NEOPLASIA

The Importance of Antibody-Specificity in Determining Successful Radioimmunotherapy of B-Cell Lymphoma

By Timothy M. Illidge, Mark S. Cragg, Harry M. McBride, Ruth R. French, and Martin J. Glennie

Radioimmunotherapy (RIT) is an expanding field and over the last few years some of the most compelling clinical data in the treatment of advanced non-Hodgkin’s lymphoma (NHL) have derived from this approach. Impressive durable partial and complete responses have been reported using a variety of antibodies, delivery schedules, radioisotopes, and doses of radioactivity.1-3 Press et al15 have adopted a myeloablative strategy with peripheral blood stem cell transplantation, whereas other groups, including Kaminski et al, using lower doses of nonmyeloablative fractionated treatments, have reported similarly impressive response rates.6-10 One feature, which appears common to all of these studies, is the exquisite sensitivity of low-grade NHL to RIT. However, the mechanisms behind these encouraging clinical responses remain largely undefined. This has meant that the optimal treatment approach for achieving remissions, including the required dose of radioactivity and the importance of the monoclonal antibody (MoAb) specificity, remains unknown.

To date, target antigens which have been used for RIT clinical trials include the major histocompatibility complex (MHC) class II allele HLA-DR10,11-13 Ig idiotype (Id),14 CD20,2-10 CD22,15,16 and CD37.3,5,17,18 The main criteria in the selection of MoAb for RIT has been that the target antigen is well expressed on the tumor, but not on critical, nonrenewable, normal tissue such as the nervous system. Recently, much of the clinical work has focused on the B-cell specific antigen, CD20. Indeed, anti-CD20 MoAb appear extremely promising for targeting B-cell lymphomas. The CD20 molecule is a plasma membrane protein that is well expressed on most B-cells, it is not expressed on hematopoietic stem cells, does not appear to undergo antibody-induced endocytosis,19 and is not shed from the plasma membrane before or after treatment.20-22 In vitro studies have shown the advantages of targeting CD20 in RIT over a range of other potential B-cell targets.20-22 However, one of the difficulties of evaluating clinical RIT data with anti-CD20 MoAb is that these MoAb are therapeutic in their own right,23,24 either through recruitment of natural effectors, such as complement, or direct cytotoxic signaling through the CD20 antigen.25 Maloney et al,23,24 have recently reported that 50% of patients achieve partial or complete remission following treatment with a human/mouse chimeric anti-CD20 MoAb. Therefore, in any RIT study it is difficult to establish which part of the clinical success is dependent on antibody effectors, antigen specificity, targeted radiation, or whole-body irradiation.

Answering such questions in a clinical setting is difficult and ideally requires good preclinical animal testing. Unfortunately most animal work to date has been restricted to human lymphoma xenografts, which suffer from the lack of crossreactivity of the treatment MoAb with normal mouse tissue and the unusual distribution of human tumors in immunodeficient animals.26 Ideally, syngeneic animal models are needed in which the tumor develops in the presence of an intact immune system and with the targeting MoAb able to cross-react with appropriate normal tissue. RIT and biodistribution work in syngeneic lymphoma models has mainly been limited to observations of radiiodinated anti-Id MoAb in the Rauscher murine T-cell erythroleukemia27-29 and the B-cell lymphoma 38Cl213,30,31 Such systems suffer a number of limitations.

In this present study we report the biodistribution, in vivo internalization, and RIT of a range of B-cell specific MoAb (anti-CD19, anti-CD22, anti-MHCII, and anti-Id) in the BCL1 B-cell lymphoma of BALB/c mice. For three of these reagents, namely anti-CD22, anti-MHCII, and anti-Id, there are similar clinical 131I-labeled MoAb either currently undergoing clinical evaluation,13,15 or that have formed the basis of recent trials.14 Currently, no anti-mouse CD20 MoAb has been developed for such work. Our results show the relative contribution of the
MoAb and the targeted irradiation to the therapeutic effects in vivo. For the first time in animals, we show at least an additive therapeutic effect between the activity of anti-Id MoAb and that of targeted irradiation, which allows the eradication of advanced tumor without toxic side effects. These results have important implications for clinical work. They suggest that the success of recent RIT in NHL may not derive solely from the targeting activity of radio-conjugated MoAb but that dose of radioactivity delivered to tumor does not correlate with tumor response. Instead the eradication of tumor appears to stem from a combination of the effect of radio-targeting activity and that of the endogenous cytotoxic activity of the carrier MoAb.

**MATERIALS AND METHODS**

**Animals and cell lines.** Ten- to 12-week-old female BALB/c mice (mean weight, 27 g; range, 25 to 30 g) were supplied by Harlan UK Limited (Blackthorn, Oxford, UK), and maintained in local animal facilities.

BCL1 is a B-cell leukemia that arose spontaneously in a 2-year-old BALB/c mouse and is transplanted in syngeneic recipients by injection of spleen lymphocytes from leukemic animals.12,13 Unlike most human lymphomas, which are disseminated, BCL1, and most other animal lymphomas develop predominantly in the spleen and then the liver with a leukemic spill in the terminal stages of the disease. Experimental results show that the BCL1 is a highly malignant tumor and as few as 10 cells can transfer the BCL1 tumor to unirradiated BALB/c mice.\(^{34}\) The BCL1-3B3 cell line is a variant of the BCL1 tumor,\(^{35}\) which is phenotypically similar but can be maintained in culture. \(\tau\)BCL1 is a recently derived variant of BCL1 that arose in this laboratory from the wild-type BCL1, and is capable of growth both in culture and also in BALB/c mice (T. Illidge, unpublished results, September 1996). Cell culture of BCL1-3B3 was performed in supplemented RPMI (RPMI containing 2-mercaptoethanol [50 µmol/L], glutamine [2 mmol/L], pyruvate [1 mmol/L], penicillin and streptomycin [100 IU/mL], fungizone [2 mg/mL], and 10% fetal calf serum [FCS] [Myoclone]) (GIBCO-BRL, Paisley, Scotland). \(\tau\)BCL1 was maintained in supplemented RPMI and 20% FCS.

**Antibody production and purification.** A list of the MoAb used in this report and their source is given in Table 1.\(^{16,17}\) The MoAb TT2-3 is an anti-MHC class II reagent that was produced in house. Its specificity was confirmed by sequential immunoprecipitation using a well recognized anti-MHC class II MoAb, N22 American Type Culture Collection (ATCC). MoAb-secreting hybridoma cells were expanded in culture using supplemented Dulbecco’s Modified Eagle’s Medium (containing the same supplements as supplemented RPMI [above], but without 2-mercaptoethanol). To purify the IgG MoAb, the culture supernatants were concentrated 20 times by membrane filtration (Millipore, Bedford, MA), precipitated with saturated ammonium sulphate, and then dialyzed and fractionated on protein G (1D3, NIMR6, and TT2-3) or protein A (N22) (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK) according to the manufacturer’s instructions. Two of the rat MoAb (Mc39-16 and Mc10-6A5) were prepared by ion-exchange chromatography on DEAE (Whatman, Maidstone, UK) as described by Elliott et al.\(^{40}\) The purity of all IgG preparations was checked by electrophoresis (Beckman EP system; Beckman Instruments, Irvine, CA).

**Iodination, labeling efficiency, and specific activity of MoAb.** Iodine-125 and iodine-131 were supplied as sodium iodide in dilute sodium hydroxide solution at pH 10 and free from reducing agent (Amersham Pharmacia Biotech UK Ltd). Purified antibodies were iodinated (in phosphate-buffered saline) using Iodobeads (Pierce Chemicals Co, Rockford, IL) according to the manufacturers’ instructions.

The labeled protein was separated from unbound iodine by gel filtration on a Sephadex G-50 column (Amersham Pharmacia Biotech UK Ltd). Between 80% and 90% of the counts of labeled antibody were precipitable with trichloroacetic acid. Labeling efficiency was determined as the amount of radioactive iodine incorporated into the recovered product as compared with the amount added to the reaction mixture. This varied between 43.5% to 56.5% for both the Iodine-125 and the Iodine-131. The specific activity, which was expressed as the amount of radionuclide attached per mg of MoAb in the final product, varied from 8.1 to 15.0 MBq/mg (0.22 to 0.40 mCi/mg), but was routinely 10 to 11.33 MBq/mg for all of the Iodine-131 therapies.

**Flow cytometry.** Splenic BCL1 cells were prepared and analyzed by direct immunofluorescence\(^{46}\) using each of the MoAb (fluorescein isothiocyanate-IgG) under investigation (Table 1).

**Binding of iodinated MoAb to the surface of BCL1.** The binding of radioiodinated MoAb to cells was determined as described previously.\(^{36}\) Briefly, \(\text{[Iodine-125]MoAb}\) were serially diluted and incubated with fresh BCL1 cells for 2 hours at 37°C in the presence of NaN\(_3\) and 2-deoxyglucose to prevent endocytosis. The cell-bound and free \(\text{[Iodine-125]MoAb}\) were then separated by centrifugation through a mixture of dibutyl phthalate: dioctyl phthalate oils (1:1:1, vol:vol). This allowed rapid separation of bound and free antibody without disturbing the binding equilibrium. The cell pellets with bound radioiodinated MoAb were counted on a gamma counter (Wallac UK Ltd, Milton Keynes, UK).

**Clearance of surface bound MoAb in vivo.** To determine the level of MoAb remaining bound to the surface of the tumor cells after treatment, surface MoAb was assessed by two-color flow cytometry using a FACS Vantage (Becton Dickinson, Mountain View, CA). Mice were treated with 0.5 mg of the appropriate MoAb or a control, nonbinding isotype-match, MoAb by tail-vein injection, 4 days after they had received 5 \(\times\) 10\(^7\) BCL1 cells by intravenous (IV) injection. Sixteen hours later mice were killed, their spleens homogenized to give a single cell suspension, and the homogenate washed twice with PBS. Surface-bound MoAb remaining after treatment was detected using the appropriate concentration of FITC-mouse anti-rat IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA). To allow gating on only the BCL1 cells and exclude normal spleen cells, phycoerythrin (PE)-labeled rat anti-BCL1 Id (PE-Mc10-6A5; Serotech, Oxford, UK) MoAb (25 µg/mL) was added after the cells had been labeled for rat IgG. The results are displayed in histograms which show FITC-anti-rat IgG binding to Id positive cells. In the case of BCL1, taken from mice treated with anti-Id MoAb, surface Id was significantly blocked or internalized by the in vivo treatment, but we were still able to achieve sufficient PE-anti-Id MoAb-staining to allow gating on the tumor cells.

**Measurement of apoptosis in vitro.** Radiation and MoAb-induced apoptosis was analyzed by the method of Nicoletti et al.\(^{45}\) Briefly,

<table>
<thead>
<tr>
<th>Antibody Clone</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Affinity (K(_{a}); M(^{-1}))</th>
<th>Saturation Level</th>
<th>Source (ref)</th>
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<td>Mc10-6A5</td>
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<td>Tenovus (36)</td>
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</tr>
<tr>
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<td>Control</td>
<td>Raty2a</td>
<td>—</td>
<td>Tenovus (35)</td>
<td></td>
</tr>
<tr>
<td>1D3</td>
<td>CD19</td>
<td>Raty2a</td>
<td>(3 \times 10^9) 0.4 \times 10^9</td>
<td>(37)</td>
<td></td>
</tr>
<tr>
<td>NIMR6</td>
<td>CD22</td>
<td>Raty1</td>
<td>(1.2 \times 10^9) 0.6 \times 10^9</td>
<td>(38)</td>
<td></td>
</tr>
<tr>
<td>T12-3</td>
<td>MHCIi</td>
<td>Raty1</td>
<td>(5.3 \times 10^9) 1.8 \times 10^9</td>
<td>Tenovus (34)</td>
<td></td>
</tr>
<tr>
<td>N22</td>
<td>MHCIi</td>
<td>Hamster IgG</td>
<td>ND ND ATCC</td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviations: ND, not determined; ATCC, American Type Culture Collection, Rockville, MD.

\(^{1}\)Estimates of the Ka values were calculated from binding curves\(^{40}\) by determining the concentration of antibody required to achieve half-maximum binding capacity.

\(^{2}\)Estimates of the average number antibody molecules bound to each BCL1 cell when the 125I-MoAb-binding curve had reach saturation.\(^{40}\)

\(^{3}\)Control MoAb was directed against the Id of the A31 tumor and did not react with BCL1.
treated samples (approximately $5 \times 10^5$ cells) were washed in PBS, resuspended in hypotonic fluorochrome solution (50 µg/mL propidium iodide (PI), 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100) and then stored overnight in the dark at 4°C to allow staining of DNA. Analysis was performed by flow cytometry on a FACScan (Becton Dickinson). PI-stained nuclei fluoresce in the red wavelength, which was analysed using a 488-nm argon laser for excitation and a 560-nm dichroic mirror and 600-nm band pass filter (bandwidth 35 nm) for detection. For comparison of results, the G1 peak in each sample was adjusted to around channel 250 on a logarithmic scale.

In vitro irradiation of cells was achieved using a Caesium source (AECL Gammacell 1,000; Toronto, Canada) at a dose rate of 2.18 Gy/min. Culture plates (24-well multi-plates; Life Technology, Paisley, UK) were coated with MoAb (5 µg/mL) in PBS for 2 hours at 37°C before addition of cells (2.5 to $5 \times 10^5$/mL). In combination experiments, cells were added immediately postirradiation. 

**Biodistribution studies.** Groups of BALB/c mice were injected through the tail vein with $10^6$ fresh BCL1 cells. They were given Lugol’s solution (5.0 mL Lugol’s stock/400 mL H2O; Lugol’s stock: 10 g KI, 5 g elemental iodine in 100 mL H2O) in their drinking water 3 days before initiation of the biodistribution or RIT. On day 14 post tumor inoculation animals received 500 µg of trace-labeled $^{125}$I-MoAb by tail-vein injection. Biodistribution of each radioactive MoAb (anti-Id, anti-CD22, and anti-MHCII) was compared with that of an irrelevant antibody, which failed to bind to the tumor cells. Animals were killed 1, 24, 48, 96, and 120 hours after receiving the radioactive MoAb. The weight and radioactive counts of the dissected organs (spleen, liver, kidneys, heart, lungs, and thymus) were measured, and the percentage of the injected dose/g of tissue (% ID/g) was calculated as described by Badger et al. In addition, blood samples were obtained by cardiac puncture immediately postmortem. Marrow samples were obtained by removing both ends of one femur and passing a 21-guage needle through the marrow cavity. The marrow sample was expressed into a preweighed test tube containing 1 mL 10% Formalin. Data were not adjusted for minor differences in individual mouse weights. The calculation of the area under the curve (AUC), used as a measure of the total delivered dose, was performed using Fig. P Scientific processor (Software Corporation, Durham, NC). 

**RIT with $^{125}$I-labeled MoAb.** For RIT experiments, groups of mice were inoculated with freshly prepared BCL1 tumor cells. The number of tumor cells given, by tail vein injection, varied according to individual experiments, and then stored overnight in the dark at 4°C to allow staining of DNA. Analysis was performed by flow cytometry on a FACScan (Becton Dickinson). PI-stained nuclei fluoresce in the red wavelength, which was analysed using a 488-nm argon laser for excitation and a 560-nm dichroic mirror and 600-nm band pass filter (bandwidth 35 nm) for detection. For comparison of results, the G1 peak in each sample was adjusted to around channel 250 on a logarithmic scale.

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**RIT with $^{125}$I-labeled and unlabeled MoAb.** We next tested the same panel of reagents in RIT against BCL1 lymphoma (Fig 2). Age-matched groups of mice received $10^6$ BCL1 cells IV followed 4 days later by various unlabeled and radioiodinated MoAb preparations. Our most striking observation was the therapeutic efficacy of TI2-3, an anti-class II MoAb (Fig 2A). While this MoAb was completely nontherapeutic when unlabeled, used as a $^{131}$I-conjugate it “cured” 85% of animals (34 out of 40). These results where achieved with around 500 µg of radiolabeled antibody, carrying approximately 5 MBq per mouse (Fig 2A). At this level no animals were lost due to irradiation. RIT experiments were generally terminated after 100 days and at this time, none of the surviving animals showed any signs of tumor, even when spleen cells were examined by flow cytometry with anti-Id MoAb. Interestingly, 16 of 18 animals from the initial $^{131}$I-anti-MHCII MoAb (TI2-3) experiment survived for more than 350 days and never developed BCL1. The lack of efficacy of the “naked” anti-MHCII MoAb (TI2-3) and the marginal therapeutic effects of nonspecific irradiation delivered by control antibody strongly suggest that the success of $^{131}$I-anti-MHCII MoAb depends on its ability to “target” irradiation to the tumor-bearing organs. 

Treatment with anti-Id MoAb produced markedly different therapeutic results. The survival curves in Fig 2A show that unconjugated anti-Id MoAb extended survival over controls by
approximately 10 days, confirming earlier work from this laboratory.\textsuperscript{36} Iodination of the MoAb increased survival to around 20 days but none of the animals treated (42 mice) achieved long-term survival. Thus, unlike the anti-MHCII MoAb, the therapeutic activity of \textsuperscript{131}I-anti-Id MoAb may have two components, one resulting directly from the MoAb, and a second from its targeted irradiation. It is important to note that tumors emerging after anti-Id treatment were phenotypically unchanged and were still bound by treatment MoAb. Thus, it would appear that despite binding at a similar level to the BCL\textsubscript{1} tumor, and being more active as an unconjugated reagent, the anti-Id MoAb is less therapeutic in RIT than the anti-MHCII MoAb.

We next extended this work to include the remainder of our panel of MoAb. Figure 2B shows that neither anti-CD19 nor anti-CD22 MoAb were appreciably active in therapy, regardless of whether or not they were radiolabeled. Previous work from this laboratory\textsuperscript{56} has shown that anti-CD19 MoAb can be therapeutic in BCL\textsubscript{1} but only when given in high amounts and over an extended period. A second anti-MHCII MoAb, N22, behaved exactly like TI2-3 and showed that, although inactive as an unconjugated reagent, it was highly effective once radioiodinated.

Thus from these RIT experiments both the anti-MHCII MoAb (N22 and TI2-3), which bound at high levels (Fig 1), were active against BCL\textsubscript{1} tumor. The reason for the poorer efficacy of anti-Id MoAb was not clear and did not seem to result from any marked difference in its binding characteristics (Table 1). We decided, therefore, to look for possible explanations in vivo.

\textbf{Biodistribution of }\textsuperscript{125}I-MoAb \textbf{in tumor-bearing animals.} Initial dosimetry work with BCL\textsubscript{1} showed that 14 days postinoculation (10\textsuperscript{6} cells), spleens were macroscopically enlarged with disrupted architecture when examined by immunohistology (data not shown). Spleens from such animals weighed 0.522 ± 0.23 g (mean ± 2 SEM), compared with only 0.15 ± 0.04 g for those from normal mice. These mice also show slight liver enlargement because of developing tumor.

To investigate the biodistribution of iodinated MoAb in such mice and estimate the total amount of irradiation delivered to the splenic tumor, we injected 500 µg of \textsuperscript{125}I-anti-CD22 (low binding), -anti-MHCII (TI2-3, high binding), or -anti-Id (high, tumor-specific binding) MoAb into 14-day tumor-bearing mice. At given times over the next 5 days the animals were killed and various organs removed to determine radioactive content. The results showed (Fig 3) that the different MoAb all accumulated rapidly in the spleen where the majority of the tumor was localized. Even just 1 hour after MoAb injection the spleens contained around 30% ID/g of tissue (anti-Id and anti-MHCII). The corresponding amount for the anti-CD22 MoAb was around 15%. Other organs, such as the liver, kidney, and lungs, carried between 10% and 15% ID/g of tissue at the same time.
point. Furthermore, unlike the results from these organs, the results for the spleen showed a large differential between uptake of the specific MoAb and uptake of the control IgG (approximately 5%), consistent with specific targeting to the tumor.

Figure 3 also shows the rate at which MoAb, once accumulated in the spleen, were cleared. This was particularly interesting with regard to the RIT. Thus, while the half-life for $^{125}\text{I}$-anti-Id MoAb was about 9 hours, that for $^{125}\text{I}$-anti-MHCII MoAb was almost 24 hours. As a result of such a large difference, the AUC for these two reagents, which gives a measure of total dose of irradiation delivered to the organ during the RIT, differ by close to 4:1. Interestingly, the anti-CD22 MoAb, which accumulates at relatively low levels in the spleen, presumably because of the relatively low expression of CD22, also has a short half-life resulting in a very small AUC and probably explaining its lack of RIT.

The total dose of radiolabeled anti-Id, anti-MHCII, and control IgG delivered to organs other than the spleen is quite similar over the 5-day period with little evidence of specific uptake. In the blood, however, we did find an extended half-life for anti-MHCII MoAb. This probably results from binding to MHCII-expressing cells, particularly B cells, in the circulation, which extends the half-life beyond that of the other MoAb.

**Clearance of tumor surface antigen by MoAb in vivo.** We next investigated whether surface antigens, once bound by treatment MoAb in vivo, are likely to remain at the surface or be endocytozed along with the treatment MoAb inside the cell. The results of such work might explain why anti-Id and anti-MHCII MoAb differed in their splenic survival and consequent AUC (Fig 3) and could have important implications for selecting target antigens in RIT.

For these experiments, BCL$_1$-bearing mice were treated with the same amount of MoAb that was used in RIT (0.5 mg/animal) of anti-Id, anti-CD19, anti-CD22, or anti-MHCII MoAb (TI2-3), and then 24 hours later splenic tumor cells were investigated for the presence of surface antibody. The level of the therapeutic MoAb on the surface of recovered tumor cells was detected by adding FITC-mouse anti-rat IgG, and then PE-anti-BCL$_1$ Id MoAb used to allow analysis (gating) of just the tumor cell population. The results (Fig 4) showed that in vivo exposure to anti-MHC II MoAb (TI2-3) did little or nothing to the expression of MHC II by BCL$_1$, and that the

![Fig 4. Changes in the level of surface MoAb after treatment in vivo. BCL$_1$-bearing mice were treated with 0.5 mg of anti-CD19, anti-CD22, anti-Id, anti-MHCII (TI2-3), or control IgG (Materials and Methods). Sixteen hours later spleen cells were taken and any MoAb remaining at the tumor surface detected by adding FITC-mouse anti-rat IgG polyclonal Ab. PE-anti-Id MoAb was also added to the staining mixture to allow gating on the tumor cells. The dotted histogram in the anti-CD22 MoAb box shows background staining with FITC-anti-rat IgG. These cells were taken from mice treated with control IgG. The solid histogram shows the maximum level of FITC-staining obtained when cells from mice treated with control IgG were stained in vitro with a saturating level of MoAb (25 µg/mL anti-CD22, anti-CD19, anti-Id, or anti-MHCII as indicated in each box). The open histograms (solid lines) show the level of MoAb remaining on the surface of tumor cells when they were recovered from mice treated with MoAb. Mice treated with anti-CD22, anti-CD19, and anti-Id MoAb show evidence of clearing in vivo; anti-MHCII MoAb does not.](image)
recovered tumor cells remained coated with MoAb. In contrast, those tumors that had been exposed to anti-Id, anti-CD19, or anti-CD22 MoAb showed clear evidence of MoAb-clearing in vivo, and carried less MoAb/cell than that on control cells stained with these reagents. For example, fresh tumor cells stained with anti-Id MoAb gave a mean fluorescence intensity that was more than 10 times higher than that on cells recovered after in vivo treatment. However, despite such losses, the cells treated with the three clearing MoAb were not completely negative for treatment MoAb, because they all stained more strongly than untreated cells stained with control MoAb (dotted histogram).

These data confirm and extend previous work showing that MoAb that are not cleared from the targets, such as anti-MHCII MoAb, are likely to be more active in RIT, than those that tend to modulate.20-22 These results are also consistent with the biodistribution studies showing the smaller AUC for the internalizing anti-CD22 and anti-Id MoAb.

**RIT of advanced BCL1 lymphoma.** In an attempt to increase tumor burden and extend the tumor model more in line with human disease, in the next set of RIT experiments treatment was delayed until day 14 of tumor development when the spleens were enlarged and tumor was beginning to appear in the liver. These experiments were confined to the anti-Id and the anti-MHCII MoAb that had been successful when used in RIT early in the disease. For this work we increased the amount of MoAb administered to 750 µg/mouse, which was conjugated to approximately 7.5 MBq to 8.5 MBq 131I. The results with unconjugated MoAb were as expected, with only the anti-Id showing any therapeutic activity (Fig 5). However, somewhat surprisingly we found that when treating at this late time point, the anti-Id MoAb gave, if anything, a more effective therapy (13 days) than had been achieved when used on day 4 of the disease (Fig 2A). The radiiodinated anti-MHCII MoAb, rather than curing mice as had been seen earlier, only extended animal survival moderately (by approximately 12 to 15 days over those in control groups), little better than unlabeled anti-Id MoAb under these conditions. However, by far the most impressive result was achieved with 115I–anti-Id MoAb, which extended the survival of all the animals to beyond 100 days with no signs of tumor. This observation was confirmed in two subsequent experiments. Thus by delaying the treatment and increasing the tumor burden it appears that the anti-Id therapy had become more effective, a result which is in direct contrast to that seen using the anti-MHCII MoAb.

To investigate the relative contribution of the anti-Id MoAb and the radiation on the improved efficacy of 131I–anti-Id when treating later in the disease, an investigation was set up using unlabeled anti-Id MoAb to directly compare the efficacy of treating on days 4 and 14 after tumor inoculation. The results from this work are shown in Fig 6 and confirm an improved survival with anti-Id MoAb when treating later in the disease. It would appear, therefore, that by delaying treatment with anti-Id MoAb for 10 days we have been able to increase survival by between 8 and 9 days. No improvement in efficacy was seen by delaying treatment with any of the other MoAb under investigation (data not shown).

**Anti-Id MoAb and irradiation provide additive cytotoxicity.** It is possible that the unexpected increase in therapeutic activity seen when delaying anti-Id MoAb treatment might relate to the growth status of the tumor cells at different times during its development. Preliminary data suggest that during the first 10 days after inoculation of a small dose of BCL1 cells (10^2/mouse) there is very little, if any, proliferation that can be detected (not shown). After this period, tumor cells are detectable in the log phase of an exponential growth curve. One possible explanation for the current results is that anti-Id MoAb is therapeutically more effective in rapidly growing cells. We next measured the sensitivity of rapidly growing BCL1 cells lines (BCL1-3B3 and BCL1) to anti-Id MoAb and external beam irradiation. Cells were exposed to MoAb, irradiation, or both, and then the level of growth arrest and DNA fragmentation assessed by flow cytometry following nuclear staining with PI. Figure 7A shows the DNA cell-cycle profiles obtained when BCL1 cells (log phase) were exposed to MoAb for 72 hours. During log-phase growth, the DNA in cells treated with control IgG was distributed with 51% to 53% in G1/G0 phase, 16% to 18% in S phase, and 12% to 13% in G2/M phase. In addition, approximately 13.5% to 14.4% of the DNA was in the sub-G1/G0 position, which represents fragmented DNA derived from cells undergoing apoptosis.41 Most MoAb under investigation (anti-CD19, anti-CD22, and anti-MHCII) had no effect on cell growth or DNA profiles. However, as expected,42 anti-Id and anti-Fcµ MoAb caused profound cell-cycle arrest (not shown).

**Fig 5.** Treatment of the BCL1 lymphoma with RIT late in the disease. Groups of 10 mice were given 10^7 cells on day 0 as usual and then left until day 14 before treating IV with radiiodinated MoAb (750 µg/animal [approximately 7.5 MBq]). The treatments included: Control IgG (○); control IgG2a (●); anti-Id (□); anti-MHC II (TI2-3; ◆), (●); and anti-MHCII (N22; ▽, ▼). The solid and open symbols represent MoAb labeled with and without radioactive iodine, respectively. Surprisingly the 131I-anti-Id MoAb provided long-term protection to all the mice.

**Fig 6.** The effect of delaying anti-Id MoAb-treatment in BCL1 lymphoma. Groups of 10 mice were given 10^7 BCL1, on day 0 and then treated with MoAb (1 mg/mouse) 4 or 14 days later as shown. For comparison, an additional group of mice was treated with 131I-anti-Id MoAb (750 µg/animal [approximately 7.5 MBq]) on day 14. Treatment groups include: Control IgG (○); anti-Id on day 4 and 14 (□); 131I-anti-Id (●); and anti-MHCII (◆) on day 14.
which was followed by an increase in DNA fragmentation (Fig 7A), indicative of apoptosis.

Cells treated with external beam irradiation underwent a number of changes in their DNA composition, including the well-described G2 cell-cycle arrest and marked DNA fragmentation (37.7% to 41.5%). However, the most interesting results were obtained when cells were irradiated and then cultured in anti-Id or anti-Fcµ MoAb. The combination of cytotoxic MoAb and irradiation resulted in greatly increased DNA fragmentation (61% to 67%), suggesting the two modalities had an additive cytotoxic activity. None of the other MoAb under study (Table 1) increased the toxic effect of irradiation. The potency of irradiation and MoAb binding to the surface Ig of BCL1 was confirmed in three separate experiments (Fig 7B) and on BCL1-3B3 cells (not shown). Finally, we asked the question whether the combination of anti-Id MoAb and external beam irradiation showed the same additive effect in vivo. Cultured pBCL1 cells were treated with 2 Gy external beam irradiation and then injected (10^6/mouse) with or without 0.5 µg/mouse anti-Id MoAb. Figure 8 shows a significant benefit (p < .01) of treating with both MoAb and irradiation over either treatment alone and suggests that this cooperative effect may play an important role in the therapeutic activity of radiolabeled anti-Id MoAb.

**DISCUSSION**

In this study we have investigated RIT in an animal model in an attempt to define the relative contributions of “targeted” low-dose irradiation and MoAb to the therapeutic effect. Dosimetry and treatment planning for therapeutic infusions of radiolabeled MoAb in lymphoma are usually performed by extrapolation from biodistribution studies with trace-labeled MoAb.3,6,8,27 This extrapolation assumes that the biodistribution of a therapeutic conjugate will be similar to that seen with trace-labeled MoAb.27 A primary aim of this investigation was to provide a model of targeted, nonmyeloablative irradiation that would accurately predict organ dosimetry for subsequent low dose RIT. We believe this is the first time a range of anti–B-cell MoAb have been compared in such a well-defined syngeneic lymphoma model using both radioiodinated and “naked” MoAb.

A number of observations have been made with regard to RIT. First, predictions initially made in vitro20-22 that strongly expressed surface B-cell antigens (MHCII and Id) are more suitable targets for RIT than weakly expressed molecules (CD19, CD22), have been confirmed in vivo. Thus tumor-bearing animals, if treated early in the disease with 131I-anti-MHCII MoAb (TI2-3 or N22), were mainly cured, while those given 131I-anti-CD19 or 131I-anti-CD22 MoAb were given very little protection, despite similar binding constants. These results appear to reflect levels of antigen expression, but are also influenced by the tendency of MoAb to clear in vivo (see below). Interestingly, under these conditions, 131I-anti-Id MoAb, which bound to BCL1 at high levels, gave only partial protection. Second, although the anti-MHCII and anti-Id MoAb bound at similar levels to BCL1, the anti-MHCII (TI2-3) delivered about four times more irradiation to tumor-bearing spleens. This result reflected its much longer half-life and may be explained by the observation that TI2-3 remains on the surface of tumor cells after MoAb treatment in vivo while Id was cleared.

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**Fig 7.** Growth arrest and apoptosis of pBCL1 cells treated with external beam irradiation and MoAb. pBCL1 cells were either treated with 2 Gy external beam irradiation, or remained untreated, before being cultured for 72 hours in 24-well plates, which were either coated (or uncoated) with MoAb as indicated. The cells were then harvested after 72 hours, stained with PI to detect DNA content, and analyzed by flow cytometry. (A) Flow cytometry histograms for one typical experiment. The horizontal bars show the gated areas representing fragmented DNA (indicative of apoptosis). The percentage of DNA within each gate is shown. (B) Average (+ SD) of three similar experiments to that in Fig 7A. The * indicates statistical significance (P < .01) over other combinations of MoAb and irradiation.

**Fig 8.** Anti-Id and external beam irradiation has a significant benefit over either treatment alone in vivo. pBCL1 are treated in vitro followed by growth in mice. Cells (10^6/mL) were either treated with 2 Gy of external beam irradiation or remained untreated before being mixed with MoAb (0.5 µg/mL) as described, before being transferred into BALB/c (5/group). Each mouse received 10^6 cells and 0.5 µg of MoAb as indicated in the key. Survival was monitored daily.
probably by endocytosis. Third, radioiodinated anti-MHCII MoAb, which were highly effective when used early in the disease (day 4), were much less effective when used against advanced tumor on day 14 and no longer provided any cures. Fourth, our most unexpected observation was that under these demanding conditions, 131I-anti-Id MoAb, which had given about 30 days protection when used on day 4, was able to cure (as determined by survival >100 days after inoculation) almost all animals when given on day 14. It would thus appear that with this particular MoAb, which was the only one investigated that had intrinsic therapeutic activity, treating later in the disease was highly beneficial. Biodistribution studies performed also on day 14, indicated that this tumor response did not correlate with the dose of radioactivity delivered to the tumor and indeed that the anti-Id MoAb delivered four times less irradiation to the tumor-bearing organs. Finally, in vitro experiments indicated an interesting additive effect between the therapeutic capacity of irradiation and the cytotoxic activity of anti-Id MoAb, so that when used in combination, these two treatments induced interesting additive effect between the therapeutic capacity of irradiation and MoAb. The growth and organ distribution of the BCL4 lymphoma has been very well characterized.33 Knapp et al34 showed that the tumor is confined mainly to the spleen until the terminal stages of the disease, when large numbers of tumor cells appear in the peripheral blood. These workers also showed that 14 days postinjection of 10^6 cells, the spleen contained approximately 30% λ- and Id-positive cells with little or no λ-positive cells in the blood. We conducted biodistribution studies on tumor at this stage of development and showed high-level uptake of trace-labeled anti-MHCII and anti-Id MoAb in the spleen, which was about six times that of a control IgG, and twice that of anti-CD22 MAb. Thus as expected, uptake was MoAb-dependent and probably related directly to binding to tumor cells and, in the case of anti-CD22 and anti-MHCII MoAb, antigen-positive normal cells. However, although the initial uptake of anti-MHCII and anti-Id MoAb was similar, biodistribution studies over the next 5 days showed that they had quite different half-lives of 24 hours and 8 to 9 hours, respectively. Integrating the AUC gives an approximation of the total dose of irradiation delivered to an organ and is commonly used to estimate this parameter.30,43 The AUC calculation using our data showed that the 125I-conjugated anti-MHCII MoAb (TI2-3) delivered a total dose of irradiation that was at least four times that delivered by the anti-Id MoAb. The explanation for the difference in half-life between anti-Id and anti-MHCII MoAb (TI2-3) is most likely related to the rate at which anti-Id MoAb was internalized leading to rapid dehalogenation.20 The rapid internalization, dehalogenation, and excretion of radioiodinated MoAb against the B-cell receptor and tumor Id compared with MoAb against MHCII have been well-documented in vitro.19,20 However, other factors may also contribute and it is possible that low levels of secreted BCL4 Id could form immune complexes with the MoAb and thereby promote degradation in FcR-bearing cells.45 Our biodistribution data for 125I-anti-MHCII MoAb confirm previous reports from similar models30 and show that the 125I-control IgG has an increased half-life over the anti-Id MoAb. Also of note was the observation that at these modest doses of radioactivity, there was no detectable bone marrow accumulation. This was in keeping with the report by Badger et al,45 which suggested that, unless larger doses, greater than 250 μCi (9.4 MBq) of radioactive iodine were used, myelosuppression is not an expected treated related toxicity in these animal models.

The results from the initial RIT with 131I-MoAb strongly implicate targeted irradiation to the tumor-bearing organs by the MHCII MoAb (TI2-3 or N22) as the major cause of the tumoricidal effects. The lack of effects seen with “naked” anti-MHCII MoAb is in agreement with previous work,36 and the negligible effects of the radiolabeled control IgG appear to exclude either a direct therapeutic effect from the MoAb or a nonspecific “total body” irradiation effect. Importantly, when interpreting these results in animal models and attempting to extrapolate to clinical RIT of B-cell lymphomas, the effects observed here with the anti-MHCII MoAb appear entirely compatible with those seen in the clinic using 131I-Lym-1 directed against the HLA-DR10 antigen.13 Like the anti-MHCII MoAb used here, the Lym-1 MoAb has had little or no therapeutic efficacy as a naked antibody in vivo and appears to be an inactive delivery vehicle for the targeting of what DeNardo et al13 describe as “systemic radiotherapy.” To increase the efficacy of this approach, DeNardo et al13 have used fractionation of RIT, which has in turn enabled an increase in the maximum-tolerated dose and response rates.

Thus our initial data underline what has been shown by others mainly from in vitro data,20-22 that a highly expressed, nonendo- cytosurface target is likely to be the most successful for RIT. The survival advantage seen here with 131I-anti-MHCII MoAb is more marked than that previously reported in this model using other delivery systems,46 or than those seen in the murine T-cell lymphoma EL4 with Yttrium 90-labeled MoAb.47 Knox et al30,31 have performed extensive investigations in the 38C13 murine B-cell subcutaneous lymphoma model showing the radiobiological effect of 131I-anti-Id MoAb and comparing its activity with dose-equivalent external beam irradiation. This study showed that, although there was a statistically significant difference between specific (131I-anti-Id MoAb) and nonspecific irrelevant (131I-control MoAb) MoAb on tumor response, the relative efficacy of the 131I-anti-Id was low, indicative of poor tumor targeting. A difficulty in interpreting such results is the lack of another anti-B cell MoAb to define the contribution that anti-Id MoAb may have had in these tumor responses.

The most unexpected observation to emerge from the current study was the capacity of 131I-anti-Id MoAb to cure animals when given as a single dose on day 14 of the BCL4 tumor (Figs 5 and 6). Part of the therapeutic activity of this derivative comes from the anti-Id MoAb, which as an unconjugated reagent gave partial protection to tumor-bearing mice and was also more active when administered as a naked antibody later in the disease (Fig 6). Considerable evidence now shows that anti-Id MoAb can be directly cytotoxic to human and mouse lymphoma cells and that this effect seems to depend on the ability of the MoAb to cross-link the surface Ig and thereby deliver transmembrane signals to the cells (Fig 7).42 Racila et al,48
working in the BCL\textsubscript{1} model, have presented strong evidence that anti-Id MoAb can provoke intracellular signals in tumor cells, which regulate their growth and can leave them in a state of dormancy for extended periods. Our own work\textsuperscript{48} shows that in a similar mouse B-cell lymphoma model, anti-Id MoAb, when administered in vivo, causes an abrupt growth arrest of tumor without immediate eradication. The reason that anti-Id MoAb might be more therapeutic when used on day 14 rather than day 4 is not clear. However, it is possible that the growth rate of the tumor may play some role in this phenomenon. For example, if BCL\textsubscript{1} cells are cycling faster on day 14 than on day 4, then it does not seem unreasonable to expect changes in their sensitivity to signaling through the B-cell receptor. The c-myc oncogene protein is expressed throughout the G1, S, and G2M stages of the cell cycle and is one of a number of genes responsible for regulating the linked pathways of proliferation and apoptosis.\textsuperscript{49} The Myc protein is not expressed in resting (G0) cells. In cycling cells, perturbations in myc levels are and apoptosis.\textsuperscript{49} Signaling through the B-cell receptor is one such way of perturbing myc, and for some time it has been known that such signaling in vitro can result in apoptosis. Therefore, if in established disease (day 14) more cells are in cell cycle, then anti-Id might be expected to be more effective. It is also quite clear that in addition to the MoAb effect, target irradiation plays an important role in providing tumor control. In our in vitro data show that anti-Id, unlike the anti-MHCII, MoAb were able to give at least an additive effect with external beam irradiation resulting in unexpected levels of apoptosis in cultured BCL\textsubscript{1} and BCL\textsubscript{3B} cells. Cells treated with a combination of irradiation and anti-Id MoAb in vitro were also less viable than cells treated with either moiety alone when inoculated into animals. This observation offers a potential explanation for the immunotherapy results. By treating later in the disease, we have increased the activity of the MoAb, which together with the effect of the targeted irradiation, can eradicate all tumor cells. The improvement in therapeutic efficacy that is provided by combined irradiation and anti-Id MoAb treatment may arise from events that occur at the transmembrane signaling stage. Recently, it has been discovered that, at least for lymphoblasts, apoptosis can be induced by irradiation with release of the intracellular messenger ceramide.\textsuperscript{51} When elevated, this molecule can induce a variety of cellular effects, including growth arrest and apoptosis. Intriguingly, signaling through the B-cell receptor, through MoAb such as anti-\textmu and presumably anti-Id, can also yield increases in ceramide. Therefore, it may be that the improved potency of the radiolabeled anti-Id MoAb is governed by an enhanced ceramide response. An alternate explanation may center on the fact that both irradiation and anti-Id MoAb treatments are capable of modulating the expression of c-myc. These two explanations may not be mutually exclusive, indeed, it may be that elevated levels of ceramide themselves translate to a more potent perturbation of myc and hence, apoptotic signaling. We believe these results may also help to explain some of the excellent therapeutic effects seen in the clinic with radiolabeled anti-CD20 MoAb. In contrast to the anti-MHCII MoAb, recent evidence suggests that anti-CD20 MoAb may be directly cytotoxic to cells and operate at least in part through a signaling pathway that induces apoptosis in target cells.\textsuperscript{25} Therefore, it seems plausible that together, targeted irradiation and anti-CD20 MoAb, may have a combined therapeutic efficacy that is greater than either treatment alone. Studies are underway to discover if any other radioconjugated MoAb benefit from the additive effects of targeted irradiation and direct Ab cytotoxicity. For example, anti-CD79 MoAb that bind to the invariant signaling chains associated with the B-cell receptor may provide an interesting target.\textsuperscript{52} These MoAb may have similar signaling properties to anti-Id MoAb, but will not suffer from the inherent problems of genetic instability and the requirement of patient-specific reagents.

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The Importance of Antibody-Specificity in Determining Successful Radioimmunotherapy of B-Cell Lymphoma

Timothy M. Illidge, Mark S. Cragg, Harry M. McBride, Ruth R. French and Martin J. Glennie