HIGHLY 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 Horssen1, 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 aspartate aminotransferase (AST) and alkyltransferase (α-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.

Residual malignant cells in autologous bone-marrow transplants, as well as in patients who suffer from leukemia and lymphoma, are responsible for relapse after transplantation. The ability to kill malignant cells specifically renders therapy with ribosome-inactivating protein (RIP) containing ITs an excellent treatment to be applied in addition to chemotherapy, radiotherapy and/or surgery. ITs consist of a targeting compound and a toxin (Preijers, 1993).

Ricin, a plant lectin, is the toxic agent most frequently used to construct ITs. Ricin occurs naturally as a heterodimer composed of the cytotoxic A chain that is linked by a disulphide bond to a galactose-residue-binding B chain (Lord et al., 1994). Substitution of the B chain by a specific targeting compound such as a monoclonal antibody (MAb) gives rise to specific cell kill by the ricin A chain. Disulphide-bridge-containing heterobifunctional linkers are often used to couple the MAb to the ricin A chain. After binding and internalization of the IT, the enzymatic ricin A chain is translocated to the cytosol. By cleaving the N-glycosidic bond of a single adenosine residue of 28S rRNA of the 60S ribosomal sub-unit (Endo and Tsurugi, 1988), ricin A catalytically and irreversibly inhibits protein synthesis of eukaryotic cells (Lord et al., 1994) and causes cell death.

The potency of ITs depends on several factors, such as the kind of antigen, the antigen density and number of internalized IT molecules (Preijers et al., 1988) and the intracellular routing and processing (Van Horssen et al., 1995). Additional factors influencing in vivo efficacy are the IT half-life, the dose-limiting non-specific toxicity, anti-IT reactions (Ghetie and Vitetta, 1994) and accessibility of the target cells. The large size of ITs may reduce the passage through the vascular endothelium into the interstitial space of solid tumors (Dedrick and Flessner, 1989).

IT treatment may be limited by non-specific toxicity of ricin A, which in man appears to be vascular leak syndrome (VLS) (Ghetie and Vitetta, 1994). In mice, VLS does not occur, due to resistance of murine endothelial cells to ricin A (Soler-Rodriguez et al., 1993). Toxicity in mice is expressed by changes in hepatic and renal characteristics and loss of body weight. The LD₅₀ for IgG-ricin A ITs varies between 0.30 and 0.72 mg IT/25 g mouse (Ghetie et al., 1991, 1992; Blakey et al., 1988).

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 Horssen 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 (Ghetie et al., 1988; Shen et al., 1988; Kreitman et al., 1993; Van Horssen et al., 1995). This potency has been shown in clinical phase I trials with CD22-deglycoesylated 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 μM 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/IcrBom-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.

Received: June 3, 1996 and in revised form July 18, 1996.
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).

Immunotoxin preparation

CD22 was coupled to recombinant ricin A chain (generously provided by Zeneca, Macclesfield, UK) by means of 4-succinimidyl-4′-[2-pyridyldithio]butyric acid (SUPT; 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 reacted with a 10-fold molar excess of SMPT (110 μM 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). 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 of 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 (10⁶) 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 x 10⁶/ml, Delaware, 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, 6, 12, 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) and 1:2 step wise in blocking buffer. Each plate contained 7 samples and one control sample, starting at 0.9 μg/ml (4 x 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 N₂H₄NO₂; 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 10⁶ 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 = ½ x length x width x height) (Tomayko and Reynolds, 1989). Treatment was started when tumor volume was 100 mm³. 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 10⁶ 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 administered 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 ID₅₀, was 3.5 × 10⁻¹¹ M (Fig. 1). In the presence of 6 mM of the enhancer NH₄Cl, which enhances the activity of ricin A ITs in ex vivo treatment of bone marrow, the ID₅₀ was 5.0 × 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 NH₄Cl.

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 for 24 hr with 10⁻⁴ M NH₄Cl. Assays were performed in triplicate. SD < 10%.

**Figure 1** - Cytotoxicity of CD22-recombinant ricin A to Ramos determined in a protein-synthesis-inhibition assay in the absence (○) and in the presence (□) of 6 mM NH₄Cl. Assays were performed in triplicate. SD < 10%.

In order to study in vivo treatment of a hematological tumor that is more comparable with a disseminated tumor in patients, we developed a model in SCID mice. Mice were inoculated i.v. with 10⁷ Ramos cells. Initially, we tested variation in tumor growth resulting in paralysis of the hind legs in all mice. Paralysis occurred in one mouse 22 days after and in the other 5 mice 23 days after inoculation of the Ramos cells. To determine the anti-tumor effect of CD22-recombinant ricin A, mice were treated i.v. 7 days after inoculation. They received PBS, 250 μg CD22 MAb, 30 or 60 μg IT once or 5 times 30 or 60 μg IT on consecutive days. The mean paralysis time (MPT) of the control group was 30.1 ± 2.5 days whereas the MPT of the group treated with the MAB was significantly increased (p = 0.045) to 36.4 ± 1.1 days (Fig. 3). Even with the lowest IT dose, treatment of the mice resulted in cure of all mice. The experiment was ended 175 days after the inoculation with Ramos cells.

**Establishment and treatment of an i.v. Ramos xenograft in SCID mice**

To determine the anti-tumor activity of CD22-recombinant ricin A in vivo, 10⁷ Ramos cells were injected s.c. in nude BALB/c mice. The time period to detect tumor growth varied among mice. Tumors developed in 64% of the mice. Treatment with CD22-recombinant ricin A was started when tumor volume was approximately 100 mm³. 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 ID₅₀ (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.

**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, 10⁷ Ramos cells were injected s.c. in nude BALB/c mice. The time period to detect tumor growth varied among mice. Tumors developed in 64% of the mice. Treatment with CD22-recombinant ricin A was started when tumor volume was approximately 100 mm³. 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 ID₅₀ (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.

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 (T½ is 12.1 hr) and slowed down thereafter (T½ is 53.6 hr). After injection of 60 μg IT, the IT concentration in serum after 72 hr was still more than 10⁻⁸ M (Fig. 5).

**Table 1** - Number of living cells per 1000 counted beads

<table>
<thead>
<tr>
<th></th>
<th>−NH₄Cl</th>
<th>+6 mM NH₄Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control sample</td>
<td>295357</td>
<td>215611</td>
</tr>
<tr>
<td>Treated sample</td>
<td>603</td>
<td>14</td>
</tr>
</tbody>
</table>

**Log kill**

<p>| |</p>
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
</tr>
<tr>
<td>4.2</td>
</tr>
</tbody>
</table>

Non-specific toxicity of IT therapy was investigated in SCID mice. After injection of 30, 60 or 5 x 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 x 60 μg), 9% in the group administrated with 5 x 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 x 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 (T½ is 12.1 hr) and slowed down thereafter (T½ is 53.6 hr). After injection of 60 μg IT, the IT concentration in serum after 72 hr was still more than 10⁻⁸ M (Fig. 5).
CURE OF SCID MICE BY CD22-RECOMBINANT RICIN A

A

B

tumorvolume (% of day 0)

days after starting treatment

2000
1600
1200
800
400
0

0 5
10
15
20

800
400
0

0 5
10
15
20

days after starting treatment

Figure 2 – Treatment of a SC Ramos xenograft in nude mice. Ramos cells (10^7) were injected s.c. and mice were treated when the tumor volume was approximately 100 mm^3. Mice were treated with PBS (●), 25 μg CD22 (■) or 30 μg CD22-recombinant ricin A (○) (a) and with PBS (●) or 5 × 30 μg CD22-recombinant ricin A (○) (b). All groups consisted of 5 mice. Tumor volume was determined daily. Difference between the control and the IT-treated group was significant (p < 0.05), starting on day 3.

LD50 of CD22-recombinant ricin A in SCID mice

The median lethal dose was determined in mice that were injected i.v. with varying doses IT. The LD50 was calculated according to the method of Weil and appeared to be 0.626 mg IT (0.104 mg ricin A) per mouse (25 g). Occasionally, the LD50 of IT in mice is determined after i.p. injection. Therefore experiments were repeated with i.p. injection of the IT. These mice survived the 2 highest doses that were injected i.v. (0.77 and 1.0 mg), and that killed them all. This indicates that the LD50 of i.p.-injected IT is more than 1.0 mg.

DISCUSSION

In this study we showed the high cytotoxic potency of CD22-recombinant ricin A to the B-cell line Ramos, with ID50 values of 3.5 × 10^{-11} M in the absence and 5.0 × 10^{-12} M in the presence of 6 mM NH4Cl. 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).

ID50 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 NH4Cl. This means that only 1 out of every 15,400 cells survived. These results showed that the

FIGURE 2 – Treatment of a disseminated Ramos xenograft in SCID mice. Ramos cells (10^7) were injected i.v. 7 days prior to i.v. treatment with PBS (●), 250 μg CD22 (■) or once or 5 times with 30 or 60 μg IT (○). All groups consisted of 5 mice, except the PBS-treated group (10 mice). Mice were monitored daily for occurrence of paralysis. The experiment represents one of 3 reproducible experiments.

FIGURE 3 – Influence of IT treatment on the body weight of SCID mice. Body weight of mice was determined almost daily after administration of 30 (■), 5 × 30 (□), 60 (●) or 5 × 60 (○) μg CD22-recombinant ricin A.
Figure 5 - Blood clearance of CD22-recombinant ricin A in SCID mice. Mice were injected i.v. with 60 μg CD22-recombinant ricin A (each group, n = 5). Blood was obtained after varying time periods and the concentration CD22-recombinant ricin A was determined in serum by ELISA, as described in “Material and Methods”. The pharmacokinetics of CD22-recombinant ricin A is biphasic, with a $T_{1/2\alpha}$ of 12.1 hours and a $T_{1/2\beta}$ of 53.6 hr.

strong protein-synthesis inhibition of CD22-recombinant ricin A results in effective cell elimination.

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 Horsen 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 mouse models. 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 μg 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 μg 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 $T_{1/2\beta}$. We used the heterobifunctional linker SMPT, 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 SMPT 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-pyridyldithio)-3-methylpropionate (Calvete et al., 1993).

Treatment appeared to be hardly toxic, even with the highest dose (5 x 60 μg), 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 will increase the chance of VLS. The LD$_{50}$ is comparable or even higher than the LD$_{50}$ found by other investigators (Ghetie et al., 1991, 1992; Blakely et al., 1988). However, we determined the LD$_{50}$ after i.v. administration of IT, whereas other LD$_{50}$ values were determined after i.p. injection. When our IT was injected i.p., even the highest dose (1 mg) did not result in the death of mice, indicating that recombinant ricin A IT is much less toxic than ITs prepared with native or deglycosylated ricin A.

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...
ACKNOWLEDGEMENTS

We thank the veterinary technicians Mr. J. Koedam, Ms. B. Van de Weem, Mr. A. Hanssen, Ms. A. Verleg, Ms. H. Janssen and Mr. W. Wilms for their help in animal handling and for care of the mice. We also thank the Department of Organic Chemistry, University of Nijmegen, The Netherlands, for synthesizing SMPT. This work was supported in part by grants from the Ank van Vlissingen Foundation and the Maurits and Anna de Kock Foundation.

REFERENCES


WELL, C.S., Tables for convenient calculation of median effective dose (LD50 or ED50) and instructions in their use. Biometrics, 8, 249–252 (1952).