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Differential Usage of the Transport Systems for Folic acid and Methotrexate in Normal Human T-Lymphocytes and Leukemic Cells

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Methotrexate (MTX) has been used as an effective anti-cancer drug for a long time. Conceptually, it is accepted that MTX and folic acid are transported by folate receptors (FRs) in cancerous cells, but the exact mechanism of MTX uptake in human leukemia is unknown. The objective of this study was to investigate different transport systems for FA and MTX, and to delineate their uptake mechanism in MOLT4, K562, Hut78 leukemia cells and normal human T cells. In MOLT4, uptake of MTX was higher than FA, similar to that of K562, Hut78 and normal T cells. In MOLT4 cells, MTX uptake was maximum at pH 7.4 whereas FA uptake was maximum at pH 4.5. Uptake of FA and MTX was significantly inhibited by anions, suggesting anion-dependent transport system. FA uptake was found to be energy dependent whereas MTX uptake was energy independent. RT-PCR and immunofluorescence results demonstrated the presence of reduced folate carrier as well as proton coupled folate transporter and absence of FR in MOLT4 and normal T cells. These data suggest the existence of two separate and independent carrier-mediated transport systems for the uptake of FA and MTX in normal and leukemic human T cells.

Key words: folate receptor, folic acid, methotrexate, MOLT4 cells, reduced folate carrier.

Abbreviations: FA, folic acid; FR, folate receptor; MTX, methotrexate; PCFT, proton coupled folate transporter; RFC, reduced folate carrier; RT-PCR, reverse transcription polymerase chain reaction; PBS, phosphate buffered saline; HBS, hepes buffered saline; DEPC, diethylpyrocarbonate; FITC, fluorescein isothiocyanate.

Folic acid (FA) and its coenzyme derivatives play a vital role in one carbon metabolism. These coenzymes provide one carbon units for the synthesis of thymidine, purines, methionine, glycine and provide a methyl group for methylation of DNA and RNA (*1*). Deranged folate metabolism has been implicated in numerous diseases, including megaloblastic anemia (*2*), carcinogenesis (*3*), cardiovascular disease (*4*) and in the occurrence of neural tube defect (*5*). Some anti-folates, most notably methotrexate (MTX), has been used as an effective anti-cancer drug in the treatment of malignancies such as leukemia, lymphoma and breast cancer (*6*). Several folate transport systems have been described in mammalian cells, including L1210 murine leukemia cells, fibroblasts, erythroid cells, hepatocytes and enterocytes (*7*, *8*). In mammalian cells, entry of FA and MTX is by two general categories of transport system namely receptor mediated and carrier mediated. The receptor-mediated uptake is by folate receptor (FR) (*9*) and the carrier-mediated one includes the reduced folate carrier (RFC) as well as the recently identified low pH transporter called proton coupled folate transporter (PCFT) (*10–12*).

Rheumatoid arthritis (RA) is an autoimmune disease, which causes destructive inflammation of both internal organs and joints (*13*). The synovial fluid of RA patients has an excess of T cells that causes inflammation. MTX along with other drugs has been widely used as a chemotherapeutic drug for the treatment of RA (*14–16*). Sulphasalazine is also used as a drug for the treatment of RA. Sulphasalazine is a potent inhibitor of the RFC and thus reduces the efficacy of MTX when used in combination therapy (*17*). Transport system and uptake mechanism of MTX in the T cells of synovial fluid has not been well understood. Many studies have investigated the mechanisms of FA uptake in various tissues such as intestine, kidney, placenta and pancreas and MTX uptake in PC-3 and CCRF-CEM cell lines (*18–23*). In CCRF-CEM cells, MTX transport is done by a carrier-mediated pathway (*23*). FA has a critical role in the growth and development of T cells, while MTX is an important agent in the treatment of T leukemia as well as RA. Still the transport system and uptake mechanism of FA and MTX in normal human T cells and leukemic cells are not well defined.

Various studies reported the over expression of FR in the malignant cells and its relatively low expression in most normal tissues (*24*). This differential expression of FR has been exploited in the development of diagnostic agents and for FR mediated drug delivery (*25*, *26*). The major aim of the present study therefore, is to identify the presence of FA and MTX transport systems in human-derived T leukemia cell line MOLT4 and to investigate the mechanism and intracellular regulation of FA and MTX uptake. As there is an excess of T-lymphocytes

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in T leukemia, in this study we used MOLT4 cell line as an *in vitro* system to investigate the transport and uptake mechanism of FA and MTX. We also compared the FA and MTX uptake in MOLT4 cells with K562 and Hut78 leukemic cells as well as normal human T cells. In this present study, we describe the uptake mechanism of FA and MTX, energy dependence during the transport, inhibition study using structural analogue and structurally unrelated anions and also RT-PCR analysis of different transport systems.

MATERIALS AND METHODS

Materials—[3′, 5′, 7, 9-^{3}H] FA (35 Ci/mmol) and [3′, 5′, 7-^{3}H (N)] and MTX (50.8 Ci/mmol) were purchased from Moravek Biochemicals, (Brea, CA). Unlabelled MTX, FA, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES), Verapamail, Mitomycin C, sulphasalazine, phytohemagglutinin (PHA), Ficoll-Hypaque and Triton X-100 were procured from Sigma Chemical Co. (USA). Antibody against FRs was obtained from Santa Cruz Biotechnology, Inc. (USA). Anti-rabbit IgG FITC-conjugated secondary antibodies were procured from Sigma Chemical Co. (USA). All other chemicals used were of molecular grade.

Cell culture—Human T-leukemia cell line MOLT4, K562, Hut78 and non-leukemic HeLa and KG1 cell lines were obtained from National Centre for Cell Sciences (NCCS, Pune, India). Cells were grown in RPMI 1640 media (Gibco-BRL) containing 2.2 μM FA supplemented with 10% fetal bovine serum (Hyclone labs), 1% (v/v) penicillin/streptomycin (5000 v/ml, 5000 mg/ml) in T-25 flasks (costar) at 37°C in a humidified 5% CO_2 atmosphere. Cells were routinely sub-cultured at the ratio of 1 : 6 dilutions. For determining the effect of FA on the uptake of [^{3}H] FA and [^{3}H] MTX, cells were grown in folate free RPMI 1640 media (Gibco-BRL) containing dialysed fetal bovine serum for three passages by slowly conditioning in the medium from physiological folate (2.2 μM) to folate free media. Peripheral blood mononuclear cells were isolated from normal human blood by using ficoll-hypaque and culture in RPMI 1640 media with 10 μg/ml PHA which helps in the proliferation of T lymphocytes. These normal human T cells were used to study to uptake of [^{3}H] FA and [^{3}H] MTX.

Uptake experiments—Cells grown in suspension were taken, centrifuged and washed with HBS buffer (135 mM NaCl, 5 mM KCl, 2.5 Mm $CaCl_2$, 0.6 mM $MgSO_4$, 6 mM glucose and 10 mM HEPES, pH 7.4) and counted using a hemocytometer. For uptake experiments, 1×10^6 cells were taken in 200 μl of HBS buffer in separate eppendorf tubes. [^{3}H] FA and [^{3}H] MTX were added to the eppendorf tubes at the onset of the experiments and incubated at 37°C. Uptake of radio labelled FA and MTX was terminated at the appropriate time by placing the tubes on ice as well as by adding 200 μl of ice cold incubation buffer (HBS buffer). Then cells were rapidly washed three times with 1 ml of ice cold HBS buffer. The cells were lysed with 50 μl of lysis buffer (10 mM EDTA, pH 12, 1% Triton X-100) and kept for 15 min on ice. Then 250 μl Aqualight Scintillation fluids (Hidex personal life sciences, Groningen, The Netherlands) was added to the lysate, mixed by vortexing and kept on ice for 20 min. The lysates were transferred to a 96 well isoplate and counted for radioactivity in a liquid scintillation counter (Model: Rigaweg 22, 9700 GE, Groningen, The Netherlands). Results were expressed as pico moles per 10^6 cells. The data were plotted using the 'origin 6.0 software' and Michaelis–Menten kinetic parameters (K_m and V_{max}) were calculated by non-linear regression curve fitting of the data to the standard equation $v = K.S. + V_{max}S/(K_m + S)$. The inhibition constant Ki was calculated by using the Dixon plot.

Effect of pH on the uptake of FA and MTX—Transport of many vitamins and drugs into the cells depends on the pH of the medium. In order to determine the optimum pH for the uptake of FA and MTX into MOLT4 cells, uptake experiments were carried out at different pH. Cells were resuspended and incubated for 30 min with [^{3}H] FA and [^{3}H] MTX at 37°C in the incubation buffer (HBS buffer) with pH ranging from 4.5 to 8.0. Radioactivity was counted as described in the earlier section.

Effect of SO_4^{2-} and PO_4^{2-} anions on the uptake of FA and MTX—To determine the effect of sulphate and phosphate anion on the uptake of [^{3}H] FA and [^{3}H] MTX, studies were conducted in the presence of different anion concentrations. Appropriate concentrations of potassium sulphate and potassium phosphate were added isoosmotically, to HEPES-Sucrose-MgO buffer (20 mM HEPES, 235 mM Sucrose, pH adjusted to 7.4 with MgO). Cells were then resuspended in HEPES–sucrose–MgO buffer. [^{3}H] FA and [^{3}H] MTX were added at the onset of the experiments and incubated at 37°C for the appropriate time. Uptake was terminated by keeping the tubes on ice followed by three washes with 1 ml of ice cold incubation medium. Cells were lysed and radioactivity was counted as described in the earlier section.

Effect of energy inhibitors on the uptake of FA and MTX—To determine the role of energy during the uptake and transport of MTX and FA, the effects of energy inhibitors on the uptake of [^{3}H] FA and [^{3}H] MTX were studied. Cells were preincubated for 1 h at 37°C with either 1 or 10 mM sodium azide, sodium cyanide or sodium arsenite prior to measuring the uptake. After incubation, radiolabelled FA or MTX was added and incubated at 37°C for 30 min. Uptake was terminated; cells were washed, lysed and counted for radioactivity as per the method described previously.

Effect of FR antibody, verapamil, mitomycin and sulphasalazine on the uptake of FA and MTX—To rule out the presence/involvement of FR mediated transport, uptake studies were performed both in the presence and absence of FR antibody. To find out the effect of DNA cross linking inhibition on the uptake of FA and MTX, uptake in presence of Mitomycin C was analysed. The role of drug efflux system in the uptake of FA and MTX in MOLT4 was studied by using verapamil, which is an efflux pump inhibitor. Cells were preincubated separately for 1 h at 37°C with FR antibody, verapamil, verapamil with FR antibody and mitomycin C prior to measuring FA and MTX uptake. After 1 h of incubation with the inhibitors, radio labelled FA or MTX was added and incubated at 37°C for 30 min. To prove that uptake

of MTX is by RFC and FA by PCFT, uptake studies were carried out in the presence of sulphasalazine, which is known as a potent inhibitor of RFC at physiological pH. After the incubation, uptake was terminated and radioactivity in the cells was counted as before.

Molecular evidence: RT-PCR analysis—Total RNA was isolated from HeLa, KG1, K562, Hut78, MOLT4 and normal blood T cells by a standard protocol using guanidinium isothiocynate (*27*). RNA was extracted by phenol–chloroform–isopropanol method, purified and dissolved in 50 μl of DEPC treated water. Purity and integrity of RNA were checked by spectrophotometer and agarose gel electrophoresis. For single strand cDNA synthesis, 2 μg of total RNA was reverse transcribed according to a standard protocol using M MuLV-Reverse Transcriptase (Finnzymes, USA). The conditions for reverse transcription were denaturation of template RNA for 5 min at 94°C and reverse transcription for 1 h at 42°C. Polymerase chain reaction amplification was performed using cDNA and primers specific for FR alpha (FRα), FR beta (FRβ), FR gama (FRγ), RFC, PCFT and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as control experiment. The forward and reverse primers for FRα were 5′-CGC TCG AGA CAG ACA TGG CTC AGC GGA TGA CAA-3′ and 5′-GCT CTA GAG GAG GTC AGC TGA GCA GCC ACA GCA-3′ which correspond to nucleotides −7 to 19 and 755 to 780, respectively, of FRα. The forward and reverse primers for FRβ were 5′-GCG CTC GAG AAA GAC ATG GTC TGG AAA TGG ATG-3′ and 5′-GCT CTA GAC TGA ACT CAG CCA AGG AGC CAG AGT T-3′ which correspond to nucleotides 255 to 280 and 1011 to 1038, respectively, of FRβ. The forward and reverse primers for FRγ were 5′-GGG GGT ACC TGC CTC CAG GAA TA-3′ and 5′-GGG CTC GAA TTG TTG GAA GAA CC-3′. The FRα and FRβ primer sequences were obtained from the previously published article by Prasad *et al*. (*28*). The forward and reverse primers for RFC were 5′-CGC AGC TTA GGC ACA GTG TCA CCT TCG T-3′ and 5′-GCG TCT AGA CCG AGA GTC ACT GGT TCA CA-3′, respectively. The forward and reverse primers for PCFT were 5′-GCT CCG CCG CGC ACG CAC AT-3′ and 5′-TCA GGG GCT CTG GGG AAA CTG C-3′, respectively (Genbank accession number NP_542400). The forward and reverse primers for GAPDH were 5′-GAA GGT GAA GGT CGG AGT-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′ which correspond to nucleotides 6–24 and 231–212, respectively (Genbank accession number NM 002046). PCR conditions were as follows: denaturation (94°C, 45 s), annealing (60°C, 45 s) and extension (72°C, 45 s) for 35 amplification cycles, followed by a final extension of 72°C for 10 min. The product was separated by gel electrophoresis using 1% agarose gel and visualized by chemiluminescence.

Immunocytochemistry studies—In order to establish the absence of known FRs in MOLT4 cells, immunocytochemistry studies were done with FRs antibody. HeLa cells, which over express FR, were taken as control. Briefly, MOLT4 and HeLa cells were grown in 35 mm disc. Cells were washed with low pH PBS buffer to remove the FA which may be attached to FR. In PBS, 4% paraformaldehyde was added and incubated for 20 min at room temperature to fix the cells. Cells were washed two times with PBS followed by 5% fetal bovine serum in PBS was added for blocking non specific binding and incubated for 1 h at 37°C. Cells were again washed two times with PBS. Anti FR antibody (1 : 50) which recognizes all the three types of receptors was added to the cells and incubated at 4°C overnight. Then cells were washed with PBS and FITC-conjugated secondary antibody (1 : 200) was added and incubated for 2 h at 37°C. Cells were washed with PBS and visualized by fluorescence microscope (Nikon Eclipse Ti, Nikon Japan).

Statistical analysis—All experiments were conducted at least in triplicates and results are expressed as mean ± SEM unless otherwise specified. Michaelis–Menten parameters K_m and V_{max} were expressed as mean ± SEM. Unpaired students' *t*-test was applied to calculate statistical significance. A difference between mean values was considered significant if $P < 0.05$. The inhibition constant Ki was calculated by using the Dixon plot.

RESULTS

Uptake of MTX and FA at different incubation time—Uptake of MTX and FA into MOLT4, K562, Hut78 and normal human T cells (Fig. 1A and B) was measured at pH 7.4 in cells that had been cultured in normal RPMI 1640 medium containing 2.2 μM FA and in cells grown in folate free RPMI 1640 medium. In MOLT4 cells, uptake of MTX was linear for the first 20 min of incubation in both normal and folate free RPMI 1640 medium (Fig. 1A), thus showing the initial rate of uptake. But in the case of FA, there was little change in the uptake at different incubation time periods (Fig. 1B). The initial rate of uptake of MTX and FA at pH 7.4 in cells grown in folate free RPMI 1640 medium was not significantly different from that of cells grown in normal RPMI 1640 medium. The initial and total uptake of MTX (V_{max} = 0.2077) was much more compared to that of FA (V_{max} = 0.0175) in MOLT4 cells (Fig.1C). In K562 cells (Fig. 1D) and Hut78 cells (Fig. 1E), uptake of MTX and FA was linear for the first 20 min and uptake of MTX was more compared with that of FA uptake. This result was same as that of MOLT4 cells. But in K562 cells, total uptake of MTX and FA is double than that of MOLT4 cells. Uptake of MTX and FA in Hut78 cells was more compared to K562 and MOLT4 cells. In normal human T cells, the MTX and FA uptake was linear for the first 20 min as in the case of MOLT4, K562 and Hut78 cells (Fig. 1F). The uptake of MTX and FA was same for the first 20 min and then the FA uptake saturates. The total MTX uptake was approximately same as that of MOLT4 but the FA uptake was more than that of MOLT4 cells.

Uptake of MTX and FA at different substrate concentration in MOLT4 cells—The initial velocity of uptake of MTX and FA by MOLT4 cells with increasing radio labelled MTX and FA concentrations was measured after 30 min of incubation with the labelled substrate. Uptake of MTX at pH 7.4 into MOLT4 exhibited component, which is saturated, having maximum transport at 30 pmol and decreased at higher concentration of

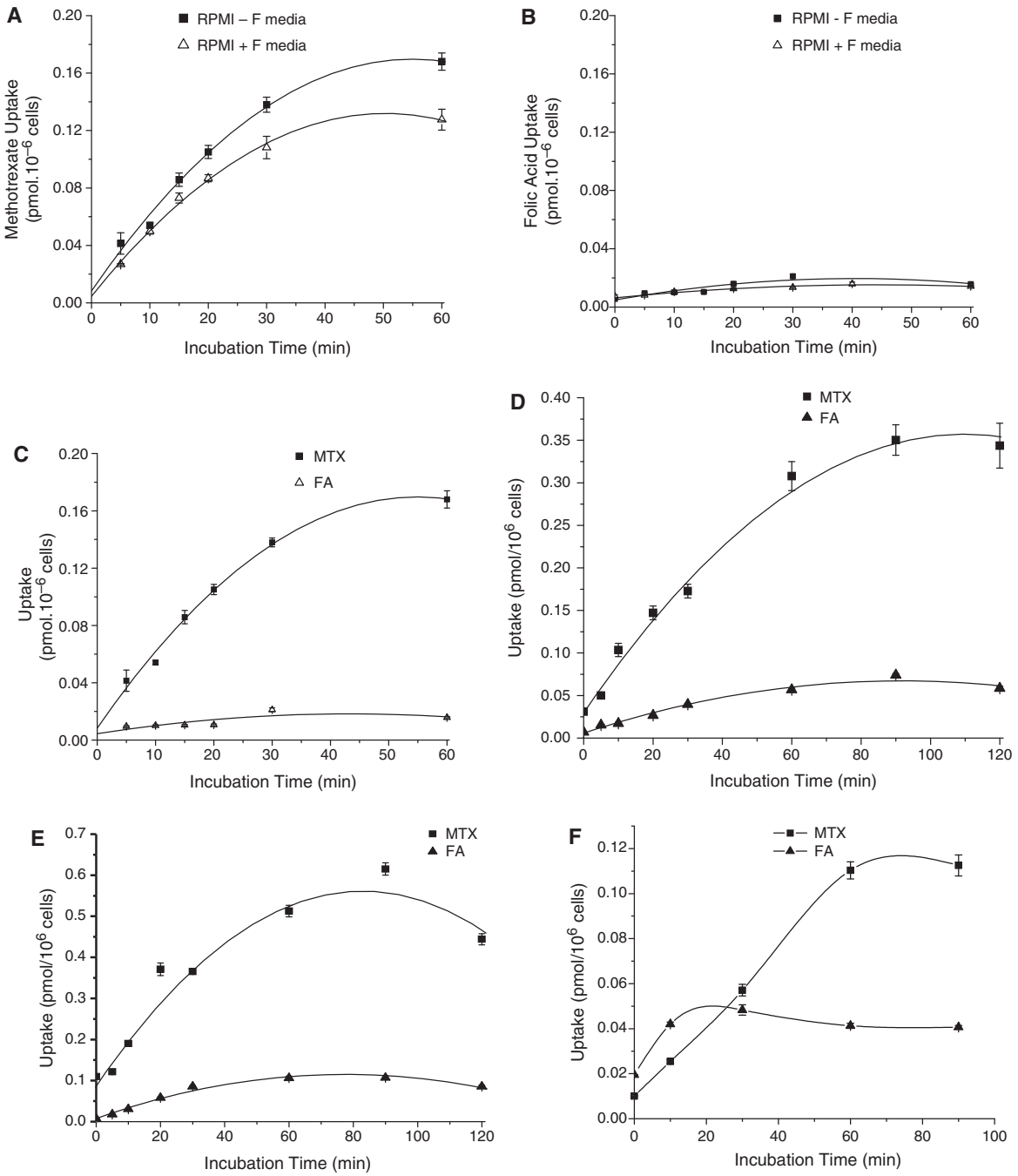

Fig. 1. **Time course of uptake of MTX and FA.** (A) Transport of MTX into MOLT4 cells versus time. Uptake of 10 pmol [^{3}H] MTX was determined, at the times indicated, as described under materials and methods. Closed squares represent uptake of MTX in cells grown in folate free RPMI 1640 media for three passages. Open triangles represent uptake of MTX in cells grown in normal RPMI 1640 media which contains 2.2 μM folate. Results are expressed as mean ± SEM (error bars) for three independent experiments. (B) Transport of FA into MOLT4 cells versus time. Uptake of 10 pmol [^{3}H] FA was determined, at the times indicated, as described under materials and methods. Closed squares represent uptake of FA in cells grown in folate free RPMI 1640 media for three passages. Open triangles represent uptake of FA in cells grown in normal RPMI 1640 media which contains 2.2 μM folate. Results are mean ± SEM (error bars) for three separate experiments. (C) Comparison of MTX and FA uptake into MOLT4 cells grown in folate free RPMI 1640 media versus time. Uptake of 10 pmol of [^{3}H] MTX and 10 pmol of [^{3}H] FA was determined, at the times indicated. Closed squares represent uptake of MTX and open triangles represent uptake of FA. (D) Transport of MTX and FA into K562 cells versus time. Uptake of 10 pmol [^{3}H] MTX and [^{3}H] FA was determined at different incubation time. Closed squares represent uptake of MTX and closed triangles represent uptake of FA. (E) Transport of MTX and FA into Hut78 cells versus time. Uptake of 10 pmol [^{3}H] MTX and [^{3}H] FA was determined at different incubation time. Closed squares represent uptake of MTX and closed triangles represent uptake of FA. (F) Transport of MTX and FA into normal human T cells versus time Uptake of 10 pmol [^{3}H] MTX and [^{3}H] FA was determined at different incubation time. Closed squares represent uptake of MTX and closed triangles represent uptake of FA. All the results are mean ± SEM (error bars) for three separate experiments.

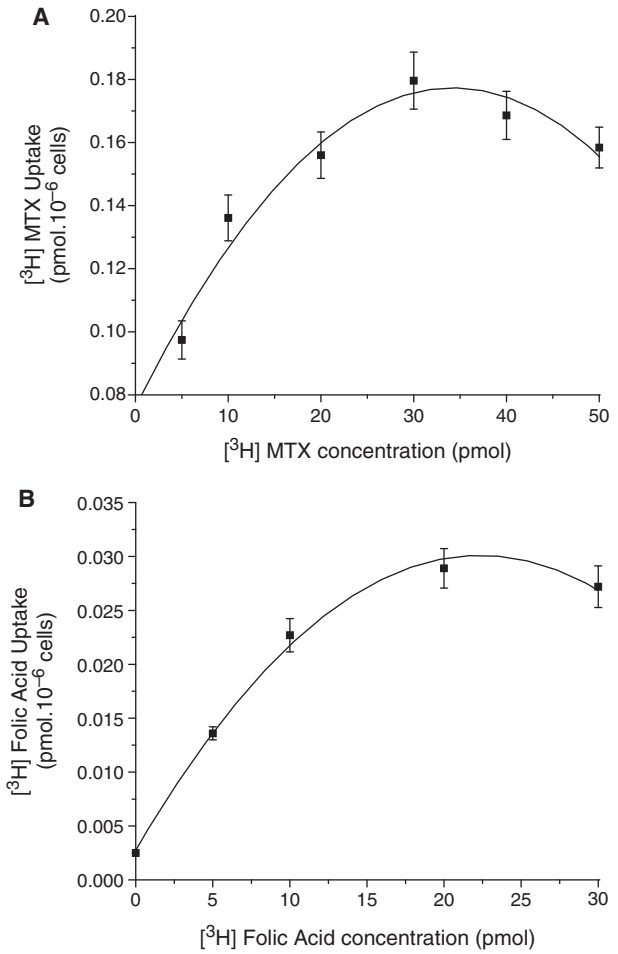

Fig. 2. **Concentration dependent uptake of MTX and FA.** (A) Concentration dependence of MTX transport into MOLT4 cells. Uptake of MTX was determined at 30 min of incubation in medium containing the indicated concentrations of labelled MTX. Results are mean ± SEM (error bars) for three separate experiments. (B) Concentration dependence of FA transport into MOLT4 cells. Uptake of FA was determined at 30 min of incubation in medium containing the indicated concentrations of labelled FA. Results are mean ± SEM (error bars) for three separate experiments.

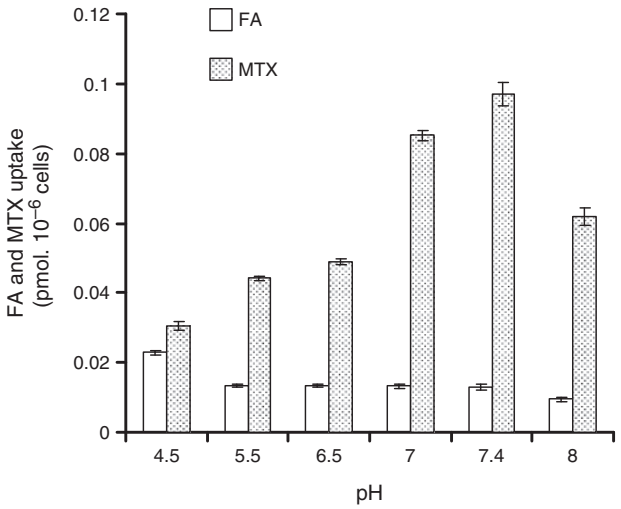

Fig. 3. **pH-dependent uptake profile in MOLT4 cells.** Uptake of MTX and FA into MOLT4 cells at different pH. MOLT4 cells grown in normal RPMI 1640 media were incubated in uptake medium at the indicated pH for 30 min with 10 pmol [^{3}H] MTX and 10 pmol [^{3}H] FA. Results are mean ± SEM (error bars) for three separate experiments.

labelled MTX in both normal and folate free medium (Fig. 2A). Figure 2B shows the uptake of FA into MOLT4 cells at different concentrations of labelled FA in normal RPMI 1640 medium. Uptake of FA increased as the concentration of FA increased upto 20 pmol and then decreased linearly.

Uptake of MTX and FA at different incubation buffer pH—Uptake of MTX and FA into MOLT4 cells was found to be pH dependent (Fig. 3). It can be seen that there was a minimum uptake of MTX at pH 4.5 and then uptake increased as pH increased up to pH 7.4. Maximum uptake of MTX was seen at pH 7.4 and then uptake decreased as pH increased to alkaline. In contrast to MTX, maximum FA uptake was observed at acidic pH 4.5 and it decreased gradually as pH increased. Minimum uptake of FA was at physiological pH and then it increased marginally as pH increased to 8.0.

Effect of structural analogue on MTX and FA transport in MOLT4 cells—To investigate the substrate specificity of the uptake of MTX and FA, we studied the effects of structural analogue *i.e.* unlabelled FA for [^{3}H] MTX and unlabelled MTX for [^{3}H] FA in MOLT4, K562, Hut78 and normal human T cells. The unlabelled competitor was simultaneously incubated with the respective radio labelled MTX and FA (10 pmol). Unlabelled FA and unlabelled MTX at a concentration of 1–100 μM were used in these studies. It was observed that there was a little inhibition in the uptake of [^{3}H] MTX into MOLT4 cells by the structural analogue FA even at concentration of 50 μM (K_i = 72) (Fig. 4A). Similarly, transport of [^{3}H] FA into MOLT4 cells was not significantly inhibited by the structural analogue MTX even at a concentration of 100 μM (K_i = 70) (Fig. 4B). These analogues could not cause a concentration-dependent inhibition of uptake. The insets in Fig. 4A and B show the Dixon plot of the data. Assuming that the observed inhibition is competitive, these plots may be used to calculate an apparent inhibition constant, K_i. This value is determined with the plot of $1/V_i$ versus concentration of inhibitor. Identical results were observed in K562 cells (Fig. 4C) and normal human T cells as MTX and FA uptake was not inhibited by their structural analogues (Table 4). In case of Hut78 cells, [^{3}H] FA uptake was not inhibited by unlabelled MTX but there was a little inhibition of [^{3}H] MTX uptake by unlabelled FA (Fig. 4D).

Effect of sulphate and phosphate ions on MTX and FA uptake into MOLT4 cells—Anions help in the transport of many compounds into cell by means of an anion-dependent transporter. To investigate the effect of structurally unrelated anions on the transport of MTX and FA, we studied the uptake of [^{3}H] MTX and [^{3}H] FA in presence of different concentrations of sulphate and phosphate ions. Uptake of both MTX and FA decreased

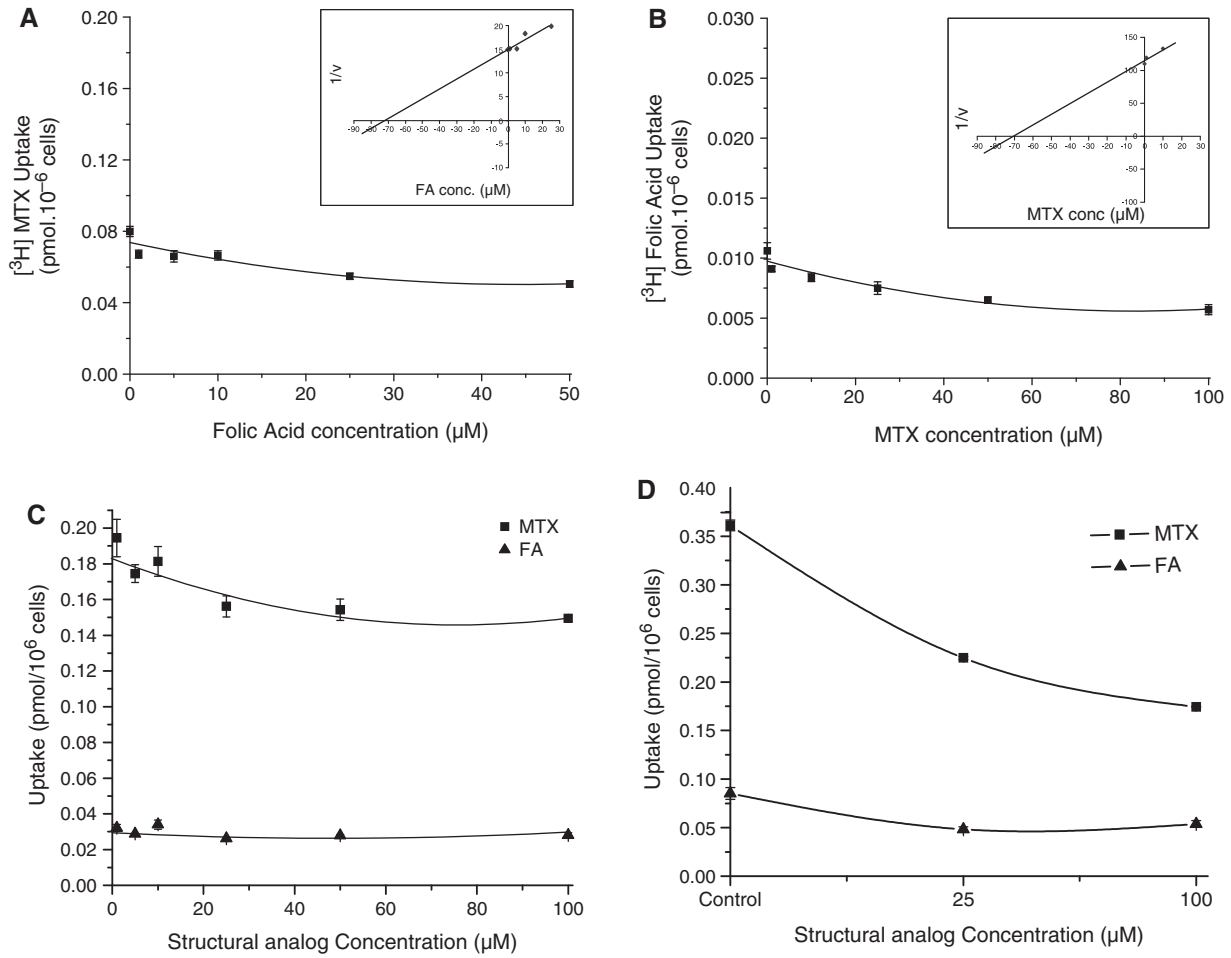

Fig. 4. **Effects of structural analogue on the uptake of FA and MTX.** (A) Effect of structural analogue FA on MTX transport into MOLT4 cells grown in normal RPMI 1640 media. Uptake of 10 pmol of [^{3}H] MTX was determined at 30 min of incubation at pH 7.4 with increasing concentrations of the structural analogue FA. Results are mean ± SEM (error bars) for three separate experiments. (B) Effect of structural analogue MTX on FA transport into MOLT4 cells grown in normal RPMI 1640 media. Uptake of 10 pmol of [^{3}H] FA was determined at 30 min of incubation at pH 7.4 with increasing concentrations of the structural analogue MTX. Results are mean ± SEM (error bars) for three separate experiments. (C) Effect of FA on the uptake of [^{3}H] MTX and MTX on the uptake of [^{3}H] FA into K562 cells grown in normal RPMI 1640 media. Uptake of 10 pmol of [^{3}H] MTX and 10 pmol of [^{3}H] FA was determined at 30 min of incubation at pH 7.4 with increasing concentrations of the structural analogue. Results are mean ± SEM (error bars) for three separate experiments. (D) Effect of FA on the uptake of [^{3}H] MTX and MTX on the uptake of [^{3}H] FA into Hut78 cells grown in normal RPMI 1640 media. Uptake of 10 pmol of [^{3}H] MTX and 10 pmol of [^{3}H] FA was determined at 30 min of incubation at pH 7.4 with increasing concentrations of the structural analogue. Results are mean ± SEM (error bars) for three separate experiments.

at an anion concentration of 5 mM and then saturated at an anion concentration of 10 mM. Inhibition of MTX uptake is found to be the same by both sulphate and phosphate ions (Fig. 5A) whereas inhibition of FA uptake is more by sulphate ion as compared with phosphate ion (Fig. 5B).

Effect of energy inhibitors on MTX and FA transport into MOLT4 cells—The effect of energy inhibitors on transport of FA and MTX in MOLT4 cells grown in normal RPMI 1640 medium is shown in Table 1. In control experiment, the MTX uptake was 0.102 pmol/10^6 cells. In the presence of 1 mM sodium azide, the MTX uptake increased slightly while in the presence of 10 mM sodium azide, MTX uptake increased up to 1.5-fold as compared with control experiment. Sodium arsenite at either 1 or 10 mM concentration had no significant effect on the MTX uptake in MOLT4 cells. Uptake of MTX in MOLT4 cells was not affected by 1 mM sodium cyanide, but at 10 mM concentration of sodium cyanide, MTX uptake increased to 2.5-fold as compared with control experiment. This result confirms that the MTX uptake increases, at higher concentration of energy inhibitors. In control experiment, FA uptake was found to be 0.0132 pmol/10^6 cells. FA uptake was decreased by 1 mM sodium azide and there was a further decrease in uptake by 10 mM of sodium azide. Uptake of FA decreased significantly at 1 mM as well as 10 mM sodium arsenite concentration. Sodium cyanide at 1 mM concentration inhibited the FA uptake, but higher degree of inhibition was observed at 10 mM

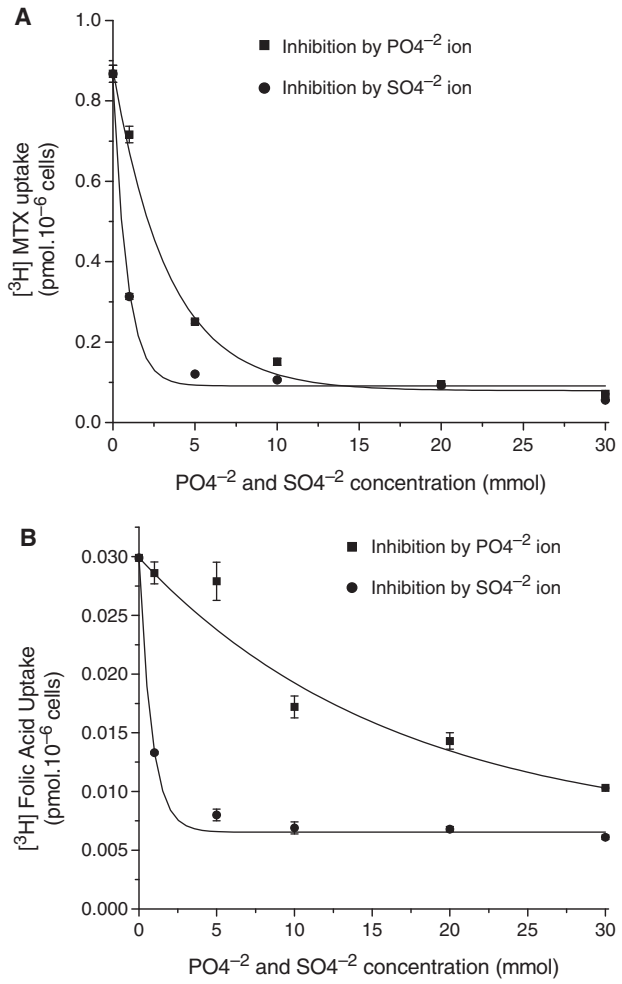

Fig. 5. **Effect of various anions on the uptake of FA and MTX.** Effects of sulphate and phosphate ions on (A) MTX or (B) FA uptake into MOLT4 cells grown in normal RPMI 1640 media. Cells were incubated for 30 min in medium of differing concentrations of the anions, in HEPES–sucrose–MgO buffer as described in material and methods. [^{3}H] MTX (10 pmol) or [^{3}H] FA (10 pmol) was added and uptake was determined after 30 min incubation. Results are mean ± SEM (error bars) for three separate experiments.

Table 1. **Effect of energy inhibitors on folic acid and MTX uptake into MOLT4 cells.**

Inhibitor	Folic acid uptake (pmol/30 min/10^6 cells)	MTX uptake (pmol/30 min/10^6 cells)
Control	0.0132 ± 0.00079	0.1021 ± 0.00412
NaN_3 (1 mM)	0.0110 ± 0.00122	0.1199 ± 0.00568
NaN_3 (10 mM)	0.0101 ± 0.00025	0.1588 ± 0.00621
$NaAsO_3$ (1 mM)	0.0091 ± 0.00037	0.1067 ± 0.004
$NaAsO_3$ (10 mM)	0.0090 ± 0.00015	0.0866 ± 0.00428
NaCN (1 mM)	0.0116 ± 0.00073	0.0889 ± 0.00128
NaCN (10 mM)	0.0089 ± 0.00035	0.2469 ± 0.00611

MOLT4 cells were pre-incubated for 1 h with the energy inhibitors prior to measuring uptake. [^{3}H] MTX or 10 pmol of [^{3}H] FA was added and uptake was determined after 30 min of incubation. Results are mean ± SEM for three separate experiments. Unpaired students' *t*-test was applied to calculate statistical significance and $P < 0.05$ was taken as statistical significance.

Table 2. **Effect of different inhibitors on folic acid and MTX uptake into MOLT4 cells.**

Inhibitor	Folic acid uptake (pmol/30 min/10^6 cells)	MTX uptake (pmol/30 min/10^6 cells)
Control	0.0130 ± 0.00084	0.1020 ± 0.00452
FR antibody (1 : 200)	0.0123 ± 0.00092	0.1019 ± 0.00834
FR antibody (1 : 1000)	0.0112 ± 0.00133	0.1016 ± 0.00645
Verapamil (50 μM)	0.0110 ± 0.00059	0.1737 ± 0.00644
Verapamil + Ab (1 : 200)	0.0120 ± 0.00061	0.1665 ± 0.0083
Verapamil + Ab (1 : 1000)	0.0110 ± 0.00117	0.1660 ± 0.00535
Mitomycin C (50 ng/ml)	0.0106 ± 0.00032	0.0968 ± 0.00323
Mitomycin C (100 ng/ml)	0.0113 ± 0.00044	0.1073 ± 0.00585

MOLT4 cells were pre-incubated for 1 h with the inhibitors prior to measuring the uptake. [^{3}H] MTX or 10 pmol of [^{3}H] FA was added and uptake was determined after 30 min of incubation. Results are mean ± SEM for three separate experiments. Unpaired students' *t*-test was applied to calculate statistical significance and $P < 0.05$ was taken as statistical significance.

Table 3. **MTX and FA uptake into MOLT4 cells at different sulphasalazine concentration in pH 7.4.**

	MTX uptake (pmol/10^6 cells)	FA uptake (pmol/10^6 cells)
Control	0.1019 ± 0.0089	0.0116 ± 0.0009
Sulphasalazine		
50 μM	0.0883 ± 0.0058	0.0115 ± 0.0006
100 μM	0.0492 ± 0.004	0.0107 ± 0.0005

Results are mean ± SEM for three separate experiments. Unpaired students' *t*-test was applied to calculate statistical significance. $P < 0.05$ was taken as statistical significance.

sodium cyanide concentration. Thus, FA uptake in MOLT4 cells is energy dependent since uptake decreases significantly at higher concentrations of energy inhibitors.

Effect of FR antibody, verapamil and mitomycin C and sulphasalazine on the uptake of FA and MTX—The effect of FR antibody, verapamil, verapamil in combination with FR antibody and mitomycin C on the uptake of FA and MTX, in MOLT4 cells grown in normal RPMI 1640 medium, is shown in Table 2. Uptake of FA was not affected in the presence of FR antibody at 1000 dilution or 200 dilutions. It was also not inhibited by verapamil, mitomycin C and verapamil in combination with FR antibody. Thus the efflux system, which was inhibited by verapamil had no effect on the uptake of FA in MOLT4 cells whereas the uptake of MTX increased in the presence of verapamil. FR antibody at 200 and 1000 dilutions and mitomycin at 50 and 100 ng/ml concentrations had no effect on the uptake of MTX. Sulphasalazine, which is an inhibitor of RFC, inhibits the MTX uptake into MOLT4 cells at pH7.4 but has no effect on the uptake of FA (Table 3). This proves that the uptake of MTX is by RFC and FA uptake is by PCFT which is insensitive to sulphasalazine at physiological pH.

Molecular evidence for the absence of FR and presence of RFC: RT-PCR analysis—The absence of the known FRs and presence of RFC and PCFT in MOLT4 cells at the mRNA level was determined by RT-PCR analysis.

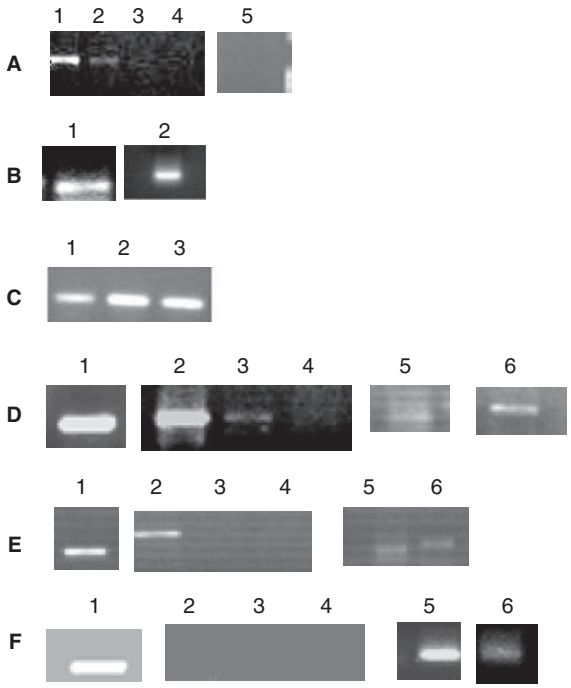

Fig. 6. **Molecular evidence for the presence or absence of FRs, RFC and PCFT in MOLT4, K562, Hut78 and normal human T cells at the mRNA level.** PCR product obtained using FRα, FRβ, RFC, PCFT and GAPDH specific primers and total RNA isolated from HeLa, KG1, MOLT4, K562, Hut78 and normal human T cells. PCR products were analysed by gel electrophoresis on 1% agarose gel. (A) Lane 1: HeLa FRα, lane 2: KG1 FRβ, lane 3: MOLT4 FRα, lane 4: MOLT4 FRβ, lane 5: FRγ. (B) Lane 1: MOLT4 RFC, lane 2: MOLT4 PCFT (C) Lanes 1–3: GAPDH of MOLT4, HeLa and KG1, respectively. (D) Lane 1: GAPDH, lane 2: Frα, lane 3: FRβ, lane 4: FRγ, lane 5: RFC, lane 6: PCFT PCR product of K562 cells. (E) Lane 1: GAPDH, lane 2: Frα, lane 3: FRβ, lane 4: FRγ, lane 5: RFC, lane 6: PCFT PCR product of Hut78 cells. (F) Lane 1: GAPDH, lane 2: Frα, lane 3: FRβ, lane 4: FRγ, lane 5: RFC, lane 6: PCFT PCR product of normal human T cells.

HeLa cells RNA yielded FRα PCR products and KG1 cells RNA yielded FRβ PCR products of expected size (~0.8 kb) with the FRα and FRβ specific primers. RNA from MOLT4 cells generate RFC and PCFT PCR products of expected size (~0.2 kb) (Fig. 6B) but failed to generate FRα, FRβ or FRγ PCR products (Fig. 6A). In control experiment with MOLT4 mRNA, GAPDH PCR product (188 bp) corresponding to the amplified GAPDH precursor mRNA by the GAPDH primer was observed (Fig. 6C). K562 cells express FRα, FRβ, RFC and PCFT (Fig. 6D). Hut78 cells express FRα, RFC and PCFT but do not express FRβ and FRγ (Fig. 6E). Normal human T cells do not express any known FRs but express RFC and PCFT same as that of MOLT4 cells (Fig. 6F).

Immunocytochemistry studies—There was no visible fluorescence observed in MOLT4 cells which were incubated with antibodies for FR, whereas HeLa cells which overexpress FR gave good fluorescence (Fig. 7). Immunocytochemistry result further proves the absence of known FR in MOLT4 cells.

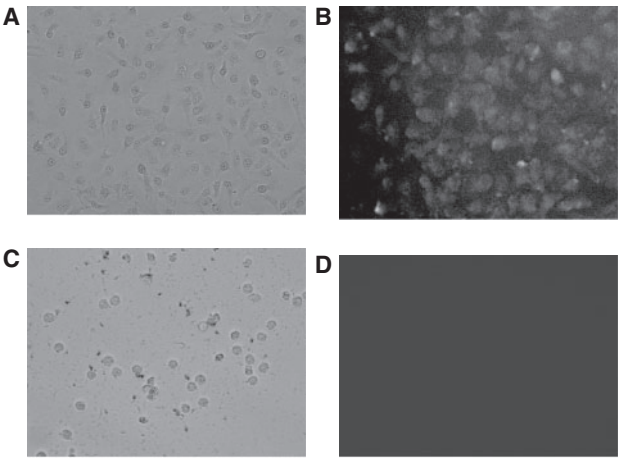

Fig. 7. **Immunofluorescence labelling of MOLT4 cells.** HeLa and MOLT4 cells were stained with FR antibody and FITC-conjugated anti rabbit IgG secondary antibody and fluorescence images were taken using fluorescence microscope as described in Material and methods section. (A) HeLa cells in normal view. (B) HeLa cells with fluorescence view. (C) MOLT4 cells in normal view. (D) MOLT4 cells with fluorescence view.

DISCUSSION

MTX has been used as a combinational drug therapy in RA and many leukemias. Our main aim was to investigate the presence of different transport systems for MTX and FA in human T leukemia cell lines. There are several reports on the transport of FA and MTX in the murine leukemia cells L1210 (*11*) and the human T-ALL CCRF-CEM cells (*24*), yet the transport mechanisms of FA and MTX in normal and leukemic human T cells are not fully understood. Studies on the transport of FA and MTX in MOLT4 and normal T cells are necessary as they will throw light on the molecular mechanism of MTX and FA transport in RA and leukemia patients.

Uptake of MTX into MOLT4 cells was found to be linear upto 20 min of incubation and hardly any change was observed in the transport of FA with different time in both normal and folate free media. It appears that uptake of MTX and FA in MOLT4 cells is different from PC-3 and intestinal cells but same as 5-methyltetrahydrofolate transport into PC-3 cells (*22*, *29*, *30*). Uptake of MTX was much higher as compared with the uptake of FA in MOLT4, K562, Hut78 and normal human T cells, indicating the presence of either two different transport systems or one transport system with variable affinity for these compounds. The total uptake of MTX in K562 and Hut 78 cells is more than that of MOLT4 cells. This may be due to the presence of FRs in these cells whereas in MOLT4 cells MTX uptake is only by carrier mediated pathway. Uptake kinetics of FA and MTX at different substrate concentrations also shows the presence of two different transport systems. Many transport systems are inhibited by structural analogues as shown by the inhibition of MTX uptake by FA, 5-methyl tetrahydrofolate and 5-formyltetrahydrofolate in PC-3 cells (*30*). But in

Table 4. **MTX and FA uptake into normal human T cells in presence of structural analogue.**

	MTX uptake (pmol/10^6 cells)	FA uptake (pmol/10^6 cells)
Control	0.0571 ± 0.0026	0.0483 ± 0.0023
FA (100 μM)	0.0520 ± 0.002	–
MTX (100 μM)	–	0.0466 ± 0.0017

Results are mean ± SEM for three separate experiments. Unpaired students' *t*-test was applied to calculate statistical significance. $P < 0.05$ was taken as statistical significance.

MOLT4, K562 and normal T cells, uptake of MTX and FA was not inhibited significantly by their analogue. This result further proves the existence of two different transport systems for FA and MTX in MOLT4, K562 and normal human T cells.

The pH profile for uptake of FA was totally different from that of MTX in MOLT4 cells (Fig. 3). In MOLT4 cells, maximum uptake of FA was at low pH, which resembled the uptake of folate in the intestine and 5-methyltetrahydrofolate in liver (*31*, *32*). MTX uptake in MOLT4 cells showed a sharp optimum at physiological pH, similar to the pH profile of isolated hepatocytes (*32*). Uptake of MTX in MOLT4 cells decreased as pH decreased to 4.5, which is same as seen in L1210 leukemia cells (*33*, *34*). This suggests that the transport of FA in MOLT4 cells may be mediated by a low pH transport system which has been recently identified as PCFT that transports FA in human embryonic kidney 293 cells and human small intestine (*12*, *35*, *36*). These data suggest that there may be two different transport systems for FA and MTX in MOLT4; one may function optimally at low pH and the other at physiological pH. It has been described earlier that MTX transport into murine leukemia cells L1210 is mediated by an anion-exchange mechanism and therefore, chloride and other anions inhibit MTX uptake (*37–39*). In MOLT4 cells uptake of MTX and FA was inhibited significantly by sulphate and phosphate anions, showing that their transport may involve anion exchange mechanism. Similar results were also reported for MTX uptake in L1210 cells by Sharina *et al*. (*40*).

MTX uptake into MOLT4 cells was not inhibited by metabolic energy inhibitors (Table 1). However; it was stimulated by high concentrations of sodium azide and sodium cyanide. Similar results were found in the uptake of 5-methyltetrahydrofolate in hepatocytes, and MTX in L1210 cells (*41*, *42*). It was also shown that the efflux of MTX in L1210 cells is energy dependent and energy inhibitors inhibit the MTX efflux (*43*). In the present study, stimulation of MTX uptake in MOLT4 cells by sodium azide and sodium cyanide may be due to the inhibition of efflux route rather than the influx one. Earlier studies in hepatocytes and L1210 cells also showed that the influx system of the folate is inhibited to a lesser degree than the efflux route (*41*, *42*). Uptake of FA into MOLT4 cell was inhibited by metabolic energy inhibitors similar to the inhibition of 5-methyltetrahydrofolate and MTX uptake in PC-3 cells by energy inhibitors (*22*, *30*). These results clearly indicate the existence of two different transport systems for the uptake of MTX and FA in MOLT4.

Uptake of FA and MTX in MOLT4 cells was not inhibited by antibodies that were raised against FR, further ruling out the involvement of receptor mediated uptake. Uptake of MTX increased in presence of verapamil, which may be due to the inhibition of efflux pump. In MOLT4 cells, uptake of MTX in presence of sulphasalazine proves that its uptake is by RFC and different from that of FA uptake pathway. RT-PCR and immunofluorescence studies showed the absence of FRs and the presence of RFC as well as PCFT in MOLT4 cells. The expression pattern of FRs, RFC and PCFT in normal human T cells is same as that of MOLT4 cells. In the case of K562 and Hut78 cells, the total uptake of FA and MTX was more than that in both normal T cells and MOLT4, which may be due to the presence of FRs as well as the folate carriers (Fig. 6). These results altogether support the existence of two different carrier-mediated transport systems for the uptake of FA and MTX in MOLT4 cells and normal T cells. FA may be transported by the low pH transporter, PCFT and MTX uptake might be through the RFC which works optimally at physiological pH.

In conclusion, our results suggest that transport of FA and MTX into human T leukemia cells (MOLT4) and normal human T cells is mediated by two different and independent carrier-mediated transport systems. FA transporter in MOLT4 cells is an energy and anion dependent component which is not affected by its structural analogue, whereas uptake of MTX into MOLT4 cells is by a carrier-mediated transport system which is independent of metabolic energy source and is anion dependent. Absence of FR and presence of RFC and PCFT as suggested by RT-PCR analysis and immunofluorescence studies confirm the presence of carrier mediated transport system for FA and MTX transport in MOLT4 and normal human T cells. This information can be used to evaluate and design the strategy to be adopted when MTX is used in the treatment of diseases like cancer and RA. This study will also help to formulate the optimum dose of MTX that may be toxic or sub-toxic to T cells in leukemic patients and other cancers.

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CONFLICT OF INTEREST

None declared.

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