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Comparison of blocked and non-blocked ricin-antibody immunotoxins against human gastric carcinoma and colorectal adenocarcinoma cell lines*

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Summary. To avoid non-specific binding of intact ricin-antibody conjugates, we prepared a new blocked thioether-linked ricin-antibody IT, in which the galactose binding site of ricin had lost the ability to bind to galactosidic residues of Sepharose 6B gel. As carrier agent, the monoclonal antibody AR-3, which defines the CAR-3 tumour-associated antigenic determinant expressed selectively on different human carcinoma cell lines, was used. Purification of the new conjugate was performed in three sequential steps: (1) by HPLC gel filtration on TSK G3000SW to remove the unconjugated ricin: (2) by affinity chromatography on Affi-Gel Blue to separate the free antibody from the conjugate and (3) by affinity chromatography on Sepharose 6B to separate the galactose-binding IT from the non-binding moiety. The cytotoxicity of the blocked and non-blocked thioether-linked IT was compared with that of classical ricin-antibody IT conjugated via SPDP and that of ricin A chain IT. The comparison was made on two different target cell lines (KATO III human gastric carcinoma and HT-29 human colorectal carcinoma) versus two control cell lines (HL-60 promyelocytic pre-leukaemic and COLO38 melanoma). The results showed that the blocked thioether IT displayed a more selective toxicity to target cells than the non-blocked IT and was much more potent than the ricin A chain conjugate.

Introduction

Immunotoxins, hybrid molecules comprising antibodies chemically coupled to toxins, have been found to selectively block the protein synthesis of the cell bearing the surface antigen against which the antibodies are directed [9, 10, 30, 31, 32]. The plant toxin ricin, a lectin with specificity for galactose-terminating glycoproteins [19], is frequently used in the synthesis of antibody-toxin conjugates [2, 3, 23, 28]. This toxin is a glycoprotein consisting of two polypeptide chains (A and B), joined by a disulphide bond. The B chain first binds to galactose-containing glycoproteins on the cell surface, then the A chain crosses the cell membrane and kills the cells by catalytically inactivating ribosomal protein synthesis [31, 32, 36].

Conjugates containing intact ricin showed high non-specific activity associated with the ability to interact with cells through the natural binding capacity of the native toxin which is generally retained unaltered in conjugates. These ITs require the presence of a high concentration of galactose or lactose which prevents non-specific binding to the cell surface galactose-containing receptor [19, 36]. An alternative way to prevent non-specific cytotoxicity is to use the ricin A chain alone, coupled to a monoclonal antibody. Such conjugates, while demonstrating high specificity, are not as toxic as whole toxin conjugates [4, 13, 29, 34, 36]. This is presumed to be due to poor internalisation reported to be related to the lack of the B chain [26].

Another approach to reducing the non-specific galactose-mediated uptake of intact ricin-antibody conjugates by non-targeted cells has been to block the galactose-binding site by linking intact ricin to antibody in such a way as to produce steric restraint [26, 32]. These blocked ITs, which apparently combine the advantage of high potency with high specificity appeared to be more effective towards Thy-1.1-expressing AKR-A lymphoma cells in tissue culture [26].

In the present work we prepared differently linked ITs made by coupling SATA-derivatized antibody to an iodoacetylated ricin (SIA-ricin) or by the classical SPDP method [5, 7, 8, 25]. The use of SATA [8] to introduce thiol groups into a monoclonal antibody has an advantage over other methods (e.g. the more common SPDP), which need dithiotreitol or other reducing reagents in order to generate the thiol group. Instead, a rather mild non-reducing reagent, hydroxylamine, was added directly to the reaction mixture of the two derivatized proteins during conjugation. In this way, since the thiol group was generated in situ during the conjugation reaction, the formation of intra-molecular cross-links, usually generated by the oxidation of the thiol groups liberated from the antibody, was avoided.

The ability of the two differently linked ITs to bind on Sepharose 6B was also tested [25] in order to separate the

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Abbreviations used: IT, immunotoxin; PBS, phosphate-buffered saline (100 m*M* sodium phosphate, 150 m*M* NaCl); SPDP, *N*-succinimidyl-3-(2-pyridyldithio)-propionate; PBS-EDTA, phosphate-buffered saline containing 1 m*M* EDTA; DMF, dimethylformamide; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SIA, *N*-hydroxuccinimidyl ester of iodoacetic acid; SATA, *N*-succinimidyl-*S*-acetylthiolacetate

blocked IT, which was not retained, from the non-blocked IT, which was bound. The specificity and cytotoxic effects of the blocked and non-blocked ITs were compared on two different carcinoma cell lines expressing the CAR-3 antigen, defined by AR-3 monoclonal antibody [20], on their surface. These lines were KATO III, a human gastric carcinoma cell line, and HT-29, a human colorectal carcinoma cell line.

The results showed that the blocked IT combined the advantage of high cytotoxicity on target cells with high specificity, evidenced by its poor cytotoxicity on the control cells.

Materials and methods

Materials

The SATA was synthesized as described by Duncan et al. [8], and SIA using the method of Higgins and Miles [12].

Methods

Monoclonal antibody. The monoclonal antibody utilized, AR-3 (IgG1), was obtained as described elsewhere [20], and was purified from mouse ascitic fluid by affinity chromatography on Sepharose CL-Protein A.

Ricin. The ricin toxin was purified using the method of Nicolson and Blaustei [18] with minor modifications.

Cell lines. Four cell lines were used: KATO III and HT-29 as target cells both expressing the CAR-3 antigen, and HL-60 and COLO38 as control cells.

Inhibition of cellular protein synthesis. Two types of cytotoxicity tests were used: one for the non-adherent cell lines KATO III and HL-60 and the other for the adherent cell lines HT-29 and COLO38. Aliquots of 2.4×10^5 non-adherent KATO III and HL-60 cells in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 1% antibiotics (penicillin, streptomycin, gentamycin) were centrifuged in polystyrene tubes. The pellet was resuspended in 1.2 ml of various dilutions of antibodies, ricin and conjugates, in the presence or absence of 100 m*M* galactose and/or 1×10^{-6} *M* AR-3. The mixtures were incubated for 3 h at 37° C with occasional shaking, and then centrifuged. The supernatant was removed and the cells washed twice before final resuspension in 1.2 ml of culture medium. Aliquots of 200 µl (4×10^4) were plated in microtitre plates and cultured for 28–30 h.

The HT-29 and COLO38 cells were dispersed with 0.25% trypsin and 0.02% EDTA. Then 3×10^4 cells in 100 µl of RPMI 1640 containing 10% FCS and 1% antibiotics per well were seeded in microtitre plates and incubated for 3 h to allow the cells to adhere. Various dilutions (100 µl) of antibody, ricin and conjugates, in the presence or absence of 100 m*M* galactose and/or 1×10^{-6} *M* AR-3, were added to each well, and plates were incubated for 20 h. The supernatants were removed and the cells washed twice and then incubated with 200 µl culture medium for another 8 h.

At the end of the incubations, in both tests, 200 µl of culture medium containing 1 µCi of ^{3}H-leucine was added to each well, and plates were incubated for an additional 16 h. The cells were collected on glass fibre filters using a Skatron cell harvester. Results were expressed as percentage ^{3}H-leucine incorporation compared to control cultures, background values being substracted.

Ricin radiolabelling. Ricin was iodinated with Na ^{125}I according to the procedure of Sadvig et al. [21].

Synthesis of ITs. A chain ricin conjugate via SPDP: The method used has been previously described [6].

Intact ricin conjugate via SPDP: The method used was essentially derived from Thorpe and Ross [25].

Intact ricin conjugate via SATA and SIA: Ricin (3.15 mg) containing 3 µCi ^{125}I ricin in PBS-EDTA was concentrated to 500 µl and 17 µl of SIA solution (11 m*M*) in dry DMF was added. After stirring for 30 min at room temperature, the reaction mixture was dialyzed for 16 h at 4° C with two changes of buffer. The iodoacetylated groups linked to the protein were calculated using a method described elsewhere [26]; the molar ratio ricin-SIA was determined to be 1:1.1. The SATA reagent (3.5 µl, 100 m*M*) in dry DMF was added to a solution of 5.2 mg of AR-3 monoclonal antibody in 1 ml of PBS-EDTA. After stirring for 10 min at room temperature, the reaction mixture was dialyzed as before. The thioacetylated groups linked to the protein were calculated using a method described elsewhere [11]. The molar ratio AR-3-SATA was determined to be 1:2.5. The two solutions were mixed and hydroxylamine (0.5 *M* plus 12.5 m*M* EDTA) neutralized to pH 7.2 with NaOH, was added (ratio v/v 1:10). After stirring for 6 h at room temperature the mixture was treated with *N*-ethylmaleimide (1 mg) in dry DMF.

Purification of ITs. The reaction mixtures were centrifuged and the supernatants applied to an HPLC column TSK G3000SW (7.5 × 600 mm) in several steps, and eluted in PBS containing 0.05 *M* galactose. The profiles of absorbance at 280 nm and radioactivity are shown in Fig. 1 A, A' for SATA-SIA and SPDP conjugates, respectively.

The fractions containing antibody and conjugate were pooled, dialyzed and concentrated to 1 ml; these preparations were loaded onto Affi-Gel Blue columns (7 × 100 mm), first eluted in sodium phosphate buffer 0.05 *M* and then in the same buffer plus 0.5 *M* NaCl and 0.1 *M* galactose (Fig. 1 B).

The SPDP and SATA-SIA conjugates were separately pooled, dialyzed and concentrated to 1 ml and loaded onto a Sepharose 6B propionic acid-treated column (10 × 50 mm) and then eluted in PBS and successively in PBS plus 0.1 *M* galactose (Fig. 1 C). The purity and the molecular weight of the conjugates were monitored by SDS-PAGE using the method developed by Laemmli [15].

Rabbit Reticulocyte cell-free assay. A reticulocyte lysate was prepared using the method of Allen and Schweet [1]. Freshly thawed samples of lysate were assayed for their ability to incorporate ^{14}C-leucine into protein as described elsewhere [16]. The lysate mixture was incubated at 37° C for 60 min and then labelled amino acid incorporation was assayed in 25 µl aliquots of the incubation mixture, on glass fibre filters, which were then counted in a beta counter.

Results

Preparation and purification of conjugates

The immunoglobulin was bound to the ricin molecule in two steps: (1) SIA reagent was reacted with the amino groups of intact ricin as described by Thorpe et al. [26] and (2) at the same time, an *S*-acetylthiolacetate residue was introduced into the immunoglobulin molecule by the reaction of SATA with the AR-3 molecule. The coupling of thiolacetylated immunoglobulin to iodoacetylated ricin is a relatively rapid reaction and was completed by mixing the two modified proteins for 6 h at room temperature in the presence of hydroxylamine. At the end of the reaction a solution of *N*-ethylmaleimide was added in order to cap the unreacted thiol groups which could interfere with the subsequent purification. For comparison a disulphide-linked AR-3-ricin IT was also prepared using SPDP-derivatized antibody mixed with the SPDP-derivatized ricin as previously described [25]; A chain ricin IT, always using SPDP as linker, was also prepared. The two crude ITs (the disulphide (I) and the thioether (II)-linked AR-3-ricin conjugates) were separately purified to remove free ricin molecules and antibody aggregates (Fig. 1 A, A').

The different IT conjugates were evaluated by SDS-PAGE under non-reducing conditions after each purification step, showing that a 1:1 ricin-antibody conjugate was the major species present. The elution profiles of the two conjugates appeared to be different since the disulphide-linked IT I showed a greater number of compounds with a higher molecular weight (molecular weight >300000) than conjugates II (Fig. 1 A, A').

In order to completely remove the free antibody from the conjugates, the two crude ITs were independently purified by affinity chromatography on Cibacron Blue coupled to agarose (Affi-Gel Blue) (Fig. 1 B). This method has recently been used to separate free antibody from ricin A chain or abrin A chain immunoconjugates in high yield and high purity [14, 33], which were usually not obtained by gel filtration. The complete removal of free antibody is a necessary step for future in vivo study of the IT since the antibody has a longer in vivo half-life than the IT [22, 35].

At this point the yields of the purified ITs were 15% for SATA-SIA IT (II) and 10% for SPDP IT (I) (percentages calculated relative to the number of antibody moles used). The two purified ITs were independently applied to an affinity column (propionic acid-treated Sepharose 6B) to test

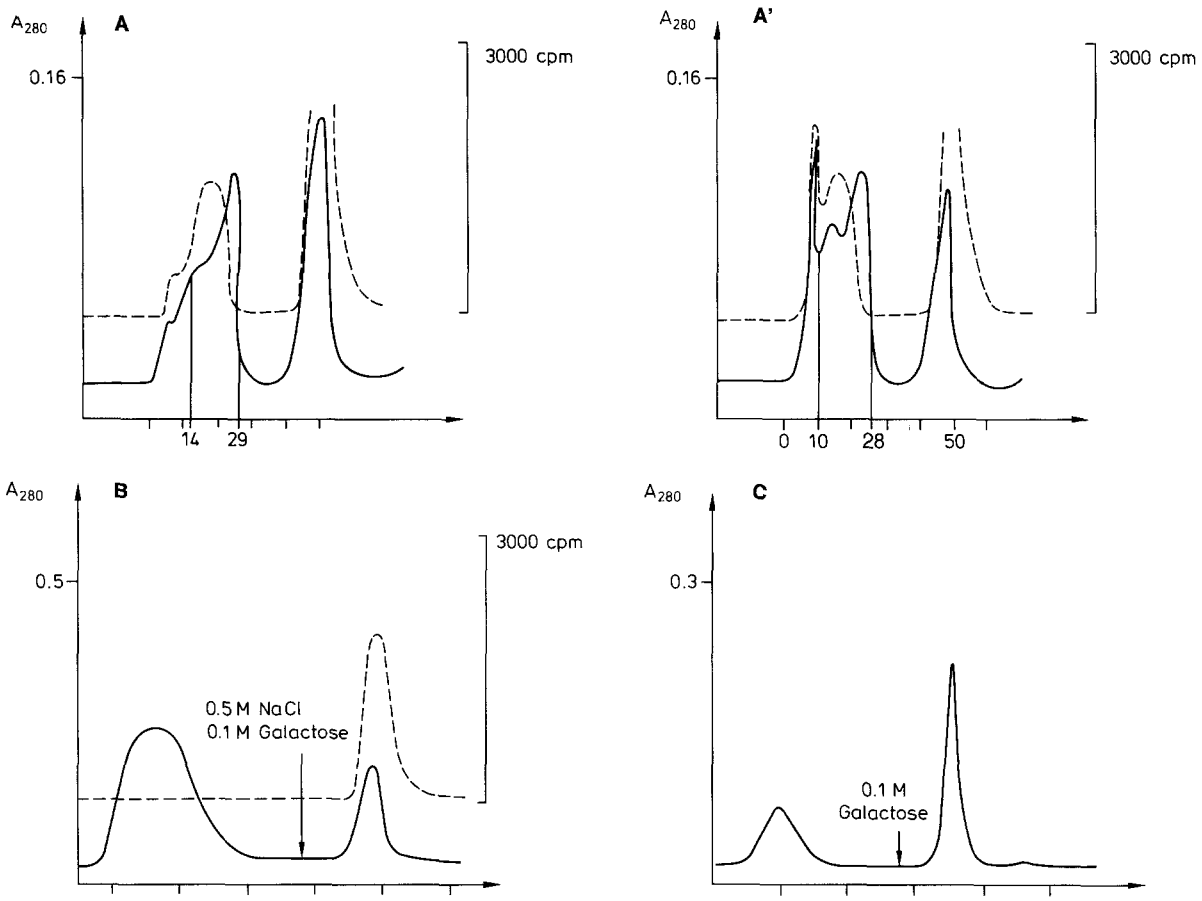

Fig. 1. Purification of SATA-SIA and SPDP ITs
(A) HPLC gel filtration of SATA-SIA IT; the reaction mixture was loaded on a TSK G3000 SW (7.5 × 600 mm), eluted with PBS containing 50 m*M* galactose over several cycles (flow rate 0.5 ml/min); 0.25 ml fractions were collected. The eluate was monitored spectrophotometrically at 280 nm (———) and on-line gamma counter (– – – –). Fractions 14 to 29 were pooled for the next purification steps. **(A')** HPLC gel filtration of SPDP IT; as described in Fig. 1 A. **(B)** Affinity chromatography of SATA-SIA and SPDP ITs fractions containing IT and unconjugated AR-3 were loaded onto an Affi-Gel Blue column (7 × 100 mm) and eluted with the same buffer containing 0.5 *M* NaCl and 0.1 *M* galactose at a flow rate 0.25 ml/min; 1 ml fractions were collected for further separation. **(C)** Affinity chromatography of SATA-SIA IT; fractions containing immunoconjugate were loaded onto a propionic acid-activated Sepharose 6B column (10 × 50 mm) and eluted first in PBS and then in PBS plus 0.1 *M* galactose at a flow rate 0.25 ml/min

the binding capacity of the conjugates, which is probably related to the presence of a free or hidden galactose-binding site.

When applied to the Sepharose 6B column, the disulphide-linked IT I was bound strongly and completely eluted by 0.1 *M* galactose. In contrast, a major fraction (60%) of the thioether-linked conjugate IIa did not bind to the Sepharose 6B and was eluted in PBS, whereas a minor fraction (40%) IIb did bind, and was eluted in PBS plus 0.1 *M* galactose (Fig. 1C). These results suggest that the disulphide bond conjugate was completely non-blocked because it was able to recognize the Sepharose 6B galactose moiety. In contrast the SATA-SIA conjugate resolved into blocked and non-blocked populations, distinguished by their ability to bind to the Sepharose 6B.

Inhibition of protein synthesis in rabbit reticulocyte lysates

As shown in Table 1 the AR-3-ricin conjugates I, IIa and IIb stronglv inhibited protein synthesis in the rabbit reticulocyte lysate assay. They reduced the rate of incorporation of ^{14}C-leucine into protein by 50% at concentrations of 2×10^{-9} *M* (I), 8×10^{-9} *M* (IIa) and 5×10^{-9} *M* (IIb). These results indicate that conjugation of ricin to the AR-3 antibody did not significantly reduce its toxicity.

Table 1. Inhibition of protein synthesis in rabbit reticulocyte lysate

Sample	IC50 (*M*)
AR-3	5×10^{-5}
Ricin	3×10^{-10}
AR-3-ricin (SPDP)	2×10^{-9}
AR-3-ricin (SATA-SIA blocked)	8×10^{-9}
AR-3-ricin (SATA-SIA non blocked)	5×10^{-9}

IC50 = concentration calculated to reduce ^{14}C-leucine incorporation by 50%

Evaluation of the specific cytotoxicity of immunoconjugates

Human gastric carcinoma-derived cell lines. The toxicity of the three conjugates I, IIa and IIb was compared by incu-

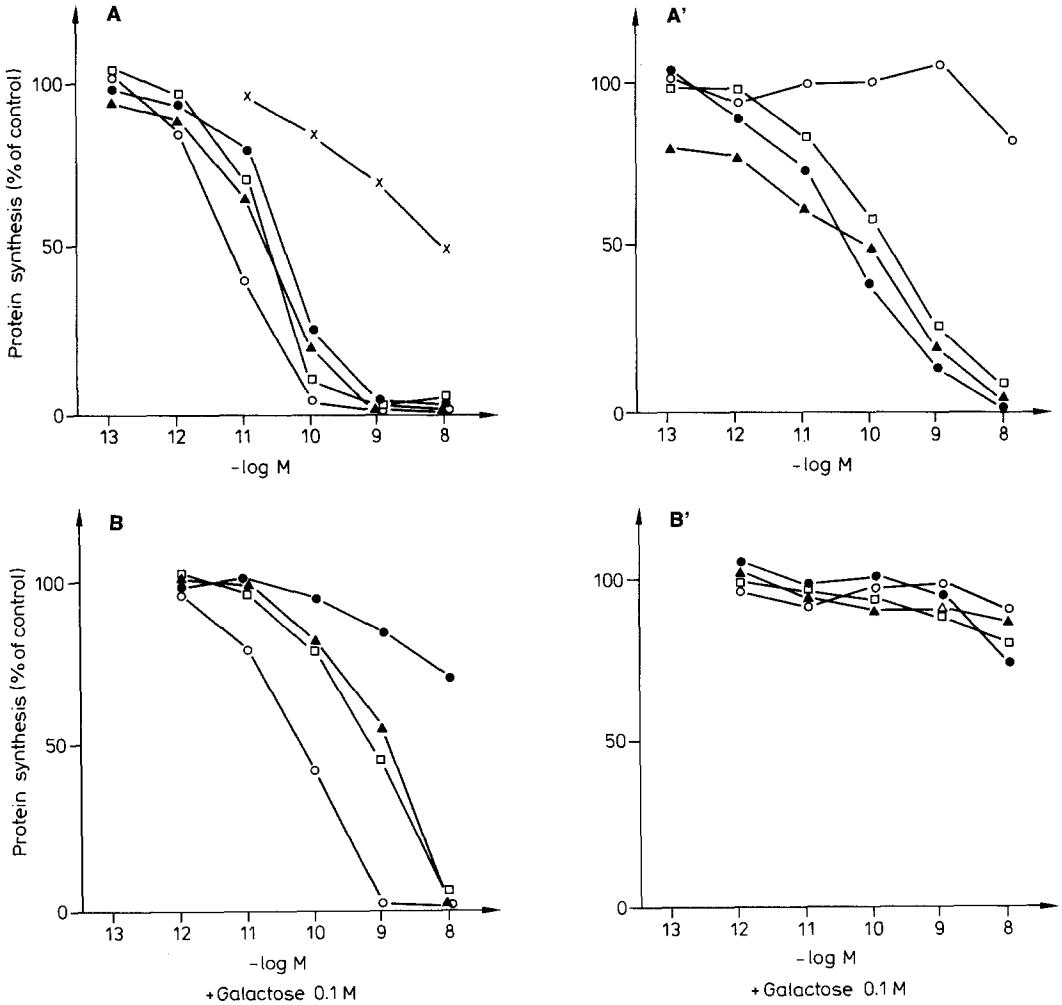

Fig. 2. Inhibition of ^{3}H-leucine incorporation in KATO III and HL-60 cells
Protein synthesis inhibition in non-adherent cells measured by the cytotoxicity test, described in *Materials and methods.* KATO III cells (A) and HL-60 cells (B) were treated with various concentrations of the following reagents: ricin (○), AR-3-ricin SPDP IT (▲), AR-3-ricin SATA-SIA blocked IT (*), AR-3-ricin SATA-SIA non-blocked IT (□), AR-3-ricin A chain IT (x). The points represent the arithmetic means of six determinations, SDs (not reported) were less than 10% of the means. Mean ^{3}H-leucine incorporation in untreated control cultures was 170,000 cpm for KATO III and 90,100 cpm for HL-60 cells. **(A)** KATO III cells in RPMI 1640 medium, **(A')** KATO III cells in RPMI 1640 medium containing 0.1 *M* galactose, **(B)** HL-60 cells in RPMI 1640 medium, **(B')** HL-60 cells in RPMI 1640 medium containing 0.1 *M* galactose

bating KATO III cells with ^{3}H-leucine in the presence or absence of 100 m*M* galactose. Addition of galactose or lactose to the cell medium was normally done to minimize interaction of the residual galactose-binding sites present in the IT molecules with their cellular receptor. The inhibition of protein synthesis in KATO III cells by thioether blocked IIa, non-blocked IIb, disulphide-linked I IT, intact ricin and ricin-A chain IT at a series of dilutions is shown in Fig. 2A. It was apparent that although the three ITs were somewhat less effective than unconjugated ricin, they retained a high degree of cytotoxicity: 50% inhibition of protein synthesis (IC50) was 2.2×10^{-11} *M* for I, 7×10^{-11} *M* for IIa and 2×10^{-11} *M* for IIb.

Figure 2A' shows that toxicity of the blocked IT IIa was not significantly affected when the protein synthesis assay was performed in the presence of galactose. In contrast, under the same conditions the cytoxicity of SPDP-linked IT I and of non-blocked IT IIb were inhibited 5- and 10-fold, whereas the toxicity of ricin was completely abolished.

To test the specificity of the ITs, further experiments were performed using HL-60 cells, which do not bear the CAR-3 surface antigen. As shown in Fig. 2B, in the absence of galactose the disulphide- and the two thioether-linked ITs were 15-, more than 800-and 10-fold less effective than the native ricin. A further comparison of the protein synthesis inhibition produced by conjugate I toward the target and control cells showed that I was 60-fold more specific for KATO III cells than for HL-60 cells. Specific cytotoxicity was more pronounced in the blocked thioether-linked IT, since at a concentration of 7×10^{-8} *M*, protein synthesis in the control HL-60 cells was 50% inhibited while a concentration of 7×10^{-11} *M* was needed to give the same level of inhibition on KATO III cells. Considering the 10-fold difference in sensitivity of KATO III and HL-60 cells to ricin the specificity of SPDP-linked I and SATA-SIA linked ITs IIa and IIb was corrected to 6-, 100- and 4-fold (Table 2) [37].

Human colon carcinoma-derived cell lines. Figure 3A–A' shows results of similar experiments performed with HT-29 cells, derived from a human colorectal carcinoma expressing high levels of CAR-3 antigen [20]. There was a dose-response inhibition of protein synthesis by the three ITs and ricin, with IC50 at a concentration of 3×10^{-11} *M* for IIa and also for unconjugated ricin. The cytotoxicity of ITs I (IC50 = 4×10^{-11} *M*) and IIb (IC50 = 1.5×10^{-11} *M*) did not significantly differ from that the blocked IT in the absence of galactose. Addition of galactose, which blocked non-antibody-mediated killing, did not decrease the activity of the blocked IT IIa (IC50 = 5×10^{-11} *M*), but reduced the toxicity of ricin alone approximately 400-fold and the toxicity of the non-blocked IT 13-fold (I) and 20-fold (IIb), respectively (Fig. 3A').

Therefore, when non-specific binding was blocked by galactose, IT IIa was 200-fold more cytotoxic than free ricin on HT-29 cells. Moreover, the presence of excess monoclonal antibody inhibited IT cytotoxicity but had no effect on ricin toxicity (data not shown), demonstrating that antibody binding is required for IT activity.

The specificity of the antibody-mediated killing capability of the different ITs was claculated by comparing their differential toxicity between target and non-target cells (Table 2), using COLO38, an established human melanoma cell line, as non-target cells. As shown in Fig. 3B, the cytotoxicity of IIa was very low (IC50 > 10^{-8} *M*), while the IC50 for ricin was -5×10^{-11} *M*, and for IT I and IIb 5×10^{-10} *M*.

Table 2. IC50 (*M*) in non-target cells vs IC50 in target cells corrected for different sensitivities to ricin

	Ricin	I	IIa	IIb
HT-29	3×10^{-11}	4×10^{-11}	3×10^{-11}	1.5×10^{-11}
COLO38	5×10^{-11}	5×10^{-10}	1.5×10^{-8}	5×10^{-10}
Difference in sensitivity	1.6			
Index of specificity (corrected)		7.8	312	21
	Ricin	I	IIa	IIb
KATO III	8×10^{-12}	2×10^{-11}	7×10^{-11}	2×10^{-11}
HL-60	8×10^{-11}	1.2×10^{-9}	7×10^{-8}	8×10^{-10}
Difference in sensitivity	10			
Index of specificity (corrected)		6	100	4

Discussion

Comparison of the two differently linked ITs showed that while the SPDP-conjugated IT I completely retained its galactose-binding ability, a major fraction of the SATA-SIA-linked IT IIa had lost the ability to link to the galactosidic gel.

The differential capacity of the three ITs (the non-blocked IT via SPDP (I), the blocked IT (IIa) and the non-blocked IT (IIb) via SATA-SIA) to inhibit protein synthesis in the target cell lines was compared. The results showed that the SATA-SIA linked AR-3-ricin IT IIa was selectively toxic to cells expressing the CAR-3 tumour-associated marker on their surface, such as the human gastric carcinoma cells KATO III and human colorectal carcinoma cells HT-29.

The inhibitory effect of conjugates I and II on protein synthesis in KATO III and HT-29 target cells and HL-60 and COLO38 control cells, was tested in the presence or absence of galactose, since under conditions where galactose saturated its binding site in the ricin B moiety of the conjugates, the aspecific lectin binding of IT will be totally inhibited, so that only their antibody specificity remained. On KATO III cells (Fig. 2A), in the absence of galactose, the three ITs appeared to inhibit protein synthesis slightly less than unconjugated ricin, whereas on HT-29 cells the cytotoxicity of the three ITs was similar to that of free ricin (Fig. 3A).

In contrast, when galactose was added to the medium, the cytotoxicity of the two non-blocked ITs I and IIb was appreciably reduced, whereas that of the blocked IT IIa was virtually unchanged (Figs. 2A' and 3A'). This provided additional evidence that binding of the blocked IT to target cells is due only to the antibody moiety, whereas that of non-blocked ITs I and IIb, which maintained the galactose-binding site unaltered, was affected by the presence of galactose.

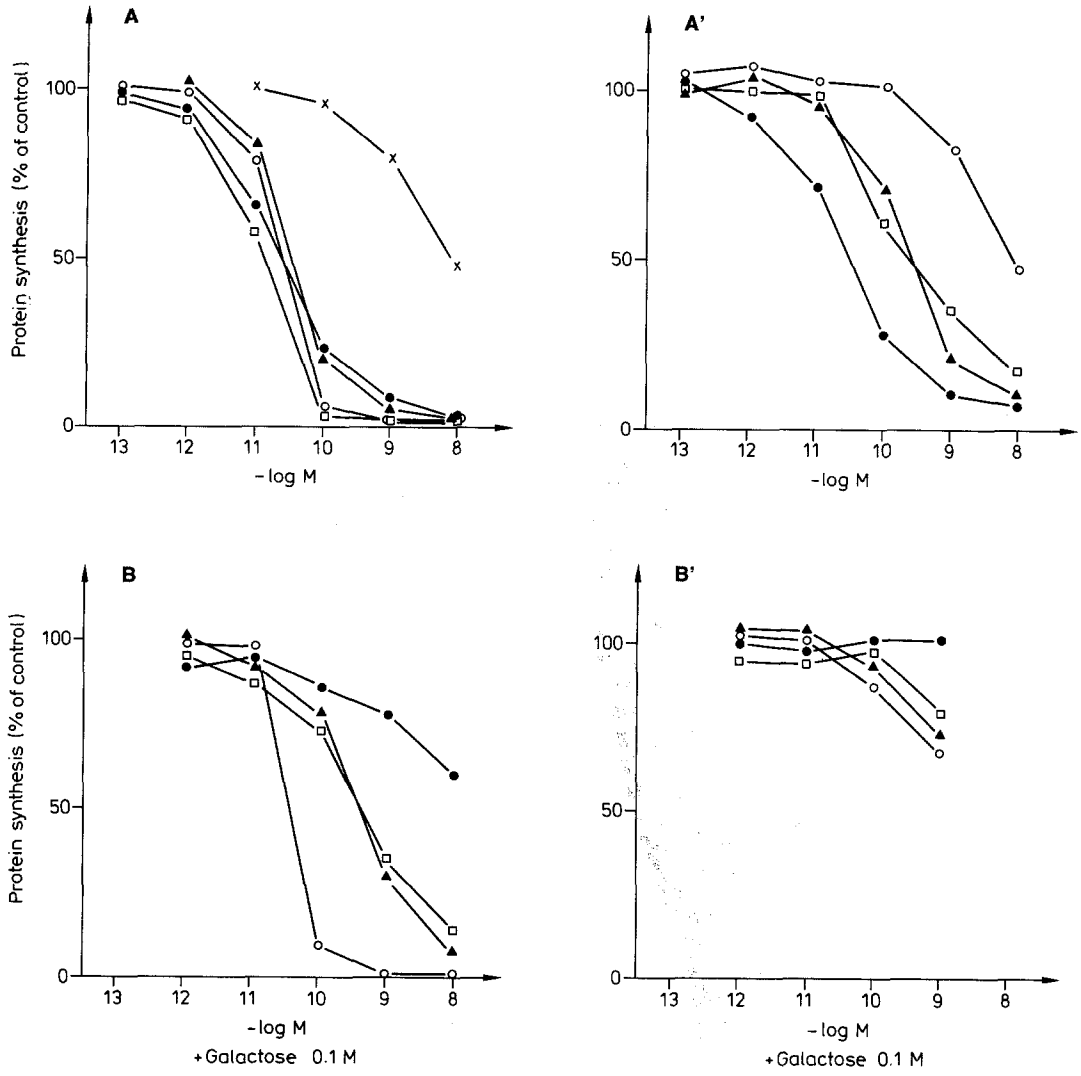

Fig. 3. Inhibition of ^{3}H-leucine incorporation in HT-29 and COLO38 cells
Protein synthesis inhibition in adherent cells measured by the cytotoxicity test, described in *Materials and methods.* HT-29 cells (A) and COLO38 cells (B) were treated with various concentrations of the following reagents: ricin (○), AR-3-ricin SPDP IT (▲), AR-3-ricin SATA-SIA blocked IT (*), AR-3-ricin SATA-SIA non-blocked IT (□), AR-3-ricin A chain IT (x). The points represent the arithmetic means of three determinations, SDs (not reported) were less than 10% of the means. Mean ^{3}H-leucine incorporation in untreated control cultures was 110,000 cpm for HT-29 and 70,000 cpm for COLO38. **(A)** HT-29 cells in RPMI 1640 medium, **(A')** HT-29 cells in RPMI 1640 medium containing 0.1 *M* galactose, **(B)** COLO38 cells in RPMI 1640 medium, **(B')** COLO38 cells in RPMI 1640 medium containing 0.1 *M* galactose

The high specificity shown by the blocked thioether-linked conjugate IIa was demonstrated by its lack of cytotoxicity against control cells, in the absence of galactose (Figs. 2B and 3B). Under the same conditions, the SPDP-derivatized conjugate I and the non-blocked IT, IIb retained significant cytotoxicity.

In contrast, in the presence of galactose, the killing effect of conjugate I and IIb on both HL-60 and COLO38 cells was completely abolished (Figs. 2B' and 3B'). These data indicate that the non-blocked ITs I and IIb retained an important non-specific activity associated with their ability to interact with cells through the natural binding capacity of the toxin moiety.

The difference in specificity between the blocked IIa and non-blocked ITs I and IIb is noteworthy and also depended on the different levels of sensitivity of targeted cells to the uncoupled toxin. When this factor was taken into account, the sensitivity index (IC50 non-target cells vs IC50 target cells corrected for the different sensitivity to ricin) of IIa ranged from 100, in the case of the gastric carcinoma cell line, to 312 for the human colorectal cell line. That for non-blocked IT varied, in the case of I from 6 (KATO III) to 7.8 (HT-29), and in the case of IIb from 4 (KATO III) to 21 (HT-29) (Table 2). This was almost certainly not due to differences in the enzyme inhibitory activity of the conjugated toxin produced by the chemical coupling, since I, IIa and IIb had virtually identical activity when tested in the cell-free protein synthesizing system (Table 1). Since the antibody activity was fully preserved in both conjugates (ELISA data not shown), it seems likely that the difference in specificity resides in the nature of the linkage between ricin and the antibody molecule. It was previously reported [2, 17] in a different tumour cell system, that the disulphide-linked ricin A chain conjugates

were much more cytotoxic that the corresponding thioether-linked conjugates. It was suggested that a stable bond (i.e. thioether) between the ricin A chain and the antibody may prevent the liberation of the A chain before its translocation from endocytic vesicles to ribosomes, after binding of the conjugate to the cell surface [32].

Nevertheless, future in vivo applications of SPDP-derivatized ricin A chain conjugates may be precluded, since this conjugate may be dissociated by a thiol exchange process or by glutathione, in the circulation [24, 27]. This suggests that the intact ricin-antibody conjugate linked via a thiother bond has a further advantage over the ricin A chain IT, since it is probably not cleavable in vivo and thus retains its ability to home to target tumour cells. Moreover, the high in vitro cytotoxicity shown by the conjugate described here, which preserves the disulphide subunit connection present in the native ricin, suggests that full capacity to release and then to translocate the toxic A chain to the ribosomes is not compromised.

In addition, as shown in Figs. 2A and 3A, the blocked IT (IIa) was more than 500-fold more cytotoxic than the ricin A chain IT, obtained by conjugation of AR-3 antibody to ricin A chain via the SPDP linkage. These results agree with the more general finding in the literature [4, 13, 29, 34, 36] that ricin A chain conjugates were less effective than intact ricin IT in killing solid tumours. On the other hand cytotoxicity of ricin A chain on HT-29 cells appeared to be in the range of results already reported [4]. The blocked IT may overcome the non-specificity of the intact ricin conjugate and may be a potential clinical agent in the treatment of certain types of neoplasm. Nevertheless further work will be required to evaluate the in vivo efficacy of blocked ITs.

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