The University of Manitoba

The Effects and Mechanism of Action of MTX in an Immunologically Active System Including an Examination of the Effects of Different Sera Used in the Cultures

by

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ABSTRACT

The effects and mechanism of action of methotrexate (MTX) in an immunologically active system including an examination of the effects of different sera used in the cultures.

DNA and RNA synthetic rates were studied following stimulation of guinea pig lymph node lymphocytes by phytohemagglutinin (PHA) or rabbit anti-guinea pig lymphocyte-serum (ALS). Incorporation of H^3-TdR decreases over the first 12 hours of culture but then rises to a peak at 48 hours with both stimulants.

Methotrexate was used to suppress DNA and RNA synthesis in both systems. DNA synthesis in ALS-stimulated cultures is suppressible only at 12 hours after ALS addition to the cultures. DNA synthesis in PHA-stimulated cultures is suppressible by MTX at any time after 6 hours incubation in PHA if the culture medium is enhanced with guinea pig serum (GPS). If the medium is enhanced with normal rabbit serum (NRS), MTX treatment produces suppression only at 6 and 12 hours after PHA stimulation. The MTX-induced suppression of DNA synthesis in 24 hour PHA cultures enhanced with GPS was preventable by the addition of thymidine and inosine to the cultures. Reversal of this suppression can occur by addition of leucovorin to cultures pretreated with MTX.

DNA synthesis in unstimulated cultures was not suppressible by MTX.

There was no correlation between the rate of DNA synthesis and the degree of suppression obtained by MTX addition.

RNA synthesis in unstimulated cultures was not suppressible by MTX addition. Peak H^3-uridine incorporation rates in PHA stimulated cultures occurred at 24 hours after addition of the stimulant and fell towards earlier levels at 48 hours. Suppression by MTX treatment in these cultures occurred only after 24 hours of stimulation. The suppression at 24 hours was preventable by purine nucleosides added simultaneously with the methotrexate.

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LITERATURE REVIEW

INTRODUCTION

With the advent and increasing popularity of transplantation it has become of greater importance to devise a method of immunosuppression that is more selective. Ideally the therapy would suppress only the immune response to a specific set of antigens, but would have no effect on immunocytes reacting with other antigenic determinants. At present, this degree of specificity exists only in cases of immune tolerance which can be induced with either very large or very small doses of the antigen (1). Drugs such as the Vinca alkaloids, cytosine arabinoside and methotrexate which are thought to kill cells most effectively when they are proliferating rapidly (2,3), approach the degree of specificity desired. All have been shown to be most immunosuppressive if given 1 to 4 days after antigen, at a time when immunocompetent cells are responding to the antigenic challenge and are beginning to proliferate rapidly. The biochemical reasons for the increased sensitivity of rapidly proliferating cells are not well understood. It has been suggested that rapidly proliferating populations simply have more cells in the drug sensitive phase of the cell cycle at any given time (2). This implies that all cells reaching the drug sensitive phase are equally sensitive to the drug whether they are drawn from a rapidly proliferating or a slowly proliferating pool. The present studies with methotrexate (MTX) indicate that kinetic considerations alone may not be enough to explain the selective action of this drug.

It is hoped that a better understanding of the mechanism of action of drugs like MTX will lead to their more effective and selective use in immunosuppression and cancer chemotherapy.

In order to explore the relationship between proliferative rate and sensitivity to methotrexate, lymph node cells from guinea pigs have been studied with respect to suppressibility of nucleoside incorporation by MTX following *in vitro* stimulation with phytohemagglutinin (PHA) or rabbit-anti-guinea pig lymphocyte-sera (ALS).

PHYTOHEMAGGLUTININ

The addition of phytohemagglutinin (PHA) to resting lymphocytes induces these cells to replicate (4,5,11). PHA is a nonspecific mitogen derived from the red kidney bean. It is a glycoprotein which binds carbohydrate moieties on the cell membrane (4). Galactose and mannose residues of the receptor molecule for PHA on erythrocyte membranes bind the mitogen. It is quite possible that other cell types also possess distinct receptor sites for PHA (52).

Once the PHA molecule binds to the surface of the lymphocyte, it initiates a complex series of biochemical events which culminate in a completed cell cycle.

Initially, changes are confined primarily to the membrane. Within the first two minutes after the addition of PHA to the cells the adenyl cyclase activity increases (5,7), there is an increased phosphate incorporation into membrane phospholipids (8,9,10,11) and an increase in the glucose transport into the cell (8). Since no RNA or protein synthesis has occurred within this two minute time span, these events appear to be due to a PHA effect which activates pre-existing enzymatic mechanisms (8). The increased synthesis of membrane phospholipids is thought to cause a rearrangement of the membrane components which may facilitate the transport mechanisms of the cell. The increased glucose transport into the cell is

thus believed to be due to an unmasking of pre-existing inactive carrier proteins (9). Since the adenyl cyclase activity of the cell increases within two minutes of the addition of PHA to the cell, the cyclic AMP level within the cell will also increase. Cyclic AMP has been associated with activation of kinase systems, histone phosphorylation and phospholipid synthesis in its role as a secondary messenger (6). It may thus serve any or all of these functions in a PHA stimulated lymphocyte. In general, the major events occurring during the first two minutes of PHA stimulation appear to be alterations in membrane structure and permeability.

Over the next thirty minutes, membrane phenomena again appear to be the primary events in a PHA stimulated lymphocyte. A 21-fold increase of phosphate incorporation into phosphatidyl inositol, but very little increase in incorporation into phosphatidyl choline or phosphatidyl serine, occurs (10). There is also an increased incorporation of oleate into lecithin (11). These membrane phenomena are thought to generally enhance endocytosis in the lymphocyte. During this first 30 minutes of PHA stimulation the RNA concentration within the cell drops 15-20%. The RNA which is being degraded is thought to be responsible for maintaining the cell in the resting state. One of the types of RNA being degraded is mRNA (14).

Within one hour of PHA addition to the cells, the majority of events are now found to be occuring within the cell rather than at the membrane surface. Phosphorylation and dephosphorylation of nuclear proteins, acetylation of histones, de novo purine synthesis and RNA synthesis can all be measured at this time (12). It would seem that activation of the genome has occured.

Protein synthesis first becomes detectable 2 hours after PHA addition to resting lymphocytes (12). At 6 hours after the addition of PHA,

the mitogen can be washed out and the cell will still continue on through a cell cycle. At this time, the cyclic AMP level within the stimulated cells will be the same or lower than the cyclic AMP level in unstimulated resting lymphocytes (5). Smith et al conclude that cyclic AMP may be essential to the initiation of the events which lead to transformation, but is not essential and perhaps even detrimental to cell division (7).

From 6 to 24 hours after PHA addition, there is a continual increase in RNA and protein synthesis, an increase in the number of ribosomes and the production of the enzymes required for a successful S-phase - thymidylate synthetase, deoxycytidine deaminase, various kinases, etc. (12). At the end of this initial twenty-four hours of PHA stimulation, the lymphocyte is ready to enter into the S-phase. Thus, the "G_1" phase in a PHA stimulated lymphocyte is 24 hours long.

The S-phase in PHA stimulated lymphocytes is characterized by DNA synthesis as well as RNA and protein synthesis. The energy for these processes is provided by an increased glycolytic rate (12). Of considerable interest is the fact that the cyclic AMP level during S-phase must remain lower than the level in unstimulated cells if the cell cycle is to progress to completion (6). A decrease in alkaline phosphatase activity is also noticed during S-phase. Alkaline phosphatase inhibits incorporation of thymidine into DNA (13).

S-phase is followed by the G_2 phase which is characterized by chromatin condensation, decreased synthesis of RNA and synthesis of the proteins of the mitotic apparatus (13).

Mitosis follows G_2 and is chacterized by a cessation of all RNA synthesis and a greatly decreased protein synthesis (13).

Two or more such cycles of growth and division may occur following the initial stimulation with PHA (12).

ANTILYMPHOCYTE SERUM

In some experiments we used antilymphocyte serum (ALS) instead of PHA as the stimulant. The lymphocytes undergo blast transformation and cell division as demonstrated by the uptake of thymidine in vitro. ALS is useful as an immunosuppressive agent. It has been shown to prevent or delay the development of cell mediated immune responses such as allograft rejection (53,54), delayed hypersensitivity reactions (54), and graft versus host reactions (55). Tolerance can also be induced by ALS treatment (26). Its immunosuppressive actions occur in both primary and secondary cellular responses. ALS is also capable of suppressing the primary humoral response to certain antigens but is less effective in depressing secondary responses (16).

ALS would seem to affect the thymus dependent pool of long lived small lymphocytes in that its effects are localized to the periarteriolar areas of the spleen and the paracortical areas of the lymph nodes (17,18). In the presence of ALS these thymus dependent regions become depleted. These findings thus explain the ability of ALS to suppress the cellular immune responses. Histological findings show that ALS has no effect on the short lived variety of lymphocytes, most of which are believed to be derived from the bone marrow. These lymphocytes are involved in the humoral immune response. Small lymphocytes of the lymph and splenic follicles, and of the germinal centres remain unaffected by ALS treatment. These regions are not considered to be thymus dependent. Thus the relative inefficiency of antilymphocyte serum in suppressing the humoral immune response can be explained (18). Antigens whose humoral antibody responses can be depressed

by *in vivo* administration of ALS are those which require the synergistic proliferation of both thymus derived T cells and bone marrow derived B cells (19,20). Thus ALS suppresses by acting primarily on the thymus derived T cells.

The actual mechanism of action of ALS is not known precisely. A number of mechanisms have been postulated which are not mutually exclusive. One popular theory proposed originally by Lance and backed by considerable evidence is that the circulating, long lived, thymus dependent lymphocytes are selectively killed by ALS (18). The cytotoxic theory proposes that the lymphocytes are destroyed by the agglutinating and cytotoxic ALS antibodies. However, cytotoxicity titers *in vitro*, do not regularly correlate with the effects of the ALS on graft survival *in vivo*. Also, not all cytotoxic antisera are active (21).

The sterile inactivation theory of ALS action claims that *in vivo* administration of the antisera results in a nonspecific and functionally sterile proliferation of lymphocytes which pre-empts all their immunologic functions (22,26).

ALS may also act as an opsonin. It may coat lymphocytes and thereby make them palatable to macrophages in the reticulo-endothelial system (RES) (23,26).

The popular blindfolding hypothesis claims that ALS coats lymphocytes in such a way as to block the antigen receptors of the cell. Thus the cell cannot recognize the antigen (24,25,26). Surface-associated immunoglobulin is not detectable by the RICA technique on mouse spleen lymphocytes after exposure of the cells to ALS. Both 7S and 19S fractions of ALS are required to obtain this effect. The blocking of the surface

immunoglobulin can not be attributed to an anti-Ig present in the ALS (25). It has been suggested that steric hindrance or allosteric effects are responsible for the blockade of the receptor sites.

ALS blocks the *in vitro* production of macrophage migration inhibitory factor (MIF) when the lymphocytes are preincubated with ALS and then washed before addition of antigen. ALS added to supernatant containing MIF does not block MIF activity by direct neutralization (27). This further suggests that ALS acts directly by blocking receptors for the antigen on the surface of lymphocytes. The macrophages are still capable of phagocytosis with ALS present (28).

Thus, a number of mechanisms of action of ALS have been postulated. Which of these is the "true" mechanism is still a controversial issue. Since each postulated mechanism of action appears possible, more than one mechanism may be operating.

The timing and dosage of ALS used in clinical situations is especially important. Timing is important since there is practically no suppression of the humoral antibody response if ALS is given after the antigen (26).

Combination therapy involving the use of both ALS and drugs like methotrexate may prove more effective and selective than the use of either therapy alone. Thus, ALS, by synchronizing cell populations, may create a situation in which MTX can function most effectively in killing immunocompetent lymphocytes (26,29).

METHOTREXATE

Throughout these studies we have used methotrexate as our

immunosuppressive agent. This drug is a folic acid analogue, and as such interferes with the cell's normal folic acid metabolism. The various interconversions involved in folic acid metabolism as well as the way in which some of these forms affect nucleic acid synthesis are shown in Figure 1 (30,31,32,33).

The end product of folic acid metabolism in the liver is normally N^5-methyltetrahydrofolate (33). This form is transported in the serum, loosely bound to a variety of serum proteins, to various tissue types. Bone marrow, gut and rapidly proliferating tissues take up this transport form and convert it to active tetrahydrofolate (FH_4) (33). This form can then be converted to N^5N^{10}-methylene-FH_4 which is used as a substrate in the conversion of deoxyuridine monophosphate to thymidine monophosphate in the presence of the thymidylate synthetase enzyme. The N^5N^{10}-methylene-FH_4 simultaneously donates two hydrogen atoms and a methyl group to deoxyuridine monophosphate. The active FH_4 can also be converted to N^5N^{10}-methenyl-FH_4 (which is instrumental in the donation of the carbon 8 atom to the purine ring), or to N^{10}-formyl-FH_4 (which similarly donates the carbon 2 atom to the purine ring). For these latter two reactions, the folate is not oxidized and as a result is left in its still active tetrahydrofolate form (FH_4). Thus, during purine ring synthesis, there is no drain on the store of active folate (FH_4), but, for every molecule of thymidine monophosphate formed, one molecule of active folate is oxidized to dihydrofolate (FH_2) which is an inactive form. Thus, this latter reaction uses up active folate (30,31).

A great deal of information is available regarding the biochemical lesions created within cells by MTX. Since methotrexate is a folic acid

analogue, it interferes with normal folic acid metabolism and thus with the synthesis of thymidine monophosphate and the purine ring. Thus in cells treated with methotrexate, RNA and DNA synthesis and thus protein synthesis are all interfered with to a large extent.

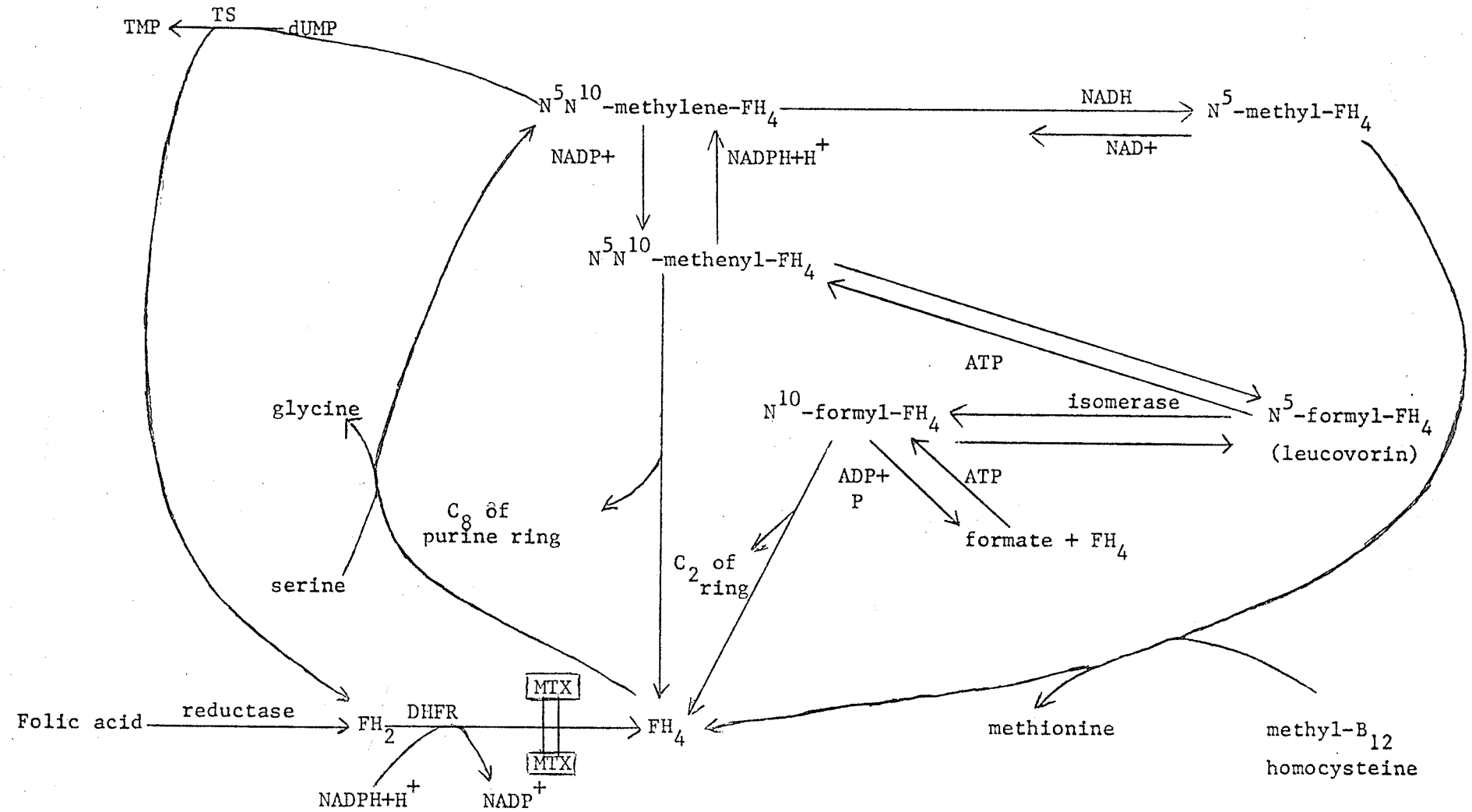
The exact interconversion in folic acid metabolism which MTX affects is well known. The drug binds to the enzyme dihydrofolate reductase (DHFR) and thereby prevents the normal binding of dihydrofolate (FH_2) to this enzyme (33,34,35). Thus, FH_2 cannot be reduced to active FH_4 when MTX is bound to DHFR. MTX functions as a "pseudoirreversible" inhibitor of DHFR at the cells pH (6.5). Only at a higher pH (7.6) can MTX be competitively removed from DHFR by high concentrations of FH_2. Thus at the cell pH, MTX has a much higher affinity for DHFR than does FH_2 and, once MTX binds, it cannot be easily removed by FH_2 (34). In humans (33,35) an increase in DHFR activity occurs after MTX treatment in normal and leukemic lymphocytes as well as in erythrocytes. Bertino calls this a type of "co-factor induction" (33). When MTX binds to DHFR, it seems to stabilize the enzyme against proteolysis and thus the enzyme activity appears to be increased.

The possibility also exists that MTX has multiple sites of action, not just as an inhibitor of DHFR. Evidence from <u>in vitro</u> studies in L-cells suggest that MTX might directly inhibit transformylase enzymes involved in de novo purine synthesis (42) and the thymidylate synthetase enzyme involved in converting deoxyuridine monophosphate to thymidine monophosphate (44). More evidence is required before any definite conclusions can be reached in this regard.

Transport of MTX across cell membranes is achieved by active

FOLATE METABOLISM

FIGURE 1



FH_4 = tetrahydrofolate

dUMP = deoxyuridine monophosphate

TMP = thymidine monophosphate

DHFR = dihydrofolate reductase

TS = thymidylate synthetase

transport (36,37,38). The transport carrier appears to have an equally high affinity for N^5-formyl-FH_4 and N^5-methyl-FH_4, the transport form of reduced folate (50). However, the affinity of DHFR for N^5-formyl-FH_4 is several orders of magnitude lower than that for MTX (38). The rate at which DHFR is inactivated by MTX binding is dependent upon the MTX influx rate in that the affinity of DHFR for MTX is several orders of magnitude greater than the affinity of the carrier for the drug. Accompanying the transport of MTX into the cell is a countertransport of exchangeable folate from the intracellular compartment.

Studies done in experimental systems have shown that the degree of cell membrane transport correlates well with the anti-tumour activity of MTX and thus, cells which transport MTX poorly are most resistant to its effects (37,39,40).

In our attempts to elucidate the mode of action of MTX, we tried to prevent MTX from exerting its effects. Various substances have been added simultaneously with MTX or within a few hours after MTX addition to cell cultures. Leucovorin (N^5-formyl-FH_4) can reverse MTX effects if given not more than 4 hours after MTX injection into a mouse (41). Similar results can be obtained <u>in vitro</u> with L-cells (42,43), the dose and timing of administration of leucovorin varying with the conditions of the culture. Leucovorin does not compete with MTX for DHFR. Instead, since leucovorin is a reduced form of folate, it can provide the MTX-treated cells with an active form of the vitamin, thus by-passing the MTX effect. Since leucovorin must be given within 4 hours of MTX administration, it seems likely that MTX-affected cells can withstand a certain period of dihydrofolate reductase inhibition, but if this period

is too long, irreversible damage ensues (41).

Attempts to prevent MTX effects by simultaneous addition of nucleosides to cell cultures have been carried out by numerous investigators. Borsa found that adding deoxyadenosine with MTX increased the amount of cell kill relative to the killing done by MTX alone. MTX, thymidine and deoxyadenosine added simultaneously resulted in complete prevention of MTX effects. Thymidine and MTX together prevent about 50% of the cell kill created by MTX alone (43). From this data Borsa claims MTX works to kill cells by the mechanism of unbalanced growth postulated by Cohen (48). This, in effect, suggests that cells treated with MTX die due to a thymine lack. Hryniuk and Bertino (45) were able to "completely prevent" the suppressive effects of $1x10^{-6}$M MTX on H^3-TdR incorporation in L5178Y cells by the simultaneous addition of $2x10^{-5}$M hypoxanthine with the MTX to the cultures. More important, the lethal effects as measured by cloning, were considerably reduced. They suggest that the cell killing not prevented by hypoxanthine is preventable by thymidine addition. However, the considerable protection from MTX-kill provided by hypoxanthine indicates that in L5178Y cells there is a purineless death as well as a thymineless death.

In all studies reported, the cell killing effects of MTX are greater in rapidly proliferating as compared to resting-state populations. Since MTX is a cycle active drug (S-phase), this is understandable. Recent studies by Hryniuk indicate however, that individual logarithmically growing L5178Y cells passing through the S-phase also suffer greater metabolic pertubations than cells from resting cultures passing through this same phase, as judged by the greater suppression by drug treatment of DNA, RNA and protein synthesis (45,46). In effect, in faster growing populations, not

only would more S-phase cells be exposed to MTX but each exposed cell is more severely disturbed and thus more likely to be killed. Thus it seems the greater kill in log cultures cannot be explained solely on the basis of the numbers of S-phase cells being exposed to the drug. Kinetic explanations alone cannot explain the increased kill.

MATERIALS AND METHODS

I. Preparation of in vitro guinea pig lymphocyte cultures

Female Hartley strain guinea pigs were purchased from Camm Research Inc. The animals were immunized in each footpad with 12.5 μg of dinitrophenol-poly-L-lysine (DNP-PLL) suspended in water and emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco). Three to six weeks after immunization axillary, cervical, inguinal, femoral and popliteal lymph nodes were removed in a sterile manner and teased into a single cell suspension in culture medium. Tissue fragments were allowed to settle and the supernatant cell suspension was pipetted off. The cells were then spun at 800 rpm for 10 minutes. The supernatant was then decanted from the cell button, the cells washed in medium once more and spun again. The number of cells was counted in a hemocytometer. Viability was determined using the trypan blue exclusion method. Average viability was approximately 70%.

The culture medium consisted of Eagles MEM for spinner cultures (Gibco) with added penicillin (50 units/ml), streptomycin (50 μg/ml), glutamine and decomplemented guinea pig serum at a final concentration of 10% in PHA stimulated cultures. Rabbit-anti-guinea pig lymphocyte-sera (ALS) or normal rabbit sera (NRS) was added to a final concentration of 10% in ALS experiments. The optimal concentration for ALS induced stimulation was shown to be either 7.5% or 10% (depending on batch). The ALS and NRS were also decomplemented.

Cultures were set up in 75x110 mm plastic tissue culture tubes (Falcon Plastics) at $4x10^6$ viable cells per tube in 2 ml of medium. Cultures were incubated at 37 ^{o}C in a moist 5% CO_2 atmosphere. The optimal stimulatory concentration of PHA (Warner Chilcott) was found to be 0.5%.

II. Methotrexate treatment

At the appropriate time after addition of the stimulant to the culture,

0.1 ml of methotrexate sodium was added to the culture tubes to give a final concentration of $1x10^{-5}$M. This was the optimal dose.

III. Deoxynucleoside pulse labelling

After 4 hours incubation with MTX, 1μCi tritiated thymidine (H^3-TdR, specific activity 1.9mCi/mmole, obtained from Amersham Searle) or 5μCi tritiated deoxycytidine (H^3-CdR, S.A. 5mCi/mmole) was added to each tube. After a futher 5 to 25 minutes of incubation, the reaction was stopped by the addition of ice cold 5% perchloric acid (PCA). A total of 8 ml of 5% PCA was added at the duration of the incubation in the label. The samples were then stored at 4°C overnight. The next day the DNA isolation procedure was begun as has been described (46). In studies of RNA synthesis, the uptake of H^3-uridine label was stopped by the addition of 8 ml of ice cold cetrimide (Eastman Kodak).

IV. DNA isolation procedure

Centrifuge tubes containing the precipitated cell substances in PCA were removed from the cold and spun at 2500 rpm for 5 minutes. The supernatant was aspirated completely. The precipitate was mixed on a vortex in another 8 ml of PCA. This was then centrifuged at 2500 rpm for 5 minutes. This procedure was repeated twice more. After the third wash in 5% PCA, the supernatant was aspirated and 0.5 ml of 5% PCA was added to the precipitate. The sample was mixed and hydrolysed at 85°C for 15 minutes. After the 15 minute hydrolysis, the samples were placed in an ice bucket for 10 minutes to stop the hydrolysis. The DNA is now found in the supernatant whereas the RNA and protein fraction of the cell are still precipitable. After 10 minutes on ice, the samples were mixed on a vortex and then centrifuged at 2500 rpm for 5 minutes. All the supernatants were suctioned off with a Pasteur pipette and placed into plastic scintillation vials (Amersham Searle) containing 20 ml of cold

scintillation fluid.

The scintillation fluid consisted of 100 mg 1,4-Bis-[2-(5 phenyl-oxazolyi)]- benzene (POPOP), 8 grams 2,5-Diphenyloxazole (PPO), 2 liters toluene and 1000 ml absolute ethanol. Each scintillation vial contained 20 ml of this mixture.

The radioactivity in each vial was counted in a "Packard Tri-Carb" liquid scintillation counter and recorded on a teletype hook-up as counts per minute. The rate of synthesis was then established using a linear regression program and an Olivetti table top computer. The rates were then established as disintegrations per minute /minute pulse /10^6 cells.

V. RNA isolation procedure

The procedure used to isolate radioactive RNA was adapted from a method described by Kennell (58). The labelling of the cells with H^3-uridine was stopped by the addition of 8 ml of ice cold cetrimide which precipitates out the nucleic acids (59). The cellular remnants in the cetrimide were transferred into centrifuge tubes. Immediately after this step, the samples were spun at 1700 rpm for 5 minutes. The supernatant was suctioned off and the precipitate was mixed on a vortex. Ten ml of ice cold 10% trichloro-acetic acid (TCA) were added to each sample and the centrifuge tubes were placed in ice for 30 minutes. The tubes were then centrifuged at 2500 rpm for 5 minutes and the supernatant was suctioned off. The samples were washed twice more with 10 ml of ice cold 10% TCA and the supernatants suctioned off. After the last wash, 0.5 ml of 0.6 N NaOH was added to each sample, the tube was mixed on a vortex and then placed in a water bath at 37°C for 60 minutes. After this, the tubes were put on ice and 0.2 ml of 50% TCA was added to each sample. The net result of these procedures is that the RNA will remain in the solution but that the DNA and protein fractions will be precipitable. The

samples were left on ice for 30 minutes. After this time, they were immediately spun at 2500 rpm for 5 minutes. The supernatant was then suctioned off and put into appropriate scintillation tubes. The scintillation fluid used was the same as that used to count the radioactive DNA particles.

VI. Rescue and saving experiments

In order to try to establish the mechanism of action of MTX in our system, we tried to prevent its immunosuppressive effects by adding nucleosides simultaneously with the MTX ("saving") or by adding calcium leucovorin to cells which had been pretreated with MTX for several hours prior to the leucovorin addition ("rescue").

The nucleosides used were: deoxyadenosine, adenosine, guanosine, inosine, hypoxanthine, and thymidine (Nutritional Biological Corporation). These were added at various concentrations and in various combinations.

The leucovorin was added at 10^{-4}M concentration in each experiment. American Cyanimide Co. supplied the calcium leucovorin as a gift.

Fractional rates were used as an index of the amount of suppression of radioactive label incorporation into DNA or RNA by MTX. Rates in the presence of MTX were expressed as decimal fractions of the control rate and thus designated as "fractional rates".

VII. Preparation of ALS in rabbits

A large number of immunizing and bleeding schedules were used for the preparation of the ALS. The method which worked best was as follows : initially $500x10^6$ guinea pig lymphocytes emulsified in complete Freund's adjuvant (CFA) were injected into the footpads. Fourteen days later the rabbit was boosted intra-muscularly with $500x10^6$ lymphocytes emulsified in CFA. Twenty-one days after the initial immunization, 50 ml of blood

was removed from the rabbit by cardiac puncture. The next day the rabbit was exsanguinated by cardiac puncture.

An alternative method involved boosting the rabbit intra-venously with $500x10^6$ lymphocytes in 1 ml of medium. The ear vein was the site of injection.

VIII. Animal handling

The guinea pigs were kept in groups of 8 to 10 in large cages and had free access to food and water.

Rabbits were individually caged and also allowed free access to food and water.

The method of immunization of the animals has been described.

EXPERIMENTAL

I. MTX effects on RNA synthesis as a function of time after PHA stimulation

Guinea pig lymphocyte cell cultures were established with 4.0×10^6 cells per tube in a volume of 2 ml of MEM, penicillin, streptomycin, and glutamine. The medium also contained 10% guinea pig serum. Rates of H^3-uridine incorporation and fractional rates of incorporation in MTX-treated cultures were obtained at 0, 6, 12, 24, and 48 hours after the addition of PHA. Control studies were done in which no stimulant was added.

In control cultures, the rates of synthesis at 0, 6, and 12 hours were not significantly different from the corresponding rates in PHA-stimulated cultures. However, in the absence of PHA, the H^3-uridine incorporation rates at 24 and 48 hours fell to levels which were significantly different ($p<0.01$) from the rates obtained at these times in PHA-stimulated cultures.

PHA stimulated cultures show a very slow increase in incorporation of the label over the first 12 hours but reach maximal rates by 24 hours. By 48 hours, the RNA synthetic rate is dropping towards early levels (Fig.2, Table 1).

The fractional rates in stimulated cultures show no MTX effects until 24 and 48 hours, the times at which RNA synthesis is at its peak. At these times MTX suppresses RNA synthesis to 61% and 57% of the rates obtainable in untreated cultures. At 0, 6, and 12 hours, the fractional rates remain essentially at 1.0 .

The fractional rates in unstimulated control cultures show no

TABLE 1

RNA synthesis following PHA stimulation(H^3-uridine)(GPS enhanced medium)

	Time in PHA(hours)				
	0	6	12	24	48
H^3-uridine incorp. rate	81.14±10.61	91.0±8.82	108.0±9.46	232.0±74.66	138.5±46.33
Fractional rate	0.95±0.07	1.20±0.17	1.16±0.13	0.61±0.06	0.57±0.05

	Control Cultures(No PHA)				
	0	6	12	24	48
H^3-uridine rate	62.25±5.54	81.43±40.52	109.45±12.61	60.0±19.11	27.66±8.08
Fractional rate	0.83±0.10	0.98±0.03	1.28±0.12	1.13±0.13	1.05±0.04

PHA data represent means and standard errors of 8 experiments.

Control data represent means and standard errors of 4 experiments.

$$\text{Fractional rate} = \frac{\text{rate incorporation in MTX treated cultures}}{\text{rate incorporation in untreated cultures}}$$

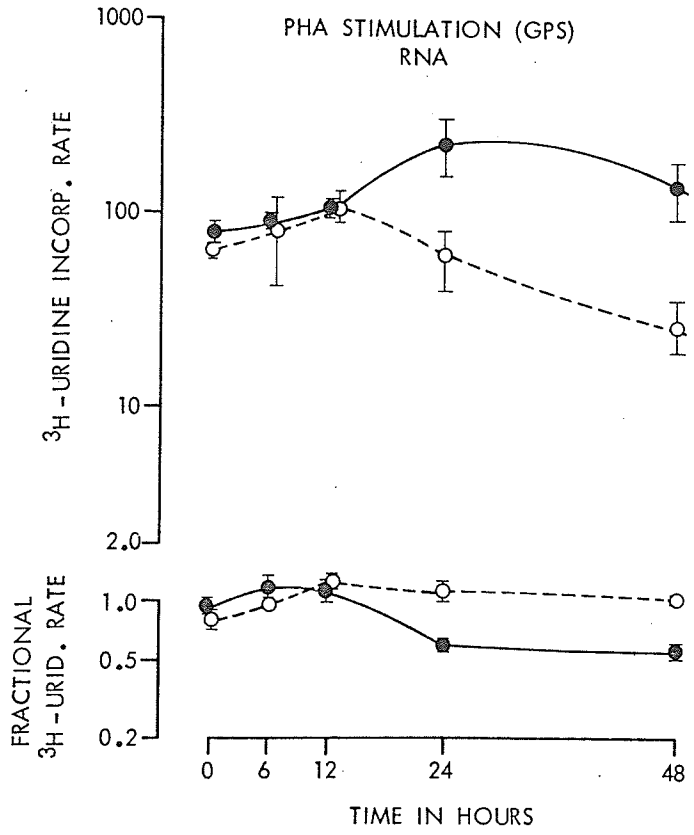


FIGURE 2 Rate of ^{3}H-uridine incorporation and fractional rate in cells cultured in GPS-supplemented medium after addition of PHA (0.5%). Broken lines represent control unstimulated cultures. Each point represents mean ± standard error.

MTX effects at any time. The fractional rate remains at 1.0 throughout indicating that MTX does not seem to suppress the spontaneous RNA synthesis occurring in these cultures.

II. MTX effects on DNA synthesis as a function of time after stimulation

1. PHA stimulation in GPS enriched medium

Rates of DNA synthesis in PHA stimulated and control (no stimulant) cultures were obtained at 0, 6, 12, 24, and 48 hours after incubation began. Fractional rates of synthesis were also obtained at these times.

Cells cultured in medium containing 10% guinea pig serum (GPS) but no stimulants show a gradual decline of H^3-TdR incorporation with time(Table 2 ,Fig. 3). In these cultures the fractional rate does not differ significantly from 1.00 (Cochrane-Cox weighted t-test).

In PHA stimulated cultures, H^3-TdR incorporation declines over the first 12 hours and is not significantly different from the rate in unstimulated control cultures. Maximum rates of DNA synthesis are reached at 48 hours in these studies (which were only carried out to 48 hours).

Significant suppression of DNA synthesis by MTX was seen in PHA-stimulated cultures at 6, 12, 24, and 48 hours as evidenced by significantly decreased fractional rates as compared to unstimulated cultures ($p<0.05$ at 6, 24, 48 hours; $p<0.01$ at 12 hours).

Thus the effect of MTX on H^3-TdR incorporation occurs as early as 6 and 12 hours after addition of PHA, at a time when the rate of DNA synthesis is minimal, and remains constant, even though DNA synthesis increases markedly at 24 and 48 hours.

TABLE 2

DNA synthesis following PHA stimulation (H^3-TdR) (GPS enhanced medium)

	Time in PHA (hours)				
	0	6	12	24	48
H^3-TdR rate	31.28±7.53	18.0±3.74	16.55±4.64	54.85±13.83	221.88±40.29
Fractional rate	1.18±0.13	0.73±0.06	0.57±0.06	0.49±0.05	0.56±0.03

	Control Cultures (No PHA)				
	0	6	12	24	48
H^3-TdR rate	45.83±8.6	13.80±2.87	8.33±3.67	2.19±0.46	1.24±0.27
Fractional rate	0.97±0.09	1.01±0.06	0.96±0.03	1.09±0.23	1.42±0.17

PHA data represent means and standard errors of 9 experiments.

Control data represent means and standard errors of 4 experiments.

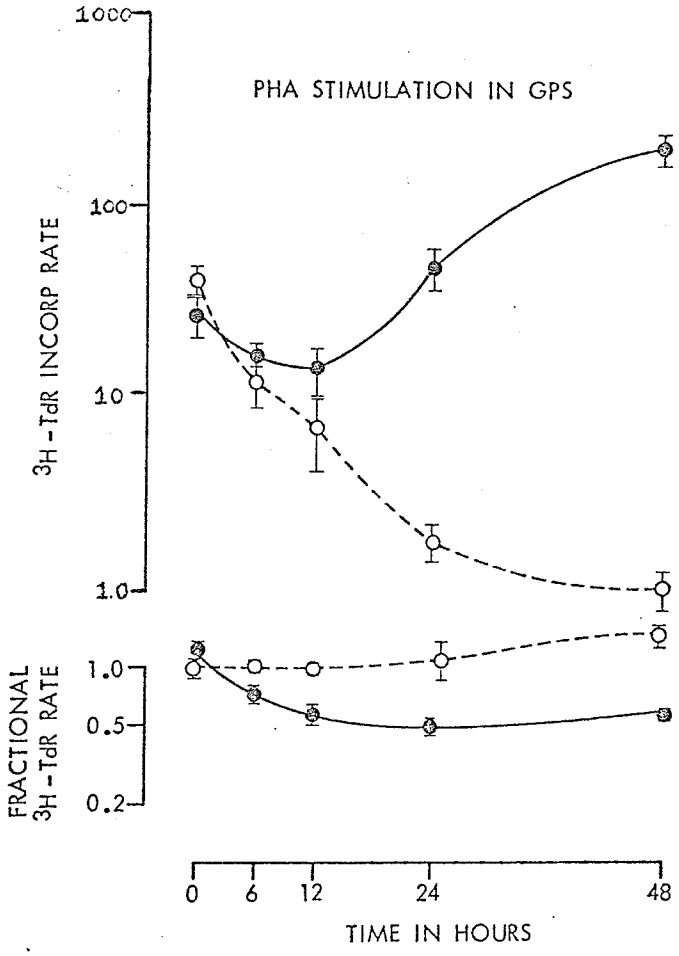


FIGURE 3 Rate of ^{3}H-TdR incorporation and fractional rate in cells cultured in GPS-supplemented medium after addition of PHA (0.5%). Broken lines represent the control unstimulated cultures. Each point represents mean ± standard error.

2. ALS stimulation

Rates of DNA synthesis in the presence of ALS or NRS were obtained at 0, 6, 12, 24, and 48 hours after the cultures were begun. Fractional rates were also obtained for MTX treated cultures grown in ALS or NRS supplemented medium. NRS cultures serve as controls.

Cultures grown in NRS supplemented medium show a steady decrease of H^3-TdR incorporation with time. When MTX is added to such cultures, the fractional rate never differs significantly from 1.00 (Fig. 4, Table 3). In other words, no stimulation or suppression of H^3-TdR incorporation occurs as a result of addition of MTX to cultures grown in NRS supplemented medium.

Cultures grown in the ALS supplemented medium show an initial decrease in H^3-TdR incorporation, with a subsequent increase beginning at 12 hours after the addition of ALS, but the rate at 12 hours is still lower than at 0 hours. By 24 hours, DNA synthesis in ALS treated cultures is proceeding rapidly and increases to a maximum rate at 48 hours.

In contrast to PHA stimulated cultures, ALS stimulated lymphocytes incubated in MTX only show significant suppression of H^3-TdR incorporation at 12 hours ($p<0.01$) while the fractional rates at 0, 6, 24, and 48 hours do not differ significantly from the corresponding NRS fractional rate or from 1.00 .

The rate of DNA synthesis at 12 hours in MTX treated cultures is 55% of the rate in the corresponding untreated cultures.

Thus in this system, the effect of MTX on H^3-TdR incorporation is restricted to a short interval of time at the beginning of the S-phase. Even when maximal proliferative activity occurs at 48 hours there is no suppressive

TABLE 3

DNA synthesis following ALS stimulation (H^3-TdR)

	Time in ALS (hours)				
	0	6	12	24	48
H^3-TdR rate	69.22±12.53	48.50±9.05	58.0±10.15	264.13±45.95	352.42±62.20
Fractional rate	0.93±0.07	0.75±0.08	0.55±0.03	0.87±0.06	1.22±0.10

	Control Cultures Time in 10% NRS (hours)				
	0	6	12	24	48
H^3-TdR rate	70.50±23.73	47.00±18.83	32.0±11.53	5.58±1.87	2.66±0.83
Fractional rate	1.00±0.06	0.91±0.05	0.92±0.03	0.82±0.14	0.86±0.11

ALS data represent means and standard errors of 8 experiments.

Control data represent means and standard errors of 6 experiments.

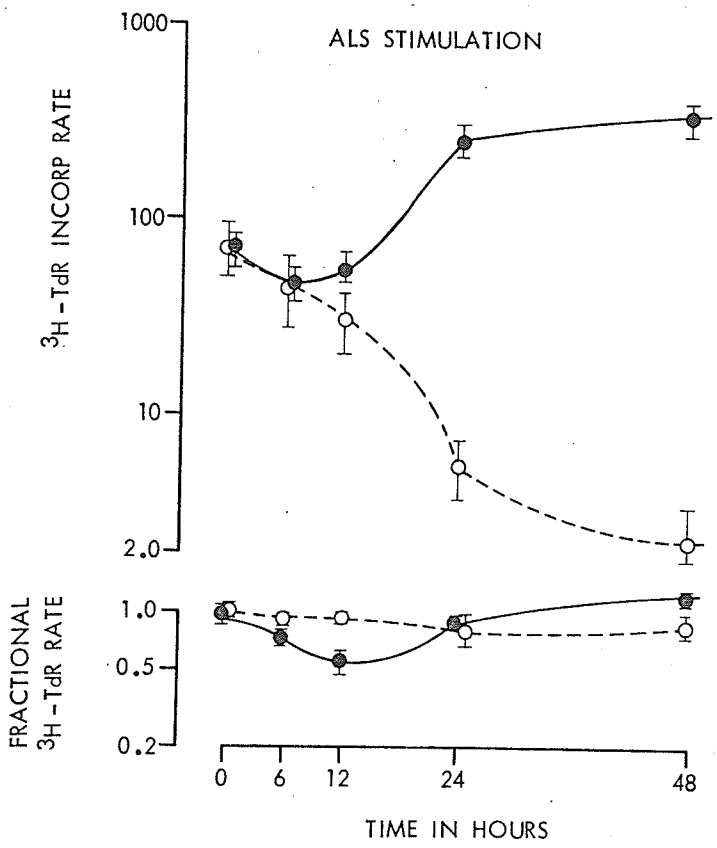


FIGURE 4 Rate of ^{3}H-TdR incorporation and fractional rate in cells cultured in ALS-supplemented medium. Broken lines represent control studies done in NRS-supplemented medium. Each point represents mean ± standard error.

effect.

3. PHA stimulation in NRS enriched medium

Rates of DNA synthesis in PHA stimulated cultures grown in medium containing 10% normal rabbit serum (NRS) were obtained at 0, 6, 12, 24, and 48 hours after incubation. Fractional rates were also obtained at these times (Fig.5, Table 4).

The pattern of incorporation of the radioactive label resembles the pattern established in the PHA stimulated cultures grown in medium enhanced with guinea pig serum. An initial decline in incorporation of ^{3}H-TdR occurs between 0 and 12 hours, followed by an increase in incorporation at 24 hours. By 48 hours, the rate of DNA synthesis is at its maximum as measured by incorporation of the label.

The fractional rate of DNA synthesis in these cultures closely resembles the pattern established in ALS stimulated cultures. Only at 6 and 12 hours is there any significant suppression of synthesis by MTX treatment. At 24 and 48 hours, the fractional rates of synthesis are essentially 1.0.

Thus, the pattern of stimulation in these cultures resembles the pattern in PHA stimulated cultures grown in GPS enhanced medium, but the effect of MTX more closely resembles that seen in ALS stimulated cultures treated with methotrexate.

TABLE 4

DNA synthesis following PHA stimulation (H^3-TdR) (NRS enhanced medium)

	Time in PHA (hours)				
	0	6	12	24	48
H^3-TdR rate	68.43±19.35	42.70±16.97	28.66±7.04	80.59±32.11	367.99±160.47
Fractional rate	1.12±0.05	0.53±0.11	0.63±0.19	0.98±0.16	0.95±0.05

PHA data represents means and standard errors of 5 experiments.

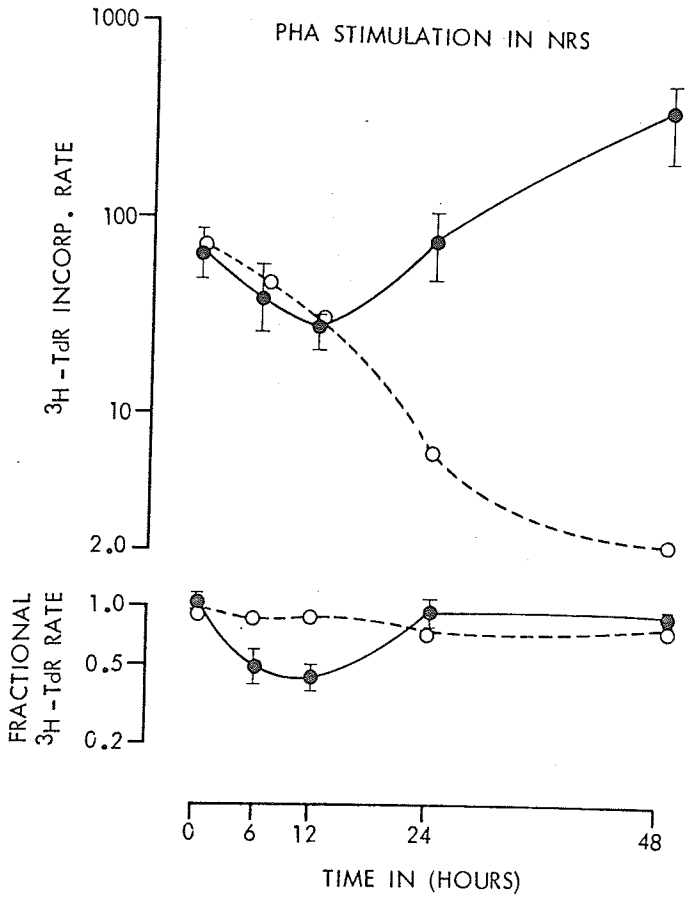


FIGURE 5 Rate of ^{3}H-TdR incorporation and fractional rate in cells cultured in NRS-supplemented medium after addition of PHA (0.5%). Broken lines represent control studies done in NRS-supplemented medium without PHA. Each point represents mean ± standard error.

III. Prevention of MTX effects on RNA synthesis

Twenty-four hours after PHA stimulation, RNA synthesis is at its peak as measured by H^3-uridine incorporation and suppression of RNA synthesis by MTX is maximal with a fractional rate of 0.61±0.06 in the MTX treated cultures.

Nucleosides at various coneentrations were added to these cell cultures at the same time as the methotrexate. The cells were then incubated in the "(MTX + Nucleoside)" mixture for the usual 4 hours before being labelled and processed. It was shown that the addition of nucleosides alone to the cultures produced either an increase or decrease in the H^3-uridine incorporation rate relative to control cultures grown in GPS enriched medium. Therefore, it is appropriate to compare the rate of synthesis in cultures treated with (MTX + Nucleoside) to the rate in cultures treated with the nucleoside alone. This eliminates the error which would occur if the nucleoside increased the incorporation rate irregardless of any " saving ".

By this technique it was shown that the addition of any one of adenosine, guanosine, inosine, hypoxanthine, or deoxyadenosine simultaneously with MTX to cultures results in the elimination of MTX suppression on RNA synthesis (Table 5, Fig. 6). When deoxyadenosine alone was added, the results were inconsistent, since only one concentration was effective in preventing the block, whereas other concentrations (both above and below the effective one) were not.

IV. Prevention of MTX effects on DNA synthesis

At 24 hours after PHA addition the rate of DNA synthesis is increasing rapidly as measured by incorporation of H^3-TdR and is suppressible

TABLE 5

RNA saving results: PHA stimulation for 24 hours

Nucleoside used	F_R of $\frac{\text{(MTX and nucleoside)}}{\text{(Nucleoside alone)}}$	F_R of $\frac{\text{(MTX alone)}}{\text{(Nucleoside alone)}}$
Mix of 4 (adeno,Hx, d.adeno,guano) each at $1x10^{-5}$ M	1.20±0.22*	0.72±0.11
Adenosine		
$1x10^{-5}$	1.09±0.17	0.69±0.10
$2x10^{-5}$	1.15±0.23	-
Inosine		
$1x10^{-5}$	0.83±0.12	0.58±0.09
$2x10^{-5}$	0.94±0.07	-
Hypoxanthine		
$1x10^{-5}$	1.10±0.13	0.54±0.08
Guanosine		
$1x10^{-5}$	0.90±0.07	0.53±0.08
d.Adenosine		
$1x10^{-5}$	0.61±0.09	0.52±0.08
$2x10^{-5}$	1.30±0.18	-
$4x10^{-5}$	0.69±0.06	0.66±0.05

* Numbers represent $\bar{x}$ ± S.D.

d.adeno- deoxyadenosine guano - guanosine
Hx - hypoxanthine adeno - adenosine

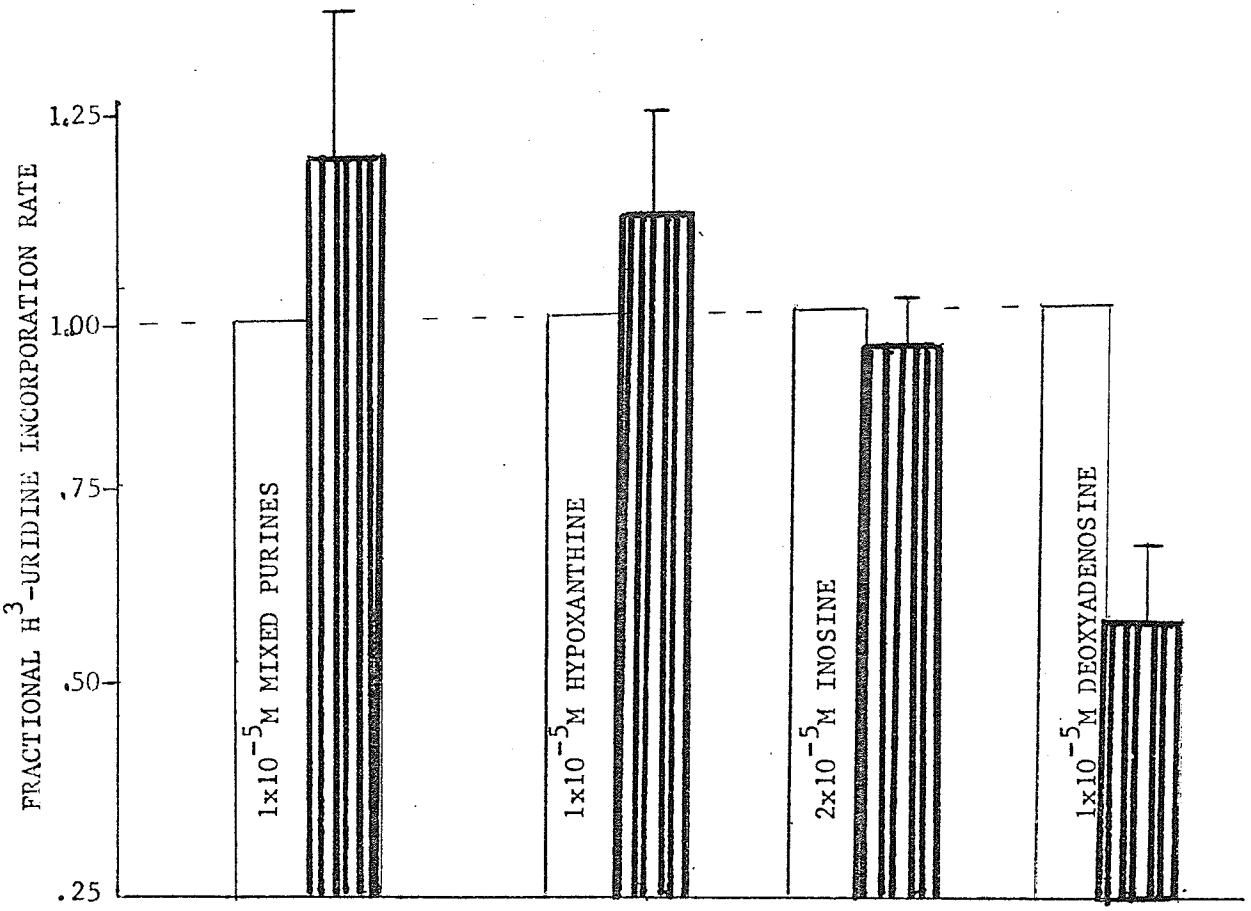


Fig. 6 Fractional rate of H^3-uridine incorporation 24 hours after PHA addition, showing the effect on cell cultures of combined treatment with MTX + various nucleosides (shaded bars) as compared to cultures treated with nucleosides only (open bars). $\bar{x} \pm$ S.D.

by MTX therapy, with an average fractional rate of synthesis of 0.49±0.05 in MTX treated cultures (Table 2).

In order to elucidate the mechanism of action of MTX in proliferating lymphocyte cultures, we attempted to prevent the MTX induced suppression of DNA synthesis by the simultaneous addition of various nucleosides.

Since the addition of nucleosides alone frequently results in an increased incorporation of labeled precursors in cultures not treated with MTX, it was necessary to use nucleoside treated cultures as controls rather than the customary "medium only" cultures.

It also became apparent that H^3-TdR could not be used as labelled precursor, since the thymidine content of the label was sufficiently high ($5x10^{-7}$M) to exert a degree of "saving" by itself (see Table 6). Therefore, we chose H^3-CdR since its metabolism is probably independent of folate pathways (30,32,33) and will therefore not interfere with the MTX effects. Thus it was hoped that the H^3-CdR would measure the combined effect of MTX on purine and pyrimidine synthesis, whereas the H^3-TdR label would measure primarily the effect of MTX on purine synthesis.

[An experiment was performed which showed that H^3-CdR was not being incorporated into RNA since there was a negligible uptake of the deoxyribonucleotide label into the RNA. This is expected since there is no known direct pathway by which a deoxyribose can be converted to a ribose form. The nucleoside must first be converted to a free base by a phosphorylase reaction and then the base must travel one of the available salvage pathways of nucleotide synthesis. In the salvage route the base must bind with a ribose form of the sugar if the resulting nucleotide is to be incorporated into RNA (30,56).]

Table 6 and Fig. 7 show that in concentrations of $2x10^{-4}M$ inosine (source of purine) will result in some saving of MTX induced suppression of DNA synthesis as indicated by an increase from 27% to 47% of the control rate of synthesis. Thymidine alone increases the rate of DNA synthesis from 13% to 60% over the control rate. It is obvious that thymidine alone or purine alone cannot completely prevent the MTX induced suppression of DNA synthesis. The combination of thymidine and inosine together will, however, result in "saving" the cultures from the MTX effects. It was shown that $10^{-6}M$ thymidine together with $10^{-4}M$ inosine represent the optimal and minimal concentrations required to offset the suppression of DNA synthesis induced by $1x10^{-5}M$ MTX (as indicated by a fractional rate of 1.00).

V. Rescue of MTX treated cultures by leucovorin

In these experiments we wished to investigate the effects of leucovorin on MTX treated cultures. It was hoped that leucovorin would be capable of reversing the effects of MTX since this vitamin (N^5-formyl-FH_4) supplies the cell with an active form of folate. The 24 hour cultures were pre-treated with MTX ($1x10^{-5}M$) for 2 or 3 hours, after which $1x10^{-4}M$ leucovorin was added for a further 2 to 4 hours. The MTX was not removed by washing prior to the addition of leucovorin.

The results are summarized in Table 7. Once again H^3-CdR was used as the label. The addition of leucovorin to cultures not treated with MTX did not affect the rate of DNA synthesis substantially. Untreated cultures were therefore used as controls and all fractional rates of synthesis are calculated relative to "untreated" control rates.

TABLE 6

DNA saving results: PHA stimulation for 24 hours (H^3-CdR)

Nucleoside used		F_R of (MTX and nucleoside) / (Nucleoside alone)	F_R of (MTX alone) / (Nucleoside alone)
Inosine	2×10^{-4}	0.47±0.06*	0.27±0.03
	2×10^{-6}	0.15±0.02	0.13±0.02
Thymidine	10^{-6}	0.37±0.06	0.14±0.02
	10^{-4}	0.60±0.09	0.13±0.01
Thymidine/ and Inosine			
10^{-7}TdR/10^{-4}Inos		0.40±0.04	0.38±0.06
10^{-6}	/10^{-4}	1.10±0.15	0.41±0.07
10^{-5}	/10^{-4}	0.88±0.09	0.34±0.05
10^{-4}	/10^{-4}	0.97±0.07	0.06±0.01
10^{-6}	/10^{-5}	0.15±0.03	-
10^{-4}	/10^{-5}	0.35±0.06	0.06±0.01
10^{-7}	/10^{-5}	0.18±0.02	-
--- Using a H^3-TdR label (5×10^{-7}M TdR)---			
4 Purine mixture 2×10^{-5}M each		1.35±0.14(H^3-TdR) 0.63±0.08(H^3-CdR)	0.56±0.06 0.78±0.08
Inosine 2×10^{-5}M		3.05±0.46(H^3-TdR)	-

* Numbers represent $\bar{x}$ ± S.D.

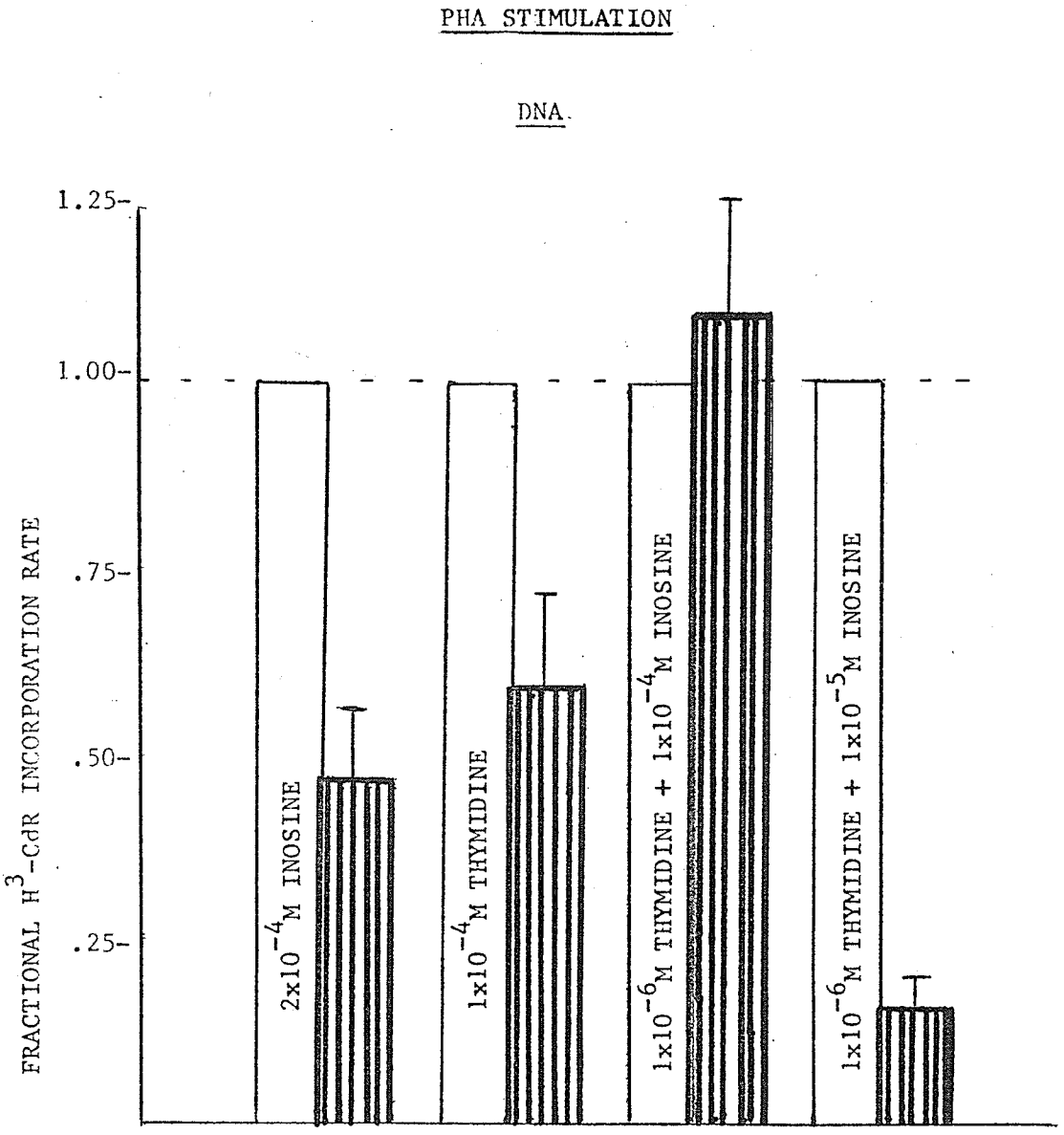


Fig. 7 Fractional rate of H^3-CdR incorporation 24 hours after PHA addition, showing the effect on cell cultures of combined treatment with MTX and various nucleosides (shaded bars) as compared to cultures treated with nucleosides only (open bars). $\tilde{x} \pm$ S.D.

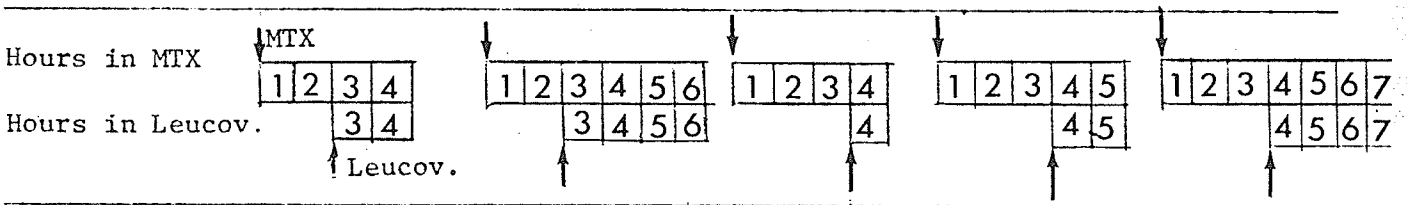
It can be seen that cultures pretreated with MTX for 2 hours may be "rescued" by a further 4 hours of incubation in leucovorin; this treatment returns the fractional rate from 0.52 to 0.87 .

In another experiment, leucovorin was added for an additional 4 hours after 3 hours preincubation with MTX and increased the fractional rate from 0.64 to 0.92, relative to the control value of 1.00 . Thus an exogenous supply of active folate is capable of reversing at least some of the biochemical effects of MTX.

In cultures where leucovorin was present for only 1 or 2 hours following pretreatment in MTX, no rescue was observed. Cultures in which leucovorin was present for 3 hours following pretreatment with MTX were technically unacceptable.

TABLE 7

Leucovorin rescue results : PHA stimulation for 24 hours
(GPS enhanced medium)



Fractional H^3-CdR rate ± S.D.					
Leucov./Medium	-	1.00±0.10	0.92±0.18	0.94±0.16	1.12±0.22
MTX/Medium	0.58±0.13	0.52±0.10	0.67±0.15	0.36±0.07	0.64±0.11
MTX+Leucov./ Medium	0.54±0.12	0.87±0.10	0.64±0.15	0.42±0.08	0.92±0.15

Arrows indicate time of addition of leucovorin relative to time of MTX addition.

DISCUSSION

I. Rate of proliferation and degree of suppressibility

It is currently believed that cells from rapidly proliferating populations are more susceptible to kill by certain antimetabolites, including MTX, simply because more cells pass through the drug sensitive phase of the cell cycle per unit time (2,47). Hryniuk has indicated, however, that logarithmically growing L5178Y cells passing through the MTX sensitive phase (S-phase) also suffer greater metabolic pertubations than do proliferating cells from resting cultures, as judged by the greater suppression by MTX treatment of DNA, RNA and protein synthesis (45). Thus the increased death of these cells following exposure to MTX is not only due to the increased rate at which they pass through the drug sensitive phase of the cell cycle but also due to the heavier damage these cells suffer with exposure. The studies reported here suggest in addition that within populations of rapidly growing cells, the rate of H^3-TdR incorporation at any given point in time correlates poorly with susceptibility to the biochemical effect of MTX.

(1) When lymphocytes are stimulated with ALS, they are most sensitive to MTX before they reach the peak of H^3-TdR incorporation. In fact, these cells are least susceptible to the biochemical effects of MTX at the height of H^3-TdR incorporation at 24 and 48 hours (fractional rates of incorporation are respectively, 0.87 and 1.22). Peak suppression of ALS cultures occurs at 12 hours (fractional rate 0.55) when the rate of DNA synthesis is just about to enter into the log phase (Table 3).

(2) Lymphocytes grown in GPS enhanced medium and stimulated by PHA are also sensitive to MTX effects before they reach the peak of H^3-TdR incorpora-

tion as indicated by significantly decreased fractional rates at 6 and 12 hours and while the cells remain suppressible when proliferative activity is maximal at 24 and 48 hours the degree of suppression is no greater than that occurring at 12 hours in these cultures (Table 2). Thus even in this system an increased rate of H^3-TdR incorporation is not necessarily associated with increased suppressibility to MTX.

(3) This is even more striking when PHA-stimulated cultures are grown in NRS enriched medium: here peak proliferative activity is not accompanied by any measurable MTX effects. Suppression occurs only at 6 and 12 hours when H^3-TdR incorporation is at its lowest (Table 4).

(4) The rates of H^3-TdR incorporation in control cultures do not differ significantly from the rates in the corresponding stimulated cultures at 6 and 12 hours, but control cultures are not suppressible by MTX addition, whereas the stimulated cultures are suppressible at these times both in PHA and ALS stimulated cultures. Thus it is not simply the rate of DNA synthesis which determines the effectiveness of MTX in PHA stimulated cultures, but some cellular event related to stimulation.

This data therefore argues against a strictly kinetic interpretation of the degree of suppression obtainable by MTX. It may also point to the possible need for critical timing of MTX therapy in relation to the administration of agents like heterologous antilymphocyte sera in vivo . However, whether or not these in vitro studies can be applied to in vivo situations is not presently known.

II. Factors influencing suppressibility

Both the type of stimulant used and the type of sera present in the

culture medium determine the effectiveness of MTX in these cultures.

When PHA is the stimulant, MTX effects on H^3-TdR incorporation are observed as early as 6 hours after stimulation and are maintained for the duration of the culture. When ALS is used to stimulate the lymphocytes, MTX exerts a suppressive effect on H^3-TdR incorporation only at 12 hours. If PHA stimulated lymphocytes are cultured in medium containing NRS, MTX effects are restricted to 6 and 12 hours, even though the rabbit serum does not appear to affect the rates of H^3-TdR incorporation.

How the rabbit serum exerts this effect is not presently known. Perhaps the rate of uptake of MTX is directly or indirectly affected by an action of the serum on the cell membrane. An indirect effect may involve suppression of the tetrahydrofolate transport system by which MTX is brought into the cell (38,39). If this is true, then the effects of MTX could be limited to a critical short period of time after the rabbit serum has made contact with the cell. If this interpretation is correct the MTX uptake in this system would not be sufficient to exert suppression of DNA synthesis at any time after 24 hours in rabbit serum. Transport studies of MTX would determine the validity of this interpretation.

An alternative but similar explanation is that rabbit serum contains a readily exhaustable factor (perhaps an antibody) which has some early sensitizing effect on the cell in the presence of stimulants such as ALS and PHA. Since this factor is found in NRS, it is probably not identical with the factor providing the stimulatory effects of the ALS.

Another possible explanation for the lack of suppression at 24 and 48 hours in cultures grown in medium containing rabbit serum is that the serum contains high concentrations of active FH_4 and/or nucleosides which would provide the cells treated with MTX an exogenous source of substrates for DNA and RNA synthesis. Recent studies suggest this is probably not the case (56,57). However if this were true, the biochemical effects of MTX could be by-passed and the techniques used would show no suppression of DNA synthesis. Why these exogenous substrates cannot "rescue" DNA synthesis in 6 and 12 hour cultures would remain unexplained. Perhaps these substrates cannot get into these cells at this time. The available literature suggests that this is not the case (8,10-12).

RNA synthesis at 0, 6 and 12 hours goes on freely in the presence of MTX (Fig. 2, Table 1). This also supports the view that the suppression of DNA synthesis at 6 and 12 hours is not due to a lack of nucleosides, or an inability of the cells to internalize any exogenous substrates. If the nucleosides are available for RNA synthesis, they should also be available for the little DNA synthesis required at these times.

III. Mechanism of action of MTX

Twenty-four hours after PHA stimulation RNA synthesis is at its peak. MTX treatment results in optimal suppression at this time. Rescue of the cultures from MTX effects on RNA synthesis was seen with all nucleosides used, in concentrations of $1x10^{-5}$M and $2x10^{-5}$M, as evidenced by a return of the fractional rate to 1.00 . Thus the addition of exogenous purines effectively masks the effects of MTX and extends the previously postulated mechanism of action for MTX to this system (33-35,45).

The results with deoxyadenosine are confusing. At $2x10^{-5}$M deoxy-

adenosine seems to "prevent" the biochemical effects of MTX but at concentrations of $1x10^{-5}$M and $4x10^{-5}$M it does not. Borsa (43) found deoxyadenosine combined with MTX to increase cell kill in L-cells, but when thymidine was also added the effects of MTX were completely prevented. It is not easily understandable why deoxyadenosine would prevent the biochemical effects of MTX on RNA synthesis in that this form of the nucleoside is incorporated into DNA rather than RNA. A mechanism whereby the deoxyribonucleoside could be converted to the corresponding ribonucleoside (involving salvage routes of nucleotide synthesis) would explain the "saving" situation that occurs with $2x10^{-5}$M deoxyadenosine. Why $4x10^{-5}$M fails to produce this same effect is not understood.

A more direct measure of a cell's capability to divide is provided by studying DNA synthesis. Cell reproduction depends on the ability to make new DNA. MTX blocks the conversion of dUMP to TMP indirectly (33,34,35) and perhaps directly (44) and thus inhibits DNA synthesis (Fig.1). At 24 hours after PHA stimulation DNA synthesis is progressing rapidly. MTX suppression of H^3-TdR incorporation at this time is also high (fractional rate is 0.49±0.05). Therefore, this time was chosen in an attempt to prevent the MTX induced suppression of DNA synthesis by the simultaneous addition of nucleosides.

In this series of studies inosine was chosen as a purine source. It can be directly phosphorylated to inosine monophosphate which serves as the normal precursor for the synthesis of both adenosine monophosphate and guanosine monophosphate (30). Thus, it provides a source of all purines.

By adding $2x10^{-4}$M inosine, the fractional rate of H^3-CdR incorporation increased from 0.27±0.03 to 0.47±0.06 (Table 5). In effect,

the addition of a high concentration of exogenous purine frees the cell to synthesize a bit more DNA (45,49). Thus, in our system, as in the L5178Y murine leukemic (49) a purine deficiency seems to be a contributory factor to the biochemical effects of MTX.

However, since exogenous purines cannot completely prevent the MTX induced suppression of H^3-TdR incorporation, a lack of thymidine probably represents the primary mechanism of MTX action on PHA stimulated guinea pig lymphocytes. This is borne out by the fact that complete saving can be achieved by adding a source of exogenous thymidine to the (MTX and purine) treated cultures. Thymidine concentrations of 10^{-6}M and inosine concentrations of 10^{-4}M give optimal results. In an effort to see whether the ratio of purine to thymidine was of prime importance, different concentrations of thymidine (10^{-7}M) and inosine (10^{-5}M) were used. This failed to prevent the MTX induced suppression of DNA synthesis (fractional rate - 0.18±0.02).

Addition of 10^{-4}M thymidine alone will increase the fractional rate of H^3-CdR incorporation from 0.13±0.01 to 0.60±0.09. Thus thymidine alone does not totally prevent MTX effects on DNA synthesis. Therefore both purine and pyrimidine metabolism are affected by MTX in the cell system, as in others studies (36,45,46).

The leucovorin rescue studies show that the biochemical effects of MTX are reversible for several hours after addition of MTX, at least in some cells. It is possible that the 3 hours pretreatment in MTX has killed a number of cells and that the effects of leucovorin rescue are expressed only in a fraction of surviving cells. Perhaps these cells

were not optimally affected by MTX. Therefore rescue by leucovorin does not necessarily mean that no cell kill had occurred.

In the L5178Y system, Hryniuk showed that the simultaneous addition of hypoxanthine with MTX completely lifts the suppression of H^3-TdR incorporation, but only partially prevents cell kill as demonstrated by cloning (49). Thus labeling experiments probably reflect MTX effects on surviving cells only.

Of interest is the seeming competitive nature of the reversal brought about by leucovorin. This has been noted in other systems as well (43,50). Two hours pretreatment with MTX followed by 2 hours more in leucovorin does not result in any rescue. However, if the initial two hours in MTX is followed by 4 hours in leucovorin, rescue results. One would not expect a competitive type of reversal if leucovorin only by-passed MTX induced inhibition of dihydrofolate reductase. Leucovorin has been shown in L1210 cells to be transported in by a carrier-mediated transport system which also brings MTX and N^5-methyl-FH_4 into the cell (50). The leucovorin is very rapidly metabolized to N^{10}-formyl-FH_4 and N^5N^{10}-methenyl-FH_4 as well as one other active folate not yet identified (perhaps N^5N^{10}-methylene-FH_4). Some of the accumulated intracellular leucovorin (or products of its metabolism) is not necessarily readily exchangeable with extracellular leucovorin present in excess. In dividing cells, the products of this leucovorin metabolism probably includes dihydrofolate, which, if in sufficient quantity, may displace MTX bound to dihydrofolate reductase (DHFR). Methotrexate which is not bound to DHFR can also be displaced from the cell upon entry and

rapid metabolism of the leucovorin (50). MTX is not rapidly metabolized in L1210 cells (50). Another possible explanation for the competitive nature of the rescue afforded by leucovorin lies in the competition for the carrier (if any MTX is extracellular when the leucovorin is added). Therefore, leucovorin not only provides the obvious by-pass of the MTX block of folate metabolism, but may also bring about the displacement of MTX from the cell in a competitive manner (50). Thus, a competitive type reversal can be explained.

The suppression of DNA synthesis resulting from three hours pretreatment in MTX can be relieved by a further 4 hours incubation in leucovorin. If the 3 hour pretreatment in MTX is followed by a further 1 or 2 hours incubation in leucovorin, no rescue results. This data agrees with a competitive nature of rescue. In cultures where no rescue is seen, the leucovorin probably has not had enough time to effectively remove enough MTX from the cells to allow uninhibited DNA synthesis.

The ultimate aim of leucovorin rescue is to bring about rescue of normal tissue but not malignant tissue, thereby increasing the therapeutic effectiveness of the anti-folate. This aim has been extended to the therapy of some human cancers with encouraging results (51). The fact that normal stimulated lymphocytes can be rescued by leucovorin in this system is particularly encouraging in the light of combined immunotherapy-chemotherapy treatments being designed and used today. Also encouraging in this regard is the complete inability of leucovorin to rescue acute leukemic blood blasts treated with methotrexate (31).

In certain malignancies, it may therefore be possible to rescue normal tissue but not the malignant tissue with leucovorin (51). The dose and time schedules of this "rescue therapy" must still be worked out for each system.

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