CARBOXYLIC IONOPHORES ENHANCE THE CYTOTOXIC POTENCY OF LIGAND- AND ANTIBODY-DELIVERED RICIN A CHAIN

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Monoclonal antibodies and receptor ligands can deliver covalently coupled toxic ricin A chain to selected cell membrane targets but, for cytotoxicity to result, internalization and passage into the cytosol must also ensue. This prerequisite binding and entry, which allows A chain to come in contact with and enzymatically inactivate ribosomes (1), also provides a safeguard against affecting innocent bystander cells that lack the appropriate receptor or antigen. We have constructed a toxic conjugate composed of human transferrin disulfide linked to ricin A chain (TF-A chain)[1] so that this receptor-mediated iron delivery pathway can be used to explore the more subtle aspects of A chain intoxication (2). While passage of transferrin into and back out of cells occurs in a matter of minutes (3, 4), the toxic conjugate required 6 h to inhibit cellular protein synthesis (2). This indicated that its speed of action was dictated by some slow or infrequent event divorced from the normal cycle. Therefore, we tested TF-A chain along with the carboxylic ionophores monensin and nigericin, which can raise the pH and otherwise disturb the stability of vesicle compartments within the cell (5). In the presence of ionophore, inhibition of protein synthesis by TF-A chain was accelerated 15-fold, occurring with a half-time ($t_{1/2}$) of only 24 min. Potency was correspondingly raised by several orders of magnitude and the number of receptor sites required for toxicity was also dramatically reduced. Ricin A chain delivered into cells by a monoclonal antibody directed against common acute lymphoblastic leukemia antigen (CALLA) determinants (6) responded to the potentiating influences of carboxylic ionophores in a similar manner.

Materials and Methods

Toxic Conjugates. Purified ricin A chain was disulfide linked to either human transferrin or mouse monoclonal antibodies and the composition of these conjugates was analyzed as previously described (2, 6). Membrane-localized ricin A chain was detected on conjugate-treated cells using a fluoresceinated monoclonal anti-A chain antibody (7) and fluorescence-activated cell sorter (FACS) analysis.

Cells. K562, a human erythroleukemia cell line (8) was maintained and used in serum-free media (2). The human leukemia CEM cell line and a variant TF-A chain–resistant

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[1] *Abbreviations used in this paper:* CALLA, common acute lymphoblastic leukemia antigen; FACS, fluorescence-activated cell sorter; TF-A chain, disulfide-linked conjugate formed with human transferrin and ricin A chain.

CEM cell line were obtained and grown in culture as reported (2). Nalm-6, a CALLA-positive human acute lymphoblastic leukemia line as well as the CALLA-negative leukemic T cell, HSB-2, and myeloid, HL-60, lines were used as previously described (6).

Cytotoxicity. Cells in appropriate media were plated in tissue culture wells (1.7 × 1.6 cm) at an initial concentration of 40 × 10^4 cells/ml. Monensin and nigericin were purchased from Sigma Chemical Co., St. Louis, MO and were diluted into media from stock solutions at 10^{-2} M in absolute ethanol. Designated amounts of toxic conjugates and ionophore were added to the 1 ml cultures and the cell number was microscopically evaluated on a daily basis (9). Growth of CEM cells was slowed by monensin (see Table I) but this effect could be avoided if, after overnight treatment, they were washed and resuspended in fresh media (see Fig. 3). Specific blocking studies were carried out by putting an excess of iron-saturated transferrin or monoclonal antibody in the cultures just before adding conjugate. Inhibition of cellular protein synthesis was evaluated using a 30-min pulse of [^{14}C]leucine; incorporation into protein was measured as previously described (9). Kinetic studies were performed by treating cells with 10^{-7} M TF-A chain for 10 min with or without monensin at 10^{-7} M and then adding excess transferrin (10^{-5} M) to block further binding. The cells were then further incubated for various times before measuring protein synthesis (9). Identically treated cells that received excess transferrin before the addition of TF-A chain served as a control.

Results and Discussion

Given the demonstration that a single toxic A chain molecule is lethal when properly situated within the cell (10, 11), a disproportionate excess of membrane-bound toxin is usually required to cause cell death. This is especially true when alternative carriers are deployed in place of the natural binding moiety (2). The time required for such toxins to affect cells is typically several hours even when all surface antigen or receptor sites are saturated by antibody and ligand-delivered A chain (2, 9, 12). Such was the case, we found, for the receptor-mediated toxicity of TF-A chain on both human T cell leukemia CEM (2) and erythroleukemia K562 cells in culture. A loss of the cells ability to synthesize protein signals the arrival of this toxin into the cytoplasm, where it inactivates ribosomes, and the kinetics of this inhibition under receptor-saturating conditions is characterized by a $t_{1/2}$ of 375 min (Fig. 1). To reach its intracellular target, we anticipated that TF-A chain would have to escape from the normal cycling pathway of transferrin entry into and exit from the cell (3, 4). This process involves passage into various compartments, the pH of which is important. We therefore tested the speed of TF-A chain action in the presence of carboxylic ionophores, which disturb the acidification and normal functioning of these vesicles.

A 15-fold rate increase was observed when monensin was included with TF-A chain (Fig. 1) and the $t_{1/2}$ (24 min) was much closer to what would be expected from the known speed of entry of receptor-bound transferrin into cells (3, 4). Monensin exerted this influence on protein synthesis only in concert with TF-A chain, suggesting that ionophore facilitates the access of A chain to ribosomes. Accelerated release from vesicles is a possible mechanism but alternative explanations are plausible. To determine the exposure time required for toxicity to occur, TF-A chain was mixed with K562 cells and, after different intervals, excess transferrin was added to stop further binding to the receptor. TF-A chain that had become attached or internalized during this time was then allowed to act for 2 h in the presence of ionophore or 20 h in its absence before measuring

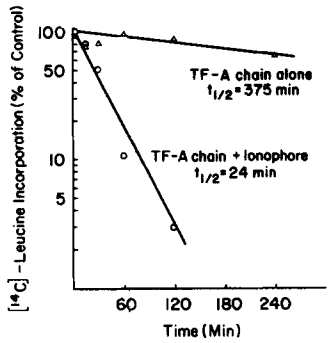

FIGURE 1. Kinetics for inhibition of cellular protein synthesis by TF-A chain. Receptor sites were saturated by treating K562 cells with 10^{-7} M TF-A chain for 10 min and then transferrin was added at 10^{-5} M to block toxin interaction with recycled receptor. The cells were then incubated for designated intervals at 37°C in leucine-free media alone (△) or with 10^{-7} M monensin (○) before pulsing with [^{14}C]leucine (30 min) and assaying incorporation. When excess transferrin was added before TF-A chain exposure, levels of incorporation were comparable to untreated cells and this served as a control for each time point.

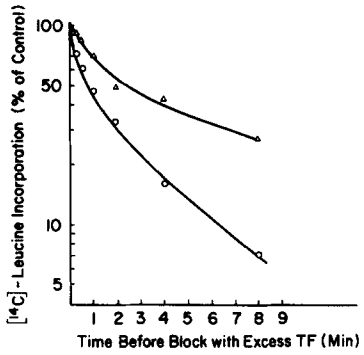

FIGURE 2. Exposure time required for toxicity. The interaction of K562 cells with 10^{-7} M TF-A chain was stopped at different times by adding a 100-fold excess of transferrin; incubation at 37°C proceeded for an additional 20 h in media alone (△) or 2 h in media with 10^{-7} M monensin (○). The cells were then pulsed with [^{14}C]leucine (30 min) and incorporation was compared with 0 time controls that were preblocked with transferrin (10^{-5} M) before addition of toxin.

protein synthesis. Cells were progressively affected as the exposure time increased (Fig. 2) and one-half of the cells had already bound or taken up a lethal amount by 50 s. When ionophore was omitted, a prolonged postincubation was required for TF-A chain to produce its effect and the shift in this curve (Fig. 2) suggests that bound toxin was not completely retained in the cell during this period.

Human leukemia CEM cells express high levels of transferrin receptor and their growth and viability is correspondingly affected by low doses of TF-A chain (Fig. 3) (2). When 10 nM monensin was included in the culture media, however, the dose response curve was dramatically shifted to 30,000 times greater potency (Fig. 3), and 50% kill was achieved by adding only five molecules of TF-A chain for each cell. The potency of this toxic conjugate on CEM cells when assisted by ionophore ($ID_{50} = 3 \times 10^{-15}$ M) was greater than that of native ricin, which is required at 1,000-fold higher levels to produce comparable cytotoxicity (2).

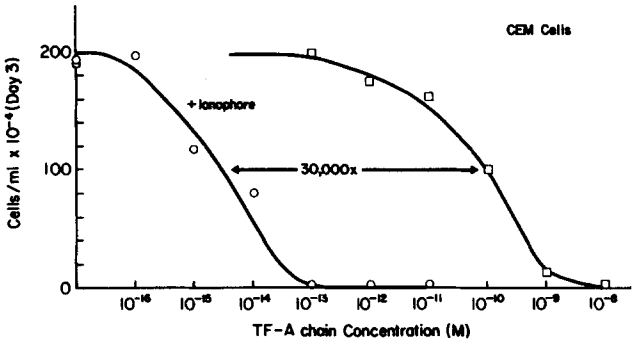

FIGURE 3. Toxicity dose response curves for TF-A chain on CEM cells in the presence and absence of ionophore. CEM cells in media alone or media containing 10^{-8} M monensin received the designated concentrations of TF-A chain. After overnight incubation at 37°C, the cells were washed, resuspended in media alone, and plated in tissue culture wells. The number of viable cells was evaluated on day 3.

It is important to determine if the action of free A chain or whole ricin is comparably influenced by ionophore. Pure ricin A chain is toxic to CEM cells at 10^{-6} M (2), indicating that at high concentrations there is nonspecific uptake of small amounts that can reach the ribosomes. In the presence of 10 nM monensin, a 10^{-8} M level of free A chain was sufficient to kill these cells and a 500-fold excess of monoclonal anti–A chain but not anti–B chain blocked this cytotoxicity. The data suggests that free A chain follows a similar intracellular pathway to that of ligand-delivered A chain, albeit with much lower internalization efficiency. The cytotoxicity of whole ricin for CEM cells was only modestly improved by using monensin (data not shown).

A previously isolated variant CEM cell line, displaying 1,000-fold resistance to TF-A chain, had a greatly diminished capacity for binding transferrin (2, 13) and this phenotypic defect allowed them to survive toxin treatment. The influence of ionophore on the time course of TF-A chain cytotoxicity was assessed using both the sensitive and resistant cell lines to see if they responded similarly (Table I). The potentiation provided by ionophore promoted more rapid lysis of sensitive CEM cells and was also able to override the resistance that reduced TF-A chain binding afforded the variant line. The inclusion of a 300-fold molar excess of unmodified human transferrin forestalled the combined cytotoxic effects of TF-A plus ionophore chain seen on day 1, but this blockade was gradually overcome by day 3 (data not shown). Since the cytotoxicity of TF-A chain alone is totally blocked by this treatment (2), it appears that ionophore reveals the few molecules that do eventually enter the cell in the presence of excess transferrin. NH_4Cl included in culture medium at 10^{-2} M failed to show effects comparable to those seen with ionophore (data not shown) even though this agent can raise vesicle pH values and influences cytotoxicity in other delivery systems (14). These results indicate that ionophore-assisted TF-A chain toxicity is still receptor mediated and that cells which bind only miniscule amounts of the toxin can be killed when ionophore is present.

A monoclonal antibody–ricin A chain conjugate developed to kill CALLA-bearing cells was shown to be highly specific but produced only partial inhibition

TABLE I

Effect of Ionophore on TF-A Chain–sensitive and –resistant CEM Cells

	Cell number per milliliter ($\times 10^{-4}$)*		
	Day 1	Day 2	Day 3
Sensitive CEM	59	106	208
Plus TF-A‡	34	7	0
Sensitive CEM + monensin§	60	74	154
Plus monensin + TF-A	0	0	0
Resistant CEM	50	80	160
Plus TF-A	53	68	178
Resistant CEM + monensin	42	60	92
Plus monensin + TF-A	19	1	0

* Started at 40×10^4 cells/ml.
‡ TF-A chain at 10^{-9} M.
§ Monensin at 10^{-8} M.

TABLE II

Cytotoxicity of Anti-CALLA–Ricin A Chain

Cell line	CALLA expression	Cells alone* (day 3)	Anti-CALLA–A chain (10^{-9} M)	Ionophore (10^{-8} M)	Anti-CALLA–A chain + ionophore	Plus excess antibody‡
Nalm 6	++++	247	80	215	0	234
CEM	±	436	439	222	34	226
HSB2	–	242	—	220	242	—
HL60	–	118	—	106	108	—

* Cell number/ml ($\times 10^{-4}$); cells were started at 40×10^4/ml.
‡ Monoclonal anti-CALLA at 1×10^{-6} M.

of protein synthesis in target cells (6). Its presence in culture medium for 2 d or more was required to totally suppress cell proliferation (6). At that time, agents such as NH_4Cl, chloroquine, and dansylcadaverine were tested in attempts to accelerate anti-CALLA–A chain action but none produced significant results. The enhancement conferred by carboxylic ionophores on transferrin receptor-mediated A chain toxicity prompted the trial of these same agents for promoting CALLA-mediated delivery into the cytosol. In fact the anti-CALLA–A chain conjugate plus monensin or nigericin gave 95% inhibition of protein synthesis when incubated for only 3 h with CALLA-positive Nalm 6 cells, whereas conjugate alone or conjugate with 10 mM NH_4Cl gave only a 30–40% reduction. It was anticipated that this potentiation by ionophore would greatly influence the speed and extent of cell kill as well as the amount of CALLA expression required to produce cytotoxicity. To test this, the viability of cell lines having different amounts of CALLA on their membrane was examined after treatment using the antibody–A chain conjugate with and without monensin (Table II). Nalm 6 cells strongly expressed CALLA and bound a substantial amount of antibody–A chain; however, even though their growth was reduced over 3 d by 10^{-9} M conjugate, complete cell kill was not achieved (6) (Table II). Proliferation of these cells remained unaffected by monensin alone but, when a combination of ionophore plus toxin was used, no cells were present on day 3 and in fact all

cells had lysed 1 d after treatment. This cytotoxicity was entirely CALLA mediated and could be prevented when all antigen sites were blocked by an excess of unmodified monoclonal anti-CALLA antibody (Table II).

The amount of anti-CALLA–A chain bound by CEM cells was barely detectable using a fluoresceinated monoclonal anti–A chain probe and analysis by cytofluorimetry. In accordance with the low level of target antigen, their growth was not affected by antibody–A chain conjugate at 10^{-9} M (Table II) or even as high as 10^{-7} M (8). This is in striking contrast to their response to TF-A chain (Fig. 3), where the abundance of transferrin receptor makes them highly susceptible. The combined effects of ionophore and the CALLA-specific conjugate overcame this antigen deficiency to produce significant toxicity (Table II). Once again, the inclusion of excess uncoupled antibody rescued these cells from the action of ionophore plus toxin and established specificity (Table II). Growth of the CALLA-negative HL60 and HSB-2 lines remained unaffected by conjugate with ionophore (Table II) and this persisted even when 10^{-7} M levels of anti-CALLA–A chain were tested.

The vast diversity in expression of surface receptors and antigens offers a means of distinguishing various cell types and the selective toxicity of A chain conjugates is based upon this feature. It has been apparent for some time now that the full toxic potential of these new conjugates has not been realized, due to their slow or inefficient passage into the cytosol. An ability to manipulate the intracellular aspects of the intoxication process is therefore important so that the speed of action and potency of these toxins can be controlled. Various agents including NH_4Cl (14), chloroquine (15), adenovirus (16), and ricin B chain (12, 17) have been used to potentiate toxic activity. The carboxylic ionophores examined in this work, however, possess some unique properties and advantages. They act catalytically and are therefore effective at much lower concentrations than NH_4Cl or chloroquine. Ricin A chain delivery systems, which have been reticent to potentiation by other agents, may be appreciably enhanced by carboxylic ionophores. Moreover, the observed increases in rate of cell kill, conjugate potency, and cell susceptibility to toxic action have been pronounced.

Summary

The highly selective cytotoxicity of site-directed ricin A chain conjugates can be potentiated by membrane-active carboxylic ionophores. The combined use of the two agents results in much faster inactivation of ribosomes and subsequent cell death and lysis. The potency of A chain cytotoxins is correspondingly increased by several orders of magnitude and cells that sparsely express the target antigen or receptor can be killed.

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