Methods for the Study of the Metabolism of Radiolabeled Monoclonal Antibodies by Liver and Tumor

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Methods for elucidating the mechanisms by which radiolabeled antibodies are taken up and accumulated in tumor and liver are reviewed. These include the use of isolated perfused rat livers, RES blockade using dextran sulfate, single and double labeled antibodies, micropore chambers for the accumulation of the interstitial fluid, and in vitro tissue culture studies of antibody metabolism. Each method has its utility, examples of which will be discussed along with the methods' limitations. All of the methods have value in furthering our understanding of the metabolism of monoclonal antibodies both in vivo and in vitro. Use of these procedures to create a greater understanding of radiolabeled antibody metabolism, hopefully, will result in improved clinically useful agents for diagnosis and therapy.

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A great deal of research during the past 30 years has been directed at understanding the control of antibody production. Relatively little effort, however, has been expended to determine the mechanisms by which antibodies are cleared from the body. It has been known for many years that serum proteins, including antibodies, are rapidly turned over in the body and that different classes of antibodies clear from the blood at different rates (*1*). The organ or organs which account for this clearance are largely unknown. Catabolism of antibodies by liver (*2–4*), spleen (*3*), lymph nodes (*3*), gut (*5*), and kidney (*6,7*) have been reported. With the advent of antibodies for use in diagnosis and therapy, an understanding of the manner by which antibodies are cleared is becoming more important. This is especially true in the case of radiolabeled antibodies where the accumulation of the radiolabel in certain organs may interfere with either the therapeutic or diagnostic utility of the antibody.

An understanding of antibody metabolism is of more than academic interest. Using our present knowledge of immunoglobulin metabolism and uptake, proteins which are either labeled with iodine-131 (^{131}I) (which generally clear relatively rapidly from the liver and tumor) or with indium-111 (^{111}In) (which accumulates in the liver) have been produced. Approximately 20% of the I.D. (injected dose) of ^{111}In-labeled antibody will accumulate in an animal or patient's liver (*9–10*). Figure 1 shows images taken of a patient given either ^{111}In- or iodine-125- (^{125}I) labeled anti-sarcoma antibody 791T/36. The rapid accumulation of ^{111}In in the liver is readily apparent. The ^{131}I-labeled antibody showed only blood pool at approximately the same time at which the ^{111}In antibody showed predominantly liver uptake (*9*). This liver accumulation results in a decrease in the availability of radiolabeled antibody for tumor targeting and in the loss of ability of the antibody to effectively detect metastases in the liver. In addition to the liver uptake, ^{111}In from antibodies has also accumulated in bone marrow (*11*). A reduction in liver and marrow uptake will be essential especially if radiolabeled antibodies will be used for tumor therapy and if isotopes such as ^{90}Y are used.

A more complete understanding of the uptake, clearance, and radioisotopic sequestration mechanisms may lead to the development of alternative labeling procedures which may circumvent some of these problems. This paper will review the various methods that have been used to elucidate the mechanisms of antibody uptake and of radioisotope sequestration by liver and tumor.

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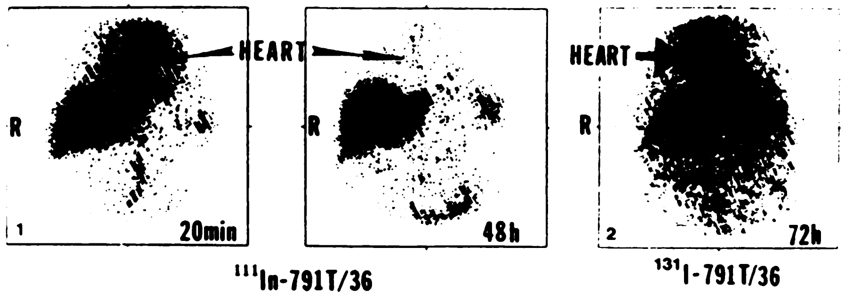

FIGURE 1
Comparison of the clinical image produced using either ^{111}In or ^{131}I label 791T136. Anterior images of the heart, liver, and spleen. From Reference (*9*) with permission.

Radiolabeling of Antibodies

The method utilized to radiolabel antibodies may play a major role in determining their subsequent uptake and metabolism by liver and tumor. Several different methods of labeling are discussed with regard to our understanding of antibody metabolism.

Internally labeled antibodies. Few studies are available in which internally labeled antibodies have been utilized for complete biodistribution studies. One example of a limited study is the work of Pollock et al. (*12*) who studied the blood clearance of various classes of murine antibodies internally labeled using sulfur-35 (^{35}S) methionine. Antibodies are labeled by growing the hybridoma in tissue culture medium containing [^{35}S] methionine which results in the incorporation of the ^{35}S into the native antibody. The blood clearance of IgM was much more rapid than the clearance of IgG. In a more complete investigation, Halpern and coworkers (*12*) studied the pharmacokinetics of selenium-75 (^{75}Se) methionine-containing anti-CEA (carcinoembryonic antigen) antibody in tumor-bearing and normal mice (Fig. 2). The data in this figure are represented as both the %I.D. per gram and %I.D. per organ. The rapid clearance of ^{75}Se from the blood over a one week period can be seen. The ^{75}Se rapidly accumulated in the liver, and the amount in this organ remained relatively constant over the seven-day period. These results differ somewhat from the pharmacokinetics observed when antibodies are labeled with either ^{125}I or ^{131}I as described below.

The internally labeled antibody (^{35}S, ^{75}Se) represents a "gold standard" to which other labeling techniques can be compared. Unfortunately, the low yield of antibody available by current in vitro techniques limits the amount of antibody produced and, therefore, the utility of the method. In most cases, comparisons are made using radioiodinated antibody as the standard. It is, therefore, important to be aware of the differences between the biodistribution of radioiodinated and internally labeled antibodies.

Radioiodinated antibodies. Many studies using murine antibodies have utilized radioiodine (^{125}I, ^{131}I, ^{123}I) as the tracer to follow the distribution of the monoclonal antibodies in animals (*8,14–18*). While these radioisotopes are useful, radioiodine is easily removed from the antibody through natural dehalogenation mechanisms (*19,20*). Therefore, the optimal method to study the distribution of a murine antibody in either a mouse or a higher animal employs internally labeled antibodies using isotopes such as ^{75}Se, ^{35}S, carbon-14, or hydrogen-3, as previously discussed.

Radioiodination is often considerably harsher than

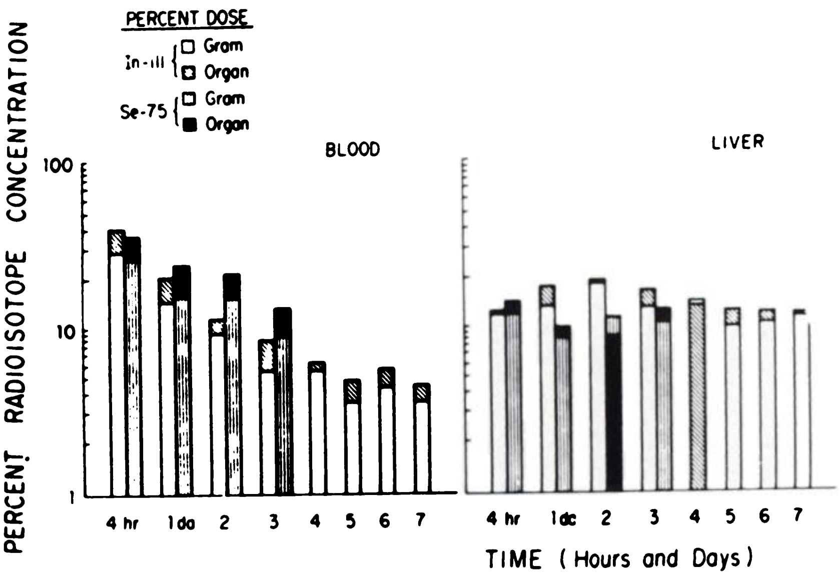

FIGURE 2
Distribution of ^{111}In and ^{25}Se monoclonal anti-CEA in normal animals. From Reference (*20*) with permission.

the internal labeling procedures and thus may result in altered biodistributions. Radioiodination protocols include the use of chloramine-T (*21*), iodogen (*22*), and iodine monochloride (*23*). Results from our laboratory of a typical biodistribution using ^{125}I-radiolabeled anti-colorectal carcinoma antibody B72.3, using the iodogen method, are shown in Table 1 and shows the rapid clearance of ^{125}I from the blood. The %I.D. per gram falls from ~40% at 1 hr to 10% I.D. per gram at 48 hr. The rapid uptake of radioiodine by the liver is also shown. One hour after injection, 10% of the I.D. per gram was found in the liver. Clearance of radioiodine resulted in ~3% I.D. per gram remaining in the liver at 48 hr. In comparison, more than 15% of the I.D. per gram of ^{75}Se from [^{75}Se]anti-CEA was found in the liver (Fig. 2).

Indium-111-labeled antibodies. The biodistribution of radiolabeled antibodies containing ^{111}In is considerably different than that of radioiodinated antibodies. This may be due to the complex handling of ^{111}In in the body. Indium can be substituted for iron in many iron-binding proteins. Iron is transported in the blood by means of transferrin and is deposited in the liver where it is found bound to ferritin (*24*). The liver has a large iron binding capacity (*25*) and, therefore, metabolism of antibodies which releases ^{111}In could result in the sequestration by ferritin and other iron-binding proteins. This is in contrast to what would be expected with radioiodinated antibodies. Metabolism and/or dehalogenation of a radioiodinated protein would result in the liberation of free radioiodine which is rapidly lost from the liver. The radioiodine would then be either sequestered in the thyroid or stomach and excreted into the urine. The results shown in Figure 2 and Table 1 support this hypothesis in that the liver uptake of ^{111}In from [^{111}In]diethylenetriaminepentaacetic acid (DTPA) B72.3 was initially high and equal to that of ^{125}I. With time, however, ^{125}I cleared from the liver while the ^{111}In concentration in the liver either remained constant or increased. It is uncertain whether the labeling process itself or sequestering of indium by the liver resulted in the high liver uptake. When the pharmacokinetics of indium labeled antibody is compared with that of internally labeled ^{75}Se-antibody (Fig. 2) identical pharmacokinetics were observed suggesting that the liver uptake was not due to the labeling process but rather to the manner by which the liver handles murine monoclonal antibodies. It should, however, be remembered that [^{75}Se]methionine can be re-utilized in the liver and reincorporated into other proteins. Therefore, these data do not definitively distinguish between metabolism of ^{75}Se and release of ^{111}In labeled from radiolabeled antibodies.

Accumulation of ^{111}In by the liver is clearly not understood. Other mechanisms, besides ligand exchange are possible. Relatively little data currently exists. Several laboratories are now exploring radiolabeled antibody metabolism. Recently, Shochat et al. studied the metabolism of [^{111}In]DTPA-labeled antibodies (*26*). Livers from guinea pigs injected with the antibodies were homogenized and the form of the radiolabel analyzed by size-exclusion HPLC. Indium-111 was found in three peaks: ferritin, intact antibody, and a low molecular weight fraction. The latter contained the majority of the ^{111}In. These data do not support the concept of liver accumulation of ^{111}In simply by a ligand exchange. Much more work needs to be done before the metabolism pathways for antibodies and their radionuclides are fully understood.

Dual labeled antibodies. To answer some of the questions relating to the difference between antibody uptake and radioisotope release due to metabolism, Khaw and co-workers used a dual-labeled antibody (*27*). The antibody, in this case the anti-breast carcinoma 103D2, was first conjugated with metal-free DTPA. The DTPA antibody complex was subsequently radioiodinated (^{125}I) and labeled with ^{111}In. The biodistributions of the ^{125}I- and ^{111}In-labeled 103D2 were remarkably similar to those seen in Table 1 for a similar experiment done with B72.3, another anticolorectal carcinoma antibody, labeled separately with either ^{111}In or ^{125}I. Again, there was a very rapid and prolonged retention of ^{111}In by the liver accompanied by the rapid accumulation and prolonged retention of ^{111}In by the spleen. Iodine-125 radiolabeled antibody was also taken up rapidly by liver and spleen, while the radiolabel left

TABLE 1
Pharmacokinetics of ^{125}I- and ^{111}In-Labeled B72.3

	Percent Injected Dose/G ± s.d. (n = 5)					
	Time (hr)					
	1		24		48	
Isotope	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In
Organ						
Blood	43.07 ± 4.04	40.01 ± 4.66	20.03 ± 11.06	15.48 ± 8.00	16.26 ± 7.46	11.23 ± 5.51
Spleen	12.03 ± 2.26	11.84 ± 2.06	6.97 ± 2.50	15.79 ± 7.43	3.86 ± 1.12	12.12 ± 7.25
Liver	9.94 ± 1.47	14.00 ± 2.25	5.40 ± 1.86	18.88 ± 9.28	3.07 ± 1.19	19.39 ± 10.97

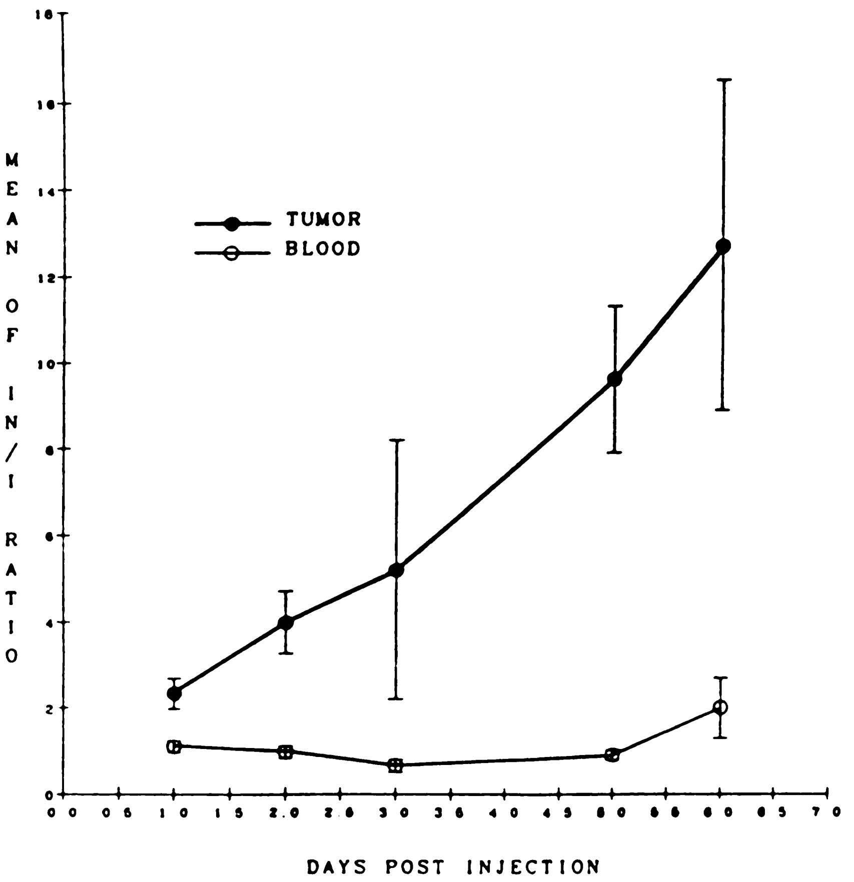

FIGURE 3
Differences in experimental tumor localization of dual-labeled 103D2. From Reference (*27*) with permission.

both organs quickly. The results of the dual label experiment are summarized in Figure 3 where the ratio of ^{111}In to ^{125}I is compared for tumor and blood over several days. A ratio of 1.0 indicates that the ^{111}In and ^{125}I were handled identically. This was true for antibody in the blood where the ratio remained ~1.0 from 1 to 6 days after antibody injection. In contrast, the tumor ratios continually rose from ~2.0 at Day 1 to 12.0 at Day 6. This increase was due to a decrease in the tumor values of ^{125}I and not to an increase in the ^{111}In values. These data, along with that from the single isotopically labeled and the internally labeled antibodies, strongly suggest that murine antibodies radiolabeled by currently available methods rapidly accumulate in the liver. Differences in liver content of radioactivity are, therefore, due to inherent differences in the manner by which the radioisotope is subsequently handled.

In Vitro Methodology for the Study of Liver Metabolism

Catabolism or dehalogenation of radiolabeled antibodies by organs other than the liver may obscure liver metabolism. Several in vitro techniques are available to isolate the role of the liver in these processes.

Isolated perfused rat livers. Use of the isolated perfused rat liver allows for the study of antibody uptake by the liver independent of protein processing by other organs (*28*). In our laboratory, studies have shown that the liver uptakes of the anti-breast carcinoma B6.2 radiolabeled with either ^{111}In or ^{125}I were identical during the 2-hr time course of the study. After this time the isolated livers may no longer function. Liver function was shown by the use of technetium-99m (^{99m}Tc) Hepatolite (hepatobiliary imaging agent) and [^{99m}Tc] Microlite (microaggregated albumin) which assess the functionality of the hepatocytes and Kupffer cells, respectively. Two hours after antibody administration, the ^{111}In and ^{125}I values found in the isolated liver were 0.61 ± 0.15 and 0.65 ± 0.17% I.D. per gram, respectively. These data showed rapid liver uptake of both ^{111}In- and ^{125}I-labeled antibody at early time points. Differences in liver uptake seen in vitro were not reproduced at times in which the isolated perfused rat liver preparation was viable.

Isolated hepatocytes and Kupffer cells. Methods are also available which utilize single cell preparations of the liver to study the cellular mechanism of hepatic uptake of radiolabeled antibody. Isolated hepatocytes are obtained in our laboratory by perfusion of the liver with collagenase according to the method of Seglen (*29*). This procedure is followed by hepatocyte enrichment of the crude cell suspension using a Percoll gradient. The resulting cell preparation specifically binds [^{99m}Tc]Hepatolite. Nonparenchymal cells, of which

Kupffer cells are the predominant cell type, may be obtained free of hepatocytes by differential centrifugation of the crude cell suspension obtained as described above. Alternatively, the crude cell preparation may be incubated with pronase which selectively digests parenchymal cells and from which "pure" Kupffer cell preparations can be obtained (*29*). Kupffer cells which stain dark brown and appear granulated in the peroxidatic reaction (*30*) can be also distinguished from hepatocytes since they do not bind [^{99m}Tc]Hepatolite. Kupffer cells are capable of binding IgG molecules through surface Fc receptors (*31*). The contribution of the Kupffer cell to liver uptake of antibody from the circulation, however, is unclear. In one study using isolated cell preparations, ^{125}I polyclonal antibody was shown to bind to hepatocytes and not to Kupffer cells (*32*) (Fig. 4).

To further investigate the role of the reticuloendothelial system (RES) in liver uptake of radiolabeled antibody, we utilized mice which had been pretreated with dextran sulfate to produce RES blockade. The extent of the resulting blockade was determined by measurement of the uptake of [^{99m}Tc]Microlite, a specific marker of RES function. Microlite uptake was markedly reduced following the dextran sulfate pretreatment (Fig. 5A). The mechanism of blockade of the RES is unknown. It may be due either to direct blockade of the Kupffer cells or to the removal of a factor necessary for RES function, e.g., fibronectin, from the blood. Regardless of the mechanism of RES suppression, we found that dextran sulfate pretreatment had no effect on liver uptake of radiolabel following injection of ^{111}In- or ^{125}I-labeled antibody (Fig. 5B). These data along with results of the isolated cell studies strongly suggest that the uptake of radiolabeled antibodies is due to binding to hepatocytes. Other factors, however, such as the type of radiolabel, presence of aggregates, colloid, or denatured protein may contribute to binding of antibody preparations to nonparenchymal cells.

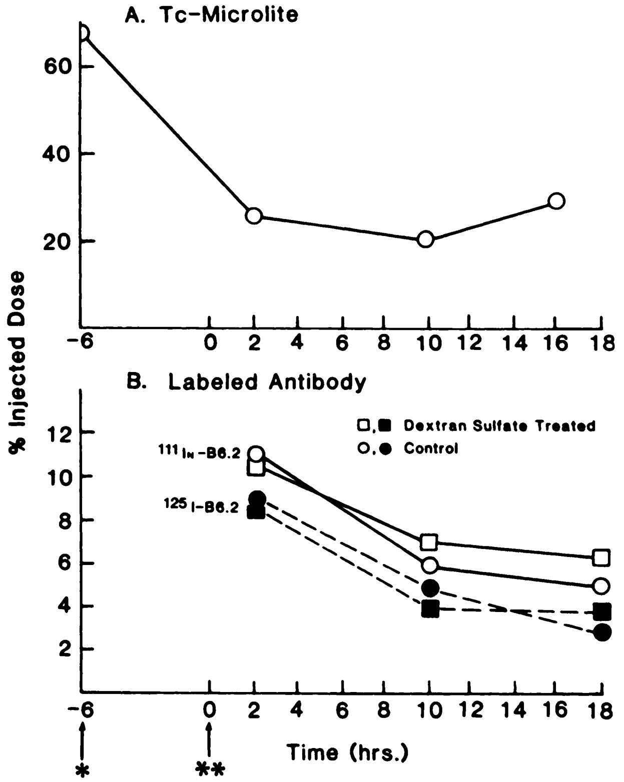

FIGURE 5
A: Liver accumulation of [^{99m}Tc]Microlite as a determinant of RES activity. B: Accumulation of either ^{111}In or ^{125}I labeled B6.2 by liver of normal and RES blocked mice. (*) Injection of dextran; (**) Injection of antibody.

Effect of Circulating Antibody-Antigen Complex

The discussion of liver uptake has focused on the accumulation of native antibody in the liver. The presence of antigen shed from tumor into serum and subsequent immune complex formation will also drastically alter the pharmacokinetics of radiolabeled antibodies. Most studies using ^{131}I anti-CEA in patients

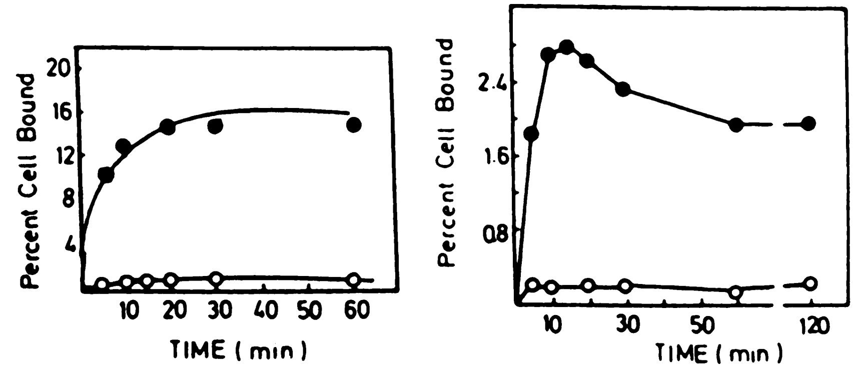

FIGURE 4
Isolated hepatocyte (•) and Kupffer cell (o) binding of polyclonal IgG. A: Aggregated IgG binding, B: monomesic IgG binding of 1–10 μg I ml of protein to 1.5 × 10^6 cells/ml at 4°C. From Reference (*32*) with permission.

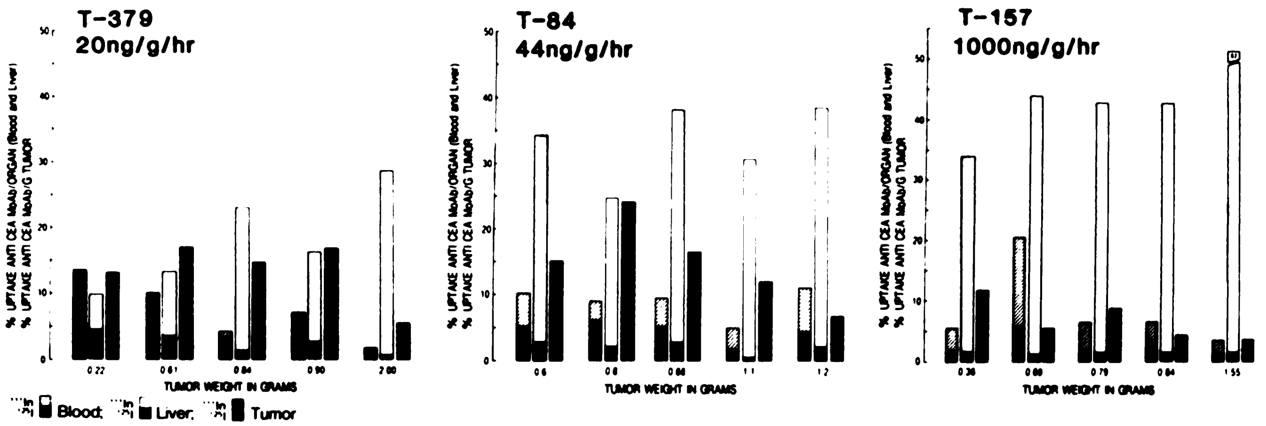

FIGURE 6
The influence of circulating shed CEA on the tumor uptake of either ^{125}I or ^{111}In anti-CEA. From Reference (*34*) with permission.

have shown little, if any, effect of the level of circulating antigen on either tumor localization or liver uptake (*33*) whereas studies in mice have shown that the level of circulating antigen have a major impact on the pharmacokinetics of radiolabeled antibody (*34*). Figure 6 is from a study by Hagan and co-workers in which the pharmacokinetics of ^{111}In and ^{125}I anti-CEA was determined in athymic mice bearing tumors which secreted different amounts of CEA. In the animals bearing lower tumor secreting levels of CEA, the blood ^{111}In-anti-CEA activity at 24 hours was ~15% I.D. per gram for both isotopes. This contrasts with a blood level of ~2% I.D. per gram for the ^{125}I-labeled anti-CEA seen in animals with high CEA secretory rates. Liver values of ^{111}In were also markedly increased in these animals. It should be noted that while the ^{111}In in the liver was markedly increased, the ^{125}I liver values were essentially the same regardless of the level of secreted CEA. Probably both antibody and antibody antigen complexes are taken up by the liver. Iodine-125, however, was removed from the antibody and secreted from the liver while ^{111}In may be removed from the antibody was sequestered in the liver and reutilized in ferritin and other iron binding proteins. These results are confirmed

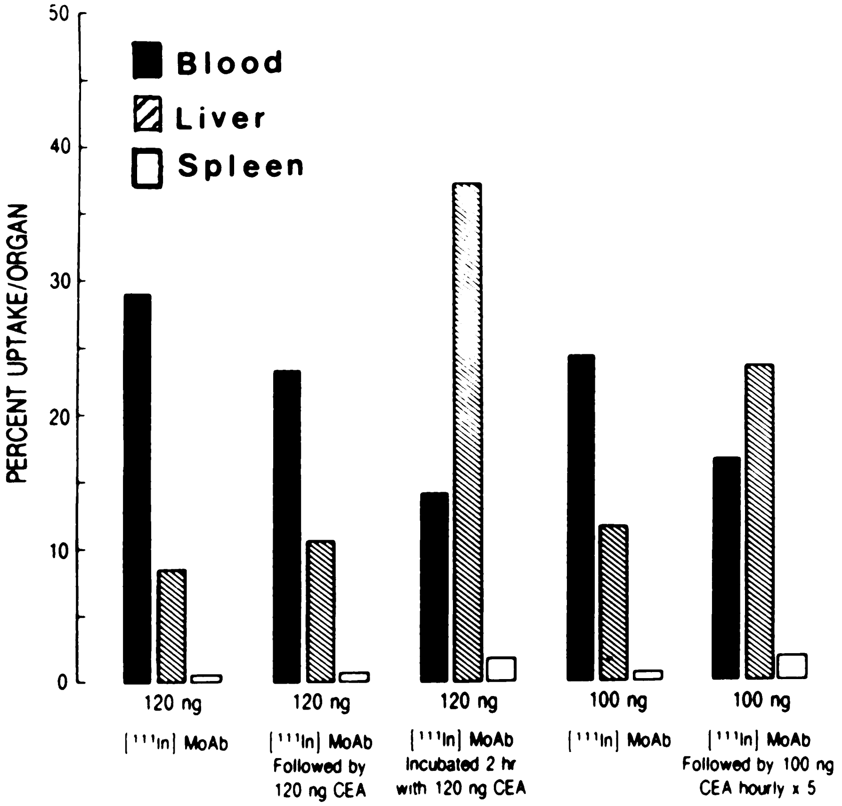

FIGURE 7
The effect of preformed CEA-anti-CEA-complexes on the distribution of ^{111}In-labeled anti-CEA. From Reference (*34*) with permission.

in another study from Hagan et al. in which nontumor bearing animals were injected with ^{111}In-labeled antibody (Fig. 7) (*35*). In this study, either ^{111}In anti-CEA alone or labeled antibody which had previously been incubated with CEA to form the antibody-antigen complex was utilized. When the antibody-antigen complex was injected into mice, the liver uptake of ^{111}In was enhanced and the blood values reduced. It is important to understand that an increased liver uptake of ^{111}In may be due to either shed antigen or to the ability of the liver to sequester antibodies. Total accumulation of antibody in the liver may appear similar regardless of the cell type (Kupffer cells, hepatocytes, or both) which initiated uptake of antibody-antigen complexes.

Antibody Metabolism by Tumors

Few studies have dealt with the metabolism of radiolabeled antibody by solid tumors. Several techniques including the analysis of fluid from micropore chambers implanted in vivo and of supernatants of tumor cells maintained in vitro are now in use. Data using these techniques will enhance our understanding of metabolism of radiolabeled antibodies by tumors.

In vivo metabolic studies. While a limited amount of information is available concerning antibody uptake and metabolism in liver, even less is known about metabolism of radiolabeled antibodies by various tumor types. Reports indicate that in virtually every case the uptake of ^{111}In into tumor was considerably greater than that of ^{125}I, as demonstrated in Figure 6 (*8,35*). The mechanism for this greater uptake may be similar to that proposed for the greater ^{111}In accretion in the liver, i.e., sequestration by iron binding proteins within the rapidly growing tumor. These explanations of increased metal binding capacity have been used to explain the relative affinity of gallium-67 for tumors (*36*). While this hypothesis is plausible, it remains unsubstantiated.

Micropore chambers. In an effort to further our understanding of the metabolism of antibodies by tumors, we have utilized a micropore chamber to sample tumor interstitial fluid (*36*). A small plastic chamber was covered on both sides with a Millipore filter which had been sterilized and had a small drain tube attached. These chambers were implanted subcutaneously in athymic mice, and tumors can be grown around the chambers (*36*). The interstitial fluid which was secreted into the chamber could then be sampled and its content assayed. We have used these chambers to help explain a major discrepancy in our experimental observations. Both LS174T (human colorectal xenograft) and A549 (human lung xenograft) carcinoma cell lines bind ^{125}I-B6.2 in vitro. The binding by A549 is to a greater degree (88.7% versus 60% by 3 × 10^7 cells) and more uniform (66.2% of the A549 cells bind antibody versus 30% of the LS174T cells) as determined by the fluorescent cell sorter. When mice bearing these tumors as xenografts were injected with ^{125}I-B6.2, only the LS174T tumors took up antibody as determined by biodistribution and imaging studies.

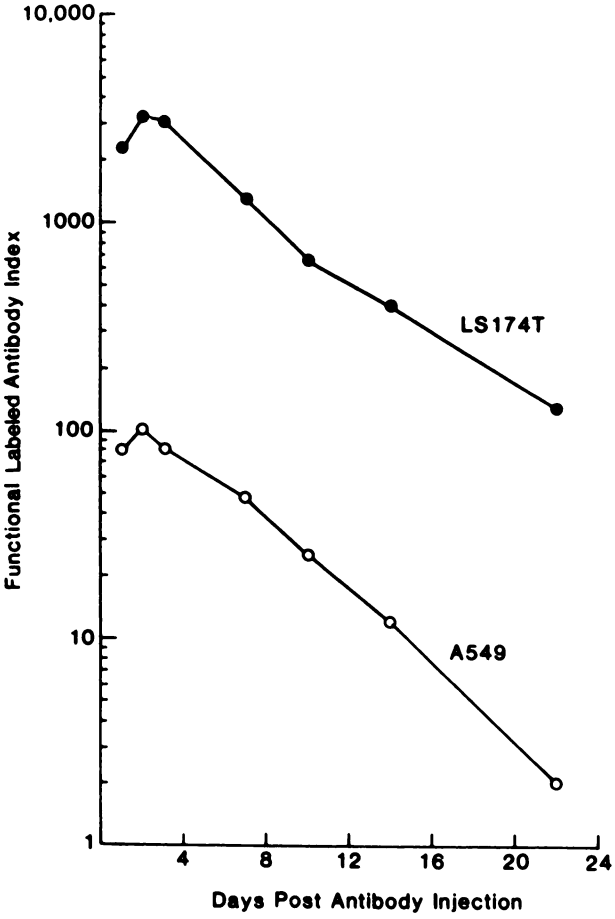

FIGURE 8
Functional labeled antibody index determined on the interstitial fluid taken from micropore chamber surrounded by either LS174T or A549 human xenografts.

We used this method to assay the interstitial fluid from chambers around which either LS174T or A549 were grown as shown in Figure 8. The interstitial fluid from these chambers was analyzed in three ways: (1) % I.D. per ml of interstitial fluid; (b) as the percent of counts found in the IgG bands of the gel; and (c) the percentage of counts which bind to LS714T cells in vitro. In this way, three different parameters of the interstitial fluid were determined: (a) the amount of label found in the fluid, (b) the amount of intact IgG in that interstitial fluid, and (c) the degree of immunoreactivity found in the sample. The product of these values results in a Functional Labeled Antibody Index (FLABI).

$$\text{FLABI} = (\%\ \text{I.D./ml}) \times (\%\ \text{IgG}) \times (\%\ \text{of counts bound to LS174T cells}).$$

This index gives a measure of the functional antibody

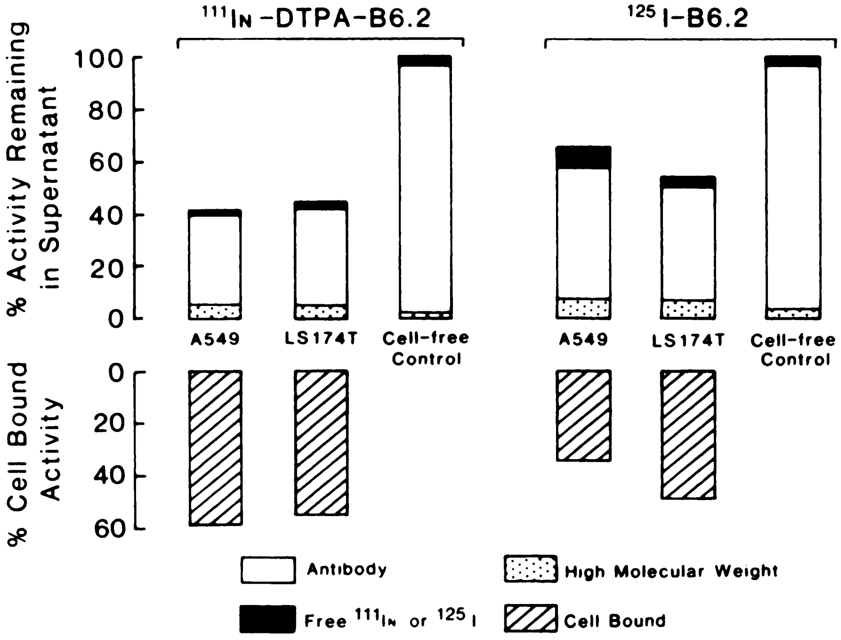

FIGURE 9
HPLC analysis of the label found in the media taken from 24 hour incubations of either ^{125}I or ^{111}In-labeled B6.2 with either LS174T or A549 cells.

secreted into the interstitial fluid (Fig. 8). A difference of almost two orders of magnitude of the index was seen between the interstitial fluids taken from LS174T and A549 tumor chambers. These results indicate that the antibody was handled differently by the two tumors. The markedly reduced FLABI of the interstitial fluid from A549 tumors suggested that A549 tumors were capable of metabolizing antibody rapidly and explains our observation that ^{125}I-B6.2 fails to accumulate in A549 tumors. Thus the chamber method has potential for expanding our understanding of the way antibodies are metabolized in various types of tumors.

In vitro metabolic studies. An example of an in vitro study of antibody metabolism is given in Figure 9. A549 tumor cells bound B6.2 in vitro extremely well while A549 solid tumors did not accumulate B6.2 when the tumor was grown in an athymic mouse (see above). When either ^{125}I- or ^{111}In-B6.2 was added to cell cultures containing either A549 or LS174T cells, both cell types bound B6.2. After incubation at 37°C for 16 hr, the supernatant was removed and the activity bound to the cells was determined by centrifugation of the incubation medium. Both A549 and LS174T cells bound ~40–60% of the radioactivity added to the medium. The supernatant was analyzed using high performance liquid chromatography (HPLC) and a TSK 250 column. Counts that were associated with antibody, free ^{111}In, ^{125}I, or a high molecular weight component (possibly indicative of shed antigen) were determined. No major differences were seen between cell preparations (Fig. 9) indicating that in vitro there was little shed antigen and that there was identical release of free ^{111}In and ^{125}I by these two preparations. In contrast, the in vivo accumulation of radiolabeled B6.2 differed drastically when injected into mice bearing either LS174T or A549 xenografts. B6.2 accumulated specifically in LS174T tumors (16.35 ± 5.22% I.D./g at 24 hr) but did not accumulate in A549 tumors. These results indicate that metabolism of antibody by the tumor cells did not play a role in the difference in in vivo accumulations seen.

SUMMARY

Various methods for studying antibody uptake and accumulation in tumor and liver have been reviewed. These methods include the use of isolated perfused rat livers, RES blockade using dextran sulfate, single and double labeled antibodies, micropore chambers for the accumulation of the interstitial fluid, and in vitro tissue culture studies of antibody metabolism. All of the methods have value in furthering our understanding of the metabolism of monoclonal antibodies both in vivo and in vitro. A greater understanding of antibody metabolism hopefully will result in improved clinically useful agents for diagnosis and therapy.

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