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Antitumour activity of a sterically blocked ricin immunotoxin on a human colorectal adenocarcinoma grafted subcutaneously in nude mice

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Summary. We prepared a ricin-antibody conjugate, lacking the ability to bind the galactosidic residues of Sepharose 6B, a so-called blocked immunotoxin. The monoclonal antibody AR-3 was cross-linked to ricin through a thioether bond. Further studies showed that the immunoconjugate suppressed the tumour growth of HT-29 cells in intraperitoneally grafted nude mice, without showing any undesirable ricin toxicity.

In this work, to demonstrate the therapeutic activity of the AR-3–ricin conjugate injected into mice bearing subcutaneous tumour, we first evaluated its pharmacokinetic behaviour and biodistribution. The behaviour of the immunoconjugate injected intravenously was almost intermediate between that of the antibody and ricin. Moreover, when the immunotoxin was intravenously administered to nude mice bearing subcutaneous tumour, no therapeutic effects appeared, in accordance with the relatively low permeability of the immunotoxin from the blood to the skin. In contrast, peritumoral treatment produced a strong reduction of the neoplastic nodules without substantial regrowth of the malignant cells. This result was also achieved when the immunotoxin treatment was performed on a well-established tumour. This finding was strictly related to the specificity of the immunoconjugate, since the analogous treatment with an irrelevant immunotoxin showed therapeutic failure.

Key words: Drug targeting – Immunotoxin – Pharmacokinetics – Antitumoral therapy

Introduction

One of the most attractive approaches in tumour therapy is the targeting of drugs delivered by tumour-specific monoclonal antibodies (mAb) [7, 11, 24]. To maintain mAb activity and produce a significant and specific cytotoxic effect, highly toxic compounds have been used as drugs [5].

Ricin is a very toxic glycoprotein, isolated from *Ricinus communis* seeds and consisting of two chains (A, B) with molecular masses of about 32 kDa and 34 kDa respectively [16, 17]. The A chain can inactivate protein synthesis, while the B chain binds galactose-containing oligosaccharides commonly expressed on the cell surface. Consequently immunotoxins made by linking ricin to an antibody are toxic but cannot be used in vivo because of the aspecific binding of the B chain [32].

A widely used approach to minimize the aspecificity of ricin in antitumour therapy has been to use only the A chain subunit. Such immunotoxins possess less aspecific toxicity but are also generally less [12] cytotoxic than whole ricin conjugates. An alternative approach is to block the saccharide-binding capacity of the B chain sterically using the mAb molecule [29].

This procedure has been successfully applied by our group [3, 4] and others [13, 19, 20, 29, 33]. We prepared a ricin-antibody conjugate, lacking the ability to bind the galactosidic residues of Sepharose 6B, a so-called blocked immunotoxin.

The mAb AR-3, directed against human adenocarcinomas of the stomach, colon, pancreas, ovary and uterus, previously derivatized with SATA (*N*-hydroxysuccinimidyl-*S*-acetyl thioacetate) was linked to ricin, iodoacetylated with the *N*-hydroxysuccinimidyl ester of iodoacetic acid (SIA), using a thioether linkage. This conjugate showed more selective toxicity to target cells than the non-blocked immunotoxin (i.e. the population of immunotoxins that have retained the ability to bind to Sepharose 6B); moreover it was more potent than the analogous ricin A chain conjugate as well as the similar disulphide-linked immunotoxin [4].

Further studies showed that the AR-3–ricin immunotoxin suppressed tumour growth in nude mice grafted intraperitoneally (i.p.) with HT-29 cells, without showing any undesirable ricin toxicity [3]. Taking steps towards the

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therapeutic application of the blocked AR-3–ricin immunotoxin, in this work we examine its pharmacokinetic parameters and tissue distribution in tumour-free mice or in athymic mice bearing an established subcutaneous (s.c.) grafted human colorectal tumour.

Materials and methods

Tumour cell line. The target cell line used was HT-29, a human colorectal adenocarcinoma expressing the CAR-3 antigen [22]; the cells were maintained in RPMI-1640 medium containing 10% fetal calf serum and 0.1% antibiotics (penicillin, streptomycin, gentamycin), in a 5% CO_2-humidified atmosphere at 37° C.

Immunotoxin preparation and toxicity assays. The monoclonal antibody was AR-3, an IgG1 that recognizes the CAR-3 antigen, widely distributed among adenocarcinomas of the stomach, colon, pancreas, ovary and uterus. The toxin was ricin, a glycoprotein extracted from *Ricinus communis* seeds. The proteins were purified and then used to prepare the immunotoxin, as described previously [4]. Briefly the two proteins were separately derivatized (ricin with SIA and AR-3 with SATA cross-linkers) and then mixed in the presence of hydroxylamine to obtain a thioether-linked immunotoxin. The reaction mixture was purified in different steps and two immunotoxin populations were separated. The major fraction (60%), referred to as blocked, was unable to bind the galactose residue of Sepharose 6B. Sodium dodecylsulphate/polyacrylamide gel electrophoresis showed a molar ratio AR-3/ricin of 1 : 1.

Toxicity assays were routinely used to test in vitro and in vivo activities, and were done as described previously [3, 4]. The cytotoxicity assay was based on incorporation of [^{3}H] leucine into cell proteins, while the LD_{50} was evaluated by the survival of mice 7 days after i.p. injection of the drugs.

Mice. Nude mice CR1: Nu/Nu(CD-1)BR and Balb/C mice, female and 1 month old, were purchased from Charles River Italia. The nude mice were kept in isolation and treated under a laminar-flow hood. They were maintained on a diet of autoclaved standard pellets and drinking water. Both kinds of mice were acclimatised for 1 week before the start of the experiments.

In vivo toxicity of immunotoxin after i.v. injection. Increasing amounts (100, 200, 300 μg/kg mouse) of blocked immunotoxin were intravenously (i.v.) injected into the tail vein of groups of five animals. The volume of each injection was 0.1 ml in phosphate-buffered saline. A control group was always set up by injecting mice with an amount of bovine serum albumin equimolar to the immunotoxin doses tested. Toxicity was evaluated from the results of autopsy and histological analysis performed on the dead or killed mice.

In vivo HT-29 graft. HT-29 cells, maintained as described above, were resuspended at a final concentration of 5×10^7 cells in 1 ml serum-free medium. A 100 μl sample of cell suspension was s.c. injected into the right flank of each mouse. Each experiment was performed with nude mice, the spleens of which had been removed when 40 days old; the mice were then irradiated (4 Gy) 48 h before cell inoculation. The tumour diameters were measured on days 4, 6, 13, 19, 29, 34, 42, 48, 54, 56, 59 and 63 to evaluate the tumour growth. Each week groups of three mice were killed and autopsied to analyse the extent of metastasis in different organs. Histological blocks of the different tissues (tumour, cutis, liver, kidney, gut, peritoneum, diaphragm and lung) were then prepared, fixed in 10% formaldehyde and embedded in paraffin. The sections were stained with haematoxylin/eosin and were also examined by an immunocytochemical method (avidin/biotin/peroxidase) [22] with AR-3 monoclonal antibody to confirm CAR-3 expression on the tumour cell surface.

Pharmacokinetic and biodistribution studies of the immunotoxin in nude mice with or without tumour. AR-3, ricin and immunotoxins were labelled with ^{125}I by the iodogen method [9] to a specific activity of 10 mCi/mg for antibody, 42 mCi/mg for ricin and 27 mCi/mg for immunotoxins. The labelled proteins were used as tracers to identify the circulatory clearance of the tested drugs.

Table 1. In vivo therapeutic protocols[a]

Protocols			Dose (μg)	Tumour-free mice[c] (%)	
	(days)			1	2
A[b]	4, 6	IT	2	100	60
B	4, 6, 12	IT	2	40	60
C	14, 16, 19	IT	2	50	40
D[b]	12, 13, 14	IT	2	50	50
E	4, 6	Not-blocked IT	1	70	NT
F	4, 6	IT+AR-3	2+200	0	0
G	4, 6	AR-3	200	0	NT
H[b]	4, 6	Irrelevant IT	2	40	5

[a] The mice were s.c. grafted on day 0 with 5×10^6 HT-29 cells. In all protocols the drugs were peritumorally injected. The results are the means of data from groups of five animals and the protocols were repeated three times. SD were less than 2.8%. NT, not tested; IT, immunotoxin

[b] Autopsy and histological checks were also performed on days 28, 46 from the graft

[c] 1, 6 days after the last injection; 2, end of experiment: 64th day

The mice (20 g weight) were treated with 2 μg protein (ten times lower for ricin because it was highly toxic) mixed with 4.5 μCi of the appropriate ^{125}I tracer. The samples were diluted in a carrier solution of phosphate-buffered saline containing 0.1% bovine serum albumin to a volume of 100 μl for both the i.v. and s.c. injection.

Blood samples of 50 μl were taken from the retroorbital plexus of groups of five animals at various times; both the Pasteur capillary and the Eppendorf vials were washed in a heparin solution 1 day before use. Each animal underwent up to four drawings, all from the same plexus. Treatment of the blood samples was similar to that described by Scott [25], with minor modifications. Blood samples were diluted into the same volume of 54 mM iodoacetamide to prevent the aggregation of the free thiol groups eventually formed. Proteins were rapidly precipitated with 1 ml cold trichloroacetic acid (12.5% w/v). The samples were held in ice until precipitated and were then stored at 4° C for 1–4 h. The samples were directly counted in a γ-counter (L'ACN, Milan, Italy) and were then centrifuged; the pellets were counted separately from the supernatants.

The trichloroacetic-acid-precipitable radioactivity was related to the total blood volume, assumed as 10% body weight [21]. The pellets, after counting, were washed: first in 8% trichloroacetic acid, then in acetone (both 4 ml), to test the completeness of the initial precipitation.

The pharmacokinetic parameters and slopes were derived using a least-square regression algorithm with a weighting function $1/(y+y)^2$ (MK Model and Sigma Plot 4.0 software). Each point of the slope was the mean of seven drawings and is represented as concentration in nmol/l plotted against time expressed in minutes.

Simultaneously, the principal organs (skin, gastrointestinal tract, kidney, liver, diaphragm, lung, heart and tumour or spleen if present) were removed at different times; they were twice washed in physiological solution, wiped, weighed and γ-counted. The counting was performed on intact organs and after shattering. The radioactivity was reported as concentration, as percentage of injected dose (ID) per gram organs versus time.

Other groups of animals, s.c. injected in the same way, were differently analysed. After killing, the organs were immediately embedded in OCT compound, placed in small steel boxes, and then frozen at –80° C. Serial 8 μm cryostat sections were cut, rapidly dried under a stream of cold dry air, and analysed by two different methods. Some slides were autoradiographed by exposure to Trimax-3M film, in autoradiographic

cassettes, and serial sections were dipped in Ilford K5 emulsion. After 90 days at 4° C the films and the emulsioned sections were respectively developed with X-OMATIC Processor (Kodak), and D19 Kodak (5 min)/Unifix (8 min) [15]. The specimens were counterstained with haematoxylin/eosin and mounted in balsam.

The withdrawings and autopsies of all these experiments were made at fixed times with respect to the route of administration: 0, 2, 4, 6, 24 h after the injection, if s.c. treated. If the experiments were done on mice bearing s.c. tumours, the immunotoxin was injected 4 days after the graft. If a second immunotoxin injection was given on day 6, the withdrawings and the biodistribution check were also carried out on days 6, 7, 8, 11 and 12 from the graft. In the case of i.v. treatment, the times fixed were: 0, 10, 30 and 45 min; 1, 1.5, 2, 3, 5, 6, 22, 24 and 48 h.

In vivo immunotoxin treatment. Several preliminary protocols (A–D) were set up to establish the most effective approach (Table 1). Each protocol was performed on groups of five animals and was repeated three times; immunotoxin administration and cell grafting were made s.c. into the right flank of nude mice, as described above. Tumour growth was checked every 3 days as described. The mice were killed at fixed times and autopsied; the diameters of the tumours, present in the tissue sections, were exactly measured with an eyepiece micrometer. After initial screening, two protocols (A, D) were adopted and extensively used in the experimental conditions described.

In vivo treatment to test the efficacy of the blocked immunotoxin. Different drugs were tested following protocol A and the experiments were performed as reported in Table 1. Protocols E–H were as follows: E, immunotoxin not blocked; F, immunotoxin blocked + excess AR-3; G, AR-3; H, irrelevant immunotoxin composed of intact ricin cross-linked via a thioether bond (SATA/SIA) to the monoclonal antibody MOv18, which does not recognize antigens on the HT-29 cell surface [14]. All groups were subjected to the autopsy and the treatment described above.

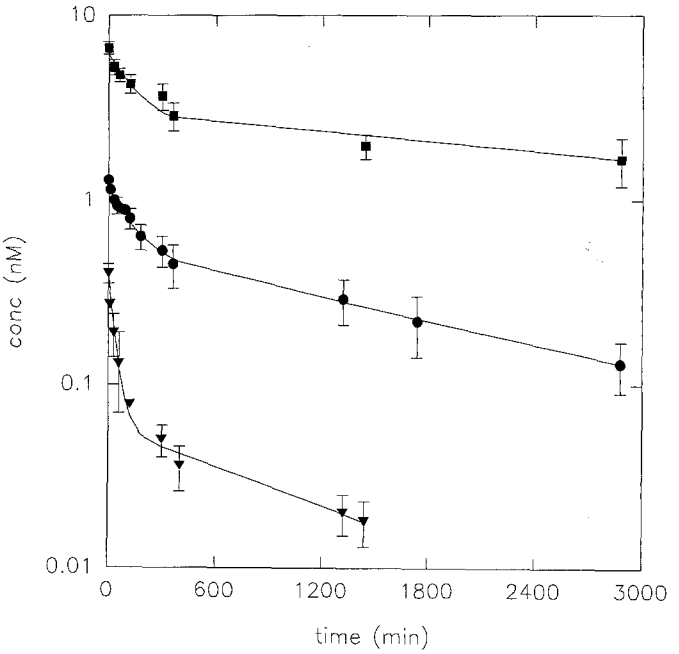


Fig. 1. Blood clearance. Balb/c mice were i.v. injected with radioiodinated AR-3 (■), AR-3–ricin immunotoxin (●) or ricin (▼). Geometric mean (*points*) and SD (*bars*) of results obtained in seven drawings

Results

Toxicity and macro-microscopic findings in nude mice

The ability of the immunotoxin to damage mouse tissues was evaluated through autopsy and histological analysis. The LD_{50} of the immunoconjugate administered i.v. was 0.1 mg/kg mouse, four times lower than that found following i.p. treatment [3]. Administering 100 µg/kg mouse, the animals showed no signs of toxicity whereas at higher doses (200–300 µg/kg mouse) the mice died 40–42 h after the injection, or were killed to limit suffering.

The autopsies showed the presence of a few white plaques mainly localized on the liver, corresponding histologically to parenchymal necrotic areas. Massive and diffuse tissue congestion was also present. To confirm these results, treated and untreated mice were simultaneously autopsied, revealing that control animals did not show any signs of toxicity either in the autopsy or in the histological analysis.

Tumour growth in nude mice

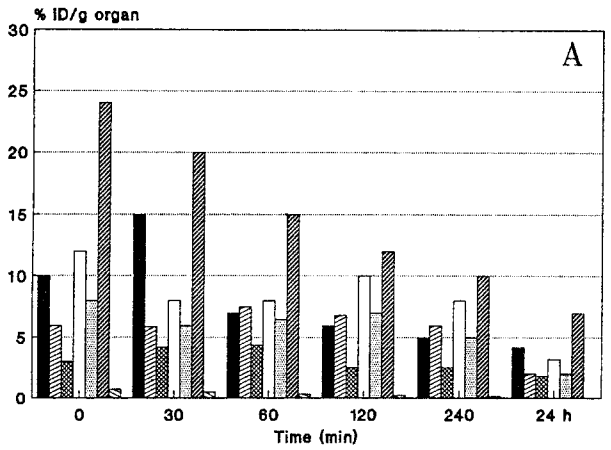
A nude mouse model in which the tumours grow as solid s.c. implanted nodules was used. Measurement of diameter, early autopsies and histological examinations of the tumour masses showed that the neoplastic cells were organized in nodules 3 days after the s.c. graft. Progressing with time, the tumour did not invade any other body compartment and later began to show central necrosis.

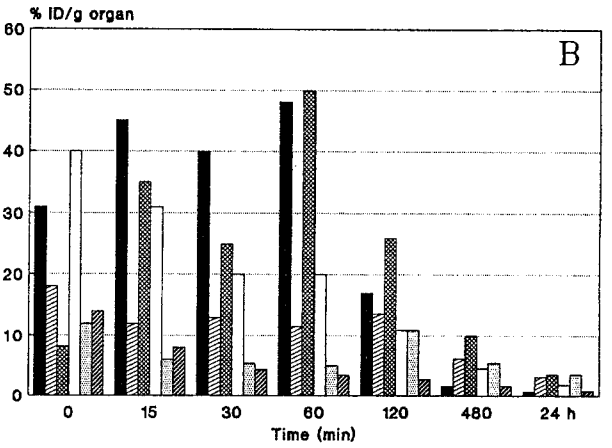
Immunocytochemical analysis of the tumour sections showed that the CAR-3 tumour-associated antigen was always expressed during the course of the experiment (from 4 to 70 days after graft). The neoplastic sections immunocytochemically tested showed positive for 100%–80% of the cells.

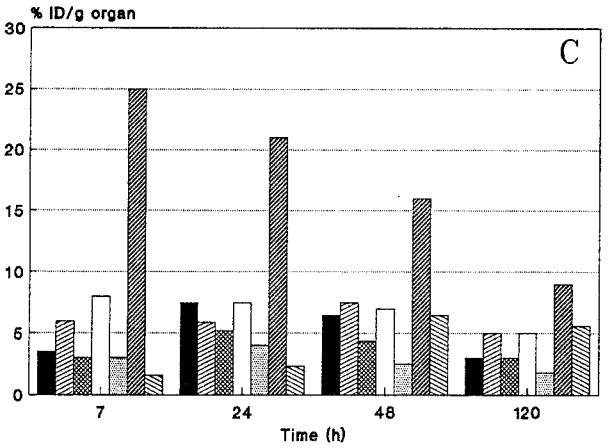
Table 2. Pharmacokinetic data for immunotoxin, antibody and ricin[a]

Protein	A (µg/ml)	B (µg/ml)	α (h^{-1})	β (h^{-1})	AUC (µg h ml^{-1})	V_d (ml)	Cl (ml/h)
AR-3	1.50	0.72	0.31	0.005	148.8	2.77	0.0134
IT	0.213	0.176	0.82	0.024	5.2	15.8	1.35
Ricin	0.0108	0.0023	3.43	0.220	0.017	52.9	11.7

[a] The values were obtained adopting a two-compartment open pharmacokinetic model: $c = Ae^{-\alpha t} + Be^{-\beta t}$ where c is the concentration at the time t, and A, B, α, β are respectively the concentrations and rate constants. SD were less than 2.5% of the mean. V_d, volume of distribution; AUC, area under the curve; Cl, clearance







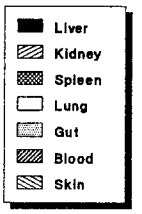


Fig. 2 A–C. Biodistribution in Balb/c mice. Means of five animals are shown. SD are not shown, for clarity, but all SD were less than 14.1%. The more interesting tissues are represented. The radioactivities of AR-3–ricin immunotoxin (**A**), ricin (**B**) and AR-3 monoclonal antibody (**C**) are expressed as percentages of the injected dose relative to 1 g organ against time

Pharmacokinetic and biodistribution study

To understand better the in vivo behaviour of the immunotoxin and the corresponding mAb and ricin moieties, we initially performed a comparison of their pharmacokinetics and biodistribution after i.v. injection in mice. The blood clearance curves relative to ricin, the antibody AR-3 and the AR-3–ricin immunoconjugate are shown in Fig. 1. The data were consistent with a two-compartment open pharmacokinetic model described by the biexponential equation $c = Ae^{-\alpha t} + Be^{-\beta t}$ where c is the concentration at time t, and A, B, and α, β are respectively the concentrations and rate constants [26].

The most important pharmacokinetic parameters of the three proteins are given in Table 2. As previously reported [8, 23], ricin showed both rapid elimination from the body ($t_{1/2}\beta = 3.11 \pm 0.05$ h) and a relatively high apparent volume of distribution ($V_d = 52.9 \pm 1$ ml), meaning that the toxin rapidly left the bloodstream and diffused into the clearance organs liver and spleen. Indeed from the biodistribution study (Fig. 2) the liver and the spleen appeared to be the major sites of clearance, trapping up to 50% of the injected ricin. In contrast the AR-3 antibody was slowly eliminated ($t_{1/2}\beta = 137.3 \pm 2.5$ h), with a reduced V_d (2.77 ± 0.06 ml) and a relatively high blood concentration (25% ID/g after 7 h). The relevant blood survival and the low clearance rate maintained the antibody in the blood (21% ID/g up to 24 h and 9% ID/g after 5 days).

The pharmacokinetic behaviour of the immunotoxin lay between those of the ricin and the mAb, although it was closer to that of the antibody, ($t_{1/2}\beta = 28.5 \pm 0.5$ h, $V_d = 15.8 \pm 0.3$ ml). The biodistribution study (Fig. 2) confirmed the pharmacokinetic observation, showing that after 30 min the immunotoxin was mostly present in the blood (20% ID/g), followed by the liver (15% ID/g) and lung (8% ID/g). After an initial decrease, less rapid than that of ricin, about 5%–10% ID/g still remained in the blood 24 h after injection. These results showed that the immunotoxin remained in the bloodstream from longer than ricin, but still at too low a level to be efficient therapeutically. Moreover, analysis of the biodistribution data suggested that the i.v. treatment with the immunotoxin was not suitable to eradicate a tumour implanted s.c. in nude mice.

In order to evaluate which kind of immunotoxin treatment could be the most favourable to eradicate tumour grafted s.c., we studied the biodistribution and blood clearance of the immunotoxin injected s.c. into mice both tumour-free and s.c.-tumour-grafted. In the absence of tumours, the immunotoxin did not permeate from the skin to the bloodstream, since no trace of radioactivity was found in the blood samples (data not shown). When a protein of lower molecular mass (the AR-3 antibody) was injected we observed a slight degree of permeability, since the mAb was weakly detectable in the blood.

When the experiments were performed in s.c.-tumour-bearing animals peritumorally injected with AR-3–ricin an

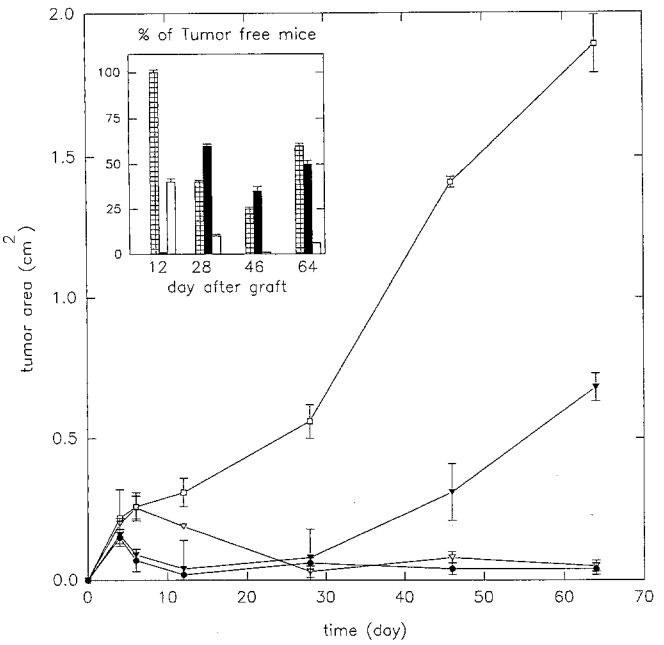


Fig. 3. Immunotoxin therapy on s.c.-tumour-bearing mouse model. The tumour development with time, after no treatment (□), immunotoxin treatment on days 12–14 (▽), immunotoxin treatment on days 4, 6 (●) or irrelevant immunotoxin treatment (▼) are shown. The inserted *histograms* represented the percentage of tumour-free mice in the days after the graft when immunotoxin had been injected on days 4, 6 (*crossed columns*), or on days 12–14 (*black columns*) or an irrelevant immunotoxin had been injected (*white columns*). Geometric means (*points*) and SD (*bars*) of results obtained as described in Table 1

irrelevant immunotoxin and AR-3, the results were similar. This suggests that the inability of the immunotoxin to reach the bloodstream from the site of injection (the s.c. grafted tumour) was not only related to the CAR-3 antigen present on the tumour but also to the molecular mass of the immunoconjugate. This finding was supported by analysis of the autoradiographies performed on different histological sections obtained from mice treated with both immunoconjugates. Both the films and the emulsions showed that only the tumour cells were positive while the other tissues tested were completely negative. Moreover the necrosis present in the tumour sections was strictly related to the use of the AR-3–ricin immunotoxin for the peritumoral injection.

Immunotoxin therapy on a tumour-bearing mouse model

As shown in Table 1, different protocols were performed to demonstrate the therapeutic activity of the immunotoxin in mice bearing an HT-29 human colorectal adenocarcinoma grafted s.c. To evaluate the development or regression of the tumour mass, the mice were sacrified at fixed times until 64 days after the graft. The degree of tumour regression was measured through autopsy and histological examination of the diameters of the tumour mass.

A preliminary study was performed on s.c.-tumour-bearing mice to determine the best therapeutic protocol, evaluating the relationship between the immunotoxin efficacy and the dimensions of the tumour mass. As shown in Table 1 with protocol A, in which the immunotoxin was peritumorally injected on the 4th and 6th days after tumour implantation, the tumour mass was completely eradicated 12 days after the graft and at the end of the experiment (64 days) 60% of the mice were still free of tumour whereas 40% displayed a very small trace of tumour regrowth (less than 0.1 cm^2). No effect at all was observed when immunotoxin injections were performed i.v. or i.p. These results were clearly in accordance with those observed in the pharmacokinetic study. Protocol B did not improve the results obtained with protocol A. With protocols C and D, developed to study the effect of immunotoxins on more developed tumoral masses, only 50% of the treated mice were tumour-free 6 days after the last injection. Nevertheless at the end of the experiment (64 days) the results were similar to that with protocol A: 60%–50% of treated mice respectively with protocols C and D showed a little tumour regrowth, while the rest appeared tumour-free.

From the screening of the preliminary studies two therapeutic protocols, A and D, were chosen, and were extensively used (Fig. 3). These two strategies appeared interesting, because the first gave a good level of tumour regression without increasing the amount of immunotoxin injected; the second demonstrated that a well-developed neoplastic mass could be attacked. During these experiments no damage in the liver, kidney or other organs was revealed by autopsy, although an eschar at the injection site was clearly evident. During the experiment the eschar regressed, cicatrizing until complete recovery. This phenomenon, which was probably an inflammatory response to the ricin toxin, also appeared when free ricin or the irrelevant immunotoxin was peritumorally injected.

Specificity of the intratumoral treatment with AR-3–ricin immunotoxin

To determine the relationship between immunotoxin efficacy and specific binding to the CAR-3 antigen expressed on HT-29 cell lines, several experiments were done (Table 1). When protocol E was followed, the efficacy of not-blocked immunotoxin was comparable to that of the blocked molecule, since at day 12, 70% of the treated mice were tumour-free. However, this protocol could not be prolonged for more than 12 days because the animals showed evident signs of ricin poisoning. The partial efficacy of the not-blocked immunotoxin was surely related both to its aspecific toxicity and to the poor permeability generally shown by the immunotoxins when s.c. injected.

More interesting were the results obtained by peritumoral injection of an irrelevant immunotoxin, unable to bind specifically to the HT-29 cell line (protocol H, Fig. 3). Initially, up to 28 days after the graft, the tumour mass decreased, as it did with the blocked AR-3–ricin. Later, the results diverged. Treatment with AR-3–ricin completely eradicated the tumour mass by the day 12, allowing a reduced neoplastic regrowth in 40% of treated mice at day 64 after the graft. In contrast, treatment with the irrelevant immunotoxin produced only 40% of tumour-free animals on day 12, besides a considerable tumoral regrowth starting on day 30 after the graft; at the end of experiment

on day 64, the slope of the graph relating tumour size and time was similar to that for untreated mice. Moreover the percentage of tumour-free animals was only 5%, as opposed to 60% with mice treated with AR-3–ricin.

Protocol F clearly showed that the efficacy of the AR-3–ricin was related to the presence in the immunoconjugate of the mAb AR-3 specifically directed against the tumour nodules grafted. Coadministration of the AR-3 antibody and the AR-3–ricin did not cause any reduction of tumour mass, and did not affect cell growth. Evidently the AR-3 antibody was able to bind the targeted tumour cells, thus inhibiting the antineoplastic activity of the immunotoxin.

No therapeutic effect was observed after injecting up to 200 μg mAb into s.c.-tumour-bearing mice (protocol G).

Discussion

In this work, to establish the therapeutic activity of AR-3–ricin immunotoxin in s.c.-tumour-bearing mice, we first evaluated its pharmacokinetic and biodistribution behaviour. The i.v. injected immunoconjugate showed behaviour intermediate between that of the antibody and the ricin. Moreover, when the immunotoxin was i.v. administered to the s.c.-tumour-bearing nude mice, there was no therapeutic effect, in accordance with the relatively low permeability of the immunotoxin from the blood to the skin. In contrast, injection near the tumour resulted in strong reduction of the neoplastic nodules without substantial regrowth of the malignant cells. This result was also obtained when the treatment was applied to a well-established tumour. This finding was strictly related to immunotoxin specificity, since analogous treatment with an irrelevant immunoconjugate was a therapeutic failure.

The tumor localization of a drug is a critical step required to achieve therapeutic effects. It is therefore necessary that the immunotoxin remains highly concentrated in the bloodstream for a time sufficient to allow binding with tumour cells. The constant $t_{1/2}\alpha$ and $t_{1/2}\beta$ of AR-3–ricin consistently increased, with respect to the ricin ($t_{1/2}\alpha$ = 0.84 h vs 0.21 h for ricin; $t_{1/2}\beta$ = 28.5 h vs 3.11 h for ricin). Ricin rapidly disappeared, since it was immediately cleared by the liver and spleen, as previously reported [8, 23]. This behaviour seems to derive both from the lectin aspecificity and from the presence of hepatic receptors for the mannose and fucose residues on the ricin [1, 2, 27]. In fact, a modified toxin, (ricin A chain, whole toxin deglycosylated, or recombinant A chain lacking the oligomannose residues), was cleared less rapidly than native ricin by the liver (2, 13, 27, 34).

In our case the linkage of the antibody to the toxin, causing steric hindrance of the galactose binding site of the ricin [4], contributed to the longer blood survival time of the immunotoxin than of the ricin. Moreover the $t_{1/2}\alpha$ and $t_{1/2}\beta$ of our immunotoxin were also similar to those of immunotoxins containing the abrin A chain or saporin toxins [10, 28, 30, 31] with a prolonged blood survival. It is generally accepted that the α phase of blood clearance is related to the liver uptake while the β phase is strictly connected to the cleavage of the linkage between the antibody and the toxin [10, 34]. Thus, immunotoxins linked by agents possessing hindered disulphide bonds, less susceptible to chemical and enzymatic reduction, showed significantly higher $t_{1/2}\beta$ values than the corresponding immunotoxins possessing a labile disulphide linkage [30, 35]. In this context, the behaviour of our blocked immunotoxin could be related to the presence of a stable thioether bond [4].

The low toxicity of the AR-3–ricin could be due to the minor uptake by the liver compared to the uptake of ricin (Fig. 2). In spite of these favourable characteristics, the blocked AR-3–ricin maintained a high toxicity when i.v. injected into mice (LD_{50} = 0.1 mg/kg mouse) with respect to the A chain immunoconjugates [6, 10, 28, 30, 31]. Consequently the blood level was limited by its toxicity and a sufficient therapeutic concentration could not be reached, as demonstrated by the poor efficacy of our immunotoxin, in the i.v. treatment of a HT-29 solid tumour s.c. grafted in nude mice. Moreover the poor immunotoxin permeability from the peritumoral s.c. area to the bloodstream and vice versa could be related to the high molecular mass (approximately 210 kDa), since the smaller mAb showed detectable permeation, also perhaps because of more prolonged blood survival (Fig. 2).

Injection of ricin-antibody conjugate into the edge of the s.c. tumour caused a rapid disappearance of tumour, although in a minor percentage of treated mice a very small neoplastic portion reappeared. This result was still true when the size of the treated tumour was greater (Fig. 3). An initial inhibition of tumour growth, caused by the irrelevant immunotoxin, was observed. This effect was related to the residual toxicity of the native ricin. However, after initial regression, the tumour growth rate increased to the level in the control mice. Moreover AR-3 – ricin was unable to eradicate the tumour in the presence of an excess of AR-3 antibody (Table 1).

These data show that the efficacy of the immunotoxin depended on specific binding of AR-3 to the CAR-3 antigen expressed on the HT-29 surface, and not on the linkage of the ricin moiety to the galactosidic residues of the target cells. Nevertheless, as previously suggested [18, 29, 33], the saccharide-binding ability of the ricin B chain should be not totally abolished by the steric hindrance of the antibody molecule on the linked ricin moiety.

Our results are similar to those found by McKenzie et al. [18], who used a similar sterically blocked whole ricin-antibody conjugate for the treatment of tumours in mice. Our immunotoxin showed a longer blood survival in both the distribution and elimination pharmacokinetic phases. These effects could be due to its major in vivo stability, related to the greater resistance of the thioether linkage to cleavage than the disulphide bond, usually employed in coupling procedures. In the latter case contamination of the immunotoxin by free mAb, derived by the cleavage of the disulphide bond, could really alter the pharmacokinetic constants.

Moreover strong clearance of the immunotoxin caused signs of toxicity in the liver and spleen; to reduce this, McKenzie used a deglycosylated immunotoxin [13]. Our group, in contrast, has shown that i.v. administration of blocked immunotoxin up to the LD_{50} dose did not damage

the major perfused organs such as the liver, kidney and spleen. Only the not-blocked immunotoxin (i.e. the population that bound to Sepharose 6B) gave signs of ricin poisoning when administered to mice.

Thus we consider that the in vivo antitumour activity of the sterically blocked ricin-mAb conjugate could depend on several factors: the nature of the antibody, the target tumour cell line, the quality of the purification procedure, the choice of an appropriate cross-linking system to conjugate the two proteins and the route of administration.

In conclusion, our studies have shown that a whole-ricin-antibody conjugate that has lost the ability to bind to the galactosidic residues of Sepharose 6B, when administered i.p. in nude mice i.p. grafted with a solid tumour [3] or peritumorally injected in a s.c. growing carcinoma, caused a nearly complete eradication of the malignancy. In contrast, after the immunotoxin had been injected i.v. in s.c.-tumour-bearing mice, no antitumoral effect was seen. Thus, while i.p. or peritumoral treatment should be devised for the therapy of intraperitoneal carcinomas or superficial tumours that are difficult to remove surgically, the major obstacle to the systemic use of intact ricin-antibody conjugates still remains the residual toxicity, which limits the dose.

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