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Recombinant antibody–metallothionein: Design and evaluation for radioimmunoimaging

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ABSTRACT **We have produced a chimeric antibody (Ab) in which metallothionein, a well-characterized biological chelator of metals, was genetically fused to the F(ab') domain of the S107 Ab heavy chain. Coexpression with the Ab light chain that conveys specificity for the synthetic antigen phosphocholine was achieved in plasmacytoma cells. Metal- and antigen-binding domains of the Ab–metallothionein hybrid function with normal avidity and specificity. Ab–metallothionein can be efficiently loaded with ^{99m}Tc and used to specifically bind phosphocholine-haptenated cells *in vitro* or to localize plasma-cell ascites tumors in mice. The approach offers potential advantages for producing radiolabeled Ab for targeted radiotherapy and diagnostic imaging.**

Radioimmunoimaging has been applied successfully to diagnose neoplastic and cardiovascular diseases and to detect infection (for review, see ref. 1). To date this application has been accomplished by chemically conjugating chelators to monoclonal antibodies (mAbs) to couple metallic radionuclides. For radioimmunoimaging, short-lived, γ-emitting radionuclides allow a high-quality scan, while minimizing the radiation dose to patients. Antibody (Ab) conjugates tagged with indium-111 or technetium-99m (^{99m}Tc) are the most widely used radioimmunoimaging agents in nuclear medicine (1). Such conjugation requires chemical modifications that may diminish immunoreactivity or lead to aggregation. Coupling also displays batch-to-batch variation, and the desired conjugate must be purified from unreacted material and aggregates. Despite these limitations, highly specific and sensitive immunoconjugates have been produced. A serious obstacle has been the development of an immune response in the recipient.

Metallothionein (MT) is a ubiquitous, low-molecular-weight, metal-binding protein that participates in metal metabolism and detoxification (2). Mammalian forms of MT bind seven ions in tetrahedral metal–thiolate clusters, including technetium and other metals useful for targeted radiodiagnosis or therapy (2, 3). The highly conserved MT structure offers the additional advantage of low-to-nonexistent immunogenicity.

We reasoned that a number of the problems noted above could be circumvented by genetically engineering a chimeric Ab–MT, assuming that expression, assembly, and dual-binding specificity could be obtained. Here we report achievement of these goals and demonstrate *in vivo* feasibility with a prototype system in which the antibody specificity is directed against a hapten, phosphocholine (PCh), and an antiidiotype, T15 (4).

MATERIALS AND METHODS

Construction of Chimeric Ab–MT. Variable-region (V) segments (V_HS107 and V_κ22) (5, 6) bind PCh and antiidiotypic antibody Ab1.2 (7). The murine constant gene C_γ2b (8) was fused to human MT (MT-II) (9) 3′ to the hinge, generating an F(ab)–MT/chimeric C_γ2b chain on appropriate RNA splicing. Wild-type V_HS107–C_γ2b, V_κ22–C_κ-containing vector, was linearized at the unique *Xho* I site within the C_H2 exon; then 16 base pairs (bp) were resected with Bal-31 to destroy the 5′ splice site. The coding exons of MT-II, present on an 1.8-kilobase-pair (kbp) *Hin*dIII–*Bgl* II fragment, were linked and inserted. Assembly of two chimeric heavy chains with two light chains generates the F(ab′)$_2$–MT.

Expression in Myeloma Cells. The J558L plasmacytoma line was transfected by electroporation, and stable clones were selected by micophenolic acid resistance (10). RNA extraction, fractionation, and blotting were done according to Kuziel *et al.* (11).

Analysis of Chimeric Protein. Affinity purification of wild-type and chimeric antibodies from culture supernatants was done as described (12). 5A5 hybridoma IgG (13) was purified (14) from serum-free medium. For immunoanalysis, samples were run under reducing or nonreducing conditions on SDS/12% PAGE and blotted to nitrocellulose (15). The Ab blot was developed by using an anti-T15 heavy chain antibody (T68.1) (16) as a primary antibody with horseradish peroxidase-coupled goat anti-mouse immunoglobulin as a secondary antibody (17). Metal-binding blots were probed with $^{109}CdCl_2$ (1 μCi/ml of 0.1 M Tris/0.05 M NaCl, pH 6.0; 1 Ci = 37 GBq) or ^{99m}Tc04 (14 μCi/ml; Syncor Nuclear Pharmacy, Dallas) by using a modification of a zinc-immunoblot protocol (18).

Binding of ^{99m}Tc-Labeled Chimeric Ab to Haptenized Cells. Derivatization of B-16F10 melanoma cells with diazophenylphosphorylcholine was done as described (19). The fraction of haptenation achieved, measured by fluorescein-activated cell sorting with fluoresceinated anti-T15 Ab Ab1.2 (6), was 20–30% of total cells (data not shown). To label Ab–MT, a fresh solution of sodium dithionite at 4 mg/ml/0.05 M glycine, pH 8.0, was added to 7–12 mCi (260–444 MBq) of sodium pertechnetate (^{99m}Tc04) in saline and allowed to incubate at room temperature for 15 min under anaerobic conditions. MT (500–800 μg) in 0.05 M phosphate-buffered saline, pH 7.0 (concentration of Ab–MT was 8.8 mg/ml), was incubated for 10 min with (reduced) ^{99m}Tc solution. Radiolabeled Ab–MT was purified by size filtration. Eighty-five percent of ^{99m}Tc activity was associated with Ab–MT fraction with a radiochemical purity >90%. Dilutions of haptenated and unhaptenated cells were incubated with 50 ng of ^{99m}Tc-labeled Ab–MT (14 μCi/μg) for 30 min at 37°C in RPMI 1640 medium/10% fetal calf serum. Cells were centrifuged, washed three times with phosphate-buffered saline, and radioactive cells were counted in a γ (NaI) well counter. Because only a fraction of the cells can be derivatized and these cannot be conveniently separated from nonhaptenated cells, binding data was corrected by using the equation X =

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Abbreviations: Ab, antibody; mAb, monoclonal Ab; MT, metallothionein; PCh, phosphocholine; V, variable region; C, constant region.

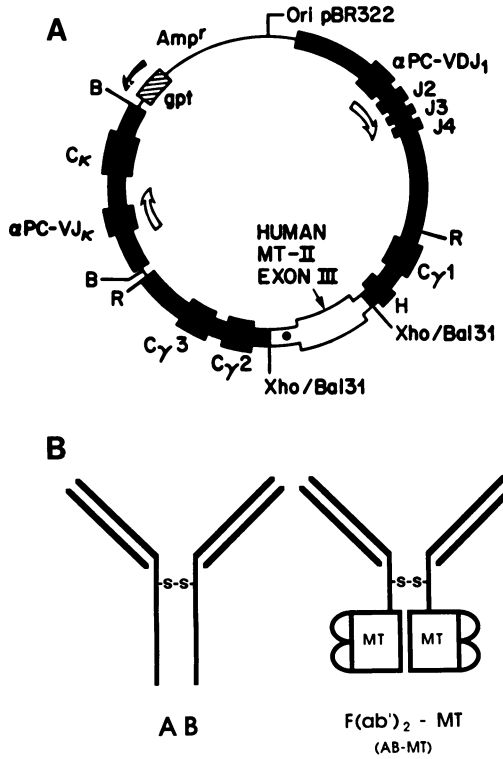

FIG. 1. Schematic of Ab–MT eukaryotic transfection vector (*A*) and predicted chimeric protein (*B*).

$(A_1 - 0.8A_2) \times 5$, where A_1 is the cpm obtained in haptenized cells (in this example, 20% haptenated) and A_2 is the cpm in nonhaptenated cells.

Quantitative Determination of Antigen and Metal Binding. Wild-type and chimeric (Ab–MT) proteins were labeled in culture to a known specific activity with [^{35}S]methionine and [^{35}S]cysteine (NEN) and then affinity purified on an antiidiotype column (12). Replicate wells of 24-well plates were coated with PCh–keyhole limpet hemagglutinin (KLH) and then loaded with ^{35}S-labeled proteins or protein labeled under maximized conditions (see above) with $^{109}CdCl_2$ (20). Wells were incubated overnight, extensively washed, and radioactivity was counted. The number of PCh-binding sites was calculated from the amount (μm) of ^{35}S bound per well, the specific activities of [^{35}S]methionine (1.68×10^7 dpm/μmol of ^{35}S) and [^{35}S]cysteine (6.21×10^6 dpm/μmol of ^{35}S), and the number of methionine and cysteine residues in a single binding site. ^{109}Cd per well was calculated from the specific activity of $^{109}CdCl_2$ (3.7×10^{10} dpm/μm) corrected for the 500-fold excess of unlabeled $CdCl_2$ present during labeling.

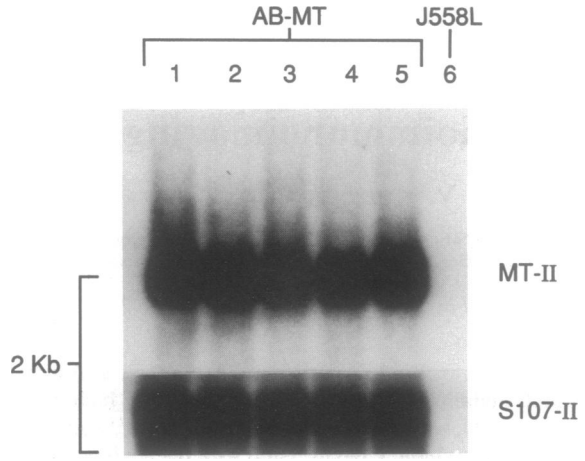

FIG. 2. Appropriate expression of Ab–MT mRNA in transfected myeloma cells. Ten micrograms from independent Ab–MT clones was loaded in lanes 1–5, and 10 μg from J558L parental cells was loaded in lane 6. The probes in the RNA analysis are to human MT-II (upper blots) and S107 V_H (lower blots). This procedure, other probings (data not shown), and mRNA sequencing confirmed that appropriate RNA splicing had occurred. Kb, kilobases.

Radioimmunoimaging. Each BALB/c mouse (≈25 g) was inoculated with $\approx 10^7$ cells i.p. After 14 days, animals bearing equivalent-sized ascites containing approximately equal concentrations of Ab (0.5–2 mg/ml) were injected i.v. with ^{99m}Tc-labeled Ab–MT (14 μCi/μg). Images shown were taken 3 hr later with a γ camera (Technicare-420; Technicare Corp., Solon, OH) that peaked at 140 kev (Tc-99m γ photopeak) and equipped with a pinhole collimator at a distance of 7–8 cm. The scintigrams were accumulated for 5–10 min. No special image processing was done, except for uniform background subtraction from the digital image.

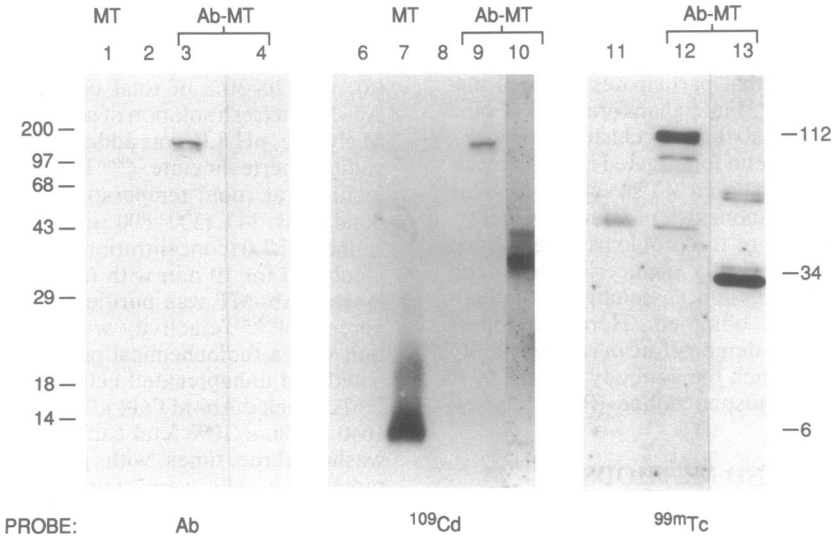

FIG. 3. Ab–MT is translated and assembled into an antigen-specific metal-binding protein. (*A*) Immunoblots of reduced (lanes 4, 10, and 13) and nonreduced (all others) proteins were probed as indicated at bottom. Lanes: 1, 6, and 11, 5 μg of C_γ2b/κ Ab 5A5 of irrelevant specificity; 2 and 7, 1 μg of bovine MT-II (Sigma); 3, 4, 9, 10, 12, and 13, 5 μg of Ab–MT; 8, 5 μg of wild-type Ab–C_γ2b. Molecular masses (kDa) (6041LA, GIBCO/BRL) are indicated at left, and apparent sizes of reduced Ab–MT (34 kDa), MT-II (6 kDa), and of nonreduced Ab–MT (112 kDa) are at right.

Table 1. Ab–MT binds antigen and metal equivalent to wild-type proteins

Protein	Met + Cys bound per site, no.	^{35}S, μM per well	PCh, μM binding sites per well	^{109}Cd, μM per well	PCh-Cd bound per binding site
Ab–$C_\gamma 2b$	11	3.4×10^{-3}	2.9×10^{-4}	1.7×10^{-4}	0.4 (±0.2)
Ab–MT	32	1.3×10^{-2}	3.2×10^{-4}	2.3×10^{-3}	6.5 (±1.9)

Wild-type (Ab–$C_\gamma 2b$) and chimeric (Ab–MT) proteins were measured for antigen (PCh–KLH) and metal ($CdCl_2$) binding in microculture wells.

RESULTS

A prototype system using V_H and V_L genes that bind the hapten PCh and the antiidiotype T15 (4) (i.e., an Ab that specifically binds the associated V_H and V_L segments of our Ab) was constructed for eukaryotic-cell expression (Fig. 1). We designed the chimera to be produced as F(ab')$_2$–MT, with the murine $C_\gamma 2b$ heavy chain 3' to the hinge replaced with human MT-II. The hybrid S107 V_H gene along with the appropriately rearranged κ chain ($V_\kappa 22$–C_κ) were transfected on the same plasmid into J558L myeloma cells that do not secrete an endogenous Ab, and long-term clones were established. Sufficient levels of apparently correctly spliced hybrid heavy chain mRNA were suggested by RNA blotting (Fig. 2) and were confirmed by sequencing PCR-amplified cDNA (data not shown).

J558L cells secreted Ab–MT protein at rates (≈10 μg/ml per 10^6 cells per 24 hr) indistinguishable from wild-type V_HS107–$C_\gamma 2b$/$V_\kappa 22$ (Ab–$C_\gamma 2b$) Ab. Ab–MT affinity purified over an antiidiotype (T15) column was analyzed on immunoblots under reducing and nonreducing conditions (Fig. 3). Sizes indicated that the hybrid heavy chain (34 kDa) was properly synthesized and that most of it assembled with $V_\kappa 22$ light chains into F(ab')$_2$–MT (112 kDa) (synonymously referred to hereafter as Ab–MT). Similar immunoblots probed with ^{109}Cd (Fig. 3) confirmed that the Ab–MT or the hybrid heavy chain alone specifically bound metal. Quantitatively, we could not distinguish the binding avidity of Ab–MT from wild-type Ab–$C_\gamma 2b$ for PCh–KLH (Table 1). Although it was not technically feasible to measure absolute metal-binding constants, the number of Cd-binding sites occupied in Ab–MT under maximum loading conditions approached the theoretical number (7) predicted from MT structure (Table 1). Acquisition of dual specificity is not unexpected, considering that appropriate immunoglobulin or MT-domain folding is retained after significant covalent and noncovalent perturbation of either native holoprotein (2, 20).

To evaluate the potential for radioimmunoimaging, we modified a method (21) (P.V.K. and A.C., unpublished work, and legend to Fig. 4) allowing us to label affinity-purified Ab–MT with ^{99m}Tc to radiochemical yields >88%. Specificity and assembly of the purified product were confirmed by immunoblotting (Fig. 3, lanes 12 and 13). We were unable to obtain naturally occurring cells that express sufficient membrane levels of antigen (PCh) or antiidiotype (T15) to serve as *in vitro* targets. Therefore, we created artificial targets by covalently coupling PCh to surfaces of lymphoid (EL4) or nonlymphoid (B-16F10) tumors. Fig. 4 shows that for B-16F10, ^{99m}Tc-loaded Ab–MT specifically binds antigen affixed to living cells in a dose-dependent fashion. This result suggests that the binding domains for antigen and metal can function in concert to identify epitopes on the cell surface under reasonably biologic conditions.

In vivo evaluation of Ab–MT was done in BALB/c mice. Peritoneal ascites was produced after 2 weeks by injecting mice (n = 5) with 10^7 Ab1.2 cells (7) that secrete T15 antiidiotypic antibodies (which for this purpose represent a specific protein antigen) or with 10^7 5A5 hybridoma cells that secrete nonspecific IgG2b/κ antibodies (13). Each of the tumor-bearing animals was injected with ^{99m}Tc-labeled Ab–MT and then was imaged with a γ camera (Fig. 5). In animals bearing an Ab1.2 ascites, scintigrams clearly visualized the liver and ascites bag as early as 1 hr after injection (data not shown). The blood background was further reduced in images obtained 3 hr after administration of Ab–MT (Fig. 5*B*). In one animal, a subcutaneous tumor that presumably had grown through metastasis was also clearly visualized (data not shown). 5A5 ascites-bearing control animals showed no clear visualization of ascites bags; most activity was localized in liver (Fig. 5*A*). These results indicate *in vivo* specific binding of ^{99m}Tc-labeled Ab–MT to antiidiotypic antibodies secreted into the ascites fluid and provide feasibility both conceptually and operationally for our approach.

DISCUSSION

Neuberger *et al.* (22) were the first to demonstrate a gene-level fusion of an antibody Fab with staphylococcal nuclease, a thiol-free secreted enzyme. In the same report, the mouse oncogene c-*myc*, which contains many thiol groups and is not secreted, was also fused to Fab. Unlike the Fab–nuclease, Fab–myc protein was unstable and heterogeneous. Fortunately MT, which is 30% cysteine, fared much better. Other reports of Ab fusions with DNA polymerase (23), plasminogen activator (24), pseudomonas exotoxin (25, 26), and CD4 (27) have demonstrated the ability to engineer bifunctionality. The specific contribution of our work is the genetic construction of a bifunctional antibody molecule that can be highly radiolabeled for specific cancer detection. An advantage of this approach is the insurance of a homogeneous, reproducible preparation that avoids the variability introduced by chemical crosslinking or proteolytic cleavage. Our strategy should achieve higher specific radiolabeling than previous approaches, whereas elimination of most C_H regions should lower immunogenicity and reduce nonspecific Fc-receptor binding.

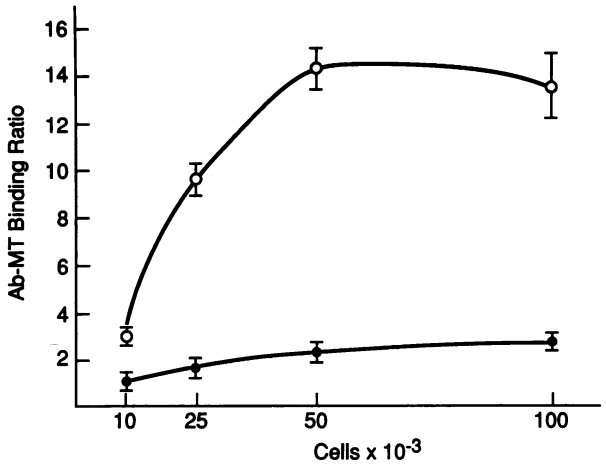

FIG. 4. ^{99m}Tc-loaded Ab–MT binds specifically to haptenized cells. B-16F10 melanoma cells, derivatized (○) or underivatized (●) with PCh, were diluted as indicated and treated with a fixed amount of ^{99m}Tc-labeled Ab–MT under viable conditions. Variability within three independent experiments is indicated by the brackets. Calculation of the Ab–MT binding ratio (y axis) is given in text.

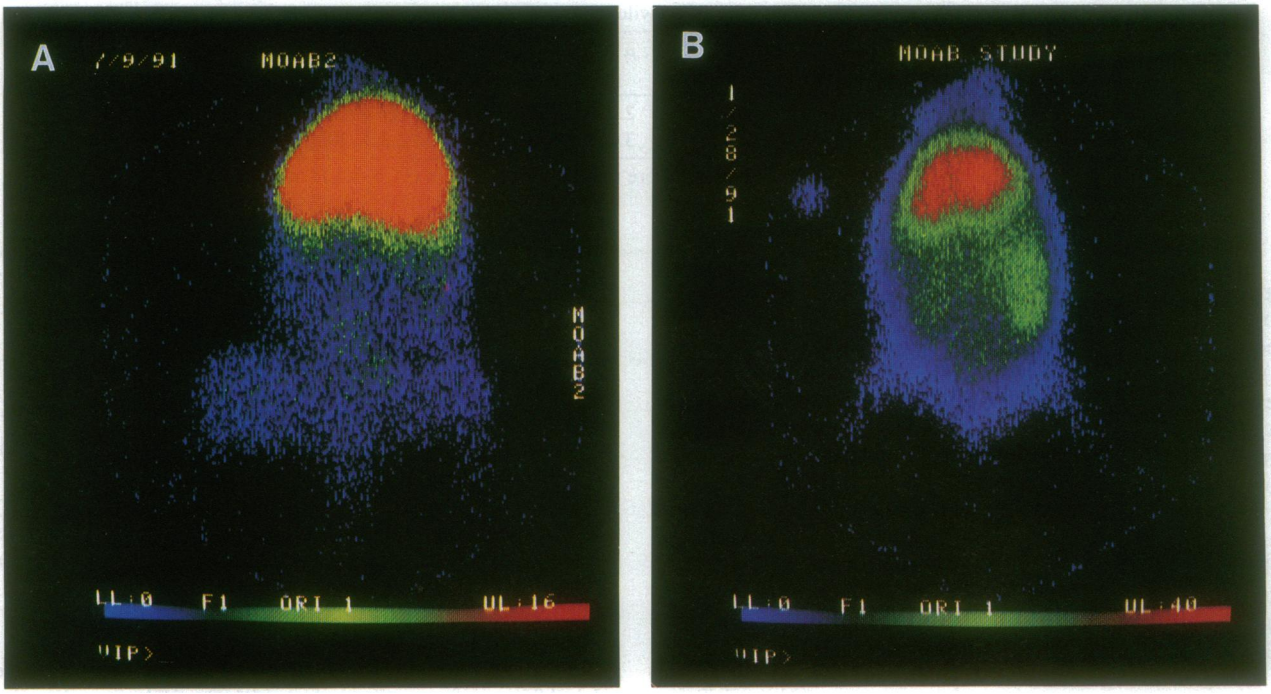

FIG. 5. Specific localization of plasma-cell ascites tumors with ^{99m}Tc-labeled Ab–MT. Three-hour images of representative BALB/c mice. Count density increases from blue to red. (*A*) Control animal inoculated with 5A5 cells secreting nonspecific antibody in the ascites; the activity appears only in liver area. (*B*) Experimental animal inoculated with cells (Ab1.2) secreting antiidiotypic antibody in ascites specific for Ab–MT V domains. Radioactivity accumulated in liver and in ascites region below liver (right side).

Because Ab–MT is a smaller molecule than native Ab, it should have more access to target cells outside the circulation and clear faster to reduce background during imaging of short-lived radioisotopes, such as ^{99m}Tc (3). However, there is high level of radioactivity accumulation in the area of the liver in both control and specific tumor-bearing animals. If an antibody or other protein is mixed with a reducing agent and sodium pertechnetate, pertechnetate is reduced and becomes associated with the protein. This chemical bond between the protein and technetium is relatively unstable *in vivo*. There is potential for formation of nanocolloid or reduced hydrolyzed form of technetium that enhances the accumulation of radioactivity in the liver. Two general approaches have been developed in labeling antibodies or proteins with technetium: Direct labeling of the native antibody (or antibody fragments) and indirect labeling with a bifunctional chelator in which one of the two reactive groups couples to the antibody and the other chelates reduced technetium. We have used MT as a protein-derived chelating agent. Our preliminary studies indicate that Ab–MT labeled with ^{99m}Tc has better *in vivo* stability compared with control antibody. In any of these approaches, accumulation of radiolabeled antibody in liver is a problem. Further modifications of the antibody may be needed to reduce the liver activity or to promote faster clearance of the radiolabel from the liver for better evaluation of metastatic diseases. Our ability to engineer bacterial expression of Ab–MT (28) should facilitate these efforts.

What remains to be established is the efficacy of recombinant Ab–MT for cell-surface-associated tumor antigens. The concentration of soluble antigen in ascites bags must certainly exceed that typically associated with membranes. However, there is reason to assume that our approach can be adapted clinically. Brown *et al.* (29) conjugated MT to an anticarcinoma antibody and labeled the complex with ^{99m}Tc. The conjugates demonstrated specificity for tumor-associated antigen. A single dose of the ^{99m}Tc-labeled Ab–MT elicited an immune response to the mouse IgG, but no detectable levels of antibody to MT were found. Higher doses of the immunoconjugate provoked no antibody titer to MT in monkeys (30), suggesting that MT metal chelates are not immunogenic.

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