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Research Article

The effect of monensin and chloroquine on the endocytosis and toxicity of chimeric toxins

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Abstract. The toxicity of two conjugates containing ribosome-inactivating proteins (RIPs, i.e. saporin and ricin-A chain x-linked to transferrin) has been measured on a prostatic cancer line (PC3) naturally overexpressing the transferrin receptor, in the presence of monensin and chloroquine. This paper investigates whether the increased toxicity of Tf-RIPs induced by monensin and chloroquine may be due to alterations of the normal endocytotic pathway of the complexes mediated by the transferrin receptor. Monensin, besides inducing alkalinization of normally acid intracellular compartments, causes an accumulation of the receptor-bound Tf-RIP in a perinuclear region contiguous to the cisternae of the trans-Golgi network. Chloroquine, though increasing the intracellular pH, seems not to modify the endocytotic pathway of these chimeric molecules. We believe that the enhanced toxicity of the Tf-RIPs may be related to intracellular alkalinization (i.e. endosomal or lysosomal pH) rather than to the effects on the recycling of transferrin receptor-bound toxins. We conclude that the efficacy of chimeric toxins may be modulated not only by the carrier used for their engineering but also by addition of drugs able to influence the stability and activation of the toxins inside the cell.

Key words. Ricin; transferrin; monensin; chloroquine; endocytosis; chimeras; cancer.

The use of cytotoxic compounds specifically targeted towards malignant cells has been studied since monoclonal antibodies against membrane proteins expressed by cancer cells and accessible from the outside have become available. Among others, conjugation of antibodies with cytotoxic proteins (immunotoxins) using chemical and recombinant techniques has been widely employed [1, 2]. Along this line of research, other chimeras with carriers differing from antibodies have been prepared employing hormones, growth factors and vitamins to deliver toxins or drugs to their intracellular target [3–7].

Ribosome-inactivating proteins (RIPs) [8] are N-glycosidases from plants which inhibit protein synthesis by irreversible chemical modification of the ribosomal RNA [9]; they have been employed not only because of their stability but also because the large variety of RIPs produced by plants warrants a multiplicity of similar

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but different conjugates to be synthesized. This may be of practical importance, since the use of artificial conjugates on humans demands a wide spectrum of 'bullets' against the same malignant cells, in view of (i) the immunological response elicited by 'extraneous' proteins systemically delivered [10] and (ii) the differential sensitivity of cancer cells to cytotoxins containing different RIPs [11]. Most naturally occurring RIPs are not toxic, since they are made up of a single catalytic chain without a proper carrier necessary for penetration into the cell [8]; on the other hand, several RIPs, including the very powerful ricin, consist of a catalytic A chain associated to a carrier (lectin) B chain. Upon binding the plasma membrane, the carrier allows the internalization of the A chain into the cell and subsequent killing. Various grades of remission, including complete remission, have been observed in clinical trials with leukaemias and lymphomas treated with cytotoxic chimeras [11, 12]; however, beneficial effects have been modest in the case of solid tumours, probably because an effective dose of the toxin could not access the target cells. In addition, nonspecific toxicity resulting from interaction with tissues other than the target was seen when high doses of toxins were employed to reach poorly vascularized solid tumours [10]. From this perspective, improving the efficacy of these artificial conjugates may be obtained by treatment of target cells with chimeric toxins in combination with drugs which are effective in enhancing toxicity.

In a previous paper [13] we studied RIP delivery using as a carrier transferrin (Tf), a protein known to transport iron in the cell by binding to a specific membrane receptor. Endocytosis of the Tf-receptor complex goes through acid compartments, where the iron is released, and the apo-Tf recycled back to the plasma membrane together with its receptor [14–18]. The number of Tf receptors expressed increases from early to late S phase of the cell cycle, indicating that it is closely related to initiation of DNA synthesis [19]. Moreover, Tf receptors are overexpressed in cancer cells, probably to meet the need of iron [20] during cell proliferation.

In this paper we present data on the cytotoxicity of two conjugates between human Tf and either saporin (Tf-Sap) or the A chain of ricin (Tf-A_{RCA}), tested on a prostatic cancer line (PC3) naturally overexpressing the Tf receptor. Previous data showed that proliferation of prostatic carcinoma cells is highly responsive to Tf [21], making the Tf receptor a reasonable target for introducing toxins into these proliferating cancer cells. We show here that Tf-Sap and Tf-A_{RCA} are both active on PC3 cells, and that their toxicity is enhanced by addition of monensin and chloroquine. Our data indicate that chimeric toxins administered with adjuvant substances (at noncytotoxic concentrations) may be potentially useful for local treatment of prostatic cancer, which is the second major cause of death among adult men in the United States [22].

Materials and methods

Saporin was purified from *Saponaria officinalis* seeds as previously described [23] and stored in aliquots at -80 °C. The A chain of the dimeric toxin ricin was purified by reduction of the disulphide bond linking the two constituent polypeptide chains, followed by affinity chromatography on a lactose-sepharose column (from Pierce) [24]. Monensin, chloroquine and human Tf were from Sigma (St. Louis, MO, USA). 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoil)sphinposine (C_6-NBD)-ceramide was from Molecular Probes (Eugene, CA, USA). All other reagents were of analytical grade.

Synthesis of the conjugates. Tf-Sap (the transferrin-saporin conjugate) was synthesized as previously described [13]. Tf-A_{RCA} (the transferrin-ricin A chain conjugate) was prepared following a similar procedure, but the final purification step was done on a blue-sepharose affinity column (Pharmacia-LKB, Uppsala, Sweden). Fluorescent derivatives of saporin and dimeric ricin, obtained as described [25], were purified by ion exchange chromatography.

Cell line. Human PC3 prostate cancer cells (obtained from the American Type Culture Collection, Rockville, MD, USA) were grown in RPMI1640 with 10% (v/v) foetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 95% air atmosphere. For drug exposure experiments, cells were plated at 1.5×10^5 cells/ml density in 24-well Costar plates and grown for 24 h before adding the drugs. Stock of monensin was prepared in EtOH. Stock solution of chloroquine was prepared in phosphate-buffered saline (PBS). The solvents produced no cytotoxic effects at the concentration used for the experiments.

Inhibition of protein synthesis. For cytotoxicity assay, cells were cultured for 1 day (1.2×10^5 cells/ml), preincubated with Tf-free medium for 2 h and then treated with serial dilutions of the chimeric proteins for 18 h at 37 °C. The medium was removed, and 0.5 ml of leucine-free medium, supplemented with ^{3}H-labelled leucine (1 μCi/ml) was added to each well. After an additional 2 h incubation time, the medium was removed, and the cells were washed twice with ice-cold PBS and once with RPMI 1640 buffered with 50 mM Hepes at pH 7.4 + 1% bovine serum albumine (HBSA) medium, then 1 ml of 10% tri-chloro acetic acid (TCA) was added to each well. The protein precipitates were dissolved by adding 0.25 ml of 0.1 M NaOH to each well. Aliquots of cell lysates (100 μl) were mixed with 5 ml of scintillation mixture, and incorporated radioactivity was measured by a liquid scintillation β counter.

The effect of monensin, chloroquine and retinoic acid was assessed by performing similar experiments. Monensin was used at 10 μM, and chloroquine and retinoic acid in doses ranging from 10 to 50 μM.

Fluorescence videomicroscopy experiments. Cells grown on coverslips (2×10^5 cells/ml) were preincubated with Tf-free medium for 2 h and then incubated with the toxins (300 nM concentration) for 1 h at 37 °C. In some experiments cells were exposed to the toxins at 4 °C for 30 min, before incubation at 37 °C.

Different concentrations (ranging from 10 to 50 μM) of monensin and chloroquine were added and maintained throughout the experiment. Cells washed with PBS were either transferred to a glass live chamber for 'in vivo' observation, or fixed with 4% *p*-formaldehyde in PBS for 20 min at 4 °C, washed with ice-cold PBS and mounted on glass slides with Aquamount (PolySciences, USA). Cell images were acquired using a fluorescence microscope (Zeiss Axiophot, Carl Zeiss, Germany) equipped with an epi-illumination lamp and connected to a videocamera controlled by an Apple IIGS computer running programs developed by our lab [26]. Images were stored and subsequently analysed by the program Image (W. Rasband, NIH, Bethesda, MD, USA).

pH measurements of intracellular acidic compartments. Endosomes containing Tf were assayed for their internal pH value by use of 5-(and-6)-carboxySNAFL-1,succinimidyl ester (SNAFL1 from Molecular Probes) covalently linked to human transferrin. Briefly, a succinimide ester derivative of the fluorescent pH indicator SNAFL1 was cross-linked to human Tf by reaction with the protein in a 5:1 molar ratio in 20 mM bicarbonate buffer, pH 8.2. Excess of the dye was eliminated by gel filtration on a Sephadex G25 column (Pharmacia). Derivatized Tf was incubated with cells at 600 nM concentration in the presence or the absence of monensin and chloroquine in the culture medium for 1 h. Images from each experiment were taken by fluorescence microscopy using the fluorescence excitation and emission filters set for fluorescein, and average relative fluorescence intensities of intracellular vesicles containing SNAFL1-Tf were calculated based on histogram analysis. The detailed procedure will be described elsewhere (Sarti et al., unpublished observations). For the calibration curve of pH, cells were loaded with SNAFL1-Tf as described above and further incubated with buffers at known pH in the presence of high K^+ (150 mM) and the ionophore nigericin (10 μM) as described elsewhere [27]. Relative intracellular fluorescences were plotted as a function of pH, and the relative fluorescence of the cells incubated with SNAFL1-Tf alone or with SNAFL1-Tf in the presence of chloroquine (10 μM) or monensin (10 μM) was reported on the graphic to extrapolate the pH value of intravesicle medium.

A similar procedure was followed to measure the pH value of acidic compartments as a whole (secondary endosomes and lysosomes). Cells were loaded for 30 min with 10 μg/ml of acridine orange as described [27]. Under these conditions the dye accumulates inside acidic compartments (particularly lysosomes), and it has a strong red fluorescence signal when excited with the blue light; furthermore, a green fluorescence is still visible as a diffused signal throughout the cytoplasm and associated with nucleic acids inside the nucleus. When cells were exposed to buffers at different pH values, the dye was released from the vesicular structures and diffused into the cytoplasm and the nucleus. Analysis made on images of single cells using the Image program allowed us to measure the number of vesicles containing acridine orange and to follow the release of the dye from these structures as a function of pH. A plot of the average number of particles per cell vs. the pH of the calibration buffer generated a standard curve on which the values obtained in control or chloroquine- or monensin-treated cells were superimposed.

Results

Inhibition of protein synthesis in PC3 cells. Incubation of PC3 cells with increasing concentrations of Tf-Sap conjugate resulted in a dose-dependent inhibition of protein synthesis; the IC_{50} (toxin concentration inhibiting 50% protein synthesis) was estimated at 10^{-8} M (fig. 1A, open circles; see also table 1). As expected, the presence of free Tf in the incubation medium reduced the toxicity of Tf-Sap (data not shown) by competing for the Tf receptor. As shown in figure 1A, both chloroquine and monensin enhanced Tf-Sap cytotoxicity (16.7- and 33.3-fold, respectively), as demonstrated by the left shift of the dose-dependent curves: the corresponding IC_{50} values are reported in table 1.

Cytotoxicity of the Tf-A_{RCA} was assessed as above (fig. 1B). As clearly shown in figure 1B and table 1, toxicity of Tf-A_{RCA} was greatly increased by addition of 10 μM monensin ($IC_{50} < 10^{-11}$ M, a 700-fold increase in toxicity), while addition of 10 or 30 μM chloroquine was ineffective (a 10-fold increase being observed only at 50 μM).

As shown in figure 1C, ricin toxicity on PC3 cells is about 40 to 50 times greater than that measured for the both Tf-Sap and Tf-A_{RCA}. It is worth noting that monensin (as well as chloroquine) had no effect on native ricin toxicity (see fig. 1C). This is in sharp contrast with the effect of the addition of drugs on the toxicity of Tf-Sap and Tf-A_{RCA}, the former approaching ricin toxicity in the presence of chloroquine and monensin and the latter being even more toxic than ricin in the presence of monensin.

Intracellular distribution of the two conjugates compared with ricin. The intracellular distribution of Tf-Sap conjugate was followed in PC3 cells by fluorescence videomicroscopy using a rhodamine-labelled fluorescent derivative of saporin for the synthesis of the chimera [13]. As shown in figure 2 (red fluorescence signal), after 60 min incubation Tf-Sap is quite uniformly distributed inside living cells and shows a very similar distribution to that of Tf alone (see fig. 3); a similar pattern was observed in other cancer cell lines [13]. Figure 2 also clearly shows that the Tf-Sap (red signal) intracellular distribution pattern is different from that of the fluorescent Golgi marker NBD-ceramide (green signal), and also different from that of the whole ricin toxin (see fig. 3), which accumulates in compact structures, previously identified as the Golgi apparatus [28].

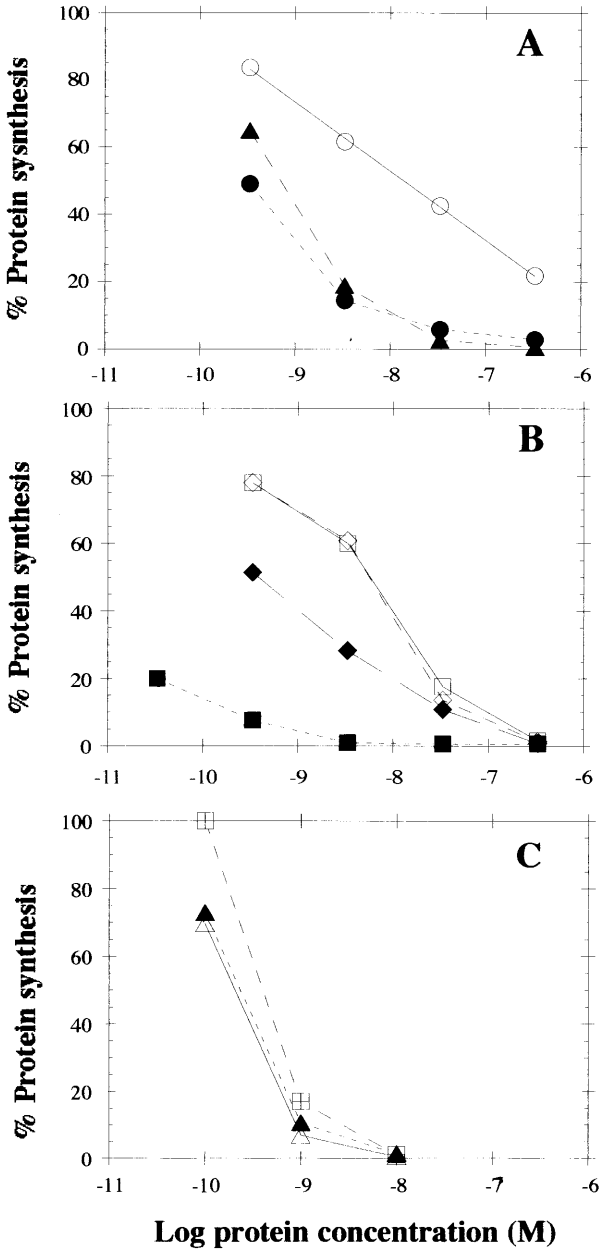

Figure 1. Inhibition of protein synthesis in PC3 cells. (*A*) Inhibition of protein synthesis in PC3 cells incubated with serial dilutions of Tf-Sap conjugate and in the presence (when indicated) of effectors. Symbols refer to PC3 cells treated with (○) Tf-Sap; (●) Tf-Sap + 10 μM monensin; (▲) Tf-Sap + 10 μM chloroquine. (*B*) Inhibition of protein synthesis in PC3 cells incubated with serial dilutions of Tf-A_{RCA} conjugate and in the presence (when indicated) of effectors. Symbols refer to PC3 cells treated with (□) Tf-A_{RCA}; (■) Tf-A_{RCA} + 10 μM monensin; (◇) Tf-A_{RCA} + 10 μM chloroquine; (◆) Tf-A_{RCA} + 50 μM chloroquine. (*C*) Inhibition of protein synthesis in PC3 cells incubated with serial dilutions of ricin and in the presence (when indicated) of effectors. Symbols refer to PC3 cells treated with (△) ricin; (▲) ricin + 10 μM monensin; (⊞) ricin + 10 μM chloroquine.

Effect of monensin and chloroquine on the intracellular distribution of toxins. Different effects were observed on addition of monensin and chloroquine to the cells before the toxins, particularly on their cellular localization. Tf-Sap distribution was altered by exposure of cells to 10 μM monensin (no differences were observed while exposing cells to monensin before or at the same time of toxin), most of the internalized conjugate being accumulated in perinuclear compact vesicular structures (fig. 3) bordering the Golgi cisternae [29]. Comparison of the endocytotic pathway of free Tf in the presence of monensin (fig. 3) shows that free Tf also accumulates in perinuclear bodies, similarly to what observed with Tf-Sap. These data indicate that the complex of the Tf receptor and its bound ligand (either Tf or Tf-Sap) is transported inside the cell and does not dissociate during accumulation into the observed perinuclear vesicles. On the basis of these observations, one would expect that these vesicles might represent the major site where the toxin is accumulated before release into the cytoplasm. But attempts to correlate the modified distribution pattern to the increase in toxicity of the conjugate induced by monensin (table 1) were frustrated by the fact that chloroquine produced no detectable change in endocytosis and intracellular accumulation (fig. 3) of Tf-Sap, though its effect on toxicity was the same as induced by monensin.

The intracellular localization of whole ricin was not altered by the presence of monensin, the toxin being delivered and accumulated in the Golgi (see fig. 3) without modification of pattern of the intracellular distribution. Since monensin has no effect on ricin toxicity (fig. 1) but is known to alter the morphology of the Golgi apparatus [30], we checked the integrity of the Golgi structure on PC3 cells treated with this drug. The distribution of the Golgi marker NBD-ceramide was not significantly changed (at least up to 1 h incubation, see fig. 4) in the presence of monensin, a result which suggests that in the PC3 cancer line this compartment is not affected by the presence of the drug.

Effect of monensin and chloroquine on the pH of intracellular vesicles. Figure 5A shows the pH titration of endosomes containing transferrin conjugated to a

Table 1. Effect of chloroquine and monensin on the toxicity of Tf-Sap, TfA_{RCA} and ricin on PC3 cells.

	Control (SE%)	Monensin at 10 μM (SE%)	Chloroquine at 10 μM (SE%)	Chloroquine at 50 μM (SE%)
Tf-Sap	10^{-8} (8.8)	3×10^{-10} (19.1)	6×0^{-10} (6.7)	n.d
Tf-A_{RCA}	7×10^{-9} (10.3)	$<10^{-11}$ (11.2)	7×10^{-9} (4.1)	6×10^{-10} (2.8)
Ricin	2×10^{-10} (12.5)	1.5×10^{-10} (12.1)	5×10^{-10} (2.3)	n.d.

IC_{50} values expressed as concentrations (M) of the toxins giving 50% inhibition of protein synthesis. The standard error (SE%) is reported, in parentheses, as a percentage of IC_{50} values.

fluorescent pH indicator (SNAFL1). As extrapolated from the calibration curve, SNAFL1-Tf is carried inside vesicles whose average pH value is 6.7. This value agrees with previously reported data [31] on the pH of fast-recycling endosomes containing Tf bound to its receptor. The presence of monensin at 10 μM concentration in the incubation medium slightly increased the pH value of these endosomes up to 6.9. Chloroquine at the same concentration was more effective in alkalinization of endosomes, although it was not possible to obtain an exact pH value (>7.5).

Figure 5B shows the pH titration of acidic cellular compartments (mainly lysosomes) following the accumulation of acridine orange inside these structures. As shown, in the control cells these vesicles are quite acid ($pH < 5$), and the presence of monensin (10 μM) allows alkalinization ($\Delta pH > 1$). Chloroquine produced an increase in the pH value of the vesicles comparable to that of monensin only at 50 μM, while at 10 μM concentration no visible change in pH (i.e. no release of the dye from the vesicles) was observed.

Discussion

This paper reports the toxic properties on human prostatic carcinoma cells (PC3) of two artificial conjugates of human transferrin (Tf) with saporin, a monomeric RIP from the seeds of *Saponaria officinalis*, and with the A chain from ricin. PC3 are Tf-receptor over-expressing cells from human prostatic carcinoma, which produces osteoblastic metastasis in advanced cancer. Preferential growth of prostatic carcinoma in a variety of bone sites, including the spine, long bones and skull may

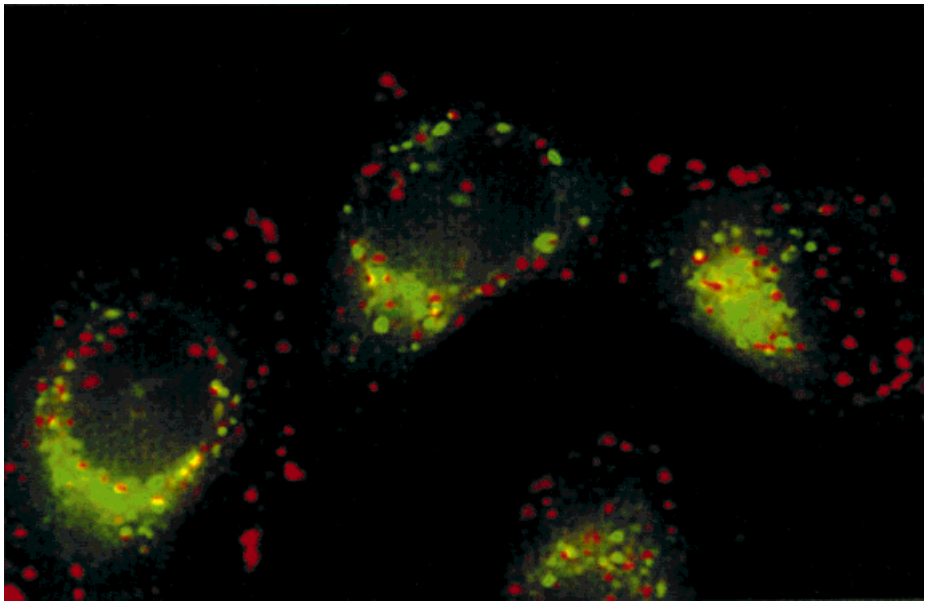

Figure 2. Fluorescence microscopic analysis of Tf-Sap and C_6-NBD-ceramide distribution in living PC3 cells. Cells grown on coverslips were incubated with 300 nM Tf-Sap for 1 h at 37 °C in D-MEM without FCS, then washed and incubated with C_6-NBD-ceramide (5 μg/ml) for 10 min at 37 °C before observation. Red fluorescence refers to Tf-Sap (recorded in the rhodamine emission region); green fluorescence refers to the C_6-NBD-ceramide (recorded in the fluorescein emission region).

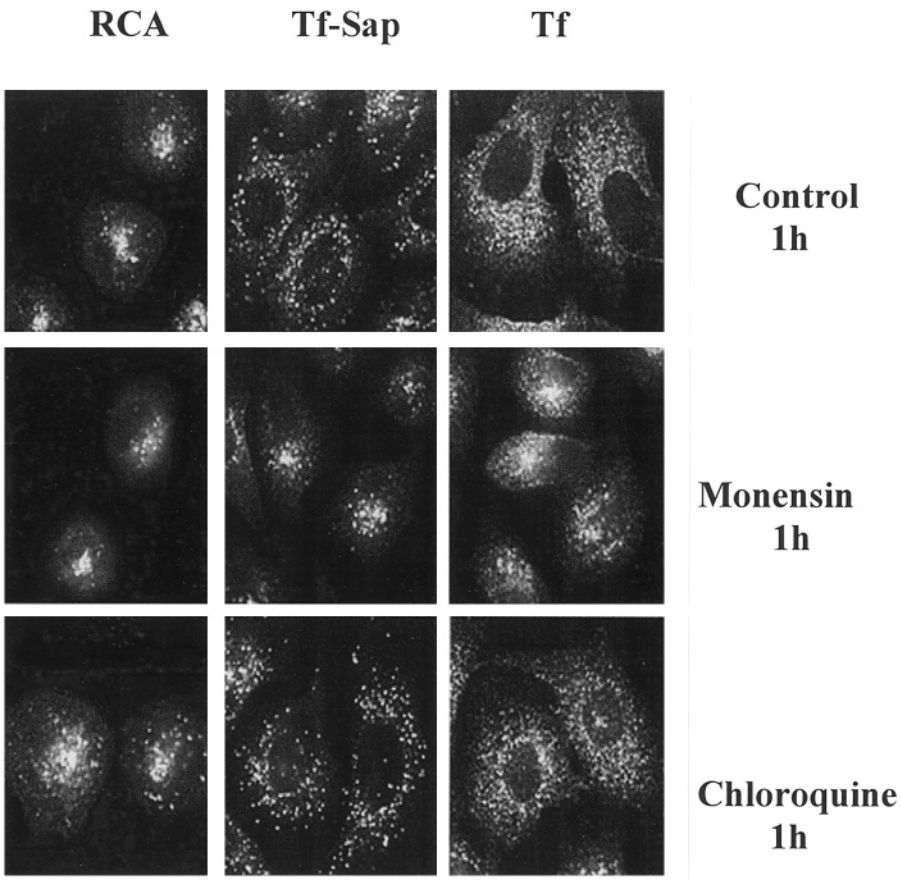

Figure 3. The effect of monensin and chloroquine on the endocytosis of Tf-Sap, Tf and ricin. Control: intracellular distribution of ricin, Tf-Sap and Tf as detected by fluorescence microscopy in PC3 cells after 1 h of incubation, in a control experiment. Monensin: effect of 10 μM monensin on the intracellular distribution of ricin, Tf-Sap and Tf. Chloroquine: effect of 30 μM chloroquine on the intracellular distribution of ricin, Tf-Sap and Tf.

be related to the finding that bone marrow contains Tf that stimulates prostatic carcinoma cell growth [21]. The two synthetic conjugates (called Tf-Sap and Tf-A_{RCA}) when tested on the PC3 cell line proved to be toxic, with comparable activity. Their toxicity was enhanced in the presence of monensin and chloroquine, known to have increasing effects on the toxicity of artificial conjugates [32]. The presence of monensin in particular increases the toxicity of Tf-Sap to levels comparable with those of the whole native ricin. Interesting and intriguing is the case of Tf-A_{RCA}, whose toxicity in the presence of monensin is even higher than that of native ricin (see table 1), though the toxic subunit (the A chain of ricin) is the same.

We investigated whether addition of monensin and chloroquine could induce changes in the endocytotic pathway of the chimeras which may account for their increase in toxicity. The fluorescence microscopy experiments shown in figure 3 indicated that monensin leads to an accumulation of the conjugate Tf-Sap in perinuclear structures, as observed with monensin-treated cells when labelled Tf alone is followed. This is consistent with the finding that monensin affects the intracellular pathway of Tf, both in free and conjugated (Tf-Sap) form.

The distribution of the Tf-Sap conjugate in the presence of monensin in PC3 cells closely resembles that of the Tf receptor in K562 cells, where previous data demonstrated [29] inhibition of Tf and its receptor recycling when cells were exposed to this drug; this effect was found to be associated with the appearance of vesicular structures containing the Tf receptor, identified as multivesicular bodies. These structures were localized very close to the TGN (trans-Golgi network) and may possibly represent an intermediate site in the recycling process of the Tf-receptor complex mediated by the Golgi apparatus.

Chloroquine induced an increase in Tf-Sap toxicity on PC3 cells comparable to that of monensin, but no changes in its intracellular distribution (e.g. no perinu-

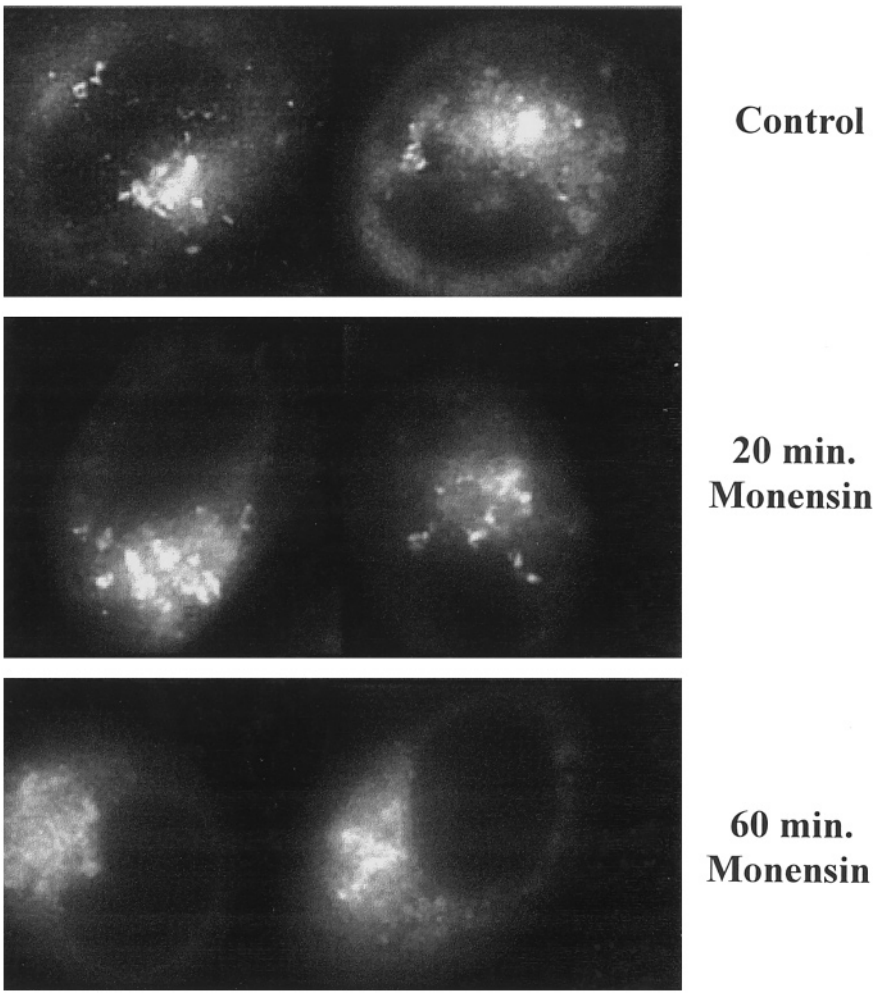

Figure 4. Effect of monensin on the intracellular distribution (on PC3 cells) of the Golgi marker C_6-NBD-ceramide (5 μg/ml) as detected by fluorescence microscopy at different time intervals. Experimental conditions as in figure 2.

clear accumulating vesicles) were observed (fig. 3). This unchanged morphological pattern agrees with the observation that chloroquine does not block the recycling of Tf on K562 cells [29].

As a control, we also tested the effect of both chloroquine and monensin on ricin endocytosis; ricin is a heterodimeric toxin probably activated in the Golgi by reduction of the disulphide between the A and B chains to allow translocation of the toxic A chain to the cytoplasm either directly or via the endoplasmic reticulum (ER). Ricin endocytosis and toxicity to PC3 cells were not affected by the presence of the drugs, and monensin treatment was not associated with changes in the intracellular distribution of the Golgi marker NBD-ceramide (see fig. 4). Thus, stability of the Golgi in PC3 upon exposure to monensin, similar to that observed with other cell lines after treatment with drugs [33], may account for the lack of effect on ricin.

We further investigated the effect of chloroquine and monensin on the pH of intracellular compartments involved in the endocytosis of conjugates. Our data show that endosomal pH (see fig. 5A) is raised by both monensin and chloroquine (though to different extent, i.e. $\Delta pH > 1$ for chloroquine and 0.2 for monensin) at 10 μM concentration. Under these conditions the toxicity of Tf-Sap is enhanced by both drugs, while Tf-A_{RCA} is not affected by the presence of chloroquine. Lysosomal pH shifts to alkaline values in the presence of monensin (see fig. 5B, $\Delta pH > 1$), but it is not altered by chloroquine at 10 μM concentration. Only at 50 μM concentration was chloroquine able to induce alkalinization as monensin. These data correlate with the effect of

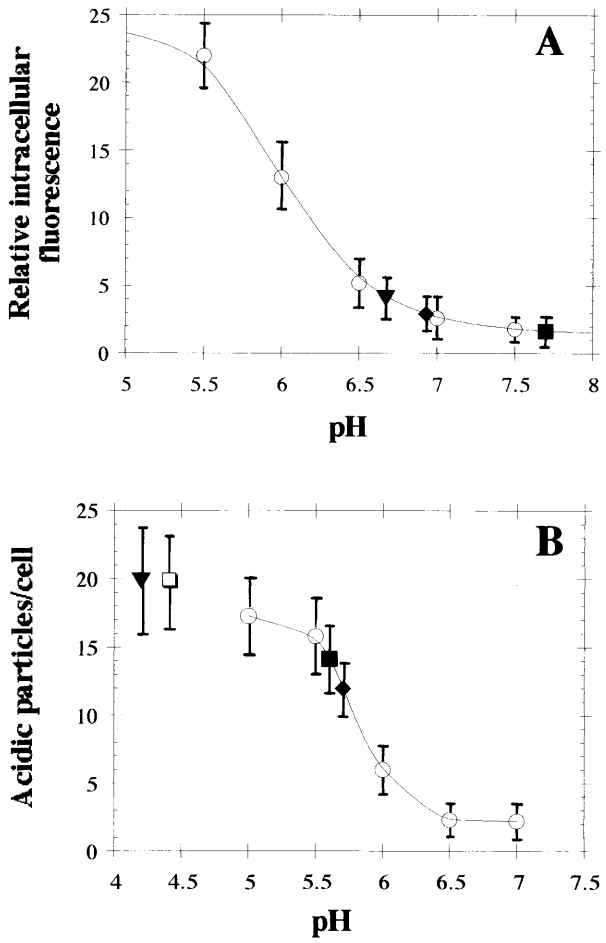

Figure 5. pH titration of endosomal and lysosomal compartments. (*A*) pH in endosomes containing Tf. The reference curve (○) was obtained by the nigericin/high K^+ method [27] using a covalent complex between the fluorescent pH indicator SNAFL1 and human Tf. PC3 cells were loaded with the fluorescent conjugate as described in figure 2 and further incubated for 5 min with the calibration buffer at known pH. Microscopic images were taken by use of the fluorescein filter set. After correction for autofluorescence intensity, data were analysed as described in the 'Materials and methods' section, and the relative fluorescence intensity for each point was plotted as a function of pH. Control cells (▼) were loaded with SNAFL1-Tf either in the absence or presence of 10 μM chloroquine (■) or monensin (◆), and images from these samples were analysed as described above. Their relative fluorescence is reported in the graph. Each point represents the average fluorescence as obtained by histogram analysis of about 200 to 250 cells. (*B*) pH in acidic endosomes and lysosomes. The reference curve (○) was obtained as described above except that acridine orange (AO) was used as fluorescent marker. Cells were incubated for 30 min with AO (10 μg/ml) before treatment with calibration buffers. The red fluorescent spots inside the cells represent acidic compartments where AO accumulates as a function of pH. Under alkaline conditions, the dye diffuses from the vesicles and stains the cell cytoplasm and nucleus. The number of fluorescent vesicles inside single cells (about 30 cells for each point) were calculated by analysis with the program Image (W. Rasband, NIH, Bethesda, MD, USA) and plotted as average values. Control cells (▲) and chloroquine- or monensin-treated cells were incubated with AO as above, except for a 1-h pretreatment with the two drugs [10 μM monensin (◆) or 10 μM (□) 50 μM (■) chloroquine] when necessary.

chloroquine on Tf-A_{RCA} toxicity, which is enhanced only at 50 μM concentration.

These experiments suggest that pH alterations of endosomal and lysosomal structures may potentiate the toxicity of Tf conjugates, but with different effects depending on the toxin bound to Tf; enhancement of Tf-Sap toxicity seems to reside in the endosomal pathway, where somehow alkalinization may favour escape of the toxin from the vesicles. Tf-A_{RCA} toxicity seems to be mainly affected by changes in lysosomal pH, suggesting protection against proteolytic degradation of the A_{RCA} molecule.

In conclusion, our data point to the following:

1) Tf conjugates are internalized and processed mainly through the endosomal system, and the Golgi apparatus seems to be excluded from this process, contrary to the case of native ricin or saporin conjugated with the B chain of ricin [13].

2) The toxicity of Tf-Sap and Tf-A_{RCA} conjugates is increased by addition of monensin and chloroquine; this phenomenon probably correlates with the alkalinization of the pH of the vesicular structures involved in the endocytosis of Tf.

3) Since the toxicity of Tf-Sap is increased by monensin as well as chloroquine, the type of intracellular accumulation of the two chimeras and the effect on Tf recycling do not account for the enhancement of toxicity.

4) Tf-A_{RCA} seems more sensitive to monensin than to chloroquine, which may correlate with the partial proteolytic activation mechanism by endosomial proteases demonstrated for A_{RCA} [34]. Should this mechanism of activation also prove to operate in PC3 cells, it would be plausible to assume that monensin somehow favours A_{RCA} activation because of longer permanence in the vesicles; since saporin is completely insensitive to proteolysis [35], the differential sensitivity of the two chimeric toxins to the presence of monensin may be thus explained.

Interpretation of these data points to a complex mechanism for toxin transport, activation and targeting inside the cell. A clear-cut conclusion of our work is that the toxin is delivered to more than one intracellular compartment before release into the cytoplasm. In fact, it is widely accepted [8, 28] that ricinlike toxins are delivered from the cell membrane to the Golgi or to the ER before reaching the ribosomes; however, toxicity is not uniquely dependent on passage through the Golgi or the ER, and indeed, endosomal structures may be equally efficient, especially if appropriate drugs are used as adjuvants. Monensin alters the normal pathway of endocytosis of Tf-conjugated RIPs, but it seems unlikely that this phenomenon is the main reason for the enhancement of their toxicity. Therefore, in agreement with previous results [13], we conclude that in the absence of drugs the nature of the carrier has an effect on

the toxicity of a given RIP. But we propose that interference of drugs such as monensin and chloroquine may allow modulation of cell-killing efficacy by changing the physiological state of some intracellular compartments. The choice of toxins to be used with specific cell lines in combination with adjuvants should be considered when application of these proteins for cancer treatment is under consideration. Heterogeneous response of tumour cells to treatment with engineered toxins or traditional chemotherapy, together with the intrinsic difficulty in specific delivery of a toxin to a receptor expressed only on the tumour cells, may suggest to employing receptors expressed at a high level on target cells and at a low level on normal cells as a complementary approach. Since the Tf receptor is overexpressed in prostatic cancer cells compared with normal ones [19], it is a possible candidate for the targeting of toxins to be employed for local intratumour therapy of this selected cancer type [36]. Enhancing the efficacy achieved by use of drugs at nontoxic concentrations may help to overcome or mitigate side-effects related to high toxin dosage. In addition, the possibility of employing different conjugates with similar toxicity for target cells seems to be of interest in circumventing immunological reactions, which is a serious drawback to the use of toxins as anticancer therapy.

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