The Combined Use of an Immunotoxin and a Radioimmunoconjugate to Treat Disseminated Human B-Cell Lymphoma in Immunodeficient Mice

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ABSTRACT

Immunotoxins (ICs) consist of a targeting moiety and a toxic moiety and have the specificity that traditional cancer therapy lacks. At appropriate doses, ICs are safe and effective in treating various cancers in experimental animals and in humans. However, because cures are rarely achieved using single agents, regimens involving combinations of agents with different mechanisms of action must be evaluated. In this study, we explored the efficacy and toxicity of a combination of two IC therapies, radioimmunotherapy (RIT) and immunotoxin (IT) therapy, to treat advanced, disseminated human lymphoma in immunodeficient mice. We proposed to use the bystander effect of RIT to reduce large tumor burdens, followed by an IT to eliminate residual tumor cells. Our results indicate that, when used alone, both RIT and IT therapy were safe and effective, but not curative. When the two therapies were combined, efficacy and toxicity became dependent on the temporal order of administration. Thus, with the doses used in this study, when RIT was administered after IT therapy, the regimen was curative. In contrast, when RIT was administered before IT therapy, the combination was highly toxic or even lethal. Both RIT and IT therapy induced pulmonary vascular leak, but with different kinetics. When RIT was given prior to IT therapy, the pulmonary vascular leak became life-threatening but not when the two agents were administered in the reverse order.

INTRODUCTION

Despite advances in the chemotherapy and radiation therapy of cancer, the lack of specificity of these agents results in significant damage to normal tissues with increased morbidity and mortality. Over the past two decades, targeted therapies using mAbs and mAb ICs have been under development (1–3).

Since 1997, two “naked” mAbs have been approved by the Federal Drug Administration for the treatment of lymphoma (chimerized Rituxan) (4, 5) and breast cancer (humanized Herceptin) (6, 7). These mAbs have the advantage of low immunogenicity, few side effects, and significant efficacy. ICs consisting of mAbs coupled to toxins, radionuclides, enzymes, or drugs are often more potent but are more difficult to develop clinically because they have significant side effects at high doses. Therefore, lower doses must be administered and clinical trials are proceeding more cautiously.

Radiolabeled mAbs and ITs are among the ICs currently in clinical trials. The former are usually mAbs labeled with β emitters, such as 131I and 90Y. The latter are mAbs coupled to a variety of native, modified, or genetically engineered plant or bacterial toxins or RIPs. Both modalities have advanced well into clinical trials (8, 9), and thus far, the most impressive clinical results have been observed in the treatment of lymphomas (10–13). Lymphomas are excellent targets for ICs, because, in comparison to solid tumors, cells are relatively accessible to systemically administered therapeutic agents (14). There are also numerous well-characterized, lineage-restricted antigens on lymphoma cells, and the normal lymphocytes killed during therapy are replaced. Furthermore, the disease itself is immunosuppressive (resulting in neutralizing antibody responses in only about 30% of the patients) (15, 16).

Clearly, future studies will involve combining various traditional and targeted therapies in an effort to eliminate large tumor masses, metastatic disease, dormant cells, and antigen-negative variants. For this reason, it is important to develop combinatorial regimens that are safe and have significant efficacy. This can be accomplished to some extent by studying human tumor xenografts in immunodeficient mice (17).

Because each targeted therapy has different advantages as well as dose-limiting toxicities, it is important to consider these in developing combinatorial regimens. For example, RIT is advantageous in treating large tumors because the radiation emitted from a few mAb-coated tumor cells can penetrate several cell diameters and kill surrounding mAb-inaccessible tumor cells [even when they lack the targeted antigen (18)]. In con-

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3 The abbreviations used are: mAb, monoclonal antibody; IC, immunotoxin; RIT, radioimmunotherapy; RIP, ribosome inactivating protein; MRD, minimal residual disease; SCID, severe combined immunodeficiency disease; PVL, pulmonary vascular leak; dgRTA, deglycosylated ricin A chain; SMPT, N-succinimidyl-oxycarbonyl-α-methyl-α-(2-pyridylidithio) toluene; PR, partial response; CR, complete response; VP, vascular permeability.
CO2 in air. Cell viability was determined by trypan blue exclusion. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 mM L-glutamine. The medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 mM L-glutamine. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

The primary goal of this study was to test the effectiveness of RIT plus IT therapy to treat disseminated human B-cell lymphomas in SCID and nude mice. Using highly sensitive methods for detecting MRD, we compared a variety of dose regimens for both efficacy and toxicity. Our studies suggest that although each modality is effective when administered alone, the combination of ITs followed by RIT is most effective and indeed curative in advanced, disseminated disease. In contrast, RIT followed by IT therapy is highly toxic and often fatal. In the latter instance, toxicity appears to be related to the fact that both therapies damage the vasculature but with different kinetics. In mice, RIT induces late PVL, whereas IT therapy induces early PVL. Thus, when RIT is given prior to IT therapy, the PVL induced by the two agents is cumulative, resulting in fatalities. In contrast, if the IT is administered first, PVL induced by the IT resolves before RIT is administered. These studies underscore the importance of scheduling in combining targeted therapy regimens and emphasize that there is increased efficacy when therapies are combined in the appropriate temporal order.

**MATERIALS AND METHODS**

**Cells.** The surface IgM-positive (IgM+) human Burkitt’s lymphoma cell line, Daudi, was maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 mM L-glutamine. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cell viability was determined by trypan blue exclusion. Cell viabilities of 90% or higher were required for injection into mice.

**Mice.** Female outbred ICR SCID mice (Taconic Farms, Germantown, NY) and athymic nude mice (Harlan) were housed in sterile cages with paper filter covers. Sterilized cages, covers, bedding, food, and drinking water were changed weekly. In the case of mice treated with RIT, the cages and bedding were changed twice a week. Mice were 6–7 weeks old at the time of tumor cell inoculation. All procedures were performed in a laminar flow hood.

**Tumor Xenografts and Experimental Models.** Two tumor models, including localized s.c. and disseminated tumors, were established in SCID and nude mice.

**Localized s.c. Tumors.** Nude (but not SCID) mice received 500 rads of whole body γ irradiation 1 day prior to tumor cell inoculation. Optimal tumor growth was achieved by injecting i.v. 2 × 107 Daudi cells into the tail vein. Body weights were followed weekly. Mice were sacrificed when hind-leg paralysis was observed or when animals lost 30% of their body weight.

**mAbs and IT.** Murine antihuman CD22 (RFB4, a gift from Dr. G. Janossy, Royal Free Hospital, London, United Kingdom), CD19 (HD37, a gift from Dr. D. Dorken, Heidelberg, Germany), and anti-CD20 (2H7; Bristol-Myers, New York, NY) mAbs were used as the targeting mAbs in this study. All mAbs were of IgG1-κ isotype. An isotype-matched irrelevant mAb, 3F12 (a gift from Dr. E. Hansen at UTSM, Medical school, Dallas, TX), was used as a control. The RFB4-dgRTA IT was prepared as described previously using purified RFB4, the SMPT cross-linker (22), and dgRTA (23). The immunoreactivity of the IT was confirmed by fluorescence-activated cell sorter analysis. The cytotoxicity of each batch of IT was determined both in *in vitro* and *in vivo*. Forty percent of the LD₅₀ was used as the therapeutic dose as previously described (24).

**Radioiodination.** Radioiodination of mAbs with Na¹³¹I was performed using the Iodogen method. Briefly, 100 μg of each mAb was mixed with 2 mCi Na¹³¹I (NEN, Boston, MA) in 50 μl PBS, pH 7.0, in a glass tube coated with 20 μg Iodogen (Pierce, Rockville, IL). The tube was then incubated in ice for 7 min, and the mixture was chromatographed on a PG-10 column (Pharmacia Biotech, Piscataway, NJ) to remove unbound Na¹³¹I. The ¹³¹I-mAb was eluted with 2 ml of PBS. The amounts of free radioiodine and the specific activities of the ¹³¹I-mAbs were determined by precipitation in 10% trichloroacetic acid. In all experiments, less than 5% of the total radioactivity was trichloroacetic acid-soluble, and the specific activity of the ¹³¹I-mAb was between 5 and 15 μCi/μg. Human serum albumin (Sigma, St. Louis, MO) was added as a carrier protein to a final concentration of 0.5% to prevent radiolysis, aggregation, and denaturation. The immunoreactivity of ¹³¹I-mAbs was unchanged, based on the fact that: (1) the binding of ¹³¹I-mAbs to Daudi cells was completely inhibited by adding cold-specific mAb but not by adding an irrelevant mAb and (2) Scatchard analyses confirmed that the affinity was unchanged.

**Therapy Protocols for s.c. Tumors.** When tumor volumes exceeded 300 mm³, mice were treated with ¹³¹I-mAbs; SCID mice received 3 μCi/g and nude mice received ~15 μCi/g by i.p. injection. ¹³¹I-mAb was mixed with unlabeled mAb and PBS so that each injection contained 100 μg mAb in 500 μl. Control animals received equal amounts of unlabeled mAbs...
Table 1  Regimens used to treat mice with disseminated tumorsa

<table>
<thead>
<tr>
<th>Regimens</th>
<th>Treatmenta</th>
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<tbody>
<tr>
<td>1</td>
<td>131I-HD37</td>
</tr>
<tr>
<td>(RIT → IT)</td>
<td>RFB4-dgRTA</td>
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<tr>
<td>2</td>
<td>RFB4-dgRTA</td>
</tr>
<tr>
<td>(IT → RIT)</td>
<td>131I-HD37</td>
</tr>
<tr>
<td>3</td>
<td>131I-HD37</td>
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<td>PBS</td>
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<td>5</td>
<td>PBS</td>
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<tr>
<td>6</td>
<td>PBS</td>
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<td>7</td>
<td>PBS</td>
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</tbody>
</table>

a Mice received i.v. injection of tumor cells on day 0 and treatments on week 2 and/or week 4.

Evaluation of Therapeutic Efficacy in SCID and Nude Mice with Disseminated Disease. SCID mice were sacrificed when hind-leg paralysis occurred, and the mean paralysis time was used to evaluate therapeutic efficacy as described previously (21). Because paralysis did not occur in all untreated tumor-bearing nude mice, mice were sacrificed when either (1) paralysis or (2) more than 30% weight loss occurred. If neither occurred, mice were sacrificed at a predetermined time as follows: (1) mice receiving regimens 3–7 (Table 1) were sacrificed on week 8; (2) mice receiving regimens 1 or 2 were randomly divided into two groups: one group was sacrificed on week 8 and the other on week 12. Gross examination, the dot blot assay, and the Daudi cell-specific, nested PCR (in order of increasing sensitivity) were used to evaluate tumor burdens in treated mice. Gross examination was first performed in all nude mice. Dot blot analysis and/or PCR were subsequently performed on DNA extracts from organs of those mice showing no evidence of tumors by gross examination. A “cure” was operationally defined as no detectable tumor cells in evaluated organs by gross examination, dot blot, and the Daudi cell-specific, nested PCR.

Preparation of Genomic DNA. Cells or organs were first incubated in 0.5 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml protease K) at 50°C overnight. Following incubation, RNA was digested by the addition of 50 μl (1 mg/ml) of RNase A. One-tenth volume of 3 M sodium acetate and 2 volumes of isopropanol were then added, and the genomic DNA was precipitated by a 20-min centrifugation at 10,000 rpm. The DNA pellet was dissolved in 200 μl sterile water.

Dot Blots. Five hundred nanograms of genomic DNA was applied to a nylon transfer membrane (Amersham Life Science Inc., Arlington Heights, IL), which was then baked in an 85°C vacuum oven for 2 h to fix the DNA onto the membrane. Prehybridization was performed by incubating the membrane with the prehybridization buffer (50% formamide, 5x SSC solution (0.75 M NaCl and 0.075 M citric acid), 5% Denhardt’s solution, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA) at 42°C for 2 h. Human DNA was detected by adding a 32P-[dCTP]-labeled human Cot-1 DNA (GIBCO BRL, Gaithersburg, MD) followed by incubation at 42°C for 8 h. The membrane was washed with 2x SSC and 0.1% SDS at 42°C for 10 min, twice, and then with 0.1x SSC and 0.1% SDS at 65°C for 30 min. Autoradiography was used to detect the binding of the probe to the human DNA.

Daudi Cell-specific, Nested PCR. A Daudi cell-specific, nested PCR was designed to detect Daudi cells remaining in the organs of treated mice. Two pairs of primers were used to amplify a 405-bp DNA fragment from the immunoglobulin kappa light chain variable region of the Daudi cell. The oligonucleotide primers (Daudi2: 5’-GCTCTGTTTGAAGT GACCTA-3’, L1: 5’-CTGGCTCCGACGTAAGGA-3’, L2: 5’-GACGTAAAGGGAGAGGAAC-3’, and R1: 5’-GTTGACGATA GTGGTG-3’) were synthesized at the Ruybern Cardiology Center facility at UTSW. The sensitivity of the PCR assay was determined by amplifying the target gene from the genomic DNA from 104 mouse cells and varying numbers of Daudi cells. To detect tumor cells in mice with disseminated Daudi tumors, 500 ng of genomic DNA extracted...
Combination Immunotherapy of B Lymphoma in Mice

In these experiments, the same agents (131I-HD37 and RFB4-dgRTA), were used to determine the maximum tolerated doses of RIT. Because of severe weight loss that occurred in animals receiving IT prior to IT therapy (RIT → IT), SCID mice treated with combination therapies were sacrificed 1 week after the completion of therapy. The lungs were weighed before and after lyophilization and the wet: dry weight ratios were calculated. This value was used as a measurement of PVL.

**RESULTS**

Therapeutic Efficacy of RIT in Mice with Large s.c. Tumors. The first objective of this study was to evaluate the ability of RIT to reduce large s.c. tumors. Preliminary studies were carried out to determine the maximum tolerated doses of RIT in SCID mice, because these mice are highly radiosensitive. Groups of 5 to 10 mice received one i.p. injection of 131I-RFB4 at 2–10 μCi/g body weight. Each injection contained equal amounts (100 μg) of RFB4 in the same volume (500 μl). The maximum tolerated dose was 3 μCi/g body weight (data not shown), bringing the total dose to 65–90 μCi per outbred SCID mouse. For nude mice, we used the doses previously described (27, 28), that is, 15–18 μCi/g body weight (~300 μCi/injection).

The therapy of mice with s.c. tumors was initiated once the tumor volume exceeded 300 mm$^3$. SCID mice received one i.p. injection of 131I-RFB4, whereas nude mice received 131I-RFB4, -HD37, -2H7, or -3F12 (Table 2). Mice in the control groups received either equal amounts of unlabeled mAb or PBS. Tumor volumes were measured twice a week for 80 days to determine efficacy.

The small dose of 3 μCi/g administered to SCID mice induced PRs for 2 weeks, but no CRs or cures were achieved. When a dose of 15–18 μCi/g was administered to nude mice, cures were achieved in 91% and 50% of the mice treated with either 131I-RFB4 or 131I-HD37, respectively. CRs were observed in the remaining mice in these two groups. 131I-2H7 was less effective, resulting in 1 CR and 8 PRs (with an 80.8 ± 10.3% reduction in tumor volume). Four of seven mice treated with the control 131I-mAb, 131I-3F12, also had minor responses, but reductions in tumor volumes were less impressive (16.8 ± 7.2%) and of shorter duration (<1 week). Minor responses were probably due to a bystander effect of the nonreactive 131I-3F12 in the circulation. In contrast, none of the unlabeled (tumor-specific or irrelevant mAbs) had any effect on tumor growth (Table 2). In summary, in these experiments, 131I-labeled tumor-reactive mAbs were highly effective in reducing large s.c. tumor burdens, and cures could be induced when large doses were used.

**Combinations of RIT and IT Therapy in SCID Mice with Advanced, Disseminated Tumors.** We next tested the combination of RIT and IT therapy in mice with more clinically relevant disseminated tumors. Growth of disseminated Daudi cells in SCID mice has been described previously (21). Briefly, 5 × 10$^6$ Daudi cells were injected i.v. into SCID mice and tumor cells grew systemically. Therapy was initiated 2 weeks after tumor cell inoculation, and 2 weeks later (week 4) the second

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average dose (μCi/μg mAb)</th>
<th>Mice</th>
<th>No. of Cures</th>
<th>No. of CRs</th>
<th>No. of PRs</th>
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<tbody>
<tr>
<td>Nude mice</td>
<td></td>
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<tr>
<td>131I-RFB4</td>
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<td>11</td>
<td>10</td>
<td>1</td>
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<tr>
<td>131I-HD37</td>
<td>3.2$^a$</td>
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<tr>
<td>131I-2H7</td>
<td>3.1$^a$</td>
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<tr>
<td>131I-3F12</td>
<td>3.2$^a$</td>
<td></td>
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<tr>
<td>RFB4</td>
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<td>7</td>
<td>0</td>
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<tr>
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<td>0</td>
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<td>0</td>
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<tr>
<td>2H7</td>
<td>0$^a$</td>
<td>9</td>
<td>0</td>
<td>0</td>
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<tr>
<td>3F12</td>
<td>0$^a$</td>
<td>3</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>PBS</td>
<td>—</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>SCID mice</td>
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<tr>
<td>131I-RFB4</td>
<td>0.8$^b$</td>
<td>9</td>
<td>0</td>
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</tr>
<tr>
<td>RFB4</td>
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<tr>
<td>PBS</td>
<td>—</td>
<td>4</td>
<td>0</td>
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</table>

$^a$ Treatment was initiated when tumor nodules reached volumes of 300–500 mm$^3$ on day 0.

$^b$ Mice were injected i.p. with 131I-mAbs or cold mAbs in total of 100 μg. Mice in the control groups received equal volumes (400–500 μl) of PBS.

$^c$ The observation period was 80 days after the completion of therapy. Cures, CRs, and PRs are defined in “Materials and Methods.”

$^d$ Dose range: 286–350 μCi.

$^e$ Dose range: 295–346 μCi.

$^f$ Dose range: 294–352 μCi.

$^g$ Dose range: 305–334 μCi.

$^h$ Dose: 100 μg cold mAbs.

$^i$ Dose range: 70–90.8 μCi.

Table 2 Inhibition of the s.c. growth of Daudi cells by tumor-specific 131I-mAbs

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from organs of treated mice was used as the template, and the same amplification procedures were carried out.

**PVL.** PVL was evaluated by measuring the amount of fluid accumulated in the lungs of treated mice (25, 26). In these experiments, the same agents (131I-HD37 and RFB4-dgRTA), doses, and regimens used in therapy experiments were administered to tumor-free SCID and nude mice. Beginning 1 week after the completion of therapy and for 4 consecutive weeks, animals were sacrificed weekly, and lungs were excised from one group of 5–10 treated animals. Because of severe weight loss that occurred in animals receiving RIT prior to IT therapy (RIT → IT), SCID mice treated with combination therapies were sacrificed 1 week after the completion of therapy. The lungs were weighed before and after lyophilization and the wet: dry weight ratios were calculated. This value was used as a measurement of PVL.
treatment was administered (Table 1). $^{131}$I-HD37 and RFB4-dgRTA were used as the therapeutic agents for RIT and IT therapy, respectively. SCID mice, which are very radiosensitive, were used in this study with the intent of developing effective and safe therapy regimens with low doses of RIT. Because we found that $^{131}$I-3F12 had only a modest and transient effect on tumor growth, $^{131}$I-3F12 was not included in subsequent experiments.

Individual treatments ($^{131}$I-HD37 or RFB4-dgRTA) administered after either 2 or 4 weeks significantly delayed paralysis (Fig. 1). When IT was administered prior to RIT, the mean survival time was further extended (Fig. 1). Unexpectedly, Regimen 1 (RIT IT) was fatal in 100% of the mice, with a mean survival time of 44.6 days. Thus, there were schedule-related toxicities when RIT and IT were combined, although both treatments were safe when used individually.

Establishment of Disseminated Tumors in Nude Mice. Nude mice are not as radiosensitive as SCID mice and can tolerate higher doses of irradiation. Disseminated tumor growth in nude mice was therefore used to test regimens involving higher doses of RIT. Optimal tumor growth in nude mice was achieved when $2 \times 10^7$ Daudi cells were injected i.v. into preirradiated mice. We consider it unlikely that this preirradiation itself influenced the toxicity of the combination therapies based on the fact that external irradiation did not induce weight loss and that the weight losses observed in the PVL study (in which both nonirradiated, tumor-free nude mice and nonirradiated SCID mice were used) were the same as those observed in the irradiated nude mice. However, unlike the situation in SCID mice, where hind-leg paralysis occurred in all mice, hind-leg paralysis was observed in only 50% of these mice. Because of this, and to evaluate therapeutic efficacy, human cell-specific dot blots and a highly sensitive Daudi cell-specific, nested PCR were developed to detect Daudi tumor cells in mouse organs. The sensitivity of the assay was determined using DNA from mixtures of $10^6$ mouse cells and various numbers of human Daudi cells. When mixed with $10^6$ mouse cells, 240 or more Daudi cells were detected using the dot blot assay. Using the Daudi cell-specific, nested PCR, one Daudi cell in $10^6$ mouse cells could be detected (data not shown).

When therapy was performed in nude mice with disseminated tumors, animals were sacrificed when any one of the following occurred: (1) hind-leg paralysis, (2) ≥30% weight loss, or (3) when mice remained healthy for 8 or 12 weeks. Gross examinations were first performed on all organs. For those mice without visible tumor nodules, DNA was extracted from various organs, and dot blot assays were performed. When dot blot assays were negative, the Daudi cell-specific, nested...
PCR was subsequently performed. A cure was operationally defined as failure to detect tumor cells by gross examination, dot blot assays, and the Daudi cell-specific, nested PCR.

Combination RIT and IT Therapy in Nude Mice with Advanced, Disseminated Tumors. Nude mice were treated as described in Table 1. Fifty percent of the 26 PBS-treated mice became paralyzed, and tumors were observed in all of the mice (Table 3, Regimen 7). Single treatments reduced paralysis to 30% (Table 3, Regimen 3) or 0% (Table 3, Regimens 4–6) of the mice. However, tumors were detected in all animals receiving single treatments by either gross examination or dot blot assays. Hence, single treatments were effective, but not curative. The antitumor effects of RIT and IT therapy, administered either at week 2 or week 4, were comparable in the treatment of advanced, disseminated disease.

In the mice treated with Regimen 2 (IT → RIT), paralysis did not occur (Table 3). Thus, on week 8, the 20 mice were divided into two groups and sacrificed either at that time (week 8) or 4 weeks later (week 12). At week 8, gross examination revealed no tumors in 10 of 10 mice. According to the protocol described above, dot blots and nested PCRs were then performed to determine whether residual tumor cells were present. Tumor cells were detected in 6 of 10 mice (Table 3) using the dot blot assay (data not shown). Of the remaining 4 mice, tumors were detected in 3 of 4 mice using the nested PCR (Table 3). On week 12, the remaining 10 mice were sacrificed. By gross examination, there were no visible tumors in the 8 mice in which dot blots or PCRs were performed. Four cures were documented by nested PCR (data not shown).

Regimen 1 (RIT → IT) was highly toxic; 9 of 19 mice died before week 8 (Table 3). The remaining 10 mice were divided into two groups with 5 mice per group. On week 8, no tumors were observed by gross examination, but residual tumor cells were detected in 4 of 5 mice. On week 12, tumors were detected in all 5 mice by either gross examination or dot blots (data not shown).

In summary, individual treatments were effective but not curative. Regimen 1 (RIT → IT) was highly toxic and often fatal, whereas Regimen 2 (IT → RIT) was curative, as determined at week 12. On week 8, the tumor burdens were comparable in surviving mice receiving either Regimen 1 or 2. However, on week 12, tumor relapses occurred in mice treated with Regimen 1 (RIT → IT) while cures were achieved in 40% of the mice receiving Regimen 2 (IT → RIT).

Toxicity of RIT and/or IT Therapy. To evaluate the toxicity of RIT in mice with s.c. tumors, decreases in body weight and peripheral WBCs were determined. In SCID mice, both weights and WBCs reached their nadirs 2–3 weeks after treatment; mice then recovered (data not shown). Weight losses were not observed in nude mice. WBCs declined and recovered in a manner similar to those observed in SCID mice (data not shown). The toxicity of RIT appeared to be transient and reversible.

Weights were followed during the therapy of disseminated tumors. The dose of RIT used in this study was well tolerated; no severe weight losses (≥20% of body weight) were observed (data not shown). Minor weight losses (5–20% of body weight) were observed in both SCID and nude mice after the administration of the IT (data not shown), but these were transient and the mice recovered after 1 week. Approximately 5% of the

### Table 3 Therapeutic effect of combinations of RIT and IT in nude mice with disseminated Daudi tumors

<table>
<thead>
<tr>
<th>Regimen&lt;sup&gt;3&lt;/sup&gt;</th>
<th>End point</th>
<th>Presence of tumors&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Paralysis</td>
<td>Death</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>No.</td>
<td>Treatment, week 2</td>
<td>Treatment, week 4</td>
</tr>
<tr>
<td>1</td>
<td>131&lt;sup&gt;a&lt;/sup&gt;I-HD37</td>
<td>RFB4-dgRTA</td>
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<tr>
<td></td>
<td>Sacrificed on week 8</td>
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<td>Sacrificed on week 12</td>
<td>5</td>
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<tr>
<td>2</td>
<td>RFB4-dgRTA</td>
<td>131&lt;sup&gt;a&lt;/sup&gt;I-HD37</td>
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<td>Sacrificed on week 8</td>
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<td>5</td>
<td>PBS</td>
<td>131&lt;sup&gt;a&lt;/sup&gt;I-HD37</td>
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<tr>
<td>6</td>
<td>PBS</td>
<td>RFB4-dgRTA</td>
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<td>7</td>
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<sup>a</sup> Mice received tumor cells on week 0, the first treatment on week 2 and the second treatment on week 4. Mice were sacrificed and gross examinations were performed under the following circumstances: paralysis, death, ≥30% weight loss, and at the predetermined terminating times (week 8 and 12). Numbers represent the number of mice in each category. Dot blots and/or PCRs were further performed on organs from mice with no visible tumor nodules. The data shown in this table represent three experiments. Two experiments were carried out with all seven groups simultaneously; in the third experiment only regimens 1, 2, and 7 were carried out simultaneously.

<sup>b</sup> Mice were considered tumor positive when tumor cells were detected in any organs including ovary, kidney, spleen, liver, lung, heart, spine, brain, and blood. + = tumor positive; − = tumor negative; n.d. = not determined.

<sup>c</sup> Due to the lack of 100% paralysis, with the exception of mice receiving combination therapies, all of the non-paralyzed mice were sacrificed on week 8 to evaluate therapeutic efficacy. For mice receiving combination therapies, the surviving mice were randomly divided into 2 groups; one group was sacrificed on week 8 and the other on week 12.

<sup>d</sup>The four mice lacked tumor cells by all 3 methods and were considered cured.
SCID mice showed a $>20\%$ loss in weight prior to the onset of hind-leg paralysis, because of the large tumor burdens. Overall, the doses of RIT and IT therapy used in this study were well tolerated, and life-threatening toxicities were not observed.

When RIT and IT therapy were combined, mice treated with Regimen 2 (IT $\rightarrow$ RIT) maintained their weights (Fig. 2, C and D). However, mice treated with Regimen 1 (RIT $\rightarrow$ IT) lost weight immediately after IT treatment (Fig. 2, A and B), and the weight losses were followed by deaths in all of the SCID mice and 47% of the nude mice. Overall, Regimen 1 (RIT $\rightarrow$ IT) caused severe and, for some mice, irreversible toxicities.

PVL. Vascular leak syndrome (VLS) is the major dose-limiting toxicity in patients receiving ITs (29). Radiation also damages vascular endothelial cells in vitro and in vivo (30). Since IT-mediated systemic VLS (weight gain and edema) is not observed in mice, PVL has been used to evaluate vascular damage mediated by IL-2 (26), and more recently by ITs containing dgA (25). In this study, we used PVL to measure vascular damage induced by RIT, IT therapy or both. Normal mice were treated according to the therapy protocols described in Table 1. At weekly intervals, mice were sacrificed and lungs were excised. The wet: dry lung weight ratios were calculated to determine the amount of fluid accumulated in the lungs. In IT-treated mice, fluid accumulation in the lungs increased 1 week after treatment and declined to baseline 2 weeks later, whereas in mice treated with RIT, the ratios did not increase until 3 weeks after treatment (Fig. 3). Using Regimen 2 (IT $\rightarrow$ RIT), PVL was moderately increased 1 week after therapy. However, during the same time interval, there was severe PVL in the mice treated with Regimen 1 (RIT $\rightarrow$ IT).

These results suggest that the kinetics of RIT- and IT-induced PVL differed. Hence, using Regimen 1 (RIT $\rightarrow$ IT), the late PVL induced by the RIT was exacerbated by the early PVL induced by the IT. In contrast, when Regimen 2 (IT $\rightarrow$ RIT) was used, IT-mediated PVL occurred early and resolved before RIT-induced PVL was initiated.

Roles of RIT and IT in the Toxicity of Regimen 1 (RIT $\rightarrow$ IT). We next explored the individual roles of RIT and IT in the toxicity induced by Regimen 1. We administered 50% of the dose of either RIT or IT. All mice survived and weight losses became less severe when the dose of RIT was reduced by 50%. All nude mice and 2 of 5 SCID mice survived (Fig. 4, A and B), that is, the survival rates were increased. In contrast, reducing the dose of the IT did not improve survival or prevent weight loss (Fig. 4, C and D). In addition, RIT mediated the toxicity of Regimen 1 in a dose-dependent manner (data not shown), that is, the weight losses, which occurred after the administration of IT, decreased as the RIT doses were reduced. Thus, RIT played the dominant role in the toxicity of Regimen 1 (RIT $\rightarrow$ IT).
Efficacy of Lower Doses of RIT followed by IT Therapy. We studied the therapeutic efficacy of the same combinations of agents in Regimen 1 and 2 using 50% of the RIT dose in nude mice (Table 4). With Regimen 1 (RIT → IT), 8 weeks after tumor cell inoculation, tumors were detected by dot blot, and, by week 12, tumor nodules were detected in all mice (60% detectable by dot blotting, and the remaining 40% detectable by PCR). There were no cures. With Regimen 2 (IT → RIT), 8 weeks after tumor cell inoculation, tumor cells were detected in 60% of the mice by dot blots and in 40% of the mice by nested PCR. At week 12, one cure (20%) was achieved, and the remaining 80% of the mice had tumors that were detectable by dot blots or nested PCRs, but not by gross examination. Using Regimen 2 (IT → RIT), the outcome of therapy was comparable when either 100% or 50% of RIT was administered. Overall, as determined by the tumor burdens on week 12, Regimen 2 (IT → RIT) had better antitumor activity than Regimen 1 (RIT → IT).

DISCUSSION

The objective of this study was to determine the optimal schedule for combining RIT and IT therapy to treat advanced, disseminated, human B-cell lymphomas in immunodeficient mice. We hypothesized that RIT followed by IT therapy was the logical order in which to combine the two therapies, because the former should debulk the tumor and the latter should eliminate MRD. To this end, we first tested the debulking ability of RIT using the 131I-labeled murine mAbs, RFB4 (anti-CD22), HD37 (anti-CD19), 2H7 (anti-CD20), or 3F12 (an irrelevant mAb) in mice with large s.c. human Daudi-cell xenografts. We next tested combinations of RIT (131I-HD37) and IT (RFB4-dgRTA) in mice with disseminated Daudi tumors. Weights, WBCs, and PVL were monitored to determine the toxicity of the treatments. The major findings to emerge from this study are as follows: (1) RIT was very effective in treating large s.c. tumors. (2) Myelo-
suppression and/or weight loss was observed in mice undergoing RIT. Both toxicities were transient and reversible. (3) Individual treatments (RIT or IT therapy) were safe and effective but not curative in mice with disseminated Daudi tumors. (4) Surprisingly, when IT therapy was administered before RIT to mice with disseminated tumors, the survival of the SCID mice was further extended, and cures were achieved in 40% of the nude mice. In addition, this regimen was safe, and the treated mice did not lose weight. In contrast, when IT therapy was administered after RIT, the regimen was lethal to all of the SCID mice and 50% of the nude mice. Weight losses occurred immediately after the IT was administered. (5) The different kinetics of PVL, which occurred after RIT (late PVL) or IT therapy (early PVL), were associated with cumulative toxicity when RIT was administered before (but not after) IT therapy. (6) By reducing the dose of RIT, but not the IT, the survival of the mice treated with RIT followed by IT, was improved. Hence, RIT dominated the toxicity of Regimen 1 (RIT → IT) and was dose-dependent. (7) The efficacy of Regimen 2 (IT → RIT) was higher than that of Regimen 1 (RIT → IT). Thus, in contrast to our initial prediction that RIT followed by IT therapy would be the better regimen, the reverse was true. Whether or not this will be the case in humans remains to be determined.

In humans, RIT has been more successful in treating bulky lymphomas than IT therapy. Using the $^{131}$I-B1 anti-CD20 mAb, myeloablative doses of RIT have been evaluated in treating advanced, relapsed B-cell lymphoma (10). Very high overall and progression-free survival rates have been reported, but autologous bone marrow or peripheral blood stem cell transplantation is required. Nonmyeloablative doses of the $^{131}$I-B1 also induced excellent responses, but fewer durable remissions or cures have been achieved (11). In contrast to the prevalent strategy of using ITs to treat MRD in mice, RIT has not been used in this setting. Thus, the efficacy of RIT on MRD in humans is unclear.

Therapy with dg-RTA-containing ITs has been more difficult to develop in humans because of VLS and hence the need to give smaller doses that cannot eliminate large tumor burdens. Nevertheless, Phase I trials with ITs have shown antitumor activity in patients with lymphoma (31, 32). Because of their dose-limiting toxicity, ITs should perform best in MRD. Indeed, in mice, IT therapy has been more successful in treating MRD than in treating large s.c. tumors (20, 33).

Although RIT and IT therapy have not been combined previously, Buchsbaum et al. (34) used a radiolabeled IT to reduce s.c. tumors. From their in vitro study (34, 35), the IT itself appeared to be responsible for the majority of the antitumor activity, because radiolabeling the IT did not increase the potency of the IT. However, no additional toxicity was reported after administrating the radiolabeled IT.
RIT and IT therapy have both been used in combination with other agents. ITs have been combined with chemotherapeutic agents and additive or synergistic effects were observed (20, 36). RIT has been combined with cytotoxic agents that also function as radiosensitizers [e.g., 5-Fluorouracil (37) and SR4233 (38)], agents that can alter the permeability of vascular [e.g., tumor necrosis factor (39) or IL-2 (40)], or hyperthermia (41) to increase efficacy. Experiments have also been carried out using agents that elevate the expression of target antigens [e.g., IFN stimulates carcinoembryonic antigen expression on colon carcinoma cells (42, 43)].

The importance of temporal order in administering different agents has been demonstrated previously. For example, DeNardo et al. (44, 45) combined 90Y-chL6 (a chimeric antibody reacting with an integral membrane glycoprotein that is expressed at high levels on human breast, colon, ovary, and lung carcinomas) with either taxol or cold ch225 (an anti-EGFR mAb) to treat breast carcinoma xenografts. Synergistic effects were observed when taxol was administered after the 90Y-mAb or when ch225 was given before 90Y-mAb. However, high mortality rates were observed when ch225 was combined with 90Y-chL6 in either temporal order. Ghetie et al. (46) have also combined ITs with various chemotherapeutic agents. Significant therapeutic benefits were achieved when the IT therapy was administered before or during chemotherapy but not after chemotherapy.

In this study, we found that, when RIT and IT therapy were combined, therapeutic efficacy was increased when ITs were administered prior to RIT. In contrast to the report by DeNardo et al. (44), in which greater toxicity occurred in either temporal order, in our study, toxicity only occurred when RIT was administered prior to IT therapy. This suggested that either RIT predisposed mice to the toxicity of the IT or that the kinetics of toxicities caused by RIT versus IT therapy were different and became cumulative only when RIT was administered first.

Changes in vascular permeability (VP) following RIT have been studied in mice with s.c. tumors (47). Radiolabeled tracers were injected into mice after the completion of RIT. The amounts of the tracer accumulating in the s.c. tumors and normal organs (liver and lung) were measured to determine changes in VP. No changes in the VP of normal organs were observed. However, RIT-induced VP changes in tumor sites varied and were idiosyncratic for the tumor, that is, VP increased in some tumors and decreased in others after RIT. In this study, instead of measuring tracer accumulation in organs of tumor-bearing mice, we measured the accumulation of pulmonary fluid, that is, PVL, in normal mice after RIT, IT therapy or both. PVL was used to measure damage to the vasculature. The kinetics of PVL were studied to further explore the differences between Regimens 1 and 2. In normal mice, PVL was induced by both RIT and IT, but with different kinetics. Hence, RIT induced late and long-lasting vascular toxicity that was exacerbated when ITs were administered. In contrast, ITs induced early PVL, which rapidly resolved so that when RIT was administered, there was no exacerbation of toxicity.

Toxicity could be avoided in some animals treated with RIT followed by IT therapy by reducing the dose of RIT by 50–75%; it could not be improved by delaying the time interval between the two therapies (data not shown) or by giving less IT. This implies that the RIT-induced damage predisposes mice to the toxicity of ITs. Thus, in this animal model, the most effective regimen is IT therapy followed by RIT.

It has been suggested that ITs can sensitize tumor cells to chemotherapeutic agents (36, 46, 48). In our experiments, it is possible that in Regimen 2 (IT → RIT), that the IT did indeed sensitize tumor cells to RIT. It is also possible that the increase in vascular permeability facilitated the penetration of radiolabeled mAbs and that the efficacy of Regimen 2 reflects the cumulative effect of two events. Although a small amount of vascular leak is probably advantageous for the extravasation of mAbs and ICs (49), severe VLS can be fatal in patients. Clearly, our results suggest that for the two therapies to be combined safely and effectively, IT therapy should be given prior to RIT. Whether this will be the case in treating advanced disseminated disease in humans remains to be determined in clinical trials.

We have also demonstrated that both cold mAbs and an irrelevant 131I-mAb were much less effective in treating large s.c. tumors. Thus, in the case of the mAbs tested in this study, both specificity and radiolabeling were optimal for their antitumor activity. However, it has been reported that some mAbs (lacking radionuclides) have good antitumor activity. Moreover, in one instance, an unlabeled anti-CD20 mAb (B1) had better efficacy than the 131I-labeled mAb in an animal model (28), although the reasons for this were not investigated. In such case,
it is possible, for example, that radiolabeling some mAbs damages portions of the mAb that are responsible for binding, signaling, or effector functions. Whatever the explanation, taken together with the results of others, our results underscore the idiosyncratic behavior of different mAbs, even against the same molecule.

In conclusion, the results of our studies demonstrate the excellent efficacy of both RIT and IT therapy in local and disseminated lymphoma in mice. They also underscore the increased efficacy achieved by combining the two therapies to treat disseminated disease. Finally, in this experimental model, the necessity of using ITs before RIT (unless the dose of the latter is reduced) can be attributed at least in part to the different kinetics of vascular toxicity induced by the two agents.

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Combination Immunotherapy of B Lymphoma in Mice


The Combined Use of an Immunotoxin and a Radioimmunoconjugate to Treat Disseminated Human B-Cell Lymphoma in Immunodeficient Mice

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