Radiotherapy in Mice with Yttrium-90-labeled Anti-Ly1 Monoclonal Antibody: Therapy of Established Graft-versus-Host Disease Induced across the Major Histocompatibility Barrier

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ABSTRACT

A monoclonal antibody recognizing Ly1, the murine homologue of CD5, was labeled with 90Y. In vivo biodistribution studies showed that 90Y-anti-Ly1 selectively localized in lymphoid tissue. Groups of B10.BR mice (H-2b) were lethally irradiated and given major histocompatibility complex-disparate C57BL/6 (H-2a) bone marrow and spleen cells to induce graft-versus-host disease (GVHD). Eight days later, mice with active GVHD were administered a single i.p. injection of 50 µCi 90Y-anti-Ly1. Fifty % of these mice were alive 2 months after treatment. Long term (>4-month) survival was significantly higher than in phosphate-buffered saline-treated mice. Survival was slightly improved in groups of mice receiving control irrelevant antibody labeled with 90Y or mice receiving free 90Y. However, survival in these groups was not significantly different from the phosphate-buffered saline-treated control group. The improved survival was supported by data showing improved mean animal weight. An anti-GVHD effect was confirmed by histopathological analysis. Unlabeled anti-Ly1 monoclonal antibody at comparable doses to 90Y-anti-Ly1 was not effective. Animals that died following 50-µCi treatment did not die of radiation toxicity, since all mice receiving 50 µCi 90Y-anti-Ly1 and Ly1 plus syngeneic bone marrow survived. The window of therapy was narrow in our studies, since 100 µCi 90Y-anti-Ly1 did not confer any survival advantage. Animals that did survive long term were studied for evidence of alloengraftment and found to have high levels of circulating donor mononuclear cells. 90Y-Anti-Ly1 localized in the spleen, thymus, liver, kidney, and bone marrow but not in the bowel, lung, muscle, or skin. Animals given similar doses of free 90Y, 90Y-anti-Ly1, or labeled irrelevant antibody eliminated free 90Y fastest, followed by 90Y-anti-Ly1 and then labeled irrelevant antibody. Hematological analysis of peripheral blood from 90Y-anti-Ly1-treated mice showed reduction in total WBC counts, absolute lymphocyte numbers, and absolute neutrophil numbers on day 24 after treatment. Myelosuppression recovered by day 38. These findings indicate that Ly1-positive cells are involved in the effector phase of GVHD and that radiolabeled antibodies may be useful as cell-specific probes for studying the GVHD network. 90Y-Anti-Ly1 protected recipients long term from lethal GVHD, and the fact that it had a rather remarkable inhibitory and selective effect on the lymphoid system of mice suggests that these agents may have broader application in the field of transplantation.

INTRODUCTION

GVHD is a pathological disease resulting from bone marrow transplantation, in which normal donor cells recognize HLA and non-HLA antigens expressed on the cells of the irradiated patient (reviewed in Ref. 1). The disease is immunological in nature, since murine studies show that T cells are involved in effector (reviewed in Ref. 2) and effector phases (3). The major target organs associated with clinical GVHD include skin, liver, and the gastrointestinal tract (reviewed in Ref. 4). However, immunosuppression, infection, and autoimmunity associated with ongoing GVHD frequently contribute to mortality.

Immunosuppressive agents such as corticosteroids, cyclosporin, and antithymocyte globulin represent standard treatment for GVHD (5–7). Although responses are frequent, survival is poor with all regimens. For example, in a multicenter trial of over 2000 patients treated with a variety of immunosuppressive agents, 48% of the patients died of causes either directly or indirectly related to acute GVHD (8). Anti-T cell antibodies are currently available for GVHD therapy. However, clinical responses to treatment with unlabeled antibodies have been incomplete (9, 10). Thus, alternative treatment strategies are desperately needed.

To explore such strategies, we labeled an anti-Ly1 MoAb with 90Y by the cyclic anhydride labeling procedure. Past studies have shown that cells responsible for GVHD induction express a surface glycoprotein called Ly1 (11). This lymphocyte surface antigen (reviewed in Ref. 12) is mostly restricted to T lymphocytes and is homologous to the CD5 structure defined in humans (13). It has been characterized as a M, 67,000 molecule with a variable extent of glycosylation (14, 15). Under certain conditions, anti-Ly1 can deliver an inductive signal to T cells that presumably mimics an undefined ligand interaction that leads to the stimulatory response by T cells (16). The advantages of 90Y for radiotherapy have been discussed in the accompanying manuscript (17). Most notably, high energy, short half-life, and short path length render it a potentially useful radionuclide for therapy.

For this study, we evaluated 90Y-anti-Ly1 in a model in which C57BL/6 bone marrow (a source of stem cells) and C57BL/6 splenocytes (a source of T cells) were transplanted into lethally irradiated B10.BR recipients that are disparate from C57BL/6 at the class I and class II regions of the major histocompatibility complex (18). We then waited 8 days, so that 90Y-anti-Ly1 could be tested against established GVHD. Most animal GVHD models are prophylactic, and to our knowledge this is the first testing of an antibody-mediated approach in an established GVHD model. We found that 90Y-anti-Ly1 protected recipients from lethal GVHD, protection was long term, and and

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1 The abbreviations used are: GVHD, graft versus host disease; ALT, alanine aminotransferase; BMT, bone marrow transplantation; FITC, fluorescein isothiocyanate; MoAb, monoclonal antibody; PBS, phosphate-buffered saline; PE, phycoerythrin; TBI, total-body irradiation; IRF, immunoreactive fraction.

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the approach shows potential for therapy as well as for determining the nature of effector cells involved in GVHD.

MATERIALS AND METHODS

Antibodies. Anti-Ly1 (from clone 53-7.3) is an IgG2a rat anti-mouse MoAb recognizing a M, 67,000 glycoprotein on mouse T cells (19). H65 is an IgG2a mouse MoAb that recognizes CDS, a M, 67,000 glycoprotein on human T lymphocytes. H65 was generously provided by Xoma Corporation (Berkeley, CA).

Mice. Female B10.BR-H-2^a and C57BL/6-H-2^a mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the NIH (Bethesda, MD). They were maintained in the University of Minnesota mouse colony. Animals were housed in conventional cages with filter lids, fed a fat-supplemented diet, and given antibiotic-supplemented water for 2 days before pretransplant immunosuppression and for 1 month after transplant. Recipients were at least 8 weeks old; donors were at least 4 weeks old.

Radiolabeling of Antibodies with \(^{3}Y\). The labeling of purified antibody with \(^{3}Y\), using the bicyclodehydride of diethylenetriaminepentaacetic acid (Pierce Chemical, Rockford, IL) as the chelating agent, has been previously described (20).

IRF. Calculation of the IRF of the labeled antibody was described in the accompanying manuscript (17). To determine specific binding of radiolabeled antibody, background or nonspecific binding was determined and subtracted. To measure nonspecific binding, increasing concentrations of Ly1-positive EL4 cells were washed and incubated with 150 \(\mu\)g/ml unlabeled anti-Ly1 to block the binding of labeled anti-Ly1. Cells were incubated for 1 h at 4°C and then spun through 84% silicone oil/16% mineral oil to separate counts in the pellet (bound counts) from counts in the supernatant (unbound counts). Percentage of binding was calculated as 
\[
\frac{cpm_{\text{pellet}} - cpm_{\text{unbound}}}{cpm_{\text{pellet}}}
\]
All points were measured in duplicate.

Biodistribution Studies. Four- to 6-week-old male C57BL/6 mice received i.p. injections of 20 \(\mu\)Ci \(^{3}Y\)-labeled antibodies. The animals were sacrificed 5 days after injection of radiolabeled antibodies. Specimens from various tissues were weighed, and the radioactivity was counted along with triplicate standards of the original injection solutions. Samples were suspended in Ecolume (ICN Biomedicals, Irvine, CA) and counted in a liquid scintillation counting unit (Beckman LS 3801) with an open window. The activity of the injected dose, in cpm, was calculated from the standards, and the activity for each tissue sample was expressed as a percentage of the injected dose/g tissue.

Blood Clearance of \(^{3}Y\)-labeled Antibodies. Between 24 and 200 h after injection of radiolabeled antibodies, five transplanted animals from each group were anesthetized and bled by retro-orbital venipuncture, at four different time points. Twenty \(\mu\)l of whole blood were aliquoted from each animal and counted in a liquid scintillation counter set on open window. Triplicate samples of the original injection solution were counted at the same time as standards, allowing the determination of the activity in 20 \(\mu\)l of blood as a percentage of the injected dose, corrected for the physical decay of the radionuclide. The individual values for the animals in each group were averaged for each time point.

Hematological Analysis. To monitor the leukocyte counts in peripheral blood at various time points after treatment, five animals from each treatment group were anesthetized, and blood was withdrawn by retro-orbital venipuncture. Leukocyte number and morphology were determined by examining Wright-Giemsa-stained blood smears.

Statistical Analysis. The computer program for compilation of the life table and statistical analysis by log-rank test was generously supplied by Dr. Bruce Bostrom, Department of Pediatrics, University of Minnesota. For comparison of biodistribution data and leukocyte counts, a Student \(t\) test was used.

Recipient Pretransplant Conditioning. Recipients were irradiated to a total dose of 8.0 Gy, using a 220-KeV GE Maximar-20 X-ray source filtered through 1 mm Al and 0.25 mm Cu, at a final absorbed dose rate of 0.4 Gy/min, as previously described (18).

BMT. Our procedure for BMT has been previously described in detail (21). C57BL/6 marrow with splenocytes (1:1 ratio) was washed once in cold medium and adjusted to 100 × 10^6 cells/ml. Twenty-five million bone marrow cells plus 25 × 10^6 splenocytes were injected into each recipient in 0.5-ml volume, via the caudal vein. Recipients were monitored daily for survival and weight loss during the 123-day observation period.

GVHD Therapy with Labeled Antibody. Recipients were given i.p. injections of either a single dose of \(^{3}Y\)-labeled antibodies 8 days after BMT or two daily doses of \(^{3}Y\)-labeled antibodies 8 and 9 days after BMT.

Flow Cytometry and Chimerism Studies. C57BL/6 spleen or thymus tissue was minced and gently forced through stainless steel screens to obtain single-cell suspensions. Following RBC lysis, cells were washed and resuspended in PBS with 5% colostrum-free bovine serum and 0.015% sodium azide. One million splenocytes or thymocytes were incubated for 15–30 min at 4°C with 0.4 \(\mu\)g of an anti-Fc receptor-specific MoAb (clone 2.4G2, generously provided by Drs. Richard Hodes and Susan Sharrow, National Institutes of Health), to prevent nonspecific binding by mouse macrophage and lymphocyte Fc receptors (22). Anti-Ly1 (clone 53-7.3) and anti-Thy-1.2 (clone 30-H12) (both provided by American Type Culture Collection, Rockville, MD) were directly conjugated to biotin or FITC, respectively (23). An irrelevant mouse myeloma protein (UPC\(_{10}\) L1tton Bionetics, Charleston, SC) was conjugated to biotin or FITC to determine the degree of background binding. Cells were then incubated with an optimal concentration of labeled antibody for 30–60 min at 4°C. Cells with biotin-labeled antibody were washed and incubated for an additional 30 min with streptavidin-PE (Molecular Probes, Eugene, OR). Cells were washed 3 times and fixed in 1% paraformaldehyde for analysis on a FACScan (Becton-Dickinson, Mountain View, CA). Background binding using UPC\(_{10}\)-biotin/streptavidin-PE and UPC\(_{10}\)-FITC was subtracted from the results obtained with the specific fluochrome-labeled antibodies, to determine the percentage of positive cells.

Chimerism of peripheral blood mononuclear cells was analyzed on day 93 after BMT. Engraftment was quantified by analyzing the cell surface binding of anti-H-2^a MoAb linked to fluorochrome (24). Anti-H-2^a (clone 11-4-1, mouse IgG2a; American Type Culture Collection) and anti-H-2^b (clone EH144, mouse IgG2b, provided by Dr. T. V. Rajan, Albert Einstein University, NY) were directly conjugated to FITC or PE, respectively. An irrelevant MoAb, 3A1E (anti-human CD7), was labeled with fluorochromes and used as the negative control. Ficol-Hypaque-purified peripheral blood mononuclear cells were treated and analyzed as described above. Zero to 1% of the control peripheral blood mononuclear cells were stained positive with the irrelevant anti-H-2 antibody linked to fluorochrome, while 99–100% of cells were positive for the relevant anti-H-2-specific antibody linked to fluorochrome.

ALT Assay. ALT is a hepatic enzyme that can be detected in the circulation following liver damage, and it has proven useful for the diagnosis of hepatic abnormalities (25). ALT levels were determined from frozen serum samples by standard spectrophotometry on a Kodak Ektachem (Eastman Kodak, Rochester, NY).

Pathological Examination of Tissues. Some preterminal mice were sacrificed. Lung and liver were placed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological assessment. Organs were scored positive for GVHD if there was perivascular infiltrate with acute necrosis (liver) or endothelialitis with a lymphocytic infiltrate (lung). In previous studies, these features were present only in mice with active GVHD and not in normal mice, irradiated nontransplanted mice, irradiated recipients of syngeneic BMT, or irradiated recipients of allogeneic marrow without supplemental splenocytes. All coded samples were read by a certified pathologist (D. C. S.).

RESULTS

Selective Binding of \(^{3}Y\)-Anti-Ly1 to EL4 Cells in Vitro. Immunofluorescence studies showed that anti-Ly1 bound to 22 ± 7% of splenocytes and 93 ± 3% of thymocytes (not shown). Anti-Thy-1.2 bound to 27 ± 11% of splenocytes and 94 ± 5%
of thymocytes. In the study described in the accompanying manuscript (17), 90Y-anti-Lyl selectively bound to EL4 cells (a murine T cell leukemia/lymphoma) but did not bind to a human T cell leukemia line, CEM. We further evaluated the selectivity of our labeled antibody in a blocking assay. Varying concentrations of EL4 cells were incubated with radiolabeled Ly1 in the presence of excess (150 μg/ml) unlabeled anti-Lyl, in an attempt to block the binding of the radiolabeled agent. In the absence of blocking antibody, we obtained 3.3, 6.8, 22.9, 42.2, and 53.9% binding to 0.5 × 10^6, 10^6, 5 × 10^6, 10^7, and 2.5 × 10^7 cells, respectively. In the presence of blocking antibody, binding was 0.5, 0.3, 1.4, 0.9, and 2.3%, respectively. These data suggest that specific binding was nearly eliminated by unlabeled anti-Lyl and are regarded as further evidence of the specificity of our reagent in vitro. The IRF values were 72 and 95% in two of three experiments. In the third experiment, we were unable to calculate the IRF because of a technical difficulty.

Biodistribution of 90Y-Anti-Lyl. 90Y-anti-Lyl was tested for biodistribution by injection of 20 μCi into a group of 10 untreated C57BL/6 mice. Five days after i.p. injection, tissues were removed and counted. Localization of radiolabeled antibody was in the spleen and thymus lymphoid organs and in bone (Fig. 1). Studies described in the accompanying manuscript showed that splenic localization was selective (17). There was also a high level of localization in the liver and kidney. Radiolabeled antibody did not localize in the bowel, blood, lung, muscle, or skin.

Pharmacokinetics. To determine the pharmacokinetics of radiolabeled antibodies in bone marrow-transplanted mice, B10.BR recipients given C57BL/6 bone marrow were bled by retro-orbital venipuncture at 23, 41, 116, and 161 h following treatment. For 90Y-anti-Lyl, percentage of injected dose/ml values were 5.5, 2.5, 0.3, and 0.1, respectively (Fig. 2). The half-life was 25.8 h, similar to the half-life values in the non-transplanted EL4 model reported in the accompanying manuscript (17). 90Y-Anti-human CDS was cleared more slowly (t1/2 = 39.8 h), and the most rapid clearance was observed with free 90Y. The percentage of injected dose/ml values for free 90Y were 0.23, 0.10, 0.03, and 0.02 at 23, 41, 116, and 161 h after treatment, respectively. At each time point, the amount of radioactivity detected in the blood of mice given free 90Y was at least 10-fold less than the amount of radioactivity detected in the blood of mice given 90Y-anti-Lyl. These data suggest that free 90Y is cleared from the blood more quickly and support the notion that the radiolabeled antibody remains intact in vivo. However, it is interesting that, when the radioactivity of all animals was estimated, using a Geiger counter, about 17 days after transplant, we detected 41,000 ± 15,000, 43,000 ± 23,000, and 31,000 ± 12,000 cpm for 90Y-anti-Lyl, 90Y-anti-human CDS, and free 90Y-treated mice, respectively (data not shown). This suggests that, while free 90Y is rapidly cleared from the blood, a large amount of free 90Y remains localized in the tissues of the mouse. On day 31 after treatment, fewer than 1,000 cpm were detected in all animals, regardless of treatment.

Hematological Analysis of Mice with Established GVHD. Transplanted mice that had been given a single dose of radiolabeled antibody were bled on days 24, 31, and 38 after BMT. On day 24, mice that had received a single 50-μCi dose of 90Y-anti-Lyl or 90Y-anti-human CDS had significantly depressed WBC counts, as compared to PBS-treated controls (Table 1). Differential counts of the blood smears showed that the reduction in WBC count was attributed to reductions in both lymphocytes and neutrophils. Treatment with single-dose 90Y-anti-Lyl reduced neutrophils by 2.3-fold and lymphocytes by 2.7-fold, compared to PBS-treated controls. By day 38, 90Y-anti-

Table 1  Hematological analysis of peripheral blood cells from mice with established GVHD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 24</th>
<th>Day 31</th>
<th>Day 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PBS</td>
<td>1.5 ± 0.6</td>
<td>1.9 ± 1.1</td>
<td>1.9 ± 1.1</td>
</tr>
<tr>
<td>90Y-Anti-Lyl (50 μCi)</td>
<td>0.7 ± 0.4*</td>
<td>2.7 ± 0.7</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>90Y-Anti-CDS (50 μCi)</td>
<td>0.6 ± 0.3*</td>
<td>3.0 ± 2.3</td>
<td>3.0 ± 2.3</td>
</tr>
<tr>
<td>90Y-Anti-Lyl (50 + 31 μCi)</td>
<td>0.2 ± 0.1*</td>
<td>1.9 ± 1.6</td>
<td>1.9 ± 1.6</td>
</tr>
<tr>
<td>90Y-Anti-CDS (50 + 31 μCi)</td>
<td>0.4 ± 0.2*</td>
<td>0.5 ± 0.4</td>
<td>0.5 ± 0.4</td>
</tr>
</tbody>
</table>

Experiment 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 24</th>
<th>Day 31</th>
<th>Day 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PBS</td>
<td>1.5 ± 0.8</td>
<td>3.7 ± 1.9</td>
<td>3.7 ± 1.9</td>
</tr>
<tr>
<td>90Y-Anti-Lyl (50 μCi)</td>
<td>3.2 ± 4.9</td>
<td>3.7 ± 3.1</td>
<td>3.7 ± 3.1</td>
</tr>
<tr>
<td>90Y-Anti-CDS (50 μCi)</td>
<td>1.0 ± 4.9</td>
<td>3.8 ± 1.9</td>
<td>3.8 ± 1.9</td>
</tr>
<tr>
<td>90Y</td>
<td>3.1 ± 2.2</td>
<td>3.8 ± 3.0</td>
<td>3.8 ± 3.0</td>
</tr>
<tr>
<td>Anti-Lyl</td>
<td>1.9 ± 1.1</td>
<td>4.6 ± 4.5</td>
<td>4.6 ± 4.5</td>
</tr>
</tbody>
</table>

* P ≤ 0.042.
I GVHD, BIO.BR recipients given C57BL/6 bone marrow and Class I and Class II Major Histocompatibility Complex Barriers. To study the ability of $^{90}$Y-labeled antibody to treat established anti-Lyl reduced neutrophils 6.9-fold and lymphocytes 4.5-fold, compared to controls. Again, on day 38, treated mice had recovered. In a second experiment, WBC counts were evaluated on days 31 and 38 after treatment. None of the $^{90}$Y-treated groups showed counts significantly different from those of PBS controls, although these groups were not significantly different at day 123 after BMT.

To provide further information on the cause of death in the $^{90}$Y-anti-Lyl-treated group, a few mice were examined for histopathological evidence of GVHD near death. Table 2 shows that histopathological analysis of control GVHD mice treated with PBS showed pathological evidence of GVHD in lung and/or liver on days 31 and 35 after transplant. In contrast, mice treated with one 50-μCi dose of $^{90}$Y-anti-Lyl on day 8 or two doses of 50 and 31 μCi on days 8 and 9 (respectively) showed no signs of GVHD 36 and 38 days after transplant. Fig. 3B shows the results of a second experiment (n = 6/group). The most notable findings in this experiment were (a) 50 μCi $^{90}$Y-anti-Lyl were effective against established GVHD, (b) 100 μCi $^{90}$Y-anti-Lyl did not improve survival (thus, the therapeutic window was quite narrow), and (c) mice given 50 μCi $^{90}$Y-anti-Lyl after syngeneic BMT showed no mortality.

Mean animal weight data are shown in Fig. 4 (n = 10–12/group). The dashed line in Fig. 4 represents PBS-treated controls and shows a progressive weight loss, beginning on day 0 of BMT and continuing to day 76 after BMT. Similar weight losses were documented in groups of control mice treated with anti-Lyl treated mice had recovered. Animals given an additional 31 μCi on day 9 also showed a significant reduction in WBC counts, compared to controls. Treatment with two doses of $^{90}$Y-anti-Lyl reduced neutrophils 6.9-fold and lymphocytes 4.5-fold, compared to controls. Again, on day 38, treated mice had recovered. In a second experiment, WBC counts were evaluated on days 31 and 38 after treatment. None of the $^{90}$Y-treated groups showed counts significantly different from those of PBS-treated mice.

Efficacy of $^{90}$Y-Anti-Lyl Therapy in Preventing GVHD Across Class I and Class II Major Histocompatibility Complex Barriers. To study the ability of $^{90}$Y-labeled antibody to treat established GVHD, BIO.BR recipients given C57BL/6 bone marrow and splenocytes were treated with radiolabeled antibodies 8 days after GVHD induction. A representative experiment, showing the actuarial survival of the treated animals given injections of 50 μCi $^{90}$Y-anti-Lyl, is shown in Fig. 3. The control mice receiving PBS (Fig. 3A) had poor survival (n = 12/group). These animals showed signs of GVHD (ruffled fur, weight loss, diarrhea, running, alopecia). Only one mouse remained alive 123 days after BMT. The best survival (50%) was obtained in the group receiving 50-μCi $^{90}$Y-anti-Lyl1 treatment. Survival in this group was significantly higher than survival in the PBS control group (P = 0.024). Survival of groups of mice treated with free $^{90}$Y or labeled irrelevant antibody was somewhat improved over that of PBS controls, although these groups were not significantly different at day 123 after BMT.

Four BIO.BR recipients were given 8.0-Gy TBI and then 25 × 10⁶ each of C57BL/6 bone marrow and spleen cells. Eight days after BMT, mice were given PBS, a single 50-μCi dose of labeled anti-Lyl1, or a single 50-μCi dose of labeled antibody followed by a second 31-μCi dose on the following day. Tissue was collected on days 31–38 after transplant. It was fixed, sectioned, stained, and examined by a certified pathologist for histopathological signs of GVHD.

**Table 2 Histopathological analysis of transplanted mice treated with $^{90}$Y-anti-Lyl**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Lung</th>
<th>Days after transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PBS</td>
<td>+</td>
<td>+</td>
<td>35</td>
</tr>
<tr>
<td>Control PBS</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>$^{90}$Y-Anti-Lyl (50 μCi)</td>
<td>-</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>$^{90}$Y-Anti-Lyl (50 + 31 μCi)</td>
<td>-</td>
<td>-</td>
<td>38</td>
</tr>
</tbody>
</table>

Fig. 3. Survival of recipients with GVHD, treated with $^{90}$Y-anti-Lyl. BIO.BR recipients were given 8-Gy TBI and then 25 × 10⁶ each of C57BL/6 bone marrow and spleen cells. In A, mice (n = 12) were given a single i.p. dose of 50 μCi $^{90}$Y-anti-Lyl1, an equivalent protein dose of unlabeled anti-Lyl1, 50 μCi $^{90}$Y-anti-human CD5 (H65; irrelevant MoAb control), 50 μCi free $^{90}$Y, or PBS, 8 days after BMT. Survival was measured daily and plotted in an actuarial manner. Only the group of mice treated with $^{90}$Y-anti-Lyl1 was significantly (P = 0.024) different from the control group treated with PBS. In B, MMT (n = 6) was performed as in A, with the exception of one group (Syn BMT), in which BIO.BR recipients were given 8-Gy TBI and 15 × 10⁶ syngeneic bone marrow cells. Eight days after transplant, these mice were given a single injection of 50 μCi $^{90}$Y-anti-Lyl1, 100 μCi $^{90}$Y-anti-Lyl1, or PBS.

Fig. 4. Mean weight of recipients with GVHD that were treated with $^{90}$Y-anti-Lyl. BIO.BR recipients from Fig. 3A, each given 8-Gy TBI and 25 × 10⁶ C57BL/6 bone marrow and spleen cells, were given a single i.p. dose of 50 μCi $^{90}$Y-anti-Lyl1, an equivalent protein dose of unlabeled anti-Lyl1, 50 μCi $^{90}$Y-anti-human CD5 (H65; irrelevant MoAb control), 50 μCi free $^{90}$Y, or PBS, 8 days after BMT. Animals (n = 12/group) were weighed daily and data were plotted as mean weight over time, in days.
unlabeled anti-Lyl, free 90Y, or control anti-human CD5 MoAb labeled with 90Y. Treatment with 90Y-anti-Lyl also resulted in a progressive weight loss that began to level at day 30 and remained at about 20 g for the duration of the experiment. The mean weight of mice treated with 90Y-anti-CD5 turned upward on day 58. This was attributed to the death of those animals suffering from GVHD. The data show that the healthiest animals were the animals treated with 90Y-anti-Lyl.

Long Term Alloengraftment in GVHD Mice Treated with Radiolabeled Antibody. Surviving animals were seroyped on day 93 to determine engraftment levels. Long term survivors which had been treated with 90Y-anti-Lyl, 90Y-H65, or free 90Y all showed evidence of complete donor chimerism, with no circulating host-type peripheral blood cells (Table 3). Similar results were obtained in a second experiment (not shown).

Further Dose and ALT Studies. To enhance the effect of day 8 treatment, an additional dose of 31 μCi 90Y-labeled antibody was given on day 9 (data not shown). The mean weight, in g, was still highest for the 90Y-anti-Lyl-treated group with a single dose, compared to a double-dose treatment. Survival was shortened by the double-dose treatment, perhaps because of increased toxicity. Serum ALT levels were measured in the mice given 50 μCi 90Y-anti-Lyl 35 days after BMT (data not shown). Levels in mice were all in the normal range, indicating that, at a dose capable of reducing GVHD, radiolabeled antibody was not hepatotoxic.

DISCUSSION

Myelosuppression is generally regarded as a major complication associated with the use of 90Y-labeled antibodies. We took advantage of the ability of 90Y-anti-Lyl to selectively localize in lymphoid tissue and to dramatically reduce lymphocyte numbers, as shown in the accompanying study of murine lymphoma (17). We found that 90Y-anti-Lyl improved the survival of mice with GVHD induced across a major histocompatibility barrier, even though we treated mice with established GVHD. Studies involving models for therapy of ongoing GVHD have been few, likely because of the aggressiveness of the disease once it has been initiated. Protection following therapy with radiolabeled antibody was long term and somewhat selective, while comparable doses of unlabeled antibody were ineffective. Improvements in survival were supported by improvements in mean animal weight showing the highest weight levels for mice treated with 90Y-anti-Lyl, even after 80 days. It is interesting that, when anti-Lyl linked to the potent catalytic toxin ricin A chain was administered in an identical manner in the same model of established GVHD, we were able to promote mean survival time by only 1 week at best, compared to control-treated mice (26). Thus, our sustained high level of long term survival with 90Y-anti-Lyl was somewhat unexpected.

GVHD is known to be an immunological disease in which T cells are clearly involved (1–3), but the role of T cells in target tissue damage, i.e., the effector phase of GVHD, is uncertain. To ensure GVHD, we injected mismatched C57BL/6 bone marrow along with splenocytes (containing an estimated 5.5 × 106 Ly-1-positive T cells) into irradiated B10.BR recipients and waited 8 days before treatment. This was ample time for the T cells to initiate responses to host H-2 and non-H-2 antigens. The median survival time of mice with GVHD in this system is 20–40 days. Clinical symptoms of GVHD in this model include weight loss, alopecia, diarrhea, and runting. Pathological symptoms include single-cell necrosis in skin and colon, crypt dropout, peritumoral infiltrate with acute necrosis in liver, endothelialitis, and infiltrate in the lung (26). The efficacy of 90Y-anti-Lyl in protecting against GVHD shows that Ly1-positive T cells are involved in the effector phase of GVHD and supports immunohistochemical studies showing that Ly1-positive cells migrate into the epidermis and mediate cutaneous GVHD (3).

Although our findings support the observation that Ly1-positive T lymphocytes are involved in the effector phase of GVHD (3) and suggest that these cells are radiation sensitive, they do not rule out the possibility that other cells may serve as secondary bystander targets. Large granular lymphocytes have also been implicated in the GVHD effector network (27).

In vitro studies showed that 90Y-anti-Lyl was selective, and in vivo studies showed that 90Y-anti-Lyl protected mice from GVHD better than any other reagent. However, selectivity was not absolute. Transient anti-GVHD effects were conferred by free 90Y, although free 90Y treatment did not result in significant GVHD protection, compared to control mice given PBS. This might be due to the more rapid blood clearance of 90Y, compared to the labeled antibody. Activity in serum 24 h after treatment with free 90Y was 10-fold less than activity after treatment with either 90Y-anti-Lyl or 90Y-anti-human CD5. Mean weight measurements of mice treated with free 90Y showed sustained low values even after 80 days, suggesting that the mice had sublethal GVHD. An upturn in the weight curves on day 60 suggested that GVHD had not occurred in mice treated with 90Y-anti-Lyl or 90Y-anti-human CD5 at this time. Taken together, our data argue that the anti-GVHD effect was not entirely due to nonspecific TBI. On day 93, mice from all groups were undergoing hair loss from the face and neck. This could indicate subclinical or chronic GVHD. However, hair loss can be radiation related as well.

Histopathological studies were performed on preterminal mice on days 31–38 after transplant. Our studies showed that dying PBS-treated GVHD control mice showed symptoms of lung and liver GVHD, while dying mice treated with 90Y-anti-Lyl showed no pathological symptoms of GVHD in liver and lung. Since WBC counts were restored at this time, it is unlikely that the animals died of myelosuppression, and the cause of their death is undefined.

The therapeutic window obtained with 90Y-anti-Lyl treatment was narrow. When we increased the single-injection dose from 50 μCi to 100 μCi (given in one i.p. injection), we lost our survival advantage, perhaps due to increased radiation toxicity. Lower maximum tolerated single-injection doses in this model (between 50 and 100 μCi), compared to the EL4 studies (200 μCi) (17), could likely be attributed to the high-dose TBI (8.0 Gy) and allogeneic BMT given only 8 days prior to radiolabeled antibody in the GVHD studies.

Table 3 Donor alloengraftment in GVHD mice treated with radiolabeled antibody

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Donor cells (%)</th>
<th>Host cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90Y-Anti-Lyl</td>
<td>6</td>
<td>96 ± 5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>90Y-Anti-human CD5</td>
<td>3</td>
<td>98 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Free 90Y</td>
<td>3</td>
<td>99 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>C57BL/6 control</td>
<td>1</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>B10.BR control</td>
<td>1</td>
<td>0</td>
<td>96</td>
</tr>
</tbody>
</table>

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GVHD is debatable. Some would argue that administration of alloengraftment devoid of GVHD (31, 32). The clinical potential of 90Y-labeled antibodies for therapy of GVHD has been associated with a high incidence of T cell depletion. In both mice (29) and humans (30), T cell depletion could be reversed with autologous marrow transplant a dog model to explore the possibility of using iodine-131 radioiodinated antibody for the immunosuppression of bone marrow recipients to evaluate 90Y-labeled anti-T cell antibodies as conditioning agents for the immunosuppression of bone marrow recipients in order to promote engraftment in murine models of alloengraftment via 90Y-anti-Lyl treatment sustained hepatic toxicity is given allogeneic transplants and radiolabeled antibody did not support.

Radiolabeled antibodies may have potential for use in conditioning regimens. Investigators (28) have recently employed a dog model to explore the possibility of using iodine-131-labeled antibodies as part of a marrow-preparative regimen to develop reagents with greater anti-leukemia effects but less toxicity than current radiochemotherapy regimens. Myelo-suppression was a complication at high doses using a labeled antibody directed to a lymphocyte adherence molecule. Lethal effects could be reversed with autologous marrow transplant support. Engraftment has become an important issue in the context of T cell depletion. In both mice (29) and humans (30), T cell deletion of donor marrow has been associated with a high incidence of engraftment problems. In our murine system, all mice surviving GVHD after 90Y-anti-Lyl treatment sustained high levels (>95%) of donor engraftment. It will also be important to evaluate 90Y-labeled anti-T cell antibodies as conditioning agents for the immunosuppression of bone marrow recipients in order to promote engraftment in murine models of aloengraftment devoid of GVHD (31, 32).

The clinical potential of 90Y-labeled antibodies for therapy of GVHD is debatable. Some would argue that administration of labeled antibodies to patients that have already received large doses of external beam irradiation would be dangerous. Others might argue that, since we are currently giving 8.5-Gy TBI for clinical conditioning at our institution (and 8.0-Gy TBI in our animal model), it would be feasible to increase the dose by giving radiolabeled antibody. However, we feel that, before 90Y-labeled antibodies are considered for clinical use, toxicity must be decreased. This may be accomplished by (a) improvements in coupling procedures (33–36) to ensure conjugate stability; (b) the use of Fab or F(ab')2 fragments to decrease nonspecific Fc binding (37, 38); (c) the use of radionuclides with shorter path lengths, such as α emitters (39), which may have fewer bystander effects; and (d) the use of recombinant cytokines to decrease myelosuppression.

In conclusion, we have shown for the first time that the ability of 90Y-anti-Lyl to selectively localize in lymphoid tissue renders it useful for GVHD therapy, even across major histocompatibility differences. Protection was long lived, despite the fact that treatment was not begun until 8 days following GVHD initiation. The broader implication is that selective targeting of the lymphoid system using radiolabeled antibodies will be useful in treating immunological diseases. Meanwhile, we believe that these reagents will be useful tools for determining which cells are important in the GVHD network.

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REFERENCES

Radiotherapy in Mice with Yttrium-90-labeled Anti-Ly1 Monoclonal Antibody: Therapy of Established Graft-versus-Host Disease Induced across the Major Histocompatibility Barrier

Daniel A. Vallera, Heinz Schmidberger, Donald J. Buchsbaum, et al.