The Processing and Fate of Antibodies and Their Radiolabels Bound to the Surface of Tumor Cells In Vitro: A Comparison of Nine Radiolabels

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Processing radiolabeled degradation products is the key factor affecting retention of antibodies within the cell. In this study, we have analyzed the processing of antibodies labeled in nine different ways. **Methods:** Antibodies were labeled with three different radioisotopes and seven different forms of ^{125}I. Eight of the radiolabels (except ^{188}Re) were conjugated to the same antibody, MA103, and tested on the renal carcinoma cell line SK-RC-18 and/or the ovarian carcinoma cell line SK-OV-6. Rhenium conjugation utilized the antibody RS7, the target cell line ME180 and three of the other radiolabels were also tested with this antibody-target cell combination for comparison. **Results:** Iodine conjugated to antibodies by conventional methods was rapidly released from the cell after antibody catabolism. In contrast, iodinated moieties, such as dilactitol-tyramine and inulin-tyramine were retained within cells four to five times longer. **Conclusions:** The use of radiolabels that are trapped within cells after antibody catabolism can potentially increase the dose of radiation delivered to the tumor, from the same amount of radioactivity deposited by a factor of four or five. The prolonged retention of ^{111}In relative to ^{125}I is not due to deiodination of iodine conjugates, but rather to intracellular retention of catabolic products containing ^{111}In, perhaps within lysosomes.

Key Words: antibody conjugates; radioimmunotherapy; catabolism of radioisotope conjugates

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The effective use of antibody conjugates for immunotherapy and immunodiagnosis depends upon knowledge of antibody pharmacokinetics. Our recent in vitro experiments demonstrated that most antibodies bind irreversibly to the cell surface, since, after free antibody was washed away, most of the bound antibody remained on the tumor cell surface until it was gradually internalized and degraded over a period of 2–3 days (*1*). The irreversible binding required bivalent interaction of the antibody with the cell surface. Experiments included a total of 15 antibodies recognizing 13 distinct antigens, including both integral membrane glycoproteins and glycoproteins bound to the membrane via glycosylphosphatidyl inositol. A generally similar pattern of processing was observed with 13 of the antibodies (*1,2*). Essentially irreversible antibody binding was also observed with erythrocyte target cells, which lack catabolic activity (*3*).

Since antibody degradation within tumor cells occurs in lysosomes, which was confirmed in our experiments by the use of lysosomal enzyme inhibitors, the fate of the radiolabeled catabolic product generated within lysosomes becomes a central issue. Lysosomes are known to be highly selective with respect to the release of molecules into the cytoplasm, and many relatively small molecules are unable to readily cross the lysosomal membrane (*4*). From a theoretical perspective, it seems that generally it would be advantageous to use radiolabels that are retained within cells after antibody degradation, especially for cancer radioimmunotherapy where a long duration of exposure is desired.

Our previous study utilized only antibodies iodinated by the chloramine-T method. It is well established that catabolism of iodinated proteins results in the generation, of iodotyrosine within lysosomes, which leaves the cell rapidly (*4–7*). Several groups of investigators have developed iodinated labels that are designed to be lysosomally trapped after antibody catabolism (*8–12*). These are conjugates of tyrosine, tyramine or other iodinatable molecules with small polysaccharides known to be indigestible and trapped within lysosomes. For most other radiolabels, there is little information regarding where or if they will be retained within the cell after antibody catabolism. Therefore, we have also examined the fate of antibodies labeled with ^{111}In, the Bolton-Hunter reagent (iodinated), and ^{188}Re, which is currently being evaluated as a potentially therapeutic isotope (*13*). It has been known for many years

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that radiometals such as ^{111}In and ^{90}Y are retained in vivo at the tumor site longer than radioiodine (*14–18*).

We also report on antibodies labeled with iodinated fluorescein derivatives, which are of particular interest due to their potential to binding to DNA (*19*). One of the fluorescein derivatives, iodinated DTAF, was recently reported to provide a virtually permanent cellular label, with a $T_{1/2}$ for release of >2 wk (*20*), and it was suggested that this label was lysosomally trapped. Rushfeldt and Smedsrod (*20*) used a protein ligand, GalNAc-BSA, which was considered to be recognized by a cell surface lectin. We have tested this ligand as well as antibodies labeled with ^{125}I-DTAF.

MATERIALS AND METHODS

Cell Lines, Antibodies and Radiolabeling

Most of the cell lines and monoclonal antibodies (Mabs) used were described previously (*1,2*). All Mabs used are mouse IgG antibodies. Mab MA103 reacts with high avidity to a high-density antigen present on all human cell lines tested, and is processed similarly to most other antibodies reacting with the cell surface (*1*). The human carcinoma cell line ME180 was obtained from the American Type Culture Collection (Rockville, MD). Radiolabeled antibodies, except for the rhenium conjugate, were routinely monitored by SDS-PAGE and autoradiography, as described previously (*21*), and at least 90% of the radiolabel was present in the IgG subunits. All conjugates were tested for stability in a tissue culture medium for at least 3 days, by precipitation at various times with either TCA or methanol, as described below. No instability of the labels was detected with the exception of the rhenium label, which is described under Results. Conventional labeling of antibodies with ^{125}I (*1*) or ^{111}In utilizing ITC-benzyl-DTPA (*14,22*) was described previously.

Rhenium-188 was conjugated to thiol groups of mildly reduced antibody, as described by Griffiths et al. (*13*), and the product was analyzed by gel filtration HPLC and ITLC as described. Bolton-Hunter iodination, using the water-soluble Bolton-Hunter reagent (Pierce Laboratories, Rockford, IL), followed Thompson et al. (*23*), as follows. To 1.0 mCi (37 MBq) ^{125}I in 0.1 ml 0.05 *M* sodium phosphate buffer, pH 7.3, was added 1.0 μl sulfo-SHPP at 0.2 mg/ml in dimethylsulfoxide, followed by 10 μl chloramine T at 5.0 mg/ml. After 30 sec at room temperature, 10 μl sodium metabisulfite at 12.0 mg/ml was added, the reaction mixture cooled in ice water and 40 μg of IgG were added. After 30 min at 0–4°C, 0.5 ml lysine at 1.0 mg/ml was added and the sample was fractionated on a gel filtration PD-10 column (Pharmacia, Piscataway, NJ) using the same phosphate buffer containing 0.25% gelatin, 10 m*M* NaI. The specific activity obtained was 1.2 mCi/mg (44.4 MBq/mg). Dilactitol-^{125}I-tyramine was conjugated to the antibody as described by Strobel et al. (*8*), producing a specific activity of 0.5 mCi/mg (18.5 MBq/mg). Inulin-tyramine was prepared following Sommerman et al. (*24*), then iodinated and conjugated to the antibody with cyanuric chloride as described (*12*).

Conjugation of antibodies with ^{125}I-fluorescein was achieved by three methods. Starting with fluorescein isothiocyanate (Sigma Chemicals, St. Louis, MO), 25 μl of 13 μg/ml in 0.05 *M* TrisHCl, pH 8.0, was added to a 0.5-ml microfuge tube that had been precoated with Iodogen (Pierce), as described by Pittman et al. (*11*). Two millicurie (74 MBq) of ^{125}I in the same buffer was added. After 30 min at room temperature, the solution was transferred to another microfuge tube containing 0.1 mg IgG in 0.2 ml 0.1 *M* sodium carbonate buffer, pH 9.5. After incubating overnight at 4°C, the conjugate was purified by gel filtration on a PD-10 column. The specific activity of the product was 0.3 mCi/mg (11.1 MBq/mg).

Conjugation of antibodies with fluorescein-β-D-galactopyranoside (Sigma F-4146) generally followed the method of Strobel et al. (*8*) developed for other galactose-containing molecules. To Iodogen-coated microfuge tubes was added 25 μl 0.5 *M* KPO_4 buffer, pH 7.0, containing 1–10 nmoles of fluorescein-galactoside (the yield and final specific activity did not substantially vary over this concentration range). After adding 1–2 mCi (37–74 MBq) of ^{125}I in 25 μl of the same buffer, samples were incubated for 30 min at room temperature then transferred to another microfuge tube containing 4 units of galactose oxidase (Sigma G-3385) in 5 μl of the same buffer. After 45 min at 37°C, 0.1 mg of IgG in 50 μl of phosphate-buffered saline was added, followed immediately by 2.1 μl of 2.0 *M* sodium cyanoborohydride. After 3 hr at 37°C, the conjugate was purified on a PD-10 column as described above. Incorporation of ^{125}I ranged from 9%–11%, and the specific activity of the product ranged from 0.8–1.0 mCi/mg (30–37 MBq/mg). To ensure that labeling was performed by the intended method, controls were performed in which galactose oxidase was omitted, which resulted in no significant incorporation.

Iodination with DTAF was modified from Rushfeldt and Smedsrod (*20*), with quantities of the reagents greatly reduced. One nmole DTAF in 25 μl 0.1 *M* sodium borate buffer, pH 9.0, was added to an Iodogen-coated tube. One millicurie (37 MBq) of ^{125}I was added in 25 μl, and incubated 30 min at room temperature. The reaction mixture was transferred to a tube containing 10 μl of $Na_2S_2O_3$ at 1.0 mg/ml. After 3 min at room temperature, 25 μg of IgG was added in ≤50 μl of phosphate-buffered saline and incubated 2 hr at room temperature. After addition of glycine to a final concentration of 50 m*M*, followed by a 30-min incubation at 37°C, the sample was purified as described above. The iodination efficiency was 8%–10%, resulting in a specific activity of approximately 3–5 mCi/mg (111–185 MBq/mg).

Antibody Retention Experiments

As described previously in detail (*1*), confluent cells in 96-well plates were incubated with 5×10^5 cpm antibody for 2 hr at 37°C, then washed four times. Two-tenths of a milliliter of tissue culture medium was added and incubation was continued for various times from 4 hr to 7 days. At various times, 0.1 ml of supernatant was collected (half of the total supernatant), and, after further washing, the cells were solubilized with 2.0 *M* NaOH. After determining the cpm in the supernatant, iodinated samples were precipitated with 5 ml cold 10% TCA, and indium- or rhenium-labeled samples were precipitated with 5 ml methanol, using 1.0 mg of bovine IgG as a carrier protein and the precipitate was collected by centrifugation for 15 min at 6,000 rpm in a Sorvall SS-34 rotor. In control wells, included in every experiment, a large excess of unlabeled antibody was added to some wells, and the cpm binding under these conditions was considered to be nonspecific; in all experiments, at least 80%–90% of activity bound was bound specifically. The specific activity of the different labels varied over approximately a sevenfold range; however, we previously found that a similar variation in antibody concentration had no significant effect on antibody processing (*1*), at least with a conventional ^{125}I label. Counts obtained with indium and rhenium were corrected for the decay from time 0, due to the relatively short half-life of these isotopes.

Biochemical Analysis of Cell Extracts Containing ^{111}In

Cells were extracted 6 days after coating with antibodies. The target cells were either SK-OV-6 or ME180, coated with either MH99 or RS7, respectively, both of which produced similar results. For analysis by SDS-polyacrylamide gel electrophoresis (*21*), cells were extracted from 96-well plates directly into the standard sample buffer. In other experiments, cells were first detached from the plastic by brief trypsinization. A membrane fraction and a soluble fraction were prepared after cell disruption by nitrogen cavitation, using 1,000 psi nitrogen, followed by differential centrifugation as described previously (*21*). For the membrane preparation, labeled cells from 51 wells of a 96-well plate were mixed with unlabeled cells from a confluent T150 flask, which were used as a "carrier."

To more simply obtain a soluble extract from suspended cells, the cell pellet was rapidly frozen and thawed four times and insoluble material was removed by centrifugation at 10,000 rpm for 10 min in a microcentrifuge. Radioactivity present in the soluble fraction was analyzed as follows: (1) HPLC gel filtration chromatography was used to analyze the molecular weight of the labeled material, using a Zorbax GF250 six exclusion column (MacMod Analytical, Chadd's Ford, PA) and a buffer of 0.2 *M* sodium phosphate, pH 7.0, 10 m*M* sodium azide. The radioactivity of the effluent was monitored with an in-line Beckman Model 170 gamma detector. (2) Instant thin-layer chromatography (ITLC) was used to distinguish between indium-DTPA, free indium and indium-DTPA bound to protein, using silica gel-impregnated glass fiber sheets (Gelman Scientific, Ann Arbor, MI). To distinguish protein-bound In-DTPA from low molecular weight degradation products, the solvent used was 10 m*M* EDTA, in which protein-bound indium remains at the origin. To distinguish free indium from a low molecular weight degradation product containing In-DTPA, the solvent used was water-to-ethanol-to-ammonia (5:2:1) in which unbound indium remains at the origin. After migration of the solvent to the top of the plate, the 10-cm long strips were cut into 1-cm sections and the radioactivity was counted.

To investigate the pH stability of ^{111}In-antibody conjugates, an aliquot was incubated in 0.1 *M* glycine HCl buffer, pH 3.0, together with 1.0 mg normal bovine IgG as a carrier protein, for 24 hr at 37°C. Samples were then neutralized with 1.0 *M* Tris HCl, pH 8.0, and nine volumes of methanol were added to precipitate the protein which was collected as described above. To ensure that the indium did not precipitate as colloidal indium (*22*), some samples were pre-centrifuged (before adding methanol), which did not remove a significant fraction of the radioactivity.

Miscellaneous Methods

Galactosamine-conjugated bovine albumin was purchased from Sigma Chemicals (#A-1159) and iodinated with chloramine-T as described above. It was used in binding and processing experiments just as described for iodinated antibodies, with galNAc-BSA used at 0.5 mg/ml in control wells to determine nonspecific binding.

RESULTS

Antibodies Labeled with ^{125}I-DLT and ^{125}I-Inulin-Tyramine

These molecules were designed to be entrapped within lysosomes (*8,25*). Antibody MA103 was labeled with iodinated DLT and tested for its processing after binding to the carcinoma cell line SK-RC-18. As shown in Figure 1, retention of ^{125}I by the cell was greatly prolonged in comparison with a conventional iodine label, and the rate of excretion of degradation products was correspondingly decreased by a factor of ~4. Loss of ^{125}I-DLT from the cell occurred with a mean half-life of 4.3 days as calculated from the data obtained on Days 1–3. This result supports previous data that antibody degradation is lysosomal and that DLT is retained by cells after degradation of the protein to which it was conjugated. Similar result were obtained with two other antibody/target cell combinations: RS7 on ME180 cervical carcinoma cells and LL2 on Raji B lymphoma cells.

The inulin-tyramine label was previously found to be retained by cells significantly better than DLT, presumably due to its larger size (*12*). In our experiments, there was little difference between the two labels (Figs. 1 and 2). These figures also illustrate a complicating factor that must

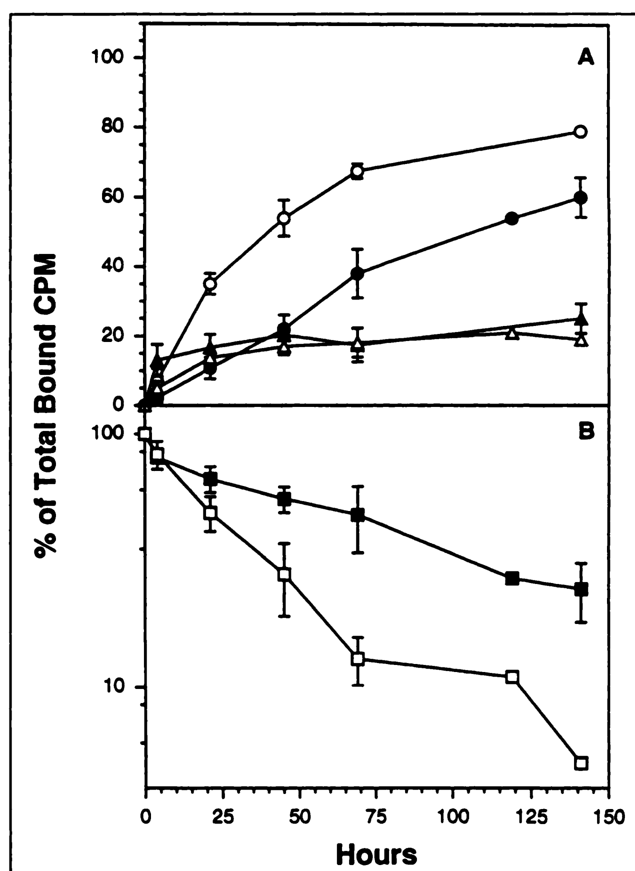

FIGURE 1. Retention and processing of antibody MA103 labeled with ^{125}I via either DLT (closed symbols) or chloramine T (open symbols), bound to the renal carcinoma cell line SK-RC-18. (A) The percentage of initially bound cpm released into the medium degraded (circles) or intact (precipitable by 10% TCA) (triangles). (B) The percentage of initially bound cpm retained by the cells. Means ± standard deviations are shown for two to three experiments, each done in triplicate. Points without error bars were included in only one experiment. The difference between the two labeling methods is statistically significant at 21, 45 and 69 hr (p values ranging from <0.05 to <0.001, by Student's t-test).

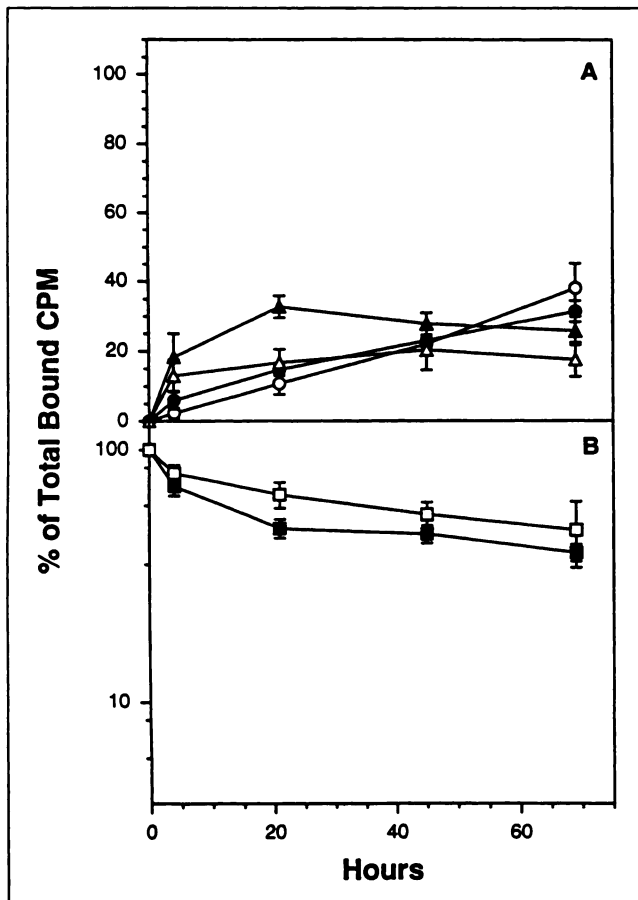

FIGURE 2. Retention and processing of antibody MA103 labeled with ^{125}I via either inulin-tyramine (closed symbols) or DLT (open symbols) bound to the renal carcinoma cell line SK-RC-18. (A) The percentage of initially bound cpm released into medium degraded (circles) or intact (triangles). (B) The percentage of initially bound cpm retained by the cells. Mean ± standard deviations are shown for three experiments, each done in triplicate. The apparent more rapid loss from the cell of the inulin-tyramine label, relative to DLT, can be attributed to the greater release of intact antibody, which was complete by 21 hr. There was not a statistically significant difference in retention $T_{1/2}$ between these two radiolabels.

be taken into consideration in interpreting these experiments. In general, a relatively small fraction of bound antibody is released intact from the cells and reaches completion rapidly, often within 4 hr and invariably within 21 hr. This release of intact antibody has been tentatively attributed to dissociation of monovalently bound antibody (*1*). The complication factor is that antibodies labeled in different ways frequently differ in the fraction of bound antibody that is released intact. For example, with the inulin-tyramine-MA103, 33% of the bound antibody was released intact, which is at least 10% higher than seen with any of the other MA103 conjugates that have been tested on the same cell line. A simple, likely explanation for this effect is that in a fraction of the antibody molecules, one binding site may be destroyed by a particular conjugation method resulting in an essentially monovalent antibody which is therefore expected to dissociate readily.

Usually lysine-directed conjugates displayed a higher percentage released intact than tyrosine-iodinated conjugates, but this was not always true and with antibody RS7, for example, the opposite effect was observed (M.J.M., unpublished data). Such variation can be attributed to the presence of different critical amino acids in the antigen-binding site of different antibodies. Variation in the percentage released intact will affect the result of processing experiments in several ways. Increased loss of intact antibody will of course result in increased loss of radioactivity from the cell. However, it will also cause a decrease in the percentage released degraded due to the fact that once antibody has left the cell surface, it is no longer susceptible to internalization and catabolism. The fact that release of intact antibody is essentially complete by 21 hr provides a simple approach to correct for this variation, namely to focus upon the rate of antibody loss from the cell after 21 hr. Thus, the rate of loss from Day 1 to Day 3 provides the best estimate of the rate of internalization, catabolism and release of the radiolabel. The mean $T_{1/2}$ for loss from the cell, from Day 1 to 3, was 104 hr for DLT and 151 hr for inulin-tyramine, as calculated from three experiments with each label. Therefore, there was a suggestion that the inulin label was retained slightly longer than DLT, although the difference in $T_{1/2}$ between these two labels was not statistically significant.

Antibodies Labeled with ^{111}In

In comparison with ^{125}I-labeled antibodies, the indium was retained strongly by the cells (Fig. 3). The rate of loss from the cell, from Day 1 to 3, was approximately fivefold slower than with a conventional iodine label. Again, the release of intact ^{111}In-MA103 was relatively high, suggesting that a lysine may be present in the antigen-combining site of this antibody. A comparison between ^{111}In-labeling and conventional ^{125}I labeling was also performed with two other antibodies; MH99 on SK-OV-6 target cells and RS7 on ME180 target cells. With these antibodies, the prolonged retention of indium was confirmed, and indium conjugation did not result in increased release of intact antibody (Fig. 3C). With MH99, >50% of the bound radioactivity was still present on the cells after 7 days.

Based on previous data indicating that antibody catabolism was due to proteolysis within lysosomes, we expected that the indium retained by the cells after 4 days would no longer be predominantly conjugated to antibody. Several approaches were used to evaluate this point. First, the cells were extracted with 2% SDS 4–6 days after the binding of indium-labeled antibody and the extract analyzed by SDS-PAGE after reduction of disulfide bonds. The radioactive material, seen in autoradiograms, migrated almost entirely as low molecular weight material at the dye front and only a trace amount migrated in the positions of intact IgG chains. This result demonstrated that the antibody had been degraded.

Analysis of cells detached from the flasks by trypsinization showed that essentially all of the radioactivity was

associated with the cell pellet, ruling out the possibility that the indium was associated with the extracellular matrix. Approximately three-fourths of the cell-associated cpm was not precipitated by methanol, confirming that it was no longer linked to antibody. To determine whether the indium was associated with either nuclei or membrane fractions, cells were disrupted by nitrogen cavitation followed by differential centrifugation. Seventy-four percent of the cpm were present in the soluble fraction, with the remainder distributed approximately equally between the nuclear and membrane fractions. Some of the counts may have been trapped nonspecifically within pelleted material. To further analyze the soluble radioactivity, viable cell pellets were disrupted by rapidly freezing and thawing four times. After centrifugation at 10,000 rpm to remove particulate material, approximately 80%–90% of the initial cpm were present in the supernatant.

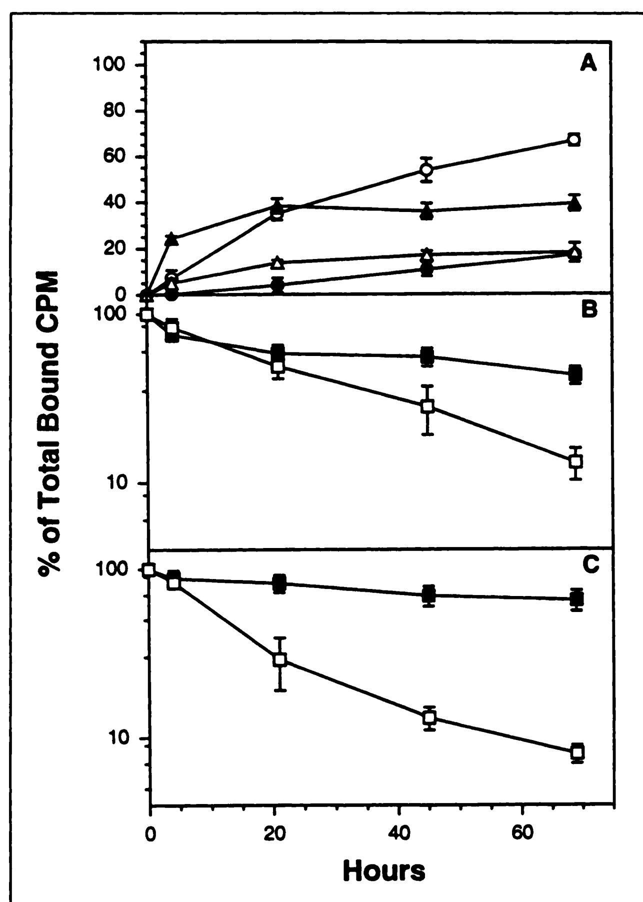

FIGURE 3. Retention and processing of antibodies labeled with either ^{111}In (closed symbols) or ^{125}I via chloramine T (open symbols) bound to carcinoma cell lines. (A, B) Antibody MA103 bound to SK-RC-18 cells. (A) The percentage of initially bound cpm released in the medium degraded (circles) or intact (triangles). (B) The percentage of initially bound cpm retained by the cells. Although the major effect observed was prolonged retention of the ^{111}In label, there was also increased release of intact ^{111}In-MA103, which was complete within 21 hr. The second effect, however, was not characteristic of the labeling procedure, since it was not observed with another antibody, MH99, tested on SK-OV-6 cells, as shown in (C), which shows the percentage of initially bound cpm retained by the cells. Means and standard deviations are shown, for three experiments, each done in triplicate. The difference between the two labeling methods is statistically significant ($p < 0.01$) at all time points after 4 hr.

When the supernatant was analyzed by gel filtration HPLC, the radioactive material eluted close to the position of In-DTPA, with no detectable peak at the elution position of IgG. In instant thin-layer chromatography (ITLC) in the presence of EDTA, the material migrated with indium-DTPA, and differently from labeled protein. In ITLC without EDTA, the material migrated like In-DTPA, and differently from free indium (data not shown). These data demonstrate that the indium is no longer bound to antibody, and is in the form of a low molecular weight degradation product. They strongly suggest that the indium remains on DTPA, although we have not excluded the possibility that it may be associated with a different low molecular weight ligand. We also investigated whether the relatively low pH of lysosomes would affect the stability of chelation. Indium-111-labeled antibody was incubated 24 hr at 37°C in 0.1 *M* glycine HCl buffer, pH 3.0, which is a lower pH than that encountered in lysosomes. Control incubations were performed in PBS. No detectable release of free ^{111}In occurred under these conditions, as detected by methanol precipitation.

Antibodies Labeled with ^{125}I-Bolton-Hunter Reagent

The iodinated Bolton-Hunter reagent has been reported to be processed differently from conventionally-linked iodine in hepatocytes, which deliver the Bolton-Hunter reagent to bile (*26*). We therefore tested MA103 labeled with the Bolton-Hunter reagent, but found that it was not processed significantly different from conventional iodine (data not shown).

Antibodies Labeled with ^{188}Re

Rhenium-188 conjugation of antibodies was recently developed as a potentially therapeutic radiolabel (*13*), since this isotope emits high-energy beta particles. In retention experiments using rhenium-labeled RS7, with ME180 target cells, rhenium was processed essentially the same as a conventional iodine label, with no indication of intracellular retention (Fig. 4). The apparent slight increase in the degradation rate of ^{188}Re, relative to ^{125}I, can be attributed to the relative instability of the radiolabel, as described below. This antibody/target cell combination was also tested with two "residualizing" radiolabels: DLT and ^{111}In-DTPA. These labels were retained by the cells much longer than the conventional ^{125}I label, producing results similar to those described above for labeled MA103.

In contrast to all other radiolabels included in this study, rhenium conjugates displayed significant lability. Control aliquots incubated in tissue culture medium decomposed at a rate of approximately 5%–15% per day, as determined by methanol precipitation, with the degradation rate being faster at earlier time points than later. This factor introduces some uncertainty in analyzing and interpreting the experiments. In general, this effect would be expected to

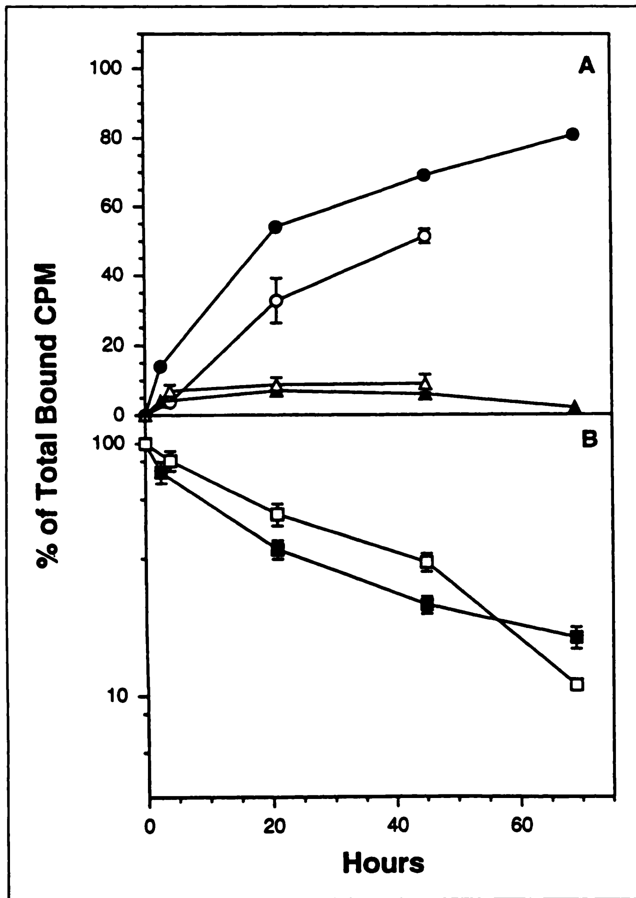

FIGURE 4. Retention and processing of antibody RS7 labeled with either ^{188}Re (closed symbols) or ^{125}I via chloramine T (open symbols), bound to the cervical carcinoma cell line ME180. (A) The percentage of initially bound cpm released into the medium degraded (circles) or intact (precipitable by methanol) (triangles). (B) The percentage of initially bound cpm retained by the cells. Nonspecific binding, determined by including a large excess of unlabeled antibody in control wells, was <4% of the total bound cpm for the ^{188}Re label. Means ± standard deviations of triplicates are shown for cell-bound cpm, and the percentages of degraded and intact cpm in the medium was calculated from means of triplicates. Similar results were obtained in a second experiment. The results are affected by spontaneous decomposition of the conjugate, as described in the text, but ^{188}Re does not appear to be retained by cells after antibody catabolism.

cause an overestimate of the rate of antibody catabolism and release by the cell. However, if it is assumed that antibody bound to cells decomposes at the same rate as antibody in solution and that the decomposition of bound antibody occurs almost entirely while the antibody is on the cell surface which would maximize the correction obtained, we can calculate corrected values of percent retained, percent released intact and percent released degraded. Even with this correction, it is clear that rhenium is not retained by the cells like indium or DLT, with at least half of the bound antibody being catabolized and released within 3 days, although it may be retained slightly better than conventional ^{125}I.

Antibodies Labeled with Iodinated Fluorescein Derivatives

In preliminary experiments, FITC-dextran (Sigma Chemicals, #FD-20s) was iodinated under the conditions normally used for iodinating proteins to demonstrate the susceptibility of fluorescein to iodination. Fifty micrograms of FITC-dextran, with an average molecular weight of 17,000, was iodinated with approximately the same efficiency, 30%–40%, as generally obtained with the same weight of IgG (data not shown). Iodofluorescein was conjugated to antibody MA103 using FITC, which was radiolabeled immediately before protein conjugation. This conjugation resulted in a relatively low specific activity, of approximately 0.5 mCi/mg (18.5 MBq/mg), but this was sufficient to determine the fate of the radiolabel after antibody binding to the cell surface. A second procedure for conjugation of iodofluorescein was developed using fluorescein-galactoside, following the method developed by Thorpe and her collaborators for DLT (*8*). Following iodination, the galactose moiety was oxidized by galactose oxidase, then conjugated to amino groups on MA103 by reductive amination via a Schiff base. This conjugation method resulted in an approximately 10% efficiency of iodination and a specific activity of approximately 1 mCi/mg (37 MBq/mg). Incorporation of these radiolabels into the IgG subunits was demonstrated by SDS-PAGE (data not shown). In two to three experiments with these radiolabels, significant differences in processing between iodo-FITC or iodo-fluorescein-galactose and conventional iodine were not detected (Table 1).

The third iodofluorescein conjugate utilized was iodo-DTAF. Iodo-DTAF labeling was considerably more efficient than any of the other nonchloramine-T iodination procedures, resulting in a specific activity of 3–5 mCi/mg (111–185 MBq/mg). Iodo-DTAF-MA103 was retained by SK-RC-18 cells slightly better than conventional ^{125}I, but not as well as In-DTPA or DLT (Table 1). Some of the degraded material in the supernatant was precipitated by 10% TCA, and we therefore used methanol precipitation for this radiolabel. In control experiments with a conventional iodine label, TCA and methanol precipitation of culture supernatants produced indistinguishable results.

These data appear to be incompatible with results of Rushfeldt and Smedsrod (*20*), and we therefore tested the same ligand used by these investigators, GalNAc-BSA, which was reported to enter carcinoma cells via a cell surface lectin. This protein was tested initially after a conventional chloramine-T iodination. It appeared to bind specifically and at relatively high levels to both carcinoma cells tested, SK-RC-18 and HeLa. In a well of a 96-well plate, 20,000–50,000 cpm were bound with a nonspecific binding of approximately 15%. However, unexpectedly, this conventionally iodinated ligand appeared to be retained strongly by cells, with essentially no release of degraded material within 3 days. Hence, the prolonged retention of this label appeared to depend not on the mode of iodination, but rather on the carrier protein. Further investigation

TABLE 1
Processing of Seven Radioconjugates of MA103

Target cell	Label	Percentage cpm retained by cells (hr)				$T_{1/2}$ (hr)
		4	21	45	69	
SK-RC-18						
	^{125}I	83.0 ± 10.5	48.7 ± 7.6	28.0 ± 8.9	13.0 ± 2.8	25
	DTAF-^{125}I	82.6 ± 1.7	54.1 ± 1.1	38.6 ± 0.9	28.5 ± 0.1	52
	DLT-^{125}I	80.7 ± 3.2	66.3 ± 7.8	55.3 ± 6.5	48.0 ± 14.1	104
	Inulin-^{125}I	71.7 ± 3.1	48.7 ± 2.1	46.3 ± 1.2	39.0 ± 3.0	151
	^{111}In	75.0 ± 5.3	58.0 ± 7.0	55.3 ± 6.8	43.0 ± 2.8	112
SK-OV-6						
	^{125}I	88.3 ± 2.5	54.3 ± 4.7	35.7 ± 4.2	25.7 ± 2.1	44
	B-H-^{125}I	76.0 ± 4.2	48.5 ± 3.5	31.5 ± 0.7	—	39
	Fluor-gal-^{125}I	88.3 ± 1.5	68.3 ± 4.6	48.7 ± 2.9	38.5 ± 3.5	58

Values shown are means ± standard deviations of two to three experiments. The $T_{1/2}$ was calculated from time points from 21 to 69 hr. ^{125}I indicates a standard iodination with chloramine-T.

indicated, however, that the ligand was binding to the plastic wells, rather than to the cells. Evidence supporting this conclusion includes the following. (1) Trypsinization of the cells, as performed previously with the ^{111}In label, did not result in the association of radioactivity with the cell pellet. Only 1%–2% of the cpm were associated with the cells and only 2% were present in the supernatant of the trypsinized, pelleted cells. In contrast, 89%–91% of the cpm remained in the "empty" wells, and were extracted with 2.0 *M* NaOH. Further experiments demonstrated that these plastic-bound counts were not extracted with 2% SDS. (2) Experiments performed with empty wells, that had no cells but had been preincubated with tissue culture medium, demonstrated very similar binding and "processing" to wells containing cells. Therefore, the lack of catabolism of this ligand can be attributed to the fact that it is binding to the plastic rather than to the cells. Additional experiments demonstrated that binding of the ligand to plastic did not require serum proteins, and that binding was increased approximately threefold in serum-free medium, suggesting that serum proteins may partially inhibit the binding.

DISCUSSION

The focus of this work is analysis of the fate of radiolabels after degradation of the antibody to which they were attached. This factor can strongly affect the dose of radiation delivered to a tumor by antibodies that have localized there, since antibody catabolism is relatively fast in comparison with the physical half-lives of the isotopes that are commonly used for radioimmunotherapy. A key aspect of this study is our attempt to select "typical" antibodies for the experiments based on our evaluation of 17 antibodies reacting with 15 distinct antigens ((*1,2*), unpublished data). Most previous studies of antibody internalization and processing have intentionally selected rapidly internalizing antibodies, which enter coated pits and the results have been considered to apply only to rapidly internalizing antibodies (*5,6,17*). Hence, a major conclusion of this work is that antibody catabolism is a critical factor not just for rapidly internalizing antibodies, but rather for all antibodies binding to the cell surface.

Table 1 provides a summary of results obtained with seven radiolabels conjugated to antibody MA103. As discussed above, we consider that the $T_{1/2}$ of the rate of loss from the cell, from 21 to 69 hr, provides perhaps the most meaningful measure of the retention of the label by the cell. The best residualizing labels had $T_{1/2}$'s four- to sixfold higher than conventional iodine, while the fluorescein derivatives were retained only slightly.

Indium was retained by tumor cells much longer than iodine, in a low molecular weight form resulting from antibody catabolism. Results consistent with this observation have been reported in many in vivo experiments: in tumor-bearing animals injected with radiolabeled antibodies, indium is retained much longer at the tumor site than iodine (*14–17*). Moreover, the retained indium was found to be in a low molecular weight form (*27*). Naruki et al. (*18*) performed in vitro experiments similar to those described here, with an anti-CD5 antibody reacting with lymphocytes and also showed the prolonged retention of ^{111}In by the cells, relative to ^{125}I. Although they did not show that the indium was retained in degraded form, they did demonstrate that release of ^{125}I was strongly inhibited by metabolic inhibitors, including chloroquine and monensin. In our experiments and in those of Motta-Hennessey et al. (*27*), the retained indium behaved biochemically like indium-DTPA. However, it must be considered probable that the DTPA remains conjugated to a lysine and perhaps to a peptide since the bond linking DTPA to lysine is not expected to be susceptible to cleavage within lysosomes. While our results do not demonstrate definitively that the indium remains bound to DTPA, this conclusion is strongly

suggested by our data and is consistent with the known stability of the chelate used, benzyl-DTPA (*28*).

The catabolism of a different but related chelate, indium bound to benzyl-EDTA, was thoroughly investigated by Deshpande et al. (*29*) using an antibody to the chelate. They found that in both urine and liver extracts, essentially all of the indium remained bound to benzyl-EDTA. While the dissociation rate of indium from benzyl-DTPA was slightly greater than that from benzyl-EDTA, the difference was only 0.6% per day (*29*). Therefore, it is unlikely that the indium dissociates significantly from DTPA under the conditions of our experiments. Given the assumption that the indium-DTPA chelate remains intact and the known highly selective permeability of the lysosomal membrane, it is likely that the indium is trapped within lysosomes, as was previously suggested by investigators working with the low-density lipoprotein receptor (*30*).

The better retention of indium by tumor cells, relative to iodine, has generally been attributed, at least tentatively, to deiodination (*15–17,31–37*). However, deiodination of intact iodinated proteins apparently has never been demonstrated. In discussing this subject, it seems necessary to first define the process: deiodination means the removal of iodine from an antibody that remains otherwise intact. If, in contrast, the entire antibody molecule is degraded, releasing amino acids and small peptides including iodotyrosine from the cell, such a process cannot be considered to be deiodination. Deiodination of the iodotyrosine may (and does) occur later (*4–6*), but this would be generally after the release of iodotyrosine from the cell, so it would have little effect on tumor-to-nontumor ratios except for the thyroid.

The enzyme which deiodinates thyroxine (which has been suggested to play a role in deiodination) is in fact known not to deiodinate iodotyrosine (*38*) and the only known iodotyrosine deiodinase is unable to act on tyrosine incorporated into proteins, but requires prior protein digestion (*38*). Furthermore, iodinated proteins have been used for many years in the analysis of the blood clearance rate of a large number of different proteins, including antibodies. The validity of using iodine as a protein tracer is supported by a wide range of experiments, and this would not be the case if extensive deiodination occurred (*39*). The significance of deiodination has been questioned previously by other investigators (*6,7,40*). Geissler et al. (*7*) compared an ^{125}I label with a biosynthetic ^{3}H label, using anti-CD3 antibodies, and found that they were catabolized and released by cells at the same rate. Moreover, they demonstrated that the major catabolic product released from cells was monoiodotyrosine. Similar results were obtained earlier from studies of the catabolism of iodinated proteins by macrophages (*4*). These data provide direct evidence that significant deiodination did not occur under the experimental conditions used and this analysis strongly suggests that, generally, deiodination is not a significant factor in experiments of this type.

Our results with rhenium-labeled antibodies are consistent with in vivo biodistribution data showing that rhenium seems generally similar to iodine in its lack of retention in the liver, kidney and tumor to which it binds (*13*). We note that a different type of rhenium conjugation has been described, which utilizes a chelator (*41*). It is clear that this label may be processed differently from the rhenium label utilized here.

The experiments with the known lysosomally trapped labels, DLT and inulin-tyramine, confirmed that these labels are retained within cells much longer than a conventional iodine label. Early studies on lysosome permeability by Cohn et al. indicated that large polysaccharides are retained longer than disaccharides (*42*), which was also suggested by Maxwell et al. (*12*). These results suggest that while DLT is retained within lysosomes, it may not be retained to the maximum possible extent. However, in our experiments there was not a significant difference in the catabolic rate between DLT, inulin-tyramine, or indium-DTPA, although there was a hint that DLT was released slightly faster than the others. None of the labels was permanently retained by the cells, since all were released in degraded form at a slow rate.

Iodofluorescein conjugates were investigated, in part because of the potential ability of these molecules to bind to DNA. The cytotoxic potency of ^{125}I incorporated into DNA is well established (*43*), tetraiodofluorescein (erythrosin B) is known to bind to the nucleus of dead cells (*19*) and diiodofluorescein is also a nuclear stain of dead cells (M.J.M., unpublished data). Once molecules escape from lysosomes, they have an opportunity to bind to DNA before exiting the cell. Additional investigations are required to determine whether iodine can be delivered to the nucleus by this approach. The DTAF label can be discussed from two perspectives. It is a fluorescein derivative, so iodinated DTAF has the potential of binding to nuclei. However, iodo-DTAF was also reported to act as a "residualizing" label by Rushfeldt and Smedsrod (*20*). Our experiments with iodo-DTAF have, however, indicated that iodinated GalNAc-BSA, the ligand used by Rushfeldt and Smedsrod, binds avidly to the plastic wells and thus is not degraded. The binding of this ligand to plastic has unusual properties such that it appears to be specific; binding was not blocked by overnight prior incubation of the wells with tissue culture medium containing 7.5% fetal bovine serum and it was >80% blocked by excess unlabeled ligand. We suggest that this binding property might be attributed to the extensive modification of the protein; all amino groups were conjugated to glyceraldehyde, and approximately 20 carboxyl groups were conjugated to the carbohydrate hapten. This interpretation is not inconsistent with the in vivo experiments of Rushfeldt and Smedsrod which utilized a labeled cell suspension. In these experiments, cells were incubated for 20 hr with the ligand so some uptake due to pinocytosis would be expected.

Lysosomally-trapped radioisotopes will be trapped not only in tumor cells, but also in tissues normally involved in antibody catabolism such as the liver, spleen and possibly

other tissues (*44*), as well as in any antigen-expressing normal cells. Hence, the actual advantage of using lysosomally-trapped labels will depend on the details of the particular study, and on which normal organs are of prime importance. For example, liver metastasis may be more difficult to detect with a lysosomally-trapped label in radioimmunodetection experiments, but metastases in other organs may be detected more readily. Regarding radioimmunotherapy, liver uptake may not be as much of a problem, considering the relative radioresistance of normal liver cells. Another complicating factor in vivo is the fate of the radiolabeled catabolic products eventually released from the cell after antibody degradation. These degradation products could potentially be taken up by other tissues. However, the excretion rate of small molecules similar to those in question has been investigated and once they reach the blood, kidney uptake and excretion in urine appear to be rapid (*8,26,29*). Indeed, metal chelators such as DTPA are used clinically to remove toxic metals from the body (*45*). However, if the metal atoms escape from the chelators, they are likely to remain in the body for a prolonged period with the site dependent on the particular metal.

In conclusion, we have demonstrated that different radiolabels conjugated to antibodies may be processed very differently after internalization and catabolism within the cell to which they initially bind. The differences described seems certain to have a major effect on the ability of such conjugates to deliver a lethal radiation dose to the cells. Novel radiolabels, such as those described here, can be designed to take advantage of such differences.

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Condensed from 15 Years Ago:

Can the Extent of Coronary Artery Disease Be Predicted from Thallium-201 Myocardial Images?

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The accuracy with which the extent of coronary artery disease can be predicted from stress ^{201}Tl myocardial images was assessed in 81 patients with chest pain. Whereas the appearance of the myocardial images was both a sensitive means of detecting coronary artery disease (images abnormal in 43 of 47 patients with abnormal coronary arteriograms) and specific in excluding it (images normal in 31 of 34 patients with normal arteriograms), there was poor correlation between the extent of disease predicted from the ^{201}Tl images arteriographic results. It is concluded that although stress ^{201}Tl myocardial imaging is a useful method for the noninvasive diagnosis of coronary artery disease, it cannot be relied upon to predict the number of abnormal vessels.

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