Immunotoxins constructed with chimeric, short-lived anti-CD22 monoclonal antibodies induce less vascular leak without loss of cytotoxicity

Xiao-yun Liu,1,† Laurentiu M. Pop,1,† John Schindler1 and Ellen S. Vitetta1,2,*

1The Cancer Immunobiology Center; 2Departments of Immunology and Microbiology; University of Texas Southwestern Medical Center at Dallas; Dallas, TX USA

† These authors contributed equally to this work.

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Abbreviations: ALL, acute lymphoblastic leukemia; AUC, area under the curve; CH2, heavy chain constant domain-2; CH3, heavy chain constant domain-3; cRFB4, chimeric RFB4; dgRTA, deglycosylated ricin toxin A chain; DLT, dose limiting toxicity; FCR, fractional catabolic rate; FcRn, neonatal Fc receptor; IT, immunotoxin; mAb, monoclonal antibody; mcRFB4, mutant chimeric RFB4; mcIT, mutant chimeric immunotoxin; MRT, mean residence time; MTD, maximum tolerable dose; NHL, non-Hodgkin's lymphoma; PBS, phosphate buffer saline; PVL, pulmonary vascular leak; rRTA, recombinant ricin toxin A chain; RTA, ricin toxin A chain; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMPT, 4-succinimidyl-oxy-carbonyl-α-methyl-α-(2-pyridyldithio)-toluene; T 1/2, half life; VLS, vascular leak syndrome

An immunotoxin (IT) constructed with RFB4, a murine anti-CD22 monoclonal antibody, and the “deglycosylated” A chain of ricin has shown activity at safe doses in patients with non-Hodgkin lymphoma and in children with acute lymphoblastic leukemia. The dose limiting toxicity is vascular leak syndrome (VLS), which appears to be due to a unique amino acid motif in the ricin toxin A (RTA) chain that damages vascular endothelial cells. We mutated recombinant (r) RTA to disable this site, but await testing of the IT prepared with this mutant RTA in humans. Another possible approach to reducing IT-induced VLS is to shorten the half-life of the IT in vivo. We previously constructed a mouse-human chimeric RFB4 by grafting the variable genes of RFB4 onto the human IgG1k constant regions. Here, we report the expansion of our panel of mutant chimeric RFB4s (mcRFB4s) that lack the ability to bind to the neonatal Fc receptor (FcRn). In comparison with cRFB4, which had a T 1/2 of 263 h, the mcRFB4s had T 1/2s ranging from 39–106 h. ITs were constructed with these mcRFB4s and rRTA. The mcRFB4-RTA ITs retained their cytotoxicity in vitro and had shorter half lives than the parental cRFB4-RTA IT. In addition, the mcRFB4 IT with the shortest T 1/2 induced less pulmonary vascular leak in mice, which we have postulated is a surrogate marker for VLS in humans.

Introduction

Immunotoxins (ITs) are hybrid molecules that consist of monoclonal antibodies (mAbs) linked to toxins or their subunits.1 ITs can bind to and kill target cells without killing the surrounding normal cells. Because of the liver’s role in clearing toxins, the maximum tolerated dose (MTD) of ricin and some first generation ITs was defined by hepatotoxicity.1,2 Second generation ITs constructed with the deglycosylated A chain of ricin (dgRTA) did not cause hepatotoxicity because the liver-binding sugars in the toxin were chemically altered.4 ITs prepared with dgRTA and several murine mAbs are extremely potent. They have been evaluated in patients with refractory relapsed lymphoma or leukemia and have shown anti-tumor activity, including partial and complete responses.5-9 The safety and efficacy of these ITs is limited by vascular leak syndrome (VLS), which is the most common dose-limiting toxicity (DLT) of ITs and is characterized by hypalbuminemia, weight gain and, in the most severe cases, pulmonary edema and hypotension.10-14

Three approaches have been tested to prevent VLS. The first approach was to use prophylactic corticosteroids. Their use did not prevent the occurrence of severe VLS and did not lead to an increase in the MTD.15

The second approach was based on observations that VLS was the result of RTA-mediated damage to vascular endothelial cells. This activity was associated with a unique amino acid sequence in...
Results

Design, construction and expression of recombinant RFB4 mutants. The major aim of this study was to construct an anti-CD22 IT that induced less PVL while retaining full cytotoxicity. Our approach was to eliminate the binding of the anti-CD22 mAb to the FcRn receptor. We generated mcRFB4 constructs by introducing mutations into the Fc region involved in the IgG-FcRn interaction.23-25 Five mutant constructs were derived from cRFB4: (1) mcRFB4-P247W with a single mutation in residue 247 (Pro247Trp), (2) mcRFB4-I253A with a single mutation in residue 253 (Ile253Ala), (3) mcRFB4-H310A with a single mutation in residue 310 (His310Ala), (4) mcRFB4-H435A with a single mutation in residue 435 (His435Ala) and (5) mcRFB4-AAA with mutations in residues 253 (Ile253Ala), 310 (His310Ala) and 435 (His435Ala).

All RFB4 constructs were expressed in CHO/DHFR cells and purified to homogeneity by affinity chromatography on Protein G-Sepharose. There were no differences in the purity or the molecular weights between the mAbs as determined by sodium dodecyl sulfate PAGE (SDS-PAGE) (Fig. 1). All mAbs had a molecular weight of 150 kDa under non-reducing conditions, while under reducing conditions all samples contained two bands of 50 and 25 kDa corresponding to IgG heavy and light chains, respectively. In addition, there were no aggregates or degradation as determined by size-exclusion chromatography (Figs. 2, S1 and S2).

Binding activity of mcRFB4 constructs for FcRns and CD22+ human B-cell lymphoma Daudi cells. The mcRFB4 constructs were designed to have a reduced affinity for FcRns. The binding of all constructs to human and rat FcRns at pH 6.0 was below the limit of detection of the assay. cRFB4 mAb and human IgG controls exhibited comparable affinity for these FcRns (data not shown).

Amino acids of IgG that are in the vicinity of the FcRn binding site are located on or around the CH2-CH3 interface.23-25 Therefore, mutation of these residues should not affect binding to CD22. The binding of mcRFB4 constructs to CD22 was evaluated using the CD22+ human B-cell lymphoma Daudi cell line. All mcRFB4 constructs exhibited similar binding to Daudi cells that was comparable to that of cRFB4 (data not shown). In summary, mutations in the FcRn binding site eliminated binding of mcRFB4 to FcRns, but not binding to the CD22 antigen.

PK and stability of mcRFB4 constructs. A mAb with impaired affinity for FcRn should have a decreased T1/2. All mcRFB4s exhibited shorter T1/2 than our previous mutants. These new mcRFB4s had decreased T1/2 in mice. Their stability in vitro and in vivo was not affected. Therefore, ITs were prepared with two mutants, mcRFB4-P247W and mcRFB4-H310A, and the wild-type cRFB4 by conjugating them to rRTA. Compared with cRFB4-rRTA, the mutant ITs had reduced T1/2 in mice, but equivalent cytotoxicity in vitro. The mcRFB4-H310A-rRTA, which had the shortest T1/2, induced less PVL in mice compared with both the cRFB4-rRTA and the murine RFB4-rRTA. 

A mAb with impaired affinity for FcRn should have a decreased T1/2. All mcRFB4s exhibited shorter T1/2 in Swiss-Webster mice (T1/2 ranged from 39 to 106 h), than cRFB4 (T1/2 was 263.7 ± 17.0 h; Table 1).

To determine whether the decreased T1/2 of mcRFB4 mutants was due to the decreased resistance to proteolysis, the in vitro and in vivo stability of mcRFB4 constructs were analyzed and also compared with the murine RFB4 mAb and with cRFB4. The 125I-labeled mAbs were incubated with pooled mouse serum in vitro for 24 h and then analyzed using autoradiography on SDS gels. A comparison of cRFB4, murine RFB4, and the mutants showed no degradation in serum (Fig. 3).

125I-labeled mAbs were injected into mice to evaluate their in vivo stability. Mouse serum was collected at various time periods (0, 3, 6, 24, 48, 72 and 120 h) post injection and analyzed using size-exclusion chromatography. All samples eluted as single sharp peaks with a similar retention times at 0 and 24 h (Figs. 4, S3 and S4). There were no differences between any constructs

RFA: Leu74-Asp75-Val76 (LDV). Altering this sequence led to reduction of pulmonary vascular leak (PVL), a surrogate marker for VLS in mice.13,16 However, mutations to this LDV sequence also caused a significant decrease in the specific cytotoxicity of the IT against tumor cells.17 Therefore, mutations were made to amino acids that were spatially near the “VLS” site. One recombinant (r) RTA mutant, rRTA-N97A reduced PVL induced by RFB4-rRTA but did not reduce the cytotoxic activity of this IT either in vitro or in SCID mice xenografted with a human lymphoma cell line.17 This IT must now be tested in humans.

The third potential approach to decreasing VLS is to reduce the in vivo half-life of ITs in order to prevent their prolonged contact with vasculature. We previously reported that an IT constructed with the Fab’ fragment of the RFB4 mAb had a shorter T1/2 and a higher MTD in humans than an IT prepared with the IgG of RFB4. The former was cleared very rapidly and gave even better clinical responses,9,19 but it was expensive to prepare. Therefore, another approach was used to reduce the T1/2. This approach involved chimerization and modification of the Fc region to shorten the T1/2.

The catabolism of IgG is controlled by the interaction of IgG with the neonatal Fc receptor (FcRn) that is expressed predominantly in vascular endothelial cells.20 Once internalized by the cells, IgG molecules bind to FcRn at the slightly acidic pH of the endosomes and they are then returned to the cell membrane where they are released into the circulation. If IgG molecules do not bind to FcRn, they are destroyed in the lysosomes.21 When a single mutation (H435A) was introduced into the FcRn binding site of chimeric RFB4 (cRFB4), the affinity of the mutant was not detectable and the T1/2 was decreased by approximately 2.6-fold.22 Furthermore, the T1/2 of a rRTA-containing IT prepared with mcRFB4 was approximately 2.3-fold shorter than the T1/2 of an IT prepared with cRFB4. The cytotoxicities of the two ITs were equivalent. These results demonstrated the feasibility of producing mAbs with mutations in and around the FcRn binding site that do not bind to FcRn and, when used to construct ITs, have reduced T1/2 without loss of cytotoxicity.

In this study, we generated a panel of mcRFB4s that contained mutations in or around the FcRn binding site in a search for mcRFB4s that had shorter T1/2 than our previous mutants. These new mcRFB4 had decreased T1/2 in mice. Their stability in vitro and in vivo was not affected. Therefore, ITs were prepared with two mutants, mcRFB4-P247W and mcRFB4-H310A, and the wild-type cRFB4 by conjugating them to rRTA. Compared with cRFB4-rRTA, the mutant ITs had reduced T1/2 in mice, but equivalent cytotoxicity in vitro. The mcRFB4-H310A-rRTA, which had the shortest T1/2, induced less PVL in mice compared with both the cRFB4-rRTA and the murine RFB4-rRTA.
Discussion

An IT constructed with the Fab’ fragment of the murine mAb, anti-human CD22 mAb, RFB4, exhibited a shorter $T_{1/2}$ in humans than the same IT prepared with the RFB4 IgG due to the lack of an Fc:FcRn interaction.9,18-21 It also had a higher MTD and gave better clinical responses in patients with B cell lymphoma. However, it was expensive to prepare and yields were low. Our goal in this study was to produce an IT constructed with a short-lived mcRFB4 IgG that would have a decreased $T_{1/2}$ and reduced toxicity in vivo. Therefore, using site-directed mutagenesis, we constructed five mcmAbs with mutations near the FcRn binding site on the heavy chain of the anti-CD22 cRFB4 mAb. These mcmAbs were then used to produce rRTA-containing ITs. The stability, $T_{1/2}$, cytotoxicity and systemic toxicity of the mAbs and ITs were evaluated.

The major findings of this study are: (1) a new single amino acid mutation, P247W, plays a role in the FcRn binding; (2)
cRFB4 and all the mcRFB4 mutants had comparable stability in vitro in mouse serum and in vivo in mice; (3) single substitutions of Fc residues on the FcRn binding sites were sufficient to eliminate detectable binding to FcRn and to reduce T1/2s in mice. Combinations of these mutations did not further reduce T1/2; (4) mcRFB4-rRTA ITs had shorter T1/2s in mice than the cRFB4-rRTA IT; (5) the in vitro cytotoxicity of mcRFB4-rRTA ITs on CD22+ Daudi cells was not affected by mutations to the FcRn binding sites and (6) compared with murine RFB4-rRTA and cRFB4-rRTA, mcRFB4-H310A-rRTA induced less PVL in mice.

The Fc residues involving the FcRn binding have been well documented.27-29 These include amino acids at positions 253, 310 and 435. Substitution of these residues significantly changed the affinity for FcRn and the half-life of IgG as demonstrated in the present study and other reports.23-25,28-30 Other amino acids around the direct Fc:FcRn interaction sites may also affect the affinity for FcRn and have been identified as candidates for mutagenesis to find IgG molecules with increased affinity for FcRn and prolonged half-life.25,31-35 Our results demonstrated that the substitution of Pro247 with a tryptophan significantly reduced the affinity for FcRn (data not shown) and reduced the T1/2 without affecting the stability. Therefore, Pro247 is a new candidate for mutagenesis to prepare mAbs with altered T1/2.

Our previous study demonstrated that a single substitution of His435 of the cRFB4 mAb with alanine diminished the binding affinity for FcRns and resulted in a shorter T1/2 in mice.22 In this study, we confirmed that mutations that eliminate detectable binding to FcRn result in shorter T1/2s. We have also investigated whether multiple mutations had an additive or synergistic effect by using the mcRFB4-AAA, e.g., mcRFB4-I253A/H310A/H435A, triple mutant. Although the mcRFB4-AAA triple mutant had a shorter T1/2 than that of mcRFB4-I253A and mcRFB4-H435A, its T1/2 was not shorter than the T1/2 of mcRFB4-H310A. It has been reported that a scFv-Fc mAb with a single H310A or H435Q mutation had a decreased T1/2, while the H310A/H435Q double mutant exhibited a synergistic effect.29,30 This might be due to the difference in the molecular mass of the mAb constructs (scFv-Fc vs. whole IgG) and the substitution at position 435 (glutamate vs. alanine). In addition, the mcRFB4-AAA construct showed similar in vivo stability with other constructs, while the stability of the H310A/H435Q was not reported.

The in vivo stabilities of mAbs with mutations to the FcRn binding region have not been documented. In this study, we radiolabeled the mcRFB4 constructs with 125I and evaluated their in vitro stability in mouse serum using radio-autographs and in vivo stability using size-exclusion chromatography. The results demonstrated that the in vitro and in vivo stability of cRFB4 and all mcRFB4 constructs were comparable. Thus, the mutagenesis performed in this study did not affect stability and the mAbs in circulation remained intact.

This study confirmed the results of previous studies that ITs have shorter T1/2s than their parental mAbs.22 The conjugation of rRTA to mAbs using SMPT as a cross-linker modifies their PK in part due to nonspecific interactions of RTA with non-FcRn receptors, such as α2-macroglobulin.36 The shorter T1/2 is not a result of the modification of the mAb molecule by the SMPT cross-linker used to prepare the IT.37 The Fc:FcRn interaction was not affected when rRTA was attached to mAbs as reported previously since the relative affinities for the FcRn of an IT constructed with rRTA and either cRFB4 or a triple mutant of cRFB4, T307A/E380A/N434A, were similar to the affinities of the two parental mAbs.22

Short-lived mAbs have shown advantages in cancer imaging and therapy. Murine mAbs have been used for conjugation to toxins or radioisotopes since they have short T1/2s in humans and thus may cause less damage to normal tissues.8-10,18,19 As another approach to shorten T1/2, ITs were constructed from small fragments of mAbs devoid of the murine Fc.19,38-43 Studies with these ITs in humans demonstrated that their T1/2s were approximately 25% of the T1/2 of ITs made with the full length mAb.44-46 These Fab’ ITs were not extensively tested to determine if their higher MTD led to a higher response rate. It is unknown if the tumor-to-background ratio of the Fab’ IT was different from those constructed with the whole IgG. Other studies have shown that reduction of T1/2 can lead to a higher tumor-to-background ratio.29,30 Our data showed that the mcRFB4-H310A-rRTA conjugate, which had the shortest T1/2, exhibited reduced toxicity in mice when compared with cRFB4-rRTA IT. This suggests that
when this IT is injected into mice xenografted with tumor, the excess non tumor bound IT should be quickly eliminated and a higher tumor-to-background ratio should be achieved.

In summary, this study demonstrates that fully active mcmAbs and mcMab-rRTA ITs with decreased serum $T_{1/2}$ and toxicity can be constructed by altering residues on or around Fc:FcRn interaction sites. Further studies will be designed to determine the in vivo anti-tumor efficacy of the short-lived mcRFB4 ITs using SCID mice xenografted with CD22+ human B lymphoma cells.

### Materials and Methods

#### Construction, expression and purification of recombinant chimeric mAbs.

We previously chimerized and expressed cRFB4 mAb in murine myeloma Sp2/0 cells. To increase the expression level, the VH and VL genes of RFB4 were amplified by polymerase chain reaction (PCR) and inserted sequentially in-frame into plZDHL, a new vector that expresses chimeric IgG1k at high levels. The new cRFB4 expression plasmid was transfected into dihydrofolate reductase-deficient Chinese hamster ovary (CHO/DHFR) cells (ATCC, Cat#CRL-9096) using Lipofectamine™ LTX reagent (Invitrogen, Cat#15338-100). Stable transfecants were selected in IMDM media (Sigma, Cat#13390) supplemented with 10% dialyzed FBS (Invitrogen, Cat#26400-036). Supernatants from each cloned cell line were tested for presence of cRFB4 by human Ig specific enzyme linked immunosorbent assays (ELISAs).

To construct mcRFB4 vectors, the gene encoding the heavy chain of cRFB4 was inserted into the pGEM-T-Easy vector (Promega, Cat#A1360). Mutation was performed by PCR using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Cat#200523) and confirmed by DNA sequencing. The primers used for mutation were as follows: (1) P247W forward, 5'-CTT CCT CTT CCC CCC AAA ATG GAA GGA CAC CCT C-3'; (2) P247W backward, 5'-GAG GGT GTC CTT CCA TTT TGG GGG GAA GAG GAA G-3'; (3) I253A forward, 5'-CAA GGA CAC CCT CAT GGC CTC CCG GAC CCC TGA G-3'; (4) I253A backward, 5'-CTC AGG GGT CCG GGA GGC CAT GAG GGT GTC CTT G-3'; (5) H310A forward, 5'-CAA GGA CAC CCT CAT GGC CTC CCG GAC CCC TGA G-3'; (6) H310A backward, 5'-CTC AGG GGT CCG GGA GGC CAT GAG GGT GTC CTT G-3'; (7) H435A forward, 5'-GA GA GGT CCG GGA GGC CAT GAG GGT GTC CTT G-3'; (8) H435A backward, 5'-GCT CCT CTG GCT GAG GGT GTC CTT G-3'.

#### Table 1. Pharmacokinetics of cRFB4 constructs in Swiss Webster mice

| mAbs             | $T_{1/2}$ (H) | AUC (H x ng/mL) | MRT (H) | FCR (day$^{-1}$) |
|------------------|--------------|-----------------|---------|------------------|
| cRFB4            | 263.7 ± 17.0 | 27,468 ± 1,627  | 375.6 ± 24.5 | 0.063 ± 0.004   |
| mcRFB4-P247W     | 106.4 ± 4.2  | 9,640 ± 475     | 144.9 ± 6.2 | 0.156 ± 0.006   |
| mcRFB4-I253A     | 58.1 ± 3.0   | 5,185 ± 104     | 72.9 ± 3.7  | 0.286 ± 0.015   |
| mcRFB4-H310A     | 39.0 ± 0.9   | 4,280 ± 139     | 47.8 ± 1.2  | 0.425 ± 0.01    |
| mcRFB4-H435A     | 59.7 ± 6.0   | 5,018 ± 510     | 72.2 ± 8.4  | 0.280 ± 0.03    |
| mcRFB4-AAA       | 45.7 ± 1.0   | 4,228 ± 145     | 53.3 ± 0.5  | 0.362 ± 0.008   |

$T_{1/2}$, half life (beta); AUC, area under the curve; FCR, fractional catabolic rate; MRT, mean residence time. Groups of five mice were used; two experiments were performed. There are statistically significant differences between all pairs of mAbs (p < 0.001) with the exception of the mcRFB4-I253A vs. mcRFB4-H435A pair.

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**Figure 3.** Autoradiograph of murine and chimeric RFB4 constructs on SDS gels. $^{125}$I-labeled mAbs were incubated for 24 h with mouse serum at 37°C and then 4–15% SDS-PAGE was performed under non-reducing (A) or reducing (B) conditions. Lane 1, murine RFB4; lane 2, cRFB4; lane 3, mcRFB4-P247W; lane 4, mcRFB4-I253A; lane 5, mcRFB4-H310A; lane 6, mcRFB4-H435A; lane 7, mcRFB4-AAA. This is one of three experiments.
Figure 4. Analysis of the stability of cRFB4 constructs in mouse serum in vivo. (A) cRFB4: time 0 h; (B) cRFB4: time 24 h; (C) mcRFB4-H310A: time 0 h; (D) mcRFB4-H310A: time 24 h. Black line: measurement of radioactivity in cpm.; red line: measurement of the absorbance at 280 nm. IgM, immunoglobulin M; IgG, immunoglobulin G; Alb, albumin. This is one of two experiments.
Supernatants from these clones were purified on Protein G-Sepharose (GE Healthcare, Cat#17-0618-04). After washing with phosphate buffered saline (PBS), pH 7.4, the bound proteins were eluted with 0.1 mol/L glycine-HCl buffer, pH 2.8.

Heavy chain mutants were then excised and used to substitute the wild-type heavy chain in the cRFB4 expression plasmid. The resulting plasmids were transfected into CHO/DHFR cells and cloned as described above.

**Figure 5.** 4–15% SDS-PAGE of murine and chimeric RFB4 ITs under non-reducing conditions. Lane 1, RFB4-rRTA; lane 2, cRFB4-rRTA; lane 3, mcRFB4-P247W-rRTA; lane 4, mcRFB4-H310A-rRTA. This is one of three experiments.

**Figure 6.** Antigen-binding activity of the ITs vs. the corresponding cmAbs as determined by FACS. (A) (▲) cRFB4; (●) cRFB4-rRTA; (B) (▲) mcRFB4-P247W; (●) mcRFB4-P247W-rRTA; (C) (▲) mcRFB4-H310A; (●) mcRFB4-H310A-rRTA. This is one of three experiments.
The eluted mAbs were dialyzed against PBS, filter-sterilized and stored at 4°C. The purity of mAbs was analyzed by SDS-PAGE and size-exclusion HPLC chromatography.

Preparation of ITs. Recombinant RTA was prepared as previously described in reference 45. cRFB4 or mcRFB4s were chemically conjugated to rRTA using 4-succinimidyl-oxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene (SMPT; Thermo Scientific, Cat#21558) as a cross-linking reagent and then purified. Briefly, SMPT, dissolved in dimethylformamide (Sigma, Cat#D227056), was added to a solution of antibody (5 mg/mL), to give a final molar ratio of linker:antibody of 5:1. After incubation for 1 h at room temperature, the derivatized protein was then mixed with reduced rRTA and then purified. Briefly, SMPT, dissolved in dimethylformamide (Sigma, Cat#D227056), was added to a solution of antibody (5 mg/mL), to give a final molar ratio of linker:antibody of 5:1. After incubation for 1 h at room temperature, the derivatized protein was purified by passing the solution through a column of Sepharose CL-6B (GE Healthcare, Cat#17-0962-25) followed by Blue Sepharose CL-6B (GE Healthcare, Cat#17-0962-25) followed by Superdex 200 (GE Healthcare, Cat#17-1043-02).

Radiolabeling. mAbs and ITs were radiolabeled with Na\(^{125}\)I (GE Healthcare, Cat#25004781) using iodination reagents (Thermo Scientific, Cat#28600) as described in reference 22. The free \(^{125}\)I was removed by centrifugation on MicroSpin G-25 columns (GE Healthcare, Cat#27-5345-01). The specific radioactivity of the labeled proteins was in the range of 5 x 10\(^{6}\) cpm/µg with less than 5% free Na\(^{125}\)I.

SDS-PAGE. The molecular weights and purity of the ITs were determined using 4–15% SDS-PAGE (GE Healthcare, Cat#17-0678-01) under non-reducing conditions. The gel was stained with PhastGel\(^{TM}\) Blue R (GE Healthcare, Cat#17-0518-01).

Cells. CD22\(^+\) Daudi cells (ATCC, Cat#CCL-213) were maintained in culture by serial passages in RPMI-1640 (Sigma, Cat#R8758) containing 10% heat-inactivated fetal bovine serum (HyClone, Cat#HY-ME-SH30070-02) and 2 mM L-glutamate (Sigma, Cat#G7513). The cells were grown in a humidified atmosphere of 5% CO\(_2\) and air. Cell viability was determined by trypan blue exclusion.

Antigen-binding activity of mAbs and ITs. The binding of mAbs and ITs to Daudi cells was evaluated using an indirect immunofluorescence assay. 1 x 10\(^{6}\) cells/100 µL were incubated with various amounts of mAbs and ITs ranging from 10\(^{-8}\) to 10\(^{-12}\) M, for 30 min at 4°C. After the cells were washed with PBS containing 0.01% sodium azide, they were incubated with FITC-labeled goat anti-human IgG (Fc specific) (Sigma, Cat#F9512) for 30 min at 4°C in the dark (0.25 µg FITC-antibody/100 µL/1 x 10\(^{6}\) cells). The samples were analyzed on Becton Dickinson FACS. The percent of fluorescent cells was plotted against the mAb or IT concentration and the concentration at which 50% of the cells were fluorescent was extrapolated from the graph.

Measurement of the relative affinity of mAbs for FcRn. Recombinant human and rat FcRns were radioiodinated and then incubated with the cRFB4 and mcRFB4 mAbs. The mAb-FcRn mixture was added to human or mouse IgG-Sepharose for separation and quantitation of bound and unbound radioiodinated mAb as described in reference 22.

PK analysis. Six–eight weeks old female BALB/c (Taconic) or Swiss Webster mice (Taconic) were used for PK analysis. Lugal solution (Sigma, Cat#L6146) was added to their drinking water to a concentration of 0.05% from 1 d prior to injection throughout the entire period of the experiment (168 h). Radiolabeled proteins were injected into the tail veins of mice in a volume not larger than 100 µL and whole body radioactivity was measured daily in an AtomLab 100 dose calibrator (Atomic Product Corporation). A computer program kindly provided by Dr. K. Vyas from Merck Sharp and Dohme Research Laboratory was used for the calculation of all pharmacokinetic parameters.

Analysis of the stability of cRFB4 and mcRFB4 mAbs. The in vitro stability of cRFB4 mAbs was analyzed by autoradiography. Sera from ten female Swiss Webster mice were pooled. Ten microliters of this serum was incubated with 2 µL of radiolabeled mAb at a specific radioactivity of 50,000 cpm/µL for 24 h under sterile conditions at 37°C. After incubation, the samples were boiled under non-reducing conditions at 100°C for 4 min. One microgram samples were loaded on a PhastGel high density gel designed for peptides with molecular weight between 1,000 and 20,000 (GE Healthcare, Cat#17-0679-01). The gel was electrophoresed using PhastSystem apparatus using the program designed for the PhastGel high density gel. The gel was stained with PhastGel Blue R. Autoradiography was performed by exposing blue basic autoradiographic film (BioExpress, Cat#F-9023) to the gel.

To test in vivo stability, radiolabeled mAbs in a volume of 100 µL were injected into the tail veins of 6–8 week old female Swiss Webster mice (Taconic). Mice were bled via their tail veins using heparinized capillary tubes at time 0 (1 min) and at 3, 6, 24, 48, 72 and 120 h after injection. A volume of 50 µL blood was diluted in 250 µL PBS and centrifuged at 4,000 RPM for 10 min at RT. Two hundred microliters of diluted sera was removed and analyzed by high performance liquid chromatography.

| ITs | IC\(_{50}\) (M) |
|-----|-------------|
| RFB4-rRTA | 1.8 x 10\(^{-12}\) |
| cRFB4-rRTA | 1.6 x 10\(^{-12}\) |
| mcRFB4-P247W-rRTA | 1.1 x 10\(^{-12}\) |
| mcRFB4-H310A-rRTA | 1.2 x 10\(^{-12}\) |

*Results of one experiment of three experiments performed.

**Table 2. Cytotoxicity of ITs on CD22\(^+\) Daudi tumor cells**

\[\text{[H]-thymidine incorporation.}\] The cytotoxicity of ITs against CD22\(^+\) cancer cells was determined by \([\text{H}]-\text{thymidine incorporation.}\) Daudi cells in complete medium (5 x 10\(^4\) cells/100 µL) were plated in 96-well plates and incubated for 20 h at 37°C with 100 µL of different concentrations of the ITs ranging from 10\(^{-9}\) to 10\(^{-12}\) M. After incubation, the cells were pulsed for 4 h with 1 µCi [methyl-\(^{3}\)H]-thymidine (PerkinElmer, Cat#NET027005MC), harvested and counted in a liquid scintillation spectrometer. \([\text{H}]-\text{thymidine incorporation at each IT concentration was compared with a baseline control value and the percent reduction at each concentration of IT was used to quantitate the cytotoxic effect, expressed as the IC\(_{50}\).}\)
measured and mice were sacrificed. Left lungs were harvested, weighed and the radioactivity of the fluid in the left lung was measured as described previously in reference 17.

Statistical tests. Two tailed t tests were used to evaluate the statistical significances of differences found in the comparisons described above. Values <0.05 were considered statistically significant.

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Supplemental Material

Supplemental material can be found at: www.landesbioscience.com/journals/mabs/article/18348

Table 3. Pharmacokinetics of ITs and their corresponding mAbs in BALB/c mice

| mAb or IT | T₁/₂ (H)       | AUC (H x ng/mL) | MRT (H)  | FCR (day⁻¹) |
|-----------|----------------|-----------------|----------|--------------|
| cRFB4     | 340.7 ± 22.9   | 47,399 ± 2,369  | 490.5 ± 32.3 | 0.049 ± 0.003 |
| mcRFB4-P247W | 183.1 ± 6.5 | 22,759 ± 824    | 261.8 ± 9.4  | 0.091 ± 0.003 |
| mcRFB4-H310A | 60.5 ± 1.7   | 8,131 ± 280     | 84.6 ± 1.9   | 0.274 ± 0.008 |
| cRFB4-rRTA | 139.7 ± 9.2   | 12,848 ± 652    | 193.8 ± 13.0 | 0.119 ± 0.008 |
| mcRFB4-P247W-rRTA | 83.6 ± 6.4 | 6,160 ± 166     | 106.8 ± 8.0  | 0.199 ± 0.016 |
| mcRFB4-H310A-rRTA | 54.4 ± 3.1   | 4,010 ± 232     | 62.3 ± 4.4   | 0.305 ± 0.017 |

*Groups of five mice were used; two experiments were performed. †There is a statistically significant difference between all pairs.

Figure 7. Pharmacokinetics of ITs and their corresponding mAbs in BALB/c mice.

(HPLC) using a TSK guard column followed by a preparative TSK-G3000SW equilibrated with PBS at pH 6.85 (Tosoh Bioscience LLC). One milliliter samples were collected and the absorbance at 280 nm and radioactivity of the samples were measured. The serum IgM and albumin peaks were identified by comparison of elution times of the samples with elution times of proteins of known molecular weights (MWs) ranging from 900-12 kDa (Sigma, Cat#MS170 and MWGF200). The corresponding radioactive counts were overlaid on the graphs of the OD₂₈₀ measurements to estimate to MWs of the iodinated protein peaks.

PVL assay in mice. Six-to-eight weeks old female BALB/c mice (Charles Rivers Labs) were used for PVL assays. Lugol solution was added to their drinking water to a concentration of 0.05% from 1 d prior to injection and throughout the entire period of the experiment (168 h). ITs at the doses of 2.5, 5.0 and 7.5 μg/g body weight/day were injected i.p daily at a constant volume of 480 μL from day one through day three. On day 3, ¹²⁵I-albumin was injected i.v. and whole body radiation was immediately measured. After 24 h, whole body radioactivity was measured and mice were sacrificed. Left lungs were harvested, weighed and the radioactivity of the fluid in the left lung was measured as described previously in reference 17.

Statistical tests. Two tailed t tests were used to evaluate the statistical significances of differences found in the comparisons described above. Values <0.05 were considered statistically significant.

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Supplemental Material

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Figure 8. Changes in the body weight of BALB/c mice after treatment with various doses of ITs. (A) 2.5 mg/kg; (B) 5 mg/kg; (C) 7.5 mg/kg; (■) RFB4-rRTA; (□) cRFB4-rRTA; (▲) mcRFB4-H310A-rRTA. This is one of two experiments.
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