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Improved Therapy by Pretargeted Radioimmunotherapy of Non-Hodgkin 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-DOTA-peptide could reduce hematological toxicity yet improve therapeutic responses, compared to conventional ⁹⁰Y-anti-CD20 IgG and a chemically-conjugated bsMAb. TF4, a humanized, tri-Fab, bsMAb with 2 Fabs binding CD20 and 1 Fab binding HSG (histamine-succinyl-glycine), 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 kidneys. At 24 h, TF4 improved tumor uptake of ¹¹¹In-HSG-peptide 2.6-fold (13% vs 5% injected-dose/g) and enhanced tumor/blood ratios more than 45-fold (770 vs 17), compared to an anti-CD20 Fab x anti-HSG Fab chemical conjugate, and by 1.6-fold (9.0% vs 5.6% injected-dose/g) and 1600-fold (522 vs 0.32), respectively, compared to radiolabeled anti-CD20 IgG. A severe (≥90%) and prolonged reduction of white blood cells 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 33-90% of the animals, even at relatively low doses, while most tumors progressed quickly without cures with ⁹⁰Y-anti-CD20 IgG. These results indicate an improved therapeutic index with pretargeting radioimmunotherapy using a DNL-constructed tri-Fab, bsMAb, as compared to conventional therapy with directly-radiolabeled antibody or with a chemically-conjugated bsMAb. These encouraging results prompt testing of these constructs for pretargeting radioimmunotherapy in patients.

Keywords

Bispecific antibody; non-Hodgkin lymphoma; pretargeting; radioimmunotherapy

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Introduction

Antibody-targeted therapies have gained an important role in hematological malignancies, with the anti-CD20 IgG, rituximab, having the greatest impact on non-Hodgkin's lymphoma (NHL) treatment, and also in other hematological tumors and even non-malignant conditions (1-6). Although rituximab has gained widespread acceptance, clinical studies have shown that radiolabeled anti-CD20 antibodies, ^{131}I -tositumomab and ^{90}Y -ibritumomab tiuxetan, have a higher objective response rate, yet these treatments are prescribed infrequently (7-9). A number of 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 hematological 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 hematological toxicity (22,23). Pretargeting systems show less hematological toxicity and improved efficacy in animal model systems, including solid and hematological 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, veltzumab (Immunomedics, Inc., Morris Plains, NJ), and a Fab' of an anti-hapten antibody, we showed improved efficacy by pretargeting a ^{90}Y -hapten-peptide over that of the ^{90}Y -anti-CD20 IgG (32). Recently, we reported a novel method of producing fusion proteins, called the "Dock-and-Lock" (DNL) procedure, that can be used to prepare recombinant, humanized, bsMAbs composed of 2 Fabs against a tumor antigen tethered to a Fab fragment of the anti-peptide/hapten antibody, as well as preparing a number of different multivalent mono- 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 HSG (histamine-succinyl-glycine) hapten-capture that together improve tumor uptake and the therapeutic index when compared to a monovalent CD20-binding, Fab' x 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 (PKA) and the anchoring domain (AD) in a reactive A-kinase anchoring protein (AKAP) (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 linked covalently 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 DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) chelate, is provided in Figure 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-D-Tyr-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂; MH⁺, 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 on line 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 was purchased from American Type Culture Collection (Manassas, VA), and grown in roller bottle cultures to a concentration of 1×10^6 before harvesting for s.c. implantation (1×10^7 cells) in 6-8-week old female BALB/c nude mice (AnNCr-nu/nu; NCI/CRL, Fredrick, MD). Within 17-19 days, 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 the Results. All agents were administered intravenously (≤ 0.2 mL). Trace amounts of the radiolabeled antibodies were supplemented with unconjugated antibody to achieve the prescribed dose (50 μ g for hA20 IgG, but for pretargeting, the bsMAb and IMP-288 doses were adjusted to yield a desired mole ratio). For therapy studies, the nmoles of IMP-288 were based on the radioactivity given, with the TF4 or control TF2 protein dose adjusted to give a final mole ratio of 20:1.

The pretargeting capability of TF4, which binds divalently to CD20, was compared to pretargeting with a hA20 Fab x m679 Fab chemical conjugate that binds CD20 monovalently. Both constructs have monovalent binding to HSG. Ramos-bearing nude mice were given 30 or 101 μ g (0.3 to 1.0 nmole) of the chemical conjugate or 118 μ g (1.0 nmole) of TF4, and 48 or 29 h later, respectively, 0.03 to 0.05 nmole (~ 30 μ Ci) of the ¹¹¹In-IMP-288 was administered. At 24 and 48 h after the ¹¹¹In-IMP-288 injection, groups of 5 mice were necropsied.

Prior to 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/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 (μ Ci/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 effective curve's terminal phase was used to estimate activity remaining in the tumor to an infinite time. The Wilcoxon 2-sample test (one-sided) was used to compare AUCs across treatment groups, as well as ratios of the AUC for the tumor and tissues.

Hematological toxicity was assessed in non-tumor-bearing, 8-week-old, female, NIH-Swiss mice. All animals were pre-bled 1 day in advance of the treatment, and then weekly thereafter. After local proparacaine anesthesia, blood (~ 70 μ L) was taken retro-orbitally and added to a red blood cell lysing buffer. The cell pellet was washed and diluted in phosphate-buffered

saline-10% formalin for flow cytometry analysis (FACSCalibur®, BD Biosciences, San Jose, CA) using preset scatter windows to discriminate mouse lymphocytes and granulocytes, as well as the complete white blood cell count (WBC). The percent change in each animal's WBCs from its baseline determination was calculated and averaged (N = 5 per treatment). Treatments consisted of an intravenous 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 mole 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 non-radiolabeled 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 crosslinking TF4 on the cell surface (39). All syringes were read for ⁹⁰Y-activity in a calibrated dose calibrator (Capintec CRC-15R Ramsey, NJ). Groups of 5 animals were housed in filtered caging units. Bedding was changed within 3-4 hours after injection of ⁹⁰Y-IMP-288 and then again the next day to remove excreted activity. Whole-body clearance was monitored by measuring the animals in the dose calibrator at 3, 24, and 48 h. Readings also were made on a syringe containing each injected dose. Tumors were measured 2 to 3 times a week using a caliper in 3 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 if (a) tumor size exceeded 3.0 cm³, (b) their body weight decreased by more than 20% of their baseline weight, or (c) 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 non-treatment-related toxicity were censored. Analysis was performed from Kaplan-Meier survival curves using the log rank test (Prism Graphpad version 4.0, San Diego, CA). Additionally, tumor quadrupling times were calculated and analyzed using a Wilcoxon two-sample comparison. For this analysis, the time required for tumors to reach 4-times the initials cm³ size was determined from the growth curves. For humane reasons, animals were not allowed to bear tumors >3.0 cm³, and because a number of 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

SE-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). SE-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 demonstrated that the product consisted solely of the constituent TF4 polypeptides (h679-Fd-AD2, hA20-Fd-DDD2 and kappa light chains) (Supplementary Fig S1C). Bispecific binding involving all three Fabs was demonstrated by plasmon resonance (Biacore, GE Healthcare, Piscataway, NJ) (Supplementary Fig. S1D).

TF4 biodistribution in tumor-bearing nude mice

^{125}I -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 x anti-HSG DNL bsMAb (35).

TF4 biodistribution was also assessed using ^{111}In -labeled TF4. ^{111}In is sequestered in tissues, thereby providing a better estimate of the total amount of protein delivered over time, while radioiodine is released, providing a better estimate of the amount of product that potentially is accessible (i.e., not internalized) (40). At 3 h, tumor and tissue uptake with ^{111}In -TF4 distributed in a proportional manner, similar to that of ^{125}I -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, ^{111}In -TF4 uptake in the tumor and normal tissues increased, while ^{125}I -TF4 decreased more than 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 was processed in the liver. The ^{111}In -TF4 concentration in the blood at 24 h was surprisingly 9-times higher than ^{125}I -TF4. Size-exclusion HPLC of serum from mice given ^{111}In -TF4 showed no evidence of dissociation or catabolic by-products at 1, 3, or 24 h (not shown). Because ^{111}In - and ^{125}I -TF4 had similar immunoreactivity, the ~5-fold higher levels of ^{111}In -TF4 in the tumor at 24 h as compared to ^{125}I -TF4 suggest that a sizable portion of TF4 delivered to the tumor (approximate one-fifth) likely was processed.

Despite a molecular size similar to IgG, ^{111}In -TF4's concentration in the blood at 24 h was nearly 2.8-fold lower than ^{111}In -hA20 IgG (6.4 ± 1.1 vs. $17.6 \pm 1.5\%$ ID/g, respectively; Table 1). Tumor uptake for ^{111}In -TF4 and hA20 IgG was similar at each interval, but unlike ^{111}In -TF4, ^{111}In -hA20 IgG uptake in the 3 major organs decreased at 24 h from measurements made at 2 h. This suggests that IgG largely remains in the blood, while 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 ^{111}In -hA20 IgG increased to $10.4 \pm 2.3\%$ ID/g at 72 h, but ^{111}In -TF4 levels decreased to $3.4 \pm 1.0\%$ ID/g at this time.

Optimizing TF4-pretargeted ^{111}In -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 prior to the injection of a fixed amount of ^{111}In -IMP-288. Figure 2 shows the uptake of ^{111}In -IMP-288 in the tumor, blood, liver and kidneys 3 h after IMP-288's injection under each of these conditions. At the 39-h interval, tumor uptake increased as the bsMAb dose increased (i.e., increasing bsMAb/IMP-288 mole ratios) to a maximum of $9.1 \pm 1.6\%$ ID/g at a 20:1 ratio (80 μg or 0.51 nmoles TF4). Blood concentrations of IMP-288 also increased, but at the 20:1 ratio, there was only $0.05 \pm 0.03\%$ ID/g, resulting in tumor/blood ratios >200:1 at 3 h post-injection. When the interval was reduced to 29 h, a 20:1 TF4/IMP-288 mole ratio again provided the highest tumor uptake ($9.7 \pm 2.4\%$ ID/g), and while blood concentration increased to $0.34 \pm 0.29\%$ ID/g, tumor/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 ($1.92 \pm 0.96\%$ ID/g), which reduced tumor/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 to the 39-h interval, and when a 40:1 mole ratio was used.

These data suggest that a 20:1 TF4/IMP-288 mole ratio provides the highest tumor uptake when used with a 29- or 39-h interval. While delaying the injection of the radiolabeled hapten-

peptide for 39 h yields higher tumor/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 in animals given 120 μ g of TF4, followed 29 h later with decreasing amounts of ^{111}In -IMP-288 to create TF4/IMP-288 mole ratios of 5:1, 10:1, and 20:1, showed proportionally higher tumor uptake as the TF4/IMP-288 mole ratio increased (at 3 h, $3.9 \pm 0.5\%$, 6.9 ± 1.7 , and $10.6 \pm 2.0\%$ ID/g, respectively), but by 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/tissue ratios over 48 h, confirmed that a 20:1 mole ratio provided the highest tumor accretion and significantly improved tumor/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 ^{111}In -IMP-288 targeting by the recombinant TF4 bsMAb, which has 2 binding arms for CD20, to the previously used hA20 Fab x m679 Fab chemical conjugate, which binds monovalently to CD20. Each system required different conditions for optimal pretargeting. For TF4, a 20:1 mole ratio and a 29-h interval was used, whereas for the chemical conjugate, the optimal pretargeting conditions had been determined previously to be 10:1 bsMAb/peptide ratio, with a 48-h interval (32). With TF4, tumor uptake of ^{111}In -IMP-288 at 24 h was $13.0 \pm 4.6\%$ ID/g with blood concentrations being only $0.02 \pm 0.01\%$ ID/g, while with the chemical conjugate, tumor uptake was $5.1 \pm 0.9\%$ ID/g with $0.32 \pm 0.14\%$ ID/g in the blood. Increasing the chemical conjugate's protein dose to a 20:1 mole ratio with the 48-h interval elevated tumor uptake to the same level as TF4 ($13.1 \pm 0.5\%$ ID/g), but at the expense of higher activity in the blood ($1.8 \pm 0.7\%$ ID/g). ^{111}In -IMP-288 was also more stably bound in the tumor pretargeted with TF4, with tumor uptake decreasing to $8.7 \pm 1.1\%$ ID/g at 48 h (33% decrease), compared to a 50% decrease when pretargeted with the chemical conjugate ($2.6 \pm 0.5\%$ ID/g at 48 h).

Biodistribution studies performed over 5 days were used to assess the therapeutic prospects of ^{111}In -hA20 IgG and the TF4-pretargeted ^{111}In -IMP-288, with Figure 3 showing the data in terms of the biological (%ID/g) and the effective uptake ($\mu\text{Ci/g}$) (complete % ID/g and tumor/nontumor values are given in Supplementary Table S1). Measurements of whole-body radioactivity showed that 90% of the radiolabeled peptide cleared within 4 h, while ^{90}Y -hA20 IgG clearance mimicked the physical decay of ^{90}Y , 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 $11.1 \pm 3.0\%$ ID/g, but blood clearance was slow, with tumor/blood ratios remaining ≤ 1 until 5 days after injection. In contrast, tumor uptake with the pretargeted ^{111}In -IMP-288 achieved a maximum level of $10.9 \pm 1.5\%$ ID/g at 3 h and then decreased over time, with a biological half-life of about 48 h. In the pretargeted animals, the concentration in the blood at 3 h was 55-fold less than the ^{90}Y -IgG, decreasing to >500 -fold less at 24 h. The pretargeted ^{111}In -IMP-288 levels in the kidneys and liver also were substantially lower than the ^{111}In -hA20 IgG, with a somewhat faster rate of clearance.

The effective clearance data shown in Figure 3 assume a maximum dose of 0.15 mCi and 0.7 mCi for the ^{90}Y -hA20 IgG and pretargeted ^{90}Y -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 ^{90}Y -hA20 IgG over the TF4-pretargeted ^{90}Y -IMP-288 (3932 vs 3641 $\mu\text{Ci/g}\cdot\text{h}$, respectively). Tumor/blood AUC ratios were 71:1 for the pretargeted procedure, compared to 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 hematological toxicity.

Toxicity

Severe ($\geq 90\%$) reductions in WBCs occurred irrespective of the ^{90}Y -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 (not shown). One animal given 0.15 mCi of ^{90}Y -hA20 IgG was removed at 2.9 weeks because of severe body weight loss ($>20\%$). Animals receiving the pretargeted ^{90}Y -IMP-288 experienced a moderate ($\leq 60\%$) 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 ^{90}Y -hA20-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 $\geq 3.0 \text{ cm}^3$ in ~ 1 week (Table 2). Of the 55 untreated tumors examined, 4 failed to progress to $\geq 3.0 \text{ cm}^3$ (7.2%) in 3 different studies. Three of them doubled in size before completely regressing, and 1 failed to grow from its starting size of 0.53 cm^3 . While the 2 control groups in Study F that received 0.5 mCi of the ^{90}Y -IMP-288 (alone or pretargeted with the irrelevant TF2 anti-CEA bsMAb) had a statistically improved survival compared to 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 to the untreated group by Kaplan Meier analysis (Study C). In contrast, all TF4-pretargeting doses of ^{90}Y -IMP-288 gave a 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 ^{90}Y -IMP-288 were tumor-free 3 months after treatment, but one of these animals was removed at 12.5 weeks due to a non-treatment-related prolapsed rectum (Study B), leaving 3/9 (33%) cures at the end of the study. Doses of $\geq 0.25 \text{ mCi}$ ^{90}Y -IMP-288 cured $\sim 50\%$ to 70% of the animals, with median survivals longer than the duration of the studies (i.e., >17.5 weeks). The number of cures increased as the ^{90}Y -IMP-288 increased, but it was also influenced by the median size of the tumors at the onset of treatment. For example, in Study A, 8/11 animals (72.7%) were cured with a pretargeting dose of 0.25 mCi ^{90}Y -IMP-288 when the median tumor size was 0.24 cm^3 , but this was reduced to 5/9 (55%) when the median tumor size was 0.51 cm^3 (Study C). A similar effect was observed in animal given 0.5 mCi of the TF4-pretargeted ^{90}Y -IMP-288 when the tumors were larger at the onset of treatment (Study C vs D). At 0.7 mCi, 90% of the animals bearing tumors with a median size of 0.9 cm^3 were cured with 22.5 weeks of follow-up. In Study C, despite the complete ablation of one animal's 0.61 cm^3 s.c. tumor within 1.7 weeks, the animal was removed at week 16 because of hind-leg paralysis and labored breathing that was subsequently confirmed histologically to be disseminated lymphoma. We speculate that during implantation, tumor cells may have been injected below the subcutaneous layer, allowing it to grow eventually in the body cavity. Given ^{90}Y 's high-beta energy and long pathlength, the microscopic deposits of tumor that might have been present in the body cavity were not effectively controlled, whereas the established s.c. tumor was killed.

Treatment with the ^{90}Y -hA20 IgG was not as effective as the pretargeting RAIT procedure. Most tumors responded initially to the treatment, but many progressed quickly (Supplementary Fig. S5). In addition, $\geq 20\%$ loss in body weight occurred in a number of 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 3 of these animals, another 2 were regressing, and 1 had progressed after a short response. In contrast, none of the animals given the pretargeted ^{90}Y -IMP-288 were removed from the study due to a treatment-related toxicity (i.e., their body weight losses were $\leq 20\%$).

Discussion

Pretargeting approaches for radionuclide therapy reduce hematological toxicity by separating the radioisotope delivery from the antibody-targeting step (22,34). 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 $17.9 \pm 2.7\%$ of the injected activity remained in the mice 3 hours after injection of the radiolabeled peptide, while with the directly labeled anti-CD20 IgG, $47.3 \pm 4.5\%$ was in the body after 3 days. This is similar to clinical results with the ^{90}Y -anti-CD20 IgG, ibritumomab tiuxetan, 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).

Since 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 $\sim 2\%$ ID/g 3 h after injection, and declines by about 40% each day thereafter. This is significantly lower than antibody fragments or small molecular weight recombinant antibodies labeled directly 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 should allow for additional escalation. While a reassessment of the maximum tolerated dose is being made, at least in this model, the current dose levels provided exceptional anti-tumor 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, could 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. Since 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, while ^{90}Y -anti-CD20 IgG was less effective and more toxic, based on body-weight loss and WBC reduction. Thus, while 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 anti-tumor responses for pretargeting, but without the severe hematological 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:blood ratios to 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% vs 5% ID/g) and enhanced tumor/blood ratios more than 45-fold (770 vs 17), compared to an anti-CD20 Fab x anti-HSG Fab chemical conjugate, and by 1.6-fold (9.0% vs 5.6% ID/g) and 1600-fold (522 vs 0.32), respectively, compared to radiolabeled anti-CD20 IgG. The 157-kD 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 IgG's longevity 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 to a directly-radiolabeled antibodies (29-31,33,45). The published results from these methods compare favorably with those reported here. 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 et al. reported tumor uptake about 5% ID/g at 24 h (33). Tumor/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/blood ratios, achieving a tumor uptake of ~10% ID/g with tumor/blood ratios of 20:1 at 3 h and more than 500:1 at 24 h. Later studies with an anti-CD20 x streptavidin fusion protein showed similar uptake in Ramos xenografts, but with higher tumor/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 some patients developed an anti-streptavidin antibody response (46). Only a single dose of 15 mCi/m² of the ^{90}Y -biotin was given under varying conditions to assess targeting, and while 2 patients experienced grade 3/4 hematological toxicity, there were confounding factors involved (multiple chemotherapy regimens or bone marrow involvement). Otherwise, no hematological toxicity occurred in 11/14 patients.

In contrast to the bsMAb pretargeting method described here, other approaches based on streptavidin conjugates/constructs have required a clearing step to ensure adequate removal from the blood. Our bispecific antibody 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 bsMAbs should reduce immunogenicity significantly.

We reported previously improved anti-tumor responses using a monovalent anti-CD20 Fab x 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 a number of different bispecific constructs that can have as many as 4 binding sites for the tumor target antigen and 2 binding sites for a hapten (36), 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:blood ratios of >200:1 at 3 hr post radiopeptide 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 the current divalent x monovalent tri-Fab construct used in combination with a divalent hapten is advantageously suited for clinical development.

A number of studies have indicated that cells bound with an anti-CD20 IgG can be killed by apoptosis, particularly when the antibody is crosslinked with a secondary antibody or other crosslinking 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 observed.

We appreciate that pretargeting is more complex than conventional 1-step radioimmunotherapy, 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: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 anti-tumor responses, with a >40% cure rate as compared the directly radiolabeled anti-CD20 IgG, which was not curative. Reducing the risk of hematological toxicity might permit pretargeting approaches to be used alongside or even combined with chemotherapy regimens. Thus, the improved therapeutic index of this procedure warrants clinical assessment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Diagram depicts the structure of tri-Fab made by DNL method, showing DDD and AD domains bearing the anti-CD20 Fabs and the anti-HSG Fab, respectively. IMP-288's structure shows the single DOTA moiety (red), 2 HSG moieties (blue) and the D-amino acids (black) that compose this hapten-peptide.

Figure 2.

Evaluation of optimal pretargeting conditions. Tumor-bearing nude mice ($N = 5$ per determination) were administered increasing amounts of TF4 (0.125 nmoles to 1.0 nmoles). At 29 or 39 h later, 0.025 nmoles (15 μ Ci) of ^{111}In -IMP-288 was given. Doses are expressed as the TF4 to IMP-288 mole ratio, ranging from 5:1 to 40:1. Values represent means $\pm$ SD. Tumor weights averaged 1.0 g for the animals studied at the 39-h interval and $\sim$ 2.0 g for the animals examined at the 29-h interval.

Figure 3. Comparison of direct and pretargeting methods. The left side-panels show the average biological data (% ID/g; mean $\pm$ SD, $n = 5$) for tumor and normal tissue uptake, and the right-side panels show the effective data (μ Ci/g; error bars not given because they were contained within the symbol). The effective data assume that 0.7 and 0.15 mCi of the pretargeting ^{90}Y -IMP-288 and ^{90}Y -IgG, respectively, were given. (see Supplementary Table S1 in for listing of means $\pm$ SD for % ID/g and tumor/nontumor ratios for the pretargeting and IgG data.)

Figure 4.

Kaplan-Meier survival curves for six separate studies summarized in Table 2. Survival was based on the time required for the tumor to reach the size of $\geq 3.0 \text{ cm}^3$. Wilcoxon two-sample analysis of tumor quadrupling time can be found in Supplementary Data Table S2.

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

	¹²⁵ I-TF4 (80 µg, 3.5 µCi) 6 h	24 h	¹¹¹ In-TF4 (50 µg, 20 µCi) 3 h	¹¹¹ In-hA20 IgG (50 µg, 20 µCi) 2 h	¹¹¹ In-hA20 IgG (50 µg, 20 µCi) 24 h
Ramos	1.7 ± 0.6	1.1 ± 0.3	1.4 ± 0.1	1.1 ± 0.2	5.6 ± 0.5
Liver	7.4 ± 0.9	0.3 ± 0.1	7.0 ± 1.1	8.1 ± 1.7	4.1 ± 0.3
Spleen	8.4 ± 1.2	0.4 ± 0.1	6.2 ± 0.6	5.6 ± 2.2	3.3 ± 0.5
Kidney	16.5 ± 2.8	0.3 ± 0.1	11.4 ± 0.9	13.7 ± 1.5	5.1 ± 0.8
Lungs	11.2 ± 1.1	0.4 ± 0.1	8.7 ± 2.1	10.4 ± 1.2	6.7 ± 1.2
Blood	41.0 ± 2.4	0.7 ± 0.1	28.7 ± 1.8	32.6 ± 2.8	17.6 ± 1.5
N	4	5	5	5	5
Tumor wt (g)	0.23 ± 0.06	0.42 ± 0.18	0.39 ± 0.04	0.96 ± 0.12	0.90 ± 0.09

Table 2

Summary of treatment outcome in six studies

Study	Group	Initial tumor size (cm ³)				Weeks follow-up [*]	Median survival [†] (weeks)	Treatment outcome		
		N	Mean ± SD	Median	Range			# Cures (%) at end of study	P-value vs. untreated	Treatment-related toxicity (#)
A	Untreated	11	0.39 ± 0.32	0.27	0.08 – 1.04	17.5	1.2	1/11 (9.1%) [‡]	-----	NA
	TF4 pretarget - 0.25 mCi	11	0.51 ± 0.64	0.24	0.08 – 2.18		undefined	8/11 (72.7%)	0.0009	0
B	Untreated	10	0.61 ± 0.14	0.58	0.44 – 0.80	16.5	1.0	0/10 (0%)	-----	NA
	TF4 pretarget - 0.15 mCi	10	0.58 ± 0.14	0.54	0.41 – 0.82		3.5	3/9 (33%) [§]	<0.0001	0 [§]
C	Untreated	10	0.58 ± 0.09	0.56	0.40 – 0.69	20.4	1.5	1/10 (10%) [‡]	-----	NA
	TF4 + cold peptide pretarget - 0.25 mCi	10	0.79 ± 0.28	0.79	0.25 – 1.18		1.2	0/10 (10%)	NS	0
D	Untreated	10	0.55 ± 0.27	0.51	0.11 – 1.06	22.5	undefined	5/9 (55.5%) ^{**}	<0.0001	0 ^{**}
	TF4 pretarget - 0.25 mCi	10	0.63 ± 0.25	0.51	0.39 – 1.17		undefined	9/10 (90%)	<0.0001	0
E	Untreated	10	0.86 ± 0.33	0.95	0.15 – 1.25	22.5	1.1	0/10	-----	NA
	TF4 pretarget - 0.5 mCi	10	0.84 ± 0.20	0.90	0.48 – 1.11		undefined	5/10 (50%)	<0.0001	0
F	Untreated	10	0.83 ± 0.23	0.90	0.45 – 1.16	22.5	undefined	9/10 (90%)	<0.0001	0
	TF4 pretarget - 0.7 mCi	10	0.90 ± 0.15	0.92	0.65 – 1.14		3.1	0/10	<0.0001	2/10
G	Untreated	10	0.94 ± 0.14	0.95	0.73 – 1.16	22.5	4.2	0/10	<0.0001	6/10
	TF4 pretarget - 0.5 mCi	10	0.83 ± 0.23	0.90	0.45 – 1.16		undefined	9/10 (90%)	<0.0001	0
H	Untreated	10	0.59 ± 0.21	0.53	0.41 – 1.12	5.0	1.2	2/10 [‡]	-----	6/10
	TF4 pretarget - 0.15 mCi	10	0.68 ± 0.14	0.68	0.51 – 0.92		3.6	0/10	0.0007	0
I	Untreated	5	0.63 ± 0.22	0.58	0.45 – 1.01	2.0	0.9	0/5	-----	0
	TF4 pretarget - 0.5 mCi	5	0.57 ± 0.17	0.54	0.40 – 0.89		1.7	0/10	0.0042	0
J	Untreated	10	0.76 ± 0.24	0.71	0.46 – 1.19	2.0	1.1	0/10	0.0128	0
	TF4 pretarget - 0.5 mCi	10	0.76 ± 0.24	0.71	0.46 – 1.19		1.1	0/10	0.0128	0

* Study terminated.

⁷ Weeks from the treatment to the time an animal was removed from study because tumor progressed to $\geq 3 \text{ cm}^3$ or toxicity.

[#] Spontaneous regression of established, untreated tumors was observed in 4 animals in 3 studies. Three of the four tumors doubled in size before regressing, the other failed to progress from its initial size of 0.53 cm^3 .

[§] Unrelated, one animal that was tumor-free was removed at week 12.5 because of prolapsed rectum; 3 other animals were tumor-free at the end of study.

// NS, not significant.

^{**} Unrelated; subcutaneous 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.