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Immunotoxin sensitivity of Chinese hamster ovary cells expressing human transferrin receptors with differing internalization rates

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Abstract Previous studies have shown that immunotoxin action is dependent upon selective binding to the target cell, internalization and then passage into the cytosol. It is important to define precisely how these critical steps are controlled so that the underlying relationship of each to high cytotoxic effectiveness is understood. In order to evaluate the contribution of internalization rate and receptor number on immunotoxin potency, the effects of an anti-(transferrin receptor, TfR)/ricin A chain immunotoxin, 7D3-A, were assessed on a parent Chinese hamster ovary cell line developed in our laboratory with no TfR (TfR^{neg}) and two lines transfected with either wild-type TfR (Tfr^{wt}) or an internalization-deficient ($TfR^{\Delta 7-58del}$) mutated human TfR. Potent, receptor-mediated cytotoxicity resulted from the action of 7D3-A on TfR^{wt} cells ($ID_{50} < 1$ nM) while both TfR^{neg} cells and $TfR^{\Delta 7-58del}$ were only minimally affected ($ID_{50} > 100$ nM). Butyrate up-regulation substantially increased receptor expression on the TfR^{wt} and $TfR^{\Delta 7-58del}$ cells, but no corresponding rise in sensitivity to 7D3-A was observed. In contrast, immunotoxin potency was increased by co-treatment of TfR^{wt} cells with the carboxylic ionophore monensin and the effect was even more pronounced for $TfR^{\Delta 7-58del}$ cells. We conclude that internalization rate or intracellular destination is a much more important determinant of immunotoxin efficacy than receptor number.

Key words Immunotoxin · Transferrin receptor · Internalization rate · Monensin · Ricin A chain

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Introduction

Immunotoxins are attractive candidates for cancer therapy because they combine the specificity of tumor cell reactive antibodies with the high cytotoxic potency of naturally occurring toxins. Numerous cell-surface antigens can be targeted by these new agents, but the high variability in their capacity for mediating cytotoxicity [1] makes it important to define the cellular factors controlling potency. Correlating immunotoxin potency with basic parameters such as receptor number, internalization rate and endocytotic pathway is important because it will suggest ways to improve their effectiveness. The signal controlling internalization of the transferrin receptor (TfR), a commonly used immunotoxin target, has been found to reside in the amino acid residues 7–58 of its cytoplasmic domain [2–8]. We therefore investigated the effects of an anti-TfR immunotoxin on cell lines expressing transfected, genetically engineered TfR with various internalization rates. Modification of immunotoxin potency was further evaluated following up-regulation of receptor number by butyrate induction and alteration of the endocytotic pathway using monensin. The relative contribution of each of these factors in the susceptibility of cells to IT action was thereby ascertained.

Materials and methods

Materials

Monensin was purchased from Sigma Chemical Co. (St. Louis, Mo.). The ionophore was prepared as a 1 mM stock solution in ethanol and diluted to an appropriate final concentration in 0.14 M NaC1, 0.01 M $NaHPO_4$, pH 7.4 (phosphate-buffered saline). Native recombinant ricin A chain was purchased from Inland Laboratories and *n*-butyric acid was purchased from Sigma Chemicals Co.

Tissue culture

The Tfr-negative mutant cell line (Tfr^{neg}) was isolated in our laboratory after mutagenesis and selection with a Tf-toxin conjugate [7, 9]. Wild-

Table 1 Comparison of transferrin-internalization rate and surface receptor expression in engineered Chinese hamster ovary lines

Cell line	Relative rate[a] of ^{125}I-Tf internalization (%)	Mean fluorescence intensity with	
		Anti-TfR mAb	MOPC-21 control mAb
TfR^{wt}	100	23	5
$TfR^{\Delta 7-58del}$	30	24	4
TfR^{neg}	0	7	6

[a] Data as reported by Girones et al. (8)

type TfR and a truncated TfR that lacks a functional cytoplasmic domain (deletion of residues 7–58) were then expressed in TfR^{neg} cells by transfection using the calcium phosphate method [7, 8]. The level of cell-surface Tfr, as measured by investigation of the ligand-binding isotherm at 4 °C, was determined to be wild-type-TfR-containing cell line (TfR^{wt}) 105 000 ± 12 000 receptors/cell and truncated-TfR-containing cell line ($TfR^{\Delta 7-58del}$) 75 000 ± 5000 receptors/cell [8].

mAb and IT

The 7D3 mAb (IgG1), directed against the human TfR, was produced in mice by injection of cultered human leukemia CEM cells [10]. Native ricin A chain (Inland Laboratories) was disulfide-linked to anti-TfR with the *N*-succinimidyl-3-(2-pyridyldithio) proprionate reagent to produce immunotoxins [10].

Immunofluorescent staining for human TfR

Cultures of Chinese hamster ovary (CHO) cells were trypsinized and a trypan blue exclusion test was performed to ensure that more than 70% of the cells remained viable. Cells (10^6) were incubated for 20 min with 10 µg either anti-TfR or MOPC21 (Sigma Immunochemicals, St. Louis, Mo.), a nonspecific murine monoclonal immunoglobulin that served as a control. The cells were washed twice in minimal essential medium (MEM) and resuspended in 1 ml phosphate-buffered saline containing 1 µg fluorescein-isothiocyanate-labeled goat anti-(mouse Ig) antibody (Becton-Dickinson, San Jose, Calif.). After two further washes, the cells were fixed in 1% paraformaldehyde. Flow-cytometric analysis was performed on a FACSCAN cell sorter (BDIS, Mountain View, Calif.). The fluorescence intensity of 10 000 viable cells was measured.

Cytotoxicity assays

Cells growing in 75-cm^2 flasks were treated with 2 ml 0.5% trypsin for 2 min, placed in 10 ml F12 medium containing 10% fetal bovine serum and spun at 1200 *g* for 10 min. The pellet was resuspended in 10 ml leucine-free MEM by passage through a 22-gauge needle and cells were seeded into microtiter plates (10^5 cells/well) in a final volume of 200 µl/well. Addition of serially diluted 7D3-A immunotoxin (10^{-7}–10^{-11} M) was made in quadruplicate. The medium was removed after an 18-h incubation at 37 °C in 5% CO_2 and replaced with leucine-free MEM containing 2 µCi/well [^{3}H]leucine (New England Nuclear Corp., Boston, Mass.). Following an additional 2-h incubation, the medium was removed and the cells were treated with 0.05% trypsin, 0.02% EDTA (200 µl/well), for 30 min. They were then collected onto glass-fiber filters, and incorporation of [^{3}H]leucine was measured by liquid scintillation counting.

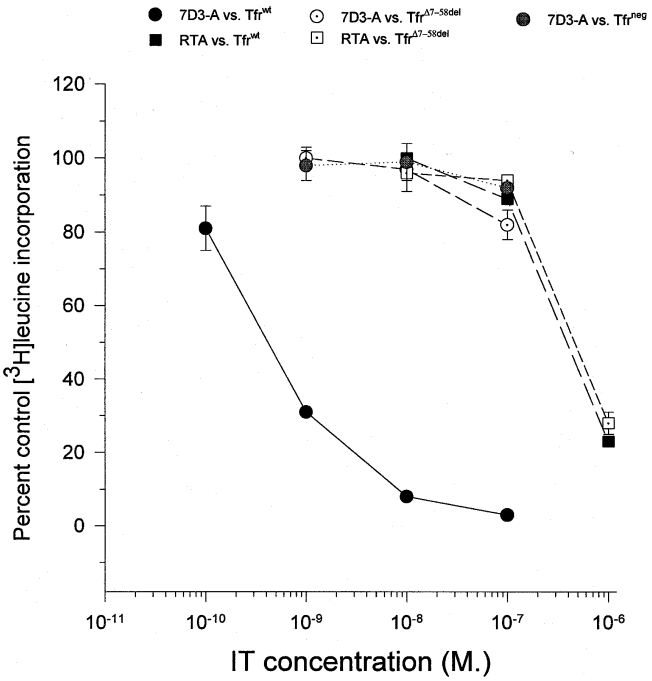

Fig. 1 Inhibtion of [^{3}H]leucine incorporation of cell lines after 18 h incubation with either the anti-TfR IT, 7D3-A, or ricin A chain. Protein incorporation of both the TfR^{neg} and internalization-defective $TfR^{\Delta 7-58del}$ cell lines is inhibited to an equal extent by both 7D3-A and ricin A chain ($IC_{50} > 100$ nM). By contrast, TfR^{wt} cells expressing wild-type TfR were much more sensitive to the effects of 7D3-A ($IC_{50} < 1$ nM)

Results

Rapid endocytosis of the TfR requires a signal sequence located at the amino terminus of its cytoplasmic domain [2–8]. This functional region was identified by transfecting a CHO cell line lacking endogenous TfR (TfR^{neg}) with normal or in-frame-deletional human TfR DNA. Measurement of TfR expression by flow cytometry shows that TfR^{neg} cells have no detectable TfR but that cell lines obtained after transfection with either wild-type or the internalization-defective Δ7–58 TfR DNA express similarly high levels of receptor (Table 1). However, only the cells possessing wild-type TfR were able to internalize bound ^{125}I-transferrin rapidly, since those bearing mutant receptors missing the functional cytoplasmic domain incorporated this radiolabeled ligand at a greatly reduced rate.

The differences in TfR internalization were reflected in the sensitivity of these cells to the anti-TfR immunotoxin 7D3-A. In 18-h cytotoxicity assays, virtually no receptor-specific protein synthesis inhibition was noted when either TfR^{neg} or $TfR^{\Delta 7-58del}$ cells were incubated with 100 nM immunotoxin. The cytotoxicity curves paralleled those obtained with purified ricin A chain alone and were probably due to non-specific uptake into cells. By contrast, the mean ID_{50} for TfR^{neg} cells expressing wild-type receptor (TfR^{wt}) was 0.4 nM, a 1000-fold increased sensitivity compared to ricin A chain alone (Fig. 1).

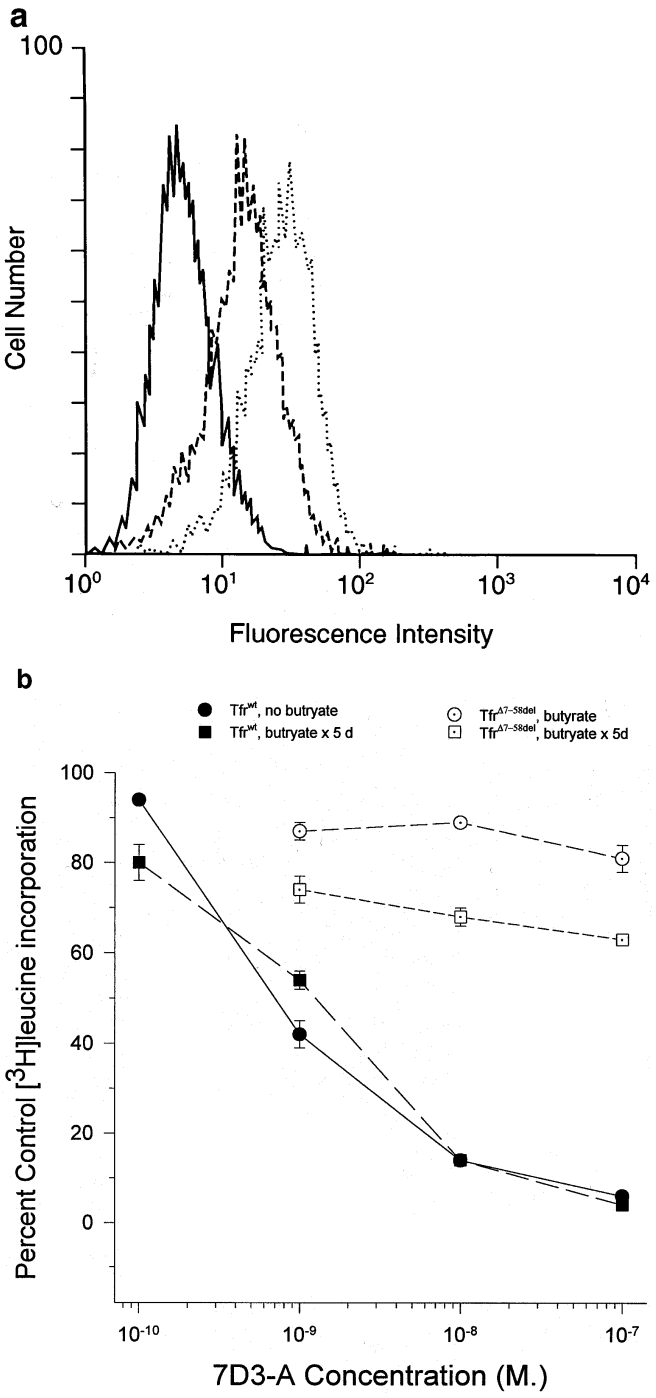

Fig. 2 **a** Results of flow-cytometric analysis of human TfR cell-surface expression in TfR^{wt} cell line. —— MOPC-21 control, - - - 7D3 anti-TfR mAb under control conditions; · · · 7D3 anti-TfR mAb after 1 week incubation in 1 mM butyrate. A similar result was obtained with $TfR^{\Delta 7-58del}$ cells. **b** Inhibition of [^{3}H]leucine incorporation by TfR^{wt} and $TfR^{\Delta 7-58del}$ after an 18-h incubation with 7D3-A before and after 1 week of growth in butyrate. No difference in cytotoxicity is noted after incubation

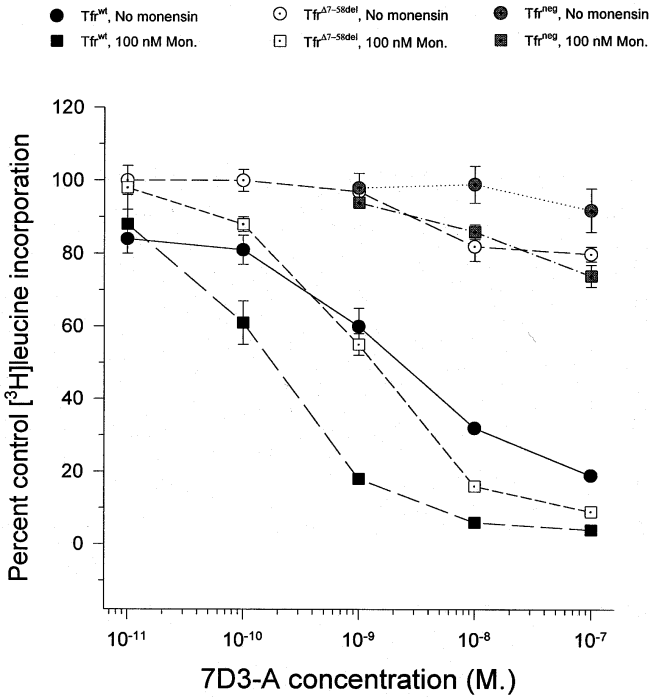

Fig. 3 Inhibition of [^{3}H]leucine incorporation by TfR^{neg}, TfR^{wt} and $TfR^{\Delta 7-58del}$ cell ines after 18-h incubation with or without 0.1 μM monensin. Significant potentiation (greater than 100-fold) is noted only for internalization-deficient $TfR^{\Delta 7-58del}$ cell line

Because the transgene is driven by the simian virus 40 early promoter, the expression of the human TfR can be increased by incubation of cells in butyrate. We therefore next asked whether immunotoxin sensitivity might be modulated by increasing the receptor number. Incubation in 1 mM sodium butyrate up-regulated receptor expression on both cell lines as assessed by flow cytometry (Fig. 2a); however, no appreciable increase in IT sensitivity was noted (Fig. 2b).

In contrast, a marked difference in immunotoxin sensitivity was noted when the cells were treated with immunotoxin plus the carboxylic ionophore monensin (Fig. 3). For TfR^{wt} cells, the decrease in ID_{50} was marginal (mean 1.0 compared to 0.6 nM, n = 4 separate experiments). In the presence of monensin, cells expressing the $TfR^{\Delta 7-58del}$ became much more sensitive to the anti-TfR immunotoxin and the ID_{50} decreased from a mean of more than 100 nM to 8.5 nM when 100 nM monensin was added (n = 2). The insensitivity of the parent TfR^{neg} cells to 7D3-A was not affected by the presence of monensin (n = 2).

Discussion

The A chain of ricin is an *N*-glycosidase that is poisonous to cells when it enters the cytosol. It inactivates ribosomes by excising a critical adenine from ribosomal RNA [11] and thereby halts protein synthesis. Endocytotic uptake of A

chain into cells is facilitated by the disulfide-linked B chain. This moiety binds to membrane receptors and may also transport the A chain into the Golgi complex, its putative point of egress into the cytosol [12, 13]. Highly selective site-specific carriers have been disulfide-linked to the A chain to replace the indiscriminate B chain binding but, for reasons that are not entirely clear, reduced cytotoxic potency and slower kinetics for killing cells often result.

Previous *in vitro* studies with immunotoxins based on ricin A chain suggest a direct relation between antigen density and immunotoxin cytotoxicity for T cell lines [14, 15] and solid tumors [16–18]. On the other hand, Preijers and colleagues [19] noted that, although a relationship existed between antigen density and cytotoxicity, this was only maintained for immunotoxins that were internalized to a high extent. Furthermore, using a variety of cell lines and immunotoxins, Goldmacher et al. [20] noted that the extent of endocytosis of the antigen-immunotoxin complex is the most important determinant of *in vitro* cytotoxicity. Other factors such as the extent of cell binding and the number of cell-surface antigens affect cytotoxicity only to the degree that they influence endocytosis.

In this study, which assessed two established CHO cell lines transfected with cDNA encoding a wild-type and internalization-deficient human TfR, we observed that immunotoxin potency was independent of receptor number. Up-regulating the receptor number with butyrate also did not affect potency, further supporting the contention that cell receptor number *per se* is not a major determinant of potency. Therefore, it seems unlikely that attempts to increase clinical immunotoxin potency by increasing receptor number will prove effective. This conclusion must be tempered, however, by the fact that the mean number of receptors per cell was in the range of 10^5 for each transfected cell line. It is very possible that receptor number might play a more crucial role if the targeted receptor was present in much lower numbers, as is the case with many cytokine receptors.

On the other hand, potency was closely correlated with the extent of internalization. This observation is supported by the marked enhancement of potency by monensin. Monensin is one of a number of diverse cytoactive agents such as NH_4Cl and retinoic acid that greatly enhance the potency of ricin A chain immunotoxin [21, 22]. At least two potentially overlapping mechanisms for this effect can be envisioned: either intracellular catabolism is inhibited or intracellular transit is accelerated. Although the gradual intracellular catabolism of cell-targeted antibodies is inhibited by micromolar concentrations of monensin [23], reduced degradation seems a less likely explanation for the increased immunotoxin cytotoxicity induced by this ionophore. Monensin greatly accelerates the action of ricin-A-chain-based toxins [21] so that over 90% inhibition of protein synthesis can be observed in only 2 h, and it is effective at a concentration (approx. $10-8$ M) that does not raise subcellular compartment pH. Furthermore, the best correlate with its enhancement of immunotoxin activity is the ability of monensin to dilate the Golgi apparatus [23, 24]. This morphological change might allow the catalytic A chain to exit from this organelle into the cytoplasm more quickly so that it can rapidly contact and inactivate its substrate, 28S ribosomal RNA [11]. In this set of cell lines, monensin's effects were much more pronounced on the internalization-deficient $TfR^{\Delta 7-58del}$ line compared to that of the line transfected with wild-type receptor. This is consistent with monensin's proposed mechanism of action of altering intracellular trafficking; in the cell line transfected with the wild-type TfR, enough internalization occurs to saturate the cell's ability to process the immunotoxin, thus adding little effect. By contrast, in the internalization-deficient line, the slowing of intracellular trafficking may be enough to result in enough ricin A chain release into the cytoplasm before the immunotoxin is inactivated.

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