials have failed due to poor tumor to non-tumor ratios. In simple one-step delivery schemes, where the radiolabeled antibody is administered, putting more radioisotopes per antibody would not be expected to improve the tumor to non-tumor targeting. Although more radioactivity may be delivered to the tumor, improved therapy would not be achieved, since more doses would also be delivered to non-tumor tissues. Generally, the tumor antigen sites are not saturated, and the additional dose could be just as well delivered by more

Table 3. Biodistribution in nude mice with human tumor xenografts 24 hours after injection of radiogold and ^{111}In conjugates. Values are expressed as percent injected dose per gram of tissue.

Tissue	Fab'-Au_{11}	F(ab)'$_2$-Au_{11}	F(ab)'$_2$-In	IgG-Au_{11}	IgG-In
Tumor	6.4 ± 1.1	8.4 ± 0.6	5.0 ± 3.2	8.0 ± 3.2	11.1 ± 1.7
Blood	3.4 ± 0.3	5.6 ± 0.4	5.8 ± 0.2	3.2 ± 0.6	4.9 ± 2.2
Spleen	2.8 ± 0.4	6.4 ± 0.8	7.2 ± 0.1	9.5 ± 0.3	13.9 ± 6.4
Stomach	0.57 ± 0.14	0.93 ± 0.19	1.0 ± 0.3	0.69 ± 0.20	1.1 ± 0.3
Kidneys	103. ± 32.	117. ± 20.	42.0 ± 2.1	17.1 ± 3.1	10.2 ± 0.5
Heart	1.7 ± 0.1	3.2 ± 0.4	3.5 ± 0.3	2.6 ± 0.4	2.9 ± 1.0
Lung	2.5 ± 0.04	4.6 ± 0.1	5.1 ± 0.8	3.4 ± 0.9	4.1 ± 0.8
Muscle	0.48 ± 0.06	0.98 ± 0.08	1.0 ± 0.1	0.80 ± 0.23	0.90 ± 0.17
Bone	0.97 ± 0.02	2.4 ± 0.2	3.4 ± 0.7	2.2 ± 0.3	6.4 ± 1.8
Gut	1.3 ± 0.1	1.6 ± 0.2	1.8 ± 0.3	1.1 ± 0.3	2.0 ± 0.3
Liver	5.6 ± 0.5	10.5 ± 0.4	10.6 ± 0.7	4.6 ± 0.7	7.9 ± 2.3

Table 4. Irradiation conditions and radiochemical yields for no-carrier added ^{199}Au Production.

Target	Irradiation Time (d)	Irradiation Yield (EOB)	Radiochemical Recovery*
^{198}Pt (3.98 mg)[b]	3.43	34.63 GBq (936 mCi)	60%
^{198}Pt (6.00 mg)[b]	5.00	77.03 GBq (2082 mCi)	85%
^{198}Pt (6.20 mg)[b]	5.00	102.1 GBq (2761 mCi)	98%
^{198}Pt (9.94 mg)[c]	3.97	26.46 GBq (751 mCi)	96%

[b]HFIR, Oak Ridge National Lab (f_n = 0.95 - 2.15 x 1015 n/cm^2·sec)
[c]HFBR, Brookhaven National Lab (f_n = 4.20 x 1014 n/cm^2·sec)
*Measured after Au/Pt separation.

$$^{198}\text{Pt} \xrightarrow[n,\gamma]{\sigma_n=3.8\text{ b}} {}^{199}\text{Pt} \xrightarrow[t_{1/2}=30.8\text{ m}]{\beta^-} {}^{199}\text{Au} \xrightarrow[t_{1/2}=3.14\text{ days}]{\beta^-} {}^{199}\text{Hg}$$

Figure 6. Preparation of high-specific activity ^{199}Au.

singly labeled radioantibody being administered, if desired. However, more selective targeting schemes that seek to improve tumor to non-tumor ratios, e.g., using 2 and 3 step procedures (A targets tumor, then radioactive B targets A), generally suffer from a loss of total tumor uptake of the radioisotope, due to limiting antigens or shorter uptake time periods. Packing more radioactive atoms per antibody could overcome this problem. Gold clusters provide a way of attaching 11 or 67 gold atoms covalently to antibodies without significant loss of immunoreactivity.

Another issue inherent in achieving this objective of delivering more radioactive atoms per antibody, is that the specific activity of the atoms must be high, or near no-carrier-added (NCA) material must be used. The previous method described for preparing Au-198/199 from direct neutron irradiation of natural gold is not suitable for making high-specific activity gold.

Preparation of high-specific activity ^{199}Au

The highest specific activity Au (~208 mCi/μg) can be achieved by irradiating enriched ^{198}Pt (Fig. 6). Only ~10^{-5} is converted to ^{199}Au, and the gold must therefore be purified from the platinum. The amount of ^{199}Au needed for a typical set of experiments is at least 300 mCi, or 1.5 μg of gold at 208 mCi/μg. The production scheme was designed around these criteria.

Methods to purify the Au from the Pt were quantita-

tively explored using various extraction and chromatography schemes (Anderson *et al.*, 1988; Kolsky and Mausner, 1993). A tributyl phosphine (TBP)-coated silica column gave the best (and most rapid) separation and radiochemical yields were > 96% with a > 10^5 separation factor (Table 4). Yields were improved from 60 to > 96% by making the silica support media used in the separation hydrophobic. Because traces of TBP were found in the final solution (15 ml, 14 N HNO_3), the solutions are now evaporated to dryness on a hot plate to remove the TBP, and the ^{199}Au then is redissolved in aqua regia. Apparatus was built to handle this separation remotely, and is now used routinely to produce very pure NCA ^{199}Au (~2 Ci at 208 μCi/μg per run).

Specific activities were calculated for all of the production runs. They averaged ~98% (205.6 μCi/μg) of the theoretical value (208 μCi/μg).

Synthesis of clusters using 1 μg of gold

To test high-specific activity ^{199}Au (where nearly all gold atoms are radioactive), it is necessary to use ~2,000 fold smaller reaction amounts than used previously. Otherwise, the radioactivity would exceed the 1 Curie level and be very difficult to handle. Previously, 2 mg of gold was typically used for a test run. Scaling reactions back by 2,000 introduces new uncertainties: will the products form efficiently, and can lower concentrations and volumes be handled without undue losses? Also, we used a precipitation step to isolate AuCN, but with such small amounts (1 μg of starting gold metal), precipitates are more difficult to form and recover.

The synthesis was changed to reduce $HAuCl_3$ directly with a triphenylphosphine to the Au^{+1} necessary to form clusters rather than producing AuCN from $HAuCl_3$. Three microliter reaction volumes were used and a 75% conversion of gold metal to gold clusters was obtained. This was a higher efficiency than we achieved earlier and the time for formation and purification was cut from 1 day to 2 hours. Labeling of a test antibody yielded ~80% coupling. All of these tests used non-radioactive gold, but were then extended with similar results to ^{199}Au.

Testing the stability of high-specific activity $^{199}Au_{11}$ clusters: they are remarkably stable

If all gold atoms are radioactive in the Au_{11} cluster, then is this cluster stable? Since the cluster contains eleven radioactive atoms, when one decays (^{199}Au has a 3.1 day half-life), does the recoil break apart the cluster, or does the resultant mercury atom make the cluster chemically unstable? Early disintegration of the cluster would cloud its use as a therapeutic agent.

The stability of high-specific activity ^{199}Au was assessed by gel filtration chromatography: various times after preparation, the clusters were chromatographed on a column with a 3,000 molecular weight exclusion limit. Since the weight of the Au_{11} cluster is ~5,000, any breakdown would be shown by trailing peaks. The high-specific activity cluster was tested immediately after its initial synthesis and purification, at which time it behaved normally (identical to a non-radioactive gold cluster). An aliquot was tested after 1 day and after 3 days; there was about a 5% degradation after 3 days. For comparison, the high-specific activity ^{199}Au was mixed with an 11-fold excess of non-radioactive gold, so that upon cluster synthesis, each Au_{11} cluster contained only one radioactive gold atom (rather than 11). This cluster showed virtually no change after 3 days.

These experiments demonstrated acceptable stability of high-specific activity clusters, an important step in validating the potential use of gold clusters for therapy.

High-specific activity ^{199}Au cluster-antibody conjugates: cell binding

At end of bombardment (eob), ~98% of the gold atoms are radioactive. After separating out the ^{198}Pt target, the gold was synthesized into undecagold clusters (Au_{11}, each containing 11 gold atoms). These were then covalently bound to 17-1A monoclonal antibody (whole IgG). After column chromatography purification, the conjugate was tested by incubating with human colon carcinoma cells (from cell culture) and with another human cell line as a control. After washing, the cells were counted to find the amount of radioactive gold delivered to each target. Serial dilutions were done to obtain a binding curve and to vary the antigen-antibody ratio.

Excellent results were obtained which showed up to 41.3% of the applied radioactivity bound to the tumor cells, with about 2-4% binding to the non-specific cells. This demonstrates the high immunoreactivity retained by the gold cluster conjugate. In particular, it shows that the use of high-specific activity clusters can deliver more radioactivity to tumor cells than with other conventional techniques, which may have important therapeutic implications.

For comparison, radioactive clusters were prepared that had 1/100th the specific activity, so that about 1 out of 100 gold atoms was radioactive. In an 11-atom gold cluster, then no more than one of the gold atoms was radioactive. This was done to see if additional problems occurred with the high-specific activity clusters; namely, did they break down more rapidly, did they damage the antibody more and cause greater loss of immunoreactivity and other such questions. The lower specific activity preparation run in parallel demonstrated a high of 51.4% binding to tumor cells with background non-specific attachment at again 2-4%. The results were very similar to the high-specific activity case and demonstrated that, under these conditions, there seem to be no adverse

Table 5. Cell binding of high and low-specific activity radioactive gold-antibody conjugates.

^{199}Au	tumor cells (% Au bound)	non-tumor cells
Low-specific activity	27	0.5
High-specific activity	37	3.9

Table 6. Biodistribution of high- and low-specific activity undecagold conjugates with MAb 17-1A at 24 hours after injection.

Tissue	Low-specific activity (% id/gm)	High-specific activity
Liver	5.2	4.4
kidney	10.3	9.9
lung	3.6	1.5
muscle	0.8	0.6
bone	1.8	2.1
tumor	6.0	4.9

effects from use of the high-specific activity clusters.

Another set of experiments using 17-1A cell binding is shown in Table 5. These results showed good activity of the conjugates with minor differences between high- and low-specific activities. The high-specific activity gold had higher uptake, but also higher background, perhaps due to radiolytic effects or some degradation.

High-specific activity ^{199}Au cluster-antibody conjugates: serum stability

Another test that a potential therapeutic immunoconjugate must pass is that it be stable in serum. The high-specific activity gold cluster conjugates along with the 1/100 specific activity cluster conjugates (described above) were incubated with fresh mouse serum and at various times later passed over a gel filtration column. If the radioactivity stayed with the IgG, the counts would appear at the IgG column retention time. If the gold-IgG conjugate had broken down in some way, some radioactivity most likely would appear at other retention times, indicating perhaps binding to serum albumin, other blood components, or free gold. Both of these preparations demonstrated excellent serum stability with ~95% of the activity remaining with the IgG peak even after 96 hours. Furthermore, the high-specific activity preparation showed virtually identical behavior to the 1/100 specific activity conjugate. These results indicate that serum stability of the gold clusters was acceptable.

High-specific activity ^{199}Au cluster-antibody conjugates: tumor animal biodistributions

Nude mice were used with human colon carcinoma xenografts. In these tests, the gold cluster conjugates were again prepared at high-specific activity (all gold atoms radioactive at eob) and 1/100 that activity for comparison. Distribution was normal for an IgG conjugate in the blood, dropping from 7.5% id/g at 24 hours to 1.0% at 96 hours. At 24 hours, the biodistribution obtained is shown in Table 6 (using MAb 17-1A). Almost identical results were obtained with high- and low-specific activity gold, indicating that the high-specific activity gold immunoconjugates perform well and are not subject to any significant additional degradation *in vivo*.

Compared to the well known In-DTPA conjugate with the same antibody (17-1A, see Table 3), the gold results were: blood 1.5x more for Au, spleen 3.0x lower for Au, stomach 1.3x lower for Au, liver 1.6x lower for Au, kidney same, heart 1.1x more for Au, lung 1.1x lower for Au, muscle 1.1x lower for Au, bone 3.6x lower for Au, tumor 1.9x lower for Au. In summary, most organs showed typical IgG immunoconjugate distributions with the spleen, liver, and bone with notably lower background using gold clusters. The tumor, however, showed lower accumulation than the In conjugate in these tests. Nevertheless, the tumor to bone ratio was 1.7 for the In and 3.3 for the Au, giving a 1.9x improvement with the use of gold clusters for this most radiosensitive tissue. These results are therefore encouraging, and tumor localization might be improved by using different antibodies.

Blocking of non-specific sites by pre-injection of non-radioactive gold clusters

Radioactivity found in other tissues besides the tumor may be due to a number of factors: the antibody may have cross-reactivity, simple dilution and diffusion, metabolism of the conjugate, breakdown of the isotope from the antibody so it is no longer tumor-targeted, opsonization, aggregation of the conjugate which usually leads to liver and spleen uptake, and stickiness of the radioisotope and/or chelate moiety to non-specific areas. In the case of gold clusters, it may be that one of these factors, namely some affinity of the cluster itself for tissues, may lead to increased backgrounds. We, therefore, postulated that a pre-injection of non-radioactive gold clusters may saturate these secondary sites and reduce background when the radioactive cluster-antibody was later injected. First, animals were tested for any acute toxicity caused by intravenous gold cluster injection. It was found that death resulted in some animals at a level of 100,000 times the amount of gold that would be injected in a typical immunotherapy trial.

Table 7. Changes in biodistribution at 24 hours with a preinjection of non-radioactive gold cluster (Au_{11}).

Tissue	Percent change with preinjection
blood	+52
spleen	−10
stomach	+9
intestine	+200
liver	−38
kidney	−39
heart	−1
lung	−18
muscle	+5
bone	−12
stumor	+8
carcass	+35

Non-radioactive gold clusters were, therefore, injected at a level of 10,000 over the antibody conjugated gold. The radioimmunoconjugate was injected 1 hour after the blocking gold. This resulted in the following changes in non-specific organ uptake 24 hours after injection, shown in Table 7. This shows an improvement for most tissues, especially the liver and kidney (38 and 39% decrease, respectively). However, the intestine (a radiosensitive organ) localization increased by a factor of 2. We can conclude from this study that some tissue backgrounds can be significantly reduced by a pre-injection blocking procedure.

Dosimetry calculation for ^{199}Au immunoconjugates

Dosimetry calculations, that integrate radiation over time and volumes, are important to predict the total dose to a tumor versus that delivered to other organs and whole body. This quantifies the results into terms that assess whether a particular immunoconjugate will be successful in therapy or whether normal tissue tolerance will be exceeded before enough doses are delivered to the tumor. Dr. Barry W. Wessels (George Washington University) has calculated maximal tumor dose achievable for radioactive gold.

Calculated tumor doses using the Wessels and Rogus dosimetry formalism (Wessels and Rogus, 1984), with $^{199}Au_{11}$ "theoretically" conjugated to IgG, F(ab')$_2$, and Fab are shown in Table 8. This uses a very good distribution obtained by Beaumier with another antibody (NR-CE-01) and isotope (^{125}I), and doses are calculated by substituting the properties of ^{199}Au for ^{125}I (Yorke *et al.*, 1991). From those data, ^{199}Au was substituted as the isotope used to give the doses to the tumor shown

Table 8. Calculated dosimetry of ^{199}Au using biodistribution obtained for ^{125}I-NR-CE-01 antibody. Dose to tumor expressed in cGy (1 cGy = 1 rad).

	IgG	F(ab)$_2$	Fab
at 300 cGy to whole body	1050	3780	2880
at 1200 cGy to kidney	4400	4500	1600

in Table 8. From these "extrapolated" data, it appears that ^{199}Au is very close to being useful in therapy, with the F(ab)'$_2$ conjugate being the best choice. (A dose to the tumor of 5,000 cGy is desirable). It should be stressed that this calculation is inaccurate, since ^{125}I will behave differently than ^{199}Au, and so may the antibody. It is shown, however, to at least give some idea of dosimetry with ^{199}Au *in vivo*.

Recalculation using other therapeutic isotopes showed radioactive gold to be one of the 7 best choices when compared to all other isotopes for therapy. Due to its shorter beta range, it should be the isotope of choice for treating micrometastases and small cell lung carcinoma. Treatment of metastases is, in fact, probably the best use of immunotherapy, where the antibodies can track down spreading cells missed by surgery.

Using our actual biodistribution collected with 17-1A IgG conjugated to $^{199}Au_{11}$, the dose to tumor at bone marrow max tolerated dose (MTD) was calculated at 1000-2000 cGy (Wessels, personal communication); this falls short of the requirements for therapy.

Reduction of kidney uptake

One feature of the gold clusters is the ability to alter the organic shell surrounding the core of gold atoms. This may be important in designing a material that has improved biodistributions, namely that the uptake in non-specific organs is reduced. Since the N-methylbenzamide-derivatized triphenylphosphines that we have normally used have given generally higher kidney accumulation of gold clusters and their conjugates than is usually seen with DTPA conjugates, another derivative was sought that would reduce kidney retention.

The elevated kidney uptake of gold clusters may be due to the thiol content of kidney tissue and its strong interaction with gold atoms. Another possibility is the ionic interaction, which has been studied by others who have shown that changing the isoelectric point by simple chemical modification of antibodies (e.g., by acetylation), alters kidney uptake (Tarburton *et al.*, 1990). We synthesized a cluster containing 21 COO^- groups on its periphery and found a dramatic effect on biodistribution (of cluster alone, no antibody attached; shown in Table 9). Significant decrements of uptake in liver and kidney

Table 9. Radioactive Au_{11} tissue distributions for cluster with 1 amino group (+1 charge), and 21 carboxyl groups (-21 charge), 24 hours after injection. No antibody was attached.

Tissue	Au_{11} (+1 charge) % id/gm	Au_{11} (-21 charge)	per cent change
blood	1.9	2.7	+42
spleen	7.5	3.5	—53
stomach	0.6	0.9	+50
intestine	1.3	1.6	+23
liver	48.0	5.4	—89
kidney	78.2	17.2	—78
heart	1.9	2.5	+31
lungs	4.9	4.3	—12
muscle	0.6	0.8	+33
bone	3.1	1.6	—48

Table 10. Biodistributions of radioactive Au_{11} conjugated to antitumor antibody using clusters that had no charge versus those with a -19 charge (carboxylated cluster).

Tissue	Au_{11} (0 charge)-Mab (% id/gm)	Au_{11} (-19 charge)-Mab
liver	10.5	9.2
kidney	25.3	26.9
bone	2.5	1.6
tumor	6.8	5.8

(89 and 78% reductions, respectively) were found with the negatively charged cluster.

The immunoconjugate using this carboxylated cluster was tested to see if significant uptake in non-tumor tissues (especially liver and kidney) could be effected. To this end, two different radioactive clusters were synthesized and coupled to a monoclonal (BR-15-6A IgG). Table 10 gives tissue distributions (mice, after 24 hours).

No significant changes were observed in this test; therefore, we conclude that use of the carboxylated cluster has little effect on the biodistribution when attached to IgG.

We also tested radioactive colloidal gold immunoconjugates, and although these were cleared by the reticuloendothelial system (RES), they showed virtually no kidney uptake, typically 0.5-1.4% id/gm. If RES uptake could be blocked, colloidal gold may provide a viable method of delivery since it avoids one of the problems with the gold cluster work, namely high kidney uptake. On the other hand, the liver is fairly radioresistant, and whether blocking the RES improves dosimetry remains to be seen.

Synthesis of other gold clusters to improve biodistributions

The gold clusters we have been using so far are made with triphenylphosphine derivatives such as the organic shell around the solid gold atom core. Even though these clusters can contain charged or polar groups and are very water soluble, the phenyl groups do impart some hydrophobic character. This may contribute to some unwanted *in vivo* binding. Therefore, we have investigated the synthesis of alkyl phosphine clusters, which are extremely hydrophilic. These are being studied further.

Single gold atom compounds: quest to improve biodistributions

In addition to the cluster approach, smaller, single gold atom compounds may be made which can be covalently coupled to antibodies. While not delivering multiple radioactive atoms per antibody, as with clusters, this method would still use ^{199}Au and retain the benefits of this isotope. Accordingly, several mono-gold compounds were evaluated. One class:

$$Au[(RPh)_2 - CH_2CH_2 - P(PhR)_2],$$

where R was COO^- or NH_2 and Ph = phenyl, was synthesized using radioactive gold. This class cleared the blood slightly faster than $Au_{11}(COO^-)_{21}$ but showed either higher kidney or liver accumulation. Other mono-gold compounds are being studied.

Colloidal gold

***In vivo* targeting of radioactive 15 nm gold colloid immunoconjugates in nude mice with human tumor implants** We have also tried intravenously administered radioactive colloidal gold-monoclonal antibody conjugates. One might reject this approach a priori, since it is known that colloidal material accumulates in the liver, spleen, bone marrow, and lungs (the target is somewhat determined by the size of the colloid). Also, the antibodies are adsorbed to the gold particles instead of being covalently attached, and may be less stable *in vivo* than the covalent conjugates. Nevertheless, colloidal gold conjugates are widely used and have a potential advantage: each gold particle contains many gold atoms, e.g., ~60,000 for a 15 nm particle, and so could deliver a much larger dose per antibody than with single isotope-

Table 11. Mouse biodistributions using ^{198}Au-15 nm colloidal gold with either tumor-specific antibody or BSA adsorbed (values are expressed as % injected dose per gram).

	24 hours		96 hours	
Tissue	F(ab)'$_2$-Au	BSA-Au	F(ab)'$_2$-Au	BSA-Au
Tumor	0.28 ± 0.27	0.11 ± 0.08	0.03 ± 0.01	0.04 ± 0.02
Blood	0.023 ± .001	0.023 ± .002	0.016 ± .003	0.017 ± .005
Spleen	16.5 ± 2.5	11.1 ± 2.6	14.3 ± 0.4	12.2 ± 2.1
Stomach	0.38 ± 0.35	0.60 ± 0.69	0.05 ± 0.01	0.15 ± 0.15
Kidneys	1.10 ± 0.33	0.57 ± 0.05	0.73 ± 0.11	0.52 ± 0.07
Heart	0.15 ± 0.08	0.22 ± 0.01	0.15 ± 0.05	0.28 ± 0.08
Lung	0.88 ± 0.04	1.53 ± 0.23	1.40 ± 0.10	1.34 ± 0.19
Muscle	0.06 ± 0.03	0.06 ± 0.03	0.06 ± 0.03	0.04 ± 0.01
Bone	4.79 ± 0.23	3.82 ± 0.39	4.47 ± 0.57	4.16 ± 0.41
Gut	0.15 ± 0.07	0.15 ± 0.07	0.14 ± 0.05	0.16 ± 0.06
Liver	123. ± 10.4	81.1 ± 4.5	111.8 ± 4.1	76.2 ± 0.7
Carcass	0.84 ± 0.21	0.57 ± 0.04	0.71 ± 0.09	0.85 ± 0.23

single antibody conjugates. Anti-CEA F(ab)'$_2$ - 15 nm ^{198}Au was used and a BSA-15 nm ^{198}Au conjugate was also injected (no antibody) as a control; Table 11 shows the results. From these data, it is clear that the colloidal gold conjugates clear the blood quickly, and that there is not much change in biodistribution from 24 to 96 hours. An exception to the stable localization is the tumor, where wash-off was substantial between 24 and 96 hours. It may also be noted that the specific conjugate with antitumor F(ab)'$_2$ and the (control) BSA had almost identical distributions, except at 24 hours there was more gold in the tumor with the specific antibody. It appears that the tumor binding or gold-antibody binding is not stable *in vivo* over time, which would account for the almost complete loss of gold from the tumor at 96 hours. As expected, the colloidal gold is largely accumulated in the liver and spleen, and secondarily in the bone and lung. As with most experiments, many other controls could be done; here, a non-specific antibody conjugated to the gold would be an interesting comparison, but was not done in this pilot study.

In comparison to the undecagold conjugates, the Au_{11} was 243 times higher in the blood at 24 hours, and had a lower amount in the liver by ~12 times; Au_{11} was 2 times lower in the bone; kidney accumulation was ~16 times higher for the Au_{11} {for IgG; worse for Fab' and F(ab)'$_2$}, and the tumor localization was 30 times higher with the Au_{11}. We may conclude from this study that the *in vivo* vascular use of colloidal gold immunoconjugates is much worse than with the Au_{11} conjugates, and that colloidal gold cannot be considered for intravenously administered immunotherapy applications, in its present formulation, since it falls far short of the necessary requirements.

Bladder carcinoma

A special case that avoids many of the intravenous delivery problems associated with antibody targeting *in vivo* was proposed by de Harven *et al.* (1992) for the therapy of superficial urinary bladder carcinoma: a radiogold antibody conjugate would be intravesically instilled via catheter into the bladder for 0.5 to 1 hour, then washed out, so only that taken up by the cells would remain. De Harven *et al.* (1992) found that biopsied tumor cells from patients took up 16 nm - Mab 48-127 antibody conjugates rapidly; non-tumor cells showed virtually no uptake. Gold particles were found not only on the surface of the tumor cells, but also in the cytoplasm. This internalization of surface antigen/antibody complex has been noted with many (but not all) tumors/antigens/antibodies. Quantification of this uptake was done by counting gold particles in thin sections, leading to a calculation of 1,000 to 12,700 gold particles per cell (E. de Harven, personal communication). In this application, one could consider using 16 nm colloidal gold conjugates, as well as other colloidal gold sizes, or the covalent gold clusters, undecagold and Nanogold. Each formulation is different and it is difficult to predict a priori which will perform the best. *In vivo* stability of these conjugates is important and needs further study. It was found, however, that the performance of the colloidal gold conjugate was unaffected by urine pH

conditions (E. de Harven, personal communication).

A pilot study with another antibody and ^{111}In has been reported using intravesical administration. Although the dose calculated that might be delivered by this antibody from the uptake measured fell below therapeutic usefulness, virtually no radioactivity was detected in the general circulation (Bamias *et al.*, 1993).

As with most antibody delivery systems, the antibody itself strongly dictates the performance of the conjugate. MAb 48-127 was developed by Fradet *et al.* (1986) after considerable screening. It is highly specific for bladder tumor cells, with negligible reactivity with normal bladder epithelium. It targets the glycoprotein gp 54 (molecular weight = 54 kD) specifically expressed on bladder tumor cells. De Harven *et al.* (1992) have provided scanning electron microscopic evidence that this antibody reacted uniquely with superficial urothelial cells with microvilli (i.e., the transformed cells), and not with normal urothelial cells, thus making it attractive for therapy.

Dosimetry for colloidal gold

An advantage of colloidal gold for therapy is its size. A 15 nm gold particle contains ~60,000 gold atoms and each immunoconjugate molecule may, therefore, deliver up to 60,000 times the dose of single antibody-radionuclide conjugates typically used. An estimate of the dose delivered to the tumor cells is as follows: since the number of colloidal gold particles per tumor cell was measured to be between 1,000 and 12,700, an average of 5,000 is used here for this calculation. Assuming a 1 g tumor mass, this would contain ~8 x 10^9 cells. The number of gold atoms contained therein would be 5,000 x 60,000 times this, or 2.4 x 10^{18}, or 8 x 10^{-4} g of gold. The total average β energy absorbed in tissue for an isotope depends upon the value of δ (gm·rad/μCi·hr), which is 0.664 and 0.182 for ^{198}Au and ^{199}Au, respectively (Kocher, 1981). Assuming low-specific activity ^{198}Au was used, 8 x 10^4 μCi/mg, the dose to the tumor would be in 24 hours:

Dose (in rad) = d · μCi·hr/gm

= 0.664 · (8 x 10^4 μCi/mg) (0.8 mg gold) (24 hr)/ (1 gm tissue);

so: Dose = 1 x 10^6 rad.

If high-specific activity gold (^{199}Au) were used, this would be 7 x 10^8 rad. Since the effective tumoricidal absorbed dose is ~10,000 rad (Bigler *et al.*, 1988), the dose delivered by the gold particles exceeds this by a factor of 100 for the low-specific activity, or 70,000 for the high-specific activity. For a 15 nm gold particle, a therapeutic dose of 10,000 rad can, therefore, be delivered by 50 particles per cell for low-specific activity gold, or 0.07 particles per cell for high-specific activity gold.

Although it appears easily possible to deliver tumoricidal doses by the means proposed, effective therapy is usually limited by tumor to non-tumor ratios. This calculation merely shows that 15 nm radioactive gold immunoconjugates are very potent, and it must be thoroughly demonstrated that they can be kept out of healthy tissue to prevent its excessive damage.

Another consideration is the dose to normal tissue incurred by the instillation of radioactive conjugate and irradiation during the incubation time. Since the range of the β's is short, only a shell in contact with the urothelium would contribute. A rough estimate would be a few percent of the material for 0.5 hour. If specific uptake was ~1-3% id/g, this would act over the gold's radioactive life, or ~10 days, so that ~200 times the background initial dose should be specifically delivered. More careful dosimetry should be done to calculate this factor.

Tumor cell binding using radioactive colloidal gold conjugates

In order to pursue this, *in vitro* cell binding of radioactive 15 nm colloidal gold conjugates was performed. This was done to obtain a quantitative measure of the binding (or total uptake) of tumor versus non-tumor cells. From these measurements, tumor to non-tumor ratios may be calculated, as well as total radioactive gold delivery. Once these figures are known, a projected dosimetry calculation can be made to estimate the dose delivered to a tumor mass, whether it would be therapeutic, and whether this strategy would be efficacious. It should be cautioned that further animal studies or other *in vivo* experiments are required to fully evaluate this method, since all too frequently, *in vitro* results do not translate directly to the *in vivo* response.

Two mg of gold foil was irradiated in a nuclear reactor, dissolved in aqua regia, dried, dissolved in concentrated HCl, dried, then resuspended in water, to produce low-specific activity gold, 80 mCi/mg, in the form of chloroauric acid. This was used to form 15 nm colloidal gold by the citrate reduction method. Conjugation to MAb 48-127 (specific for human superficial bladder carcinoma) proceeded by adjustment of the pH of the gold to 6-9 (in various experiments), and adding an amount derived from the salt protection graph; 12 μg/ml was used. The conjugate was then further protected with BSA (1% final), and purified from unbound antibody by centrifugation. Cell binding experiments were carried as described earlier. Binding results are illustrated in Figure 7.

Frequently, in this type of assay, the control cell

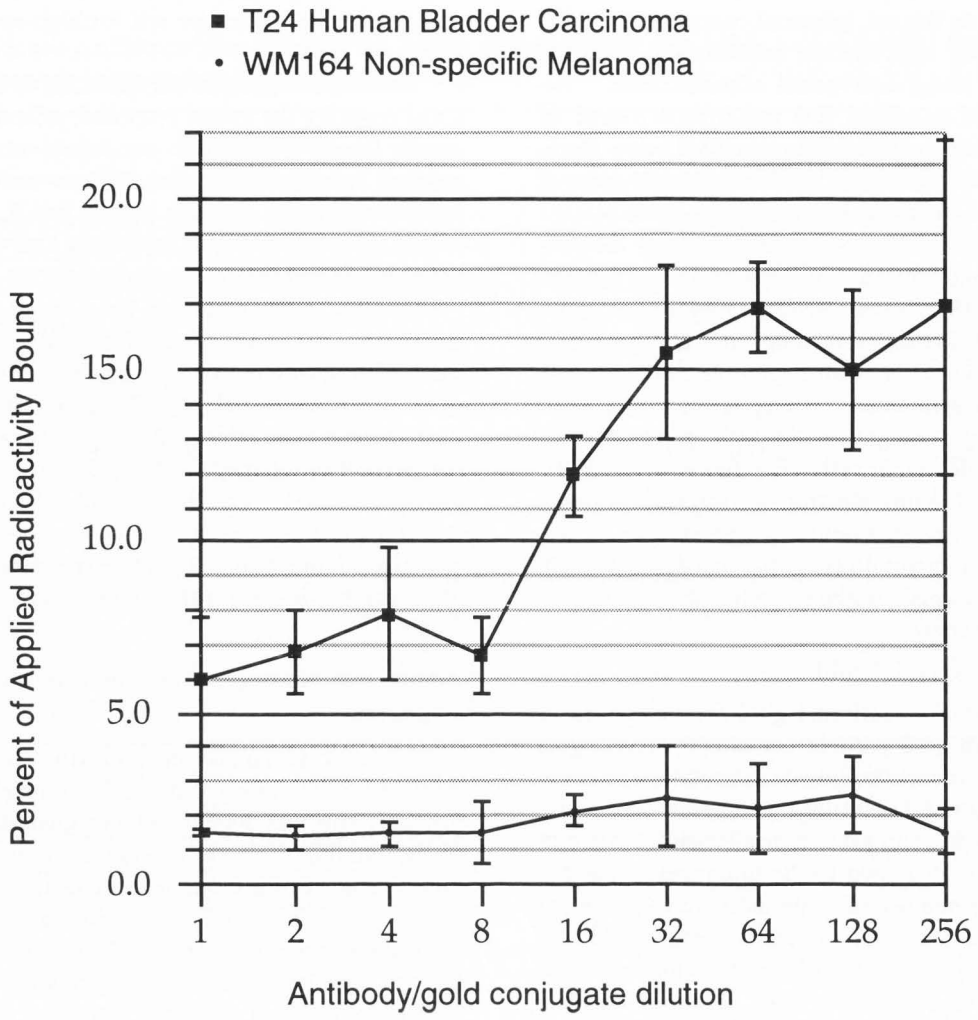

Figure 7. Cell binding of MAb 48-127 adsorbed to radioactive 15 nm ^{198}Au colloid. T24 is the antibody target cell, a human superficial bladder carcinoma, and WM164 is a non-specific human cell line.

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binding remains approximately fixed, as it does here, but the specifically bound antibody shows a more sigmoidal shape with lower binding at low antibody dilution (where there is antibody excess), and an asymptotically approached higher value at high antibody dilution, where there is antigen excess, and nearly all active antibodies can bind.

In this cell binding experiment, ~200 μCi/ml was used; 50 μl was applied to 250,000 cells. Sixteen percent uptake corresponds to ~3,000 gold particles per cell in the first dilution and a dose delivered of 9 x 10^5 rad (in 24 hours). This exceeds the calculated therapeutic level, so less gold or lower specific activity gold could be used. The tumor to non-tumor ratio was ~6.8, so for safe therapy, the dose would have to be reduced to limit that received by normal tissue. It should be pointed out that such *in vitro* results do not directly translate into identical *in vivo* results, and further testing in animal models is required.

Conclusion

Radioactive Au-198/199 has excellent radionuclidic properties for therapy to locally irradiate and kill tumor cells. Methods have been developed to produce and purify these isotopes in high yield. The use of gold coupled to antitumor antibodies has been studied using various gold formulations from single gold atom compounds to gold clusters to gold colloids. Studies were conducted *in vitro* and *in vivo* and indicate some of the problems and promises of the various gold conjugates. Preliminary data suggest that immunotherapy of bladder carcinoma may be feasible using radioactive gold.

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Discussion with Reviewers

Reviewer III: The author states that gold-199 emits a gamma energy suitable for imaging. While gold-198's gamma energy of 412 keV can be imaged, its energy is considerably higher than that normally used for imaging studies.

Author: Gold-198 is not optimal for imaging, but would permit localization studies to be done in a patient before administering high therapeutic doses, using the same isotope and labeling method. Gold-199 may also be used, which has lower gamma energies, 159 and 208 kV (see Table 1).

Reviewer III: The tumor accumulation of a radiolabeled MAb is dependent on the form; while $F(ab')_2$ fragments may yield peak accumulation at 22 hours, it is not the case for intact IgG, which often yields higher levels in the tumor at 48 hours than at 24 hours.

Author: It is true that the smaller antibody fragments localize more quickly, but the actual time seems to be dependent on the antibody (fragment and subclass), animal, and perhaps, even the conjugate moiety. Four antibodies (IgG1, IgG2a and IgG3) were time tested in a mouse with murine tumor, and three showed peak tumor localization at ~20 hours, with one peaking at 48 hours (Andrew *et al.*, 1990). In humans, an anti-melanoma IgG labeled with ^{111}In showed a peak tumor accumulation consistently at 3 days in 5 patients, whereas a ^{131}I labeled Lym-1 anti-lymphoma IgG showed peak tumor uptake after ~27 hours (DeNardo GL, DeNardo SJ, Macey DJ, Mills SL (1988) Quantitative pharmacokinetics of radiolabeled monoclonal antibodies for imaging and therapy in patients. In: Radiolabeled Monoclonal Antibodies for Imaging and Therapy. Srivastava SC (ed.). Plenum Press, New York. pp. 293-310).

Reviewer III: The data presented in Table 2 shows good specificity as claimed in the text; however, the percentage of binding is very low. The vast majority of the activity does not bind to the tumor cells. Has the immunoreactivity of 85% of the MAb been lost when the gold is conjugated to the MAb?

Author: Many MAbs show decreased immunoreactivity when radiolabeled with various chelators and conjugate moieties, compared to low level ^{125}I labeling. Values of immunoreactivity of 15-25% are not uncommon, whereas some antibodies retain high levels (> 75%). The 17-1A test antibody used here unfortunately falls in the first category, but follows along with results seen for many other chelators and metals seen with this antibody.

Reviewer III: A higher number radioactive gold molecules per cluster does not mean that there will be a better delivery of radioactivity to tumor cells. What is important is the relative localization to tumor versus normal cells. The amount of activity per MAb molecule may not be relevant. I am not aware of any system in which the amount of MAb administered to a patient saturates the tumor binding sites.

Author: The tumor to non-tumor ratio is one of the most important values; having more gold atoms per antibody should not affect the antibody distribution, so it would not alter this ratio. Graded dose studies have indicated that tumor antigen is not saturated, so administering more antibody with one radionuclide per antibody or administering lower antibody amount with more radionuclides per antibody should be equivalent. A main problem with radioimmunotherapy is that the biodistributions achieved yield a tumor to non-tumor ratio too low for effective therapy in many cases. Other multistep strategies (i.e., avidin-antibody is first administered, then after waiting ~5 days, since the non-tumor washout is faster than that on the tumor, a biotin-radionuclide is administered which quickly localizes and capitalizes on the better tumor to non-tumor ratio) typically have very reduced final radionuclide uptake. In these cases,

if each antibody or directing moiety contained more radionuclides, the delivered dose could be boosted to therapeutic levels.

Reviewer III: The text states that the low specific activity preparations had 51.4% binding to tumor cells; Table 5 shows 27%. Why do the lower specific activity conjugates have less binding to the tumor cells than the high specific activity conjugates?
Author: Each cell binding experiment is run in duplicate, so the values given are the average. Separate experiments often give different average values, indicating the variability of the assay or preparation. Given this, the difference in the high and low specific activity preparations (37% and 27-51%, respectively), does not appear to be highly significant without further measurements and statistics.

Reviewer III: The author states that the use of pretargeted clusters shows an improvement for most tissues. What is very notable is that the pretargeting resulted in a 200% change in the intestines which are a radiosensitive organ.
Author: This is an important observation, but the experiment illustrates that pretargeting (administration first of non-radioactive gold clusters) can significantly change the biodistribution.

Reviewer III: Dosimetry calculations are made by extrapolating from the biodistribution of iodinated MAb. These estimates can not be accurate because of the localization of the radiometals and radioactive gold to normal tissues is very different from that of the iodine, which does not localize in many normal tissues. Any estimates of dosimetry of normal tissues will be inaccurate; one example is the dosimetry of normal kidney where the radioactive gold localizes at higher levels than even indium.
Author: There are clear limitations, as you point out, of taking the biodistribution for an iodinated antibody and theoretically substituting gold for the iodine. The point of this exercise was more to compare the dosimetry of various isotopes, assuming the antibody distribution was the same. Conjugation techniques and chelators have been improved to the point where it is, in fact, possible to get similar biodistributions with the same antibody and different radionuclides, and it may be also possible to develop gold to this point.

Reviewer III: If the RES system can be effectively blocked, what will happen to the gold labeled MAb? How will this affect the normal tissue distribution, and thus, the toxicity to normal tissues? The liver is a fairly radioresistant tissue. The blocking of the RES may make the dosimetry worse, not better.
Author: With the colloidal gold-F(ab')$_2$, ~100% i.d./g localized in the liver; this was most of the radioactivity. To make this preparation useful, it would appear that the liver uptake should be blocked in some fashion, and more would then be expected on the tumor. This parallels the work with liposome delivery, where most goes to the liver, and after RES evasion or blocking, more goes to the tumor with better tumor to non-tumor ratios.

Reviewer III: In Table 11, the appropriate control for these studies is an F(ab')$_2$ molecule from a control antibody, not BSA.
Author: As with all experiments, there are many controls that one can imagine. Initial limited controls are often chosen because of availability, as was done here. The use of BSA has some merit in that it does not have a targeting property, it is about the same weight as the antibody fragment, and its *in vivo* properties are known. Further controls should include the one you suggest.

Reviewer III: The author should note that the internalization of MAbs is dependent to a large degree on the MAb, not the tumor type. The fact that many tumors will internalize a given antibody is a property of the MAb and the antigen primarily, not the tumor type.
Author: Except that different tumor types often have different antigens. It is interesting that for many tumor types, antibodies have been isolated that are highly internalized (while some are not), so use of internalization for delivery appears feasibly in a general way.

Reviewer III: What is the stability *in vivo* of the gold colloid MAb preparations?
Author: Serum stability studies were not done, and further experiments would have to be designed to determine the fate of the antibody-gold conjugates *in vivo*.

Reviewer III: The calculations for the tumor dosimetry are interesting, but what about the dose to normal bladder from the radioactivity in the fluid instilled?
Author: This is an important point, since if 10% of the radioactivity was taken up by the tumor cells, then the radiation they would receive over 10 hours would be the same (roughly) as that received by normal cells by the complete preparation (100%) in 1 hour when it is instilled and is incubating, before flushing. The situation would be better, however, since the gold radiation has a range of only 460 μm, so that having it on or in the tumor cell would have a direct effect, whereas gold dispersed in a bulk fluid (~100 ml) in the bladder would mostly be attenuated and diluted and should have a minimal effect. Further dosimetric calculations must be done to quantitate this initial dose, but it is clear that a short

incubation time, and, as usual, a high tumor to non-tumor uptake ratio, would improve the tumor specific dose.

Reviewer III: One cannot determine the tumor to non-tumor ratio based on *in vitro* studies. The biodistribution is rarely as good as the *in vitro* binding studies.
Author: Although *in vivo* distributions are generally worse than *in vitro* results, the *in vitro* studies have their place in screening preparations, and in calculating tumor cell to non-tumor cell ratios at that point.