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In vivo cytotoxic efficacy of immunotoxins prepared from anti-CD5 antibody linked to ricin A-chain

Odile Rostaing-Capaillon and Pierre Casellas

Department of Immunology, Sanofi Recherche, 371 rue du Professeur J. Blayac, F-34 184 Montpellier Cedex 4, France

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Summary. The antitumoral efficacy of various anti-CD5 immunotoxins, prepared with whole monoclonal antibody (mAb), F(ab′)$_2$ or Fab fragment linked to native ricin A-chain (RTA) or partially deglycosylated ricin A-chain (dRTA), was examined in vivo in ascitic nude mice bearing a large burden of Ichikawa human tumour cells. We first demonstrated that after systemic administration of IgG-RTA or F(ab′)$_2$-dRTA, the cytotoxic activity of immunotoxin molecules specifically bound to tumour cells was preserved. Secondly we showed, by using different immunotoxins with various targeting capacities, that their cytotoxic effect in vivo was related to the number of immunotoxin molecules bound per cell. However, even when antigen saturation was achieved after i.p. injection, the cytotoxic effect did not exceed 53% of the tumour burden. By contrast, when the immunotoxin was administered i.p. or i.v. with the enhancer monensin conjugated to human serum albumin and injected i.p., 90% of the tumour cells were killed. This potentiating effect was demonstrated even when the tumour localisation was as low as 5% of the saturation level. Such an effect could be completely prevented by addition of unconjugated monoclonal antibody, demonstrating the specificity of the immunotoxin-induced cytotoxicity in the presence of the enhancer. However this enhancement was demonstrated whatever the route of immunotoxin administration, i.p. or i.v., but was only observed when the enhancer was injected i.p. and not i.v.. These results emphasize the importance of optimizing the therapeutic course to improve the antitumoral efficacy of immunotoxins.

Key words: Immunotoxins – Monensin – Anti-CD5 antibody

Offprint requests to: P. Casellas

Introduction

Immunotoxins are cytotoxic agents with a high specificity particularly adapted to the therapy of metastatic cancers [13, 2]. Indeed, the linkage of a toxin to a mAb that recognizes an antigen on tumour cells confers to the toxin a specificity that is lacking in traditional anticancer treatments [17].

However, the antitumoral efficacy of immunotoxins in vivo has not always been demonstrated, probably because of a low targeting capacity after systemic administration [8, 9, 23, 11, 1, 16]. In a previous paper, we quantitatively studied the tumour localization of various immunotoxins, prepared by linking anti-CD5 mAb to ricin A-chain, in an intraperitoneal tumour model developed in the nude mouse [21]. We demonstrated that saturation of target antigens was only observed after i.p. injection. The tumour localization after systemic administration was related to the immunotoxin structure and an optimal localization was obtained with F(ab′)$_2$ fragments linked to partially deglycosylated ricin A-chain (RTA).

We examine here the cytotoxic potency of these immunotoxins. The model chosen consisted of a BALB/c nude mouse bearing a wide xenograft composed of 900×10^6 human leukemic cells, in order to mimic the clinical situations [18]. This contrasts with previous experiments for quantitative analysis of immunotoxin cytotoxic efficacy where the tumour burden represented fewer than 10^7 cells [26, 24, 10].

In this paper, we first address the question of the functional integrity of immunotoxin molecules associated with tumour cells after in vivo administration. Then we study the antitumoral effect of immunotoxins in vivo and, finally, the potentiating effect of monensin conjugated to human serum albumin was examined.

We show that most of the immunotoxin molecules preserve their cytotoxic activity after tumour localization and that the antitumoral effect, which is limited, even when all target antigens are saturated, can be strongly improved by simultaneous i.p. administration of monensin linked to human serum albumin.

Table 1. Anti-CD5 immunotoxin characterization

Immunotoxin[a]	mAb	Isotype	Affinity (M^{-1})	Coupling ratio	IC_{50}[b] (pM)
T101(IgG)-RTA	T101	IgG_{2aK}	10^{10}	1.5	0.12
T101(Fab)-RTA	T101	IgG_{2aK}	10^9	2.0	0.80
F111.98[F(ab′)2]-dRTA	F111.98	IgG_1	5×10^9	1.9	0.28

[a] RTA, ricin A-chain; dRTA, partially deglycosylated ricin A-chain

[b] IC_{50} = concentration required for 50% inhibition of the maximal proliferative response. In vitro toxicity was determined in the presence of 50 nM monensin

Materials and methods

Cell line. The Ichikawa cell line of human T-ALL origin was provided by S. Watanabe, Tokyo, Japan [28]. Line IP12/2 is a subclone of Ichikawa cells and grows in vitro as well as in vivo. This cell line carries the CD5 antigen and the number of sites has been determined as 30000/IP12/2 cell according to the method previously described [19]. Cells were cultured in a 5% CO_2 atmosphere at 37° C in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Flow Labs) and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin).

Establishment of tumours in mice. Female BALB/c (nu/nu) mice (Iffa-Credo, Lyon, France), 6–8 weeks old, were used. They were housed in sterile filter-top cages with sterile bedding. The Ichikawa cell suspension (25×10^6 cells in 0.2 ml) in Hanks solution was injected i.p. into the mice. Ascites appeared 20–25 days later in 70%–80% of the animals and were maintained by serial transplantations. After ten transplantations, fresh cultured cells were used to obtain new ascites. The ascitic volume, 20–25 days post-injection, varied from 1 ml to 5 ml/mouse. The ascitic fluid contained $(250-350) \times 10^6$ cells/ml and 95% of these tumour cells were CD5-positive as determined by flow cytometry.

Monoclonal antibodies. Two different anti-CD5 mAbs were used that recognize different epitopes on the CD5 antigen [3]. T101 is a mouse IgG2a purchased from Hybritech Inc. (San Diego, USA) and F111.98 is a mouse IgG1 (Sanofi, France). F(ab′)2 and Fab fragments were produced by digestion with pepsin and papain respectively. F(ab′)2 fragment was purified by gel filtration (ACA 44, IBF) and Fab fragment by ion-exchange chromatography on DEAE-Trisacryl (LKB) [25].

Toxins and immunotoxins. Ricin A-chain was purified as previously described [27]. Modification of the ricin A-chain carbohydrate moiety was achieved using a mixture of sodium metaperiodate and sodium cyanoborohydride [21]. Fucose, xylose and mannose residues were removed, whereas *N*-acetylglucosamine residues were not altered [6]. The inhibitory activity of RTA was preserved during the deglycosylation procedure. RTA or partially deglycosylated (dRTA) was conjugated to mAb or to the corresponding F(ab′)2 and Fab fragments by a disulphide bridge, using the heterobifunctional *N*-succinimidyl 3-(2-pyridyldithio)propionate reagent, according to the method previously described [12]. These immunotoxins will be referred to as T101(IgG)-RTA, T101(Fab)-RTA and F111.98[F(ab′)2]-dRTA. F111.98 mAb was used to prepare the dRTA conjugate because this mAb was the most easily available to us in large amounts. The biochemical and biological properties of these immunotoxins are summarized in Table 1.

Potentiating agents. Monensin was purchased from Calbiochem. In order to reduce its plasma clearance, monensin was conjugated to human serum albumin (HSA). A thiol group was introduced into monensin by reaction with *S*-acetylmercaptosuccinic anhydride and subsequent treatment by hydroxylamine. Activated thiol groups were generated in HSA by reaction with *N*-succinimidyl-3(2-pyridyldithio)propionate (SPDP). Incubation of modified monensin and HSA resulted in the substitution of two monensin molecules per HSA (HSA-mo_2).

Quantification of intact immunotoxin molecules taken up by tumour cells. Immunotoxin molecules associated with ascitic tumour cells were quantified using a fluorometric two-site enzymometric assay as described previously [21]. Briefly, ascitic fluid was collected at different times after immunotoxin injection and cells were washed to remove unbound immunotoxins. Then 10^7 cells were homogenized to release cell-associated immunotoxins by treating the cells for 30 min at 4° C with 500 µl detergent mixture consisting of 1% Triton X-100 (Sigma), 0.5% NP40 (Sigma) and 0.5% sodium deoxycholate (Merck) in phosphate-buffered saline (PBS) supplemented with 3.7 mg/ml iodoacetamide and 2 mM phenylmethylsulphonyl fluoride (Sigma). The lysate was centrifuged for 10 min at 1000 *g* before quantification of solubilized immunotoxins and 90% of the immunotoxin molecules were recovered in the supernatant, as determined using a ^{125}I-labelled immunotoxin. The number of immunotoxin molecules per cell (n_{IT}) was calculated from the concentration (c_S) of solubilized immunotoxin in the supernatant of the lysate as follows:

$$n_{IT} = \frac{c_s(\text{mol/ml}) \times N_A}{2 \times e \times n}$$

where e is the lysis efficiency ($e = 0.9$), N_A is the Avogadro number and n is the number of homogenized cells.

The fluorometric immunoenzymometric assay (IEMA), developed to quantify solubilized intact immunotoxins comprised two steps: a specific binding of immunotoxins to purified sheep anti-RTA antibodies and then detection of bound immunotoxin molecules with an anti-(mouse IgG) conjugated to β-galactosidase. Anti-RTA antibodies were adsorbed for 20 h at room temperature on a solid phase composed of plastic studs on lids of Nunc TSP screening system (Polylabo). The studs were saturated with fetal calf serum for 2 h. Specific binding of immunotoxins was then complete after 3 h of incubation at room temperature. The immunoadsorbent was washed in 10 mM PBS pH 7.4 and dipped into wells containing the immunoenzymatic conjugate, i.e., F(ab′)2–β-galactosidase from sheep anti-(mouse IgG), for detection of the immunotoxin (Amersham).

After incubation for 3 h at room temperature and washing of the solid phase in PBS, the bound conjugate was incubated with a substrate solution of 0.2 mg/ml 4-methylumbelliferyl β-D-galactoside (Sigma), in the dark for 1 h at 37° C. Then 100 µl 100 mM glycine/NaOH buffer, pH 10.3, was added to each well. Fluorescence intensity was measured with a fluoroscan device (Flow laboratories, Mac Lean, UK). The wavelengths for excitation and emission of 4-methylumbelliferone, a product of the enzymatic reaction, are 355 nm and 480 nm. An absolute scale of fluorescence intensity was used, with a range of 0–10000 fluorescence units.

As few as 400 RTA-immunotoxin molecules could be detected per cell with this method. The within-run and between-run variation coefficients of the assay were both below 10%, in the range of 1–300 ng/ml RTA. A biological activity assay was also performed to compare the measurement of T101(IgG)-RTA with that obtained by IEMA. Briefly, samples containing various amounts of immunotoxin were incubated with 4×10^4 IP12/2 cells for 18 h at 37° C. Protein synthesis was then measured by [^{14}C]leucine incorporation into cell protein during 5 h. Immunotoxin concentrations were related to the inhibition of protein synthesis. Similar values were observed with the IEMA and the biological activity assay: the correlation coefficient and the slope of the regression line were 0.997 and 1.32 respectively (Fig. 1).

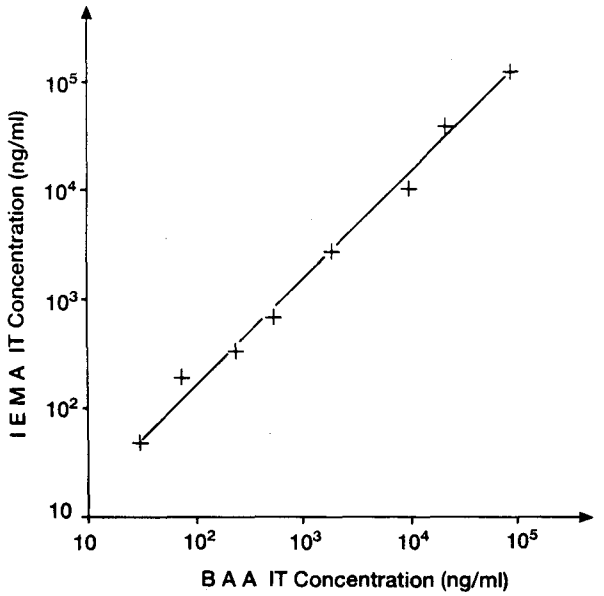

Fig. 1. Correlation between the immunoenzymometric assay (IEMA) and the biological activity assay for anti-CD5 immunotoxin quantification. Samples were loaded with various amounts of immunotoxin. Concentrations of intact immunotoxin were determined by IEMA and by measurement of cytotoxic activity on IP12/2 cells

Quantification of active immunotoxin molecules bound to tumour cells. To determine the number of active immunotoxin molecules taken up by tumour cells in vivo, the cytotoxic effect of the bound molecules towards these cells was measured in vitro in the presence of 80 nM monensin and compared to the cytotoxic effects obtained with known immunotoxin concentrations added in vitro to tumour cells from an untreated mouse.

Calibration curves were established using cells collected before immunotoxin administration as follows: the control immunotoxin (50 µl) was added to 8×10^4 cells/well (100 µl) and monensin (25 µl) was introduced into the cultures at a final concentration of 80 nM. Cells were incubated for different periods of time, indicated in Fig. 2, at 37° C in a 5% CO_2 atmosphere. The cultures were pulsed with 2 µCi [^{14}C]leucine/well during the last hour incubation and incorporation was measured after cell harvesting, by counting the filters in a liquid scintillation counter (1205 Betaplate, LKB).

The numbers of active bound molecules were calculated from the immunotoxin concentration introduced in the culture (T) according to the mass action law, $K_a = B/F(C-B)$, with $B + F = T$; where K_a stands for the affinity constant of the conjugated mAb, B for the concentration of bound immunotoxin molecules, F for the concentration of soluble immunotoxin molecules, T for the total immunotoxin concentration, and C the concentration of CD5 antigens.

The number of active molecules bound per cell (n_{act}) was determined using the following formula:

$$n_{act} = B \times n/C,$$

where n represents the number of CD5 antigens per cell.

To quantify active molecules associated with tumour cells in vivo, 100 µl ascitic fluid was tapped at various times after i. v. injection. Cells were diluted and plated at 8×10^4 cells/well in 150 µl and monensin was added. Control cells from an untreated mouse were included in the test, and incubation was performed as described above. Results are expressed as a percentage of the control. The number of active molecules bound in vivo was deduced from the [^{14}C]leucine incorporation using the calibration described above.

Cytotoxic activity of immunotoxins in vivo. Samples of 150 µg whole mAb or 100 µg F(ab')$_2$ or Fab fragment conjugated to RTA or dRTA were i. p. or i. v. injected into each mouse to study the antitumoral effect of these immunotoxins. The injected dose was reduced three times for simultaneous injection with 80 µg monensin coupled to HSA, to prevent non-specific toxicity of the mixture. The equivalent of 50 µg mAb or 35 µg of the fragments was therefore injected along with HSA-mo$_2$, and 50 µl ascitic fluid was tapped 5 h and 24 h after injection. The cell density and viability were determined by trypan blue exclusion. Viable tumour cells were plated in flat-bottomed 96-well tissue-culture plates at 8×10^4 cells/well in 100 µl RPMI-1640 medium and were incubated for 3 h at 37° C in the presence of 2 µCi [^{14}C]leucine/well. Controls were obtained with cells from untreated mice. Cells were then harvested and [^{14}C]leucine incorporation was measured as described above. The percentage of killed cells after immunotoxin treatment in vivo was calculated as the sum of the percentage of stained dead cells and the percentage of intoxicated cells that did not incorporate [^{14}C]leucine:

$$\text{Killed cells (\%)} = \text{stained cells (\%)} + \frac{1}{100}\,(100 - \text{percentage stained cells}) \times \text{non-incorporating cells (\%)}.$$

Results

Potential cytotoxic activity of immunotoxin molecules taken up by tumour cells

In a previous paper, the number of intact immunotoxin molecules localized on a tumour cell was determined at different times after immunotoxin injection [21]. In that study, tumour cells from the ascitic fluid were first washed and lysed and the solubilized immunotoxins were quantified in the lysate supernatant using a fluorometric two-sites immunoenzymometric assay (IEMA). In this assay, anti-RTA antibodies adsorbed on a solid phase and anti-(mouse IgG) conjugated to β-galactosidase were used to

Table 2. Number of intact immunotoxin molecules per tumour cell after in vivo administration[a]

Immunotoxin and route of administration		Number of immunotoxin molecules per cell: time after injection				
		10 min	30 min	1 h	5 h	24 h
T101(IgG)-RTA	i. v.	510	590	670	1270	1600
T101(Fab)-RTA	i. v.	590	2700	6500	3900	1350
F111.98[F(ab')$_2$]-dRTA	i. v.	590	3100	4990	6900	3230
	i. p.	ND	34000	31400	15900	ND

[a] The number of immunotoxin molecules was determined after lysis of 10^7 cells in 500 µg detergent mixture (1% Triton X-100, 0.5% NP40, 0.5% sodium deoxycholate). The concentration of solubilized immunotoxin in the cell lysate was measured by immunoenzymometric assay (IEMA). ND, not determined

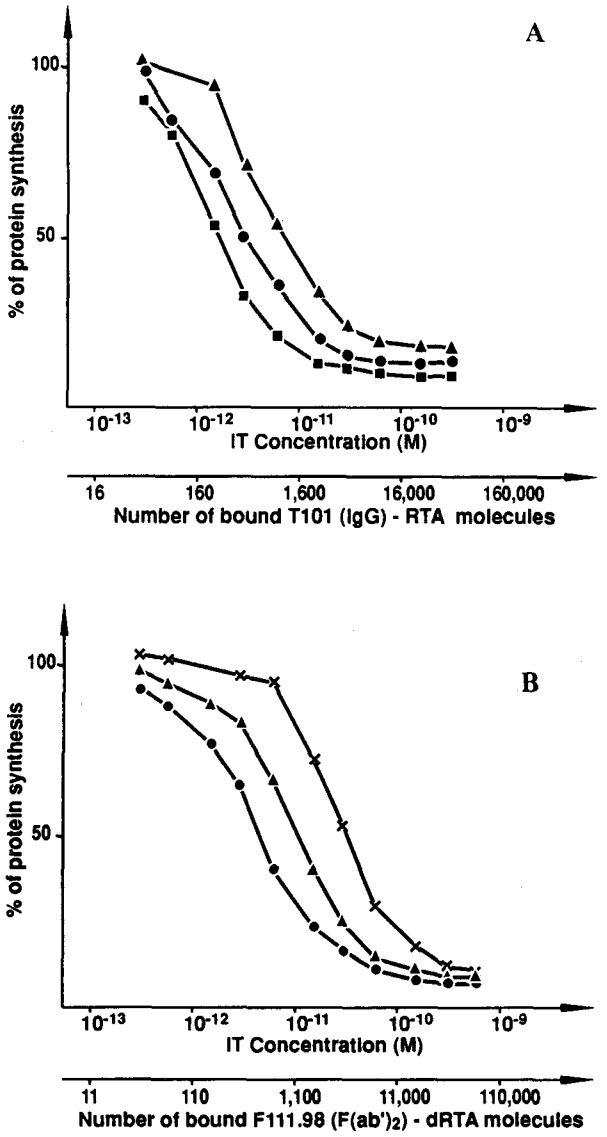

Fig. 2. Calibration curves for the determination of the number of active molecules of T101(IgG)-RTA (**A**) or F111.98[F(ab′)2]-dRTA (**B**) per tumour cell. IP12/2 cells (8×10^4) were incubated with various immunotoxin concentrations in the presence of 80 nM monensin, during 3 h (×), 4 h (▲), 5 h (●), 6 h (■). *RTA*, ricin A-chain; dRTA, partially deglycosylated ricin A-chain

detect immunotoxin molecules. Immunologically intact immunotoxin molecules bound to the cell surface or soon internalized were thus quantified by this method and the data are summarized in Table 2.

To address the question whether immunotoxin molecules associated with tumour cells after i.v. injection still presented a cytotoxic activity towards these cells, their capacity to inhibit protein synthesis was measured in vitro. To gain access to a measurable cytotoxic effect, even when the tumor cells were loaded with a small number of immunotoxin molecules, this determination was carried out in the presence of the potentiating agent, monensin. The study was performed with T101(IgG)-RTA and F111.98-[F(ab′)2]-dRTA to compare immunotoxins with opposite binding capacities after i.v. administration.

The inhibition of protein synthesis was expressed as the number of active immunotoxin molecules, using standard curves set up as follows: IP12/2 cells from an untreated mouse were incubated in the presence of various immunotoxin concentrations and protein synthesis was measured in the presence of 80 nM monensin. The inhibition of protein synthesis, induced by the various immunotoxin concentrations, was then related to the number of active immunotoxin molecules bound per cell from calculations based on the affinity constant of the mAb and CD5 antigen density, as described in Materials and methods.

To increase the reading frame of the number of active molecules, three standard curves were run in parallel for each immunotoxin according to different incubation times: 3, 4, 5 or 6 h. Standard curves, shown in Fig. 2A, B, were obtained with T101(IgG)-RTA and F111.98[F(ab′)2]-dRTA respectively. This allowed a minimal detection of as few as 100 molecules/cell to a maximum of 9000 molecules, which is the range of intact molecules per cell determined by IEMA after i.v. injection (Table 2).

As shown in Table 3, 70%–100% of the intact T101(IgG)-RTA molecules associated with tumour cells have retained their cytotoxic activity, as well as 40%–74% of the F111.98[F(ab′)2]-dRTA molecules. These results demonstrate that the biological activity of immunotoxin molecules bound to tumour cells after systemic administration, was not basically altered. Furthermore, these molecules retained their ability to be enhanced by monensin.

Table 3. Comparison between the number of intact immunotoxin (IT) molecules and the number of active molecules per cell

Injected immunotoxin		Time after i.v. injection				
		10 min	30 min	1 h	5 h	24 h
T101-(IgG)-RTA	Intact IT molecules/cell[a]	510	590	670	1270	1600
	Active IT molecules/cell[b]	400	580	480	1400	1580
	Active IT molecules/cell[c] (%)	78±9	99±3	71±5	111±11	99±2
F111.98-[F(ab′)2]-dRTA	Intact IT molecules/cell[a]	590	3100	4990	6900	3230
	Active IT molecules/cell[b]	220	ND	2200	2900	2400
	Active IT molecules/cell[c] (%)	38±20	ND	44±10	42±3	74±22

[a] The number of intact immunotoxin molecules/cell was determined by IEMA

[b] The number of active immunotoxin molecules was determined by measurement of their cytotoxic effect in vitro

[c] Percentages of active molecules are expressed as means ± standard deviation

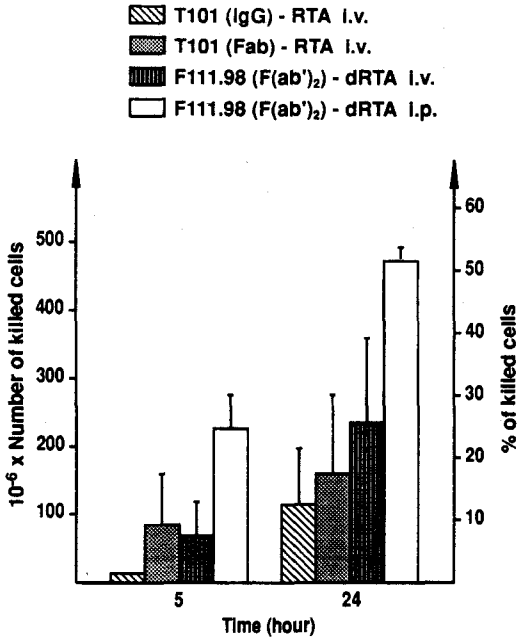

Fig. 3. Antitumour effect of anti-CD5 immunotoxins. The equivalent of 150 μg IgG or 100 μg $F(ab')_2$ or Fab was injected per mouse. Results are presented as the percentage of cells killed determined as described in Materials and method and as the number of cells killed, assuming a tumour burden of 900×10^6 cells in the peritoneal cavity. Results represent the mean value of at least two mice.

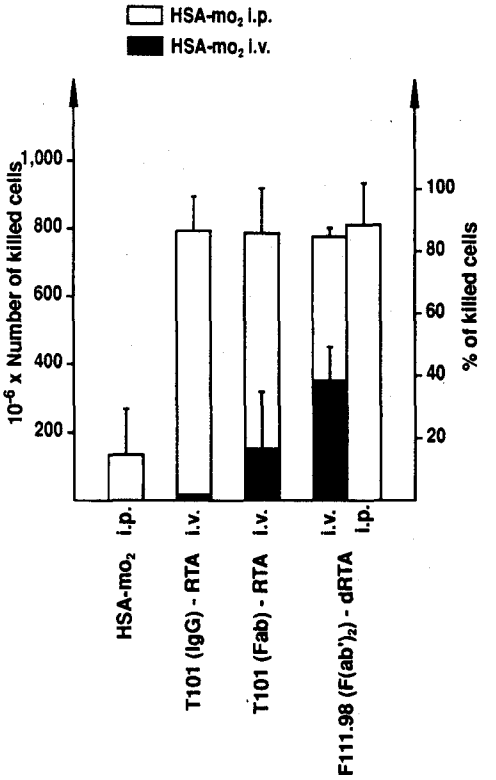

Fig. 4. Antitumour effect of anti-CD5 immunotoxins in the presence of monensin conjugated to human serum albumin (*HSA-mo2*). Mice were injected with the equivalent of 50 μg IgG or 35 μg $F(ab')_2$ or Fab and with 80 μg monensin conjugated to HSA. Results are presented as the number of killed cells determined as described in Materials and methods

Antitumoral effect of anti-CD5 immunotoxins

We next compared the in vivo cytotoxic efficacy of three anti-CD5 immunotoxins with different structures and various targeting capacities. Equivalent amounts of antibody binding site [150 μg IgG or 100 μg $F(ab')_2$ or Fab fragment conjugated to 45, 57 and 120 μg RTA respectively] were injected into ascitic-tumour-bearing mice; 9×10^8 IP12/2 tumour cells were disseminated in the peritoneal cavity and 95% were viable cells and incorporated [^{14}C]leucine in vitro to a similar extent to IP12/2 cells maintained in culture.

Because of the high tumour burden and because very few cells are sufficient to kill the animal, the antitumour effect could hardly be evaluated from survival curves. As a consequence, the measurement of the antitumoral efficacy of these immunotoxins was performed by the ex vivo determination of the percentage of intoxicated cells in the ascitic fluid, 5 h or 24 h after immunotoxin injection. Because the morphological integrity of immunotoxin-treated cells was expected to decline only over a course of several days [5], precluding the possibility of a direct determination of cell death by a dye-exclusion test, immunotoxin-intoxicated cells were evaluated by measuring the level of protein synthesis inhibition.

Results are shown in Fig. 3. After i.v. injection of the immunotoxin, the cytotoxic effect increased between 5 h and 24 h in every case and no additional activity was observed beyond 24 h (data not shown). With T101(IgG)-RTA, which has the lower targeting capacity, the maximal number of cells killed was 110×10^6, representing 12% of tumour cells. T101(Fab)RTA and F111.98[$F(ab')_2$]-dRTA, having higher tumour localizations, expressed a higher cytotoxic activity, since 1.2- to 2-fold more cells were intoxicated. When the latter immunotoxin was administered i.p., which allowed saturation of all accessible CD5 antigens, the tumour cell killing was enhanced by a factor of 2.

These data strongly suggest that the cytotoxic efficacy of the immunotoxins was related to the number of molecules bound per cell. However, even when saturation of CD5 antigens was achieved (i.e. after i.p. injection), the highest number of cells killed did not exceed 53% of the tumour cells in the ascitic fluid, which is far from a complete eradication of the tumour burden. A drastic improvement of the cytotoxic efficacy of immunotoxins in vivo is still required. We therefore examined the effect of a potentiating agent in vivo.

Antitumoral efficacy of immunotoxins in the presence of monensin in vivo

The cytotoxic activity of T101(IgG)-RTA, T101(Fab)-RTA and F111.98[$F(ab')_2$]-dRTA was measured in the presence of monensin in vivo. In order to increase the plasma half-life of monensin, which is extremely short, less than 2 min [4, 15], it was coupled through its primary hydroxyl group to human serum albumin (HSA) as described in Materials and methods. Two monensin

Table 4. Specificity of immunotoxin cytotoxicity in the presence of monensin conjugated to human serum albumin (HSA-mo2)

Injected products[a]			Cells killed 24 h after IT injection (%)
F111.98 mAb[b] i.p.	F111.98[F(ab')2]-dRTA i.v.	HSA-mo2 i.p.	
–	+	+	88.4±0.2
+	+	+	1.2±1.0

[a] 500 μg mAb, 30 μg F(ab')2 fragment conjugated to dRTA and 80 μg monensin conjugated to HSA were injected
[b] The mAb was administered 30 min before the immunotoxin (IT)

molecules were conjugated per HSA. This modified monensin showed no significant loss of its potentiating activity in vitro, whereas its half-life in blood in vivo was increased 100 times [6]. Samples of 50 μg IgG or 35 μg F(ab')2 or Fab fragment conjugated to ricin A-chain were simultaneously administered per mouse with the equivalent of 80 μg monensin.

The HSA-mo2 conjugate was first injected i.p., whereas the immunotoxin was administered i.v. at the same time (Fig. 4). The ex vivo measurement of the cytotoxic effect 24 h later showed a dramatic antitumour effect since 86% to 90% of tumour cells were killed. A similar enhancement by HSA-mo2 was found with all the immunotoxins tested, even with the T101(IgG)-RTA, which showed the lowest targeting capacity. As shown with the F111.98[F(ab')2]-dRTA, when immunotoxin was co-injected i.p. with HSA-mo2, identical results were obtained.

By contrast, only 15% of the cells were killed by i.p. injection of HSA-mo2 alone (Fig. 4) and the cytotoxic effect of F111.98[F(ab')2]-dRTA [30 μg conjugated F(ab')2 fragment] co-injected with HSA-mo2 was specifically blocked by i.p. injection of F111.98 mAb (500 μg) 30 min before immunotoxin injection (Table 4). These data demonstrate that the high antitumour effect, observed when HSA-mo2 was injected with the immunotoxin, was related to the immunotoxin specificity.

After simultaneous i.v. injection of immunotoxin and HSA-mo2 (Fig. 4), the antitumour effects were similar to the cytotoxic activities observed without potentiation. These results indicate that the efficacy of the potentiation by HSA-mo2 is tightly related to the route of administration of the enhancing agent.

Discussion

In this paper, we quantified the cytotoxic potency of various anti-CD5 immunotoxins directed against a wide tumour burden, in a model of a human xenograft inoculated i.p. into the nude mouse. We have previously studied in this model the targeting capacity of immunotoxins with different structures by the determination of the number of intact molecules bound per cell, using an immunoenzymometric assay [21].

This analysis was completed here by comparing, after immunotoxin administration in vivo, the number of intact immunotoxin molecules associated with tumour cells, to the number of active molecules determined from their cytotoxic activities established in vitro. This study, carried out with two different immunotoxins – T101(IgG)-RTA and F111.98[F(ab')2]-dRTA – showed that 40%–100% of the bound molecules following i.v. administration retained their cytotoxic properties, demonstrating that the enzymatic activity of the ricin A-chain was preserved in animals.

The antitumoral effect of these molecules in animals was measured by the ex vivo quantification of killed cells, 24 h after immunotoxin injection. Cell death by intoxication with RTA-immunotoxin depends on the inhibition of protein synthesis, which occurs rapidly after immunotoxin internalization, whereas cell degradation is a relatively slow process [14]. Then, to evaluate immunotoxin activity in vivo, we measured the protein synthesis 24 h after injection.

The antitumour potency of immunotoxins administered in animals had to be quantified ex vivo rather than in vivo. Indeed, the antitumour efficacy in such a model cannot be evaluated by the survival of the mice because of the large tumour burden. As a matter of fact, few mice survive because even a few cells are sufficient to kill the animal.

This study showed that after injection of the immunotoxin alone, the number of intoxicated cells is related to the number of immunotoxin molecules per cell: the maximal effect was obtained after saturation of the target antigens (30000 antigens/cell). However, even after antigen saturation, only 53% of the tumour cells were killed. The cytotoxic activity of anti-CD5 immunotoxins was not high enough to eradicate more than 500×10^6 tumour cells disseminated in the peritoneal cavity of the nude mouse.

On the other hand, we showed that the use of a potentiating agent in vivo such as HSA-mo2 dramatically improves the antitumoral efficacy of immunotoxins. A potentiating effect was demonstrated after i.p. injection of HSA-mo2 directly at the tumour site. Despite the 3-fold lower doses of the injected immunotoxin, the number of killed cells after i.v. injection was increased 7 times in the case of T101(IgG)-RTA and 3.5 times with F111.98[F(ab')2]-dRTA. Even with less than 3000 active molecules/tumor cell, 90% of the cells from the ascitic fluid could be killed, which corresponded to 810×10^6 cells. However, no potentiation was observed after i.v. injection of HSA-mo2. The discrepancy between the potentiating capacities of monensin after i.p. or i.v. administration could be related to different pharmacokinetics of the immunotoxin and HSA-mo2. If their rates of tumour localization are different, these molecules may reach the tumour cells at different times. Recently published data showed that monensin conjugated to HSA was cleared from the plasma of BALB/c mice with a half-life of 0.5 h [7], whereas we demonstrated in a previous paper that the half-life of F(ab')2-dRTA was

9 h. Furthermore, we demonstrated in vitro that the potentiation was 10 times lower when monensin was added 24 h after the immunotoxin rather than 1 h or 5 h later (data not shown). Similar results were obtained with ammonium chloride [20]. These results suggest that optimum conditions for the use of a potentiating agent in vivo must be determined to obtain the best potentiating effect. The respective delay of in vivo tumour localizations of the immunotoxin and the enhancer is an important parameter to consider.

The potentiating effect of monensin in vivo has already been demonstrated following intratumoral co-injection of immunotoxin and monensin. A 1.5-fold increase of the median mouse survival time was observed with a monensin-linoleate conjugate injected intraperitoneally with a RTA immunotoxin directed against transferrin receptor [11]. No enhancing effect was observed with non-conjugated monensin. However Roth et al. showed that the development of pulmonary metastasis was decreased by i.p. injection of monensin in mice treated i.v. with an immunotoxin composed of a whole mAb coupled to native ricin A-chain [22]. These results are in agreement with our findings, which emphasize the importance of monensin pharmacokinetics for immunotoxin enhancement in vivo.

A few systematic studies have been carried out to improve the procedure of treatment with a cocktail of an immunotoxin and a potentiating agent. However, these results indicate that RTA-bound immunotoxin molecules are active in vivo, can be potentiated by monensin or HSA-mo_2 and that 800×10^6 tumour cells, localized in the peritoneal cavity of mice, can be killed, even with a saturation level of target antigens lower than 10% of the maximum level.

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