Antibody Localization to B-Cell Lymphoma Xenografts in Immunodeficient Mice: Importance of Using Residualizing Radiolabels[1]

Rosana B. Michel, Rita Ochakovskaya, and M. Jules Mattes[2]

Center for Molecular Medicine and Immunology, Belleville, New Jersey 07109

ABSTRACT

Antibody (Ab) localization to Raji B-cell lymphoma xenografts in severe combined immunodeficient (SCID) mice was investigated using three Abs: anti-CD20; anti-CD147; and anti-MHC class II. These antigens are all high-density cell surface antigens, and the Abs are all considered to be slowly internalized and catabolized, with catabolism primarily due to the basal turnover rate of cell surface constituents. Unexpectedly, specific Ab uptake was demonstrated only when residualizing labels were used. The residualizing labels tested were ^{111}In-benzyl-diethylenetriaminepentaacetic acid and [^{125}I]iodo-dilactitol-tyramine, whereas the nonresidualizing label was a conventional iodine label. In contrast, *in vitro* experiments demonstrated very slow catabolism of the same Abs. These data strongly suggest that Ab catabolism is much more rapid *in vivo* than *in vitro* and has a strong impact on Ab accumulation in the tumor. If autologous human tumors are similar to these xenografts, then there should be a large advantage in the use of residualizing radiolabels for radioimmunotherapy.

INTRODUCTION

Whereas considerable success has been achieved in the clinical use of radiolabeled Abs[3] to treat B-cell lymphoma (1–5), there are relatively few data from animal models to support this approach, especially data on specific Ab uptake in tumors. There are several reasons for this situation. (*a*) Once clinical efficacy is demonstrated, animal studies may seem unnecessary. (*b*) It may be considered that mouse or rat xenograft models are so different from the clinical situation, due to the lack of normal antigen-positive cells in the animals, that there is little value in developing such a model. Whereas these two points are valid to some extent, it remains true that development of a reliable animal model can greatly aid in the development of the optimal therapeutic strategy. (*c*) Technical difficulties with the available animal models have probably discouraged their use. Some tumors grow inconsistently, making it difficult to obtain a group of animals with tumors of similar size. Some tumors grow too rapidly, which can make both Ab localization and therapy experiments more difficult (6). The most widely used model is the relatively cumbersome method developed by Leonard *et al.* (7), who used nude mice given multiple doses of external beam irradiation, and coinjected the mice with irradiated human fibrosarcoma cells, which act as feeder cells. It was recently reported that tumor growth is improved in mice with multiple immunological defects (8), but these mice are expensive.

In this report, we describe a problem that is likely to be partially responsible for apparent poor Ab localization to B-cell lymphoma xenografts, namely, rapid catabolism of Abs after binding to the cells. This occurs not only with rapidly internalized Abs but apparently with all Abs binding to the cell surface in the two xenograft models tested. It represents a considerable obstacle to the accumulation of a conventional iodine radiolabel by the cells, but the problem is circumvented by the use of residualizing radiolabels. Residualizing labels are defined as those that are trapped in lysosomes, as catabolites, after degradation of the Abs to which they were conjugated. For example, ^{111}In-benzyl-DTPA and [^{125}I]iodo-DLT are residualizing labels, whereas a conventional iodine label is not. In most cases, a conventional iodine label is the first label that is used to evaluate Ab uptake (9–11). The magnitude of this problem is such that there was no detectable specific Ab localization of the three Abs tested, namely anti-CD20, anti-CD147, and anti-MHC class II antigen, when labeled with conventional iodine. The first two of these are considered to be noninternalizing or slowly internalizing (12, 13) and were selected for this study for this reason. Anti-MHC class II Abs are also often considered to be noninternalizing (14), but we have shown that the Ab used here, L243, is internalized and catabolized at a slow rate (15). Because the L243 antigen level on the cell surface is very high, approximately 3.2×10^6 sites/Raji cell (16), and, *in vitro*, 56% of bound L243 remains on the cell surface 2 days after binding (15), it was expected that a large amount of the Ab would remain on the cell surface at the time of dissection in Ab localization experiments. In contrast, when residualizing radiolabels were used, high specific uptake occurred with all three Abs. The only likely explanation of these results is that catabolism of bound Ab was rapid.

These experiments primarily used a new animal model. Our previous studies had used Ramos cells growing s.c. in nude

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[2] To whom requests for reprints should be addressed, at Center for Molecular Medicine and Immunology, 520 Belleville Avenue, Belleville, NJ 07109. Phone: (973) 844-7013; Fax: (973) 844-7020; E-mail: mjmattes@gscancer.org.
[3] The abbreviations used are: Ab, antibody; DTPA, diethylenetriaminepentaacetic acid; DLT, dilactitol-tyramine; SCID, severe combined immunodeficient.

mice (6, 17), but the disadvantages of this model were inconsistency of tumor growth and exceedingly rapid growth after tumor appearance. Thus, tumors became nearly 2 g within a week of appearance, and it was difficult to test a range of time points using tumors of a reasonably small size. The model used herein, which is better and simpler, is Raji cells growing s.c. in SCID mice. Tumor growth is slow (approximately 26 days are required for good-sized tumors to develop), but it is relatively consistent.

MATERIALS AND METHODS

Abs and Radiolabels. We previously described the source of hybridomas L243 (anti-MHC class II antigen; Ref. 15), 1F5 (anti-CD20; Ref. 18), and MA103 (anti-CD147; Ref. 19). Abs, all of which are IgG2as, were purified by affinity chromatography on protein A-Sepharose as described previously (13). They were labeled with ^{125}I and ^{131}I by the chloramine-T method and labeled with ^{111}In with the chelator isothiocyanate-benzyl-DTPA, as described previously in detail (20). Procedures for labeling Abs with ^{125}I- or ^{131}I-DLT were also described previously (21). The specific activities were 10–20 mCi/mg for conventional iodine labeling, approximately 1 mCi/mg for DLT and 5–20 mCi/mg for ^{111}In. Labeled preparations were analyzed by instant thin-layer chromatography, gel filtration HPLC, or both, by methods that have been described previously (22), to determine the level of radioactivity not bound to the Ab, which was always <10% and usually <5%. Representative preparations of radiolabeled Abs, with each radiolabel, were tested for immunoreactivity (percentage bindable), by incubating them with a large excess of cells. Control tubes had excess unlabeled Ab added to block specific binding and therefore indicate the level of nonspecific binding: specific binding was calculated by subtraction. The immunoreactivity was not significantly affected by the particular labeling method used. The range of specific binding was 50.6–74.8% for 1F5 and 48.9–50.6% for MA103. Two batches of L243 were used, which varied significantly in their immunoreactivity: the batch used for the ^{111}In experiment had 56.3–58.6% immunoreactivity; whereas the batch used for the DLT experiment was only 19.0–21.0% immunoreactive (probably due to a low Ab concentration in the ascites fluid that was the original source). In all double-label Ab localization experiments, the same batch of Ab was used for both radiolabels. The second batch of Ab was adequate for the purposes of this study to show the difference between radiolabels, but it is likely that use of a more reactive Ab would result in a higher percentage uptake by the tumor and higher tumor:nontumor ratios.

Tumor Cells and Animal Models. The cell lines used were the B-cell lymphomas Raji, RL, Ramos, and Daudi. The origin of these lines was described previously (21). Cells were grown routinely in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 12.5% fetal bovine serum, penicillin, streptomycin, glutamine, and sodium pyruvate (Life Technologies, Inc.). Cell lines were tested routinely for *Mycoplasma* contamination using the Mycotect Assay (Life Technologies, Inc.) and were negative. CB.17 SCID mice were obtained from the National Cancer Institute Animal Production Program (Frederick, MD). At 5–6 weeks of age, they received s.c. injection in a shaved area of the back with 2.5×10^6 Raji cells grown in tissue culture. When tumors were approximately 0.2–0.4 g in size (day 25–27, in the experiments reported here), mice received i.v. injection with radiolabeled Ab. For the tumor growth studies, tumor size was estimated as length × width × depth.

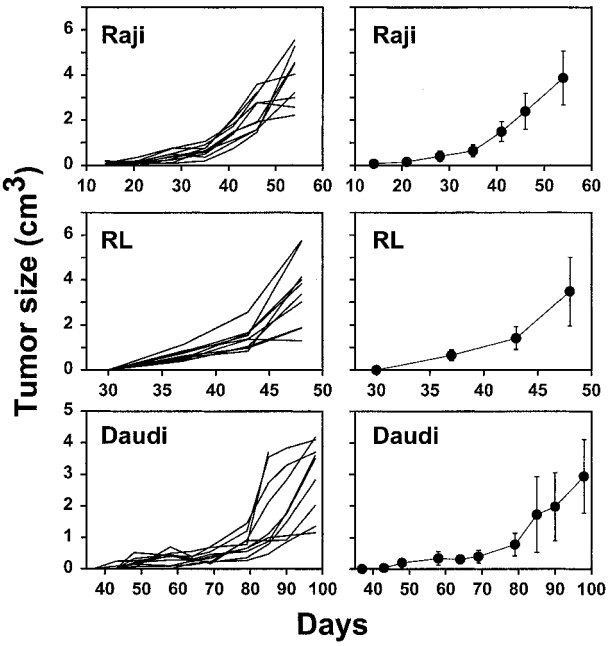

Fig. 1 Growth of human B-cell lymphoma cell lines in SCID mice. The tumors tested were Raji, RL, and Daudi, as indicated (*left panels*). Tumor growth in individual mice is shown (10 mice/group) (*right panels*). Means and standard deviations are shown.

Ab Localization Experiments. Ab localization experiments were performed as described previously (6). Briefly, mice received i.v. injection with approximately 10 μCi of Abs labeled with ^{131}I, ^{125}I, or ^{111}In. At various times, mice were sacrificed, and the dissected organs were weighed and counted for radioactivity. Counts were corrected for cross-over in the gamma counter windows. Because the Abs used were all IgG2as, all mice received coinjection with 0.1 ml of serum from normal CF1 adult female mice (Charles River Laboratories, Wilmington, MA). This serum provides normal IgG2a, which blocks splenic uptake that is probably due to Fcγ receptor CD64 (23). The rationale for this procedure is further discussed under "Results." Statistical comparisons used Student's *t* test.

RESULTS

Growth of B-Cell Lymphoma Xenograft in SCID Mice. Three B-lymphoma cell lines were tested for their growth s.c. in SCID mice. Raji, RL, and Daudi cells all grew well, and their growth rates are shown in Fig. 1. Four to five weeks were required for substantial tumor sizes (>0.1 g) to be reached with all Raji and RL tumors, whereas Daudi growth was even slower, with 9–10 weeks required for substantial tumor sizes. In a more recent experiment, Daudi tumors developed approximately 2 weeks earlier than this (data not shown). Tumor growth was relatively consistent, with all mice developing progressing tumors. It appeared that tumors grew slowly until they reached a size of approximately 0.6–1.4 g (depending on the particular

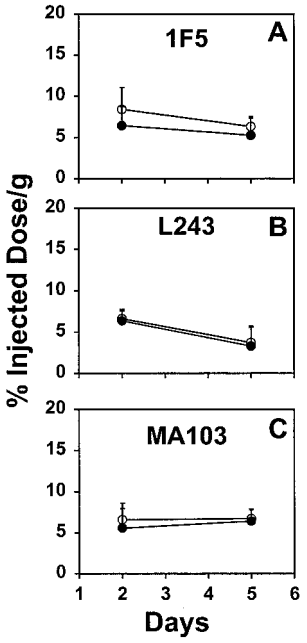

Fig. 2 Localization of three Abs to s.c. Raji xenografts in SCID mice. The Abs tested were (*A*) 1F5 (anti-CD20), (*B*) L243 (anti-MHC class II), and, (*C*) MA103 (anti-CD147), all IgG2as, and all were labeled with ^{125}I by conventional iodination. In each experiment, the same mice received injection of a control nonreactive IgG2a Ab, MX352a, labeled with ^{131}I by conventional iodination. Values shown are the mean ± SD of the % injected dose/g tumor for the specific Ab (●) and the nonreactive Ab (○) for groups of five to eight mice. Results shown are representative of at least two experiments performed with each radiolabel. Mean tumor sizes were as follows: 1F5 day 2, 0.224 ± 0.108; 1F5 day 5, 0.249 ± 0.138; L243 day 2, 0.329 ± 0.195; L243 day 5, 0.270 ± 0.102; MA103 day 2, 0.235 ± 0.171; and MA103 day 5, 0.252 ± 0.144.

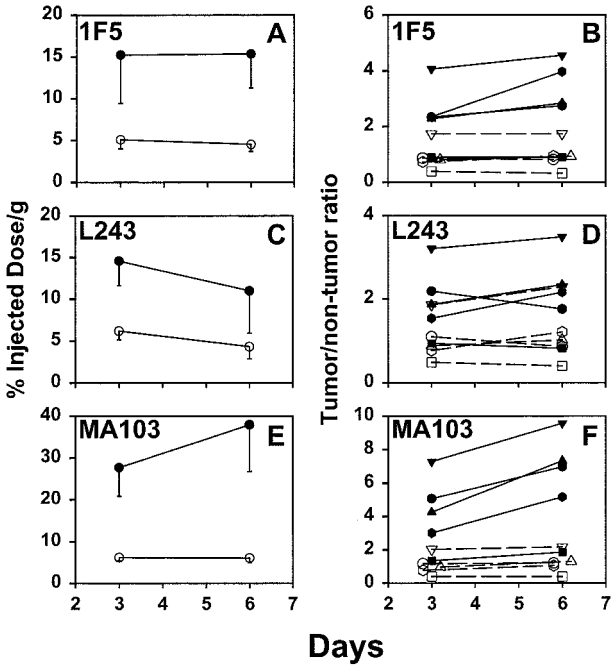

Fig. 3 Localization of three Abs to s.c. Raji xenografts in SCID mice, using a residualizing label, iodo-DLT, for both the specific and nonreactive Ab. Other details are as described in Fig. 2, except that both the tumor uptake (*A*, *C*, and *E*) and the tumor:nontumor ratios (*B*, *D*, and *F*) are shown. The tissues included are the liver (*circles*), spleen (*squares*), kidney (*triangles*), lungs (*inverted triangles*), and blood (*hexagons*). Results with the specific Abs are shown by the *filled symbols*, and results with the nonreactive Ab are shown by the *open symbols*. Values shown are means for groups of four to eight mice. SDs are omitted for clarity from the panels on the *right*. Mean tumor sizes were as follows: 1F5 day 3, 0.204 ± 0.095; 1F5 day 6, 0.410 ± 0.089; L243 day 3, 0.229 ± 0.067; L243 day 6, 0.177 ± 0.088; MA103 day 3, 0.185 ± 0.084; and MA103 day 6, 0.142 ± 0.066. Results shown are representative of at least two experiments performed with each radiolabel. To make the symbols more clearly visible, some of the values are shifted slightly to the *right* or *left*.

tumor), and then they often began to grow more rapidly. For Ab localization experiments, to obtain at least 10 mice bearing tumors of an acceptable size (0.05–0.5 g), 15 mice typically received injections, and generally 12–13 mice were usable. Occasional mice (<10%) developed ascites and multiple large i.p. solid tumors. It is uncertain whether this resulted from a defective s.c. injection of tumor cells (with some cells injected into the peritoneal cavity) or from metastasis of the tumor from the s.c. site. Such mice were excluded from the data presented.

Ab Localization to s.c. Xenografts of Raji B-Cell Lymphoma in SCID Mice. Three Abs labeled with ^{125}I by conventional iodination were tested for localization to Raji s.c. xenografts growing in SCID mice. A control nonreactive Ab of the same subclass labeled with ^{131}I was coinjected into the same mice. As shown in Fig. 2, there was no specific tumor uptake of any of the Abs tested. We note that the slight, nonsignificant difference seen with Ab 1F5 (Fig. 2*A*) shows a small advantage of the nonspecific Ab, and this is due to slightly faster blood clearance of 1F5. There are a number of possible explanations for a lack of specific uptake. To investigate this point, the same experiment was repeated, except that we used a residualizing iodine label, iodo-DLT. This label is trapped within lysosomes after catabolism of the Ab to which it was conjugated (24). As shown in Fig. 3, specific tumor uptake was clearly seen with these labels. Uptake was highest with the Ab MA103 but was also substantial with the other two Abs. The increase in tumor uptake due to the use of specific Ab was as follows: 1F5 day 3, 3.0-fold; 1F5 day 6, 3.4-fold; L243 day 3, 2.4-fold; L243 day 6, 2.6-fold; MA103 day 3, 4.5-fold; and MA103 day 6, 6.3-fold. All differences between the specific and nonreactive Abs were statistically significant ($P < 0.01$) at both time points. Fig. 3 also shows tumor:nontumor ratios in this experiment, and these ratios were also much higher with the specific Abs than with the nonreactive Ab. In addition to the tissues shown, muscle was also assayed in these experiments: tumor:nontumor ratios were too high to be plotted on these graphs, but a similar advantage of the specific Ab was demonstrated. Similar results with muscle, showing the low level of Ab uptake and the consequent high tumor:nontumor ratios, were described previously (17). To quantitate the advantage of the specific Ab, we calculated the specificity index, which is defined as the (tumor:nontumor ratio with the specific Ab)/(tumor:nontumor ratio with the nonreactive Ab). These values, presented in Table 1, show the advantage of the specific Ab relative to the nonreactive Ab. The lower

Table 1 Specificity index for I-DLT-labeled Abs localizing to s.c. Raji tumors in SCID mice

Tumor-bearing mice received injection with the indicated specific Ab labeled with ^{125}I and a nonreactive, isotype-matched control Ab labeled with ^{131}I and were dissected at the time indicated. Other data from the same experiments are shown in Fig. 3. The specificity index = (tumor:nontumor ratio with the specific Ab)/(tumor:nontumor ratio with the nonreactive Ab).

	Specificity index					
	Liver	Spleen	Kidney	Lung	Blood	Muscle
1F5 day 3	2.69	2.31	2.81	2.34	3.22	2.66
1F5 day 6	3.35	2.81	3.05	2.62	4.22	3.97
L243 day 3	1.99	1.92	2.13	1.73	2.00	1.94
L243 day 6	2.05	2.05	2.29	1.52	1.79	1.83
MA103 day 3	4.33	3.38	4.38	3.59	3.91	4.53
MA103 day 6	5.77	4.77	5.65	4.41	4.92	5.89

specificity indices for L243, relative to the other Abs can be attributed, at least in part, to the lower immunoreactivity of this Ab as described in "Materials and Methods."

In all experiments, the spleen showed substantial nonspecific uptake of the Abs, which resulted in lower tumor:normal tissue ratios for the spleen than for the other normal tissues, as shown in Fig. 3. This can be attributed to a low level of IgG2a-specific uptake by the spleen for the following reasons. (*a*) It was shown that in nude mice with low levels of endogenous IgG2a, there is splenic uptake of injected IgG2a and IgG2b, probably due to the high affinity Fcγ receptor CD64 (23). (*b*) Similar splenic uptake occurs in SCID mice (25), as would be expected, because IgG2a levels are considerably lower in SCID mice than in nude mice. (*c*) To circumvent this problem, all mice received coinjection with 0.1 ml of normal CF1 mouse serum, together with the radiolabeled Ab. The normal CF1 mouse serum provided a source of irrelevant IgG2a and IgG2b intended to block the receptor. In fact, this coinjection largely blocked the rapid blood clearance of the Abs that would otherwise occur (25), although it did not completely prevent a low level of splenic uptake. (*d*) Such uptake did not occur in previous experiments using IgG1 Abs in the same experimental model (data not shown).[4]

To demonstrate more clearly the advantage of the residualizing labels, double-label experiments were performed with a conventional iodine label and a residualizing label injected into the same mice. Whereas both residualizing labels and conventional iodine labels were used in the previous experiments, a double-label experiment in the same mice provides a large advantage in demonstrating differences, primarily because variability in tumor size is no longer a factor. Such experiments were performed with two residualizing labels: ^{125}I-DLT (for MA103) and ^{111}In-benzyl-DTPA (for 1F5 and L243). As shown in Fig. 4, the residualizing labels provided a large advantage over the conventional iodine label, consistent with the previous results, in both tumor uptake and tumor:nontumor ratios. The increase in tumor uptake due to the use of the residualizing label was as follows: 1F5 day 2, 4.43-fold; 1F5 day 5, 5.48-fold; L243 day 2, 2.36-fold; L243 day 5, 3.09-fold; MA103 day 3, 3.23-fold; and MA103 day 6, 3.10-fold. The difference in tumor uptake was statistically significant ($P < 0.01$) at all time points with all three Abs.

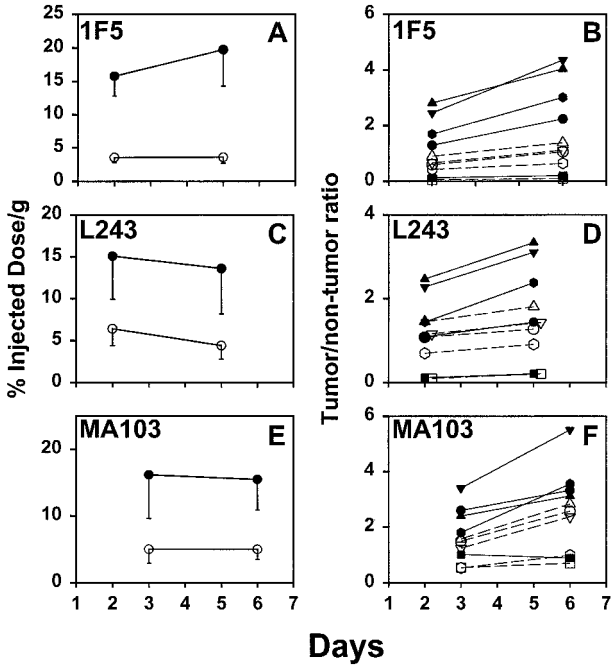

Fig. 4 Localization of three Abs to s.c. Raji xenografts in SCID mice, using a residualizing label coinjected with a conventional iodine label. The residualizing label used was either ^{111}In-benzyl-DTPA (for 1F5 and L243) or [^{125}I]iodo-DLT (for MA103). Other details are as described in Fig. 3. Results with the residualizing label are shown by the *filled symbols*, and results with the conventional iodine label are shown by the *open symbols*. Values shown are means for groups of five to seven mice. SDs are omitted for clarity from the panels on the *right*. Mean tumor sizes were as follows: 1F5 day 2, 0.311 ± 0.131; 1F5 day 5, 0.304 ± 0.135; L243 day 2, 0.210 ± 0.096; L243 day 5, 0.356 ± 0.125; MA103 day 3, 0.334 ± 0.089; and MA103 day 6, 0.507 ± 0.198. Results shown are representative of at least two experiments performed with each radiolabel. To make the symbols more clearly visible, some of the values are shifted slightly to the *right*.

Residualizing labels are trapped not only within lysosomes of the tumor cells, but also within lysosomes of any other cell that catabolizes the Ab. Such tissues are, primarily, the liver, spleen, and kidney. This uptake occurs similarly with IgG1s as well as IgG2as and occurs in normal mice as well as in immunodeficient mice (26). These sites of normal IgG catabolism were originally identified in the rat (27). In Fig. 5, we show that this expected retention in the liver, spleen, and, to a lesser extent, kidney also occurred in our experiments, using ^{111}In-1F5 as an example. In this figure, to show the difference between the two radiolabels, we plot the ratio of the (percentage of injected dose/g of tissue with ^{111}In): (percentage of injected dose/g of the same tissue with ^{125}I). If there is no catabolism of the Ab within a particular tissue, then there will be no advantage of the residualizing label, and the ratio will be 1.0. This ratio was approximately 2 for the liver and the spleen. Because the increase in the tumor uptake was approximately 5-fold, there was

[4] M. J. Mattes, unpublished data.

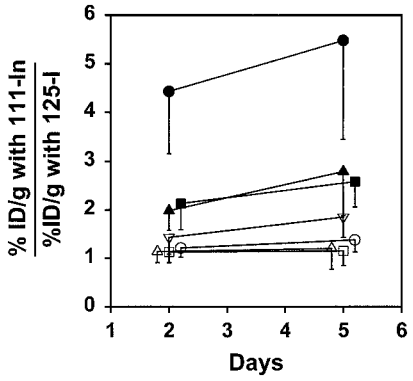

Fig. 5 The difference between residualizing and nonresidualizing radiolabels in their accumulation in tumor and normal tissues. Mice bearing s.c. Raji tumors received injection of a mixture of Ab 1F5 labeled with either ^{125}I or ^{111}In-benzyl-DTPA. At the time points indicated, mice were sacrificed, and tissues were monitored for radioactivity. Other data from the same experiment are shown in Fig. 4, *A* and *B*. Tissues shown are the tumor (●), liver (■), spleen (▲), kidney (▽), lungs (○), blood (□), and muscle (△). The *Y* axis shows the ratio of the (% injected dose/g with ^{111}In)/(% injected dose/g with ^{125}I). Values shown are the means ± SDs. To make the symbols more clearly visible, some of the values are shifted slightly to the *right* or *left*.

still an increase in the tumor:liver and tumor:spleen ratios resulting from the use of the residualizing label.

Experiments with Xenografts in Nude Mice. To demonstrate that the results obtained were not unique to this experimental model, similar experiments with 1F5 were performed with Ramos B-cell lymphoma tumors growing in nude mice, a model that we have described in detail, using other Abs (6, 17). The disadvantage of this model is the extremely rapid tumor growth, as well as the considerable variation in tumor size between animals. Results obtained with this model were consistent with those described above. More specifically, with 1F5, there was no or very little specific uptake of a conventional iodinated Ab, whereas the same Ab labeled with the residualizing label DLT showed substantial specific uptake (data not shown). The increase in tumor uptake due to use of the residualizing label was 3.46-fold at day 3 and 6.34-fold at day 6. As a further comparison of the two tumor models, SCID mice bearing Raji tumors received injection of radiolabeled anti-CD74 (LL1), a rapidly internalizing Ab that had been used extensively with the Ramos model (17). LL1, labeled with either a residualizing or a nonresidualizing radionuclide, demonstrated a level of uptake very similar to that described previously with Ramos tumors (data not shown). We conclude that the two tumor models, which are very different in growth rate, are similar in their level of Ab uptake and catabolism, at least under the conditions tested.

DISCUSSION

The major conclusion of this study is that with all three of the Abs tested, Ab accretion in the tumor required use of a residualizing radiolabel. The immunoreactivity of the Abs was the same regardless of the labeling method used, so the results cannot be a consequence of Ab damage resulting from labeling. Whereas most of our experiments used a single experimental model, Raji s.c. tumors in SCID mice, some experiments with 1F5 were also performed using Ramos s.c. tumors in nude mice, and essentially the same results were obtained. In addition, our findings can potentially explain published results that were obtained with other experimental models, as discussed further below. Therefore, we suggest that the conclusions are generally true for human lymphoma xenografts in mice, although exceptions are possible. It is important to emphasize, as discussed in the "Introduction," that the Abs used were considered to be noninternalizing or slowly internalizing and that there is abundant evidence that any Abs binding to the cell surface would be catabolized at least as rapidly as anti-CD147 (13, 19, 28). Therefore, the conclusions are expected to be applicable to all Abs binding to the cell surface.

To explain these results, it should be emphasized that, in our experience, all Abs bound to the cell surface are gradually internalized (13, 19, 28). There is a minimum rate of Ab catabolism that is probably due to the basal turnover rate of cell surface constituents, with a $t_{1/2}$ of ≥2 days *in vitro*, depending on the particular cell line. In previous tests of a large number of cell lines, anti-CD147 was always catabolized at a rate less than or equal to the catabolic rate of any other Ab binding to the same cell line, which suggests that catabolism of this Ab reflects membrane turnover. From this perspective, the simplest explanation for our data is that membrane turnover on B-cell lymphoma xenografts is relatively fast. It is therefore interesting to note that *in vitro* membrane turnover is unusually slow on B-cell lymphomas. Fig. 6 summarizes our results using anti-CD147 with 35 cell lines. These data suggest that the rate of membrane turnover is related to the histological type of the cell line, and B-cell lymphomas had the slowest catabolic rate, with EBV-transformed B-lymphoblastoid cell lines having a slightly higher catabolic rate. Catabolism of anti-CD147 by Raji cells *in vitro* occurred at a rate of only approximately 3.5%/day, which is too low to account for the results obtained *in vivo*. Therefore, we can tentatively conclude that the Ab catabolic rate *in vivo* is much greater than that *in vitro*. The simplest explanation is that membrane turnover is faster *in vivo* than on the same cell line *in vitro*. Whereas data on this point are limited, there is some evidence that rapidly growing cells have slower membrane turnover than more slowly growing cells (29), perhaps because cells growing rapidly cannot spare membrane components for turnover. Raji cells *in vitro* under our culture conditions have a rapid growth rate, with a doubling time of approximately 16 h, and it is likely that they multiply much more slowly *in vivo*. Another possible explanation for our results is that the Abs are catabolized not by the tumor cells themselves but instead by macrophages that may be attracted by the Abs coating the tumor cells. The macrophages may catabolize either the Ab alone or the entire tumor cell. We do not consider this a likely possibility, but it has not been excluded.

Whereas this effect was not previously recognized, most of the published data are not inconsistent with these conclusions. Specific Ab uptake in tumors has been claimed, but the specificity of uptake was often not fully established. First, if a nonreactive Ab of the same subclass is not tested as a control, it is impossible to know whether binding is specific or nonspecific. Second, it is well established that Ab uptake, expressed as % injected dose/g, is generally strongly dependent on tumor size, with uptake being higher in smaller tumors. If tumor sizes in individual experiments are not given, the data cannot be

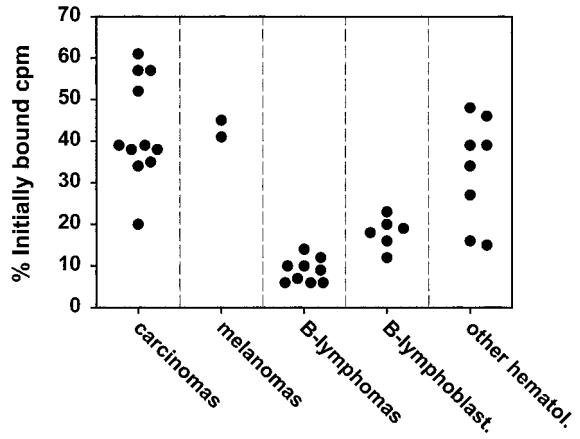

Fig. 6 Catabolism of ^{125}I-labeled Ab MA103 (anti-CD147) bound to the surface of 35 different tumor cell lines. The values shown are the percentage degraded and released into the medium as low molecular weight catabolites over a period of 2 days. Each *point* represents data from one cell line and is the mean of at least two separate experiments, each done in duplicate or triplicate. These data are mostly compiled from previously described experiments (13, 19, 40, 41). The tumor cells tested were as follows: carcinomas SK-OV-6, SK-RC-18, HeLa, LS174T (20%), LoVo, MDA-MB-468, MDA-MB-231, T47D, HT-29, TT, and ZR-75-30; melanomas SK-MEL-1 and SK-MEL-37; B-cell lymphomas Raji, Ramos, Daudi, RL, SU-DHL-4, SU-DHL-6, NC-37, Namalwa, and BJAB; B-lymphoblastoid cell lines RPMI 1788, RPMI 7666, GK-5, E.H. IV, IM-9, and SKW6.4; and other hematological tumors [Jurkat, U-937, MOLT-4, U-266 (16%), HL-60, K-562, TF-1, HUT-102 (15%)]. In this list, specific values for individual cell lines are given in *parentheses* only for those cell lines that are significantly different from the rest of the histological group. Because some of our previously published data were marred by *Mycoplasma* contamination (42, 43), we note that all data presented above were from *Mycoplasma*-free cultures.

evaluated. The best control for this variable, which also controls for many other variables, is to perform double-label experiments, with both the specific and the nonreactive Ab tested in the same animal, as done here, but such experiments have only rarely been done.

With anti-CD20 Abs, the most convincing localization experiment was the double-label experiment of Buchsbaum *et al.* (9), but specific Ab uptake in a nude mouse model was very low (only approximately 50% higher than uptake of the nonreactive Ab). In other experiments with the same tumor model, uptake of another specific Ab, anti-CD10, appeared to be considerably higher, but double-label experiments were not performed with this Ab. The same laboratory later demonstrated that anti-CD20 Ab uptake was increased by preinjection of the same Ab, unlabeled (10), but the specificity of this uptake was not established by the use of an isotype-matched nonreactive Ab. Another Ab to the MHC class II antigen, Lym-1, has been widely used in patients (3). Whereas some Ab localization experiments with Lym-1 in mice were performed (30), a nonreactive control Ab was not included, so the specificity of uptake is uncertain. ^{67}Cu-labeled Lym-1 showed no greater tumor uptake than ^{131}I-Lym-1 (31), which we cannot explain, because the ^{67}Cu label is expected to be a residualizing label. Localization of anti-CD19 was also found to be at a low level (11). In addition, the studies of Schmid *et al.* (32), which suggest that Abs are unable to penetrate certain B-cell lymphoma xenografts, may have been affected by Ab catabolism.

Despite the lack of impressive Ab localization data, effective radioimmunotherapy of B-cell lymphoma s.c. xenografts in nude mice has been described. Effective therapy of course implies, although it does not directly prove, that the Ab localized specifically to the tumor. Buchsbaum *et al.* (33), using ^{131}I-anti-CD37, reported a small therapeutic effect that did not occur with a nonreactive Ab. However, the unlabeled Ab also had a substantial effect, and the combination of unlabeled Ab plus radiolabeled nonreactive Ab was not tested, leaving open the possibility that the effect of the radionuclide was nonspecific. Wei *et al.* (34) used ^{131}I-anti-CD22, -CD19, and -CD20 and obtained effective therapy with the first two. Because a nonreactive control Ab labeled similarly had very little effect, the therapy apparently was specific. The control mentioned above was not done, but this control seems less essential if the unlabeled Abs have no effect, as was the case here (because the Abs tested were all IgG1s). Thus, these results appear to be inconsistent with ours, but the inconsistencies raise a number of issues that can and should be experimentally addressed. Because Wei *et al.* (34) did not perform biodistribution studies, it is not known whether the therapeutic effect correlated with radiolabel uptake in the tumor. The fact that anti-CD22 was much more potent than anti-CD20 seems surprising because the antigen density of CD20 is much greater than that of CD22 on Daudi cells (12), and it would therefore not be expected that anti-CD22 uptake would be higher. At this time, we can only speculate that because Ab uptake and catabolism are metabolic processes, they probably are regulated by various physiological signals. Thus, the use of sublethally irradiated mice by Wei *et al.* (34) to allow tumor growth may be important. It seems possible that the irradiation itself might affect the catabolism of Abs binding to the tumors by some indirect metabolic effect. Alternatively, the fact that sublethal irradiation was required demonstrates that there was a natural antitumor mechanism in these animals. This mechanism, gradually recovering after irradiation, may affect the tumor cells such that the rate of Ab catabolism is reduced. Wei *et al.* (34) also used much higher Ab protein doses (100 μg/mouse) than used in our experiments, which may enhance Ab uptake, as discussed below. DeNardo *et al.* (35) used ^{67}Cu-anti MHC class II; because this is probably a residualizing radiolabel and because it was more effective than an ^{131}I conjugate, the results are generally consistent with ours.

Whereas it is reasonable to suggest that all Abs used to target tumors would benefit from the use of a residualizing label because some catabolism will occur, the total lack of detectable uptake with the conventional iodine label was surprising. The results strongly suggest that internalization and catabolism of the Abs are quite rapid *in vivo*. Still, these antigens are expressed on the cell surface, so why isn't Ab bound to the cell surface detected? For Ab to bind to the cell surface, a major physiological obstacle is the movement of Ab from blood to the interstitial fluid adjacent to the target cell. This process is relatively slow, requiring several days for equilibrium to be reached (36). If the catabolic rate is faster that the rate of Ab binding to the cell surface, then little Ab will ever be present on

the cell surface, and it will accumulate only intracellularly (if residualizing radiolabels are used).

Our previous work with B-cell lymphoma xenografts was with Abs to CD22 or CD74, using the Ramos xenograft model. These Abs are known to be internalized and catabolized rapidly. Specific uptake by lymphoma xenografts was demonstrated (6, 17), and this was shown to be entirely dependent on the use of residualizing labels. With conventional iodine labels, there was no specific Ab uptake. Thus, the results were very similar to the results described herein. Whereas CD20 is commonly described as a noninternalizing Ab, our experimental data indicate a significant level of catabolism *in vitro* (19). Using Ramos cells, anti-CD20 was catabolized significantly faster than MA103 or anti-CD45, suggesting that it is catabolized due to an active and specific process, in addition to basal membrane turnover. On Raji cells *in vitro*, there was catabolism of 35.7 ± 9.1% of the bound Ab in 45 h, compared with only 7.0% catabolism of bound anti-CD147 under the same conditions.[4]

When B-lymphoma tumor cells were collected directly from s.c. tumors growing in mice that had received injection of large amounts of Ab L243, near-saturation of cell surface antigen on viable tumor cells was demonstrated by immunofluorescence with a fluorescein-conjugated goat antimouse IgG (17). Because these experiments demonstrated the presence of intact Ab on the cell surface, the results appear to be contradictory to the data presented above. However, a major difference was in the amount of Ab injected. In the saturation experiments with unlabeled Ab, up to 5.0 mg of Ab were required per mouse to achieve near-saturation. In contrast, the Ab localization experiments used only approximately 1–10 μg of Ab. This large difference in Ab dose can potentially explain the difference in the results because the higher dose will ensure that all antigen on the cell surface is bound by Ab, essentially as soon as it appears.

There is some precedent for these results. Stein *et al.* (26) compared the advantage of residualizing radiolabels with two Abs, a rapidly internalizing Ab (anti-EGP-1) and a slowly internalizing Ab (anti-EGP-2). Unexpectedly, the advantage of the residualizing label was equally strong in both cases. These results presented less of a dilemma than the results presented here because catabolism of both Abs *in vitro* by the lung carcinoma cell line used was relatively fast, but they support the basic conclusion that *in vitro* results may not always be indicative of Ab processing *in vivo*.

A key question is whether similar events occur in patients. Whereas the answer is not known, there is some evidence that this is the case. DeNardo *et al.* (14) compared uptake in patients of Lym-1 (anti-MHC class II) conjugated to either ^{67}Cu, via the chelator BAT, or ^{131}I and found much higher uptake of the Cu, due to longer retention in the tumor. Whereas Lym-1 had previously been considered a noninternalizing Ab, the authors suggested that the results were probably due to internalization and catabolism of the Ab, which would be consistent with our results. Anti-CD20 Abs conjugated with radiometals (^{90}Y or ^{111}In) have been injected into many patients, as has ^{131}I-anti-CD20, but we are not aware of a comparison in uptake between the different radiolabels. Clearly, our results suggest that there should be a large advantage of residualizing labels, such as ^{111}In and ^{90}Y, for both imaging and therapy.

An advantage of the DLT label used in these experiments should be recognized. Use of both ^{125}I and ^{131}I together allows evaluation of two residualizing labels in the same mice, as in Fig. 3, which is not readily achieved with other residualizing radiolabels. This labeling method is complex and has a low efficiency of only 5–10% but yields a good-quality product sufficient for Ab localization studies. Therapy experiments in mice were performed with ^{131}I-DLT conjugates (37), but clinical work would require a labeling method for residualizing iodine that is more efficient. Such methods are under development (38, 39). Alternatively, radiometals such as ^{90}Y could be used. Finally, the animal model described here has considerable advantages over other B-cell lymphoma xenograft models, with one of the primary advantages being its simplicity. Hudson *et al.* (8) grew Raji and other B-cell lymphomas in SCID mice, but their interest was mainly in rapid tumor growth, which was achieved in mice having a greater degree of immunodeficiency. Thus, their experiments were terminated at day 22, a time when many Raji tumors, in our experiments, are just beginning to appear. The relatively slow tumor growth might be considered a disadvantage, but it also provides a number of advantages, because (*a*) exact timing of Ab injection is not critical; (*b*) time points out to 2 weeks after tumor appearance could be used, which allows more accurate dosimetry calculations; (*c*) therapy is more likely to be successful; and (*d*) slower growing tumors are likely to provide a better model for human disease. We expect that this model will be useful in the further development of Abs for diagnosis and therapy of B-cell lymphoma.

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Antibody Localization to B-Cell Lymphoma Xenografts in Immunodeficient Mice: Importance of Using Residualizing Radiolabels

Rosana B. Michel, Rita Ochakovskaya and M. Jules Mattes

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