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Monensin is Obligatory for the Cytotoxic Action of a Disulfide Linked Methotrexate-Anti-Transferrin Receptor Conjugate

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SUMMARY: Methotrexate (MTX) in the form of a γ-cysteinylglycine derivative was disulfide linked to a monoclonal antibody reactive with the human transferrin receptor to give an antibody-MTX conjugate (anti-TfR-MTX). Antibody directed delivery of MTX to cell surface receptors was readily detected by flow cytometry using an anti-MTX antibody plus a secondary fluorescent antibody probe. Despite the presence of ample drug on the cell membrane, the conjugate alone was not cytotoxic over the course of several days. Expression of specific toxic activity, however, was obtained in conjunction with the carboxylic ionophore monensin, in whose presence anti-TfR-MTX displayed an IC_{50} of 8×10^{-8} M. These results suggest that the ionophore causes antibody-drug conjugate to bypass the normal transferrin receptor cyclic pathway, allowing sufficient drug to reach, bind to, and inactivate intracellular dihydrofolate reductase.

Specific delivery of toxic molecules into cells by receptor mediated endocytosis has been of interest as a means of achieving selective cytotoxicity. Complexities encountered within the cell interior, however, can have a profound influence upon the ultimate toxic activity of these intracellular agents. For example, in studies designed to deliver disulfide linked toxic ricin A chain into cells via the transferrin receptor mediated pathway, it was found that transferrin-A chain or anti-transferrin receptor-A chain conjugates alone killed cells slowly ($t_{1/2}$ = 300 min) (1,2) compared to the known speed with which native receptor and ligand are internalized ($t_{1/2}$ = 8 min) (3). Kinetics of cell kill and cytotoxic potency, however, were greatly improved by using carboxylic ionophores (4) to modulate intracellular events and cause the cytotoxic conjugates to have $t_{1/2}$ values of 10-15 min during the inactivation phase (1,2). In this study, a disulfide derivative of MTX [(MTX-γ-CysGly)$_2$] was synthesized, reduced to MTX-γ-CysGly,

Abbreviations: PBS, 10 mM phosphate, 0.14 M NaCl, pH 7.2; MTX, methotrexate; (MTX-γ-CysGly)$_2$, disulfide derivative of MTX; anti-TfR, antibody directed against the human transferrin receptor; anti-TfR-MTX, conjugate formed by disulfide linkage of MTX to anti-TfR.

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and disulfide linked to a monoclonal antibody directed against the human transferrin receptor. The cytotoxic activity of the conjugate was tested both in the presence and absence of carboxylic ionophores to determine if its intracellular disposition was similarly important for the expression of lethal activity.

EXPERIMENTAL PROCEDURES

Synthesis of (MTX-γ-CysGly)$_2$. N-Ethyl-N,N-diisopropylamine (1.57 ml, 9 mmol) and diethyl phosphorocyanidate (1.33 ml, 9 mmol) were added consecutively to a stirred suspension of 4-amino-4-deoxy-N^{10}-methylpteroic acid (1.08 g, 3 mmol) (5) in dry N,N-dimethylformamide (150 ml). After 4 h at room temperature, glutathione tetraethyl ester (1.09 g, 1.5 mmol) (6) and another portion of N-ethyl-N,N-diisopropylamine (1.57 ml, 9 mmol) were added to the dark-yellow solution. The reaction was followed by TLC on silica gel (Whatman MK6F) with 15:5:1 $CHCl_3$-MeOH-AcOH as the developing solvent. The product appeared as a bright-yellow spot with R_f 0.5. After 24 h at room temperature, the reaction mixture was concentrated to dryness by rotary evaporation at 40-45°C, and the residue was chromatographed on a silica gel column (50 x 5 cm) which was packed in $CHCl_3$ and eluted first with 95:5 $CHCl_3$-MeOH (1.5 lit) and then with 9:1 $CHCl_3$-MeOH (2 lit). Fractions containing the product were pooled and evaporated to a yellow powder (1 g, 47% yield); IR (KBr) 3360, 2980, 1740, 1640-1610, 1520, 1450 cm^{-1}; NMR ($CDCl_3$-CD_3OD) δ 1.2 (t, 12H, $CH_2\underline{CH_3}$), 3.2 (s, 3H, NCH_3), 3.8 (broad, 2H, 9-CH_2), 4.1 (1, 8H, $\underline{CH_2}CH_3$), 6.65 (d, 2H, 3'- and 5'-H), 7.6 (d, 2H, 2'- and 6'-H), 8.15 (s, 1H, 7-H). Anal. Calcd for $C_{58}H_{74}N_{20}O_{14}S_2 \cdot 1.3CHCl_3 \cdot 2CH_3OH$: C, 47.23; H, 5.39; N, 17.97; S, 4.11. Found: C, 47.09; H, 5.57; N, 17.99; S, 4.21.

The above tetraester (1.56 g, 1 mmol) was taken up in 50% ethanol (75 ml), and 2-mercaptoethanol (2.5 ml) was added, followed by barium hydroxide octahydrate (0.63 g, 2 mmol). The reaction mixture was stirred overnight, a second portion of barium hydroxide octahydrate (0.63 g, 2 mmol) was added, and stirring was continued for another day. The progress of hydrolysis was monitored by TLC on silica gel with 3:2:1 BuOH-AcOH-H_2O as the developing solvent. The product gave a bright-yellow spot with R_f 0.28. When hydrolysis appeared to be complete, solid sodium sulfate (0.5 g) was added and stirring was continued for 1 h. The precipitated barium sulfate was removed by suction filtration, and the filtrate was concentrated to dryness. The residue was chromatographed on a column of DEAE-cellulose (30 x 5 cm, bicarbonate form) packed in 1% 2-mercaptoethanol and eluted first with 1% ammonium bicarbonate (500 ml) and then with 2% ammonium bicarbonate (750 ml). Appropriate fractions were pooled and lyophilized to obtain nearly pure product (1 g, 73% yield). For final purification, a portion (0.25 g) of this material was applied onto a Biogel P-2 column (100 x 1.25 cm) which was packed and eluted with distilled water. The major band was collected and rechromatographed on the same column. Lyophilization of pooled fractions of TLC-homogeneous product afforded a bright-yellow powder (0.18 g, 52% yield); IR (KBr) 3400, 3200, 1600, 1400 cm^{-1}. Anal. Calcd for $C_{50}H_{58}N_{20}O_{14}S_2 \cdot 8.75H_2O$: C, 43.36; H, 5.49; N, 20.22; S, 4.63. Found: C, 43.75; H, 5.80; N, 19.95; S, 5.13.

Preparation of Anti-TfR-MTX. An IgG_1 monoclonal antibody directed against human transferrin receptor was produced by somatic cell hybridoma techniques (7) following injection of mice with cultured CEM human lymphoblastic leukemia cells. The antibody was purified by ammonium sulfate fractionation and S-300 gel filtration before reaction with N-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia) to add ca. 3 pyridyldithiopropionate groups per IgG molecule (8). (MTX-γ-CysGly)$_2$ was reduced by dissolving 1.4 mg (1 μmole) in 200 μl of water and adding 10 μl (140 μmole) of 2-mercaptoethanol. The mixture was dried in a small tube using a centrifugal vacuum apparatus (Savant), redissolved in water, and redried to remove all the 2-mercaptoethanol. The remaining solid was dissolved in water, and its thiol content was estimated with Ellman's reagent (9) to be ca. 10^{-3} M. This preparation (100 μl containing

0.1 μmole of SH) was added to the pyridyldithiopropionate substituted antibody (0.02 μmoles in 500 μl PBS) and the disulfide-thiol exchange reaction was allowed to proceed overnight at room temperature. The anti-TfR-MTX conjugate was isolated by passage through a G-25 column and, after filter sterilization, the MTX content was estimated spectrophotometrically from the absorbance at 370 nm to be between one and two molecules per antibody.

Cell Surface Binding. CEM cells (2 x 10^6) were treated with 100 μl of PBS alone or PBS containing 0.5 μM anti-TfR or anti-TfR-MTX for 30 min at 0^oC before washing with PBS. The cells were then treated with 100 μl of a 1/50 dilution of rabbit anti-MTX antiserum, Ra73-7c (10) for 30 min at 0^oC, washed, and finally reacted with 100 μl of a 1/50 dilution of fluoresceinated sheep anti-rabbit IgG. The washed cells were then analyzed on an Epics C flow cytometer (Coulter Electronics) which measured the fluorescence intensity distribution of 10,000 cells on a log scale.

Cytotoxicity Assays. CEM cells in serum-free medium (11) were plated in tissue culture wells (1.7 x 1.5 cm) starting at a density of 4 x 10^5 cells/ml. Designated amounts of anti-TfR-MTX or anti-TfR were added to the 1 ml cultures and monensin (Sigma) was added from a 10^{-3} M ethanolic stock solution to give a final concentration of 10^{-8} M. The number of viable cells was monitored over the course of several days by removing a 100 μl aliquot of the suspended cells and counting them in the presence of trypan blue using a light microscope and hemocytometer. The cytotoxicity of free MTX and of (MTX-γ-CysGly)$_2$ was determined in a similar manner using CEM cells grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37^oC in an 8% CO_2 humidified atmosphere as previously described (12). IC_{50} values were determined from dose response curves and averaged from triplicate experiments.

RESULTS AND DISCUSSION

MTX kills cells by virtue of its ability to bind tightly to the active site of dihydrofolate reductase, thereby causing depletion of reduced folates required for the synthesis of nucleotide precurors of DNA. Coupling of MTX to antibodies via the glutamate moiety has been accomplished by a variety of covalent linkage methods (13-21), and the resulting conjugates have displayed moderate cytotoxic activity in vitro suggesting that the drug can reach its intracellular target. It has generally been assumed that conjugated MTX binds to membrane sites, enters cells, and eventually reaches the lysosomes, where it is cleaved from its carrier by hydrolytic enzymes. Such a release step would allow free drug to interact with its target; however, alternate mechanisms of action for such conjugates are possible. The cyclic pathway associated with transferrin receptor mediated entry apparently avoids lysosomal compartments since neither the receptor nor ligand is degraded and transferrin is not found in the high-density subcellular fractions (2,22). It was of interest to determine if a disulfide linked anti-TfR-MTX conjugate would kill cells, since previous studies have shown that similarly coupled protein toxins can reach their targets in the cytosol upon entry into cells via this transferrin receptor mediated pathway (2,11,23,24).

A bis(γ-cysteinylglycine) derivative of MTX (Fig. 1) was reductively cleaved and the resulting thiol was disulfide coupled to a pyridyl disulfide substituted monoclonal antibody directed against human transferrin receptor. The anti-TfR-MTX conjugate attached to transferrin receptors on the surface of CEM cells after treatment at 0^oC, since

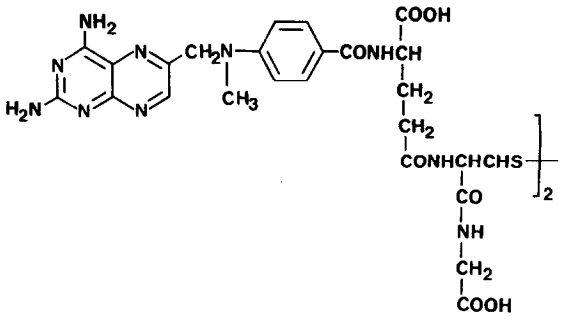

Figure 1. Structure of (MTX-γ-CysGly)2

membrane localized MTX molecules were revealed using an anti-MTX antibody plus a secondary fluorescent antibody probe (Fig. 2). Whereas 81% of anti-TfR-MTX treated cells showed positive fluorescence, only 6% of cells exposed to non-coupled anti-TfR instead of the conjugate were in this region, which was the same as background levels obtained with PBS treated controls (data not shown).

Even though substantial amounts of antibody linked MTX were delivered to the surface of CEM cells by anti-TfR-MTX, these cells were not killed despite continuous exposure to conjugate at a concentration of $5x10^{-7}$ M for 3 days (Table I). In contrast, CEM cells were killed by non-coupled (MTX-γ-CysGly)2 or MTX, which gave IC_{50}'s of

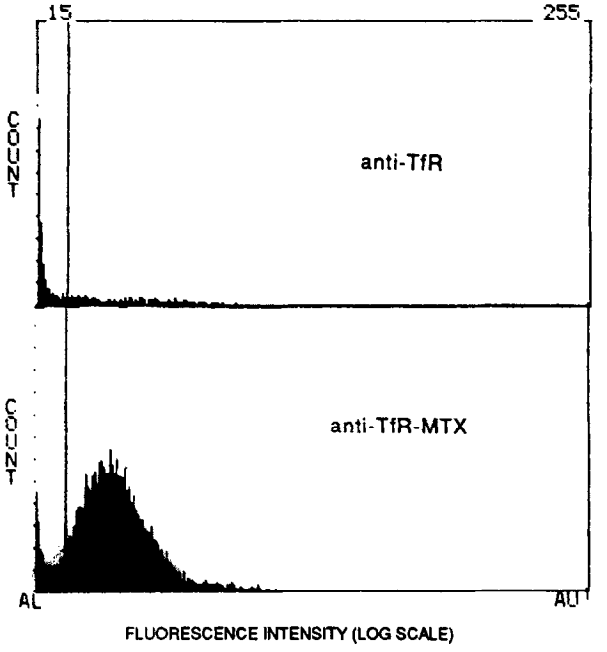

Figure 2. Flow cytometric distribution of CEM cells exposed to anti-TfR (top) or anti-TfR-MTX (bottom) and then treated with rabbit anti-MTX followed by fluoresceinated sheep anti-rabbit IgG.

Table I. Cytotoxicity of anti-TfR-MTX in the Absence and Presence of Monensin

Additions	CEM cells/ml x 10^{-4}		% Growth Inhibition
	Day 0	Day 3	
Cells alone	40	360	0
+ anti-TfR-MTX ($5x10^{-8}$ M)	40	280	22
+ anti-TfR-MTX ($5x10^{-7}$ M)	40	270	25
Cells + Monensin ($1x10^{-8}$ M)	40	310	0
+ anti-TfR-MTX (5 x 10^{-8} M)	40	170	45
+ anti-TfR-MTX (5 x 10^{-7} M)	40	10	97

$4.5x10^{-7}$ M and $2x10^{-8}$ M, respectively. This indicated that antibody bound MTX was not effectively reaching its target within the cell via the transferrin receptor mediated route. In an effort to manipulate the intracellular fate of anti-TfR-MTX, cells were simultaneously treated with the conjugate plus low concentrations of the carboxylic ionophore monensin. Under these conditions anti-TfR-MTX not only slowed the growth of the cells but was actively lethal at the higher concentrations tested (Table I). A dose response curve was generated in the presence of 10^{-8} M monensin and an IC_{50} of $8x10^{-8}$ M was obtained for the conjugate (Fig. 3). The effect of the ionophore was specific for

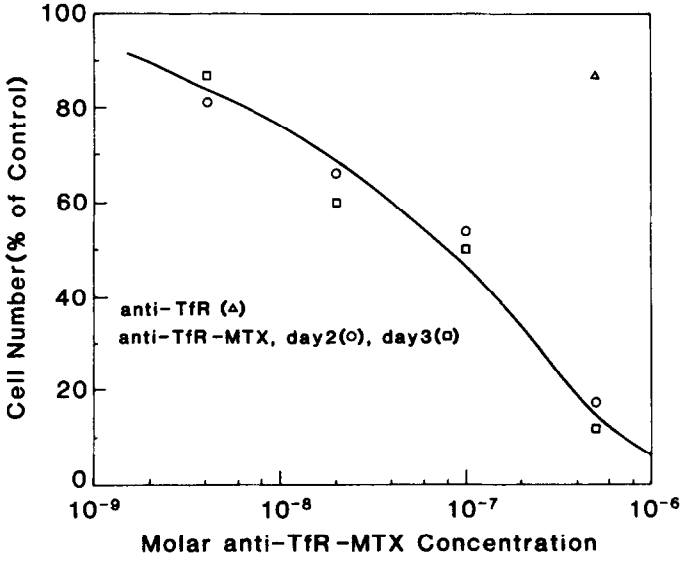

Figure 3. Inhibition of CEM cell growth with anti-TfR-MTX or anti-TfR in the presence of 1 x 10^{-8} M monensin.

coupled anti-TfR-MTX, since monensin did not improve the cytotoxic activity of free MTX or (MTX-γ-CysGly)$_2$ (data not shown). Moreover, non-coupled anti-TfR-MTX antibody at 5×10^{-7} M in the presence of monensin did not provide substantial inhibition of cell growth (Fig. 3), indicating that cytotoxicity was due to MTX delivered by the antibody.

In contrast to these results with the disulfide linked conjugate, an anti-TfR-MTX conjugate formed by the active ester method and containing 5 moles of MTX/mole of antibody was reported to be toxic to CEM cells in the absence of monensin (IC_{50} of 2.2×10^{-7} M) (13). This difference might be due to the method of MTX linkage, the extent of MTX substitution, the recognition of distinct receptor epitopes by the two antibodies, or the presence of aggregated conjugate molecules which might be diverted to lysosomes. In this regard it is important to note that fidelity to the natural transferrin cyclic pathway was retained when toxic ricin A chain was disulfide linked to anti-TfR antibodies or to transferrin by the methods used in this work for MTX coupling (2). It is likely therefore that toxic conjugate molecules which enter cells via the transferrin receptor mediated pathway do not normally gain rapid access to their targets in the cytosol without the aid of agents such as monensin, toxin B chains, or others. These potentiators may either directly facilitate translocation of molecules through the membrane of vesicle compartments or redirect the intracellular distribution of these conjugates to unique regions which allow expression of their lethal activity. Whether or not cleavage of the disulfide bond is a prerequisite for the cytotoxic action of the anti-TfR-MTX conjugate also remains an unanswered question. In this regard it is noteworthy that even though the relative inactivity of antibody-ricin A chain conjugates formed with a thioether rather than disulfide bond has led to the inference that toxicity is dependent on reductive cleavage, no direct evidence for intracellular release of A chain has been found (2).

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