The Chinese Journal of Cancer Research 10(2):84—93, 1998

SPECIFIC UPTAKE OF MONOCLONAL ANTIBODY-CONJUGATED METHOTREXATE BY HUMAN LYMPHOCYTIC LEUKEMIC B CELLS

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Objective: To analysis the uptake of free MTX and MTX conjugated to tumor specific monoclonal antibody by target and non-target cells. Methods: The folate antagonist methotrexate (MTX) was conjugated to two monoclonal antibodies (Mab) directed against human chronic lymphocytic leukemia (CLL), Dal B01 and Dal B02, by an active ester method. Both conjugates were more cytotoxic toward the target tumor cell line D10-1 than to the non-target cell line MOLT-3, and Dal B02-MTX conjugate was more inhibitory to D10-1 cells than free MTX in a 6 h pulse exposure assay. Results: Drug uptake studies revealed that D10-1 cells took up much more Dal B01 and Dal B02-conjugated MTX than free MTX. The amounts of drug taken up by D10-1 cells incubated with Dal B01 and Dal B02-conjugated MTX were always 3 to 5-fold higher than that taken up by MOLT-3 cells, although the latter took up more drug when incubated with free MTX. Furthermore, tumor cells incubated with Dal B01 or Dal B02-conjugated MTX retained much larger amounts of drug for a prolonged period of time than those incubated with free MTX. Conclusion: The enhanced specific cytotoxicity of Dal B01 and Dal B02-MTX conjugates toward target tumor cells is therefore likely due to (I) delivery of larger amounts of MTX to target cells when the drug is conjugated to Mab; (ii) longer retention of Mab-conjugated MTX by target cells; and (iii) slow, prolonged release of MTX from the surface-bound or endocytosed conjugates, rendering them into a sustained release dosage form.

Accepted November 5, 1997

Key words: Monoclonal antibody; Methotrexate; Immunoconjugates; Drug uptake; Specific Cytotoxicity; Chronic lymphocytic leukemia.

The folate antagonist methotrexate (MTX) is widely used to treat human cancer, including leukemias and lymphomas. However, like all chemotherapeutic agents, MTX is as cytotoxic to rapidly dividing normal cells, e.g., those in bone morrow and intestinal epithelium, as it is to cancer cells. The idea of targeting a cytotoxic agent to a tumor using monoclonal antibodies (Mab) against tumor associated antigens (TAA) as carriers has provided an approach by which cancer cells might be selectively eradicated with minimal effects on normal cells[1,2] We previously covalently coupled MTX to an antibody against a TAA on the surface of mouse EL4 lymphoma cells and demonstrated that this conjugate inhibited tumor more effectively *in vivo* than did free MTX or MTX conjugated to a nonspecific IgG.[3] We also observed that tumor cells take up more MTX conjugated to antitumor antibodies than either free MTX or MTX conjugated to a nonspecific IgG, both *in vitro* and *in vivo*. More recently, we produced two Mab directed against human B cell chronic lymphocytic leukemia (CLL), Dal B01 and Dal B02[4] and showed that these two Mab could selectively localize to CLL xenografts in nude mice.[5] Dal B01 and Dal B02 cross-competes for binding to m.w. 22,000 and 33,000 antigens on the surface of human CLL B

cells.[4] While both Mab bind to CLL cells with comparable affinity, Dal B02 has an immunoreactive fraction (IRF) of about 70% which is 2.5-fold higher than Dal B01. The amount of Dal B02 localized in tumors is also about 2-fold higher than that of Dal B01 when the Mab were injected intravenously into CLL xenograft-bearing nude mice.[5] In this study, we have conjugated MTX to Dal b01 and Dal B02 and compared both conjugates with free MTX on their cytotoxicity toward target vs nontarget cells. We have also studied the kinetics of uptake and efflux of free and Mab-conjugated MTX in both target and non-target cells. Our results demonstrate that target tumor cells take up more Mab-conjugated MTX than free MTX, and are more sensitive to Mab-MTX immunoconjugates than nontarget cells.

MATERIALS AND METHODS

Tumor Cell Lines and Antibodies

The details of the EBV-transformed human B-cell CLL line EBV-CLL-1 from a CLL Rai stage II patient[6,7] and of its subclone D10-1 with partial duplication of chromosome 1q, i.e., 46, XY, dup(1) (q11—q32), has been described.[8,9] The human T cell leukemia cell line MOLT-3 was from American Type Culture Collection (Rockville, MD). Both tumor cell lines were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (ICN, Costa Mesa, CA).

The murine Mab, Dal B01 (IgG2a, k) and Dal B02 (IgG1,k) that bind to m.w. 22,000 and 33,000 antigens on the surface of human B-cell CLL cells, were produced in our laboratory as described.[4] The purity of both antibody preparations was analyzed by SDS-PAGE,[10] and the protein concentration was determined by Lowry's method.[11] Both Mab have comparable binding affinity (i.e., 10.7—12.7×10—9 M-1) toward D10-1 cells, and the IRF was 28.3±2.6% and 73.9±3.9% for Dal B01 and Dal B02, respectively.

Preparation of Dal B01-MTX and Dal B02-MTX Conjugates

MTX was conjugated to Dal B01 and Dal B02 via an active ester linkage. The method reported by Kulkarni et al.[3] was followed with minor modifications. Briefly, a mixture of 0.76mg of MTX (Sigma) or [3',5',7-^{3}H]-MTX (Amersham Canada Ltd., Okaville, Ontario), 0.21 mg of N-hydroxysuccinimide (NHS) and 0.38 mg of dicyclohexylcarbodiimide (DCC) were dissolved in 0.2ml of dimethyformamide (DMF) at 4 ℃ and stirred for 18 h at room temperature. After removal of precipitates, the mixture (MTX-active ester derivative, MTX-AE) was added dropwise to a stirred solution of Dal B01 or Dal B02 (5.0mg, 3.3×10-5mmol) in 1.2ml of PBS/DMF(5:1). The reaction was carried out for about 4 h after which the solution was centrifuged at 20,000×g for 10 min. The supernatant was passed through an Econo-Pac P6 Sephadex G-25 column (Bio-Rad, CA) preequilibrated with PBS and the protein fractions were collected and pooled. The protein concentration in the conjugate was determined by Lowry's method[11] and the MTX content was calculated using ε 373nm (1cm)=7100 M-1. The molar incorporation of MTX into Mab can thus be determined.

Retention of Antibody and Drug Activities in Dal B01-MTX and Dal B02-MTX conjugate

Antibody activity was determined by indirect immunofluorescence staining using D10-1 as the target cells.[12] A dihydrofolate reductase (DHFR) inhibition assay was carried out according to the method of Peterson, et al.[13] to determine retention of MTX activity after conjugation to Mab. Briefly, to a 5 ml cuvette, reagents and test samples were added to a required volume of distilled water in the order as given: 1.0ml of 1.5M sodium acetate buffer (pH 6.0), 1.0 ml of 1.8 M KC1, 150 ml of 10 mM NADPH (Sigma), 0 to 100 ml of MTX or its Mab conjugates in 0.01 M PBS (2×10—5 to 2×10—6 M with respect to MTX), 10 ml (0.01 unit) of freshly diluted bovine liver DHFR (Sigma). The DHFR was introduced and mixed gently with a teflon adder-mixer and incubated at 22℃ for 1 to 3 min. The reaction was initiated by the addition of 100 ml of 1 mM dihydrofolic acid (Sigma). The change in the absorbance at 340 nm per min was calculated from the recorder tracing during a 3 min period. At least four concentrations of each test sample were assayed. The IC50 values (i.e., drug concentrations giving 50% inhibition of enzyme activity) were obtained from semi-logarithmic plots of percent of DHFR activity as a function of MTX (free or conjugated) concentration.

Determination of Cytotoxicity of Dal B01-MTX

and Dal B02-MTX Conjugates

For determination of IC50 values (i.e., drug concentrations giving 50% inhibition of cell proliferation) of each conjugate, the effects of continuous exposure for 72 h and pulse exposure for 6 h of target or non-target cells to the conjugates were investigated.

Continuous exposure for 72 h

Aliquots of 3×104 D10-1 cells (antibody-reactive cell line) or MOLT-3 cells (a control cell line that does not bind to Dal B01 and Dal B02) in 100 ml medium were plated in each well of 96-well culture plates and incubated in 37 ℃ 95% air/5% CO2 incubator overnight. Different amounts of free MTX or its conjugates in 100 ml medium were added in triplicate and the cells were incubated for an additional 72 h after which the total number of cells in each well were counted with a Coulter Counter (Model Zf, Coulter Electronics, Inc., Hialeah, FL). The inhibition of proliferation was determined on the basis of proliferation of cells in control preparation that contained plain medium only.

Pulse exposure for 6 h.

The procedure was carried out as described above except that the cells were incubated with MTX or its conjugates for only 6 h after which the cells were washed 3 times with plain medium. The cells were then incubated in 200 ml fresh medium for an additional 72 h and the total cell numbers were counted.

Time-course of Uptake of Free [^{3} H]-MTX, Dal B01 and Dal B02-conjugated [^{3} H]-MTX by D10-1 and MOLT-3 Cells

Twenty millions of D10-1 or MOLT-3 cells were incubated with 5 mM of free [^{3} H]-MTX, Dal B01 or Dal B02-conjugated [^{3} H]-MTX in 1.5 ml RPMI 1640 medium at either 37 ℃ or 4 ℃. At intervals of 0, 1, 3, 5, 7, 10, 15, 30, 45, 60, 90 and 120 min (for free MTX) or of 0, 5, 15, 30, 45, 90, 180 min (for conjugated MTX), aliquots of cells were taken out and washed 6 times with cold PBS containing 0.1% BSA and 0.01% sodium azide. The cell pellet was dissolved in 1.0 ml cell lysing buffer (10 mM Tris. HCl containing 1 mM EDTA, 0.1 M NaCl and 0.05% SDS). Half of the cell lysate was used for protein estimation by Lowry's method[11] while the other half was mixed with 3 ml of liquid scintillation cocktail (Ready SafeTM, Beckman, Fullerton, CA). [^{3} H] activity was counted with a liquid scintillation system (Beckman LS-7000, Beckman).

Uptake of Free [^{3} H]-MTX, Dal B01 and Dal B02-conjugated [^{3} H]-MTX at Different Extracellular Concentrations by D10-1 and MOLT-3 Cells

One to 2 million D10-1 or MOLT-3 cells in separate test tubes containing 100 ml plain RPMI 1640 medium were incubated at 37℃ or 4℃ for 2 h with different extracellular concentrations (varying from 0.078 to 40 mM) of free [^{3} H]-MTX, Dal B01 or Dal B02 conjugated [^{3} H]-MTX after which the cells were washed 6 times with cold PBS containing 0.1% BSA and 0.01% sodium azide. The cells were then processed for determination of cell-associated [^{3} H] activity as described above.

Efflux of [^{3} H]-MTX for D10-1 and MOLT-3 Cells Incubated with Free [^{3} H]-MTX

Twenty million D10-1 or MOLT-3 cells were incubated with 5 mM of free [^{3} H]-MTX in 1.5ml RPMI 1640 medium at 37℃ for 2 h after which the cells were washed 6 times with cold PBS containing 0.1% BSA. The cells were then incubated at 37℃ in 1.5ml fresh medium devoid of free MTX for up to 120 min. At intervals of 0, 1, 3, 5, 7, 10, 15, 30 45, 60, 90,120 min, aliquots of cells were taken out and washed 3 times with cold PBS containing 0.1% BSA and 0.01% sodium azide. Cell-associated [^{3} H] activity was then determined.

Release of [^{3} H]-MTX from D10-1 Cells Incubated with Dal B01-[^{3} H]-MTX or Dal B02-[^{3} H]-MTX Conjugate

Ten millions of D10-1 cells were incubated with 5 μM of Dal B01 or Dal B02-conjugated [^{3} H]-MTX at 37℃ for 2 h after which the cells were washed 4 times with PBS. The cells were reincubated at 37℃ in the presence or absence of 50-fold higher protein concentration of appropriate unconjugated Dal B01 or Dal B02. At intervals of 0, 5, 10, 15, 30, 60 min, aliquots of cells were taken out, washed and cell-

associated [^{3}H] activity was determined.

RESULTS

Preparation of Dal B01-MTX and Dal B02-MTX conjugates

The NHS active ester based method entails activation of the carboxyl group of MTX by means of NHS and DCC in DMF and then reaction of the MTX-AE with nucleophilic groups in the Mab in an aqueous medium at neutral pH. Conjugation of MTX to Dal B01 and Dal B02 did not result in significant precipitation of the protein. The IgG/MTX molar ratio in both the Dal B01-MTX and Dal B02-MTX conjugates was approximately 1 molecule of IgG to 5 to 6 molecules of MTX. No significant difference in antibody reactivity was observed among parent Mab and their MTX conjugates. The titer (i.e., antibody concentration required to stain 50% of D10-1 cells) was 0.3 mg/ml for Dal B01 and Dal B01-MTX, and 0.1mg/ml for Dal B02 and Dal B02-MTX conjugate as determined by indirect membrane immuno-fluorescence.

In vitro Cytotoxicity of Dal B01-MTX and Dal B02-MTX Conjugates

When cells were exposed continuously for 72 h to test agents *in vitro*, free MTX was the most effective inhibitor of proliferation for both D10-1 (IC50, 0.012 μM) and MOLT-3 cells (IC50, 0.005 μM). The addition of free Dal B01 to MTX did not significantly affect the cytotoxicity of the free drug toward both D10-1 and MOLT-3 cells. Considering the target D10-1 cells, Dal B02-MTX conjugate (IC50, 0.07 μM) was 8.5-fold more potent than Dal B01-MTX, but was almost 6-fold less inhibitory than free MTX. MOLT-3 cells were 2.5-fold more sensitive to free MTX than D10-1 cells. In contrast, Mab-linked MTX was much less toxic toward the non-target MOLT-3 cells. The IC50 values of Dal B01-MTX and Dal B02-MTX toward MOLT-3 cells were 0.65 μM and 0.054 μM, which were 127 and 10-fold higher than that of free MTX, respectively. The selectivity ratios of Dal B01-MTX and Dal B02-MTX conjugates to target D10-1 cells over non-target MOLT-3 cells were 2.7 and 1.8, respectively.

In another assay, the cells were exposed to test agents for only 6 h after which the cells were washed thrice and then incubated in drug free medium for an additional period of 72 h. The results of this pulse exposure assay (Table 1B) are somewhat different from those obtained in the continuous exposure assay. Considering the target D10-1 cell, the Dal B02-MTX conjugate (IC_{50}, 0.81μM) was only 1.7-fold more potent than free MTX (IC_{50} , 1.41μM), and Dal B01-MTX conjugate (IC_{50}, 3.9μM) was only 2.8-fold (instead of 50-fold in the continuous exposure assay) less inhibitory than free MTX. The cytotoxicity of both the Dal B01-MTX and Dal B02-MTX conjugates to non-target MOLT-3 cells was significantly reduced in this pulse exposure assay (IC_{50} values were>8μM for Dal B01-MTX and 3.8μM for Dal B02-MTX, which were >50-fold and 22-fold higher than that of free MTX, respectrively). The selectivity ratios of the conjutages to target D10-1 cells over non-target MOLT-3 cells were significantly increased in this puse exposure assay. These results indicate a selective cytotoxicity of Dal B01-MTX and Dal B02-MTX conjugates toward target D10-1 cells.

Design of Drug Uptake Experiments

To determine whether conjugation of MTX to anti-tumor Mab will lead to the uptake of larger amount of MTX by target cells, it is necessary to compare the kinetics of uptake and efflux of Mab-conjugated MTX with that of free MTX. In this study, experiments were carried out to determine the (i) uptake of free[^{3}H]-MTX from D10-1 and MOLT-3 cells after incubated with free [^{3}H]-MTX; and (iii) release of free [^{3}H]-MTX or [^{3}H]-MTX containing antibodies (or their fragments) from target D10-1 cells after incubated with Dal B01-[^{3}H]-MTX, either free or conjugated to Mab, were distinguished from the internalized [^{3}H]-MTX by subtracting cell-associated [^{3}H] activity after incubation at 37℃ will include both surface-bound and endocytosed [^{3}H]-MTX but the cell-associated radioactivity at 4℃ will measure only the cell surface bound drug.

Time Course of Uptake of Free [^{3}H]-MTX, Dal B01 and Dal B02-conjugated [^{3}H]-MTX by D10-1 and MOLT-3 Cells

Both D10-1 and MOLT cells took up much more free drug at 37℃ than at 4℃(Figure 1). The uptake of free [^{3}H]-MTX by D10-1 cells was rapid and leveled

off at about 15 min. In contrast, MOLT-3 cells took about 45 to 60 min to reach the plateau. The maximum amount of cell-associated [^{3}H]-MTX was 17.4 pmol MTX/mg protein (net uptake, 11.7 pmol MTX/mg protein) in D10-1 cells, and 23.4 pmol MTX/mg protein (net uptake, 19.8 pmol MTX/mg protein) in MOLT-3 cells.

Binding of Dal B01-[^{3}H]-MTX and Dal B02-[^{3}H]-MTX conjugates to D10-1 cells was rapid (Figure 2). The maximum binding (i.e., plateau) was reached within the first 5 min of incubation either at 4℃ and 37 ℃, and then remained unchanged during the incubation period of 3 h. The difference in the amount of uptake of Dal B01 and Dal B02-conjugated [^{3}H]-MTX between D10-1 cells incubated at 4℃ and 37℃ was not as prominent as that in uptake of free [^{3}H]-MTX. The maximum amount of cell-associated [^{3}H]-MTX was approximately 550 pmol MTX/mg protein in D10-1 cells incubated with Dal B01-[^{3}H]-MTX and 2100 pmol MTX/mg protein in D10-1 cells incubated with Dal B02-[^{3}H]-MTX conjugate; this is 30-fold and 120-fold higher than the amount of MTX in D10-1 cells incubated with free [^{3}H]-MTX, respectively.

Rate of Uptake of Free [^{3}H]-MTX, Dal B01 and Dal B02-conjugated [^{3}H]-MTX at Different Extracellular [^{3}H]-MTX Concentrations

Figure 3 shows the uptake of [^{3}H]-MTX, either free or conjugated to Dal B01 or Dal B02, by D10-1 and MOLT-3 cells at different extracellular [^{3}H]-MTX concentrations at either 4 ℃ or 37 ℃ for 2 h. Comparison of the net uptake of Dal B01 and Dal B02-conjugate [^{3}H]-MTX (Figure 3A and 3B) with that of free [^{3}H]-MTX by 10-1 cells (Figure 3C) revealed that the uptake of the Mab-conjugated drug exceeded that of free drug at equivalent extracellular drug concentrations by approximately 10 to 20-fold for Dal B01-[^{3}H]-MTX and 30 to 40-fold for Dal B02-[^{3}H]-MTX conjugates. The cell-associated [^{3}H]-MTX in D10-1 cells incubated with Mab-[^{3}H]-MTX conjugates leveled off (i.e., reached a plateau) at an extracellular concentration of 20 μM of [^{3}H]-MTX for Dal B01-[^{3}H]-MTX and 5 μ M of [^{3}H]-MTX for Dal B02-[^{3}H]-MTX conjugate. The maximum amount of cell-associated [^{3}H]-MTX was 1000 pmol MTX/mg protein in D10-1 cells incubated with Dal B01-[^{3}H]-MTX conjugate, and 4000 pmol MTX/mg protein in D10-1 cells incubated with Dal B02-[^{3}H]-MTX conjugate. The maximum net uptake was approximately 250 pmol MTX/mg protein and 862 pmol MTX/mg protein in D10-1 cells incubated with Dal B01-[^{3}H]-MTX and Dal B02-[^{3}H]-MTX conjugates, respectively. Though MOLT-3 cells took up slightly more free [^{3}H]-MTX (Figure 3C), the non-target took up much less (i.e., 3 to 5-fold less) Dal B01 and Dal B02-conjugated [^{3}H]-MTX than did the target D10-1 cells (Figure 3). However, the fact that non-target MOLT-3 cells also took up a substantial amount of [^{3}H]-MTX suggests there may also be nonspecific binding and uptake of Mab-[^{3}H]-MTX conjugates by tumor cells.

Efflux of Free [^{3}H]-MTX from D10-1 and MOLT-3 Cells

For inhibition of cell proliferation, the intracellular concentration of MTX has to exceed the level of intracellular target enzyme DHFR. Hence it is important to know how much MTX remains inside the exposed cells (bound to DHFR) after incubation with a defined extracellular concentration of MTX. In this study, efflux of [^{3}H]-MTX from D10-1 and MOLT-3 cells was determined after loading the cells with free [^{3}H]-MTX at an extracellular concentration of 5 μM at 37 ℃ for 2 h. The amounts of [^{3}H]-MTX that remained associated with D10-1 or MOLT-3 cells at various times after incubation in MTX-free efflux medium were determined. Since MTX binds stoichiometrically to intracellular DHFR, the molar amount of MTX that remains inside cells after the completion of efflux equals to that of intracellular DHFR. As shown in Figure 4, MOLT-3 cells initially took up more free [^{3}H]-MTX than did D10-1 cells (23 pmol MTX/mg protein in MOLT-3 cells versus 18 pmol MTX/mg grotein in D10-1 cells). The efflux of free MTX from D10-1 cells stopped at about 10 to 15 min after incubation in the efflux medium (which is earlier than the time needed by MOLT-3 cells which stopped at about 45 to 60 min), after which the cell associated [^{3}H]-MTX remained at a constant level. At the end of efflux, the levels of intracellular [^{3}H]-MTX was 11 pmol MTX/mg protein for D10-1 cells, and 8 pmol MTX/mg protein for MOLT-3 cells. These results suggest that the level of intracellular DHFR in D10-1 cells is higher than that in MOLT-3 cells.

The Fate of Dal B01 and Dal B02-conjugated [^{3}H]-MTX Associated with D10-1 Cell

To examine the fate of cell-associated Dal B01-[^{3}H]-MTX and Dal B02-[^{3}H]-MTX conjugates, D10-1 cells were preincubated with the conjugates at 37℃ for 2 h and then reincubated at 37℃ in conjugate-free efflux medium in the presence or absence of a large excess of appropriate unconjugated Dal B01 or Dal B02 for 60 min. Aliquots of cells were taken out at indicated intervals and the cell associated [^{3}H] activity was determined.

When Mab-[^{3}H]-MTX conjugate preincubated D10-1 cells were reincubated in efflux medium at 37 ℃ without unconjugated Mab, there was a rapid release of [^{3}H] activity from the cells during the first 15 min of incubation, following which a relatively constant level of cell-associated [^{3}H] activity was established (Figure 5). At the end of 60 min incubation, about 77.% and 63.6% of initial cell-associated [^{3}H] activity remained associated with D10-1 cells preincubated with Dal B01-[^{3}H]-MTX and Dal B02-[^{3}H]-MTX conjugates, respectively. The amounts of cell associated MTX at the end of incubation were 317 pmol/mg protein in D10-1 cells preincubated with Dal B01-[^{3}H]-MTX and 1080 pmol MTX/mg protein in D10-1 cells preincubated with Dal B02-[^{3}H]-MTX. Both Dal B01-[^{3}H]-MTX and Dal B02-[^{3}H]-MTX conjugate preincubated D10-1 cells showed a similar rapid release of cell-associated radioactivity in the presence of a large excess of appropriate unconjugate Dal B01 or Dal B02 (Figure 5.) This process proceeded rapidly and completed within 30 min, after which the cell associated [^{3}H] activity remained at constant levels. At the end of incubation, approximately 15% of initial cell-associated [^{3}H] activity remained associated with cells preincubated with Dal B01-[^{3}H]-MTX conjugate and 20% of initial cell-associated [^{3}H] activity remained associated with cells preincubated with Dal B02-[^{3}H]-MTX conjugate.

DISCUSSION

We here demonstrated that MTX could be rendered selectively cytotoxic to human CLL B cells by covalently coupling to Mab against TAA on the surface of the target cells. As a step toward understanding specific tumor inhibition by Mab-MTX conjugates we compared uptake of free MTX and MTX conjugated to tumor specific Mab by target and non-target cells. The results reported here show that target D10-1 cells took up much more Dal B01 and Dal B02-conjugated MTX than free MTX. In contrast, the non-target MOLT-3 cells took up 3 to 5-fold less Mab-conjugated MTX than did D10-1 cells although the former took up more free MTX.

Measurement of cell-associated radioactivity after incubation of cells with conjugates at 37℃ will include both cell surface binding and uptake; those at 4℃ will be just cell surface binding since endocytosis does not occur at 4℃. For accurate determination of endocytosed Mab-[^{3}H]-MTX conjugates (i.e., net uptake), the radioactivity bound on cell surface must be subtracted from the total cell-associated radioactivity. The cell surface bound radioactivity was estimated by incubation of cells with the Mab-[^{3}H]-MTX conjugates at 4℃ and subsequently processed at this temperature for analysis. If the cell-associated radioactivity measured for each specific incubation time at 4℃ were assumed to be the amount not endocytosed at 37℃, then net uptake would be calculated by subtracting each 4℃ value from the corresponding 37℃ value.

Several factors would determine the amount of cell-associated radioactivity after incubation with Mab-[^{3}H]-MTX conjugates at 37℃. These factors include (I) specific binding of the conjugates to tumor antigen(s) on cell surface and subsequent endocytosis and/or shedding; (ii) non-specific binding to cell surface and endocytosis by mechanisms independent of specific transport carriers, e.g., pinocytosis; (iii) efflux of free [^{3}H]-MTX or [^{3}H]-MTX containing small fragments of antibody produced by intracellular catabolism; and (iv) catabolic release of free [^{3}H]-MTX or [^{3}H]-MTX containing fragments of antibody mediated by proteolytic enzymes on the surface of tumor cells. Thus our calculation for determining net uptake of MTX conjugates by tumor cells are only approximations and will neither take into account the amount eliminated by efflux nor distinguish nonspecific uptake from specific uptake. The non-target MOLT-3 cells therefore were used in this study to estimate the amount of nonspecific uptake of Mab-[^{3}H]-MTX conjugates.

Our results show that the D10-1 cells took up much more Dal B01 and Dal B02-[^{3}H]-MTX than free [^{3}H]-MTX. Factors that determine the uptake of free MTX include the availability of a transport system, the drug concentration, the membrane potential and the ionic milieu. The uptake kinetics of Mab-MTX conjugates differed from those of free MTX (Figure 1 and 2), so it is unlikely that the Mab-conjugated MTX

was bound and transported into the cells by the same system for free MTX. Several studies have shown that Mab-MTX conjugates are endocytosed by target cells via receptor-mediated endocytosis and transported to the lysosomal compartment where digestion of the conjugates releases free drug or it derivatives. Furthermore, the non-target MOLT-3 cells took up much less Mab-conjugated [^{3}H]-MTX than did the target D10-1 cells although the former took up more free [^{3}H]-MTX (Figure 1 and 3). These results suggest that the increased uptake of Dal B01 and Dal B02-

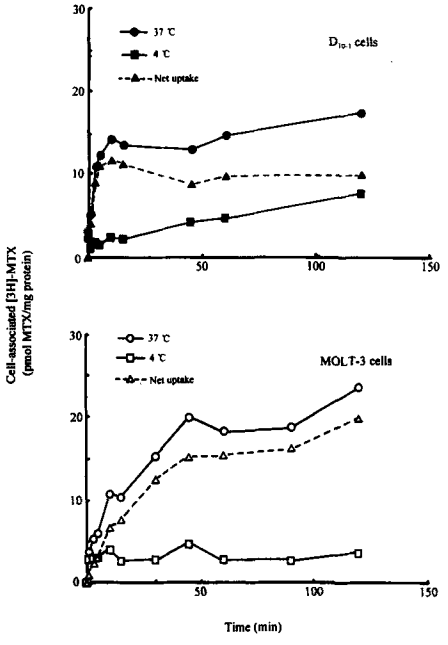

Fig. 1. Time-course of uptake of free [^{3}H]-MTX by D10-1 and MOLT-3 cells. Ten million cells were incubated with [^{3}H]-MTX at an extracellular concentration of 5 ˚M either at 4℃ or 37℃ for up to 120 min. Aliquots of cells were taken out at indicated intervals and cell-associated [^{3}H] activity was determined.

conjugated [^{3}H]-MTX by D10-1 cells was an antibody-dependent specific event. The higher uptake of MTX when conjugated to anti-tumor Mab has also been reported by several other investigators using different Mab and tumor cell lines. Consistent with our previous report, the DHFR inhibitory capacity of Dal B01 and Dal B02--conjugated MTX was approximately 15—20% of that of free MTX.

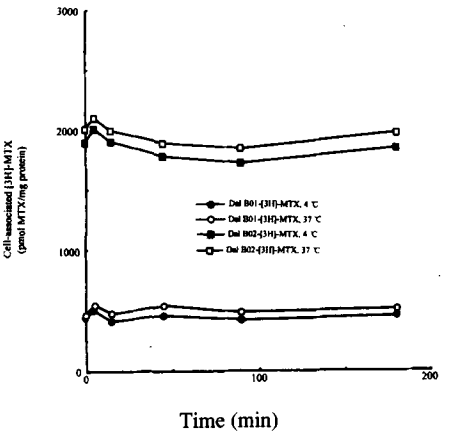

Fig. 2 Time-course of uptake of Dal B01 and Dal B02-conjugated [^{3}H]-MTX by D10-1 cells. Ten million D10-1 cells were incubated with 5˚M of Dal B01 or Dal B02-conjugated [^{3}H]-MTX at either 4˚C or 37˚C for a period of up to 180 min. Aliquots of cells were taken out at indicated intervals and cell-associated [^{3}H] activity was determined.

The loss of potency of MTX after conjugation to Mab could have been more than compensated for by the much higher uptake of Mab-conjugated MTX by the target cells (Figure 3). This was supported by the observation that Mab-MTX conjugates were much more cytotoxic to D10-1 cells than to MOLT-3 cells; and in the 6 h pulse exposure assay, Dal B02-MTX conjugates were much more cytotoxic to D10-1 cells than to MOLT-3 cells; and in the 6 h pulse exposure assay, Dal B02-MTX conjugate was even more cytotoxic to target cells than free MTX. D10-1 cells took up much more Dal B02-conjugated [^{3}H]-MTX than Dal B01-conjugated [^{3}H]-MTX at equivalent extracellular drug concentrations (Figure 2 and 3), and are more sensitive to Dal B02-[^{3}H]-MTX conjugate than to Dal B01-MTX conjugate. Furthermore, the amount of Dal B02 localized in tumors is about 2-fold higher than that of Dal B01 when the Mab were injected intravenously into CLL xenograft-bearing nude mice. Take together these results indicate that Mab preparation with higher IRF are likely to lead to the binding of a larger proportion of Mab-MTX conjugates and hence higher uptake of Mab-conjugated MTX by target cells. It is noteworthy that the non-target MOLT-3 cells also took up a substantial amount of Mab-MTX conjugates (Figure 3). This is likely due to nonspecific uptake by pinocytosis as observed with various serum proteins, and is

responsible for the cytotoxicity of the conjugates toward MOLT-3 cells observed in proliferation inhibition assays.

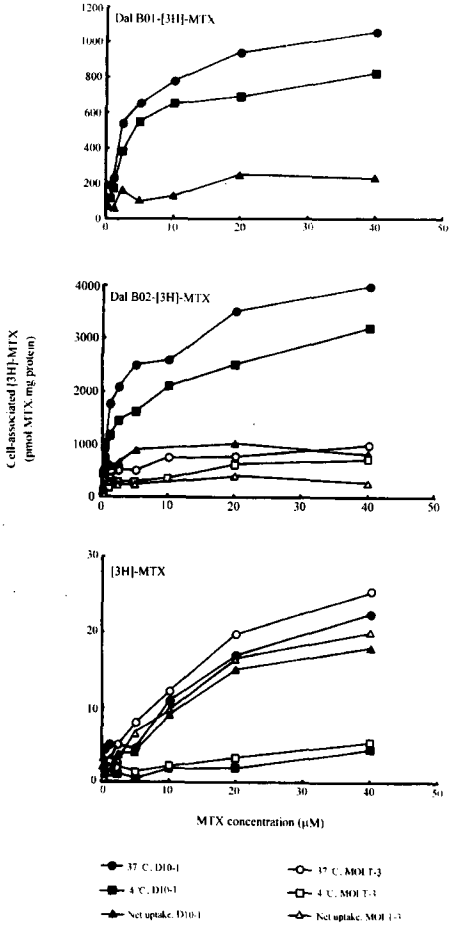

Fig. 3. Uptake of free [^{3}H]-MTX, Dal B01 and Dal B02-conjugated [^{3}H]-MTX by D10-1 and MOLT-3 cells. One million cells were incubated with various extracellular concentrations of [^{3}H]-MTX, Dal B01 and Dal B02-conjugated [^{3}H]-MTX at either 4℃ or 37℃for 2 h after which cell-associated [^{3}H] activity was determined.

Release of Dal B01 and Dal B02-conjugated [^{3}H]-MTX from D10-1 cells was also different from that of free [^{3}H]-MTX. The efflux of [^{3}H]-MTX from D10-1 cells preincubated with free [^{3}H]-MTX stopped at ~10 min after incubation in efflux medium at 37℃ with a steady state of intracellular MTX of 11 pmol MTX/mg protein. The release of [^{3}H]-MTX from conjugate-preincubated D10-1 cells also stopped at 10-15 min after incubation in efflux medium. However, the steady state levels of cell-associated MTX at the end of efflux were much higher in these D10-1 cells, i.e., 317 pmol MTX/mg protein in D10-1 cell incubated with Dal B01-[^{3}H]-MTX conjugate and 1080 pmol MTX/mg protein in D10-1 cell incubated with Dal B01-[^{3}H]-MTX conjugate and 1080 pmol MTX/mg protein in D10-1 cells incubated with Dal B02-[^{3}H]-MTX conjugate (i.e., 29 and 98 times higher than that in D10-1 cells preincubated with free [^{3}H]-MTX, respectively). Approximately 17% of these cell-associated Dal B01-[^{3}H]-MTX conjugates and 22% of cell-associated Dal B02-[^{3}H]-MTX conjugates could have been endocytosed since they could not be displaced by a large excess amount of appropriate unconjugated Mab (Figure 5). By coupling Mab to colloidal gold particles (diameter~15 nm), we were able to observe surface binding and the subsequent internalization of gold-labeled Mab by D10-1 cells by utilizing transmission electromicroscopy. Our results

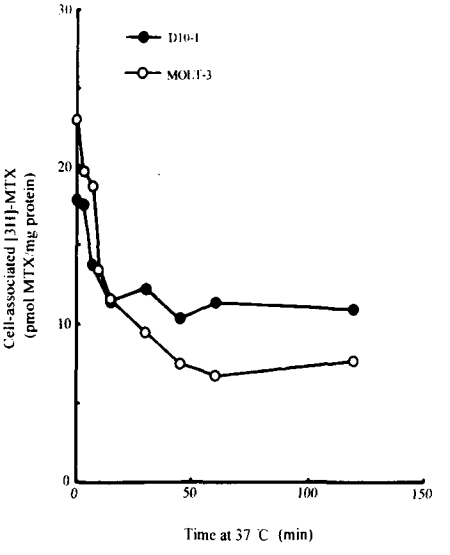

Fig. 4. Efflux of [^{3}H]-MTX from D10-1 and MOLT-3 cells. Ten million D10-1 or MOLT-3 cells were incubated with [^{3}H]-MTX at an extracellular concentration of 5*M for 2 h at 37℃. At the end of incubation, the cells were washed 6 times with cold PBS and reincubated in MTX-free efflux medium at 37℃ for a period of 120 min. Aliquots of cells were taken out at indicated intervals and the cell-associated [^{3}H] activity was determined.

demonstrated that approximately 10% of cell-associated Dal B01 and 17% of cell-associated Dal B02 were internalized after incubation at 37℃ for 2 h (manuscript in preparation). Take together these results indicate that a small proportion of cell-associated Dal B01-MTX and Dal B02-MTX conjugates were internalized by target D10-1 cells after incubation for 2 h at 37℃.

MTX binds to its target enzyme DHFR and inhibits the conversion of dihydrofolic acid to tetrahydrofolic acid. Besides the level of DHFR, the amount of uptake and the intracellular steady-state level of free MTX also influence the sensitivity of cells to this agent. Studies with free MTX have shown that cytotoxic effect correlates with the excess of drug overt the stoichiometric DHFR level and the duration of exposure. Since MTX binds stoichiometrically to intracellular DHFR, the molar amount of MTX that remains inside the MTX-preloaded cells after the completion of efflux equals to that of intracellular DHFR. In our efflux study with free MTX, D10-1 cells retained more MTX than did MOLT-3 cells (Figure 4), indicating that D10-1 cells possess a higher level of intracellular DHFR than MOLT-3 cells. We also observed that D10-1 cells took up less amount of free MTX than did MOLT-3 cells (Figure 1). These features suggest that D10-1 cells would be less sensitive to free MTX than MOLT-3 cells. This was confirmed in the cytotoxicity assay which shown that the IC50 value of MTX for D10-1 cells was 3-fold (in the 72 h continuous exposure assay) to 8-fold (in the 6 h pulse exposure assay) higher than the value for MOLT-3 cells. In contrast, D10-1 cells were far more sensitive to Mab-MTX conjugates than MOLT-3 cells. The selectivity ratio of the conjugates to D10-1 cells over MOLT-3 cells was as high as 43 in the 6 h pulse exposure assay. These results suggest the cytotoxicity of the conjugates toward D10-1 cells is an antibody-dependent specific event. This specific cytotoxicity is likely due to (i) delivery of larger amounts of MTX to target cells when the drug is conjugated to Mab (Figure 3); (ii) longer retention of Mab-conjugated MTX by target cells (Figure 5); and (iii) slow, prolonged release of MTX from the surface-bound or endocytosed conjugates, rendering them into a sustained release dosage form.

Rate of internalization is considered one of the important factors in selecting an antibody as carrier of cytotoxic agents for targeted cancer therapy.[14-17] Efficient internalization of carrier antibodies is

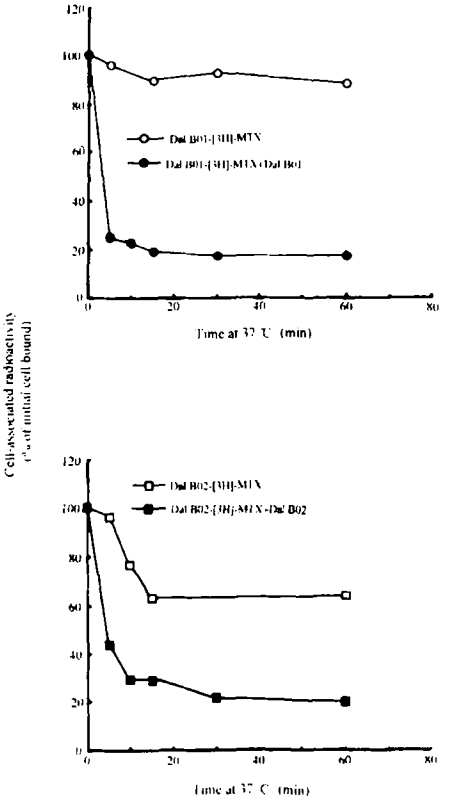

Fig. 5. The fate of D10-1 cell-associated Dal B01-[^{3}H]-MTX and Dal B02-[^{3}H]-MTX conjugates. D10-1 cells were preincubated with Dal B01-[^{3}H]-MTX or Dal B02-[^{3}H]-MTX conjugate for 2 h at 37℃ after which the cells were washed 4 times with cold PBS. The cells were then reincubated in fresh conjugate-free medium at 37℃ for up to 60 min in the absence or presence of a large excess of appropriate unconjugated Dal B01 or Dal B02. Aliquots of cells were taken out at indicated intervals and cell-associated [^{3}H] activity was determined.

advantageous for cytotoxic agents exerting their action only in the interior of the cells, e.g., toxins, some chemotherapeutic agents such as MTX, and Auger-emitter radioisotopes such as ^{125}I. On the other hand, internalization may not be essential when the antibody is used as carrier for agents exerting their cytotoxic potential on the surface of tumor cells, e.g., chemotherapeutic agents such as doxorubicin[18] and b- and/or g-emitter radioisotopes such as ^{131}I and ^{90}Y, and as antibody-enzyme conjugates for prodrug activation.[19] Based on our studies above, we speculate that Dal B02 is a good candidate carrier for delivering

b- and/or g-emitter radioisotopes and chemotherapeutic agents such as doxorubicin because of its high specificity,[4] high IRF and antigen binding affinity,[5] large number of antigen sites and low rate of internalization (Z.Z and T.G., unpublished data). In fact, we have observed that Dal B02, when labeled with radioisotope $^{131}I^{20}$ or conjugated with doxorubicin,[21] could significantly prolong the survival of human CLL xenograft-bearing nude and SCID mice, and in some cases, eradicate the tumors and result in complete cure.

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