Evaluation of CD20, CD22, and HLA-DR Targeting for Radioimmunotherapy of B-Cell Lymphomas

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Abstract
Despite the promise of radioimmunotherapy using anti-CD20 antibodies (Ab) for the treatment of relapsed patients with indolent non-Hodgkin lymphoma (NHL), most patients treated with conventional doses of $^{131}$I-tositumomab or $^{90}$Y-ibritumomab eventually relapse. We did comparative assessments using conventional radioimmunotherapy targeting CD20, CD22, and HLA-DR on human Ramos, Raji, and FL-18 lymphoma xenografts in athymic mice to assess the potential for improving the efficacy of radioimmunotherapy by targeting other NHL cell surface antigens. Results of biodistribution studies showed significant differences in tumor localization consistent with variable antigenic expression on the different lymphoma cell lines. Interestingly, the radioimmunoconjugate that yielded the best tumor-to-normal organ ratios differed in each tumor model. We also explored administering all three $^{111}$In-1,4,7,10-tetra-azacylododecane $N,N,N,N'$-tetraacetic acid antibodies in combination, but discovered, surprisingly, that this approach did not augment the localization of radioactivity to tumors compared with the administration of the best single radioiodated Ab alone. These data suggest that conventional radioimmunotherapy using anti-CD20, anti–HLA-DR, or anti-CD22 Abs is effective when used singly and provides targeted uptake of radiolabel into the tumor that is dependent on the levels of antigen expression. Improvements in tumor-to-normal organ ratios of radioactivity cannot be achieved using directly labeled Abs in combination but may be afforded by novel pretargeting methods.

Introduction
Immunotherapies using monoclonal antibodies (Ab) targeting B-cell surface antigens have been widely accepted for the treatment of non-Hodgkin lymphomas (NHL), with anti-CD20 Abs being most commonly employed (1). Rituximab, a chimeric anti-CD20 Ab, has provided the best clinical results to date with single-agent remission induction rates of >60% in patients with indolent lymphomas and 30% to 35% in relapsed aggressive lymphomas (2–4). Despite the promise of therapy with unmodified Abs, only 6% to 20% of patients achieve complete remissions (CR) and most patients treated with an anti-CD20 Ab alone relapse (5). To overcome the potential limitations of unconjugated Abs, anti-CD20 Abs conjugated to $^{131}$I or $^{90}$Y have been tested and shown to produce higher overall response and CR rates compared with unlabeled Abs (6–9). The majority of patients treated with conventional doses of radiolabeled anti-CD20 Abs, however, also eventually relapse. Escalating the dose of targeted radioimmunotherapy to myeloblastic levels has further improved response rates. Objective remissions have been seen in 85% to 95% of relapsed lymphoma patients receiving high-dose $^{131}$I-tositumomab (Bexxar), including CR rates of 75% to 80% (10–13). This myeloblastic approach, however, has relied on autologous stem cell transplantation to reconstitute hematopoiesis, and while effective, the toxicity associated with the transplantation is substantial.

In addition to targeting the CD20 antigen for Ab-mediated immunotherapy of B-cell lymphomas, several Abs targeting other B-cell surface antigens have been tested either alone or conjugated to radionuclides. Radiolabeled Ab conjugates targeting the CD19 antigen have been explored in preliminary radioimmunotherapy trials (14). Radioimmunotherapy with anti-idiotypic Abs have shown promise, but have not been widely investigated because of logistic and financial issues related to the production of patient-specific Abs (15). Prior reports have shown that CD20, CD22, and HLA-DR are all expressed at relatively high densities on the majority of B-cell lymphomas (16, 17). The Lym-1 Ab targeting a class II HLA-DR antigen and LI2/epratuzumab targeting CD22 have had the most success in radioimmunotherapy clinical trials (18–23). In this report, we have compared CD20, CD22, and HLA-DR targeting with directly labeled Abs, either singly or in combination, to determine whether we can further improve tumor-to-normal organ ratios, and thus potentially achieve the excellent outcomes of high-dose radioimmunotherapy without the attendant toxicities. We have done comparative biodistribution experiments using conventional one-step radioimmunotherapy in athymic mice bearing either Burkitt’s (Ramos and Raji) or transformed follicular (FL-18) lymphoma xenografts. In vitro cell binding experiments suggest that significant associations exist between levels of CD22, CD20, and HLA-DR expression. In vivo biodistribution studies indicate that the optimal target may vary among different lymphoma cell lines. Moreover, we show that a combination of directly labeled Abs targeting the CD20, CD22, and HLA-DR antigens on the surface of Ramos, Raji, and FL18 lymphoma cells does not provide any additional benefit over targeting each antigen alone. These results suggest that the slow clearance of unbound radioiodated Abs from the circulation and the resultant high levels of background radioactivity remain major obstacles to the optimal implementation of radioimmunotherapy in NHL because these pharmacokinetic features limit the tumor-to-normal organ ratios of absorbed radiation that can be achieved.
Materials and Methods

Cell lines. The human Ramos and Raji Burkitt's lymphoma cell lines were obtained from the American Type Culture Collection (ATCC). The transformed follicular FL-18 cell line was a gift from David Maloney [Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA]. Cell lines were maintained as previously described (24). Cell viability exceeded 95% by trypan blue exclusion.

Antibodies and production of DOTA-Ab conjugates. The 1F5 hybridoma cell line expressing the murine immunoglobulin G2a (IgG2a) antihuman CD20 Ab was a gift from Clay Siegall (Seattle Genetics, Seattle, WA). The HD39 hybridoma expressing the murine IgG1 anti–HLA-DR Ab and a hybridoma expressing a nonspecific IgG2a negative control Ab, HB-8181, were obtained from ATCC. The HD39 Ab was produced by injecting the hybridoma into pristane-primed mice to generate ascites. The Ab was purified by protein A immunoabsorption and column chromatography. All other Abs were produced from the respective hybridomas using a hollow fiber bioreactor system in the monoclonal Ab production facility at the FHCRC. DOTA-Ab reagents were conjugated as described previously (24).

Radiolabeling. 111In (Nordion) radiolabeling of intact 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid antibodies (DOTA-Abs) for conventional radioimmunotherapy was done as described (24). Radiochemical purity was typically >99% as determined by HPLC, and labeling efficiencies were >90%. DOTA-biotin was synthesized as described (24).

Cytometric cell binding analysis of 111In, HD39, and Lym-1 Abs. Ramos, Raji, or FL-18 cells (1 × 10^6) in 2% fetal bovine serum (FBS)-PBS were incubated with 10 μg of each DOTA-Ab conjugate for 30 min at 4°C and washed with 2% FBS-PBS. A second incubation for 30 min at 4°C was done using 5 μg of secondary Ab, horse anti-mouse FITC. Following a second wash, the cells were fixed in 2% paraformaldehyde in PBS and analyzed in a FACScan II flow cytometer (Becton Dickinson Labware). Mean fluorescence intensity was determined using CellQuest Pro 5.2 software.

Mouse studies. Female BALB/c nu/nu mice, aged 6 to 8 weeks, were obtained from Harlan Sprague-Dawley and Charles River Laboratories and housed under protocols approved by the FHCRC Institutional Animal Care and Use Committee. Ramos, Raji, or FL18 cells (1-1.2 × 10^7) were injected s.c. in the right flank – 10 days before therapy to obtain lymphoma xenografts. Mice with similar, palpable tumors were chosen for the studies. For single Ab biodistribution studies, mice were injected i.v. via the tail vein with 1.4 nmol of radioiodinated Ab (215 μg; 50 μCi). For combination studies, tumor xenograft–bearing mice were coinjected with an equimolar mix (1.4 nmol of each conjugate) of all three conjugates (4.2 nmol of total conjugates). In each experiment, mice were also coinjected with 400 μg of an irrelevant IgG2a Ab (HB8181) to block nonspecific binding of the 1F5, HD39, and Lym-1 Abs to Fc receptors (25). Mice were bled from the retro-orbital venous plexus, euthanized, and tumors and normal organs (lung, liver, spleen, stomach, kidneys, small intestine, and colon) were harvested, weighed, and gamma counted for 111In activity 24 and 48 h after the 111In-DOTA-Ab injections. The percent- injected dose of 111In per gram (%ID/g) of blood, tumor, and normal organs was calculated after correcting for radioactive decay using an aliquot of the injectate. Tumor-to-normal organ ratios of absorbed radioactivity were also calculated. Control groups were injected with radiolabeled isotype-matched, nonbinding control 111In-DOTA-HB8181 Ab conjugate.

Results

In vitro Binding of Anti-CD20, Anti-CD22, and Anti–HLA-DR Abs to Human Lymphoma Cells

The anti-CD20, anti-CD22, and anti–HLA-DR DOTA-Ab conjugates were tested for binding to the FL18, Ramos, and Raji cell lines by flow cytometry. These studies showed that the DOTA-Ab conjugates displayed different binding patterns on the three lymphoma cell lines (Fig. 1). The DOTA-1F5 Ab bound to Ramos cells with the highest mean fluorescence intensity (MFI), whereas the DOTA-Lym-1 and DOTA-HD39 Abs exhibited MFIs that were 22% and 13%, respectively, of the DOTA-1F5 Ab value. In contrast, the highest level of binding to Raji cells was seen using the DOTA-Lym-1 Ab, followed by the DOTA-1F5 Ab (MFI, 33% of DOTA-Lym-1 MFI). The DOTA-HD39 Ab conjugate exhibited minimal binding to Raji cells (4% of DOTA-Lym-1 MFI). The DOTA-Lym-1 and DOTA-1F5 Ab conjugates bound the FL-18 cell at comparable high levels, whereas the DOTA-HD39 anti-CD22 Ab exhibited a MFI >90% lower than the mean MFI of the other two Abs.

Comparative Biodistributions of 111In in Tumor-Bearing Mice Treated with Directly Labeled 111In-DOTA-1F5, 111In-DOTA-HD39, or 111In-DOTA-Lym-1 Abs

Groups of five mice were injected i.v. with either 1.4 nmol of directly labeled 111In-DOTA-1F5, 111In-DOTA-Lym-1, or 111In-DOTA-HD39 Ab. Mice receiving each 111In-DOTA-Ab were also coinjected with 400 μg of a control IgG2a Ab, HB-8181, to prevent nonspecific binding of each DOTA-Ab to Fc-receptors in the spleen and marrow (25). Mice were sacrificed 24 and 48 h following 111In-DOTA-Ab (Fig. 2) administration. At each time point, control animals injected with the nonbinding 111In-DOTA-HB8181 Ab had negligible tumor uptake of radioactivity in Ramos, Raji, and FL-18 xenografts (2.0 ± 1.3%, 2.6 ± 0.7%, 1.2 ± 0.3% ID/g at 48 h, respectively), demonstrating the specificity of targeting (Fig. 2D).

Anti-CD20 conventional radioimmunotherapy: 111In-DOTA-1F5 targeting Ramos, Raji, and FL-18 xenografts in athymic mice. Similar biodistributions of 111In were obtained in Ramos, Raji, and FL-18 tumors following conventional radioimmunotherapy employing 111In-DOTA-1F5 Ab (Fig. 2A). After 24 and 48 h, peak levels in Ramos tumors were 6.0 ± 2.3% ID/g and 4.8 ± 2.0% ID/g with 111In-DOTA-1F5 Ab, respectively. In Raji tumor xenografts, uptake at 24 and 48 h were 9.2 ± 1.6% and 9.7 ± 2.6% ID/g, respectively, whereas in FL-18 tumor xenografts, 111In-DOTA-1F5 Ab resulted in 7.2 ± 2.0% and 7.1 ± 2.2% ID/g at 24 and 48 h, respectively. The levels of radioactivity in normal organs at 24 h using directly labeled 111In-DOTA-1F5 Ab ranged from 0.8% ID/g...
in colon to 11.9% ID/g in kidney. In addition, circulating blood levels of radioactivity after 24 and 48 h were relatively high in mice receiving $^{111}$In-DOTA-1F5 Ab. For example, in mice bearing Raji tumors, levels in blood of $^{111}$In-DOTA-1F5 Ab were 8.3 ± 1.4% and 6.6 ± 0.8% ID/g at 24 and 48 h, respectively.

**Anti–HLA-DR conventional radioimmunotherapy**: $^{111}$In-DOTA-Lym-1 targeting Ramos, Raji, and FL-18 xenografts in athymic mice. In a similar manner, we compared $^{111}$In-labeled anti–HLA-DR radioimmunotherapy in mice bearing Ramos, Raji, or FL-18 human lymphoma xenografts. Mice were treated with equimolar doses of $^{111}$In-DOTA-Lym-1 Ab as described in Materials and Methods. The amount of radioactivity localizing to Ramos tumors was considerably less than the amounts in Raji or FL-18 tumors when the $^{111}$In-DOTA-Lym-1 Ab was employed for tumor targeting (Fig. 2B). Consistent with antigenic expression patterns for these cell lines (Fig. 1), peak radioactivity levels 24 h after delivery of $^{111}$In-DOTA-Lym-1 Ab in Raji and FL-18 tumors were 12.3 ± 3.1% and 13.7 ± 3.4% ID/g, respectively. By comparison, biodistributions of radioactivity seen in Ramos tumors were 3.5 ± 0.6% ID/g at 24 h using the $^{111}$In-DOTA-Lym-1 Ab. Directly labeled $^{111}$In-DOTA-Lym-1 Ab yielded tumor uptakes of 14.7 ± 3.9% and 11.5 ± 5.6% ID/g uptake in Raji and FL-18 tumors at 48 h, respectively, but only 4.8 ± 2.5% ID/g uptake in Ramos tumors at the same time point (Fig. 2B).

**Anti-CD22 conventional radioimmunotherapy**: $^{111}$In-DOTA-HD39 targeting Ramos, Raji, and FL-18 xenografts in athymic mice. Because most anti-CD22 Abs are rapidly internalized into mature and malignant B lymphocytes after binding to cell surface CD22 (17), we investigated if the short residence time of anti-CD22 (HD39) Ab on the cell surface would affect the ability to localize $^{111}$In-DOTA-HD39 Ab to Ramos, Raji, and FL-18 tumor xenografts (Fig. 2C). Localization of radioactivity to Ramos xenografts using $^{111}$In-DOTA-HD39 Ab was near background at 24 h (2.2 ± 0.4% ID/g) and 48 h (2.5 ± 0.1% ID/g). In contrast, the use of $^{111}$In-DOTA-HD39 Ab resulted in uptakes of 6.1 ± 1.2% ID/g in Raji tumors and 9.7 ± 4.0% ID/g in FL18 tumors at 24 h. The corresponding 48-h uptakes were 7.3 ± 0.6% ID/g in Raji tumors and 7.3 ± 1.0% ID/g in FL18 tumors (Fig. 2C).

Biodistributions of $^{111}$In in mice bearing Ramos, Raji, or FL-18 tumors treated simultaneously with a combination of directly labeled $^{111}$In-DOTA-1F5, $^{111}$In-DOTA-HD39, and $^{111}$In-DOTA-Lym-1 Abs. In an attempt to deliver escalated doses of
radiation to determine if additive or synergistic effects could be imparted to Ramos, Raji, and FL-18 tumor xenografts, we did combination experiments targeting all three antigenic sites simultaneously. Equimolar amounts of each directly labeled $^{111}$In-DOTA-Ab conjugate were coinjected into tumor-bearing mice. Therefore, the total amount of each individual conjugate was equivalent to the amount used in the single-agent biodistribution experiments, and thus, thrice the total amount of protein was used in directly labeled combination experiments. As before, the HB8181 control Ab was coinjected to block nonspecific Fc receptor interaction of the IgG2a DOTA conjugates. The combination of $^{111}$In-DOTA-1F5, $^{111}$In-DOTA-Lym-1, and $^{111}$In-DOTA-HD39 Abs did not achieve additive levels of radioactivity in any of the three types of tumor xenografts (Fig. 3). For example, the combination of the three radiolabeled DOTA-Ab conjugates in mice bearing Ramos tumors resulted in tumor biodistributions that were similar to the single doses of each DOTA-Ab conjugate, yielding $7.5 \pm 5.3$%ID/g with the conjugate combination at 48 h compared with $4.8 \pm 2.0$%ID/g using $^{111}$In-DOTA-1F5 Ab alone, $4.8 \pm 2.5$%ID/g using $^{111}$In-DOTA-Lym-1 Ab alone, and $2.5 \pm 0.1$%ID/g with $^{111}$In-DOTA-HD39 alone (Fig. 3A). Similarly, mice bearing Raji tumors treated with the triple (i.e., 4.2 nmol) $^{111}$In-DOTA-Ab combination had a tumor concentration of $9.4 \pm 2.5$%ID/g at 48 h compared with $9.7 \pm 2.6$%, $14.7 \pm 4.9$%, and $7.3 \pm 0.6$%ID/g in Raji tumors after 48 h when $^{111}$In-DOTA-1F5 Ab, $^{111}$In-DOTA-Lym-1 Ab, or $^{111}$In-DOTA-HD39 Ab were used as single agents, respectively (Fig. 3B). Using FL-18 tumors, mice that received the $^{111}$In-DOTA triple-Ab combination accumulated $11.2 \pm 7.2$%ID/g in tumor after 48 h compared with $7.1 \pm 2.2$% (DOTA-1F5 Ab), $11.5 \pm 5.6$%ID/g (DOTA-Lym-1 Ab), and $7.3 \pm 1.0$% (DOTA-HD39) in tumors of mice receiving the three DOTA-Ab conjugates singly (Fig. 3C).

The data from the combination studies suggest that there may be a significant increase in the amount of radioactivity deposited in normal organs compared with normal organ doses delivered when each DOTA-Ab conjugate was used separately. In mice bearing Ramos tumors, the concentration of radioactivity in the liver was $7.8 \pm 2.8$%ID/g after 48 h compared with a lower concentration when each DOTA-Ab conjugate was used individually ($3.7 \pm 0.3$%ID/g in liver; Fig. 3A). Thus, for the Ramos model, the increased radiation delivered to normal organs using the combination of radioimmunoconjugates may lead to increased toxicities compared with the use of the individual DOTA-Ab conjugates, without a commensurate increase in the delivery of radioactivity to tumor sites.

### Figure 3.

Comparative biodistributions using conventional radioimmunotherapy with all three $^{111}$In-DOTA-Ab conjugates given either alone or in combination. For the combination studies, mice bearing Ramos (A), Raji (B), or FL-18 (C) tumor xenografts were simultaneously injected i.v. with a combination of 1.4 nmol each of $^{111}$In-DOTA-1F5, $^{111}$In-DOTA-Lym-1, and $^{111}$In-DOTA-HD39 Abs. Mice were euthanized 24 and 48 h after injection of the $^{111}$In-DOTA-Ab cocktail, and tumor and organs were harvested and analyzed as for the studies in Fig. 2. Each graph shows results 48 h after injecting $^{111}$In-DOTA-1F5 Ab alone (A), $^{111}$In-DOTA-Lym-1 Ab alone (B), and all three used in combination at 1.4 nmol/conjugate (C).
Ramos and 1:2:1 in both Raji and FL-18 tumors. Conversely, tumor-to-normal organ ratios using $^{111}$In-DOTA-Lym-1 Ab were best in the mice bearing Raji xenografts where the tumor-to-stomach and tumor-to-kidney ratios were 2:1 and 3:1, respectively (Fig. 4B). Ratios were least favorable in mice that received the $^{111}$In-DOTA-HD39 Ab; animals bearing FL-18 tumor xenografts that received $^{111}$In-DOTA-HD39 Ab had a tumor-to-stomach ratio of 4.7:1 and tumor-to-kidney ratio of 0.8:1, respectively (Fig. 4C).

As described in a separate report submitted concurrently with this study (26) we have explored a pretargeting strategy in an attempt to decrease the levels of radiolabeled Ab remaining in the bloodstream before delivery of the therapeutic radionuclide (27–35). This pretargeted method employs streptavidin conjugated to the Ab, followed by delivery of a radiolabeled biotin conjugate. We have also used a biotinylated polymeric, $N$-acetyl-galactosamine-clearing agent to further decrease the levels of Ab-streptavidin conjugate persisting in the circulation before delivery of the radiobiotin to reduce the nonspecific radiation exposure from blood-borne radiolabeled Ab. Compared with ratios obtained using conventional directly labeled anti-CD20, anti–HLA-DR, and anti-CD22 Abs at 24 and 48 h, pretargeted radioimmunotherapy using IF5 Ab-streptavidin, Lym-1 Ab-streptavidin, or the HD39 Ab-streptavidin followed by $^{111}$In-DOTA-biotin yielded superior tumor-to-blood and tumor-to-normal organ ratios of radioactivity due to the rapid clearance from blood of each conjugate after the administration of the clearing agent (Fig. 5). In contrast to the conventional radioimmunotherapy biodistribution experiments, a maximum of only 2.3 ± 0.8% ID/g was present in blood after 24 h using any of the pretargeted Ab-streptavidin conjugates, decreasing to a maximum of 1.3 ± 0.8% ID/g in blood after 48 h. Ratios were most favorable using the pretargeted Lym-1 Ab-streptavidin conjugate in mice bearing FL18 tumor xenografts (Fig. 5B). Using the pretargeting approach with the Lym-1 Ab-streptavidin, the tumor-to-blood ratio after 48 h was 23:1 in contrast to only 1.6:1 when using conventional $^{111}$In-Lym-1 Ab. Likewise, the tumor-to-kidney ratio at 48 h using the pretargeting approach was 9:1 in comparison to 0.9:1 for conventional $^{111}$In-Lym-1 Ab.

**Discussion**

Although the CD20 antigen has proven to be a promising target for radioimmunotherapy for NHL, other Abs targeting B-cell surface antigens have also been conjugated to radionuclides and tested for their potential to improve the efficacy of radioimmunotherapy. Goldenberg et al. have evaluated an anti-CD22 $^{90}$Y-labeled humanized Ab, epratuzumab, in patients with NHL and found that this radioimmunoconjugate can result in significant antitumor responses (22, 23). Interestingly, the accumulation of $^{90}$Y-epratuzumab at tumor sites did not correlate with individual tumor responses, and the degree of tumor response could not be predicted based on the estimated tumor dose delivered. These data suggest that anti-CD22 radioimmunotherapy may use other mechanisms of tumor control, perhaps similar to those mechanisms of antitumor activity seen with the use of unconjugated B-cell Abs. Class II HLA-DR antigens have also been targeted using Lym-1 (18–21, 36) and Hu1D10 Abs (37, 38) with success in both clinical and preclinical studies. DeNardo et al. have shown that conjugation of a Lym-1 Ab to a radionuclide will augment potency of chemotherapeutic regimens (39) and other unconjugated Abs when used in combination (40). Moreover, it was shown that radiolabeled Lym-1 Ab has the ability to overcome resistance of unconjugated Abs to lymphoma cells in vitro, suggesting a distinct therapeutic advantage of radioimmunotherapy compared with the use of unconjugated Abs in chemotherapy-resistant, but radiosensitive relapsed NHL. Although initial clinical investigations of anti–HLA-DR radioimmunotherapy used Lym-1 Ab conjugated to $^{131}$I, recent explorations have focused on Lym-1 Abs conjugated to either $^{90}$Y (39, 40) or $^{67}$Cu (19). In this report, we have compared conventional radioimmunotherapy approaches targeting the CD20, CD22, and

![Figure 4. Tumor-to-normal organ ratios for conventional radioimmunotherapy with all three $^{111}$In-DOTA-Ab conjugates given either alone or in combination. Mice were treated as described in the legend of Fig. 3. Tumor-to-normal organ ratios of each $^{111}$In-DOTA-Ab given alone or in combination are shown for the 48-h time point after injection of radioactivity. Animals bearing either Ramos (A), Raji (B), or FL-18 (C) tumor xenografts received either $^{111}$In-DOTA-IF5 Ab alone (C), $^{111}$In-DOTA-Lym-1 Ab alone ( ), $^{111}$In-DOTA-HD39 Ab alone ( ), or all three used in combination at 1.4 nmol/conjugate ( ). Results are shown in each cell line: Ramos (A), Raji (B), and FL-18 (C).](www.aacrjournals.org)
HLA-DR antigens to determine the most appropriate molecular target for radioimmunotherapy. We have shown that the effectiveness of the targeting Ab is dependent on the cell surface expression of each antigen as well as the tumor xenograft employed. The three radioimmunoconjugates investigated delivered variable doses of radiation to tumors in vivo, which correlated with the levels of target antigen expression. For example, the localization of radioactivity in Raji tumor xenografts was superior using an $^{111}$In-anti–HLA-DR Ab compared with using $^{111}$In- Abs targeting CD20 or CD22. These results were consistent with the higher relative expression patterns of HLA-DR compared with CD20 and CD22 in Raji tumor cells. In a similar manner, significantly higher levels of radioactivity were delivered to Ramos xenografts in vivo using an $^{111}$In-anti-CD20 Ab compared with $^{111}$In-anti-CD22 due to significant differences in the antigenic surface expression levels for CD20 and CD22 on Ramos cells in vitro. In this study, we also investigated combining all three directly labeled Abs to determine if radioimmunotherapy can be further improved by targeting multiple antigens simultaneously. The results from these studies surprisingly suggested that the amount of radiation specifically delivered to tumor sites could not be augmented by using a combination of all three $^{111}$In-labeled Abs at equimolar doses. When compared with targeting each antigen alone, radioimmunotherapy targeting CD20, CD22, and HLA DR antigens in combination lead to inferior tumor-to-normal organ ratios due to the relatively high background exposure of normal organs to radioactivity. Although we have seen no evidence of cross-blocking or steric hindrance in preliminary studies among simultaneously bound Ab molecules, it is possible that down-modulation of antigenic expression may be partially responsible for the results observed with Ab combinations as shown by Carnahan et al. (41, 42).

Several recent studies have documented the promise of pretargeted radioimmunotherapy as a method to improve target-to-nontarget organ ratios of absorbed radiation compared with ratios obtained using conventional radioimmunotherapy. Pretargeted radioimmunotherapy attempts to limit the radiation exposure of normal organs and, thus, the attendant associated toxicities, while maintaining or amplifying the delivery of radiation therapy to tumor sites (35). Our group and others have studied a novel pretargeting approach using Ab constructs conjugated to streptavidin that bind to radiolabeled biotin as a means to improve target-to-nontarget ratios of absorbed radiation doses. This is accomplished by reducing the relatively protracted circulating half-life of conventional radiolabeled Abs that results in nonspecific exposure of normal organs to radioactivity (24, 25, 31, 43–46). This pretargeted radioimmunotherapy approach has been shown to improve the ratios of radiation delivered to tumors compared with normal organs in both preclinical and clinical models of solid tumors as well as hematologic malignancies. In particular, we have shown reduced toxicity and markedly enhanced efficacy using pretargeted anti-CD20 Ab-streptavidin conjugate compared with directly labeled anti-CD20 Ab in mouse lymphoma xenograft studies (25, 47). Pantelias et al. explored the use of anti-CD20, anti-CD22, and anti–HLA-DR Ab-streptavidin conjugates for pretargeted radioimmunotherapy (26). As seen with the conventional radioimmunotherapy results presented in this study, the best tumor uptake and tumor-to-normal organ ratios of radioactivity varied depending on the target antigen expression of the cell line employed. The best tumor-to-normal organ ratios of absorbed radioactivity using the pretargeted radioimmunotherapy approach were also observed when single Ab-streptavidin conjugates were employed rather than combination therapy with all three conjugates. We report here our comparison of the one-step conventional radioimmunotherapy and pretargeted radioimmunotherpy approaches for targeting the CD20, CD22, and HLA-DR antigens by evaluating tumor-to-normal organ ratios of radioactivity (Fig. 5). The results presented in this study confirm the

**Figure 5.** Tumor-to-normal organ ratios for conventional $^{111}$In-DOTA one-step–labeled 1F5, Lym-1, and HD39 Abs or $^{111}$In-DOTA biotin pretargeted with 1F5 (A), Lym-1 (B), and HD39 (C) Ab-streptavidin chemical conjugates in mice bearing Ramos, Raji, and FL-18 xenografts. Mice were treated as described in the legend of Fig. 1. Tumor-to-normal organ ratios of each administered $^{111}$In-DOTA-Ab or $^{111}$In-DOTA biotin are shown for the 48-h time point after injection of radioactivity.

advantages of pretargeted radioimmuno-therapy compared with conventional radioimmuno-therapy. The significant improvement in the therapeutic index for lymphoma therapy using pretargeted radioimmuno-therapy is due to the rapid clearance of each Ab-streptavidin constructs from the blood and nonspecific normal organs compared with the slow clearances observed with conventional radioimmuno-therapy.

Despite the promise of pretargeting, however, additional variables must be considered to optimally improve the efficacy of radioimmuno-therapy. These parameters include the accessibility, shedding, cell surface density, and heterogeneity of expression of the targeted antigen. The binding site specificity, immunoreactivity, in vivo stability, avidity, and affinity of the monoclonal Ab also are likely to be critical factors in determining the maximal effectiveness of radioimmuno-therapy. B-cell antigens on the surface of human lymphoma cells have been shown to have distinct variation among several B cell–specific Abs in their rates of internalization, degradation, and dissociation from the cell surface (16, 17). For example, prior studies targeting the CD45 antigen have indicated that an anti-CD45 Ab was minimally internalized or shed from the cell surface. This was in contrast to the cell surface retention of an anti-CD20 Ab that was minimally internalized, but displayed a significant rate of passive dissociation from the surface of human lymphoma cells. Preliminary radioimmuno-therapy studies using CD45 targeting have yielded comparable results to those achievable with anti-CD20 Ab-mediated radioimmuno-therapy in murine preclinical models, at least in part, to the properties of the target antigen at the cell surface (24).

We and others have used radiolanthanides as the radionuclide in radioimmuno-therapy models because of the experience with these radionuclides and the appeal for therapeutic administrations brought about by their ease of use in outpatient nonmyeloablative settings. Preclinical and clinical studies using 90Y have suggested improved therapeutic ratios due to its higher energy and shorter half-life (2.7 days) compared with other β-emitting radionuclides such as 131I, which has a longer a physical half-life (8.1 days) and problematic gamma rays. Although most radiolanthanides emit gamma rays, their emission energies do not require isolation of patients. In fact, some radiolanthanides, such as 177Lu, have half-life (2.7 days) compared with other β-emitting radionuclides such as 131I, which has a longer a physical half-life (8.1 days) and problematic gamma rays. Although most radiolanthanides emit gamma rays, their emission energies do not require isolation of patients. In fact, some radiolanthanides, such as 177Lu, have gamma emissions that allow imaging on standard gamma cameras.

Although greater β-ray tissue penetration may be highly favorable for deposition of radiation in large tumor masses, this factor must be balanced by the desire to limit localization of the radiation effects to antigen-positive cells and to avoid unduly irradiating the normal bystander cells. In an attempt to avoid the relative non-specific cytotoxicity of β-emitting constructs due to the cross-fire effect, the α emitters 213Bi and 225Ac that have shorter path lengths (μm) have recently been explored as radiolabels to treat hematologic malignancies (48, 49). Bismuth-labeled biotin has been investigated in pretargeting radioimmuno-therapy experiments targeting human carcinoma xenografts that resulted in significantly higher tumor-to-nontumor targeting ratios than those achieved with a directly labeled Ab (50). Despite the therapeutic effect seen using 213Bi-DOTA-biotin, however, the main toxicity was renal, suggesting that renal protection agents may be useful in this setting.

In conclusion, this study highlights the importance of screening the antigenic expression on lymphomas to select the most superior reagents for radioimmuno-therapy. Radioimmuno-therapy targeting of tumor masses with anti-CD20, anti-CD22, and anti-HLA-DR Abs results in rapid and high concentration of radioactivity into tumor where the ability of each radioimmunoconjugates to localize to human NHL tumors in vivo is dependent on the antigenic expression on the NHL cell surface. This study also provides data relevant to clinical situations suggesting that the biology of the lymphoma tumor type and the specific antigen-Ab combination employed are critical for achieving the most favorable therapeutic ratios. These results further suggest that combinations of radiolabeled Abs may not be superior over the use of a single radioimmunoconjugate and may not be warranted in the clinical arena. The amount of radiation that can be delivered to these single target antigens using a directly labeled Ab, however, will still be limited by toxicities due to the suboptimal therapeutic index (target-to-nontarget ratio) currently achievable with conventional radioimmuno-therapy methodologies. Therefore, in view of the compelling advantages for pretargeted radioimmuno-therapy to further improve this index, we have begun translating this approach to human studies. Pretargeted radioimmuno-therapy has the potential to allow for delivery of higher absorbed radiation doses to target tissues with minimal increase in toxicity, and such a result could diminish relapse rates leading to improved survival.

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