Antibody-induced intracellular signaling works in combination with radiation to eradicate lymphoma in radioimmunotherapy

Yong Du, Jamie Honeychurch, Mark S. Cragg, Mike Bayne, Martin J. Glennie, Peter W. M. Johnson, and Tim M. Illidge

Radioimmunotherapy (RIT) has emerged as an effective treatment for lymphoma, however the underlying mechanisms are poorly understood. We therefore investigated the relative contributions of antibody and targeted radiation to the clearance of tumor in vivo, using 2 different syngeneic murine B-cell lymphoma models. Although RIT with 131I–anti–major histocompatibility complex class II (MHCII) was effective in targeting radiation to tumor, no improvement in survival was seen by escalating the radiation dose alone and there were no long-term survivors. In contrast, using the combination of 131I anti-MHCII in the presence of unlabeled anti-idiotype (anti-Id), 100% prolonged disease-free survival was seen in both B-cell lymphoma models at the higher radiation dose. Using in vivo tracking we show that treatment with radiation plus anti-Id monoclonal antibody (mAb) results in a substantially greater reduction of splenic tumor cells than with either treatment alone. Prolonged survival could also be achieved using 131I anti-MHCII plus the signaling anti-CD19 mAb. Furthermore, the ability of these anti–B-cell mAbs to improve survival with targeted radiotherapy appeared to correlate with their ability to initiate intracellular signal transduction. Together these data illustrate that using 1 mAb to target radiation to tumor and a second to induce cell signaling is an effective new strategy in RIT. (Blood. 2004;103:1485-1494)

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Introduction

Monoclonal antibodies (mAbs) offer new therapeutic choices for patients with hematologic malignancies. Although the single-agent activity of most mAbs has generally been modest and short lived, the different mechanisms of action and nonoverlapping toxicity of mAbs has enabled them to be combined with chemotherapy and radiotherapy to greatly improve response rates. Radioimmunotherapy (RIT) involves the conjugation of radioisotopes to mAbs and has resulted in significantly superior response rates to unlabeled mAbs. A variety of different mAbs, delivery schedules, radioisotopes, and doses of radioactivity have been used in RIT, resulting in impressive partial and complete responses in the treatment of non-Hodgkin lymphoma (NHL). It now seems highly likely that RIT will play a significant role in the treatment of some NHL patients following the United States Food and Drug Administration (US FDA) approval of 90Y-ibritumomab tiuxetan and 131I-tositumomab.

Despite these clinical successes there remains considerable uncertainty regarding the optimal treatment approach in using RIT in NHL patients. One such issue is whether a radiation dose response exists and whether higher or lower doses of radioactivity should be used. Press and colleagues have adopted a high-dose myeloablative approach with peripheral blood stem cell transplantation support. In contrast, other groups including Kaminski and coworkers have elected to use a lower nonmyeloablative dose. Both approaches have resulted in impressive durable clinical responses and there is currently no randomized evidence to support the superiority of either approach. Using higher doses of radiation and stem cell transplantation does, however, create a number of radiation protection and logistical issues that make widespread use of this approach more difficult. A clear benefit of using higher doses of radiation would therefore have to be demonstrated before higher doses of radiation could enter routine clinical practice.

Furthermore, the mechanisms involved in clearance of tumor by RIT remains poorly understood and some mAbs, such as Lym-1 (binding to human leukocyte antigen [HLA]–DR) have often been considered simply as vectors for delivering radiation to tumor or “systemic radiotherapy.” Recent preclinical and clinical data have, however, clarified that some mAbs are intrinsically therapeutically active and may operate by interacting with the host immune system or through a direct cytotoxic effect via cell surface signaling. An understanding of the mechanisms underlying the clinical responses is likely to be important in the further development and optimization of this treatment approach. Dissecting the mechanism of action of RIT and attributing the relative importance of mAbs, targeted radiation, and nonspecific whole-body irradiation to the therapeutic efficacy is difficult or impossible in a clinical setting. Such fundamental issues are therefore ideally approached in syngeneic tumor models. In this situation tumor develops in the presence of an intact immune system and the targeting mAb is able to cross-react with appropriate normal tissues in a manner analogous to the clinical situation and contrasting with the limitations of human severe combined immunodeficiency (SCID) xenograft models where there is no such host immunity or specific cross-reaction of mAbs with normal tissues.

We have previously reported the importance of antibody specificity in the successful eradication of tumor with RIT. In this...
study, we have demonstrated that a radiation dose response exists for RIT in the presence of cell surface signaling in B-cell lymphomas in vivo. We have also characterized the relative contributions made by targeted irradiation and mAb effector mechanisms using two different mAbs to fulfill different functions and work cooperatively to successfully eradicate lymphoma in vivo. In this situation, the characteristics of one mAb are exploited to target radiation effectively to the tumor while the second mAb provides intracellular signaling by ligating key cell surface receptor molecules. We have shown that both anti-idiotypic (anti-Id) and anti-CD19 mAbs initiate cell surface signaling and this appears to dramatically enhance the clearance of tumor when combined with targeted radiation delivered by $^{131}$I-anti—major histocompatibility complex class II (MHCII). We believe that these results provide new insights into the mechanisms of action of RIT and should influence the design of further clinical studies integrating combinations of mAbs to successfully treat lymphoma.

Materials and methods

Animals and cell lines

Mice were supplied by Harlan UK Limited (Blackthorn, United Kingdom) and maintained in local animal facilities. BCL1, and A31 are B-cell lymphomas that arose spontaneously in mice and are maintained by in vivo passage in BALB/c and CBA/H mice, respectively. nBCL1 is a variant of the BCL1 lymphoma that can be maintained in culture as well as in vivo.20 Cell culture was performed in RPMI (Life Technologies, Paisley, United Kingdom) supplemented with glutamine (2 mM), pyruvate (1 mM), penicillin and streptomycin (100 IU/mL), fungizone (2 µg/mL), 2-mercaptoethanol (50 µM) (VWR International, Poole, United Kingdom), and 10% fetal calf serum (FCS; Myoclone; Life Technologies).

Antibodies and iodination

A list of the mAbs and their sources is given in Table 1.21-23 Details of their production and purification is given elsewhere.17 The $^{125}$I and $^{131}$I radioisotopes were supplied from Amersham International (Little Chalfont, United Kingdom). The mAbs were iodinated using Iodobeads (Pierce Chemicals, Rockford, IL) according to the manufacturer’s instructions and dialyzed to remove unbound iodine. Labeling efficiency varied between 82.5% and 98.5% as measured by thin layer chromatography and high performance liquid chromatography. The specific activity was 37 to 185 MBq/µg (1.0-5.0 mCi/µg). The immunoreactivity of iodinated antibodies was estimated by integrating the area under the retention curve.26

Biodistribution studies

Groups of BALB/c or CBA/H mice were injected via the tail vein with $10^5$ BCL1, or $10^6$ A31 cells, respectively. Mice were given Lugol solution (0.125 g KI, 0.0625 g elemental iodine in 100 mL water) in their drinking water 3 days before the biodistribution. Ten days after tumor inoculation animals received 500 µg of $^{125}$I-labeled mAbs by intravenous injection. Biodistribution of each radioactive mAb (anti-Id, anti-CD19, anti-MHCII) was compared with an isotype-matched control antibody. Animals were killed 1, 8, 24, 48, and 96 hours after receiving the radioactive mAbs and the blood, spleen, liver, kidneys, and lungs were assessed.

The weight and radioactive counts of the dissected organs were measured, and the percentage of the injected dose per gram of tissue (% ID/g) was calculated as described by Badger et al.25 The effective half-life and biodistribution of the labeled antibodies were determined. Assuming total absorption of the nonpenetrating radiation component with a homogeneous distribution, both the whole body and the organ doses were calculated by integrating the area under the retention curve.26

Immunotherapy

Age- and sex-matched mice were inoculated by intravenous injection with $10^6$ A31 or $10^5$ BCL1 lymphoma cells on day 0 and treated with external beam radiation therapy (EBRT) plus mAb, or mAb alone, 10 days later. EBRT was delivered using a modified 225 kV X-ray unit (Gulmay Medical, Chertsey, United Kingdom) at a dose rate of 0.11 Gy/minute. Intraintravenous mAb was given 4 hours after EBRT. Animals were fed acidic water (pH 2.5; 1 N HCl) supplemented with neomycin sulfate (2 g/L; Sigma-Aldrich, Poole, United Kingdom) starting 3 days prior to EBRT and continuing for 2 weeks afterward to prevent the risk of opportunistic infection. In addition, animals were housed in a sterile maximizer unit (Techniplast, Northants, United Kingdom).

For RIT experiments, 500 µg (anti-CD19, anti-CD38, anti-MHCII, or anti-CD3, Id) or 100 µg (anti-Id) $^{131}$I-labeled mAbs carrying 1.85 MBq to 18.5 MBq was injected intravenously per mouse. For therapies combining RIT with unlabeled mAbs, mice were injected intravenously with unlabeled anti-BCL1, and anti-A31 Id antibodies (doses as mentioned above) 2 to 3 hours prior to the injection of the $^{131}$I-labeled anti-MHCII. Parallel groups of mice were treated with unlabeled antibodies alone or $^{131}$I anti-MHCII alone for comparison. For all RIT therapies mice were given Lugol solution (as described in “Biodistribution studies”).

Animal survival was monitored daily, and the results were analyzed using the $\chi^2$ test of Peto.27 Animal immunotherapy was approved by the local ethical committee and was performed under a United Kingdom Home Office project license.

Hematologic toxicity of RIT

RIT toxicity experiments were carried out to evaluate the hematologic impact of the $^{131}$I-labeled anti-MHCII antibody. Groups of BALB/c mice (12 mice/group) were injected via the tail vein with $10^5$ BCL1 cells and given Lugol solution. Ten days after tumor inoculation animals received 500 µg of either 9.25 MBq or 18.5 MBq $^{131}$I-labeled anti-MHCII mAbs by intravenous injection 2 to 3 hours after unlabeled anti-BCL1. Parallel groups of untreated or non-tumor-bearing mice were given the same therapy as controls. Animals were killed on days 1, 7, 14, 21, 28, and 35 after receiving the radioactive mAbs. Blood samples were obtained from cardiac puncture and analyzed using a Sysmex XE-2100 Blood Cell Analyzer (Sysmex, Milton Keynes, United Kingdom).

In vivo tracking

To measure tumor growth following treatment and chart the progress of any immunologic response, groups of CBA/H mice were inoculated with $10^6$ A31 tumor cells intravenously on day 0 and treated on day 10 with EBRT, mAb (anti-Id, 100 µg intravenously), or a combination of both. Mice were cycled 5 days after treatment, and the spleens were removed, homogenized, and then assessed by flow cytometry. Tumor cells were detected by staining using fluorescein isothiocyanate (FITC)--labeled Mc39-16 (rat anti-A31 Id)27 and phycoerythrin (PE)--labeled-ID3 (rat anti-mouse CD19). Cells were stained with mAbs (50 µg/mL) on ice for 20 minutes, washed, and analyzed on a FACS Calibur (Becton Dickinson, Mountain View, CA). To characterize the immunologic response to treatment, homogenates were labeled with either PE-YTS169 (rat anti-mouse CD8) or FITC-YTA1.3.2

Table 1. Antimouse monoclonal antibodies used in this work

<table>
<thead>
<tr>
<th>Antibody clone</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Affinity, $K_d$ M$^{-1}$</th>
<th>Saturation level</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc10-6A5</td>
<td>BCL1 Id</td>
<td>Rat2a</td>
<td>$1 \times 10^9$</td>
<td>0.9 $\times 10^6$</td>
<td>George et al21</td>
</tr>
<tr>
<td>Mc39-16</td>
<td>A31 Id</td>
<td>Rat2a</td>
<td>$5 \times 10^9$</td>
<td>0.4 $\times 10^6$</td>
<td>Tenovus</td>
</tr>
<tr>
<td>ND3</td>
<td>CD19</td>
<td>Rat2a</td>
<td>$3 \times 10^9$</td>
<td>0.4 $\times 10^6$</td>
<td>Kroep et al22</td>
</tr>
<tr>
<td>NZM6</td>
<td>CD22</td>
<td>Rat1</td>
<td>$1.2 \times 10^9$</td>
<td>0.6 $\times 10^6$</td>
<td>Torres et al23</td>
</tr>
<tr>
<td>TIL-3</td>
<td>MHCII</td>
<td>Rat1</td>
<td>$5.3 \times 10^9$</td>
<td>1.8 $\times 10^6$</td>
<td>Illidge et al24</td>
</tr>
<tr>
<td>1A568</td>
<td>CD38</td>
<td>Rat1</td>
<td>$1.5 \times 10^9$</td>
<td>0.4 $\times 10^6$</td>
<td>Parkhouse</td>
</tr>
</tbody>
</table>

*$^*$Estimates of the $K_\alpha$ were calculated from binding curves by determining the concentration of antibody required to achieve half-maximum binding capacity.

†Estimates of the average number.

‡Generous gift from Michael E. Parkhouse (Pirbright Laboratory, Woking, United Kingdom).
Antihamster IgG was added at a

Where appropriate, hyper-

Where cells were then

For the assay, 250 μg/mL of mAbs was added, and the assay was

Figure 1. EBRT and anti-Id mAb therapy in BCL1 and A31 lymphoma. Groups of 5 age- and sex-matched BALB/c (A) and CBA (B) mice were inoculated with BCL1 (105) or A31 (106) lymphoma cells, respectively, by intravenous injection on day 0. Mice were

Results

External beam radiation therapy (EBRT) and anti-Id mAbs have an additive effect in vivo

In our previous work we showed that 131I anti-Id was effective at

determined by the number of splenic CD8+ or CD4+ T cells, respectively.

Phosphotyrosine detection by Western blot

Up-regulation of intracellular protein tyrosine phosphorylation was mea-

Measurement of intracellular-free calcium

Intracellular-free calcium was measured using the cell-permeable fluorescent probe Indo-1-AM (Sigma-Aldrich) using well-established methodology. Cultured πBCL1 lymphoma cells (105/mL) were washed twice in serum-free media and loaded with Indo-1-AM at 5 μM for 30 minutes at 37°C. Cells were then washed twice in serum-free RPMI and stored in the dark at room temperature until required. For the assay, 250 μL of cells was warmed to 37°C, 2 μg/mL of mAbs was added, and the assay was immediately analyzed using flow cytometry on a FACSCalibur (Becton Dickinson). Where appropriate, hyper-cross-linking sheep antirat or rabbit antihamster IgG was added at a final concentration of 20 μg/mL immediately before analysis. Intracellular calcium mobilization was estimated by determining the fluorescence ratio of Indo-1bound calcium (398 nm, FL5) to free calcium (482 nm, FL4) against time. The response was measured over 300 seconds and recorded using the Cell Quest software (Becton Dickinson).

Immunoprecipitation analysis for tyrosine phosphorylation of Syk

To detect Syk tyrosine phosphorylation, Triton X-100 lysates prepared as detailed in the previous paragraph were first incubated with 4G10 (2 μg/sample) at 4°C overnight. Protein G-coated Sepharose beads (Amersham Biosciences, Buckinghamshire, United Kingdom) preblocked with 5% (wt/vol) BSA were then added to the samples (15 μL/sample) and incubated for a further 60 minutes at 4°C. The resulting beads were then washed 4 times with cold lysis buffer and boiled in sample buffer. The precipitated proteins were separated by SDS-PAGE and immunoblotted as described in the previous paragraph with the anti-Syk antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of intracellular-free calcium

Intracellular-free calcium was measured using the cell-permeable fluorescent probe Indo-1-AM (Sigma-Aldrich) using well-established methodology. Cultured πBCL1 lymphoma cells (105/mL) were washed twice in serum-free media and loaded with Indo-1-AM at 5 μM for 30 minutes at 37°C. Cells were then washed twice in serum-free RPMI and stored in the dark at room temperature until required. For the assay, 250 μL of cells was warmed to 37°C, 2 μg/mL of mAbs was added, and the assay was immediately analyzed using flow cytometry on a FACSCalibur (Becton Dickinson). Where appropriate, hyper-cross-linking sheep antirat or rabbit antihamster IgG was added at a final concentration of 20 μg/mL immediately before analysis. Intracellular calcium mobilization was estimated by determining the fluorescence ratio of Indo-1-bound calcium (398 nm, FL5) to free calcium (482 nm, FL4) against time. The response was measured over 300 seconds and recorded using the Cell Quest software (Becton Dickinson).

Results

External beam radiation therapy (EBRT) and anti-Id mAbs have an additive effect in vivo

In our previous work we showed that 131I anti-Id was effective at clearing tumor in the BCL1 model and providing long-term survival. In the current study we attempted to separate the actions of mAbs from that of the radiation and initially investigated this using EBRT and unlabeled anti-Id mAbs in two different syngeneic B-cell lymphoma models (A31 and BCL1). Mice were inoculated with either 105 (BCL1) or 106 (A31) tumor cells and treated 10 days later with a single fraction of 5 Gy whole-body EBRT. In both tumor models, EBRT alone resulted in only a modest improvement in animal survival of around 10 to 20 days (Figure 1A-B). Larger doses of EBRT coupled with bone marrow transplantation did not significantly increase survival over and above the level seen with 5 Gy (data not shown), confirming the similar resistance of both lymphoma models to EBRT alone. In the BCL1 model, anti-Id mAb alone produced an increase in survival of around 8 days. The addition of anti-Id mAb to EBRT produced a small increase in survival of around 3 to 5 days longer than that seen with EBRT alone and around 10 days longer than with anti-Id alone (P < .01). In contrast, the A31 tumor was much more sensitive to the effects of anti-Id mAb and improvements in survival of around 40 days were seen over control animals with mAb alone, in keeping with results previously published by our group. However, when anti-Id mAb was given in combination with 5 Gy EBRT, long-term survival (> 100 days) was observed (Figure 1B).

A radiation dose response exists for RIT of lymphoma in the presence of anti-Id mAb

Given the modest improvement in survival achieved with EBRT and mAb in the BCL1 tumor model, we next investigated whether using RIT to deliver targeted radiation could improve upon the results achieved with EBRT. We have previously shown that 131I-conjugated anti-MHCII mAb was an extremely effective mAb for delivering radiation to tumor in the BCL1 model. We therefore
investigated the therapeutic effect of using the combination of 131 I anti-MHCII mAb and unlabeled anti-Id mAb (Figure 2A-B). These experiments led us to make two important observations. First, there does not appear to be a radiation dose response with targeted radiation alone, as similar levels of animal survival were seen with 9.25 and 18.5 MBq of 131 I anti-MHCII mAb in both the A31 and BCL1 models. It is important to note that the unlabeled anti-MHCII mAb had no therapeutic effect and that 2.31 MBq of anti-MHCII mAb had a similar poor performance to that achieved with 18.5 MBq of 131 I-labeled control (irrelevant) mAb of around 5 to 7 days further protection than that seen with untreated animals (data not shown). Therefore although a dose response was seen, as the dose of 131 I anti-MHCII was increased from 2.31 MBq to 9.25 MBq and survival improved to around 15 to 20 days over controls, no improvement in survival was seen in either the A31 or BCL1 tumor models above the 9.25-MBq dose level. This lack of efficacy confirms the effective tumor targeting of the 131 I anti-MHCII and argues against a nonspecific whole-body irradiation effect. The second most striking observation was the impressive therapeutic efficacy of combining 131 I anti-MHCII with unlabeled anti-Id mAb (Figure 2A-B). In contrast to the results seen with the combination of EBRT and anti-Id in the BCL1 model, when 9.25 MBq 131 I anti-MHCII was given instead of EBRT with unlabeled anti-Id, an increase of approximately 30 days survival was seen over and above that seen with either the 131 I anti-MHCII or anti-Id alone. Furthermore, in the presence of anti-Id mAb there was a clear radiation dose response with animal survival improving as the dose increased from 9.25 MBq to 18.5 MBq 131 I anti-MHCII. While there were no long-term survivors seen with 9.25 MBq 131 I anti-MHCII plus anti-Id, when the dose of 131 I was increased to 18.5 MBq, 100% of animals survived longer than 100 days. This important observation was also reproduced using the A31 model and a similar dose response was again seen, with 18.5 MBq 131 I anti-MHCII plus anti-Id treatment consistently producing 100% long-term disease-free survivors (Figure 2B).

Biodistribution and myelotoxicity of 131 I anti-MHCII RIT

We next wished to investigate whether the impressive therapeutic results seen using 131 I anti-MHCII plus anti-Id compared with EBRT plus anti-Id in the BCL1 model were due to an increased delivery of targeted radiation by the 131 I anti-MHCII mAb to the tumor-bearing organs. We had previously shown that MHCII had a higher level of surface expression than CD19 or Id. In addition we demonstrated in vivo that the MHC antigen complex unlike CD19 or Id did not modulate on binding mAbs.16 We therefore hypothesized that anti-MHCII would deliver more radiation to the tumor-bearing organs and thus performed biodistribution experiments to calculate the radiation dose delivered to both normal and tumor-bearing organs. Figure 3 demonstrates the biodistribution of a panel of B-cell–specific mAbs given 10 days after tumor. At specific times, over the next 4 days (Figure 3), the animals were
cullled and the major organs removed to determine the percent injected radioactive dose per gram (% ID/g) of organ tissue. The ability of a single trace-labeled (0.74 MBq) dose of 500 μg of 125I-labeled anti-MHCII to selectively target the major tumor-bearing organ (spleen) is clearly shown. In contrast, the B-cell-specific mAbs (anti-CD19 and anti-Id) were considerably poorer at delivering radiation to the spleen and provided similar targeting of radiation (% ID/g) to that seen with an irrelevant isotype-matched (control) mAb. The approximate radiation doses delivered by these mAbs were calculated using the medical internal radiation dosimetry (MIRD) method and the doses are presented in Table 2. The results confirm the ability of anti-MHCII mAbs to deliver larger doses of radiation per MBq of radiolabeled mAbs infused to the spleen and less nonspecific irradiation to the whole body. For an infusion of 18.5 MBq 131I anti-MHCII, the dose delivered to the spleen is estimated to be around 18 Gy, which is 3 to 6 times greater than that delivered by anti-CD19 (5.14 Gy), the irrelevant (4.5 Gy), and the anti-Id (3.4 Gy) mAbs (Figure 3A-D). This contrasts to the whole-body dose where anti-MHCII delivered around 2.9 Gy, whereas the other mAbs delivered larger nonspecific doses: anti-Id (4.4 Gy), anti-CD19 (7.25 Gy), and the irrelevant mAb (5.8 Gy). Biodistribution studies performed in advanced disease (day 18) showed generally similar results with the exception that despite the presence of a greatly increased tumor burden, the dose of radiation delivered by anti-Id was actually decreased (data not shown). We repeated the same biodistribution studies in the A31 tumor model and found that the % ID/g of tissue studied for the anti-MHCII mAb was almost exactly the same as for the BCL1 model (data not shown).

We next investigated the hematologic toxicity of 131I-labeled anti-MHCII plus anti-Id, which we had shown was highly effective at clearing lymphoma in vivo (Figure 2). The results are shown in Figure 4 and demonstrate that the toxicity of such treatment is mild, dose dependent, and reversible. The hemoglobin level is not significantly affected (Figure 4A), while there is a dose-dependent decrease in the platelet and white cell counts as the dose of 131I is increased from 9.25 to 18.5 MBq (Figure 4B-C). For 18.5 MBq 131I anti-MHCII plus anti-Id mAb, there is substantial fall in the platelet count from around 500 10^9/L to 200 10^9/L, which recovers to pretreatment level by 21 days after the therapy. A dose-dependent decrease in the lymphocyte count can be seen as early as 1 day after RIT, remaining suppressed for 14 days, with initial recovery being seen by day 21. In contrast, neutrophils appear more resistant to the effects of 131I-labeled anti-MHCII with the total numbers unaffected (Figure 4D).

### Table 2. Internal radiation dosimetry of RIT-treated mice (131I mAb, 0.5 mCi): Gy (mean ± range)

<table>
<thead>
<tr>
<th>Control</th>
<th>Anti-MHCII</th>
<th>Anti-Id</th>
<th>Anti-CD19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole body</td>
<td>5.80 ± 0.17</td>
<td>2.90 ± 0.11</td>
<td>4.44 ± 0.23</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.50 ± 0.20</td>
<td>18.00 ± 1.02</td>
<td>3.40 ± 0.21</td>
</tr>
<tr>
<td>Liver</td>
<td>3.04 ± 0.27</td>
<td>1.82 ± 0.17</td>
<td>5.14 ± 0.34</td>
</tr>
<tr>
<td>Lung</td>
<td>7.63 ± 0.36</td>
<td>4.54 ± 0.22</td>
<td>8.57 ± 0.25</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.04 ± 0.15</td>
<td>3.80 ± 0.14</td>
<td>2.75 ± 0.12</td>
</tr>
</tbody>
</table>

Radiation dose delivered by RIT does not correlate with survival

Having demonstrated that the panel of anti–B-cell mAbs delivered very different doses of radiation to the tumor-bearing organs, we next sought to determine whether a relationship existed between the radiation dose delivered to tumor-bearing organs and the therapeutic results observed. Under conditions similar to those seen during the biodistribution studies, mice received 10^6 BCL1 cells intravenously and were treated 10 days later with unlabeled or 9.25 MBq 131I-labeled mAbs. Figure 5 confirms that while unlabeled anti-MHCII mAb gives no protection over controls, both anti-Id and anti-CD19 produce a small therapeutic effect, increasing survival with anti-Id by around 8 days. This activity was, however, further enhanced by the conjugation of 9.25 MBq 131I to the anti-CD19 mAb. Interestingly, all 3 radioimmunoconjugates produced a similar level of therapeutic performance (Figure 5). Therefore, despite 131I–anti-CD19 and 131I–anti-Id delivering significantly less radiation dose to tumor, these radioimmunoconjugates provided a similar level of therapy to 131I anti-MHCII. These data indicate that there did not appear to be a relationship between radiation dose delivered with single mAb and therapeutic response seen. The radiolabeled irrelevant mAb provided approximately 5 days improvement in survival over control animals as before (data not shown).
Anti-Id and radiation have a synergistic effect in clearing tumor in vivo

We next wished to further explore the mechanisms that might underlie the efficacy of combining radiotherapy and unlabeled anti-Id mAbs in clearing tumor in vivo that we had previously established (Figures 1B and 2). Tracking experiments were therefore performed to monitor the differences in tumor clearance in mice treated with radiation and anti-Id compared with mice treated with either treatment alone. In addition the possibility that these effects might be due to an up-regulated immune response were investigated. These experiments were performed in the A31 model where we had observed such impressive clearances of tumor with EBRT and anti-Id mAbs (Figure 1B) due to the problems of handling RIT mAb ex vivo. Mice were inoculated with $10^6$ tumor cells intravenously on day 0 and treated as before on day 10 with anti-Id mAb alone or in combination with EBRT. Animals were subsequently killed on day 5 after treatment and the number of splenic tumor cells was determined using flow cytometry. Figure 6 shows that the tumor cell kill is similar in the 5 Gy– and anti-Id–treated groups, but there is a highly significant ($P < .01$) increase in tumor cell kill when these two treatments are given in combination. To assess whether this clearance of tumor may be due to activation of an immune response, we assessed the number of splenic CD4$^+$ and CD8$^+$ T cells in mice from each treatment group (Figure 6B). These data reveal that the number of CD4$^+$ and CD8$^+$ T cells actually decreases in mice treated with a combination of anti-Id and EBRT and suggests that the profound clearance of tumor does not appear to be due to a host cellular immune response but rather is the result of a synergistic or additive action between radiation and mAb.

Anti-Id and radiation have a synergistic effect in clearing tumor in vivo

We then went on to investigate whether the long-term clearance of tumor achieved with anti-Id in combination with $^{131}$I anti-MHCII could be reproduced with other B-cell–specific mAbs, regarding the potential problems of translating these results with anti-Id mAb to the clinic. Figure 7 shows that when anti-CD19 mAb was substituted for anti-Id and used in combination with $^{131}$I anti-MHCII in the BCL 1 model, impressive tumor protection was also seen of around 35 days longer than with control animals and 15 days longer than $^{131}$I anti-MHCII alone ($P < .01$). Despite the CD38 antigen being expressed on B cells at similar levels to CD19 (Table 1), enhancement in therapy with $^{131}$I-labeled anti-MHCII
can be grown in vitro) shortly after treatment with the same panel of mAbs. Figure 8D reveals profiles of FL5/FL4 versus time, with an increase in the ratio of FL5/FL4 indicating a rise in intracellular-free calcium levels. These data show that anti-Id induces profound calcium flux in the absence of hyper-cross-linking, but as with the PTP experiments above, hyper-cross-linking is required for the induction of calcium flux with anti-CD19. In this assay, hyper-cross-linked anti-MHCII mAb also induces a degree of calcium flux that is not seen with either the anti-CD22 or the control mAb (Figure 8D).

Discussion

In this study we have investigated the mechanisms operating in RIT of B-cell lymphoma in two syngeneic models and have attempted to define the contributions of “targeted” irradiation and the intrinsic mAb effector mechanisms to the long-term clearance of tumor in vivo. We have shown that the successful clearance of tumor in RIT can be provided by using two different mAbs working in combination, whereby one mAb is more effective at targeting the radiation to tumor and the second mAb initiates intracellular signaling. Finally we have shown that a radiation dose response exists for RIT of B-cell lymphoma in the presence of a signaling mAb.

We have previously shown the importance of mAb specificity to the therapeutic outcome of RIT in the BCL1 tumor model.16 In the current study we have expanded that initial work and using MIRD dose calculations have determined the total radiation dose delivered by a panel of B-cell antigen–targeting mAbs in vivo. Although the anti-MHCII and anti-Id mAbs bound to BCL1 tumor at similar levels,16 the anti-MHCII mAb delivered approximately 3 to 6 times more irradiation to the spleen. This observation appears to be explained by the fact that anti-MHCII mAb has a longer half-life and remains on the surface of tumor cells in vivo, while the anti-Id is cleared rapidly.16 The rapid clearance of anti-Id mAb and poor delivery of radiation to tumor by anti-Id, we believe, is due to rapid endocytosis and dehalogenation of 131I, in keeping with previously published work on anti-μ mAb.32,33

The requirement for individual patient dosimetry and its role in predicting response to RIT is currently a controversial issue in clinical RIT.2,34,35 Our studies indicate that there is a poor correlation between the radiation dose delivered by RIT and the therapeutic outcome. Even accurate radiation dosimetry appears inadequate at predicting the tumor response. Thus we found a similar level of performance for the three different radioimmunoconjugates 131I-labeled anti-CD19, anti-Id, and anti-MHCII mAbs in our B-cell lymphoma models, despite widely different radiation dose delivery to tumor-bearing organs. This may be explained by the fact that the tumor cell kill induced by the mAb is not quantified by such radiation dosimetry. We therefore conclude that for the radiolabeled anti-CD19 and anti-Id mAbs there appears to be two components to the therapy, namely mAb effector mechanism and targeted radiotherapy, with only the targeted radiation being measured by the dosimetry.

Although the lymphoma models used in this study have different growth patterns from human lymphomas, the mAbs used in this work all target antigens where equivalents have been tested in clinical trials.6,13,36-38 Indeed, the 131I–Lym-1, which has been extensively investigated in clinical trials,11,15 has very similar characteristics to the 131I anti-MHCII used in this study. We believe that we have made an important observation and proof of principle here and that our results using therapeutically active mAbs demonstrate that RIT is more than simply targeted radiation.
Furthermore, this study may help to clarify why clinical RIT tumor dosimetry using radiolabeled anti-CD20 mAb in B-cell lymphoma frequently fails to correlate with the clinical responses seen, as the tumor dosimetry is unable to quantify the anti-CD20 mAb effector mechanisms.

From these experiments, it appears that larger doses of targeted $^{131}$I are best delivered by a mAb directed against a highly expressed target antigen that fails to modulate upon binding, such as the MHCII complex. However, it is clear from this study that radiation alone, whether targeted using $^{131}$I-labeled anti-MHCII mAbs or delivered by EBRT, is inadequate in producing long-term clearance of tumor. For RIT or “systemic radiotherapy,” no improvement in survival was seen as the radiation dose was increased above 9.25 MBq $^{131}$I-anti-MHCII. However, when external EBRT or $^{131}$I-labeled anti-MHCII mAb was combined with a signaling mAb, significant improvements in the therapy were seen, resulting in long-term survivors in the anti-Id plus $^{131}$I-labeled anti-MHCII mAb groups. Interestingly, under these circumstances in the presence of anti-Id mAb, we found that a radiation dose response existed for targeted radiation. We found this dose response to be reproducible in two different B-cell lymphoma models, underlying the potential importance of this observation.

This study suggests that the substantial increases in therapeutic effect seen with the addition of anti-Id and anti-CD19 to targeted radiotherapy occurs because these mAbs initiate intracellular signal transduction. Previous work from our group has shown that the anti-Id and anti-CD19 performed relatively poorly in complement-dependent cytotoxicity (CDC) and Ab-dependent cellular cytotoxicity (ADCC) assays but these mAbs were the most therapeutically active. In contrast, the anti-MHCII mAb was the most potent in the CDC and ADCC assays but provided no protection in vivo. These data argue that Fc-dependent effector mechanisms as measured in these assays cannot themselves explain the therapeutic activity of mAbs. However, other in vitro assays revealed that mAbs directed to the BCR complex were particularly active at inducing growth arrest of tumor cell lines, presumably through intracellular signaling. We now provide direct evidence that anti-Id and anti-CD19 mAbs, but not other mAb specificities (CD38, MHCII), initiate intracellular signaling shown by both up-regulation of protein tyrosine phosphorylation and calcium flux.

Figure 8. Measurement of intracellular signaling. (A) Western blot analysis of protein tyrosine phosphorylation was performed using fresh BCL1 cells as described in “Materials and methods.” Lane 1 indicates anti-μ (IgM heavy chain); 2, anti-Id; 3, anti-MHCII; 4, anti-CD19; 5, anti-CD22; 6, anti-CD38; 7, control; and 8, molecular weight marker. Anti-μ and anti-Id as single mAb demonstrate intracellular signaling through the surface Ig receptor (BCR) as measured by the induction of cellular protein tyrosine phosphorylation. Hyper-cross-linking of primary mAb with sheep antirat IgG demonstrates signal transduction in anti-CD19 and anti-CD22 as well as via the BCR. (B) Addition of radiation enhances the level of signaling observed through these molecules. (C) Immunoprecipitation of phosphorylated proteins using 4G10, followed by immunoblotting with anti-Syk mAb, revealed the approximately 70-kDa phosphorylated protein is likely to be Syk. (D) Measurement of intracellular calcium flux in lymphoma cells after hyper-cross-linking mAb. BCL1 lymphoma cells were loaded with Indo-1-AM and then incubated with either mAb alone (2 μg/mL) or mAb plus an appropriate secondary polyclonal anti-IgG (20 μg/mL). Profiles of FL5/FL4 are shown versus time. Control or irrelevant IgG was a nonbinding anti-Id mAb (to the A31 lymphoma) in both sets of experiments.
It is well documented that signals transmitted by the BCR control the fate of normal and neoplastic B cells and that mAbs directed to this receptor can induce apoptotic cell death.\(^3\),\(^4\) Furthermore it is well established that signals from the BCR can be modulated by coreceptors such as CD19\(^4\) and that anti-CD19 mAbs are capable of transmitting growth inhibitory/apoptotic signals, when sufficiently cross-linked.\(^4\) Presumably therefore, the signals induced by these 2 mAbs are similar and are generated through a shared pathway. Our Western blot analysis and subsequent immunoprecipitation suggests that the protein tyrosine kinase Syk is involved in this signal transduction. Phosphorylation of Syk is one of the key steps in the complex signaling cascades activated following BCR ligation and is an important trigger for intracellular calcium flux.\(^3\) This is again in keeping with our data, where there is a good correlation between the degree of calcium mobilization observed and the extent of Syk phosphorylation. Moreover, the strength of intracellular signal obtained appears to be proportional to the amount of BCR cross-linking.\(^4\) We observed that anti-Id mAb in the treatment of follicular lymphoma, where the response seen is proportional to the strength of anti-CD19 mAb is related to the signaling activity of these reagents and that the response seen is proportional to the strength of intracellular signal delivered. These results appear entirely compatible with the clinical results published by the Stanford group using anti-Id mAb in the treatment of follicular lymphoma, where the clinical response appeared to correlate with the degree of signal transduction.\(^2\)

This study does not exclude the possibility that other host immune effector mechanisms, such as T cells, may act as an important component of clinical RIT. However, in our models, the long-term clearance of tumor by radiation and anti-Id does not appear to involve a T-cell response. Indeed, the number of CD4\(^+\) and CD8\(^+\) T cells is actually lower in mice treated with EBRT and anti-Id than in untreated controls. From previous studies, we know that the tumor itself can stimulate an increase in CD4\(^+\) and CD8\(^+\) cells.\(^4\) This decrease may therefore reflect a reduction in the amount of tumor (and therefore antigen) present due to the potent cytoxic activity of the combination therapy, as well as the direct immunosuppressive effect of the EBRT itself.

In conclusion, we have demonstrated that the biodistribution of a single radioabeled mAb does not predict tumor response when a significant biologic contribution is derived from a signaling mAb. Furthermore, we have shown that successful eradication of lymphoma in RIT consists of targeted irradiation and mAb signaling. Long-term clearance of tumor in vivo can be achieved by combining a therapeutically active signaling mAb that is itself poor at delivering radiation with a mAb that is a more effective vector for targeting radiation to tumor. Importantly, this tumor eradication is achieved with minimal and reversible hematologic toxicity. Our results confirm that RIT is much more than targeted radiation and provide a scientific rationale to support the use of selecting combinations of mAbs in RIT rather than using a single mAb. This study also suggests that a radiation dose response may exist for RIT in B-cell lymphoma, when the targeted radiation is augmented by the presence of a signaling mAb. We have demonstrated for the first time in vivo that this combination approach is effective in RIT and we believe that these provocative data indicate that this type of approach could be readily translated to the clinic.

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Antibody-induced intracellular signaling works in combination with radiation to eradicate lymphoma in radioimmunotherapy

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