Biochemical Pharmacology, Vol. 38, No. 23, pp. 4321–4325, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INTERACTION OF D, L-*erythro*- AND D,L-*threo*-γ-FLUOROMETHOTREXATE WITH HUMAN LEUKEMIA CELL DIHYDROFOLATE REDUCTASE

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(*Received* 6 *January* 1989; *accepted* 24 *May* 1989)

Abstract—Gamma-fluoromethotrexate (FMTX) is a poorly glutamylated mimic of the anti-cancer drug methotrexate (MTX) which is useful in studies of the roles of MTX poly-γ-glutamates. A second chiral center occurs at C-4 of the 4-fluoroglutamate used to synthesize FMTX and, as a consequence, FMTX occurs as both D,L-*erythro* and D,L-*threo* diastereomers. The interaction of both diastereomers with intracellular dihydrofolate reductase has been examined in the human leukemia cell line CCRF-CEM, using a centrifugal column technique. Measurements of the rate at which radiolabel was displaced from [^{3}H]MTX-saturated dihydrofolate reductase following suspension of the cells in unlabeled drug indicated that MTX and the *erythro* isomer of FMTX gave essentially the same rate of displacement; the rate of displacement by the *threo* isomer of FMTX was slower, but the interpretation of these data was ambiguous since the rate of transport of *threo*-FMTX may have been limiting. In reciprocal experiments in which dihydrofolate reductase was saturated with [^{3}H]*erythro*-FMTX, the *erythro* isomer and MTX again behaved equivalently in terms of displacement. When dihydrofolate reductase was saturated with [^{3}H]*threo*-FMTX, the radiolabel was clearly displaced at a much faster rate than either other radiolabel regardless of whether the displacing agent was MTX or the isomer. These results indicate a distinct stereospecificity for interaction of inhibitor with dihydrofolate reductase in which the *threo* isomer has a faster off-rate. Of the two FMTX diastereomers, the *erythro* isomer thus most closely mimics the properties of MTX.

The role(s) of the poly(γ-glutamyl)metabolites of MTX§ in the cytotoxic mechanism and in the therapeutic efficacy of this agent are currently topics of wide interest. The results from a number of laboratories using different model systems (reviewed in Refs 1 and 2) are consistent with these metabolites being involved in retaining intracellular concentrations of MTX in excess of those in the extracellular space and above the level of DHFR, as is required for cytotoxicity [3]. MTX polyglutamates may also directly inhibit sites in folate metabolism other than DHFR, such as thymidylate synthase [4] or aminoimidazolecarboxamide ribotide transformylase [5], thereby increasing the blockade of the pyrimidine and purine biosynthetic pathways caused by the MTX-induced decrease in reduced folate co-factors. These conclusions have been supported, in general, by investigations in which the rate and/or amount of synthesis of MTX polyglutamates has been correlated with the property of interest.

Another strategy for investigating the significance of MTX polyglutamates in cytotoxicity and therapeutic efficacy is the use of a nonglutamylatable mimic of MTX in comparative studies with MTX itself [6–8]. Such a mimic should have biochemical properties identical to those of MTX, except that it would be unable to form polyglutamate derivatives. The difference in the effects on cytotoxicity or efficacy between MTX and the mimic under appropriately controlled conditions would be directly attributable to the effects of the polyglutamates.

A mimic has been prepared by the substitution of 4-fluoroglutamate [9] for glutamate in MTX, thereby creating the analog FMTX [6]. Our initial studies were performed with FMTX that was a mixture of four isomers since the FMTX was chemically synthesized [6] from D,L-*erythro*,*threo*-4-fluoroglutamate. These studies indicated that FMTX was poorly glutamylated; however, its other biochemical properties were very similar to those of MTX [6]. Subsequent studies of the individual *erythro* and *threo* diastereomers [8] indicated that, although both inhibited isolated DHFR to the same extent as MTX (by IC_{50}) and both were poor substrates for glutamylation, the *erythro* isomer was transported more nearly like MTX.

Since the usefulness of either *erythro*-FMTX or *threo*-FMTX in the comparative studies referred to above is critically dependent on the fidelity of their biochemical properties to those of MTX (except for glutamylation), we have continued our studies of these properties in detail. We have focused on DHFR since DHFR is the principal cellular target

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§ Abbreviations: MTX, methotrexate (4-amino-10-methylpteroyl-glutamic acid); FMTX, γ-fluoromethotrexate [4-amino-10-methylpteroyl(D,L-*erythro*,*threo*-4-fluoroglutamic acid)]; eFMTX, D,L-*erythro*-γ-fluoromethotrexate [4-amino-10-methylpteroyl-(4-fluoro[2*S*,4*R*;2*R*,4*S*] glutamic acid)]; tFMTX, D,L-*threo*-γ-fluoromethotrexate [4-amino-10-methylpteroyl-(4-fluoro [2*S*,4*S*;2*R*,4*R*] glutamic acid)]; DHFR, dihydrofolate reductase (EC 1.5.1.3); and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

of MTX [10]. Both diastereomers of FMTX inhibit isolated DHFR with IC_{50} values equivalent to that of MTX [8]; IC_{50} values, however, offer only a rough estimate of "stoichiometric" interactions of inhibitors with this enzyme. As a more sensitive measurement of the interaction of the *erythro* and *threo* isomers of FMTX, we therefore chose to follow the approach used previously with MTX by Cohen *et al.* [11], in which dissociation of radiolabeled ligand from DHFR is measured in intact cells.

MATERIALS AND METHODS

Uptake, efflux, and displacement. Drug uptake was performed at CCRF-CEM [12] cell densities of 2×10^7 cells/ml in RPMI 1640 (GIBCO) supplemented with 10% (v/v) of horse serum (GIBCO) and 20 mM HEPES-NaOH, pH 7.5, held at 37° as previously described [8]. Cells were preloaded with [^{3}H]MTX (5 min; 2 μM; 1.5 μCi/ml; 700 cpm/pmol), [^{3}H]eFMTX (15 min; 4 μM; 0.5 μCi/ml; 90 cpm/pmol), or [^{3}H]tFMTX (20 min; 4 μM; 0.5 μCi/ml; 80 cpm/pmol) to achieve saturation of DHFR with minimal unbound drug; the time required was determined in preliminary studies, and DHFR saturation was verified in each experiment. At the end of the pre-loading period, the cells were diluted 2.5-fold in iced complete medium, chilled, and centrifuged at 4° (5 min; 1000 *g*). The supernatant fraction was removed and the cell pellet was triturated in 40 ml of iced complete medium and recentrifuged. The resulting cell pellet was rapidly dispersed in a volume of complete medium (37°) which approximately restored the original cell density; the actual cell density was determined. Aliquots of the cell suspension were removed at 0, 15 and 30 min. After the 30-min efflux period, the cell suspension was divided and the appropriate concentration of displacement agent was added. At 5, 10, 20, 30, and 45 min, duplicate 1-ml samples of cell suspension were removed from each treatment, washed free of extracellular drug with repeated iced saline washes [8], and stored frozen (−90°) until processed for total and DHFR-bound drug (below). Dilution in iced medium and chilling during the wash were essential to stopping further uptake and to preventing the metabolism of intracellular [^{3}H]MTX to polyglutamates. If this washing was performed at room temperature, up to 34% of the drug could be converted to metabolites (data not shown).

Total and DHFR-bound intracellular drug. Cell pellets from transport studies were stored until processing (≤3 days). When processed, tubes containing the cell pellets were placed in ice water and 1 ml of iced lysis buffer (modeled after Fry *et al.* [13]) was added. Lysis buffer contained 50 mM citric acid–sodium citrate, pH 6.0, 50 mM 2-mercaptoethanol, 50 μM NADPH, and 2 mg/ml bovine serum albumin (fraction V; Sigma Chemical Co., St Louis, MO) and was made immediately before use to minimize nonenzymatic hydrolysis of NADPH. Following disruption of the cell pellet with a pasteur pipette, cells were quantitatively lysed by 1 cycle of freezing and thawing using a dry-ice/methanol bath. The supernatant fraction was obtained by centrifuging at 1860 *g* for 10 min. Total intracellular label was obtained by mixing 350 μl of the supernatant fraction with 150 μl of 50 mM citric acid–sodium citrate, pH 6.0, and 5.5 ml of liquid scintillation counting fluid and quantitating in a liquid scintillation counter (Beckman model LS 1801). DHFR-bound radiolabel was measured using a centrifugal column procedure [8, 13] to separate free and bound drug. Centrifugal columns were 5-ml disposable plastic syringes (Becton-Dickson) plugged with a porous polyethylene disc (70 μm pore size; Bel-Art, Pequannock, NJ) covered with a Whatman GF/C filter disc, both cut with a number 8 cork borer. Syringes were filled to the 5 ml line with Sephadex G-25 (medium; Pharmacia, Piscataway, NJ) equilibrated with 50 mM citric acid–sodium citrate, pH 6.0. The resultant columns were centrifuged dry (1000 *g*; 5 min). An aliquot (500 μl) of the cell supernatant fraction (above) was layered evenly onto the gel bed, the column was re-centrifuged, and the pass-through containing DHFR-bound radiolabel was collected directly into a miniature scintillation vial and quantitated as above. Control experiments [8] indicated that this procedure quantitatively measured the total and DHFR-bound intracellular material. Half-time of displacement and k_{off} values were calculated from linear regression analysis of plots of the logarithm of DHFR-bound radioactivity versus time [11].

Analysis of metabolism and degradation of radiolabel. Intracellular drug was analyzed for metabolites and degradation products as previously described [14].

Materials. MTX was the gift of the Lederle Division of American Cyanamid. MTX containing racemic, D,L-glutamate rather than the normal L-glutamate was obtained from the Aldrich Chemical Co. (Milwaukee, WI). The D,L-*erythro* and D,L-*threo* diastereomers of FMTX and their tritiated counterparts were synthesized as previously described [8]. In this synthetic scheme, the D- and L-enantiomers of each diastereomer would be pronounced in a 1:1 ratio. [^{3}H]MTX was purchased from Moravek Biochemicals, Inc. (Brea, CA).

RESULTS

Uptake of [^{3}H]*MTX to saturate DHFR.* CCRF-CEM cells exposed to 2 μM [^{3}H]MTX for 5 min (Fig. 1) accumulated intracellular drug concentrations of 9.1 pmol/10^7 cells, exceeded the level of DHFR, and saturated the high affinity binding capacity of DHFR (7.4 pmol/10^7 cells). At longer periods of uptake (10–30 min), there was an increase in unbound drug but the level of DHFR-bound [^{3}H]MTX remained constant (not shown). Using 10 min of uptake, this level of unbound drug allowed partial conversion of the intracellular drug to a polyglutamate form, 4-amino-10-methylpteroylglutamyl-γ-glutamate, and, at the end of efflux or at the end of the control displacement period, 14–15% of the intracellular radiolabeled drug was present as this metabolite. Using 5 min of influx, ≥94% of the intracellular label was recovered as MTX at the end of efflux or displacement, while less than 4% was recovered as the metabolite. Since at 5 min, there were not significant levels of metabolites or degradation products of radiolabeled MTX, and DHFR was saturated,

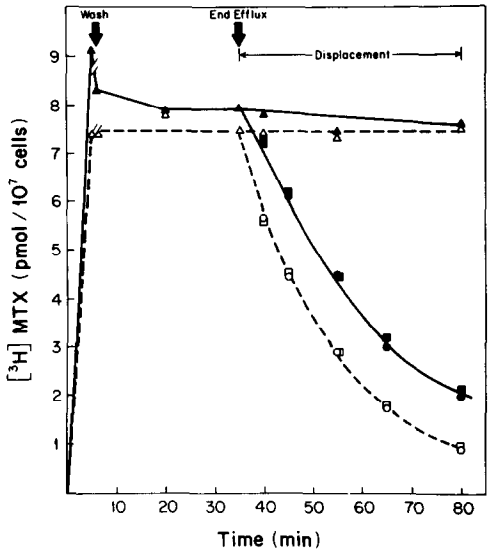

Fig. 1. Time course in CCRF-CEM cells for uptake, efflux, and displacement of [^{3}H]MTX by MTX or FMTX isomers. During uptake, 2 μM extracellular [^{3}H]MTX was present. Closed symbols are used for total intracellular drug under the particular condition, while open symbols are DHFR-bound drug only. Triangles (△) stand for drug during uptake and efflux, and also for drug in the water displacement control. Circles (○) stand for ^{3}H-labeled drug in the presence of 10 μM extracellular MTX. Squares (□) stand for ^{3}H-labeled drug in the presence of 20 μM extracellular D,L-eFMTX (10 μM active isomer). All points are averages of duplicate determinations.

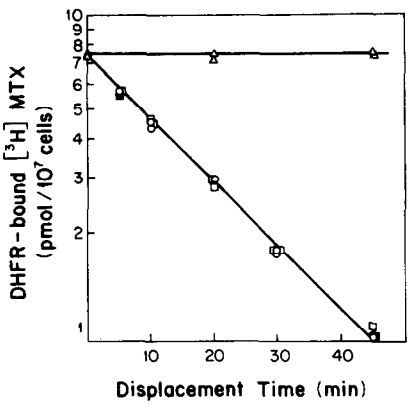

Fig. 2. Displacement of [^{3}H]MTX from intracellular DHFR by MTX or eFMTX. DHFR was saturated with [^{3}H]MTX and quantitated as described in the legend of Fig. 1 and in Materials and Methods. Displacement of this radiolabel by 10 μM MTX (○), 20 μM eFMTX (10 μM active isomer) (□), or the volume equivalent of water (△) was determined as a function of time.

5 min was chosen as an appropriate influx time. When cells exposed for 5 min were washed and placed in drug-free medium (Fig. 1), the total intracellular drug declined after 30 min to a steady state (7.9 pmol/10^7 cells) which approximated the level of DHFR-bound [^{3}H]MTX.

Displacement of [^{3}H]MTX from intracellular DHFR. If nonradioactive extracellular MTX (10 μM) was added to cell cultures containing [^{3}H]MTX saturated DHFR, but no unbound drug, there was a first order loss of both total (Fig. 1) and DHFR-bound (Figs 1 and 2) [^{3}H]MTX over a 45-min displacement period. By 45 min over 87% of the [^{3}H]MTX had been displaced from DHFR. If nonradioactive eFMTX* (10 μM) was added, there was again displacement of both total (Fig. 1) and DHFR-bound (Figs 1 and 2) [^{3}H]MTX which followed a time course nearly identical to that of MTX and reached a similar level after 45 min. Control cells showed constant levels of both total and DHFR-bound [^{3}H]MTX during the entire displacement period (Figs 1 and 2). Similar studies of displacement of radiolabel from [^{3}H]MTX-saturated DHFR were undertaken over a range of extracellular concentrations of MTX, eFMTX, or tFMTX. The results, quantitated as the half-time of displacement (Table 1), demonstrated that, at extracellular concentrations as low as 2 μM, eFMTX and MTX behaved very similarly. Displacement of [^{3}H]MTX by tFMTX had a significantly longer half-time at the highest concentration tested and, therefore, lower concentrations were not tested. Plots of log DHFR-bound [^{3}H]MTX versus time when tFMTX was the displacing agent displayed slight curvature, and this may reflect some difference in the binding of tFMTX to DHFR. Higher concentrations of the individual isomers were not tested, in order to conserve material, but increasing the extracellular concentration of MTX or the mixed diastereomer *erythro,-threo*-FMTX [6] to 20 μM did not affect the half-time of displacement.

The *erythro* and *threo* diastereomers of FMTX

Table 1. Half-time of displacement of [^{3}H]MTX from intracellular CCRF-CEM DHFR by MTX or FMTX isomers

Extracellular concentration (μM)	Half-time value (min)		
	MTX	eFMTX	tFMTX
10	15.8 ± 0.8*	16.1 ± 0.9†	21.4
5	16.9	17.5	ND‡
2	19.4	20.2	ND‡

All determinations, except as noted, are averages of closely agreeing duplicates. Half-time values were determined by linear regression analysis of plots of the logarithm of DHFR-bound drug versus time (0–45 min of displacement). DHFR-bound drug was determined as described in Materials and Methods.

* Value is average ± SD (N = 5).

† Value is average ± SD (N = 3).

‡ ND = not determined.

* The eFMTX and tFMTX diastereomers are each mixtures of D- and L-enantiomers; the concentration presented, however, is that of the L-enantiomer only (i.e. one half of the total drug concentration assuming an equal distribution between D- and L-forms). The D-enantiomer of each diastereomer was assumed to be biologically inactive since the MTX analog containing D-glutamate has no biological activity ([8, 15]; see text below).

Table 2. Half-time of displacement of [^{3}H]FMTX isomers from intracellular CCRF-CEM DHFR by MTX or FMTX isomers

	Extracellular concentration (μM)	Half-time value (min)		
		MTX	eFMTX	tFMTX
(A)	DHFR saturated with [^{3}H]*erythro*-FMTX			
	10	19.6; 20.0	20.3; 21.3	ND*
	5	19.3; 19.1	21.2; 20.1	ND*
	2	20.7 ± 1.0	22.3 ± 2.8	ND*
	0.5	27.4; 29.0	33.5; 31.3	ND*
(B)	DHFR saturated with [^{3}H]*threo*-FMTX			
	10	9.8; 9.8	ND*	12.9; 13.2

Determinations from two separate experiments are presented; at the 2 μm value in experiment A the two values for MTX varied significantly so the experiment was repeated (presented as average ± SD, N = 3). Half-time values were determined by linear regression analysis of plots of the logarithm of DHFR-bound drug versus time (0–45 min of displacement). DHFR-bound drug was determined as described in Materials and Methods.

* ND = not determined.

used in these experiments are each pairs of enantiomers, i.e. D,L-*erythro* (2*S*,4*R* and 2*R*,4*S*) and D,L-*threo* (2*S*,4*S* and 2*R*,4*R*). Since displacement of radiolabel from [^{3}H]MTX-saturated DHFR by D,L-MTX (4 or 20 μM) or L-MTX (2 or 10 μM) was comparable (data not shown), we consider it very likely that the D-*erythro* and D-*threo* isomers of FMTX have no effect in this system. Our data with racemic MTX is consistent with the data of Cramer *et al.* [15], suggesting that the D-isomer of MTX is biologically inert, presumably because it is not transported. This indirect evidence suggested that D-*erythro*-FMTX and D-*threo*-FMTX would play no part in displacement because they would not enter the cells. Direct proof of this requires pure L-enantiomers of FMTX; the synthetically challenging preparation of these enantiomers is currently underway.

Uptake of [^{3}H]*eFMTX or* [^{3}H]*tFMTX to saturate DHFR.* The *erythro* isomer of FMTX is taken up more efficiently than the *threo* isomer, but both are somewhat less efficient than MTX [8]. Thus, the uptake time required for each [^{3}H]isomer at 2 μM extracellular concentration to saturate DHFR was determined experimentally, 15 min for *erythro* and 20 min for *threo* (data not shown). In these cases, the presence of unbound drug following uptake was not of such concern since these analogs are poorly glutamylated, if at all [8]. Similar to [^{3}H]MTX (Fig. 1), the 30-min efflux period left DHFR saturated with radiolabeled drug, but removed all unbound material (data not shown).

Displacement of [^{3}H]*eFMTX or* [^{3}H]*tFMTX from intracellular DHFR.* Displacement studies of radiolabeled FMTX-isomers from DHFR were performed at various concentrations of unlabeled MTX or FMTX-isomer. The results (Table 2) demonstrated that the [^{3}H]*erythro* isomer was displaced from DHFR slightly more slowly than was [^{3}H]MTX (Table 1); MTX and the *erythro* isomer were again equivalent as displacing agents except at very low concentrations (0.5 μM) where transport of the *erythro* isomer might be limiting [8]. The [^{3}H]*threo* isomer was displaced from DHFR much more readily than either [^{3}]MTX or [^{3}H]*erythro*-FMTX. This ready displacement was seen whether the displacement agent was MTX or unlabeled tFMTX. In the absence of MTX or isomer as displacing agent, there was no loss of either radiolabeled isomer from DHFR (data not shown), similar to the case with [^{3}H]MTX (Figs. 1 and 2).

DISCUSSION

Earlier studies of FMTX, either with a diastereometric mixture [6] or with purified *erythro* and *threo* diastereomers [8], compared the interaction with DHFR of these mimics to that of MTX by examining inhibition of isolated enzyme [6, 8] and by measuring the degree of association of drug with intracellular DHFR under conditions where drug was limiting [8]. Each type of measurement indicated that the isomers were both equivalent to MTX. Simple measurement of inhibition of DHFR activity (by IC_{50} value) is not as definitive as determination of K_i; however, K_i values for stoichiometric inhibitors are difficult to determine with accuracy. The association of drug with intracellular DHFR under conditions of limiting drug [8] measures only one part of this complex interaction. Further insight could be obtained by measurement of the dissociation rate of the inhibitor–DHFR complex.

The studies of Cohen *et al.* [11], in which displacement by extracellular unlabeled MTX of [^{3}H]MTX bound to DHFR was measured, were the first to demonstrate the reversibility of MTX binding to intracellular DHFR and to measure k_{off} values. Under appropriate conditions, the general approach of this classic paper can be used to examine the interaction of any DHFR inhibitor with intracellular DHFR. For this approach to be valid it is critical that transport of unlabeled displacing agent not be limiting and that sufficient intracellular unlabeled drug be present to block re-binding of radiolabeled drug to DHFR once it dissociates. The finding that the half-times of displacement were the same over a 2- to 5-fold concentration range of displacing agent (Tables 1 and 2) supported the contention that transport was not limiting at the higher concentrations.

In addition, reciprocal displacement experiments in which each radiolabeled drug was used, in turn, to saturate DHFR allowed differences in transport properties of the FMTX isomers to be rigorously controlled for. The first order decrease in DHFR-bound radiolabel without any time lag (Fig. 2) indicated that significant re-binding of radiolabel did not occur. In the present work, the approach of Cohen *et al.* [11] was also modified by using short uptake times and iced washes to remove extracellular radiolabel in order to eliminate [^{3}H]MTX polyglutamate synthesis, which might confound the results, and by measuring both total and DHFR-bound intracellular radiolabel. Measurement of the DHFR-bound fraction eliminated potential interference by radiolabeled drug which dissociated but did not efflux from the cell (see Fig. 1).

The results of these reciprocal displacement studies (Tables 1 and 2) demonstrated that, under conditions where transport was not limiting, the half-time of displacement (and thus the k_{off}) of the *erythro* isomer of FMTX was essentially the same as that of MTX. The half-time values decreased as the extracellular concentration increased until the process appeared saturated; similar saturation was noted in the L1210 system by Cohen *et al.* [11]. The k_{off} value determined here at saturation for MTX (4.4×10^{-2}/min) was also similar to that reported by Cohen *et al.* [11] in the original work with the L1210 cell line (3.8×10^{-2}/min).

In contrast, the interaction of the *threo* isomer with DHFR was different than that of MTX. The half-time for the displacement of [^{3}H]MTX by the *threo* isomer (Table 1) was significantly longer than by either MTX or the *erythro* isomer. This could be the result of either a weaker interaction with DHFR or it could be that the poorer transport of the *threo* isomer [8] made its concentration limiting, even at a 10 μM extracellular concentration. The reciprocal experiment, however, was unambiguous (Table 2). The [^{3}H]*threo* isomer was rapidly displaced from DHFR by either MTX or nonradioactive tFMTX. Any limitation of transport of the nonradioactive displacing tFMTX could, in this case, lead only to an overestimate of the half-time of displacement. A comparison of the displacement half-times of [^{3}H]eFMTX or [^{3}H]tFMTX in the presence of nonradioactive MTX, where transport cannot be an issue, clearly showed that [^{3}H]tFMTX was more readily displaced.

These studies demonstrate that eFMTX dissociated from DHFR at a rate very similar, if not identical, to that of MTX, whereas tFMTX dissociated significantly faster. These results indicate that human leukemia cell DHFR exhibits subtle specificity at the γ-COOH for binding of classical antifols since only the diastereomeric configuration of eFMTX and tFMTX is different. Taken together with earlier data [8] showing that the transport kinetics of eFMTX are also more similar to those of MTX, it can be concluded that, except for glutamylation, eFMTX is a closer mimic of MTX than is tFMTX.

Acknowledgements—This work was supported in part by Grants CA13038, CA24538 (J. J. M.), and CA28097 (J. K. C.) from the National Cancer Institute, Public Health Service, Department of Health and Human Services, and Institutional Research Grant IN-54Z from the American Cancer Society (J. J. M.). J. J. M. is a Leukemia Society Scholar. The secretarial assistance of Mae Brown is gratefully acknowledged.

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