Improved Antitumor Effects of Immunotoxins Prepared with Deglycosylated Ricin A-Chain and Hindered Disulfide Linkages

Philip E. Thorpe, Philip M. Wallace, Philip P. Knowles, Michele G. Reif, Alex N. F. Brown, Graham J. Watson, David C. Blakey, and David R. Newell

Drug Targeting Laboratory, Imperial Cancer Research Fund, P. O. Box 123, Lincoln’s Inn Fields, London WC2A 3PX, England

ABSTRACT

A monoclonal anti-Thy-1.1 antibody (OX7) was coupled to either native or chemically deglycosylated ricin A-chain (dgA) using one of two different cross-linking agents. One cross-linker, N-succinimidyl-2-pyridylcarbonylethylcarbonyl-o-(2-pyridylthio)toluene (SMPT), generates a sterically hindered disulfide bond which is relatively resistant to reduction, whereas the other, 2-iminothiolane hydrochloride, generates an unhindered disulfide bond with greater lability. A two-compartment pharmacokinetic model was used to analyze the blood levels of each immunotoxin and its breakdown product (free antibody) after i.v. injection into mice. Immunotoxins prepared with SMPT broke down in vivo 6.3-fold more slowly than those prepared with 2-iminothiolane hydrochloride, and immunotoxins. This antibody was cleared 2- to 3-fold more rapidly from the bloodstream than those containing dgA. As a result, 24 h after injection, 16% of the OX7-SMPT-dgA remained in the blood as compared with 0.4 to 2.5% of the other immunotoxins. Immunotoxins prepared with dgA were about 3-fold more toxic to mice than those prepared with native A-chain, whereas immunotoxins prepared with SMPT were only slightly more toxic than those prepared with 2-iminothiolane hydrochloride. When equivalent toxic doses of the immunotoxins were administered i.v. to mice which had been given injections of Thy-1.1* AKR-A/2 lymphoma cells, the OX7-SMPT-dgA gave the best antitumor effect. A dose equivalent to one-seventh of the median lethal dose of the IT-A was a significant improvement in the therapeutic index of the immunotoxins in AKR-A/2 tumor-bearing mice.

INTRODUCTION

Recent studies on the fate of antibody-ricin A-chain immunotoxins in rodents have demonstrated that their therapeutic activity is diminished by rapid elimination from the bloodstream (1-3). Less than 1% of the injected IT-A remains in the bloodstream 24 h after i.v. administration as compared with more than 30% of native antibody. The rapid elimination is due to a combination of two factors, namely entrapment by cells of the liver and breakdown of the IT-A.

Hepatic entrapment of IT-As occurs because liver cells have receptors for the mannosse- and fucose-terminating oligosaccharides that are present on the A-chain moiety (4-6). In the rat, the liver cells which take up ricin A-chain are in the nonparenchymal cell fraction and are predominantly reticuloendothelial cells (7), whereas in the mouse, both the parenchymal and the nonparenchymal cells are involved (5, 8). To overcome this problem we developed a method for chemically modifying the mannosic and fucose residues on the A-chain (9). ITs prepared with deglycosylated ricin A-chain (IT-dgAs) show greatly reduced liver entrapment (1, 8), improved tumor localization (10), and enhanced antitumor effects in vivo (11).

Breakdown of ITs occurs because the two most commonly used disulfide crosslinking agents, SPDP and 2IT, generate bonds between the antibody and the A-chain which are prone to reduction in vivo (1, 3, 12-15). This instability leads to two problems: (a) there is less intact IT available to kill the tumor cells; (b) the released antibody can compete with the IT for binding to tumor cells and reduce the antitumor effect (16). The problem of instability cannot be surmounted by using a non-disulfide bond to form the IT-A because release of the A-chain by reduction within the cytoplasm appears to be necessary for cytotoxicity (17). We therefore synthesized a new coupling agent, SMPT, which introduces a sterically hindered disulfide linkage in which a methyl group and a benzene ring protect the disulfide bond from reduction (18). ITs prepared with SMPT have improved stability in vivo and, importantly, show no loss of cytotoxic activity in vitro. A similar coupling agent, SPDB, producing a disulfide bond with an adjacent methyl group but lacking a benzene ring has been described by Worrell et al. (19).

In the present study, we have compared the pharmacokinetics and antitumor activity of IT-As and IT-dgAs prepared with SMPT or 2IT. Our results show that the antitumor activity of the ITs in a mouse T-cell lymphoma model is significantly improved both by using the more stable coupling agent and by deglycosylating the A-chain. Although the new IT constructs are more toxic to mice, the improvement in their antitumor activity significantly exceeds the increase in their toxicity and hence the new ITs have increased therapeutic indices.

MATERIALS AND METHODS

Materials. Tissue culture medium (RPMI 1640) and fetal calf serum were purchased from Gibco-Biocult, Ltd. (Paisley, Scotland). Microplates with 96 flat-bottomed wells were purchased from Flow Laboratories (Irvine, Scotland). Sodium [125I]-iodide (IMS 30) and L-[4,5-3H]leucine (TRK 170) were purchased from Amersham International (Amersham, England). The Iodo-Gen reagent for protein iodination was obtained from Pierce (U.K.), Ltd. (Chester, England).

Sephacryl S-200, Sepharose 4B, Sephadex G-25 (fine grade), and Blue Sephadex CL-6B were purchased from Pharmacia, Ltd. (Milton Keynes, England). 2IT was purchased from Sigma Ltd. (Poole, England). All other reagents were of analytical grade.
Antibodies. The hybridoma cell line, MRC OX7, secreting a mouse IgG1 subclass antibody to the Thy-1.1 antigen, was kindly provided by Dr. A. F. Williams (MRC Cellular Immunology Unit, University of Oxford). Details of its derivation have been published by Mason and Williams (20). The hybridoma cell line, LICR-LON-R10, secreting a mouse IgG1 subclass antibody to human glycophorin was kindly supplied by Dr. P. A. W. Edwards (Ludwig Institute, Sutton, England). Monoclonal antibodies, OX7 and R10, were purified from the blood and ascitic fluid of hybridoma-bearing BALB/c mice by the method of Mason and Williams (20).

Tumor Cells. The Thy-1.1+ AKR-A lymphoma cell line was obtained from Professor I. MacLennan (Department of Experimental Pathology, Birmingham University, Birmingham, England). It was recloned to remove a mutant subpopulation which was resistant to ITs prepared using the SPDP reagent but sensitive to ITs prepared using the 2IT reagent (12). The recloned line is designated AKR-A/2.

Purification of A-Chain. Crushed castor beans (Ricinus communis) from Central Africa were a gift from Croda Premier Oils, Ltd., Hull, England. Ricin was extracted from the beans by the method of Cumber et al. (21). The toxin was split by reduction into its component chains and the A-chain was extensively purified by the method of Fulton et al. (22). The LD50 of the A-chain in BALB/c mice was 30 mg/kg.

Preparation of dgA. A solution of ricin (2.5 mg/ml) in 0.2 M sodium acetate buffer, pH 3.5, was treated for 1 h at 4°C with sodium metaperiodate and sodium cyanoborohydride at final concentrations of 10 and 20 mM, respectively, as described by Thorpe et al. (9). The dgA was separated from the B-chain and extensively purified by the method of Fulton et al. (22). The deglycosylation procedure destroys approximately 50% of the mannose and most of the fucose residues present on the A-chain. The N-acetylglycosamine and most of the xylose residues are unaffected (23). Deglycosylation does not affect the amino acid composition of the A-chain or reduce its ability to inhibit protein synthesis in a cell-free system (9). The LD50 of the dgA in BALB/c mice was 15 mg/kg.

Preparation and Purification of ITs. Native A-chain and dgA were linked to OX7 or R10 antibody by means of two different coupling agents, SMPT and 2IT. Full details of the procedures for synthesizing the SMPT reagent and using it to form ITs have been published previously (18), as have those for using 2IT (12).

Briefly, antibodies were treated for 1 h at room temperature with SMPT or with 2IT and Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) to introduce an average of between 1.5 and 1.8 activated disulfide groups per molecule of protein. The derivatized antibodies were mixed with a 2.5-fold molar excess of freshly reduced native A-chain or dgA, concentrated to approximately 1.5 mg of total protein per ml, and allowed to react for 1 to 3 days at room temperature.

The ITs were purified by chromatography on columns of Sephacryl S-200 to remove free A-chain and material with a molecular weight >210,000. Chromatography on Blue Sepharose was then used to remove free antibody (24). As shown previously by polyacrylamide gel electrophoresis in SDS, the ITs consisted predominantly of a single component with a molecular weight of 180,000 corresponding to one molecule of antibody linked to one molecule of A-chain (1, 12, 18). Immunoprecipitation analyses confirmed that the M, 180,000 material seen on the gel contained both mouse immunoglobulin and A-chain (1).

The antibody components fully retained antigen-binding activity, as judged by FACS analyses on AKR-A/2 cells at saturating and sub-saturating concentrations. The A-chain components, after release from the antibody by reduction with diithiothreitol, fully retained their ability to inhibit cell-free protein synthesis in rabbit reticulocyte lysates (25).

The ITs have the structures shown in Fig. 1.

OXT-SMPT-A : \[
\text{NH-COCH}_3 \quad \text{CH}_2 \quad \text{A} \quad \text{man} \quad \text{luc}
\]

OXT-2IT-A : \[
\text{NH-COCH}_2\text{CH}_2\text{CH}_2SS \quad \text{A} \quad \text{man} \quad \text{luc}
\]

OX7-SMPT-dgA : \[
\text{NH-COCH}_3 \quad \text{CH}_2 \quad \text{A} \quad \text{man} \quad \text{luc}
\]

OX7-2IT-dgA : \[
\text{NH-COCH}_2\text{CH}_2\text{CH}_2SS \quad \text{A} \quad \text{man} \quad \text{luc}
\]

Fig. 1. ITs compared in this study.

then added to each culture and the radioactivity that the cells incorporated was measured 24 h later (25).

FACS Analyses. AKR-A/2 cells from tissue culture and tumor cells from the peritoneal cavity of mice were suspended at 2 x 10^7 cells/ml in PBS containing 5 mg/ml BSA (PBS-BSA). The suspensions were distributed in 100-μl volumes into 2-ml tubes. To each tube were added 100 μl of a 10% (v/v) solution of normal rabbit serum in PBS-BSA. The tubes were kept on ice for 30 min after which 100 μl OX7 or R10 antibody at 200 μg/ml were added. The contents of the tubes were mixed, and the tubes were kept on ice for 30 min. The cells were then washed twice and resuspended in 100 μl of a 5% (v/v) solution of fluorescein isothiocyanate-labeled F(ab’)2 fragments of rabbit anti-mouse IgG (Gibco-Biocult) in PBS containing 10% (v/v) normal rabbit serum. The cells were kept on ice for 30 min, washed twice, and finally resuspended in 1 ml PBS-BSA containing 0.02% (w/v) sodium azide.

A Becton-Dickinson FACS 440 was used to analyze 10,000 cells from each sample, and the results were stored in a Tektronix 4052 computer. The median fluorescence intensity of the cells incubated in R10 was subtracted from that of cells incubated in OX7, to obtain the median fluorescence intensity attributable to antigen binding; the difference was then expressed as a percentage of the median fluorescence intensity attributable to antigen binding in the parental AKR-A/2 line.

Blood Clearance Measurements. ITs, OX7, native A-chain, and dgA were iodinated with carrier-free ^125I to a specific activity of 10^7 cpm/μg with the use of the Iodo-Gen reagent according to the manufacturer’s recommended procedures. Groups of 3 to 5 adult male BALB/c mice weighing about 25 g each were given i.v. injections of 10 μg of the radiiodinated proteins. Samples of blood were drawn from the tail vein of the mice after various time intervals. The radioactivity in the bloodstream to yield a M, 150,000 component corresponding to free antibody (1, 12, 18). This was confirmed by immunoprecipitation experiments which demonstrated
The $M$, 150,000 component was unreactive with anti-ricin antibodies but reactive with anti-mouse immunoglobulin (1). Free A-chain or dgA was not observed probably because it is rapidly cleared (5).

The autoradiographs were scanned with a densitometer and the areas under the $M$, 180,000 and $M$, 150,000 peaks were divided by the total area under all the peaks to determine the proportion of radioactivity in the plasma that corresponded to intact IT and free antibody. Calibration experiments had previously shown that the area under each peak as determined by densitometry was directly proportional to the radioactivity it contained. Analysis of the ITs by SDS-polyacrylamide gel electrophoresis under reducing conditions showed that the specific activity of the released antibody was somewhat less ($9.1 \times 10^6$ cpm/µg) than that of the intact IT ($10 \times 10^6$ cpm/µg). These specific activities were then used to calculate the amount of the intact IT in the blood and the amount which had broken down to give free antibody at various times after injection. Blood levels were expressed as a percentage of the injected dose assuming that the mice had a blood volume of 2.18 ml/25 g body weight (26).

Pharmacokinetics. The levels of ITs, OX7, and A-chains in the plasma of individual animals were analyzed using the two-compartment open pharmacokinetic model shown in Fig. 2. The analysis was performed with the use of a computerized nonlinear least-squares regression analysis of the plasma concentration versus time data after injection of OX7. The AUC (OX7) levels were best described by the biexponential equation

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where $B$ is $C_0$ (the concentration at time 0) – $A$. These analyses yielded the half-lives of the materials in the $\alpha$ and $\beta$ phases of clearance and the concentration constants $A$ and $B$. The microscopic rate constant $K_{10}$ for the overall elimination of the injected IT was calculated using standard equations (29). The volume of distribution at steady state ($V_d$), the AUC, and the mean residence time were also calculated (30).

The rate at which ITs split up to give free antibody was determined as follows. The fraction of IT which had split up ($F_{spl}$) is given by the ratio of the AUC for AB at infinite time after administration of IT to the AUC for an equivalent molar dose of native OX7 antibody (30), i.e.,

$$F_{spl} = \frac{\text{AUC (AB)}}{\text{AUC (OX7)}}$$

The AUC (OX7) was calculated from the values generated by the nonlinear least-squares regression analysis of the plasma concentration versus time data after injection of OX7. The AUC (OX7) at infinite time is given by

$$\text{AUC (OX7)} = \frac{A}{\alpha} + \frac{B}{\beta}$$

The AUC (AB) was calculated by determining $\text{AUC}_{\infty}(AB)$ using

$$\text{AUC}_{\infty}(AB) = \frac{C_t}{\beta}$$

where $C_t$ is the plasma concentration of AB at the final time point $t$ and $\beta$ is the $\beta$ phase rate constant for OX7.

The microscopic rate constant, $K_{10}$, is the sum of all the rate constants for processes which result in irreversible loss of IT from the system. Thus

$$K_{10} = K_{spl} + K_{el}$$

where $K_{spl}$ is the rate constant for the splitting of IT to AB and $K_{el}$ is the sum of the rate constants for elimination of IT by processes other than splitting. The values of $K_{spl}$ and $K_{el}$ were calculated as follows: Since

$$F_{spl} = \frac{K_{spl}}{K_{10}}$$

then

$$K_{spl} = K_{10} \times \frac{\text{AUC (AB)}}{\text{AUC (OX7)}}$$

and

$$K_{el} = K_{10} - K_{spl}$$

The half-life with which the IT split up to AB is given by

$$t_{1/2, spl} = \log_2 \frac{K_{el}}{K_{spl}} = \frac{0.693}{K_{spl}}$$

The half-life with which the IT is eliminated by processes other than splitting is given by

$$t_{1/2, el} = \frac{\log_2}{K_{el}} = \frac{0.693}{K_{el}}$$

The above analysis makes three assumptions. Firstly, it assumes that the antibody released from the IT distributes and is eliminated at the same rate as native OX7. This assumption is validated by the fact that OX7 derivatized with 2IT or SMPT followed by reduction with dithiothreitol is cleared from the bloodstream at a rate identical to that of native OX7 and by the fact that the values of $K_{10}$ for AB released from ITs are identical to that of $K_{10}$ for native OX7 (results not shown). The second assumption is that conversion of IT to AB is a first order or pseudo-first order process. This would be true, for example, for glutathione-mediated splitting since the plasma levels of glutathione are in large excess over those of the IT (31). The finding that IT-As are stable in whole blood in vitro (1) does not conflict with this possibility because, in vitro, glutathione is very rapidly eliminated by processes not involving oxidation by molecular oxygen (31). Conversion of IT to AB would also be a first order or pseudo-first order process if it were enzyme mediated, provided that the enzyme obeyed Michaelis-Menten kinetics and that the levels of IT were well below the $K_m$. The third assumption is that the elimination of IT and the breakdown of IT to AB occur in the central compartment (i.e., in the blood, or in tissues in immediate contact with the blood).

Antitumor Experiments. Groups of 8–10 female AKR/c mice (Cumberland Farms, Tennessee) aged 8–12 weeks were given i.p. injections of 10³ AKR-A/2 cells from tissue culture. One day later, the animals were given i.v. injections of 0.33 nmol/25 g body weight of IT-As or IT-dgAs (containing 10 µg of A-chain or dgA) or with equivalent amounts of OX7, A-chain, or dgA. The animals were weighed three times a week to monitor i.p. tumor growth and were sacrificed for humane reasons when the animals' body weight had increased by 20% or more. The animals would have lived approximately 5 days longer if they had been allowed to die from their disease. The “survival” times...
DEGLYCOSYLATED IMMUNOTOXINS WITH STABLE LINKAGES

In another experiment, groups of 10 AKR/c mice were given cell injections as above and 1 day later were given an i.v. dose of IT corresponding to one-seventh of the LD50. The doses given per 25 g body weight were: 0.26 nmol OX7-SMPT-dgA (containing 8 µg dgA); 0.33 nmol OX7-2IT-dgA (containing 10 µg dgA); 0.83 nmol OX7-SMPT-A (containing 25 µg A-chain). Later, when the body weight of the animals had increased by 20% through tumor growth, the ascitic tumor was drained from the peritoneum. The tumor cells were analyzed for expression of the Thy-1.1 antigen by indirect immunofluorescence on the FACS and their sensitivity to ITs and ricin was determined in [3H]leucine incorporation assays as described above.

RESULTS

Toxicity of ITs to AKR-A/2 Lymphoma Cells in Vitro

ITs prepared by linking OX7 to native A-chain or dgA by means of the SMPT or 2IT coupling agents had identical cytotoxic activities in vitro. All four ITs reduced the [3H]leucine incorporation of AKR-A/2 cells by 50% at a concentration (the IC50) of 1.1–1.4 x 10^-12 M (Table 1). Thus strengthening the linkage in the IT and deglycosylating the A-chain did not reduce the cytotoxic potency of the ITs, in accordance with previous observations (1, 18). The ITs were about 20-fold more potent than ricin which had an IC50 of 2.5 x 10^-11 M.

The toxic effects were specific. Neither unconjugated OX7 nor control ITs prepared from the R10 antibody, which does not bind to the cells, were toxic at 10^-4 M. Unconjugated dgA and native A-chain had IC50 values of 10^-7 M.

Pharmacokinetics and Stability of ITs in Vivo

In Fig. 3 are shown the blood clearance curves for the different ITs in normal mice, and in Tables 2 and 3 are listed various pharmacokinetic parameters determined using a computerized two-compartment pharmacokinetic model. Four major findings emerged which confirm and extend prior observations (1, 3, 8, 10, 12, 15, 18).

1. IT-dgAs prepared with SMPT or 2IT were cleared from the bloodstream less rapidly than the corresponding IT-As. The half-lives (t1/2)el for the elimination of ITs by routes other than splitting were 5.2–8.9 h for the IT-dgAs and 1.3–2.2 h for the IT-As (Table 3). Twenty-four h after injection the amounts of the IT-dgAs in the blood exceeded those of the corresponding IT-As by 6–9-fold (Fig. 3; Table 2). As shown previously (1, 8), the slower clearance of the IT-dgAs is due to the fact that deglycosylation of the A-chain prevents sugar-mediated recognition and entrapment of the ITs by cells in the liver and elsewhere which have receptors for the oligosaccharides present on ricin A-chain. In support of this, the volumes of distribution (Vd) of the IT-As at steady state (151–237 ml/kg) were greater than those of the IT-dgAs (78–116 ml/kg) and native OX7 (98 ml/kg) suggesting that the IT-As distribute into tissues whereas the distribution of IT-dgAs and OX7 is restricted to the blood and extracellular fluids.

2. The IT-As and IT-dgAs prepared with SMPT broke down in vivo to free antibody 6.3 times more slowly than the corresponding ITs prepared with 2IT (Table 3). The half-lives of splitting of the ITs prepared with SMPT were 43–49 h as compared with 7–8 h for the ITs prepared with 2IT. Because of the reduced tissue entrapment of the IT-dgAs, a greater fraction of the injected dose was available for splitting than of the IT-As. The fraction of the injected IT-dgAs that split up was 2.5–3-fold greater than that of the IT-As (Table 3).

3. Strengthening the linkage in the ITs increased the AUC to an extent slightly less than that of deglycosylating the A-chain (Table 2). The AUCs of the IT-As and IT-dgAs prepared with SMPT were 2.0–2.4-fold higher than those of the corresponding ITs prepared with 2IT. The difference in the AUC of IT-dgAs versus the corresponding IT-As was 2.9–3.4-fold. Thus, OX7-SMPT-dgA, having the advantage of both the stable linkage and the dgA, had an AUC 7-fold higher than that of OX7-2IT-A which had neither advantage. This difference becomes more pronounced when comparing the actual levels of the two ITs in the blood at the 24-h and 48-h time points after injection, when OX7-SMPT-dgA was present at levels 40- and 130-fold higher than OX7-2IT-A, respectively.

4. The pharmacokinetic properties conferred on the ITs by dgA and SMPT were different. Deglycosylation elevated the blood levels of the ITs most markedly during the initial 24-h period after injection and thereafter had little effect, whereas the effect of strengthening the linkage was progressive with time and was most evident at later time points after injection (Fig. 3). This difference is apparent from the mean residence times of the ITs (i.e., the time point at which the AUC is divided into two equal portions). For example, the mean residence time of OX7-2IT-dgA was 8.1 h whereas that of OX7-SMPT-A was 16.7 h yet the former had a 1.5-fold greater AUC than the latter (Table 2).

![Graph](image-url)
DEGLYCOSYLATED IMMUNOTOXINS WITH STABLE LINKAGES

Table 2 Pharmacokinetic data for ITs, antibody and A-chains

<table>
<thead>
<tr>
<th>Material injection</th>
<th>( t_{1/2} ) (h)</th>
<th>( t_{1/3} ) (h)</th>
<th>A</th>
<th>B</th>
<th>( V_d ) (ml/kg)</th>
<th>Mean residence time (h)</th>
<th>AUC (( \mu g/ml \times h ))</th>
<th>% of blood level at 24 h (% of ( C_0 ) value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX7-SMPT-A</td>
<td>1.0 ± 0.2*</td>
<td>20.7 ± 2.7</td>
<td>94.5</td>
<td>5.5</td>
<td>237</td>
<td>16.7</td>
<td>28.3</td>
<td>1.7</td>
</tr>
<tr>
<td>OX7-SMPT-dgA</td>
<td>0.8 ± 0.1</td>
<td>20.9 ± 0.7</td>
<td>67.4</td>
<td>32.6</td>
<td>116</td>
<td>28.1</td>
<td>97.2</td>
<td>16.0</td>
</tr>
<tr>
<td>OX7-2IT-A</td>
<td>0.6 ± 0.1</td>
<td>7.4 ± 0.8</td>
<td>93.5</td>
<td>6.5</td>
<td>151</td>
<td>5.4</td>
<td>14.4</td>
<td>0.4</td>
</tr>
<tr>
<td>OX7-2IT-dgA</td>
<td>1.6 ± 0.3</td>
<td>8.3 ± 0.7</td>
<td>77.7</td>
<td>22.3</td>
<td>78</td>
<td>8.1</td>
<td>41.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Ricin-A-chain</td>
<td>0.08 ± 0.01</td>
<td>2.1 ± 0.3</td>
<td>88.2</td>
<td>11.8</td>
<td>219</td>
<td>2.4</td>
<td>4.4</td>
<td>N.D</td>
</tr>
<tr>
<td>dgA</td>
<td>0.11 ± 0.09</td>
<td>2.4 ± 0.3</td>
<td>83.6</td>
<td>16.4</td>
<td>171</td>
<td>2.9</td>
<td>6.7</td>
<td>N.D</td>
</tr>
<tr>
<td>OX7</td>
<td>3.0 ± 0.3</td>
<td>114 ± 2.3</td>
<td>60.0</td>
<td>40.0</td>
<td>98</td>
<td>158</td>
<td>641</td>
<td>38.0</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Table 3 Splitting and elimination rates of ITs

<table>
<thead>
<tr>
<th>Material injection</th>
<th>( % C_0 ) value</th>
<th>( F_{ad} )</th>
<th>( t_{adpl} ) (h)</th>
<th>( t_{ad} ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX7-SMPT-A</td>
<td></td>
<td>0.05 ± 0.01</td>
<td>42.5</td>
<td>2.2</td>
</tr>
<tr>
<td>OX7-SMPT-dgA</td>
<td></td>
<td>0.15 ± 0.01</td>
<td>49.1</td>
<td>8.9</td>
</tr>
<tr>
<td>OX7-2IT-A</td>
<td></td>
<td>0.16 ± 0.03</td>
<td>6.7</td>
<td>1.3</td>
</tr>
<tr>
<td>OX7-2IT-dgA</td>
<td></td>
<td>0.40 ± 0.06</td>
<td>7.8</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 4 Toxicity of ITs to mice

<table>
<thead>
<tr>
<th>IT</th>
<th>LD50 mg/25 g mouse</th>
<th>LD50 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX7-SMPT-A</td>
<td>1.05</td>
<td>42.0</td>
</tr>
<tr>
<td>OX7-SMPT-dgA</td>
<td>0.34</td>
<td>13.6</td>
</tr>
<tr>
<td>OX7-2IT-A</td>
<td>1.29</td>
<td>51.6</td>
</tr>
<tr>
<td>OX7-2IT-dgA</td>
<td>0.42</td>
<td>16.8</td>
</tr>
</tbody>
</table>

* i.p. route. Toxicity for the i.v. route was approximately twice as great.

Fig. 4. Antitumor effects of ITs at equal dosage. Groups of 8 to 10 AKR/c mouse were given i.p. injections of 10^8 AKR-A/2 cells (approximately 10^9 lethal doses) and 1 day later were given a single i.v. injection of 0.33 nmol of the following: OX7-SMPT-dgA (C), OX7-SMPT-A (☆), OX7-2IT-dgA (△), or OX7-2IT-A (△). Another group of animals received an equivalent volume of diluent alone (□). Analogous ITs prepared with R10, native A-chain, dgA, native OX7, and mixtures of unconjugated OX7 and A-chains were all without protective effect at equivalent dosage and are not shown. The two recipients of OX7-SMPT-dgA that died did not have peritoneal tumors and the cause of their death is unknown. The remaining mice were sacrificed when their body weight had increased by 20% as a result of tumor growth in the peritoneum.

L50 Determinations in Mice

The IT-dgAs were 3-fold more toxic to mice than the corresponding IT-A's. In contrast, the ITs prepared with SMPT were only 1.25-fold more toxic than the corresponding ITs prepared with 2IT (Table 4).

Antitumor Effects

The antitumor activity of the ITs was determined in AKR/c mice which had been given i.p. injections of 10^8 AKR-A/2 cells 1 day previously. The AKR/c mice express Thy-1.2 rather than Thy-1.1 on their T-cells and other tissues; thus the Thy-1.1 antigen expressed by the AKR-A/2 lymphoma cells is operationally a tumor-specific antigen in AKR/c mice.

In the first series of experiments, groups of mice were treated i.v. with 0.33 nmol of ITs (containing 10 µg of A-chain or dgA). The OX7-SMPT-dgA was the most powerful antitumor reagent (Fig. 4). None of the group of nine animals developed peritoneal tumors although two of the animals later died from unknown causes. (Deaths were probably not due to toxicity since normal animals given the same dose of IT all survived.) This suggests that the IT had destroyed virtually all the tumor cells originally injected since, in calibration experiments, it was established that just one cell injected i.p. is lethal. The OX7-SMPT-dgA was significantly more effective than OX7-2IT-dgA (\( P = 0.0001 \)) or OX7-SMPT-A (\( P = 0.02 \)) in which groups 8 of 9 and 6 of 9 animals developed peritoneal tumors, respectively.

The least effective IT was OX7-2IT-A which was significantly (\( P < 0.005 \)) inferior to all the other ITs. The two animals that died in the OX7-SMPT-dgA recipients were included as “events” in these calculations of statistical significance. Analogous differences were obtained when the experiment was repeated.

The antitumor effects of the ITs were specific since analogous ITs prepared with R10 (an antibody of irrelevant specificity) showed no antitumor activity. Similarly, equivalent doses of OX7 alone, native A-chain or dgA alone, or mixtures of OX7 and A-chain did not protect mice against tumor growth.

In order to compare the therapeutic indices of the three most effective ITs, one-seventh of the LD50 of OX7-SMPT-dgA, OX7-SMPT-A, or OX7-2IT-dgA was administered i.v. to mice which had received 10^8 AKR-A/2 cells 1 day previously. OX7-SMPT-dgA gave the best antitumor effect (Fig. 5a), extending the median survival time of the animals by 15 days. From calibration experiments performed as published previously (35), this corresponds to the eradication of 99.999% of the tumor cells originally injected. The antitumor effect of the OX7-SMPT-dgA was significantly better than that of OX7-2IT-dgA (\( P < 0.001 \)) which extended the median survival time of the animals by 4.5 days and resulted in no long term survivors. It was, however, significantly (\( P = 0.2 \)) better than that of OX7-SMPT-A which extended the median survival time of the animals by 5.5 days and resulted in 20% long term survivors.

The tumors which developed in mice treated with one-seventh of an LD50 of IT were analyzed for the expression of Thy-1.1 by indirect immunofluorescence and for sensitivity to the different ITs by in vitro cytotoxicity experiments. As shown in Table 5, all but one of the tumors that developed in the group
No obvious patterns emerged in the types of mutants that survived exposure to the different ITs or in the degree of their resistance. None of the mutants analyzed had greater sensitivity to one IT construct than another, showing, for example, that the mutants which survived exposure to the SMPT-linked ITs did not have an inability to split the SMPT linkage and release the A-chain within the cytosol.

DISCUSSION

The major finding to emerge from the present study is that the antitumor activity of an IT in a mouse T-cell lymphoma model is increased by using a more stable disulfide cross-linker, SMPT, to prepare the IT and by deglycosylating the A-chain to reduce hepatic entrapment.

A single i.v. injection of 0.3 nmol of OX7-SMPT-dgA into mice bearing peritoneal AKR-A/2 lymphoma cells eradicated 99.999% of the tumor cells whereas the same dose of OX7-2IT-A eradicated 99% of the tumor cells. Both the SMPT cross-linker and dgA contributed to the superior antitumor activity of OX7-SMPT-dgA. This was shown by the fact that ITs making use of either SMPT or dgA but not both had antitumor activities which were intermediate between those of OX7-SMPT-dgA and OX7-2IT-A.

Importantly, the antitumor activity of OX7-SMPT-dgA was significantly better than those of OX7-2IT-dgA and OX7-SMPT-A even when the ITs were administered to mice at doses representing an equal proportion (one-seventh) of the LD50. Thus the therapeutic index of the IT (i.e., the ratio of its efficacy to its toxicity to animals) was increased by using the SMPT cross-linker and dgA. The latter result is in agreement with the recent report by Fulton et al. (11) that the therapeutic index of Fab'-A ITs in mice is increased about 3-fold by deglycosylating the A-chain. In these studies, the advantages of IT-dgAs were evident even in mice with large tumor burdens. In several other studies, ITs prepared from ricin A-chain (e.g., Refs. 11 and 36-41) and other ribosome-inactivating proteins (e.g., Refs. 12, 15, 25, 35, and 42-46) have been demonstrated to have specific antitumor activity in murine leukemia and solid tumor models. It is likely that in these models too it would be advantageous to deglycosylate the A-chain or use the SMPT cross-linker or both.

The improved antitumor activity of the ITs can be explained by their longer in vivo half-lives which gave them more time to locate and kill the tumor cells. Also, the 6.3-fold greater in vivo stability of the ITs prepared with SMPT reduced the amount of free antibody that was released which, as shown previously (17), can compete for the target antigens. Strengthening the linkage and deglycosylating the A-chain each increased the blood levels of the ITs by a similar amount; when both were used, the increase was substantial. The AUC for OX7-SMPT-dgA was 7-fold higher than that for OX7-2IT-A and, at the 24-h time point, 16% of the injected OX7-SMPT-dgA remained in the blood as compared with 0.4% of OX7-2IT-A. This indicates that OX7-SMPT-dgA is the most stable and long lived IT thus far described.

The toxicity of the IT-dgAs to mice was about 3-fold higher than that of the corresponding IT-As and correlated with their 2.9-3.4-fold greater AUC values. In contrast the toxicity of the ITs prepared with SMPT was only marginally greater than that of the ITs prepared with 2IT despite the fact that they, too, had 2.0- to 2.4-fold greater AUC values. This paradox might be explained by differences in the pharmacokinetic properties conferred on ITs by dgA or SMPT. Deglycosylation confers most of the mice treated with OX7-SMPT-dgA to have arisen from mutants that were wholly or partially resistant to the ITs. In the groups treated with OX7-2IT-dgA and OX7-SMPT-A, 3 of 10 and 2 of 8 animals, respectively, had IT-resistant tumors. None of the animals in the control groups developed resistant tumors. The reason why fewer OX7-SMPT-dgA recipients developed tumors in the experiment shown in Fig. 4 than in that shown in Fig. 5 is probably because the AKR-A/2 line was used sooner after recloning; thus fewer mutant cells had had time to emerge.

In Fig. 5b, the survival data depicted in Fig. 5a have been censored by excluding mice whose tumors were insensitive to ITs in vitro. When this was done, the difference in antitumor activity of OX7-SMPT-dgA versus OX7-SMPT-A became significant (P < 0.02), and that of OX7-SMPT-dgA versus OX7-2IT-dgA remained significant (P < 0.002).

In conclusion, the therapeutic index of ITs is significantly improved both by the use of the stable SMPT cross-linker and by deglycosylation of the A-chain.

Mutant Tumor Cells Surviving Exposure to ITs in Vivo

Three types of AKR-A/2 mutants survived exposure to the ITs in vivo to produce progressively growing tumors (Table 5).

Thy-1.1-negative Mutants. Cells from these tumors produced fluorescence profiles after staining with OX7 identical to that produced by the control antibody, R10, as determined by the FACS.

Mutants with Low Levels of Thy-1.1 Expression. Cells from these tumors produced median fluorescence intensities after staining with OX7 of less than 20% that of parental AKR-A/2 cells, as determined by the FACS.

Mutants with Deficient Internalization of ITs. Cells from these tumors expressed normal or nearly normal levels of Thy-1.1, were fully sensitive to ricin, yet had partial or complete resistance to ITs. Thus they appear to have had some lesion in the machinery necessary for endocytosis of the ITs or for translocating the A-chain moiety into the cytosol.

In Fig. 5, antitumor effects of ITs at dosage corresponding to an equal proportion (one-seventh) of the LD50. Groups of 10 AKR/c mice were given i.p. injections of 10^7 AKR-A/2 cells (10^7 lethal doses) and 1 day later were given a single i.v. injection of the following: 0.26 nmol OX7-SMPT-dgA (C), 0.83 nmol OX7-SMPT-A (D), 0.33 nmol OX7-2IT-dgA (O), or diluent alone (•). Mice were sacrificed at the times indicated when their body weight had increased by 20% as a result of tumor growth in the peritoneum. In a, all mice sacrificed are counted as "events." In b, only those mice whose tumors were sensitive to ITs in in vitro cytotoxicity assays are counted as "events." The animals whose tumors were IT resistant are included as "at risk" until the time of sacrifice and then are censored.
of its advantageous effect on the blood levels of ITs during the initial 24-h period after injection; thereafter, the blood levels of the native and dgA ITs decline at similar rates. In contrast, the difference in blood levels of ITs prepared with SMPT as opposed to 2IT develops gradually and progressively throughout the lifetime of the ITs in the animal. Thus the explanation that we favor is that there is a threshold level above which ITs cause irreparable damage to a life-sustaining tissue in the mouse. Therefore, it is the initial high blood levels of an IT during the first few h after injection that determine its toxicity to the mice rather than the lower levels at later times after injection. Deglycosylation primarily influences the former and thus increases the toxicity to mice whereas linkage stability primarily influences the latter and has little effect on the toxicity to mice.

All but one of the tumors that developed in the OX7-SMPT-dgA recipients and a few of those that developed in the OX7-2IT-dgA and OX7-SMPT-A recipients were mutants that were resistant to the ITs. This is an important result because it demonstrates that a single dose of the IT had eradicated all the IT-sensitive cells in the animals, leaving only resistant mutants. Three types of mutants were observed. The first two expressed little or no Thy-1.1 antigen. These mutants were probably of the types shown by Hyman and Trowbridge (47) to have alterations in the gene encoding Thy-1.1, in its controlling elements, or in the enzymes which must process the high mannose-type oligosaccharides on the molecule for it to be transported to the cell surface. The third type of mutant observed in the present study expressed Thy-1.1, in its controlling elements, or in the enzymes which must process the high mannose-type oligosaccharides on the molecule for it to be transported to the cell surface. The third type of mutant observed in the present study expressed Thy-1.1 and was sensitive to ricin but appeared to have some lesion in the machinery necessary for endocytosing the IT or for translocating the A-chain moiety into the cytosol. These mutants are different from the mutants identified by Hyman and Trowbridge (47) to have alterations in the gene encoding Thy-1.1.

In conclusion, the therapeutic index of ITs is increased by using the more stable SMPT linkage and by deglycosylating the A-chain. ITs using both modifications should have increased efficacy in situations where it is important to maintain high levels of IT for prolonged periods in order to attain maximal tumor access.

ACKNOWLEDGMENTS

We thank Dr. Ellen Vitetta and Dr. Andy Creighton for their valuable comments on this manuscript, Drs. Bianca De Stavola and Sharon Love for helping us with the statistical analyses, Dr. Leigh Hart for helping us with the pharmacokinetic algorithms, and Audrey Becket for her excellent secretarial assistance.

REFERENCES


33. Well, C. Tables for convenient calculation of median-effective dose (LDM or ED50) and instructions in their use. Biometrics, 8: 249-252, 1952.


Improved Antitumor Effects of Immunotoxins Prepared with Deglycosylated Ricin A-Chain and Hindered Disulfide Linkages


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/22/6396

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.