HIGHERLY POTENT CD22-RECOMBINANT RICIN A RESULTS IN COMPLETE CURE OF DISSEMINATED MALIGNANT B-CELL XENOGRAFTS IN SCID MICE BUT FAILS TO CURE SOLID XENOGRAFTS IN NUDE MICE

Peter J. Van Horsen, Frank W.M.B. Preijers, Ypke V.J.M. Van Oosterhout, and Theo De Witte

Department of Hematology, University Hospital St. Radboud, Nijmegen, The Netherlands.

The highly specific cytotoxic action of ribosome-inactivating protein (RIP) containing immunotoxins (ITs) makes IT therapy a promising approach to eliminating residual malignant cells. We investigated the cytotoxicity of the IT CD22-recombinant ricin A to the B-cell-line Ramos in vitro and in vivo. Cytotoxicity of CD22-recombinant ricin A in vitro was very high as expressed by the very low 50% inhibition dose (ID50) of 3.5 × 10⁻¹¹ M. Cytotoxicity was increased 7 times in the presence of the cytotoxicity enhancer NH4Cl. The ultimate kill of Ramos cells by CD22-recombinant ricin A was high (2.7-log kill) and was increased strongly in the presence of NH4Cl (4.2-log kill).

Anti-tumor activity in vivo was investigated by i.v. treatment of solid s.c. Ramos xenografts in nude BALB/c mice. A single dose did not inhibit tumor growth. Treatment on 5 consecutive days resulted in evident tumor reduction. In one mouse, tumor could no longer be detected on the 6th day after starting treatment. However, after 8 days tumor volumes increased again. Anti-tumor activity was more pronounced in a disseminated tumor model in SCID mice. IT treatment (i.v.) 7 days after i.v. inoculation with Ramos cells resulted in cure of all mice.

Non-specific toxicity was low. Alanine aminotransferase (ALT) levels in serum were elevated temporarily. Serum values of γ-glutamyl transferase (γ-GT), bilirubin and creatinin did not change. Body weight was also transiently reduced. The LD50 in SCID mice after i.v. administration was high (0.626 mg IT per mouse). The clearance rate in SCID mice, as determined by ELISA, was biphasic.

In order to develop an effective treatment with IT of patients suffering from B-cell malignancies, a suitable tumor-model must be studied. CD22 MAbs bind to the B-lymphocyte adhesion molecule (Bl-CAM) expressed on the surface of B cells from the pro-B-cell stage to the mature B-cell stage (Kehrl et al., 1994). After binding, the Bl-CAM/IT complex is rapidly internalized and Bl-CAM is re-expressed (Van Horsen et al., 1995; Shih et al., 1994), indicating its potential as target for IT therapy. Many investigators have shown, in vitro and in vivo, that CD22 ITs have strong anti-B-cell potency (Ghete et al., 1988; Shen et al., 1988; Kreitman et al., 1993; Van Horsen et al., 1995). This potency has been shown in clinical phase I trials with CD22-deglycosylated ricin A in over 100 patients with B-cell lymphoma (Vitetta et al., 1991; Amlot et al., 1993; Sausville et al., 1995).

We investigated the efficacy of CD22 coupled to recombinant ricin A to the Burkitt lymphoma cell line Ramos in vitro and in vivo. IT treatment of solid SC tumors in nude mice and of disseminated tumors in SCID mice was compared in different administration schedules, in order to study the influence of the accessibility of tumor cells. We treated the SCID mice after clear establishment of the tumor cells. The survival and pharmacokinetics and toxicity of CD22-recombinant ricin A were evaluated.

MATERIAL AND METHODS

Cell lines

The Burkitt lymphoma cell line Ramos was cultured in medium consisting of RPMI 1640 (Flow, Irvine, UK) supplemented with 10% heat-inactivated FCS (Integro, Zaandam, The Netherlands), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Flow) in a humidified incubator with 5% CO₂ in air at 37°C. Cells were maintained in log phase.

Animals

Seven- to eight-week-old nude BALB/c (BALB/c nu/nu) mice (male; University of Nijmegen, The Netherlands) and 7- to 9-week-old SCID (C.B.-17/ICR/Scid-scid) mice (male; Bomholtgård Breeding and Research Centre, Ry, Denmark) were used. Mice were housed in filtercap cages (microisolators) placed in laminar air flow (LAF) cabinets. Mice were fed irradiated food and acidified drinking water. All manipulations with the animals were performed in the LAF cabinets.

1To whom correspondence and reprint requests should be addressed, at Department of Hematology, University Hospital St. Radboud, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: 31 24 354 2080.

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Monoclonal antibody (MAb)  
The murine MAb CD22 (CLB-B-ly/1; IgG1; CLB, Amsterdam, The Netherlands) was produced in a hollow-fibre bioreactor and was purified from the supernatant by affinity chromatography using staphylococcal protein A-Sepharose (Pharmacia, Uppsala, Sweden).

Immunoconjugate preparation  
CD22 was coupled to recombinant ricin A chain (generously provided by Zeneca, Macclesfield, UK) by means of 4-succinimidyl-4′-maleimidocarbonyl-methyl-α-[2-pyridyldithio]toluene (SMPT; synthesized by the Department of Organic Chemistry, University of Nijmegen, The Netherlands) according to Thorpe et al. (1987). Briefly, 10 mg CD22 (in 1 ml 25 mM sodium borate, pH 9) was treated with a 10-fold molar excess of SMPT (110 μg of 1 mg/ml SMPT solved in N,N-dimethylformamide; DMF, Merck, Darmstadt, Germany) with gentle rotation during 1 hr at 20°C. Free SMPT and DMF were removed by gel filtration on a Sephadex G25 (Pharmacia, Uppsala, Sweden) column. To introduce a free sulfhydryl group, ricin A was incubated in 50 mM DTT for 30 min at 20°C. DTT was removed by gel filtration on the G25 column. The modified MAb was incubated with a 2.5 molar excess ricin A for 72 hr at 20°C, resulting in a coupling ratio (ricin A:MAb) of 1:1. Free ricin A was separated from the IT by gel filtration on a Sephacryl S200 High Resolution (Pharmacia) column.

Protein-synthesis-inhibiting activity of CD22-recombinant ricin A  
Cells (10^5) in culture medium were incubated with various concentrations of CD22-recombinant ricin A (10^-13 to 10^-8 M) in the presence or absence of 6 mM NH4Cl in a final volume of 200 μl. Incubation was performed in 96-well microtiter plates (Costar, Cambridge, MA) in triplicate for 24 hr at 37°C. Subsequently, 0.5 μCi [3H]leucine (Amersham, Aylesbury, UK) was added. After 24 hr, cells were harvested and the radioactivity was measured. Cytotoxicity was expressed as the percentage of [3H]leucine incorporation with regard to untreated cells corrected for non-specific incorporation determined by incubating cells in the presence of 1 mM cycloheximide (Boehringer, Mannheim, Germany).

Cell kill by CD22-recombinant ricin A  
Cell kill was determined according to a method described by Van Oosterhout et al. (1994). Briefly, cells were incubated with or without 10^-8 M CD22-recombinant ricin A in the presence and absence of 6 mM NH4Cl for 24 hr. Thereafter, cells were washed and incubated in fresh culture medium for 3 days. Then, 45 min before flow-cytometric analysis (Epics Elite, Coulter, Hialeah, FL) propidium iodide (PI; 20 μg/ml) was added to stain dead cells, and calcein (20 μg/ml) was added to stain living cells. Inert beads (5 × 10^5/ml, DNA-Check, Coulter) were added to permit quantification of cell kill. In the samples, the number of living cells was quantified by simultaneously measuring an appropriate number of beads. The number of living cells per 1000 beads of a treated sample was compared with the number of living cells per 1000 beads in the control sample, to calculate the factor of depletion.

Pharmacokinetics of CD22-recombinant ricin A following i.v. administration  
IT (60 μg) in 0.1 ml PBS was injected into the tail vein of SCID mice. After 5, 10, 15 and 30 min, and 1, 2, 4, 8, 16, 24, 48 and 72 hr, blood samples were taken by orbital puncture. After clotting for one hour, serum was separated and stored at -20°C. Serum concentrations of CD22-recombinant ricin A were determined in an enzyme-linked immunosorbent assay (ELISA).

ELISA for determination of serum levels of CD22-recombinant ricin A  
An ELISA slightly different from that described by Calvete et al. (1993) was used to quantify CD22-recombinant ricin A concentrations in serum. Briefly, 96-well flat-bottomed plates (Costar) were coated (16 hr at 4°C) with 100 μl rabbit anti-ricinus communis lectin (Sigma, Poole, UK) diluted 1:500 in PBS. Thereafter the plates were blocked with PBS containing 1% (w/v) gelatin (blocking buffer). After 30 min incubation at room temperature, plates were washed 5 times with PBS containing 0.05% (w/v) Tween-80. Plates were incubated for 1 hr with 100 μl serum samples (pre-diluted 20 times in PBS) diluted 1:2 step wise in blocking buffer. Each plate contained 7 samples and one control sample, starting at 0.9 μg/ml (4 × 10^-9 M). After washing 5 times, 100 μl of horseradish-peroxidase-labelled goat-anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) 1:500 diluted in blocking buffer, was added for another hour. After washing, the colour reaction was performed with the tetramethyl-benzidine (TMB) Microwell Peroxidase Substrate System (Kirkegaard and Perry, Gaithersburg, MD) and stopped after 5 min with 100 μl N H2NO3 the A450 nm was then determined (EAR 400 Easy reader Titertek, SLT, Groedig/Salzburg, Austria).

Establishment of the Ramos xenograft tumor model in nude mice and IT therapy  
Ramos cells were washed and re-suspended at 10^6 cells/ml in PBS. Cells were injected (0.1 ml) s.c. and the mice were monitored daily for developing tumors. Tumor volume was measured by sliding callipers (volume = 1/2 × length × width × height) (Tomayko and Reynolds, 1989). Treatment was started when tumor volume was 100 mm^3. Mice were treated i.v. with 30 μg IT once or 5 times on consecutive days. Control mice were injected with PBS or 25 μg IT. Each group consisted of 5 mice. Statistical significance was evaluated by Student's t-test.

Establishment of the Ramos xenograft tumor model in SCID mice and IT therapy  
Ramos cells were prepared as described for nude mice. Mice were inoculated i.v. with 10^6 cells 7 days before treatment. The control mice (10 mice) were injected with PBS. Mice were treated i.v. with 30 or 60 μg IT once or 5 times with 30 or 60 μg IT on consecutive days (5 mice per group). One group was treated with 250 μg CD22 MAb. Mice were observed daily for the onset of posterior paralysis and killed at occurrence. The mean paralysis time (MPT) was used to express efficacy of treatment. Statistical significance was evaluated by the 2-tailed log-rank test.

Toxicity of IT therapy  
SCID mice administrated once or 5 times with 30 or 60 μg (3 mice per group) were observed for IT toxicity. At days 3, 7, 12 and 21 after starting treatment, 200 μl blood was collected by orbital puncture. After clotting for 1.5 hr, serum was isolated and renal and hepatic enzymes were determined with the Hitachi 747 (Boehringer). Creatinin levels were determined to examine renal activity and bilirubin, ALAT and γ-GT for hepatic damage. Body weight of all mice was determined at least 3 times a week at the beginning of the experiment, once a week at the end of the experiment.

Determination of LD50  
SCID mice were injected i.v. with 0.47, 0.60, 0.77 and 1.0 mg IT and observed for death. The LD50 was calculated according to Weil (1952).

RESULTS  
Cytotoxicity of CD22-recombinant ricin A  
Treatment of Ramos cells with CD22-recombinant ricin A resulted in strong inhibition of protein synthesis. Cytotoxicity,
expressed by ID$_{50}$ was $3.5 \times 10^{-11}$ M (Fig. 1). In the presence of 6 mM of the enhancer NH$_4$Cl, which enhances the activity of ricin A ITs in ex vivo treatment of bone marrow, the ID$_{50}$ was $5.0 \times 10^{-12}$ M (Fig. 1). IT concentrations of $10^{-9}$ M or higher resulted in complete inhibition both in the absence and in the presence of NH$_4$Cl.

Although inhibition of protein synthesis is informative regarding IT activity, it does not reflect the ultimate cell kill. Therefore we determined elimination of cells by IT in a quantitative flow cytometric assay. Cells were incubated during 24 hr with $10^{-8}$ M IT. After a retention period of 3 days, samples were analyzed. The number of eliminated target cells was determined by comparing the number of living cells (calcine-positive and PI-negative) in the treated sample and the control sample for each 1000 beads added to the samples. Beads were detected separately from the cells by flow cytometry. Treatment of Ramos cells with CD22-recombinant ricin A resulted in a 2.7-log kill in the absence and a 4.2-log kill in the presence of NH$_4$Cl (Table 1).

### Establishment and treatment of an s.c. Ramos xenograft in nude BALB/c mice

To determine the anti-tumor activity of CD22-recombinant ricin A in vivo, $10^7$ Ramos cells were injected s.c. in nude BALB/c mice. The time period to detect tumor growth varied among mice. Tumors developed only in 64% of the mice. Treatment with CD22-recombinant ricin A was started when tumor volume was approximately 100 mm$^3$. In the first experiment, mice were treated i.v. with 30 μg IT, 25 μg CD22 MAb or PBS as control (Fig. 2a). Increase of the mean tumor volume proved that a single administration of 30 μg IT or 25 μg CD22 MAb had no significant effect on tumor growth. From each group, one Ramos xenograft was isolated and cells were assessed for CD22 antigen expression and susceptibility to CD22-recombinant ricin A IT. Cells isolated from treated and untreated xenografts expressed comparable CD22 antigen but lower amounts, as compared with cells in a parallel in vitro culture. However, cytotoxicity of CD22-recombinant ricin A to the xenograft-derived Ramos cells was higher than to the cells in culture, resulting in an approximately one log lower ID$_{50}$ (data not shown).

In a second experiment, mice were treated on 5 consecutive days with PBS or 30 μg IT (total dose 150 μg). Anti-tumor activity was more pronounced. From day 3, tumor growth was significantly reduced ($p < 0.05$). Not only was tumor growth inhibited but even tumor size was temporarily reduced (Fig. 2b). Out of 5 mice, 1 had no detectable tumor during the first 2 days after IT treatment. Tumors started to grow again on the third or fourth day after treatment.

### Cytotoxicity of CD22-recombinant ricin A to Ramos
treated in SCID mice

To determine the blood clearance rate of CD22-recombinant ricin A in SCID mice, after injection of 30, 60 or 5 × 30 or 60 μg IT liver and renal enzymes in serum and total body weight were determined in follow-up. Creatinin, bilirubin and γ-GT levels did not change but ALAT levels were temporarily elevated and normalized within 3.5 weeks (data not shown). Body weight decreased depending on the dose used. It was reduced 18% in the highest dose group (5 × 60 μg), 9% in the group administered with 5 × 30 μg and 5% or less in the groups tested with lower doses. The reduction lasted 9 weeks in the group injected with the highest dose (5 × 60 μg) to 3 days in the group injected with the lowest dose (30 μg IT) (Fig. 4).

### Pharmacokinetics of CD22-recombinant ricin A in SCID mice

To determine the blood clearance rate of CD22-recombinant ricin A, SCID mice were injected i.v. with 60 μg IT. After varying periods of time, blood was isolated and the IT concentration was determined in serum by ELISA, as described in “Material and Methods”. The decrease of IT in serum could be distinguished in 2 phases (Fig. 5). The clearance of IT was fast during the first 16 hr (T$_{1/2}$a is 12.1 hr) and slowed down thereafter (T$_{1/2}$b is 53.6 hr). After injection of 60 μg IT, the IT concentration in serum after 72 hr was still more than $10^{-6}$ M (Fig. 5).
CURE OF SCID MICE BY CD22-RECOMBINANT RICIN A

In this study we showed the high cytotoxic potency of CD22-recombinant ricin A to the B-cell line Ramos, with ID_{50} values of 3.5 \times 10^{-11} M in the absence and 5.0 \times 10^{-12} M in the presence of 6 mM NH_{4}Cl. Previously we have shown that CD22-recombinant ricin A was fast-acting and that the cytotoxic potency of CD22-recombinant ricin A depends on intracellular processing rather than on antigen density and the number of internalized molecules (Van Horssen et al., 1995). Therefore CD22-recombinant ricin A is highly cytotoxic even to cells that express low numbers of antigens (Van Horssen et al., 1995).

ID_{50} values do not predict the elimination of target cells. Therefore we determined the effective cell kill induced by IT treatment in a quantitative flow cytometric assay. Treatment of Ramos cells with 10^{-8} M CD22-recombinant ricin A resulted in a 2.7-log elimination in the absence and a 4.2-log elimination in the presence of NH_{4}Cl. This means that only 1 out of every 15,400 cells survived. These results showed that the
Many investigators have shown that CD22 ITs have strong anti-B-cell potency (Ghetie et al., 1988; Shen et al., 1988; Kreitman et al., 1993; Van Horssen et al., 1995). In mice, i.p. treatment with CD22 IT prevented or caused complete regression of measurable SC tumors, but failed to induce cure (Kreitman et al., 1993). CD22-deglycosylated ricin A treatment i.v. 24 hr after i.v. inoculation of tumor cells resulted in a more pronounced anti-tumor effect. Time before onset of paralysis was significantly postponed, but mice were not cured (Ghetie et al., 1991, 1992, 1994). These results gave rise to phase I studies in man, in which partial responses and one complete response were obtained (Vitetta et al., 1991; Amlot et al., 1993). In another phase I study, continuous infusion of CD22-deglycosylated ricin A resulted in 4 partial and 3 minor responses (Sausville et al., 1995).

To compare the in vivo efficacy of CD22-recombinant ricin A with compact growing and disseminated malignant B-cells, we used 2 xenograft model systems. In a nude-mice model, Ramos cells were injected s.c., resulting in a solid tumor. Tumor take was successful in only 64% of the animals, and the onset of detection varied extensively. This may have been due to the remaining immunologic system in the nude mice. After establishment of the tumor, the growth rate was comparable between individual mice, indicating that the immunologic system was completely suppressed in the tumor-bearing mice. Treatment with IT resulted in 4 partial responses and one complete response when the mice were treated 5 times with 30 \( \mu \)g IT on consecutive days. However, tumor volumes increased 3 days after the last IT administration.

The potency of CD22-recombinant ricin A to eliminate disseminated malignant B-cells was studied in a SCID-mice model. This model mimics well B-cell leukemias in humans. Mice injected i.v. with Ramos developed disseminated disease, resulting in paralysis of the hind legs (Ghetie et al., 1990). Tumor take was 100% in this model, with a small variation in paralysis time. MAb treatment postponed the MPT, and IT treatment with doses as low as 30 \( \mu \)g cured all mice.

The better results of CD22-recombinant ricin A treatment of disseminated tumors as compared with those for treatment of solid tumors may be explained by differences in the number of tumor cells at the moment of starting treatment or in accessibility of the cells. The large size of ITs constructed with intact MAbs may result in reduced penetration in a solid tumor (Dedrick and Flessner, 1989). Smaller CD22 IT, containing antibody fragments coupled to a truncated form of Pseudomonas exotoxin, have been shown to be more effective in eradicating solid tumors in spite of the lower affinity due to the monovalent binding (Kreitman et al., 1993). The higher affinity of intact MAb ITs may make them superior for elimination of easily accessible target cells (Ghetie et al., 1991). In contrast, fragmented ITs have higher tumor-penetration capacity, resulting in greater elimination of solid tumors (Kreitman et al., 1993). In the SCID model, target cells home mainly on to blood, bone marrow and lymphoid tissues. The IT, which is also administered i.v., may more easily reach those cells than cells in a poorly vascularized solid tumor.

The blood clearance of CD22-recombinant ricin A was determined in SCID mice. The pharmacokinetics were biphasic, with a \( T_{1/2\alpha} \) of 12.1 and a \( T_{1/2\beta} \) of 53.6 hr. These half-life times were much longer than IT half-life times in mice found by other investigators (Fulton et al., 1988). The \( T_{1/2\alpha} \) of these ITs ranged from 11 to 51 minutes and the \( T_{1/2\beta} \) varied from 4 to 22 hours (Fulton et al., 1988). The differences in \( T_{1/2\alpha} \) may be explained by the type of ricin A chain that was used. We used aglycosyl recombinant ricin A, while Fulton et al. (1988) used native or deglycosylated ricin A. As a consequence, our IT would be less rapidly cleared by the liver, due to the differences in glycosylation of the ricin A moiety. The stability of the IT affects the \( T_{1/2\alpha} \). We used the heterobifunctional linker SMP, which contains a hindered disulphide bond, while Fulton et al. (1988) used SPDP, which lacks this hindrance. The enhanced in vivo stability of ITs produced with SMP was showed by Thorpe et al. (1987). Long half-lives (\( T_{1/2\alpha} 2.8 \) and \( T_{1/2\beta} 33 \) hr) were also found for the IT ICI-D0490 constructed with the same recombinant ricin A and the methyl-hindered linker N-succinimidyl 3-(2-pyridylidithio)-3-methylproprionate (Calvete et al., 1993).

Treatment appeared to be hardly toxic, even with the highest dose (5 \( \times \) 60 \( \mu \)g), suggesting that high doses of CD22-recombinant ricin A can be administered. This may improve the outcome of therapy. The long half-life may be an explanation for the low toxicity, due to lower accumulation of IT in the liver, which in mice is a target for IT toxicity. In man, however, prolonged contact of endothelial cells with ricin A would be less rapidly cleared by the liver, due to the differences in glycosylation of the ricin A moiety. The stability of the IT affects the \( T_{1/2\alpha} \). We used the heterobifunctional linker SMP, which contains a hindered disulphide bond, while Fulton et al. (1988) used SPDP, which lacks this hindrance. The enhanced in vivo stability of ITs produced with SMP was showed by Thorpe et al. (1987). Long half-lives (\( T_{1/2\alpha} 2.8 \) and \( T_{1/2\beta} 33 \) hr) were also found for the IT ICI-D0490 constructed with the same recombinant ricin A and the methyl-hindered linker N-succinimidyl 3-(2-pyridylidithio)-3-methylproprionate (Calvete et al., 1993).

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We conclude that the CD22-recombinant ricin A IT used in our study is highly cytotoxic in vitro and in vivo in a model of disseminated growing tumor cells. However, cells in solid tumors are less well eliminated, which may be due to the lower penetrating capacity of intact MAb IT. The clearance of the IT from the blood and the non-specific toxicity are low. Therefore we propose that the CD22-recombinant ricin A IT used in our study is promising for in vivo treatment of patients suffering from B-cell leukemias and lymphomas. Efficacy of treatment
might be improved by a combination with fragmented CD22 IT that has higher tumor-penetrating capacity.

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