Microscopic Intratumoral Dosimetry of Radiolabeled Antibodies Is a Critical Determinant of Successful Radioimmunotherapy in B-Cell Lymphoma

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Abstract

Radioimmunotherapy is a highly effective treatment for some hematologic malignancies; however, the underlying mechanisms of tumor clearance remain poorly understood. We have previously shown that both targeted radiation using 131I-labeled anti–MHC class II (MHCII) monoclonal antibody (mAb) plus mAb signaling with unlabeled anti-idiotype are required for the long-term clearance of tumor in syngeneic murine lymphoma models. In this study, we have investigated how the microdistribution of the targeted radiation component of this combination affects the long-term clearance of lymphoma. 131I-labeled mAb targeting CD45 and MHCII antigens was found to deliver similar doses of radiation to tumor-bearing organ using conventional dosimetry (~1.0 Gy per MBq when 131I was labeled to 500 μg mAb and given i.v., per mouse), but when used as radiation vectors in combination therapy only, 131I-anti-MHCII plus anti-idiotype produced long-term survival. The profound differences in therapy did not seem to be dependent on levels of 131I-mAb tumor-binding or antibody-dependent cytotoxicity. Instead, the microscopic intratumoral dosimetry seemed to be critical with the 131I-anti-MHCII, delivering more concentrated and therefore substantially higher radiation dose to tumor cells. When the administered activity of 131I-anti-CD45 was increased, a radiation dose response was shown in the presence of anti-idiotype and long-term survival was seen. We believe that these new insights should influence the selection of new antigen targets and the design of dosimetric methods in radioimmunotherapy of lymphoma. [Cancer Res 2007;67(3):1335–43]

Introduction

The use of monoclonal antibody (mAb) to deliver radioisotopes selectively to tumors is theoretically appealing, and over the last decade, considerable progress has been made in the field of radioimmunotherapy. Despite a wide variety of mAbs, radionuclides, and study designs, a common theme to emerge from clinical trials investigating radioimmunotherapy in relapsed non-Hodgkin’s lymphoma has been the high response rates with durable remissions seen for significant numbers of patients (1). Based on some of these early-phase clinical studies, two radioimmunoconjugates, 90Y-ibritumomab tiuxetan (Zevalin) and 131I-tositumomab (Bexxar), were approved by the Food and Drug Administration for the treatment of relapsed follicular non–Hodgkin’s lymphoma. There is currently considerable optimism that radioimmunotherapy will play an important part in the future management of non–Hodgkin’s lymphoma, and further studies are ongoing, which will help further define the role for radioimmunotherapy (2).

Most lymphomas are extremely radiosensitive and generally have good blood perfusion for the delivery of radioimmunoconjugates. These favorable characteristics are thought to be important factors relating to the high response rates that have been seen with radioimmunotherapy in lymphomas (3–5). However, the importance of biodistribution studies and tumor dosimetry to the overall therapeutic response seen in radioimmunotherapy of lymphoma has been very difficult to clarify and remains highly controversial (6–8). Some investigators have advocated that biodistribution studies and tumor dosimetry are critically important and correlate well with clinical responses, whereas others have expressed the view that no such relationship exists (6–11). Indeed, radioimmunotherapy is still regarded by some as little more than sophisticated total body irradiation with little role for antibody targeting and mAbs are considered simply as vectors for delivering of "systemic radiotherapy" (12).

Currently little is known about the intratumoral biodistribution of radiolabeled mAb in lymphoma (4, 13). Theoretically, the highly heterogeneous cumulated radiation activity distribution that is often encountered with targeted radiolabeled mAb is likely to lead to marked differences in the absorbed radiation doses and in turn therefore to affect differences in individual cell survival across the tumor. However, conventional clinical gamma camera imaging used to estimate dosimetry typically has limited spatial resolution and is unable to reveal the intratumoral mAb distribution at the microscopic level. Consequently, a considerable number of assumptions have to be made when interpreting dosimetric data and these include a homogeneous intratumoral or intraorgan distribution. Such gamma camera imaging–based dosimetry is thus unsatisfactory because they have considerable limitations in terms of spatial resolution. Therefore, it is difficult or impossible in a clinical setting to determine the accurate distribution pattern of the infused mAb inside the tumors or normal organs at a cellular level and correlate the dosimetry with the tumor response.

Such fundamental mechanistic issues can however be approached in appropriate syngeneic tumor models. We have previously investigated the biodistribution, radiation dosimetry, and therapeutic efficacy of a panel of B-cell–targeting mAbs in the two syngeneic murine lymphoma models: BCL2 and A31 (14). We found 131I-labeled anti–MHC class II (MHCII) mAb to be an excellent vector for delivering "systemic radiotherapy" as it targets
a highly expressed, weakly modulating antigen and thus is able to deliver high doses of radiation to the tumor (14). Long-term clearance of tumor was however found to be dependent on the combination of targeted radiation using 131I-labeled anti-MHCII mAb plus antibody signaling with unlabeled anti-idiotype. Here, we have investigated the importance of intratumoral distribution of radiolabeled mAb and therefore the radiation "microdosimetry" to therapeutic outcome, comparing our established anti-MHCII vector with anti-CD45, which is another highly expressed antigen that is currently being evaluated in clinical radioimmunotherapy.

We investigated the ability of 131I-anti-CD45 to deliver systemic radiotherapy and compared the intratumoral distribution of these i.v. administered 131I-labeled mAbs at a microdosimetric level. We have correlated these findings with conventional tumor dosimetry and therapeutic effects of the radioimmunonocjugates within the same syngeneic lymphoma model. Here, we clarify that, for 131I-labeled mAb, it is the microscopic intratumoral distribution of the radiolabeled mAb that determines the long-term clearance of tumor. We believe that these new insights should influence the design and interpretation of further clinical studies and the ongoing development of new dosimetric methods to assess tumor dose in radioimmunotherapy.

Materials and Methods

Animals and cell lines. BALB/c mice were supplied by Harlan UK (Blackthorn, United Kingdom) and maintained in local animal facilities. BCL1, murine B-cell lymphoma line is maintained by in vivo passage in BALB/c mice (15). πBCL1 is a transformed variant derived from the BCL1 lymphoma that can be maintained in liquid culture grown in RPMI 1640 (Life Technologies, Paisley, United Kingdom) supplemented with glutamine (2 mmol/L), pyruvate (1 mmol/L), penicillin and streptomycin (100 IU/mL), 10% FCS (Life Technologies), and 50 mmol/L 2-mercaptoethanol (British Drug House, Poole, United Kingdom; refs. 14, 16).

Antibodies and iodination. mAbs used in this study include Mc10-6A5 (rat anti-BCL1, idiotype, IgG2a), Mc39-16 (rat anti-A31 idiotype, IgG2a), 1D3 (rat anti-mouse CD19, IgG2a), and TI2-3 (rat anti-mouse MHCII, IgG1). Details of the production and purification of these mAbs have been described previously (14, 17). The rat anti-mouse CD45 mAb YW62.3.2 (IgG2b) was kindly provided by Dr. Stephen Cobbold (Therapeutic Immunology Group, Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom). The carrier-free 125I and 131I radioisotopes were supplied by Amersham International (Little Chalfont, United Kingdom), and the mAbs were iodinated using Iodo-Beads (Pierce Chemical, Rockford, IL) as described previously (14). For the cell binding and biodistribution assays, mAbs were labeled with 125I instead of 131I. High labeling efficiency was consistently achieved (95.5–98.5%), measured by high-performance liquid chromatography. The immunoreactivity of iodinated mAb was monitored by cell binding assay as described (14, 18) using πBCL1 cells.

Binding density of radiolabeled mAb. The binding density of the mAb was assessed as before (17). Briefly, 125I-labeled mAbs were serially diluted before incubating with 10^7 πBCL1 cells in 500 μL complete medium for 2 h at 37°C. Endocytosis of cell-bound mAb was prevented by NaN3 (15 mmol/L) and 2-deoxyglucose (50 mmol/L). Following incubation, 200 μL cells were rapidly centrifuged through a 1.1:1 (v/v) mixture of dibutylnitrate-dioctyl phthalate oils, separating from unbound mAb, and the cell pellets were counted for cell-bound activity on a gamma counter (Wallace Compu gamma, Perkin-Elmer Life Sciences, Boston, MA). Assuming all the mAbs have same molecular weight (1.5 × 10^6), the cell binding curves of the mAbs were plotted as molecules of mAb per cell against the concentration of mAb.

Antibody-dependent cellular cytotoxicity assay. Antibody-dependent cellular cytotoxicity (ADCC) activity was assessed by 51Cr release assay (17). πBCL1, target cells were labeled with 51Cr and resuspended at 10^5/mL, and 50 μL aliquots were mixed with mAb in individual wells of a 96-well U-bottomed plate (Life Technologies) for 15 min on ice. Effector cells were human peripheral blood mononuclear cells (PBMC) added at an E/T ratio of 50:1 to give a final volume of 200 μL. The plates were centrifuged at 200 × g for 5 min, incubated for 6 h at 37°C in 5% CO2 incubator, and then centrifuged at 500 × g for 5 min before harvesting 100 μL of the supernatant to calculate the specific 51Cr release. Maximal release was determined using target cells to which 150 μL of 1% NP40 had been added, and the percentage specific release was calculated as follows: % specific release = [(sample release – background release) / (maximum release – background release)] × 100. All samples were done in triplicate.

Biodistribution studies and organ dosimetry estimation. Groups of BALB/c mice (15 mice per group) were inoculated by tail vein injection with 10^5 BCL1 cells. Ten days after tumor inoculation, animals received 500 μg of 125I-labeled mAb by i.v. injection. Thyroid uptake was blocked by Lugol’s solution (0.125 g KI, 0.0625 g elemental iodine in 100 mL water) given in the drinking water 3 days before the infusion of radiolabeled mAb. Biodistribution of radioactive mAb (anti-idiotype, anti-CD19, anti-MHCII, and anti-CD45) was compared with nonbinding control antibody. Animals were sacrificed 1, 8, 24, 48, and 96 h after receiving the radioactive mAb. The weight and radioactivity of the whole body and dissected organs (blood, spleen, liver, kidneys, and lungs) were measured, and the percentage of the injected dose per gram of tissue was calculated (19). Assuming total absorption of the nonpenetrating radiation component with a homogeneous distribution, both the whole body and the organ doses were calculated using the medical internal radiation dose (MIRD) method (14, 20, 21).

Radioimmunotherapy. Groups of age- and sex-matched mice were inoculated by i.v. injection with 10^7 BCL1 cells and treated 10 days later. For radioimmunotherapy experiments, 500 μg of 9.25 to 37.0 MBq 131I-labeled mAb were injected i.v. per mouse. For therapies combining radioimmunotherapy with unlabeled mAb, mice were injected i.v. with 500 μg of unlabeled anti-BCL1 idiotype antibody 2 h before the 131I-labeled mAb. Unlabeled mAbs or 131I-labeled control mAbs were used as controls. Lugol’s solution was given in all therapies. The BCL1 tumor develops predominantly in the spleen with a leukemic spill in the terminal stages of the disease (22). Without sufficient therapy, tumor-inoculated BALB/c mice die of tumor progression (rapid progressing splenomegaly). Animal survival was monitored daily and the results were analyzed using the χ2 test of Peto (23). Animal immunotherapy was approved by the local ethical committee and done under a United Kingdom Home Office license.

Cellular localization of i.v. administered mAb. Mice were inoculated with tumor and treated on day 10 as for the biodistribution studies. Radioimmunotherapy-treated and control animals were sacrificed 2 h after injection, and spleen biopsies were fixed overnight in acetone and embedded in glycol methacrylate resin as described (24). Essentially, spleens were taken immediately after culling, cut into small pieces of 2 mm × 2 mm × 4 mm in size, and fixed in acetone containing 2 mmol/L phenylmethylsulfonyl fluoride (350 mg/100 mL) and 20 mmol/L iodoacetamide (370 mg/100 mL) overnight at −20°C. After fixation, the acetone was replaced with methyl benzoate and left at room temperature for 15 min before adding processing solution (5% methyl benzoate in polyethylene glycol 400) at 4°C for 48 h for polymerization. Sections (2 μm) were then cut using glass knives on a dedicated resin microtome (Leica 2065 microtome, Leica UK, Milton Keynes, United Kingdom) for staining. The localization of the i.v. administered mAb was revealed by staining the sections with biotinylated mouse anti-rat antibody and subsequently applying the streptavidin biotin-peroxidase complexes and diamobenzidine tetrahydrodichloro substrate as described (24). Images were acquired by a Nikon Eclipse E600 microscope and a Nikon Coolpix 950 digital camera (Nikon Corp., Tokyo, Japan). The percentages of the immunohistochemically positively stained cells were observed and measured using the Zeiss KS400 imaging analysis package (Carl Zeiss Ltd., Welwyn Garden City, United Kingdom).
Results

Anti-MHCII and anti-CD45 mAb deliver similar doses of radiation to tumor-bearing organs. To assess the optimal target for an antibody vector in delivering radiation to tumor-bearing organs in vivo, we compared the biodistribution of a panel of mAbs against a range of B-cell antigens (anti-CD19, anti-CD45, anti-MHCII, and nonbinding control). The approximate radiation doses delivered by these mAbs were calculated for whole body, lung, liver, kidney, and spleen as previously described (14). Figure 1 shows the biodistribution of anti-CD19, nonbinding control mAb, anti-MHCII, and anti-CD45 presented as the percentage of injected radioactive dose per gram of organ tissue. Administration of a single trace dose (0.74 MBq) of 125I-labeled 500 μg of anti-MHCII or anti-CD45 mAb resulted in selective targeting of radiation to the spleen, the main tumor-bearing organ (Fig. 1C and D). This is in stark contrast to the B-cell–specific anti-CD19 mAb, which was considerably poorer and provided similar targeting of radiation (percentage of injected radioactive dose per gram of organ tissue) to that seen with the nonbinding control mAb (Fig. 1A and B).

The calculated radiation doses confirm that the anti-MHCII and anti-CD45 mAbs deliver similar doses of radiation per MBq of radiolabeled mAb infused to the spleen and other organs as well as equivalent doses of nonspecific irradiation to the whole body. An infusion of 18.5 MBq 131I-anti-CD45 mAb delivered ~ 18.5 Gy to the spleen and 3.42 Gy to the whole body compared with around 18 and 2.9 Gy, respectively, for the anti-MHCII mAb. Both mAbs delivered around three to five times greater doses of targeted radiation to the spleen than either the anti-CD19 (5.13 Gy) or the control mAb (4.5 Gy). Full details of radiation dosimetry are summarized in Supplementary Table S1.

Anti-MHCII but not anti-CD45 mAb is therapeutically effective as a vector for targeting radiation. We next compared the therapeutic efficacy of 131I-anti-MHCII and 131I-anti-CD45 mAbs for targeting radiation to tumor. Mice were inoculated with 10⁶ BCL1 cells i.v. and treated 10 days later with anti-CD45 or anti-MHCII mAb (unlabeled or 131I-labeled: 18.5 MBq) in the presence or absence of unlabeled anti-idiotype (500 μg/animal). When given as single agent, unlabeled anti-MHCII or anti-CD45 mAb failed to
enhance survival over controls, whereas the anti-idiotype mAb gave a modest increase in survival of ~8 days (Fig. 2). Likewise, treatment with either 131I-anti-MHCII or 131I-anti-CD45 alone provided only a moderate level of protection of around 15 to 20 days. In keeping with our previous results, 18.5 MBq of 131I-labeled anti-MHCII plus unlabeled anti-idiotype produced 100% long-term animal protection (>100 days). In contrast, none of the animals treated with 18.5 MBq of 131I-labeled anti-CD45 and unlabeled anti-idiotype became long-term survivors, with animals having a median survival time of ~30 days over controls (P < 0.02). This difference was unexpected given that the anti-CD45 mAb seemed to deliver a similar radiation dose to tumor as the anti-MHCII mAb when assessed by conventional biodistribution experiments (Fig. 1). It is worth mentioning that the radiiodinated mAb immunoreactivity as monitored by γBCL1 cell binding showed no detectable damage throughout therapy experiments.

**Cell surface binding and ADCC of mAbs.** We next investigated whether the differences in therapeutic outcome seen between the anti-MHCII and anti-CD45 mAb could be explained based on either cell surface binding density or mAb effector function. First, the cell binding characteristics of the panel of radiolabeled rat anti-mouse mAbs were assessed. Figure 3A shows binding curves done at 37°C. CD45 showed a high level of surface expression on BCL1 cells at around 6 x 10^5 mAb molecules bound per cell, which was comparable with the level of MHCII (approximately 7.8 x 10^5 mAb molecules bound per cell). These levels were around twice that seen for the antitumor idiotype mAb (around 3.5 x 10^5 molecules bound per cell) and four to five times that seen for the anti-CD19 mAb (approximately 1.5 x 10^5 molecules bound per cell). Overall, this gave binding levels in the order of MHCII = CD45 > idiotype > CD19 in keeping with our previously reported data (17) and failed to provide an explanation for the marked therapeutic differences seen between the 131I-anti-MHCII and 131I-anti-CD45 radioimmunoconjugates when combined with anti-idiotype.

It is also possible that the ability to trigger innate effector mechanisms may differ between the two mAbs. To address this, we compared the ability of our panel of mAbs to mediate ADCC using human PBMC. As shown in Fig. 3B, mAbs of all specificities were able to induce some degree of lysis. However, those targeting antigens expressed at the highest levels (anti-MHCII and anti-CD45 mAb) showed the greatest level of ADCC activity with ~25% specific chromium release, whereas those against antigens expressed at lower levels showed less activity (~8% for anti-CD19 mAb; P < 0.05). Again, there was no discernible difference between the anti-MHCII and anti-CD45 mAb (P > 0.5), suggesting that other factors may have a greater influence on therapeutic efficacy.

**Microdosimetric analysis of intratumoral localization of mAb in radioimmunotherapy of lymphoma.** We next examined whether “microdosimetric” differences in the delivery of radio-labeled mAb to tumor could account for the contrasting therapeutic activity between the two radioimmunoconjugates. Immunohistochemical studies were done to investigate the intratumoral distribution of i.v. infused mAbs within a BALB/c spleen 10 days after inoculation with tumor. In keeping with the biodistribution studies, the specific binding of i.v. injected mAb to target cells occurred as early as 1 h after injection and peaked at 8 h. Figure 4A shows the selective binding of the anti-idiotype mAb to a cluster of tumor cells (Fig. 4A, arrow) 2 h after i.v. injection. This represents ~3% of the total cells (as determined by the Zeiss KS400 imaging analysis package). Addition of a nonbinding control mAb (anti-A31 idiotype) before the application of the mouse anti-rat antibody or application of additional anti-BCL1 idiotype in vitro before the secondary antibody failed to reveal any additional staining (data not shown), indicating that all the BCL1 tumor cells were accurately targeted by the infused anti-idiotype mAb.

We next investigated the microdistribution of the anti-MHCII mAb. Figure 4B shows that ~35% of the cells were stained positive covering a large portion of the section denoting the B-cell area of the spleen. Application of an anti-CD3 mAb resulted in positive staining of the residual spleen section, representing the T-cell area (Fig. 4C). Importantly, the area stained by the anti-MHCII mAb encompasses that stained by the anti-idiotype mAb (Fig. 4A), suggesting that the initial site of tumor growth and residency is exclusively within the B-cell zone. In contrast, the anti-CD45 mAb showed a more homogeneous distribution with ~80% of total splenocytes stained positive (Fig. 4D). The staining patterns of i.v. infused and in vitro applied mAbs were also compared for all specificities with results showing no difference (data not shown).Taken together, these data clearly show that the localization of the anti-MHCII and anti-CD45 mAb is substantially different. It seems therefore that, whereas the anti-CD45 mAb may deliver targeted

![Figure 2](image-url)
radiation homogeneously within the spleen, the anti-MHCII mAb delivers the radiation more specifically to the tumor-bearing areas of the spleen, thus effectively delivering higher doses of radiation to the tumor. Although there is no established methodology available to accurately differentiate the radiation doses delivered to tumor cells by the anti-MHCII and anti-CD45 mAb at such a microscopic level, if we assume that the volume of the B-cell zone comprises approximately half of the spleen as revealed by the immunohistochemical study, the anti-MHCII mAb could potentially deliver double that of the anti-CD45 mAb as estimated from conventional dosimetric methods.

Figure 5A shows the level of anti-BCL1 idiotypic binding to tumor cells in advanced-stage disease (18 days after inoculation), comprising ~45% of the total splenocytes. This area coincides with the anti-MHCII staining, which was predominant throughout the B-cell areas, such as the marginal zone (Fig. 5B). Approximately 65% of the total splenocytes were stained positive for anti-MHCII, indicating that, as the BCL1 tumor advanced, the percentage of MHCII-positive cells increased (compared with 35% in early-stage disease; Fig. 4B). This was in contrast to T-cell areas (Fig. 5C), which were reduced to clusters around the central arteriole, with the overall percentage of CD3-positive cells vastly lower in late-stage disease (15% of total splenocytes versus 45% in early stage). The anti-CD45 mAb again provided homogeneous pan-lymphocyte staining, and the differences in the mAb microdistribution are maintained in more advanced tumor development (Fig. 5D).

Long-term survival is achievable by increasing the activity of 131I-labeled anti-CD45 mAb in combination with anti-idiotype. The immunohistochemical studies suggest that the radiation dose delivered by the anti-MHCII mAb to tumor is much
higher than that delivered by the anti-CD45 mAb. We hypothesized that improved clearance of tumor and survival could be achieved by increasing the administered activity of 131I-anti-CD45 mAb in an attempt to deliver similar radiation doses to tumor as those achieved with the anti-MHCII mAb. Mice were inoculated with tumor and treated as before. In keeping with previous results, treatment with 18.5 MBq of 131I-anti-MHCII plus anti-idiotype mAb resulted in long-term protection to 80% of animals, whereas treatment with 18.5 MBq of 131I-anti-CD45 in combination with anti-idiotype gave ~40 days of protection over controls (Fig. 6A). Mice receiving 131I-mAb as a single agent showed ~20 days of enhanced survival over controls even at the higher activity of 28.0 MBq. However, when the activity of 131I-anti-CD45 mAb was increased (to 28 MBq) and given in combination with the anti-idiotype mAb, 60% of animals survived >100 days (Fig. 6A). This level of survival was comparable with that achieved with the lower (18.5 MBq) activity of 131I-anti-MHCII and indicates that increasing the effective radiation dose delivered (with 28 MBq) to the tumor cells overcomes the less favorable mAb microdistribution and results in long-term tumor protection not seen with the lower doses ($P < 0.01$).

When the activity of 131I-anti-CD45 mAb was further increased to 37.0 MBq with and without anti-idiotype, radiation toxicity was observed, with between 20% and 40% of animals experiencing toxicity-related deaths, characterized by rapid weight loss, and these animals died around 20 to 25 days after tumor inoculation without signs of tumor development (Fig. 6B). To specifically address the question of the potential interaction between the anti-idiotype mAb and anti-CD45 or anti-MHCII mAb, we have compared the therapeutic effect of combination mAb therapy. The addition of unlabeled anti-MHCII or anti-CD45 mAb to the anti-idiotype mAb results in identical therapeutic results (data not shown). We conclude from these experiments that it is therefore most unlikely that any in vivo interaction is occurring between anti-idiotype and other mAbs used to target irradiation.

### Discussion

In this study, we have investigated the relationship between tumor dosimetry and successful clearance of lymphoma in radioimmunotherapy. We have shown that, although radioimmunoconjugates of different specificity (131I-anti-MHCII and 131I-anti-CD45) seem to deliver similar doses of radiation to tumor by established dosimetric techniques, there were profound differences in the therapeutic activity between the two mAbs when combined with unlabeled anti-idiotype mAb. Our data suggest that variation in the microscopic radiation dosimetry seems critical to the long-term clearance of tumor. The more therapeutically effective 131I-anti-MHCII delivers a substantially higher radiation dose to tumor by virtue of more specific tumor targeting in contrast to the 131I-anti-CD45 where much of the targeting is to adjacent cells and not directly to tumor. By increasing the administered activity of the 131I-anti-CD45 radioimmunoconjugate, it is possible to increase the dose to tumor and enhance survival, albeit at the cost of increased toxicity secondary to the less specific tumor targeting.

In the initial development of radioimmunotherapy, several target antigens were investigated in vitro and their properties were assessed predominantly on the ability of the mAb to deliver radiation (25, 26). From these studies, some of the characteristics thought to be important for successful radioimmunotherapy were considered to be the number of mAb binding sites, endocytosis rates, degradation rates, and the immunoreactivities and isotype of the mAb (25, 26). These preclinical studies identified CD45 and MHCII as potentially good B-cell antigen targets, and anti-CD45 was shown to be stably retained on the surface of lymphoma cells without appreciable internalization or shedding (26). Both targets have subsequently undergone extensive clinical evaluation (12, 27). These biological characteristics suggested that radiolabeled mAbs targeting CD45 could deliver a high dose of radiation to the hematopoietic tissues and CD45 was one of the first antigens to emerge as a potential target for radioimmunotherapy in hematologic malignancies. Both 131I-labeled and 90Y-labeled anti-CD45 mAbs have been investigated to treat B-cell malignancies (27–29) as well as the HLA-DR antigen of MHCII, which has also been extensively investigated in clinical trials (12, 30).

Our results show a lack of correlation between the conventional whole-organ biodistribution dosimetry data and the therapeutic outcome in a syngeneic murine B-cell lymphoma model. These significant therapeutic differences led us to explore potential differences in mAb binding level, mAb effector function, and, finally, the tumor microdosimetry of the radioimmunoconjugate as a potential explanation for these therapeutic differences. We found no significant difference in either antibody binding levels or ADCC to account for the profound differences observed in therapeutic outcome. We therefore sought to explore potential important differences in radioisotope delivery that could explain these therapeutic differences at a microscopic level.

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Figure 5. A to D, immunohistochemical analysis of the microdistribution of i.v. administered mAb in late-stage BCL1 lymphoma. Groups of age- and sex-matched BALB/c mice were inoculated with 10⁵ BCL1 cells i.v. and treated with 125I-labeled mAb (500 μg/mouse) 18 d later. Animals were sacrificed 2 h after i.v. injection of mAbs. Arrows, tumor cell clusters. Magnification, ×100.
Unlike external beam radiotherapy, the range and quality of radiation delivered by radioisotopes critically depends on the type of emissions from that particular radioisotope but is typically of short path length. Even for the more energetic $\beta$ emissions from commonly used radioisotopes, such as $^{90}$Y, the dose delivered is at the microscopic level and 90% is delivered within 5 mm, which equates to around a few hundred cell diameters. For $^{131}$I, this is considerably less with 90% of the $\beta$ emissions delivered within 0.8 mm and a mean range of just 0.4 mm, equating to ~20 cell diameters. Therefore, microdistribution at the cellular level is required to accurately assess the radiation delivered by the infused radiolabeled mAb. We chose to investigate the delivery of mAb using immunohistochemical techniques.

In this study, we have been able to show the precise targeting of vector mAb to the cells expressing the target antigen in a syngeneic B-cell lymphoma model, where the tumor cells have a predilection for the spleen. The immunohistochemical studies on splenic sections revealed large differences in the intratumoral distribution patterns of these mAbs. Whereas the anti-CD45 mAb dispersed homogeneously within the spleen, the anti-MHCII mAb specifically targeted the B-cell and tumor cell regions. Consequently, we were able to determine that the dosimetry results derived from conventional biodistribution assays seemed to substantially underestimate the real radiation dose delivered to the tumor cells by anti-MHCII mAb. The conventional biodistribution assays assume homogeneous distribution of mAb and are therefore more likely to accurately estimate the actual radiation dose delivered by anti-CD45 mAb than the more selective delivery to the B-cell zones of the anti-MHCII. This indicates that some mAb may deliver higher radiation doses than those calculated using the MIRD estimated radiation doses. This increase in tumor dose delivered by anti-MHCII mAb importantly seems to result in an improved survival and long-term clearance of tumor that is not seen with the same administered activity using the $^{131}$I-anti-CD45 mAb. Our results suggest that conventional biodistribution approaches are inadequate in accurately reflecting the microscopic tumor dosimetry of $^{131}$I-labeled mAb in lymphoma.

Dewaraja et al. recently reported the mismatch between tumor-absorbed dose and the therapeutic response seen after radioimmunotherapy and concluded that this difference might be related to inaccuracies in activity quantification and dose estimation. By using patient-specific, three-dimensional, single-photon emission computed tomography imaging–based dosimetry method, they found that the conventional MIRD-based dosimetry method could potentially have underestimated the tumor-absorbed dose by up to 35% for smaller tumors of 7 mL in size and underestimate the absorbed dose by 12% for bigger tumors of 16 mL in size (31). Although this type of approach seems to be an improvement over currently adopted methodology, it still does not address the actual intratumoral mAb microdistribution.

Intratumoral distributions of mAb have been previously observed by autoradiography techniques (32, 33). The poor spatial resolution of this technique, however, has restricted the observed magnification to around 2- to 4-fold, which is insufficient to that

Figure 6. Radiation dosimetry plays a critical role in the success of radioimmunotherapy in BCL1 lymphoma. A, groups of age- and sex-matched BALB/c mice (five mice per group) were inoculated with $10^5$ BCL1 cells i.v. and treated 10 d later by i.v. injection of $^{131}$I-labeled anti-MHCII or anti-CD45 mAb (doses shown) with and without the addition of unlabeled anti-idiotyp. By increasing the dose of radiation carried by the anti-CD45 mAb partner (28 MBq) to compensate for the differences in mAb microdistribution and dosimetry, long-term survival was achieved in 60% animals. This level of survival was comparable with that achieved by the anti-MHCII mAb (80% long-term survival). Representative of three identical experiments. B, however, further increase of the $^{131}$I activity to 37 MBq, 20% and 40% of animals died of radiation toxicity, characterized by rapid weight loss and early death with no signs of tumor development in $^{131}$I-anti-CD45 and $^{131}$I-anti-CD45 plus anti-idiotypic groups, respectively, whereas 60% of animals became long-term survivors in the $^{131}$I-anti-CD45 plus anti-idiotypic group.
required to observe mAb localization at the cellular level (34–36). The recent introduction of digital autoradiography and radioautoluminography technique has made the observation of intratumoral distribution of radiolabeled mAb more convenient but with a similar limitation to the spatial resolution seen with autoradiography and inability to observe microscopic localization of mAb (37–43). With our technique using increased magnification, the mAb distribution was observed to localize to particular cell clusters within the tissue, and with sections of 1 to 2 μm, it is technically feasible to achieve six to seven sequential sections for each single tumor cell. We have thus been able to correlate the microscopic intratumoral distribution of i.v. infused mAb with conventional dosimetry and therapeutic outcome. These data strongly suggest that the dosimetric differences found at microscopic level may determine the radioimmunotherapy therapeutic efficacy of mAb.

We were able to show that, by increasing the activity of the 131I delivered by the anti-CD45 mAb vector, this increased radiation dose to tumor cells resulted in similar levels of long-term protection to those seen with 18.5 MBq of 131I-anti-MHCII. However, the lack of more specific tumor targeting by the anti-CD45 mAb, which bound to both the T cells and B cells in comparison with the anti-MHCII, which bound only to B cells, resulted in increased nonspecific radiation-related toxicity as the administered radiation activity was increased. As the dose of 131I-anti-CD45 mAb was increased further to 37.0 MBq of 131I, the toxicity was increased in both groups with and without unlabeled anti-idiotype and between 20% and 40% of animals experienced toxicity-related deaths.

In conclusion, this study further emphasizes the critical importance of antigen/antibody selection in radioimmunotherapy for effective clearance of lymphoma. We have shown that the different intratumoral biodistribution patterns of mAb used as radiation vectors led to important differences in tumor radiation dosimetry, which in turn results in dramatically different therapeutic outcomes. These data suggest that microdosimetric differences detected at a cellular level are critical to long-term clearance of tumor and that conventional MIRD-based dosimetry methods are inadequate in providing accurate tumor dosimetry and in predicting therapeutic response. We believe that these results provide new insights into the mechanisms of action of radioimmunotherapy in lymphoma and should influence and encourage the design of further more accurate dosimetric studies in clinical studies and optimize patient-specific delivery.

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