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HETEROGENEITY OF METHOTREXATE BINDING IN HUMAN COLON TUMOR CELLS

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ABSTRACT

[BH]-methotrexate binding at pH 5.0 and pH 7.2 by the cytosol of tumor tissues and the surrounding normal areas of the gastrointestinal tract of patients suffering from colon or gastric cancer has been used to identify In these cells the presence of a binder of methotrexate having low-affinity for this drug in addition to the enzyme dihydrofolate reductase. Scatchard analysis of the [3H]- methotrexate binding data by a colon tumor sample also reveals that there are two binders of this drug present In the cytosol of these cells. The association constant (Kass) for one binder of methotrexate is = 5.6 x 10 M1 while the Kass for the second binder is = 1.0 x 106 M1. The two binders do not differ very much in their apparent molecular weight. Upon isoelectric focusing, the tumor cell cytosol resolves into 4 major isoproteins each having the ability not only to bind [H]- methotrexate but also reduce [H]- pteroylglutamic acid to H]- tetrahydropteroylglutamic acid. This suggests that the two binders of methotrexate may be the two forms of dihydrofolate reductase having different affinities for this anticancer drug(JPMA 41:136, 1991).

INTRODUCTION

Dihydrofolate reductase (DHFE., EC. 1.5.1.3) plays a central role in the pathway of pyrimidine and purine biosynthesis and, therefore, is a target enzyme in cancer chemotherapy. The anticancer drug methotrexate (MTX) produces cytocidal effect by binding to DHFR and blocking its action. There have been quite a few reports on the heterogeneity of the enzyme in terms of its binding to MDC in human as well as murine leukemia cells[1-7] and mammalian liver[8,9]. Colorectal carcinoma is one of the most common solid tumors in adults[10]. Since MTX as a single agent has never been considered to be of much value in the management of advanced colorectal carcinoma, we attempted to find out whether these cells contain another binder of MTX having low affinity for this drug and, therefore, would not respond to MDC therapy.

MATERIALS AND METHODS

[^{3}H] MTX with a specific activity 11.8 Ci/mmole and [^{3}H] pteroylglutamic acid ([^{3}H]PGA) with a specific activity 34 Ci/mmole were purchased from Amersham/Searle. [^{3} H]MTX was purified by chromatography on a Bio Gel P-30 column. NADPH, pteroylglutamic acid (PGA), MDC, pH ampholytes (Ampholines), phenylmethylsulfonylfluoride (PMSF), Norit A Neutral charcoal, dextran (molecular weight 10,000), bovine serum albumin, dextran blue, cytochrome C, horse raddish peroxidase, ethanolamine were purchased from Sigma Chemical Company. Trasylol was obtained from Mobay (New York). Preparation of tissue extracts Human tumor tissues (colon cancer) and the normal tissues (surrounding the tumor), excised at the time of surgery were homogenized as described previously[11], but with a slight modification that the homogenates were prepared using 3 ml of 0.6 M sodium citrate buffer, pH 7.2, for everygm of tissue. Since these tissue cells contain lysosomal

enzymes, trasylol (1000 KIU/1), PMSF (3.5 mg/i) and 0.02% sodium azide were included in the buffer used for making homogenates. These homogenates were then centrifuged at 105,000 x g for 1 hr and the cytosol obtained was analyzed for [3H]MTX binding at pH 5.0 and pH 7.2.

[^{3}H]MTX binding studies

Binding of [^{3}HJMTX by the tissue extracts was carried out by a procedure as described by Rothenberg et al[12]. A total reaction volume of 0.5 ml in 0.06 M citrate, pH 4.8 or pH 7.4, containing 48 uM NADPH, 5.8 mM 2-mercaptoethanol, 2.2 nM [^{3}H]MTX, 0-22 nM unlabelled MDC and 50 ul cell cytosol were incubated at room temperature for 30 minutes. The reactions were then stopped by the addition of 0.4 ml of one percent Norit. A neutral charcoal in 0.5 percent dextran (molecular weight 10,000). After centrifugation, radioactivity in 0.5 ml of supernatant solution representing the enzyme bound [^{3}H]MTX was counted in an LS-3801 Spectrometer (Beckman Instruments, Palo Alto, CA) using 5 ml of 3a70 scintillation fluor (Research Product International, USA). A blank containing all constituents of the reaction mixture except the cytosol was run with each assay series to determine the radioactivity not removed by charcoal in the absence of the cytosol preparation. This radioactivity was subtracted from the experimental samples to determine the net counts per minute of bound [^{3}H]MTX. Preliminary experiments indicated that the coefficient of variation for separating bound and free MDC by dextran-coated charcoal was between 2.1% and 3.8% when the binding activity was greater than 5% of the total MDC.

Gel Column Chromatography

Sephadex G-75 was equilibrated with 0.05 M Tris HCI buffer, pH 7.4, containing 0.02% sodium azide and packed in a column of size 0.7 cm x 50 cm. Half ml of colon tumor cytosol was applied to the column and eluted at a flow rate of 4.8 mI/hr. Fractions in a volume of 0.5 ml were collected and assayed for [3H]MDC binding at pH 7.2. Dextran blue, pure goat liver DHFR (Mr 20,000), horse raddish peroxidase (Mr 40,000), cytochrome C (Mr 12,400) and3H2O were used as column markers.

Isoelectric Focusing

A colon tumor cytosol sample was analyzed by isoelectric focusing in a standard LKB 1 10-mI column packed with 1% (W/V) ampholytes at a pH range of 3.5 10 in a zero to 50% sucrose gradient. The sample (1 ml) was dialyzed against distilled water for 4 hrs to remove electrolytes prior to application to the column. The dialyzed sample and the 75% of total ampholytes used were added to the solution which was adjusted to contain 50% sucrose (“dense solution”). The remaining ampholytes (25%) were mixed with “light solution” comprising of 5% sucrose. The electrode solution for the cathode (25 ml) was 0.262 M. ethanolantine in 60% sucrose. The electrolyte solution for the anode (20 ml) was 0.173 M H3P04 prepared in distilled water. A fraction of this solution, enough to completely cover the respective electrode was placed on top of the column. The column was run for 20 hrs at a maximum power of 5W with the maximum voltage set at 1.6 KV. Two mgs. of pure hemoglobin was added alongwith the sample as a marker for focusing. The column was eluted at a constant flow rate of 100 mI/hr and 0.9 ml fractions were collected. Every other fraction was dialyzed against 0.025 M potassium phosphate buffer, pH 7.4 containing 0.025 M potassium chloride for 20 hrs to remove Ampholines and then analyzed for [^{3}H]MTX binding12 and for the reduction of [^{3}H]PGA to [3H]-tetrahydro-PGAB.

RESULTS AND DISCUSSION

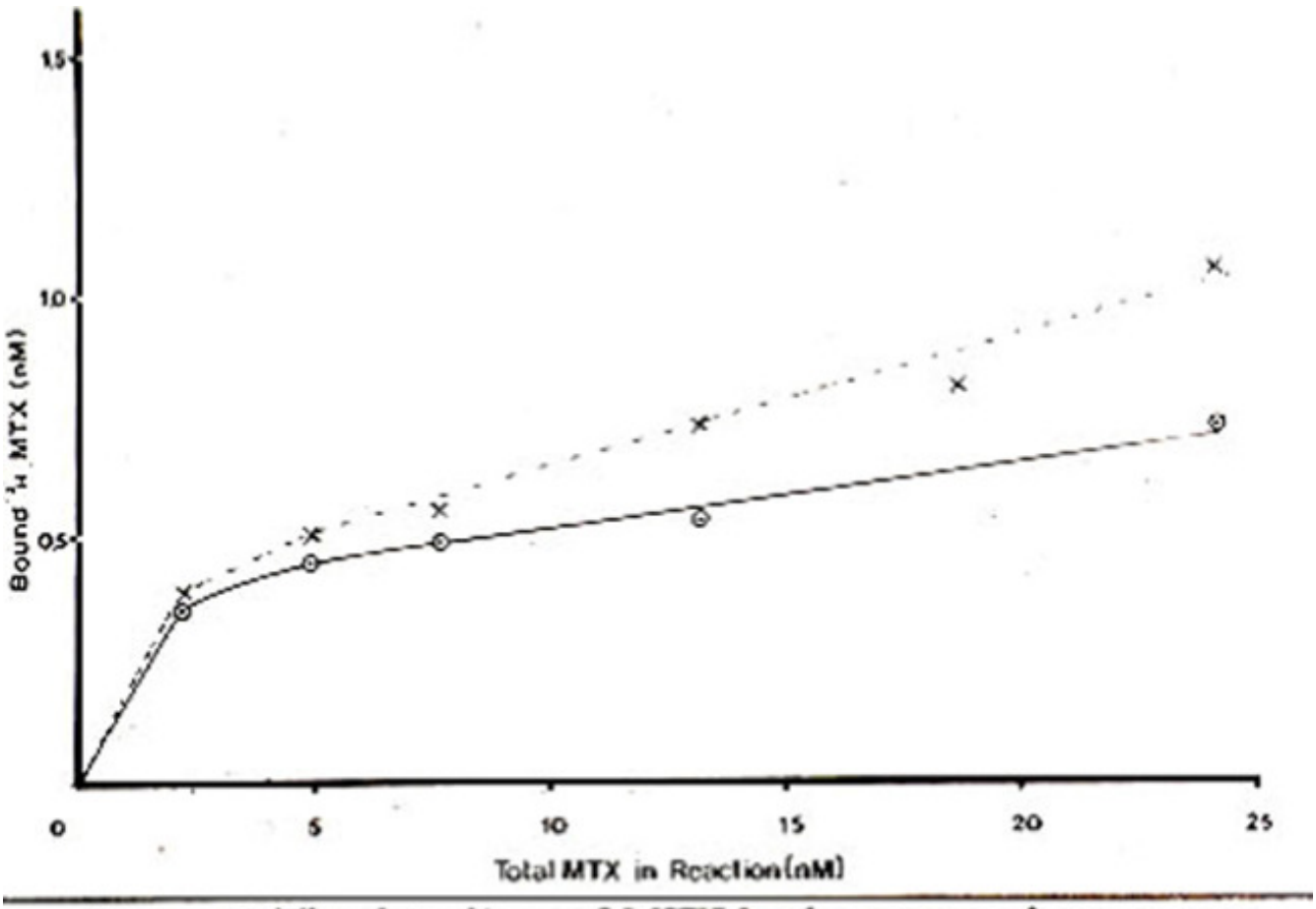

Figure 1.The binding of MTX by human colon tumor cytosol at pH 5.0 and pH 7.2. The total amount of MTX bound was obtained by multiplying the total MTX concentration in the reaction by the percent of [^{3}H]MTX bound. For details of the procedure see the Materials and Methods section.

Figure 1 shows the binding of [^{3}H]MTX at pH 5.0 and pH 7.2 by the colon tumor cytosol. At pH 5.0, the maximum concentration of [^{3}H]MTX bound was 0.73 nM as against 1.06 nM at pH 7.2 when the total MDC concentration in the reaction was 24.2 nM. There was nearly 45% increase in the [^{3}H]MTX binding at pH 7.2. Since DHFR has been shown to have a higher affinity for MDC at acidic pH and a lower affinity for the inhibitor at pH 7.2[14], increased MDC bindingat pH 7.2 as compared to at pH 5.0 suggests that in addition to DHFR there is an additional species of binding sites participating in the reaction. Had there been only one form of the binder of this drug in these cells, the binding of [^{3}H]MTX at saturating concentration of total MDC in the reaction would have been the same at pH 5.0 and pH 7.2. A number of tumor tissues of gastrointestinal tract (GIT) and the surrounding normal areas of GIT were analyzed for [^{3}H]MTX binding at pH 5.0 and pH 7.2. As shown in Table,

TABLE. [^{3}H]MTX binding by various normal and cancer tissue extracts (cytosol).

	Patient	Tissue	pgBound/mg protein at:	
			pH 5.0	pH 7.2
1.	S.J.	Normal colon	12	19
		Colon tumor	6	10
2.	Q.K.	Normal stomach	29	59
		Gastric tumor	79	87
3.	S.S.	Normal	26	56
		Adenocarcinoma	46	51
4.	A.A.R.	Normal caecum	50	55
		Caecal tumor	74	64
5.	M.A.	Normal	11	27
		Caecal tumor	21	21
6.	S.A.	Rectal tumor	61	52
7.	M.J.S.	Normal	31	65
		Gastric tumor	19	57
8.	P.D.	Normal	36	45
		Caecal tumor	35	46
9.	F.K.	Normal	87	127
		Colon tumor	164	195
10.	S.	Normal	11	20
		Rectal tumor	24	29
11.	O.H.K.	Normal	16	24
		Caecal tumor	19	36
12.	M.I.S.	Normal	19	27
		Colon tumor	34	42
13.	G.Z.A.	Normal	21	30
		Colon tumor	38	54
14.	A.H.B.	Normal	13	20
		Colon tumor	9	22

it is evident that in most of the tissues examined, there is more binding of [^{3}H]MTX at pFI 7.2 as compared to pH 5.0. The difference in the total binding of [^{3}H]MTX at the of drug bound to by one of the colon tumor cytosol samples at increasing concentrations of the unlabelled MDC in the reactions.

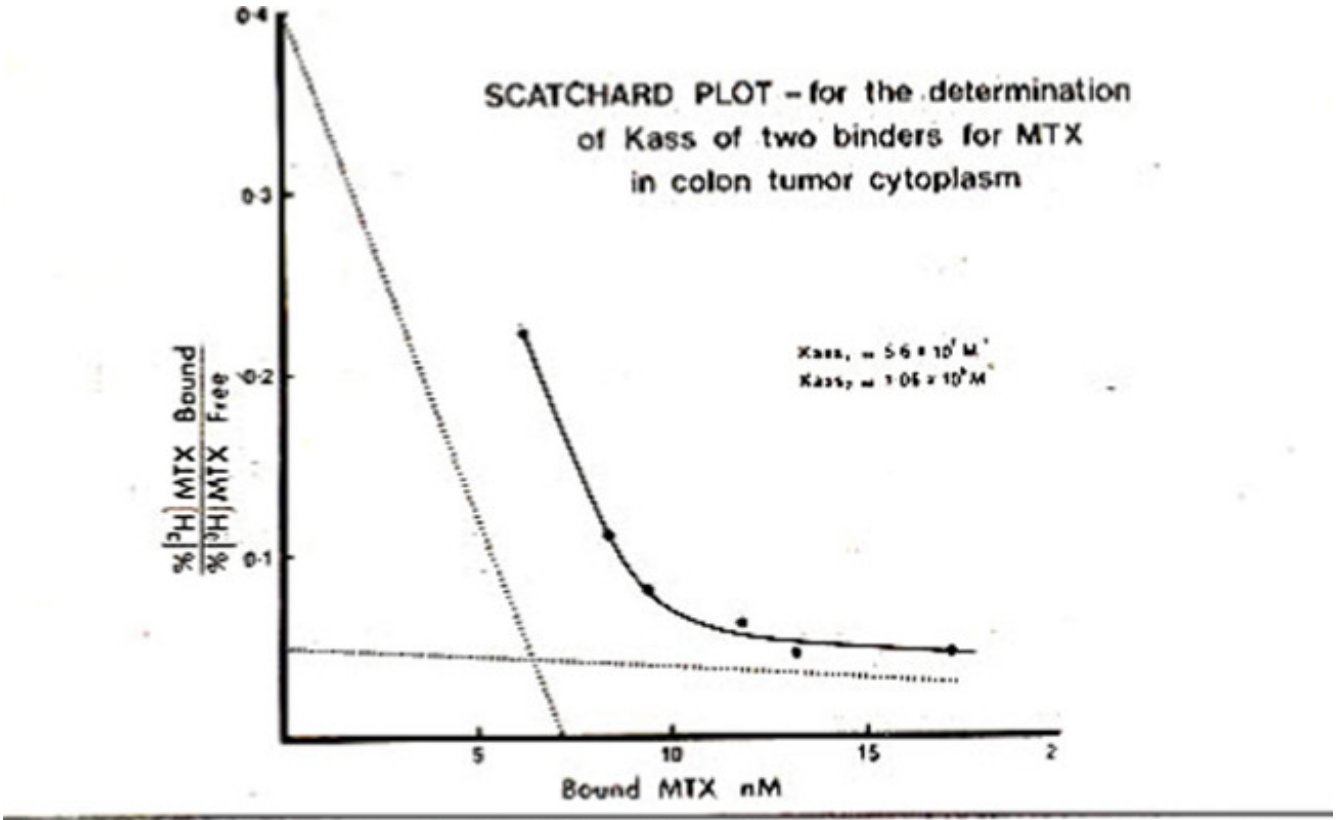

Figure 2.Scatchard plot for the determination of Kass (association constant) for the two binders of MTX in a sample of colon tumor cytosol. [^{3}H]MTX binding at 7.2 by this sample was carried out according to the procedure described in the Materials and Methods section.

Figure 2 shows that one of the binders of MDC had an association constant (Kass) = 5.6x 107 M-1, while the other one had a Kass = 1.06x 106 M-1. These values are close to the two Kass values reported for the two binders of MTX in human leukemia cells7. We could not separate the two binders of MTX by gel-filtration chromatography because a single peak of [^{3}H]MTX binding vas obtained when colon tumor cytosol was applied to Sephadex G-75 column suggesting that the two binders may not be differing much in their apparent molecular weight which appears to around 20,000 (Figure 3).

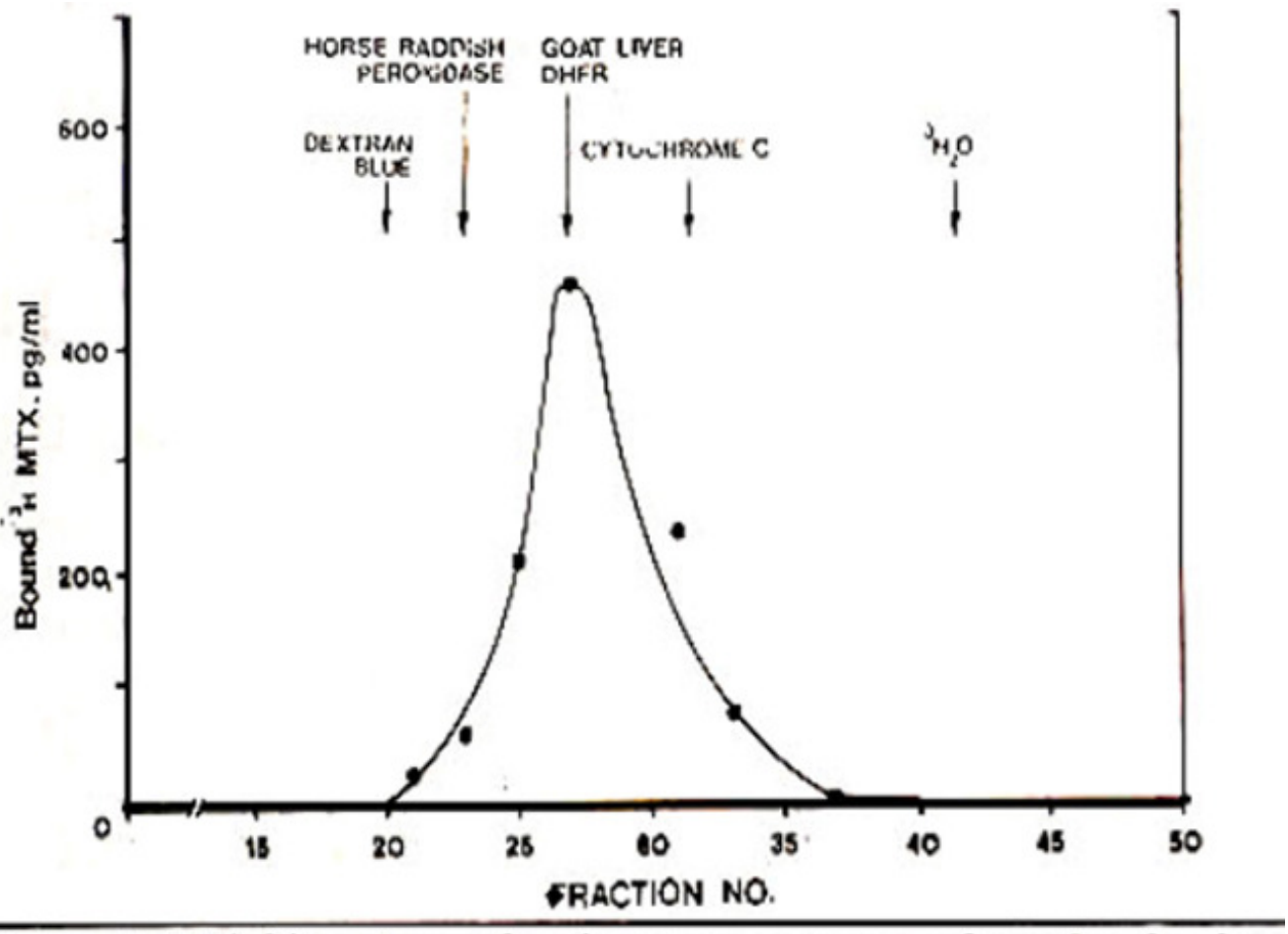

Figure 3.Gel-filtration of colon tumor cytosol on Sephadex G-75. Details have been provided in the Materials and Methods.

Previous multiple forms of DHFR in various types of cells, i.e., mouse fibroblasts, Escherichia coli, L1210 and L5178 Y leukemia cells and goat liver cells, indicate that their molecular weights are either identical[16,17] or very closely similar[3,4,9]. In our study the major limitation in terms of separating and characterizing these two binders of MDC has been the small amount of enzyme in these human tissues. At the present moment we cannot be absolutely certain that the low affinity binder of MDC is in fact a form of DHFR having weaker affinity for this antifolate. However, the isoelectric focusing profile of the colon tumor cytosol (Figure.4)

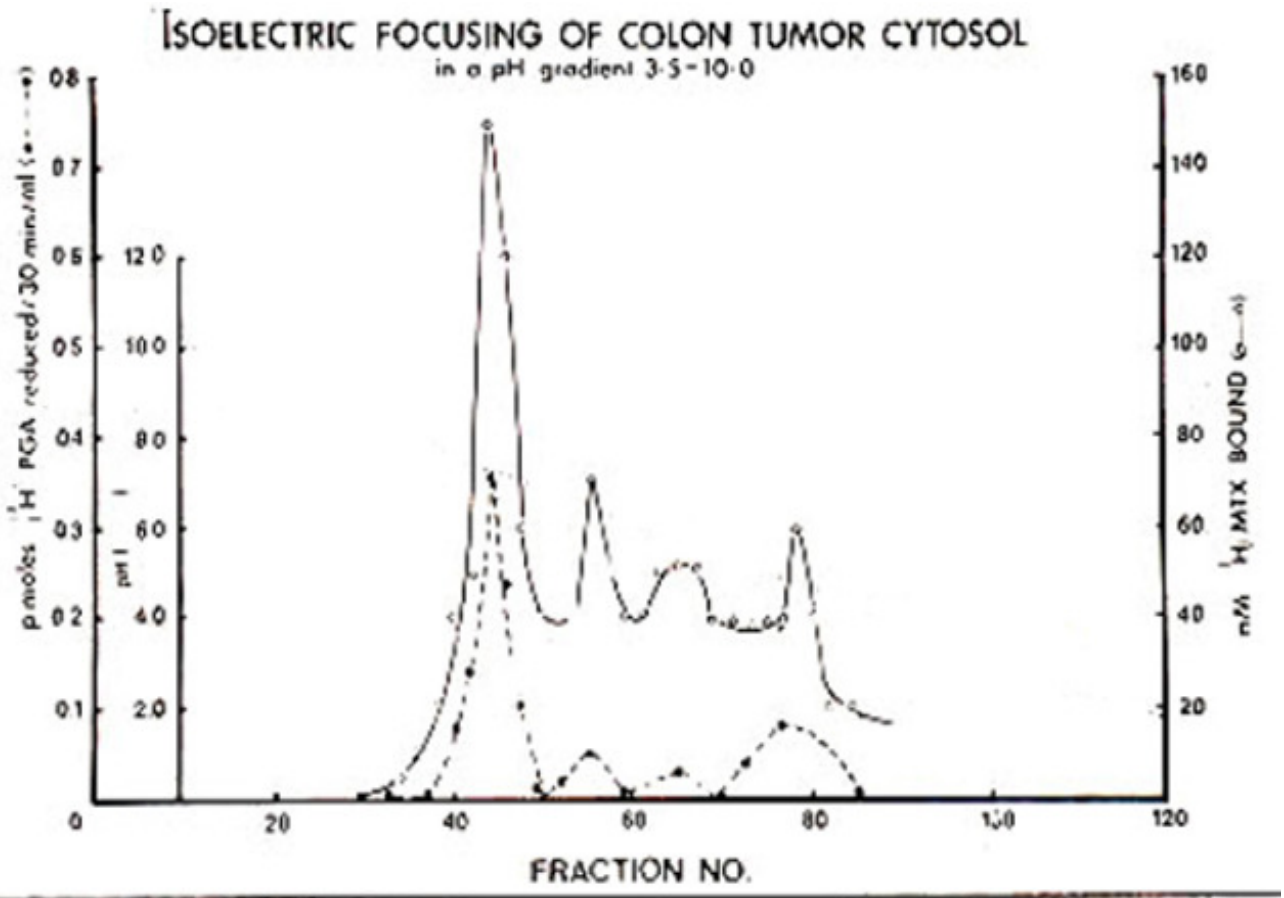

Figure 4.Isoelectric focusing of a sample of human colon tumor cytosol. Procedure details have been given in the Materials and Methods section.

shows 4 major isoproteins of dihydrofolate reductase activity with their pIs 7.3, 6.5, 5.7 and 4.7, respectively. These isoproteins were obtained by analyzing the post-focusing fractions not only for [^{3}HJMTX binding at pH 7.2 but also for [^{3}H]PGA reduction to [^{3}H]-tetrahydro-PGA. The fact that the four enzyme activity peaks co-elute exactly with the four [^{3}H]MTX binding peaks suggests that the low-affinity binder of [^{3}H]MTX in colon tumor cells is probably another form of DHFR with altered affinity for this anti-cancer drug. Since these cancer patients had never been treated with MTX, the presence of a low affinity form of DHFR in their tumor cells indicates that insensitivity to MDC is an intrinsic phenomenon in certain types of cells. Similar findings have been reported by Dedhar et al[6] for blast cells of acute myelogenous leukemia patients. MDC as a single agent has never been considered effective in the management of colorectal or gastric carcinoma and that may have been due to the fact that such cells might be containing a low affinity form of DI IFR. It has been reported by Jackson and Harrap[18] and White and Goldman[19] that no more than 5% of the folate reductase activity is required to generate sufficient tetrahydrofolate cofactors to maintain cell viability. Thus. if these cancer cells contain a small amount of low affinity form of DHFR, it would be sufficient to maintain this de novo DNA synthesis, even if all of the high affinity form of the enzyme has been inactivated by MTX. There fore, in these cells unless very high doses of MTX are used, MDC in conventional or intermediate doses would be unlikely to produce any good results. There have been quite a few reports of sequential combination chemotherapy with MTX and 5-fluorouracil in the management of colorectal

cancer[10,20,22], but in these cases the role of MDC is more in terms of enhancing the binding of 5-fluorodeoxy-uridylate (FdUMP) to thymidylate synthetase rather than completely knocking off the activity of DHFR[23,24]. In other words, MDC in these cells has a synergistic effect on the intracellular utilization of 5-fluorouracil[25,26]. Thus, heterogeneity of MDC binding in the colon tumor provides us with a plausible explanation about the ineffectiveness of MDC in the treatment of such tumors. However, very high doses of MTX which have rarely been attempted in the management of colorectal carcinoma, in theory at least, may be of value in obtaining the desired therapeutic responses.

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