Improved Therapeutic Results by Pretargeted Radioimmunotherapy of Non–Hodgkin’s Lymphoma with a New Recombinant, Trivalent, Anti-CD20, Bispecific Antibody

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Abstract

We examined whether a pretargeting method using a new recombinant anti-CD20 bispecific antibody (bsMab) followed by ⁹⁰Y-1,4,7,10-tetraazacyclododecane-N,N',N''-N'''-tetraacetic acid (⁹⁰Y-DOTA)-peptide could reduce hematologic toxicity yet improve therapeutic responses compared with conventional ⁹⁰Y-anti-CD20 IgG and a chemically conjugated bsMab. TF4, a humanized, tri-Fab bsMab with two Fab binding CD20 and one Fab binding histamine-succinyl-glycine (HSG), developed by the dock and lock (DNL) method, was tested in nude mice with Ramos B-cell lymphomas. Optimal pretargeting required a 29-h interval between TF4 and ⁹⁰Y-DOTA-HSG, and 20-fold more moles of TF4. TF4 cleared more rapidly from the blood than anti-CD20 IgG, with early processing in the liver, spleen, and kidney. At 24 h, TF4 improved tumor uptake of ¹¹¹In-HSG-peptide 2.6-fold [13% versus 5% injected dose per gram (ID/g)] and enhanced tumor to blood ratios >45-fold (770 versus 17), compared with an anti-CD20 Fab × anti-HSG Fab chemical conjugate, and by 1.6-fold (9.0% versus 5.6% ID/g) and 1.600-fold (522 versus 0.32), respectively, compared with radiolabeled anti-CD20 IgG. A severe (≥90%) and prolonged reduction of WBCs was observed at the maximum dose of ⁹⁰Y-anti-CD20 IgG, whereas pretargeting resulted in a ≤60% transient drop. TF4 pretargeting resulted in highly significant improvement in survival, curing 33% to 90% of the animals, even at relatively low doses, whereas most tumors progressed quickly without cures with ⁹⁰Y-anti-CD20 IgG. These results indicate an improved therapeutic index with pretargeted radioimmunotherapy (RAIT) using a DNL-constructed tri-Fab, bsMab, compared with conventional therapy with directly radiolabeled antibody or with a chemically conjugated bsMab. These encouraging results prompt testing these constructs for pretargeting RAIT in patients. [Cancer Res 2008;68(13):5282–90]

Introduction

Antibody-targeted therapies are important in the management of hematologic malignancies, with the anti-CD20 IgG rituximab having the greatest effect in non–Hodgkin’s lymphoma (NHL) treatment but also in other hematologic tumors and even nonmalignant conditions (1–6). Although rituximab has achieved widespread acceptance, clinical studies have shown that radio-labeled anti-CD20 antibodies, ¹³¹I-tositumomab and ⁹⁰Y-ibritumomab tiuxetan, have a higher objective response rate, yet these treatments are prescribed infrequently (7–9). Several factors may be responsible, including the pivotal trial findings that duration of response and time to progression were not improved. Unlike rituximab, anti-CD20 radioantibodies cause severe and protracted hematologic toxicity in many patients. However, there are an increasing number of reports showing a higher portion of durable complete responses, with very promising results when used in frontline and in consolidation settings (4, 10–21). Recent results with radioimmunotherapy (RAIT) of NHL suggest that this modality is gaining an increased role in the management of patients with B-cell NHL (2).

Pretargeting methods separate the delivery of the antibody from the radionuclide, which significantly reduces the residence time of the radionuclide in the blood, the major cause of dose-limiting hematologic toxicity (22, 23). Pretargeting systems show less hematologic toxicity and improved efficacy in animal model systems, including solid and hematologic tumors (24–33). We have been pursuing a pretargeting approach based on a bispecific antibody (bsMab) for localization of a radiolabeled hapten-peptide (34). Using a bsMab prepared by chemically coupling a Fab’ fragment of a humanized anti-CD20 antibody, veltuzumab (Immunomedics, Inc.), and a Fab’ of an anti-hapten antibody, we showed improved efficacy by pretargeting a ⁹⁰Y-hapten-peptide over that of the ⁹⁰Y-anti-CD20 IgG (32). Recently, we reported a novel method of producing fusion proteins, called the “dock and lock” (DNL) procedure, which can be used to prepare recombinant, humanized, bsMabs composed of two Fabs against a tumor antigen tethered to a Fab fragment of the antipeptide/hapten antibody, as well as preparing several different multivalent monospecific or bispecific antibodies (35, 36). Herein, we report the development and testing of a DNL tri-Fab recombinant construct for pretargeting CD20⁺ lymphomas and using the histamine-succinyl-glycine (HSG) hapten capture that together improve tumor uptake and the therapeutic index when compared with a monovalently CD20-binding Fab’ × anti-hapten Fab’ chemical conjugate or a directly radiolabeled anti-CD20 IgG, suggesting that this method is promising for clinical evaluation.

Materials and Methods

DNL technology. The basis of the DNL method is the exploitation of the specific protein-protein interactions occurring between the dimerization and docking domain (DDD) in protein kinase A and the anchoring domain (AD) in a reactive A-kinase anchoring protein (35, 36). With the DNL method, two types of modules, one containing the DDD and the other the
AD, are generated and subsequently combined to form noncovalent complexes, which are then covalently linked via disulfide bonds by incorporating cysteine residues into the natural sequences of DDD and AD. A diagrammatic depiction of the DNL fusion protein involving a tri-Fab construct, having two Fabs binding to CD20 and one to the peptide HSG, as well as the HSG peptide conjugated with the 1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetic acid (DOTA) chelate, is shown in Fig. 1.

**Antibody and peptide preparations.** Using DNL, the preparation of the h679-Fab-AD2-pdHL2 mammalian expression vector, the purification of the h679-Fab-AD2 fusion protein, the preparation of DOTA-conjugated IMP-288 peptide [DOTA-n-Tyr-n-Lys(HSG)-n-Glu-n-Lys(HSG)-NH₂; MHT, 1453], and the humanized anti-CD20 IgG hA20 (veltuzumab) have been described (35, 37, 38). Detailed descriptions of the preparation of the hA20-Fab-DDD2 DNL module and the subsequent assembly of TF4, as well as radiolabeling procedures, are provided in the Supplementary Online Materials and Methods.

**Animal studies.** All studies were performed after approval of the local animal care and use committee. The Ramos human B-cell lymphoma line (AnNCr-nu/nu; NCI/CRL). Within 17 to 19 d, tumors became visible and grew thereafter at a rapid rate. Most studies were initiated once the tumors were -1 cm in diameter.

The dosing, tumor size, and number of animals for each study are provided in the tables, figures, or in Results. All agents were administered i.v. (±0.2 mL). Trace amounts of the radiolabeled antibodies were supplemented with unconjugated antibody to meet the prescribed dose (50 μg for hA20 IgG, but for pretargeting, the bsMab and IMP-288 doses were adjusted to yield a desired molar ratio). For therapy studies, the moles of IMP-288 were based on the radioactivity given, with the TF4 or control TF2 protein dose adjusted to give a final molar ratio of 20:1.

The pretargeting capability of TF4, which binds divalently to CD20, was compared with pretargeting with a hA20 Fab × m679 Fab chemical conjugate that binds CD20 monovalently. Both constructs have monovalent binding to HSG. Ramos-bearing nude mice were given 30 or 101 μg (0.3–1.0 nmol) of the chemical conjugate or 118 μg (1.0 nmol) of TF4, and 48 or 24 h later, respectively, 0.03 to 0.05 nmol (±30 μCi) of the 111In-IMP-288 was administered. At 24 and 48 h after the 111In-IMP-288 injection, groups of five mice were necropsied.

Before necropsy, animals were anesthetized, bled by cardiac puncture, and then euthanized. Tumors and tissues were weighed and then counted in a gamma counter with appropriate 125I and 111In windows. The tissue radioactivity was divided by its weight and then by the total injected activity to derive the percent injected dose per gram tissue (% ID/g). Tumor to tissue ratios were calculated from the respective % ID/g data. Area under the curve (AUC) analysis was determined when multiple intervals were examined. Biological (i.e., % ID/g) or effective (μCi/g) data were fit to a single-rate exponential decay function. The exception was the effective AUC data for the tumors in animals given the 90Y-hA20 IgG, which had an initial uptake over the first 48 h (trapezoidal fit), followed by an exponential decay thereafter. The slope of the terminal phase of the effective curve was used to estimate activity remaining in the tumor to an infinite time. The Wilcoxon two-sample test (one sided) was used to compare AUCs across treatment groups as well as ratios of the AUC for the tumor and tissues.

Hematologic toxicity was assessed in nontumor-bearing, 8-wk-old, female, NIH-Swiss mice. All animals were prebled 1 d in advance of the treatment and then weekly thereafter. After local proparacaine anesthesia, blood (±70 μL) was taken retro-orbitally and added to a RBC lysing buffer. The cell pellet was washed and diluted in PBS-formalin for flow cytometry analysis (FACSCalibur, BD Biosciences) using preset scatter windows to discriminate mouse lymphocytes and granulocytes, as well as the complete WBC count. The percent change in the WBCs of each animal from its baseline determination was calculated and averaged (n = 5 per treatment). Treatments consisted of an i.v. injection of 0.15 or 0.175 mCi of 90Y-hA20 IgG or 0.25, 0.5, or 0.7 mCi of 90Y-IMP-288, pretargeted 29 h earlier with TF4 (20:1 molar ratio). These doses were selected based on prior experience suggesting that animals would tolerate the highest planned activity (28, 32).

Six separate studies were performed to assess the therapeutic activity of 90Y-hA20 IgG or TF4-pretargeted 90Y-IMP-288 at various dose levels and with a variety of controls. Each study was controlled internally with an untreated group of animals. Other controls included 90Y-IMP-288 alone, 90Y-IMP-288 pretargeted with the irrelevant TF2 anti–carcinoembryonic antigen (CEA) bsMab, and nonradiolabeled IMP-288 pretargeted with TF4 (i.e., TF4 and IMP-288 doses equivalent to 0.5 mCi 90Y-IMP-288 treatment). The last control group was included because *in vitro* studies had indicated...
that the divalent IMP-288 hapten-peptide could induce apoptosis in a lymphoma cell line as a consequence of cross-linking TF4 on the cell surface (39). All syringes were read for 125I activity in a calibrated Capintec CRC-15R dose calibrator. Groups of five animals were housed in filtered caging units. Bedding was changed within 3 to 4 h after injection of 125I-IMP-288 and then again the next day to remove excrated activity. Whole-body clearance was monitored by measuring the animals in a dose calibrator at 3, 24, and 48 h. Readings also were made on a syringe containing each injected dose. Tumors were measured two to three times a week using a caliper in three perpendicular planes and expressed as cm3. Studies typically started when the majority of the tumors were minimally 1.0 cm in the longest diameter. Animals were removed from study (a) if tumor size exceeded 3.0 cm3, (b) if their body weight decreased by >20% of their baseline weight, or (c) for other health-related reasons. Survival analysis was based on the time required for the tumor to reach a size of 3.0 cm3 (tumor progression). Animals removed based on treatment- or nontreatment-related toxicity were censored. Analysis was performed from Kaplan-Meier survival curves using the log-rank test (Prism GraphPad version 4.0). Additionally, tumor quadrupling times were calculated and analyzed using a Wilcoxon two-sample comparison. For this analysis, the time required for tumors to reach four times the initial cm3 size was determined from the growth curves. For humane reasons, animals were not allowed to bear tumors >3.0 cm3, and because several tumors exceeded 0.75 cm3 at the start of the study, in these cases the quadrupling time was given as the time to reach 3.0 cm3. For animals that had no visible tumor at the termination of the study, we assigned the termination date at the worst case scenario quadrupling time. Finally, animals that were removed from the study for reasons other than tumor progression used the time of their removal as the quadrupling time.

Results

Characterization of TF4. Size-exclusion high-performance liquid chromatography (HPLC) analysis of the IMP-291 affinity-purified fraction resolved a major peak (TF4), a lower molecular weight species representing unreacted h679-Fab-AD2 (~5%), and some high molecular weight multimer/aggregate (Supplementary Fig. S1A). Size-exclusion HPLC analysis of Q-Sepharose–purified TF4 (1.2 g or 83% yield) showed a single protein peak with a retention time consistent with a 157-kDa protein (Supplementary Fig. S1B). SDS-PAGE showed that the product consisted solely of the constituent TF4 polypeptides (h679-Fd-AD2, h2A0-Fd-DDD2, and κ light chains; Supplementary Fig. S1C). Bispecific binding involving all three Fabs was shown by Biacore (Supplementary Fig. S1D).

TF4 biodistribution in tumor-bearing nude mice. 125I-labeled TF4 cleared rapidly from the blood of mice, decreasing to <1.0% ID/g within 24 h (Table 1). At 1 h, the highest organ uptake was the kidneys, but activity decreased rapidly until, at 24 h, there was <0.5% remaining in any of the major organs. This distribution is similar to that reported previously for TF2, an anti-CEA × anti-HSG DNL bsMAB (35).

TF4 biodistribution was also assessed using 111In-labeled TF4. 111In is sequestered in tissues, thereby providing a better estimate of the total amount of protein delivered over time, whereas iodine is released, providing a better estimate of the amount of product that potentially is accessible (i.e., not internalized; ref. 40). At 3 h, tumor and tissue uptake with 111In-TF4 was distributed in a proportional manner, similar to that of 125I-TF4 at 1 and 6 h, with a high concentration in the blood and the highest uptake in the kidneys, and similar amounts in the liver and spleen. However, by 24 h, 111In-TF4 uptake in the tumor and normal tissues increased, whereas 125I-TF4 decreased >10-fold. These data suggest that TF4 is processed in the kidneys, liver, and spleen, but based on tissue weights (e.g., ~0.15, 1.1, and 0.1 g, respectively), most were processed in the liver. The 111In-TF4 concentration in the blood at 24 h was surprisingly nine times higher than 125I-TF4. Size-exclusion HPLC of serum from mice given 111In-TF4 showed no evidence of dissociation or catabolic by-products at 1, 3, or 24 h (data not shown). Because 111In-TF4 and 125I-TF4 had similar immunoreactivity, the ~5-fold higher levels of 111In-TF4 in the tumor at 24 h compared with 125I-TF4 suggest that a sizable portion of TF4 delivered to the tumor (approximately one fifth) likely was processed.

Despite a molecular size similar to IgG, the concentration of 111In-TF4 in the blood at 24 h was ~2.8-fold lower than 111In-hA20 IgG (6.4 ± 1.1% versus 17.6 ± 1.5% ID/g, respectively; Table 1). Tumor uptake for 111In-TF4 and hA20 IgG was similar at each interval, but unlike 111In-TF4, 111In-hA20 IgG uptake in the three major organs decreased at 24 h from measurements made at 2 h. This suggests that IgG largely stays in the blood, whereas TF4 is removed and processed by the major organs over the first 24 h. This rapid blood removal affects maximum tumor accretion. For example, tumor uptake for 111In-hA20 IgG increased to 10.4 ± 2.3% ID/g at 72 h, but 111In-TF4 levels decreased to 3.4 ± 1.0% ID/g at this time.

Optimizing TF4-pretargeted 111In-hapten-peptide. Prior experience indicated that the radiolabeled hapten-peptide injection should be delayed until the bsMAB blood concentration is <1.0% ID/g (41). Initial studies examined increasing amounts of TF4 given at 29 or 39 h before the injection of a fixed amount of 111In-IMP-288. Figure 2 shows the uptake of 111In-IMP-288 in the

Table 1. Biodistribution (% ID/g) of radiolabeled TF4 and hA20 IgG in nude mice bearing s.c. Ramos tumors

<table>
<thead>
<tr>
<th></th>
<th>125I-TF4 (80 μg, 3.5 μCi)</th>
<th>111In-TF4 (50 μg, 20 μCi)</th>
<th>111In-hA20 IgG (50 μg, 20 μCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Ramos</td>
<td>1.7 ± 0.6</td>
<td>2.7 ± 0.4</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>7.4 ± 0.9</td>
<td>4.1 ± 0.7</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.4 ± 1.2</td>
<td>5.4 ± 1.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>16.5 ± 2.8</td>
<td>5.7 ± 1.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Lungs</td>
<td>11.2 ± 1.1</td>
<td>6.3 ± 1.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Blood</td>
<td>41.0 ± 2.4</td>
<td>17.1 ± 3.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>0.23 ± 0.06</td>
<td>0.44 ± 0.13</td>
<td>0.42 ± 0.18</td>
</tr>
</tbody>
</table>


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tumor, blood, liver, and kidneys 3 h after the injection of IMP-288 under each of these conditions. At the 39-h interval, tumor uptake increased as the bsMAb dose increased (i.e., increasing bsMAb to IMP-288 molar ratios) to a maximum of 9.1 ± 1.6% ID/g at a 20:1 ratio (80 µg or 0.51 nmol TF4). Blood concentrations of IMP-288 also increased, but at the 20:1 ratio, there was only 0.05 ± 0.03% ID/g, resulting in tumor to blood ratios >200:1 at 3 h after injection. When the interval was reduced to 29 h, a 20:1 TF4 to IMP-288 molar ratio again provided the highest tumor uptake (9.7 ± 2.4% ID/g), and whereas blood concentration increased to 0.34 ± 0.29% ID/g, tumor to blood ratios still averaged 30:1 at 3 h. Increasing the bsMAb dose to 40:1 did not improve tumor uptake but substantially increased blood concentrations (1.92 ± 0.96% ID/g), which reduced tumor to blood ratios to ~5:1. Higher liver uptake was associated with higher levels of peptide in the blood and could reflect the clearance of bsMAb-hapten-peptide complexes. Kidney uptake was less affected by changing pretargeting conditions, although there was a trend toward slightly higher accretion at the 29-h interval compared with the 39-h interval and when a 40:1 molar ratio was used.

These data suggest that a 20:1 TF4 to IMP-288 molar ratio provides the highest tumor uptake when used with a 29- or 39-h interval. Although delaying the injection of the radiolabeled hapten-peptide for 39 h yields higher tumor to nontumor ratios, previous experience suggested that the hapten-peptide should be injected at the earliest opportunity to ensure optimal tumor retention over time (41). An evaluation of tumor retention over 48 h, tumor uptake decreased 59%, 51%, and 45%, respectively (Supplementary Fig. S2). Higher concentrations in the liver and blood also occurred as the ratio increases, but kidney uptake was relatively unaffected. Comparisons of the individual tissue AUC, as well as AUC tumor to tissue ratios over 48 h, confirmed that a 20:1 molar ratio provided the highest tumor accretion and significantly improved tumor to blood ratios (all P values ≤0.02), thereby justifying the selection of a 20:1 ratio at the 29-h interval for the best pretargeting results.

A comparison was made of 111In-IMP-288 targeting by the recombinant TF4 bsMAb, which has two binding arms for CD20, to the previously used hA20 Fab × m679 Fab chemical conjugate, which binds monovalently to CD20. Each system required different conditions for optimal pretargeting. For TF4, a 20:1 molar ratio and a 29-h interval were used, whereas for the chemical conjugate the optimal pretargeting conditions had previously been determined to be 10:1 bsMAb to peptide ratio, with a 48-h interval (32). With TF4, tumor uptake of 111In-IMP-288 at 24 h was 13.0 ± 4.6% ID/g with blood concentrations of only 0.02 ± 0.01% ID/g, whereas with the chemical conjugate tumor uptake was 5.1 ± 0.9% ID/g with 0.32 ± 0.14% ID/g in the blood. Increasing the protein dose of the chemical conjugate to a 20:1 molar ratio with the 48-h interval elevated tumor uptake to the same level as TF4 (13.1 ± 0.5% ID/g) but at the expense of higher activity in the blood (1.8 ± 0.7% ID/g). 111In-IMP-288 was also more stably bound in the tumor pretargeted with TF4, with tumor uptake decreasing to 8.7 ± 1.1% ID/g at 48 h (33% decrease) compared with a 50% decrease when pretargeted with the chemical conjugate (2.6 ± 0.5% ID/g at 48 h).

Biodistribution studies performed over 5 days were used to assess the therapeutic prospects of 111In-hA20 IgG and the TF4-pretargeted 111In-IMP-288, with Fig. 3 showing the data in terms of the biological (% ID/g) and the effective uptake (µCi/g;
complete % ID/g and tumor/nontumor values given in Supplementary Table S1). Measurements of whole-body radioactivity showed that 90% of the radiolabeled peptide cleared within 4 h, whereas 90Y-hA20 IgG clearance mimicked the physical decay of 90Y, with <5% of the total injected dose being removed from the body at 48 h (Supplementary Fig. S3). For the hA20 IgG, tumor uptake progressed gradually, reaching a peak by day 5 of 11.1 ± 3.0 % ID/g, but blood clearance was slow, with tumor to blood ratios remaining ≤1 until 5 days after injection. In contrast, tumor uptake with the pretargeted 111In-IMP-288 achieved a maximum level of 10.9 ± 1.5% ID/g at 3 h and then decreased over time, with a biological half-life of ~48 h. In the pretargeted animals, the concentration in the blood at 3 h was 55-fold less than the 90Y-IgG, decreasing to >500-fold less at 24 h. The pretargeted 111In-IMP-288 levels in the kidneys and liver also were substantially lower than the 111In-hA20 IgG, with a somewhat faster rate of clearance.

The effective clearance data shown in Fig. 3 assume a maximum dose of 0.15 and 0.7 mCi for the 90Y-hA20 IgG and pretargeted 90Y-IMP-288, respectively, based on toxicity data (see below). The pretargeting method delivered 10 to 100 times more radioactivity to the tumor in the first 24 h compared with directly radiolabeled anti-CD20 IgG and continued to have higher levels in the tumor over 72 h. AUC analysis of the effective curves for the tumors showed a slight advantage for the 90Y-hA20 IgG over the TF4-pretargeted 90Y-IMP-288 (3,932 versus 3,641 A Ci/g/h, respectively). Tumor to blood AUC ratios were 71:1 for the pretargeted procedure compared with 1.6:1 for the anti-CD20 IgG. These data indicate that pretargeting is at least as effective as directly radiolabeled IgG but with much less hematologic toxicity.

Toxicity. Severe (≥90%) reductions in WBCs occurred irrespective of the 90Y-hA20 IgG dose administered, with the nadir lasting 1 to 2 weeks and requiring 7 to 8 weeks for full recovery (Supplementary Fig. S4). Lymphocytes and granulocytes experienced similar losses (data not shown). One animal given 0.15 mCi of 90Y-hA20 IgG was removed at 2.9 weeks because of severe body weight loss (>20%). Animals receiving the pretargeted 90Y-IMP-288 experienced a moderate (≤60%) decrease in WBC and had a more rapid recovery period, lasting no more than 4 weeks, depending on the dose administered. No animals experienced severe body weight loss. Previously, we defined the maximum tolerated dose for a different pretargeted 90Y hapten-peptide in nude mice at 0.7 mCi, with dose-limiting renal toxicity seen at 0.9 mCi (28), and therefore elected not to escalate further.

Efficacy. Supplementary Figure S5 illustrates the growth curves obtained in one of the studies (Study D; Table 2) from which Kaplan-Meier survival curves were generated (Fig. 4). The s.c. Ramos tumors progressed rapidly in the untreated animals, reaching ≥3.0 cm³ in ~1 week (Table 2). Of the 55 untreated tumors examined, 4 failed to progress to ≥3.0 cm³ (7.2%) in three different studies. Three of them doubled in size before completely regressing and one failed to grow from its starting size of 0.53 cm³. Although the two control groups in Study F that received 0.5 mCi of the 90Y-IMP-288 (alone or pretargeted with the irrelevant TF2 anti-CEA bsMab) had a statistically improved survival compared with their untreated group [by Kaplan-Meier (Table 2) and Wilcoxon analysis (Supplementary Table S2)], the overall survival was increased by <1.0 week, and there were no cures. Unlabeled IMP-288 pretargeted with TF4 did not improve survival compared with their untreated group [by Kaplan-Meier (Table 2) and Wilcoxon analysis (Supplementary Table S2)].
with the untreated group by Kaplan-Meier analysis (Study C). In contrast, all TF4 pretargeting doses of 90Y-IMP-288 gave highly significant survival extension but more importantly resulted in cures for ≥4 months. Four of 10 animals given just 0.15 mCi of TF4-pretargeted 90Y-IMP-288 were tumor-free 3 months after treatment, but one of these animals was removed at 12.5 weeks due to a nontreatment-related prolapsed rectum (Study B), leaving five other animals tumor-free at the end of study. For example, in Study A, 8 of 11 animals (72.7%) were cured with a pretargeting dose of 0.25 mCi 90Y-IMP-288 when the median tumor size was 0.24 cm³, but this was reduced to 5 of 9 (55%) when the median tumor size was 0.51 cm³ (Study C). A similar effect was observed in animal given 0.5 mCi of the TF4-pretargeted 90Y-IMP-288 when the tumors were larger at the onset of treatment (Study C versus D). At 0.7 mCi, 90% of the animals bearing tumors with a median size of 0.9 cm³ were cured with 22.5 weeks of follow-up. In Study C, despite the complete ablation of the 0.61-cm³ s.c. tumor of one animal within 2.0 weeks, the animal was removed at week 16 because of hind leg paralysis attributed to spread of tumor in the body cavity occurred. Five other animals were tumor-free at the end of study.

### Table 2. Summary of treatment outcome in six studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>Initial tumor size (cm³)</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
<th>Weeks follow-up*</th>
<th>Median survival† (wk)</th>
<th>No. cures (%) at end of study</th>
<th>P vs untreated</th>
<th>Treatment-related toxicity (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Untreated</td>
<td></td>
<td>11</td>
<td>0.39 ± 0.32</td>
<td>0.27</td>
<td>0.08–1.04</td>
<td>17.5</td>
<td>1.2</td>
<td>1/11 (9.1%)</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>TF4 pretarget (0.25 mCi)</td>
<td></td>
<td>11</td>
<td>0.51 ± 0.64</td>
<td>0.24</td>
<td>0.08–2.18</td>
<td></td>
<td>Undefined</td>
<td>8/11 (72.7%)</td>
<td>0.0009</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Untreated</td>
<td></td>
<td>10</td>
<td>0.61 ± 0.14</td>
<td>0.58</td>
<td>0.44–0.80</td>
<td>16.5</td>
<td>1.0</td>
<td>0/10 (0%)</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>TF4 pretarget (0.15 mCi)</td>
<td></td>
<td>10</td>
<td>0.58 ± 0.14</td>
<td>0.54</td>
<td>0.41–0.82</td>
<td></td>
<td>3.5</td>
<td>3/9 (33%)</td>
<td>&lt;0.0001</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>Untreated</td>
<td></td>
<td>10</td>
<td>0.58 ± 0.09</td>
<td>0.56</td>
<td>0.40–0.69</td>
<td>20.4</td>
<td>1.5</td>
<td>1/10 (10%)</td>
<td>—</td>
<td>NA</td>
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<tr>
<td></td>
<td>TF4 + cold peptide</td>
<td></td>
<td>10</td>
<td>0.79 ± 0.28</td>
<td>0.79</td>
<td>0.25–1.18</td>
<td></td>
<td>1.2</td>
<td>0/10 (10%)</td>
<td>NS</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TF4 pretarget (0.25 mCi)</td>
<td></td>
<td>10</td>
<td>0.55 ± 0.27</td>
<td>0.51</td>
<td>0.11–1.06</td>
<td></td>
<td>Undefined</td>
<td>5/9 (55.5%)</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>TF4 pretarget (0.5 mCi)</td>
<td></td>
<td>10</td>
<td>0.63 ± 0.25</td>
<td>0.51</td>
<td>0.39–1.17</td>
<td></td>
<td>9/10 (90%)</td>
<td>&lt;0.0001</td>
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<tr>
<td>D</td>
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<td></td>
<td>10</td>
<td>0.86 ± 0.33</td>
<td>0.95</td>
<td>0.15–1.25</td>
<td>22.5</td>
<td>1.1</td>
<td>0/10</td>
<td>—</td>
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<tr>
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<td>TF4 pretarget (0.5 mCi)</td>
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<td>10</td>
<td>0.84 ± 0.20</td>
<td>0.90</td>
<td>0.48–1.11</td>
<td></td>
<td>Undefined</td>
<td>5/10 (50%)</td>
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<td>TF4 pretarget (0.7 mCi)</td>
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<td>0.83 ± 0.23</td>
<td>0.90</td>
<td>0.45–1.16</td>
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<td>9/10 (90%)</td>
<td>&lt;0.0001</td>
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<td>IgG (0.150 mCi)</td>
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<td>10</td>
<td>0.90 ± 0.15</td>
<td>0.92</td>
<td>0.65–1.14</td>
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<td>3.1</td>
<td>0/10</td>
<td>&lt;0.0001</td>
<td>2/10</td>
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<td></td>
<td>IgG (0.175 mCi)</td>
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<td>0.94 ± 0.14</td>
<td>0.95</td>
<td>0.73–1.16</td>
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<td>4.2</td>
<td>0/10</td>
<td>&lt;0.0001</td>
<td>6/10</td>
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<td>E</td>
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<td>9</td>
<td>0.59 ± 0.21</td>
<td>0.53</td>
<td>0.41–1.12</td>
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<td>5.0</td>
<td>1.2</td>
<td>2/10</td>
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<td>IgG (0.15 mCi)</td>
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<td>0.68 ± 0.14</td>
<td>0.68</td>
<td>0.51–0.92</td>
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<td>3.6</td>
<td>0/10</td>
<td>0.0007</td>
<td>6/10</td>
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<tr>
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<td>Untreated</td>
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<td>5</td>
<td>0.63 ± 0.22</td>
<td>0.58</td>
<td>0.45–1.01</td>
<td>2.0</td>
<td>0.9</td>
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<tr>
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<td>90Y-IMP-288 (0.5 mCi)</td>
<td></td>
<td>10</td>
<td>0.57 ± 0.17</td>
<td>0.54</td>
<td>0.40–0.89</td>
<td></td>
<td>1.7</td>
<td>0/10</td>
<td>0.0042</td>
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<tr>
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<td>TF2 pretarget (0.5 mCi)</td>
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<td>10</td>
<td>0.76 ± 0.24</td>
<td>0.71</td>
<td>0.46–1.19</td>
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<td>1.1</td>
<td>0/10</td>
<td>0.0128</td>
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</table>

Abbreviations: NA, not available; NS, not significant.

* Study terminated.
† Weeks from the treatment to the time an animal was removed from study because tumor progressed to ≥3 cm³ or toxicity.
‡ Spontaneous regression of established, untreated tumors was observed in four animals in three studies. Three of the four tumors doubled in size before regressing, and the other failed to progress from its initial size of 0.53 cm³.
§ Unrelated; one animal that was tumor-free was removed at week 12.5 because of prolapsed rectum; three other animals were tumor-free at the end of study.
¶ Unrelated; s.c. tumor in one animal was ablated, but on week 16, hind leg paralysis attributed to spread of tumor in the body cavity occurred. Five other animals were tumor-free at the end of study.

""
Fig. S5). In addition, ≥20% loss in body weight occurred in several animals, forcing their removal in <3 weeks. For example, in Study D, two of the animals given 0.15 mCi of $^{90}$Y-hA20 IgG were removed within 1 week because of >20% loss in body weight, but their tumors were regressing at the time. Six animals given 0.175 mCi of $^{90}$Y-hA20 IgG had to be removed because of excessive weight loss. Tumors had completely regressed in three of these animals, another two were regressing, and one had progressed after a short response. In contrast, none of the animals given the pretargeted $^{90}$Y-IMP-288 was removed from the study due to a treatment-related toxicity (i.e., their body weight losses were ≤20%).

Discussion

Pretargeting approaches for radionuclide therapy reduce hematologic toxicity by separating the radioisotope delivery from the antibody-targeting step (23, 34). The radionuclide is administered on a small molecule that escapes from the blood quickly into the extravascular compartment, where it binds rapidly to the pretargeted antibody, and then just as quickly is cleared from the body. In the model presented, only 17.9 ± 2.7% of the injected activity remained in the mice 3 h after injection of the radiolabeled peptide, whereas with the directly labeled anti-CD20 IgG 47.3 ± 4.5% was in the body after 3 days. This is similar to clinical results with the $^{90}$Y-anti-CD20 IgG ibritumomab where the vast amount of radioactivity remains in the body (8). The rapid clearance permits higher levels of radioactivity to be given with pretargeting, and the efficient capturing in the tumor translates to radioactivity levels in the tumor for pretargeting to often equal or higher levels than delivered by a directly radiolabeled IgG (24, 34). Because most of the radioactivity is removed by urinary excretion, renal toxicity is expected to be dose limiting, which has been confirmed in animal and clinical studies (28, 42). Kidney uptake in mice is only ~2% ID/g 3 h after injection and declines by ~40% each day thereafter. This is significantly lower than antibody fragments or small molecular weight recombinant antibodies directly labeled with a radiometal (43). Previously, we found 0.7 mCi to be the maximum tolerated dose in mice (28) and therefore limited dose escalation in this study to this level. However, we have observed 30% lower renal uptake with $^{111}$In-IMP-288 than with $^{111}$In-IMP-241 that was used in the earlier study, which might allow for additional escalation. Although a reassessment of the maximum tolerated dose is being made, at least in this model, the current dose levels provided exceptional antitumor responses, resulting in cures in the majority of the animals even at relatively low radiation amounts given by pretargeting as single doses. Repeated dose cycles, as may be desired in patients, may provide even better efficacy results and need to be examined.

The effective AUC for tumor uptake measured over 5 days suggested that the $^{90}$Y-hA20 IgG was somewhat higher than the pretargeted $^{90}$Y-peptide at their respective maximum doses. This estimate reflected an extrapolation over an infinite time. Because IgG uptake in the tumor was slow, with essentially a flat slope at the end of the 5-day monitoring period, the level of radioactivity in
the tumor over the remaining time was based mostly on the physical decay of $^{90}$Y. However, the dose rate of the radiation exposure at these later times would be much lower than that delivered earlier. In this regard, the effective AUC over the first 5 days clearly favored the pretargeted $^{90}$Y-peptide, with uptake being 10- to 100-fold higher than with IgG within the first 24 h. The therapeutic results substantiated this finding, with the pretargeted radiopeptide curing at least 40% of the animals at doses far less than the maximum tolerated level, whereas $^{90}$Y-anti-CD20 IgG was less effective and more toxic based on body weight loss and WBC reduction. Thus, although the extrapolated AUCs for the tumor with both procedures were similar, the more rapid and higher amount of radioactivity delivered to the tumor clearly translated into significantly improved antitumor responses for pretargeting but without the severe hematologic toxicity observed with the $^{90}$Y-IgG.

The superiority of this pretargeting procedure using a DNL-prepared tri-Fab bsMAb against CD20 is appreciated by comparing its tumor accretion and tumor to blood ratios with those of the bivalent chemical conjugate and the directly labeled anti-CD20 IgG. At 24 h, TF4 improved tumor uptake of $^{111}$In-HSG-peptide 2.6-fold (13% versus 5% ID/g) and enhanced tumor to blood ratios >45-fold (770 versus 17), compared with an anti-CD20 Fab × anti-HSG Fab chemical conjugate, and by 1.6-fold (9.0% versus 5.6% ID/g) and 1,600-fold (522 versus 0.32), respectively, compared with radiolaabeled anti-CD20 IgG. The 157-kDa TF4 clears very quickly in mice for its size. With such rapid clearance, there is no need for a clearing agent in mice. Because this construct lacks the Fc portion of an immunoglobulin that is responsible for the longevity of IgG in the blood (44), the pharmacokinetic behavior of this construct in humans is uncertain.

The other pretargeting procedure based on streptavidin and using anti-CD20 and antibodies against other targets associated with lymphoma also has been used successfully in preclinical models to improve therapeutic responses when compared with a directly radiolabeled antibodies (29–31, 34, 35). The published results from these methods compare favorably with those reported herein. For example, maximum uptake in Ramos xenografts reported for $^{111}$In-biotin using a chemical conjugate of the 1F5 anti-CD20 IgG coupled to streptavidin was ~13% ID/g, holding relatively steady over 24 h (31). However, using the same pretargeting system and tumor model, Subbiah and colleagues reported tumor uptake ~5% ID/g at 24 h (33). Tumor to blood ratios were 3:1 to 5:1 for this conjugate at 24 h, whereas we have optimized our system for the highest possible tumor to blood ratios, achieving a tumor uptake of ~10% ID/g with tumor to blood ratios of 20:1 at 3 h and >500:1 at 24 h. Later studies with an anti-CD20 × streptavidin fusion protein showed similar uptake in Ramos xenografts but with higher tumor to blood ratios (~50:1) than reported for the chemical conjugate 24 h after the labeled biotin was injected (45). Pilot clinical studies also were performed with a streptavidin construct that indicated that some patients developed an anti-streptavidin antibody response (46). Only a single dose of 15 mCi/m$^2$ of the $^{90}$Y-biotin was given under varying conditions to assess targeting, and although two patients experienced grade 3/4 hematologic toxicity, there were confounding factors involved (multiple chemotherapy regimens or bone marrow involvement). Otherwise, no hematologic toxicity occurred in 11 of 14 patients. In contrast to the bsMAB pretargeting method described herein, other approaches based on streptavidin conjugates/constructs have required a clearing step to ensure adequate removal from the blood. Our bsMAB method simply delays so that the primary targeting construct can clear sufficiently from the blood, administering the radiolabeled hapten-peptide later with less concern for the formation of stable complexes of the radiolabeled product that would otherwise form with biotin/streptavidin. Indeed, the basis for the affinity enhancement system used by this pretargeting procedure is that the binding of the radiolabeled divalent hapten to the bsMAB in the tumor is far stronger than to the bsMAB in the blood (47). However, the primary incentive for using a bsMAB pretargeting approach over one that uses streptavidin is because streptavidin has been proven to be quite immunogenic, whereas exclusive use of humanized bsMAb should significantly reduce immunogenicity.

We reported previously improved antitumor responses using a monovalent anti-CD20 Fab × anti-HSG Fab chemical conjugate, and here we show with the novel DNL recombinant construct that binding divalently to CD20 improves tumor accretion and retention. The DNL procedure can be used to make several different bispecific constructs that can have as many as four binding sites for the tumor target antigen and two binding sites for a hapten and also has distinct advantages of facile production, high protein yields, simple purification, and flexibility (36). The higher molecular weight of these constructs might extend blood clearance times, or their size might impede migration into the tumor, but this was not found in these studies, where the TF4 cleared faster than IgG and the radiation delivered to tumor was at least as high as directly labeled IgG. Indeed, this rapid clearance from blood while retaining binding to tumor (~11% ID/g) resulted in tumor to blood ratios of >200:1 at 3 h after radiotope injection when optimal conditions were used. At 24 h, TF4 pretargeting resulted in 2.6-fold improved tumor accretion, and 30-fold less blood radioactivity, than achieved with the bivalent chemical conjugate. Thus, we believe that the current divalent × monovalent tri-Fab construct used in combination with a divalent hapten is advantageously suited for clinical development.

Several studies have indicated that cells bound with an anti-CD20 IgG can be killed by apoptosis, particularly when the antibody is cross-linked with a secondary antibody or other cross-linking agents (48, 49). In these studies, however, mice treated with a single dose of TF4 followed by unlabeled IMP-288 had progressive growth similar to the untreated animals, indicating that the delivery of radiation to the tumor was the primary mechanism responsible for the responses seen.

We appreciate that pretargeting is more complex than conventional one-step RAIT and thus may experience even more resistance than the current practice of RAIT in NHL therapy. However, if higher radiation doses can be delivered to tumors selectively, with truly superior tumor to nontumor ratios, resulting in lower myelosuppression and other organ toxicities, then it may offer truly substantive therapeutic advantages over current RAIT, including the prospect of attaching radionuclides with other energies of possible interest.

In conclusion, a novel recombinant bsMAB construct divalently binding to CD20 and having specificity for a unique hapten, HSG, has shown improved binding over a monovalent bsMAB chemical conjugate, with the ability to significantly improve antitumor response with a >40% cure rate compared the directly radiolabeled anti-CD20 IgG, which was not curative. Reducing the risk of hematologic toxicity might allow pretargeting approaches to be
used along with or even combined with chemotherapy regimens. Thus, the improved therapeutic index of this procedure warrants further investigation.

Acknowledgments

References

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Robert M. Sharkey, Habibe Karacay, Samuel Litwin, et al.


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