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The Mechanism of Action of Methotrexate

II. Augmentation by Vincristine of Inhibition of Deoxyribonucleic Acid Synthesis by Methotrexate in Ehrlich Ascites Tumor Cells

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SUMMARY

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Incorporation of deoxyuridine into DNA persists in Ehrlich ascites tumor cells *in vitro* even after high-affinity intracellular binding sites have been saturated with methotrexate (MTX). Further suppression of deoxyuridine metabolism requires exposure of cells to extracellular MTX to achieve intracellular MTX levels in excess of the tightly bound fraction. Vincristine (10 μM) augments the steady-state level of intracellular MTX, and this is associated with an increased inhibition of deoxyuridine incorporation into DNA. Exposure of cells to vincristine in the absence of MTX, or under conditions in which high-affinity binding sites are completely associated with MTX but intracellular MTX in excess of this component is not present, did not alter the incorporation of deoxyuridine into DNA over 35 min. These observations relate to other studies which suggest that vincristine acts as a metabolic poison to inhibit energy-dependent processes which limit the net accumulation of MTX, an effect which occurs in the absence of an alteration in the unidirectional influx or the extracellular MTX level. These data further indicate a crucial role for intracellular MTX in excess of the usual tightly bound fraction in the inhibition of DNA synthesis, and suggest a rational basis for the design of chemotherapeutic regimes with MTX and vincristine.

INTRODUCTION

The accompanying paper (1) presented evidence which suggested that an important determinant of inhibition by methotrexate of deoxyuridine incorporation into DNA is the level this agent achieves within the intracellular compartment in excess of the amount required for complete association with high-affinity binding sites. Other studies have indicated that this exchangeable[3] component

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[3] "Exchangeable" methotrexate will refer to that component of intracellular MTX in excess of the tightly bound fraction. This includes MTX which is osmotically active and MTX which is loosely bound within the cell. "Free" MTX will

of intracellular MTX[4] is depressed by energy-dependent processes, since a variety of inhibitors of anaerobic and/or aerobic metabolism enhance the net uptake of MTX and produce a large increase in the steady-state level of exchangeable intracellular MTX (2, 3). Recently Zager *et al.* (4) observed that vincristine augmented net uptake of MTX into L1210 leukemia cells and increased the cytotoxicity of MTX to this tumor *in vivo* and *in vitro*. This interaction between MTX and vincristine was further characterized in the Ehrlich ascites tumor (5). Vincristine appeared to alter MTX uptake in a manner similar to that observed for a variety of metabolic poisons, except that it did not alter the unidirectional influx of MTX; net uptake of exchangeable MTX was associated with a fall in the unidirectional efflux of this agent (5). This effect of vincristine was partially selective, in that MTX uptake in the L-cell mouse fibroblast was much less sensitive to vincristine than in the Ehrlich ascites tumor. The mechanism of this effect of vincristine appeared to be different from its known cytotoxic effects: tight binding to microtubular elements (6), with inhibition of spindle formation (7–9) and the arrest of cell division in metaphase (10, 11).

In view of the apparent role of exchangeable intracellular MTX in the inhibition of deoxyuridine metabolism (1) and the increase in this component of intracellular MTX by vincristine (4, 5), studies were undertaken to determine whether the augmentation of exchangeable intracellular MTX is accompanied by increased inhibition of deoxyuridine incorporation into DNA in the Ehrlich ascites tumor. In addition, this interaction between vincristine and MTX provided an experimental approach in which the intracellular MTX level could be increased under conditions in which the unidirectional influx and the extracellular MTX levels are unchanged, permitting an opportunity for further verification of the critical role of exchangeable intracellular MTX in the inhibition of deoxyuridine metabolism. A brief report on these studies has been published (12).

refer to that component which is osmotically active within the intracellular water and contributes to the intracellular electrochemical potential for MTX.

[4] The abbreviation used is: MTX, methotrexate, 4-amino-N^{10}-methylpteroylglutamic acid.

METHODS

Cells, medium, experimental and analytical techniques. Ehrlich ascites tumor cells were harvested from CF_1 mice 7–10 days after intraperitoneal inoculation and washed at least three times in a bicarbonate-buffered electrolyte solution to remove contaminating red blood cells and residual ascitic fluid as previously described (5, 13). For experimentation, the cells were suspended in modified Eagle's medium, without folates or serum (1), to a final cytocrit of less than 4%. Experimental designs are indicated under RESULTS and are further described along with the analytical techniques and methods of data analysis in the accompanying paper (1).

Chemicals. The source and methods of purification of labeled and unlabeled MTX have been described (14). [2-^{14}C]- and [6-^{3}H]-Deoxyuridine were obtained from New England Nuclear Corporation and were employed at final concentrations of 0.06 μM and 1.2 μM, respectively. Vincristine sulfate was supplied through the generosity of Dr. Robert Hosley of the Eli Lilly Research Laboratories, Indianapolis.

Identification of intracellular radioactivity. It was previously demonstrated by DEAE-cellulose column chromatography that over the interval of these experiments Ehrlich ascites tumor cells do not metabolize MTX in either the presence or absence of 10 μM vincristine (5). After incubation of Ehrlich ascites tumor cells with labeled deoxyuridine for 1 hr, more than 90% (in three experiments) of the radioactivity associated with the washed trichloracetic acid precipitate was extracted by a 30-min incubation with 0.4 N $HClO_4$ at 70°.

RESULTS

Effect of vincristine on net MTX uptake. Figure 1 illustrates the association of MTX with Ehrlich ascites tumor cells under the experimental conditions in which the effects of vincristine and/or MTX on deoxyuridine

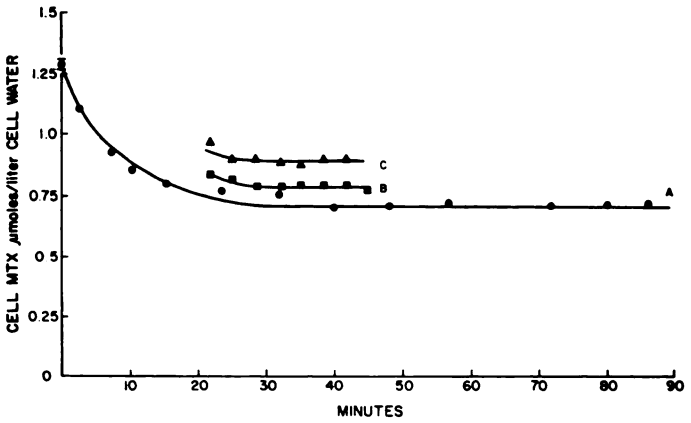

FIG. 1. *Effect of vincristine on the steady-state intracellular methotrexate level*

Cells were incubated with 6 μM MTX for 5 min at 37°, then separated by centrifugation, washed twice with 0° buffer, divided into three portions, and resuspended in MTX-free medium (A) or medium containing 0.12 μM MTX with (C) or without (B) 10 μM vincristine. The initial decline in the intracellular MTX level was determined in cells exposed to MTX-free medium. In the other suspensions intracellular MTX was determined over the interval in which the subsequent incorporation of deoxyuridine into DNA would be measured, as in the protocol described in the text and Fig. 2. The intracellular MTX level prior to resuspension (zero-time point) is the mean ± standard error of three determinations. The tightly bound MTX component was determined from the average of the intracellular MTX values over 57–85 min following resuspension in MTX-free medium.

metabolism were subsequently measured (see next section). Cells were incubated with 6 μM MTX for 5 min at 37°, following which the cell fraction was separated, divided into three equal portions, and resuspended in MTX-free medium (A) or medium containing 0.12 μM MTX in the presence (C) or absence (B) of 10 μM vincristine. Between 20 and 50 min later the level of intracellular MTX was determined. This procedure produced an initial intracellular MTX level which exceeded the capacity of the high-affinity binding sites, to assure saturation of these sites, following which the extracellular MTX level was decreased to permit the intracellular level to fall to a steady state. From six such experiments the average initial intracellular MTX level in excess of the high-affinity binding capacity was 0.93 ± 0.18 μM. When extracellular MTX was 0.12 μM, the average intracellular MTX level in excess of the usual binding capacity was 0.16 ± 0.04 μM. In the presence of 0.12 μM MTX and 10 μM vincristine, this component of intracellular MTX was doubled to 0.32 ± 0.07 μM. Two intracellular MTX components can be identified from the unidirectional efflux of MTX into MTX-free medium: exchangeable intracellular MTX, represented by the rapid-exit component ($t_{1/2}$ = 7.02 ± 0.89 min), and the tightly bound intracellular fraction. The rate of efflux of exchangeable intracellular MTX is about one-third that observed for L-cells (1).

Effect of vincristine on MTX inhibition of deoxyuridine incorporation into DNA. Figure 2 is a representative experiment which illustrates the effect of vincristine on deoxyuridine metabolism in the presence and absence of intracellular MTX. A cell suspension was divided into two portions: A, the control, and B, which was exposed to 6 μM MTX. After a 5-min incubation at 37°, the cell fractions were separated and washed, and each was further subdivided into two equal portions, I and II. BI and BII were suspended into medium containing 0.12 μM MTX, while AI and AII were again suspended in MTX-free medium. Both AII and BII were exposed to 10 μM vincristine. The cell suspensions were incubated for 15 min at 37°, following which deoxyuridine was added to each suspension and the incorporation of radioactivity into the trichloracetic

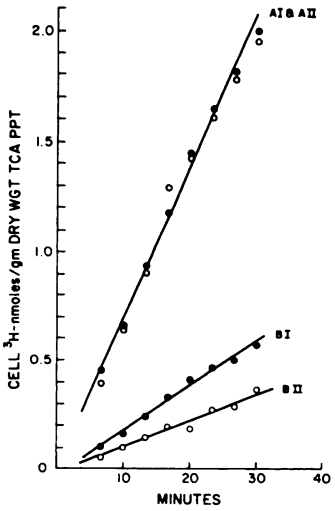

FIG. 2. *Effect of vincristine on methotrexate inhibition of deoxyuridine incorporation into DNA*

Cells were divided into two portions. A cells were suspended in MTX-free medium, and B cells were exposed to 6 μM MTX. After a 5-min incubation at 37°, the cells were separated by centrifugation and washed twice with 0° buffer; then each portion was divided into two subportions, I and II. BI and BII were suspended in medium containing 0.12 μM MTX. AI and AII were suspended in MTX-free medium. Both AII and BII were exposed to 10 μM vincristine. After a 15-min incubation at 37° all the cell suspensions were exposed to deoxyuridine at zero time, and the rate of incorporation of radioactivity into the 0° trichloracetic acid (TCA) precipitate was monitored.

acid precipitate was monitored 7–30 min later. In the absence of MTX vincristine did not alter the rate of deoxyuridine incorporation into DNA. From the average incorporation rates in eight such experiments, deoxyuridine metabolism in the presence of vincristine alone (AII) was 98.16 ± 4.37% of the rate in the absence of this agent (AI), a difference which is not significant ($p > 0.5$). When 0.12 μM MTX was present (BI) incorporation was 42.62 ± 4.95% of that of the control cells, but metabolism of deoxyuridine was not completely abolished. In the presence of MTX and 10 μM vincristine (BII) there was a further 40.35 ± 4.02% reduction in the rate of deoxyuridine metabolism as compared to MTX alone (BI) ($p < 0.001$). Vincristine also increased MTX inhibition of deoxyuridine incorporation into the trichloracetic acid supernatant fraction ($p < 0.01$ in four experiments). The results were qualitatively similar whether [^{14}C]- or [^{3}H]deoxyuridine was employed at their respective concentrations.

Role of intracellular MTX in excess of tightly bound fraction in interaction between MTX and vincristine. To determine whether the vincristine-induced augmentation of inhibition of deoxyuridine metabolism by MTX is related to the presence of intracellular MTX in excess of the usual tightly bound component or requires only the complete association of MTX with high-affinity binding sites, the following experiment was conducted. A cell suspension was incubated in medium containing 1.4 μM MTX for 6.5 min. This resulted in the saturation of high-affinity binding sites, but with the generation of less exchangeable intracellular MTX than in the protocols detailed above. The cells were then washed, divided into two portions, and resuspended in medium in the presence (B) or absence (A) of 1.4 μM MTX. After a 21-min incubation the cells were washed again and resuspended in fresh media of the same respective compositions. Twenty-one minutes later the wash and resuspension were repeated, A and B were each subdivided into two suspensions, I and II, and AII and BII were exposed to 10 μM vincristine. All four suspensions were then exposed to deoxyuridine, and 7–30 min later the incorporation of deoxyuridine into DNA was monitored. The second wash and the long interval prior to addition of deoxyuridine were required to assure adequate loss of exchangeable intracellular and extracellular MTX in the group A cells, since the exit half-time for this component is 7 min. A representative experiment is illustrated in Fig. 3. In five such experiments the rate of deoxyuridine metabolism in the absence of exchangeable intracellular MTX was unaffected by vincristine ($p > 0.5$ on comparing the difference between the incorporation of label into DNA in the presence and absence of vincristine between 18 and 30 min after addition of deoxyuridine). However, as indicated in both the large graph and inset of Fig. 3, when exchangeable intracellular MTX was present vincristine produced a

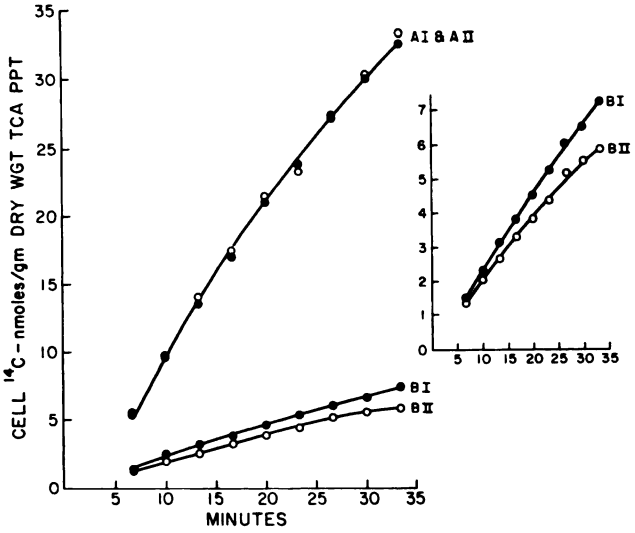

FIG. 3. *Effect of intracellular methotrexate in excess of the usual tightly bound fraction on the augmentation of methotrexate inhibition of DNA synthesis by vincristine*

Cells were incubated with 1.4 μM MTX for 6.5 min at 37°, then separated by centrifugation, washed twice with 0° buffer, and divided into two portions, A and B. Portion A was resuspended in MTX-free medium; B was suspended in medium containing 1.4 μM MTX. After 21 min of incubation at 37°, the cells were separated, washed, and resuspended in media of the same compositions. After an additional incubation at 37° for 21 min, the cells were washed again and resuspended in similar media. Portions A and B were each divided into two subportions, I and II. AII and BII were exposed to 10 μM vincristine, following which all suspensions were exposed to deoxyuridine and the incorporation of radioactivity into the 0° trichloracetic acid (TCA) precipitate was measured. The inset represents an expansion of lines BI and BII.

28.73 ± 1.95% fall in the incorporation of deoxyuridine into DNA over this interval. The delay in this effect is ascribed to the time required for sufficient levels of intracellular MTX to accumulate after the addition of vincristine (5). This figure also indicates that, as observed for L-cells (1), exchangeable intracellular MTX markedly inhibits deoxyuridine incorporation into DNA (BI) in comparison to the rate of metabolism when high-affinity binding sites are saturated with MTX but negligible exchangeable MTX is present (AI).

DISCUSSION

MTX and the naturally occurring folates share a carrier transport route in many normal and malignant mammalian cells. This transport system has a high-affinity for MTX and the reduced derivatives of folic acid, but a very low affinity for folic acid itself (13, 14–20). The accumulation of folates within the intracellular compartment appears to be limited by energy-dependent processes, since metabolic poisons enhance the net uptake of MTX (2, 3), folic acid (20), and N^5-methyltetrahydrofolate (21). Recently vincristine was noted to increase net uptake of MTX into the L1210 leukemia cell (4), and the mechanism of this effect was characterized in the Ehrlich ascites tumor (5). The similarities between the effects of vincristine (5) and known metabolic poisons (2), such as azide, iodoacetate, and anaerobiosis, on MTX uptake, as well as the observation that stimulation of net MTX uptake by vincristine is partially reversed by energy substrate, suggested that vincristine acts in this context as a metabolic poison to inhibit those processes which limit the accumulation of MTX within the cell (5). The same report (5) suggested further that the mechanism by which vincristine enhances MTX uptake is different from the mechanism by which this agent produces mitotic arrest: tight binding to microtubular elements (6) with the inhibition of spindle formation (7–9). This

was based upon the observations that the effect of vincristine was reduced in the presence of glucose and that vincristine did not alter the net uptake of MTX in L-cells at a concentration at which cell replication ceased (5).

The accompanying paper (1) demonstrates that deoxyuridine incorporation into DNA in L-cells continues even after high-affinity intracellular binding sites have been saturated with MTX, and that complete suppression of deoxyuridine incorporation can be achieved only when cells are exposed to extracellular MTX levels sufficient to produce intracellular MTX in excess of this amount. Since alterations in exchangeable intracellular MTX required comparable alterations in the extracellular MTX level, experimental approaches were developed to verify that the depression in deoxyuridine metabolism was related to the presence of exchangeable intracellular rather than extracellular MTX. Those studies suggested that the exit rate of intracellular tetrahydrofolate cofactors was not increased by extracellular MTX, providing evidence against a transconcentration phenomenon; and countertransport between extracellular MTX and intracellular tetrahydrofolates also appeared to be excluded. The data suggested that extracellular MTX did not depress the membrane transport of deoxyuridine (1). Our present findings indicate that Ehrlich ascites tumor cells also require sustained exposure to MTX for maximum suppression of deoxyuridine incorporation into DNA, and emphasize the critical role of intracellular MTX, since the increase in net MTX uptake into Ehrlich ascites tumor cells induced by vincristine was accompanied by a fall in the rate of deoxyuridine metabolism under conditions in which the extracellular MTX level was unchanged. Furthermore, since vincristine does not alter the unidirectional influx of MTX in Ehrlich ascites cells (5) and deoxyuridine incorporation into DNA was measured under nearly steady-state conditions for intracellular MTX, the vincristine effect is related neither to alterations in the rate of MTX influx into the cell nor, on the same basis, to an increase in the exit rate of tetrahydrofolates. Indeed, since MTX and the tetrahydrofolates appear to share the same transport carrier and respond similarly to metabolic poisons (2, 3, 20, 21), it is likely that the exit of intracellular tetrahydrofolates is slowed by vincristine; this is under further study. The effect of vincristine on deoxyuridine metabolism is not related to inhibition of deoxyuridine transport into the cell, since deoxyuridine metabolism is not inhibited by vincristine in the absence of MTX. Vincristine does not result in metabolism of MTX (5), so that its effects cannot be related to the accumulation of metabolites of MTX. Alterations in the metabolism of deoxyuridine by vincristine cannot be due to changes in the influx of folates into the cell, since, as in the studies with L-cells (1), the medium was free of folates, and vincristine does not alter this transport parameter of this carrier system (5). Although high levels of vinblastine were reported to inhibit incorporation of thymidine into DNA (22), vincristine did not alter deoxyuridine metabolism in the absence of MTX or when the only MTX present within the cell was tightly bound. It is possible, however, that the vincristine effect on deoxyuridine metabolism could be due to inhibition of the utilization of tetrahydrofolate cofactor stores rather than to the augmentation of the net intracellular MTX level per se. This effect might not be measurable when there is abundant synthesis of tetrahydrofolates, but only become apparent when exchangeable intracellular MTX is present, tetrahydrofolate production is reduced, and the utilization of cellular stores becomes critical.

Intracellular MTX in excess of the tightly bound fraction was assumed to be free and osmotically active within the intracellular water (14). However, the apparent interaction between MTX and a low-affinity intracellular site, which results in inhibition of deoxyuridine metabolism (1), indicates that a component of exchangeable intracellular MTX is bound and does not contribute to the free intracellular pool. The increased net uptake of exchangeable MTX by vincristine or azide was attributed to the intracellular accumulation of free MTX (2, 5), and the effect of these agents was related

to an alteration in energy coupling to the membrane transport system (2, 5, 16). A model to account for uphill transport of MTX even in the presence of metabolic poisons was proposed (16). The augmentation of MTX inhibition of deoxyuridine metabolism by vincristine may be related to the inhibition of an energy-dependent exit mechanism, with an increase in the level of free intracellular MTX resulting in the association of a small component of the total exchangeable intracellular MTX with a low-affinity target site. Alternatively, vincristine or other metabolic poisons may increase the affinity of this target site for MTX, with the increased level of intracellular MTX due, to a large extent, to intracellular binding. Indeed, the exit kinetics for MTX (2) or folic acid (20) in L1210 leukemia cells in the presence of azide is compatible with increased binding to a low-affinity site, although there would still be an increase, albeit smaller, in the free component (2).[5] Intracellular binding of MTX could be related to the cellular oxidation-reduction potential. The affinity of dihydrofolate reductase for MTX in some cell-free systems depends upon association with TPNH (23–26). Free MTX may be required for maximum inhibition of deoxyuridine incorporation into DNA by permitting binding to dihydrofolate reductase species unassociated with TPNH. It is possible that an increase in the level of reduced coenzymes due to the depressive effects of vincristine on aerobic energy metabolism (5, 27) may result in an increase in the affinity of dihydrofolate reductase species for MTX or an increase in the total number of binding sites which may associate with MTX. However, the relevance of these observations to the TPNH levels and dihydrofolate reductase species in the intact mammalian cell has not been established.

These studies may be relevant to the clinical use of MTX and vincristine. Our observations provide a rational basis for the design of anticancer regimens with MTX and vincristine and may account for the increased toxicity of MTX to the L1210 leukemia with the combined use of these agents (4). The toxicity of the *Vinca* alkaloids is related to binding to specific microtubular elements, an effect which may not be the basis of the alterations induced in the uptake of MTX (5); hence *Vinca* analogues may be identified which lack toxicity to marrow or nerve tissue but retain the capacity to augment MTX uptake. Finally, as suggested previously (5), the different sensitivities of the L-cell and Ehrlich ascites tumor to the effects of vincristine on MTX uptake raise the possibility that there may be selective effects on MTX uptake in host-vs.-tumor tissues which can be exploited in the clinical use of these agents.

[5] Efflux of exchangeable intracellular MTX in cells loaded with MTX in the presence of azide consists of two components (Figs. 8 and 9 of ref. 2). In the analysis of these data it was suggested that both exit components represent free MTX, but from cells with different capacities for maintaining high gradients across the cell membrane. Alternatively, the slow-exit component might be due to slow dissociation from low-affinity sites within the cell. However, even according to this analysis, the level of free intracellular MTX (rapid-exit component) is increased by azide to a level about 3 times greater than that predicted for a passive system, so that the data continue to suggest that azide augments uphill transport of MTX into the cell, and the model previously proposed would still be relevant (2, 16). Earlier work suggested that azide (2) and vincristine (5) may reversibly increase a small, tightly bound, intracellular MTX component. This may, however, be related to small residual amounts of MTX in the extracellular compartment during washout studies, with concurrent residual loose binding or uphill transport of MTX within the cell. This is under investigation.

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