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COMPARATIVE EFFECTS OF SELECTED ANTIFOLATES ON TRANSFORMING HUMAN LYMPHOCYTES AND ON ESTABLISHED HUMAN LYMPHOBLASTIC CELL LINES

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Abstract—When 15 nM methotrexate was added to the medium in which human peripheral lymphocytes stimulated with phytohemagglutinin were incubated, it caused a 50 per cent decrease in the maximum number of blasts produced, in the number of cells in mitosis and in the incorporation of [^{3}H]deoxyuridine into DNA. However, [^{3}H]thymidine incorporation into DNA was increased by methotrexate concentrations up to 0.5 mM. When 50 nM methotrexate was present continuously, blast formation, mitosis and deoxyuridine incorporation were virtually abolished, but if this concentration was present only during the induction phase (the first 24 hr), the subsequent effect on blast proliferation was slight. In contrast, 24-hr exposure during the proliferative phase (days 3–5) severely affected blast proliferation. The effects of methotrexate were largely reversible by thymidine, but hypoxanthine or purine nucleosides had no significant effect so that the metabolic block appears to be entirely at thymidylate synthetase under the experimental conditions. The inhibitory effects of ten other antifolates on transforming lymphocytes were determined and, with one exception, their relative effectiveness was found to be as predicted from inhibitory effects on highly purified bovine dihydrofolate reductase. The growth of four established lines of human lymphoblastic cells was inhibited to essentially the same extent by six of the antifolates, and these cells were only slightly less sensitive to the antifolates than were the transforming normal lymphocytes.

Results have been obtained in mice [1], rats [2] and dogs [3, 4] indicating that, after bone marrow transplantation, treatment with methotrexate (4-amino-4-deoxy-10-methyl-folic acid) enhances the chances of these animals of surviving graft-vs-host disease. Consequently, methotrexate has been used as an immunosuppressant in marrow transplants in man in a limited number of cases of aplastic anemia, acute lymphoblastic leukemia and acute myeloblastic leukemia [5–8]. Marrow transplantation is in principle also applicable to the treatment of other hematologic malignancies. Since the efficient suppression of graft-vs-host disease is essential for successful marrow transplantation, it is desirable to accumulate data which may provide a rationale for choosing the optimum immunosuppressant for this purpose, and the effect of immunosuppressants on T-lymphocyte transformation *in vitro* supplies data relevant to this purpose.

The first object of this investigation was to study in some detail the effect of methotrexate on human lymphocytes stimulated by phytohemagglutinin (PHA). Although lymphocyte response to this lectin differs in some respects from the transformation produced by the antigens on the surface of allogeneic cells, PHA has the advantage that it specifically activates thymus-derived lymphocytes [9–12], which are responsible for the cell-mediated immune response in graft-vs-host disease [13–16]. Second, we have compared the effects of methotrexate on PHA-stimulated lymphocytes with the effects of representatives of several other classes of antifolates, with the purpose of determining whether any was more effective than predicted by its inhibition of mammalian dihydrofolate reductase. Third, we have compared the ID_{50} for various antifolates on PHA-stimulated lymphocytes with the values on four humal lymphoblastic cell lines.

MATERIALS AND METHODS

Except for methotrexate, which was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, antifolates were generously provided through the courtesy of the following: Dr. J. J. Burchall of the Wellcome Research Laboratories, Raleigh, N. C., who provided the pyridopyrimidines, the triazine and pyrimethamine; Dr. J. A. Montgomery of the Southern Research Institute, Birmingham, Ala., who provided the deazapteridines; and Dr. J. R. Dice of Parke, Davis & Co., Ann Arbor, Mich., who provided the quinazolines. NADPH and agarose-hexane-NADP were obtained from PL Biochemicals, Milwaukee, Wisc. Penicillin-streptomycin, colcemide, 1% acetoorcein, neomycin, fetal calf serum and Eagle's minimal essential medium (MEM) containing glutamine were purchased from Grand Island Biological Co., Grand Island, N.Y. PHA was from Burroughs-Wellcome Co., Greenville, N.C., and NCS tissue solubilizer from Amersham–Searle Corp., Arlington Heights, Ill. [^{3}Hmethyl]thymidine (sp. act. 51.4 Ci/m-mole) and [6-^{3}H]deoxyuridine (sp. act. 16 Ci/m-mole) were purchased from New England Nuclear, Boston, Mass., and Schwartz–Mann, Orangeburg, N.Y. respectively. These compounds were 99 per cent pure when purchased, and the radiopurity was checked monthly. Human pooled plasma was generously provided by

the Blood Bank of the Veterans' Administration Hospital, Iowa City, Ia.

Preparation of cultures of PHA-stimulated lymphocytes. Peripheral blood (15–30 ml) was drawn from healthy human subjects into heparinized vacutainers, and allowed to sediment for 2 hr at 37°. The buffy coat was removed, and the leukocytes were recovered as a pellet by centrifugation at 48 *g* for 10 min. The leukocytes were resuspended in 10 ml MEM, which was supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). The lymphocyte concentration was determined by diluting 10 μl of the cell suspension with 0.19 ml of 4% (v/v) acetic acid containing 30 μg of crystal violet/ml and counting in a hemocytometer. In some experiments T-lymphocytes were purified from leukocytes by passage through a nylon column by the method of Eisen *et al.* [17] as modified by Greaves and Brown [18]. The T-lymphocytes were recovered in 70.5 per cent yield. Examination after Giemsa staining showed that no monocytes were present, and immunofluorescent staining [19] showed that B-lymphocytes were also absent, but 9 per cent of the lymphocytes did not form spontaneous rosettes with sheep red blood cells [20]. There were also a significant number of red cells present. Triplicate cultures were prepared, each containing 10^5 lymphocytes suspended in a mixture of 0.8 ml MEM and 0.2 ml of human pooled plasma with the further addition of 100 units penicillin and 100 μg streptomycin. Cultures were incubated in the presence of the optimum level of PHA, which was found to be about 8 μg/ml whether determined by the effect on mitotic index, the blast count or the incorporation of thymidine or deoxyuridine. Antifolates were added in a small volume of MEM at the beginning of the incubation period. Incubation was carried out in a water-saturated atmosphere of 5% CO_2–95% air at 37°.

Incorporation of thymidine and deoxyuridine into DNA. After incubation of PHA-stimulated lymphocytes for a suitable period (68 hr unless otherwise specified), [^{3}H]thymidine or [^{3}H]deoxyuridine was added to each culture in a volume of 25 μl to give a final concentration of 0.11 μM and a total radioactivity of 1 μCi. The cells were pulsed for 30 min, after which time they were harvested by centrifugation and frozen. After thawing, the nuclear material was precipitated with 5 ml of cold 5% (w/v) trichloroacetic acid. The precipitate was recovered by centrifugation and washed once with 5% trichloroacetic acid and twice with cold absolute methanol and then solubilized with 0.3 ml of NCS tissue solubilizer at 51° for 45 min; the radioactivity was counted in 6% liquifluor in toluene.

Determination of the mitotic index. After incubation of PHA-stimulated lymphocytes for a suitable period (68 hr unless otherwise specified), 10 μl of colcemide solution (0.04 μg) was added to cultures and incubation continued for 4 hr, so that cells undergoing mitosis were arrested at metaphase. The supernatant was then aspirated off, the cells were stained in 1% aceto-orcein dye, and the mitotic figures and total cells were counted. From 1000 to 1500 cells were counted from each of the triplicate cultures.

Measurement of viability. The viability of cells in culture was measured using the dye exclusion technique [21]. Cultures of 1 ml were centrifuged for 10 min at 48 *g* after an appropriate period of culture. After removing 0.8 ml supernatant, 0.70 ml of 0.154 M saline buffered to pH 7.25 with 0.01 M phosphate and 0.10 ml of 0.4% (w/v) erythrosin B was added to the remaining suspension. Total cells and the stained cells were counted within 4 min in a hemocytometer.

Blast counts in cultures of PHA-stimulated lymphocytes. Blasts, which have a diameter about twice that of the nontransformed lymphocytes, were counted in a hemocytometer. In cultures with antifolates present, this was carried out after a period of incubation (usually 5 or 6 days, Fig. 1) when the number in the control culture (without antifolate) had reached a maximum (5.5 to 8.5 × 10^5/ml). Cetrimide (0.1 ml of a solution containing 10 mg/ml in 1% acetic acid) was used to disperse cell aggregates in 0.2 ml cell suspension before counting. The blasts were found to have surface characteristics of T-lymphocytes with a few null-lymphocytes present but negligible numbers of B-lymphocytes. These details will be described in full elsewhere.

Growth of established lines of human lymphoblasts. Four human lymphoblastic cell lines were obtained from other laboratories. Line CCRF-CEM was established by Foley *et al.* [22] from the peripheral blood of a 3-year-old girl with acute lymphoblastic leukemia. Cultures were kindly provided by Dr. Ward D. Peterson, Jr. (The Child Research Center of Michigan, Detroit). Other lines were obtained from Dr. Joan Stadler of the Department of Genetics, Iowa State University. Line L33-6-1 was a subline of PGL33H originally established in Dr. John W. Littlefield's laboratory (Genetics Unit, Massachusetts General Hospital, Boston) from the peripheral blood of a female patient with infectious mononucleosis. Line E34 was derived by Dr. Stadler from lymphocytes from a normal male donor by transforming them with Epstein–Barr virus; line HMW9 is a mutant of E34, lacking the enzyme hypoxanthine-guanine phosphoribosyltransferase.

Lymphoblasts of CCRF-CEM were maintained as stationary suspension cultures in 250-ml Falcon plastic flasks containing Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% (v/v) heat-inactivated fetal calf serum. Fresh medium was added every 2 or 3 days and the cell density of cultures was kept at 0.5 to 3 × 10^6 cells/ml. Lymphoblasts of L33-6-1, E34 and HMW9 were grown as stationary suspension cultures in 100-ml screw-cap glass bottles containing RPMI 1640 medium plus 15% (v/v) heat-inactivated fetal calf serum. Fresh medium was added every 2 days; the cell density of cultures was maintained at 0.2 to 1 × 10^6 cells/ml.

In experiments to determine the effect of antifolates on growth, cell suspensions (2.5 ml) containing five different drug concentrations were each incubated in Falcon plastic flasks (25 cm^2) at 37° in humidified air containing 5% CO_2. The control was without drug, and each series was done in triplicate. The initial density of all lines except CCRF-CEM was 2 × 10^5 cells/ml; the CCRF-CEM initial density was 5 × 10^5 cells/ml. After 3 days of incubation the cell density was determined with the use of a Coulter counter (model B).

Calculation of ID_{50}. For computing ID_{50}, average

blast densities (expressed as percentage of control) and the logarithms of the corresponding drug concentrations were fitted to a straight line by a least squares program. Only data corresponding to concentrations in a 10-fold range centered on ID_{50} were used.

Inhibition of bovine liver dihydrofolate reductase by antifolates. The reductase was purified and assayed as described by Peterson *et al.* [23] with the modification that after the pterolyllysine-Sepharose column the enzyme was further purified, and folate was removed, by passage through an agarose-hexane-NADP column. Details of the latter step will be described later. In experiments to determine I_{50} (the concentration of drug giving 50 per cent inhibition), the inhibitor was incubated with all the components of the standard assay system except dihydrofolate for 2–3 min at 37° before the reaction was initiated by addition of dihydrofolate. Dihydrofolate was prepared as previously described [24].

RESULTS

Time course of blast proliferation, mitotic index and incorporation of thymidine and deoxyuridine into DNA in PHA-stimulated lymphocytes. With the experimental conditions chosen, the time course for the transformation of PHA-stimulated lymphocytes was as shown in Fig. 1. The number of blasts reached a maximum on days 5–8 of incubation, and at this time about 95 per cent were T-lymphocytes as determined by formation of spontaneous rosettes with sheep red blood cells. When purified T-lymphocytes were used, the commencement of blast proliferation and the peak of blast count occurred at the same times as with the unfractionated leukocyte preparation, but the blast count reached only about 63 per cent of that obtained when unfractionated leukocytes from the same donor were used. Mitosis reached a maximum at 4 days of incubation, thymidine incorporation into DNA at 5 days, and deoxyuridine incorporation at 6 days. The number of nonviable cells (as measured by dye exclusion) decreased only slightly over the entire period of incubation (for example, 98.2 ± 0.7 per cent on day 1 and 83.6 ± 2.5 per cent on day 8).

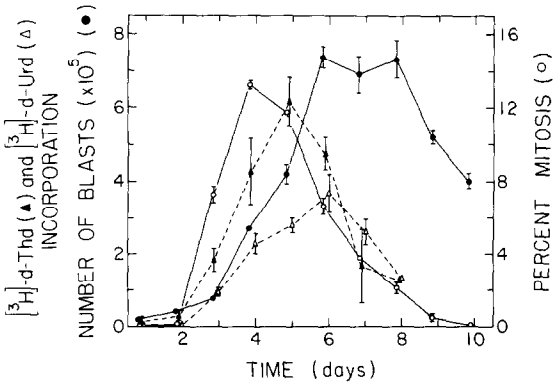

Figure 1. Time course of transformation by PHA-stimulated lymphocytes. The optimum amount of PHA (8 μg) was added at zero time and as cells were subsequently incubated at 37°, measurements were made of the number of blasts, the per cent of cells in mitosis, and the incorporation of [^{3}H]thymidine and of [^{3}H]deoxyuridine (expressed as pmoles/10^5cells/hr).

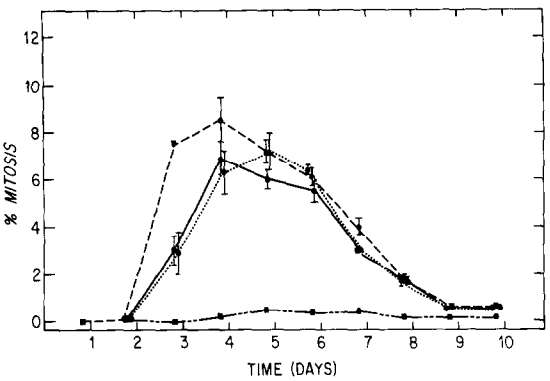

Fig. 2. Inhibition of mitosis of PHA-stimulated lymphocytes by methotrexate. The methotrexate concentrations were (----) 0, (·····) 0.5 nM, (——) 5 nM, and (— — —) 50 nM. Bars show standard deviations.

Effect of methotrexate on mitotic index. Figure 2 shows the effects of various methotrexate concentrations on mitosis in stimulated lymphocytes. Mitosis was completely inhibited throughout the 10 days of incubation by methotrexate at a concentration of 5 μM or higher, and even at 50 nM the number of mitoses was very small. Lower concentrations (5 and 0.5 nM) were less inhibitory and produced their greatest effect (60 per cent) on day 3.

Effect of methotrexate on incorporation of thymidine and deoxyuridine into DNA and on blastogenesis. Since 5 nM methotrexate significantly depresses mitosis, the effect of this concentration on nucleoside incorporation was investigated. There was little effect on [^{3}H]thymidine incorporation, but a significant decrease (about 40 per cent) in [^{3}H]deoxyuridine incorporation occurred at 140 and 166 hr, although not at 184 hr of incubation. In further experiments with cells pulsed at 68 hr, 50 nM methotrexate was found to abolish deoxyuridine incorporation whereas thymidine incorporation was not inhibited even at concentrations up to 0.5 mM. Instead, moderate concentrations of methotrexate produced a significant activation of thymidine incorporation, the maximum effect (88 per cent) being produced by 50 nM methotrexate.

The magnitude of the inhibitory and activating effects of methotrexate and the drug concentration at which the maximum effect is produced depend to some degree on the individual donor of the lymphocytes. However, despite considerable individual variation, the pattern of the effects was fundamentally the same in all cases.

Blast formation was almost abolished when methotrexate was present throughout the culture period at a concentration of 50 nM or higher, whereas at 5 nM or lower concentrations the effect was negligible.

Effects of varying the period of exposure of cells to methotrexate. In the experiments described above, methotrexate was present throughout the period of culture. When methotrexate is administered *in vivo*, however, it is present only transiently in the serum. The effect of shorter exposure to methotrexate was investigated therefore by adding methotrexate at various times after stimulation by PHA (Fig. 3). Methotrexate at concentrations of 5 μM and 0.5 mM

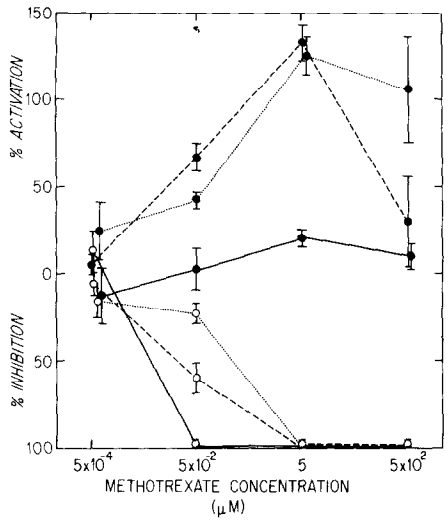

Fig. 3. Effect of time of exposure of PHA-stimulated lymphocytes to methotrexate on the incorporation of [^{3}H]thymidine (●) and [^{3}H]deoxyuridine (○) into DNA. Methotrexate was added to each of the triplicate cultures at 0 time (——), 64 hr (– – –) or 66 hr (·····) of incubation. Labeled nucleosides were added at 68 hr and incubated with the cultures for 30 min prior to harvest. Further experimental details are described in Methods. The per cent inhibition and activation were calculated from the number of counts incorporated into DNA as compared with the control culture.

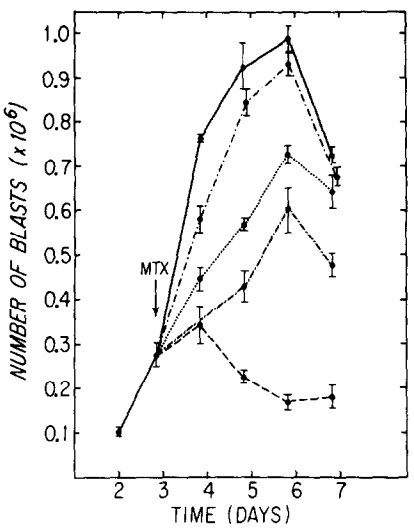

Fig. 5. Effect on blast formation of exposure for various periods to methotrexate added 3 days after stimulation. Methotrexate (50 nM) was added on day 3 to all the cultures except for the control (——). The drug was removed by washing the cells with MEM at 37° after 6 hr (–·–·–), 12 hr (·····) or 24 hr (–/–/–/) of incubation, and the washed cells were resuspended in supernatant medium containing no methotrexate from other lymphocytes that had been stimulated at the same time as the experimental cultures. In one culture (– – –) the methotrexate was not removed.

completely inhibited [^{3}H]deoxyuridine incorporation into DNA even when the cells were exposed to the drug for only 2 hr. However, 50 nM methotrexate produced considerably less inhibition in 2 or 4 hr than in 68 hr. Conversely, methotrexate produced the most activation of thymidine incorporation in the short periods of treatment.

In another experiment on blast formation, 50 nM methotrexate was added to PHA-treated lymphocytes on days 0, 1, 2 and 3, respectively, and subsequent blast formation was measured (Fig. 4). This concentration of methotrexate arrested blast formation almost immediately in all cases, even when methotrexate was absent throughout the induction phase.

A somewhat different procedure used to test the effect of exposure to methotrexate for different times is described in Fig. 5. Here 50 nm methotrexate was added to cultures 3 days after PHA stimulation, and the drug subsequently removed from the cells after

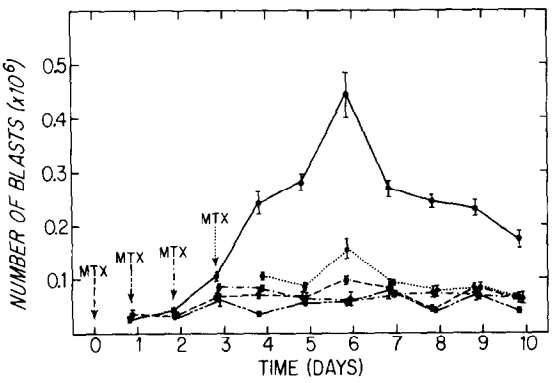

Fig. 4. Effect of blastogenesis in PHA-stimulated lymphocytes of methotrexate added on different days of incubation. PHA was added to each culture on day 0, and 50 nM methotrexate was added on day 0 (– – –), day 1 (— — —), day 2 (–·–·–) or day 3 (·····). The control (——) contained no methotrexate.

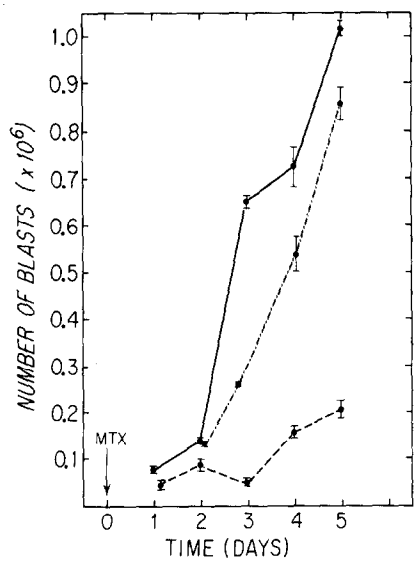

Fig. 6. Effect on blast formation of exposure for various periods to methotrexate added at the time of stimulation. Methotrexate (50 nM) was added to all the cultures except for the control (——). After 24 hr (–/–/–/), the drug was removed as in Fig. 5. The drug was not removed from some cultures (– – –).

Table 1. Systematic names of antifolates

Code No. or trivial name	Systematic name
BW 58-283b	5-methyl-6-sec-butyl-2,4-diaminopyridopyrimidine
BW 60-205d	5-methyl-6-benzyl-2,4-diaminopyridopyrimidine
BW 57-43	5-*N*-(*p*-*n*-butylphenyl)-2,4-diamino-6,6-dimethyl-1,6-dihydro-1,3,5-triazine
Pyrimethamine	5-(*p*-chlorophenyl)-6-ethyl-2,4-diaminopyrimidine
SRI 4736	*p*-[[(5,7-diaminopyrido[3,4-b]pyrazin-3-yl)methyl]methylamino]-benzoic acid
SRI 4754	*N*[*p*-[[(5,7-diaminopyrido[3,4-b]pyrazin-3-yl)methyl]methylamino]-benzoyl]glutamic acid
SRI 4782	*N*-[*p*-[[(6,8-diaminopyrido[2,3-b]pyrazin-2-yl)methyl]methylamino]benzoyl]glutamic acid
SK 29,861	*N*-[*p*-[[(2,4-diamino-5-chloro-6-quinazolinyl)methyl]amino]benzoyl]-L-aspartic acid
SK 29,836	*N*-[*p*-[[(2,4-diamino-5-methyl-6-quinazolinyl)methyl]amino]benzoyl]-L-aspartic acid
SK 29,758	*N*-[*p*-[[(2,4-diamino-6-quinazolinyl)methyl]amino]benzoyl]-L-aspartic acid

various further intervals. In order to supply lymphokines, the washed cells were suspended in medium from other cultures which did not contain methotrexate, and incubation was continued. Exposure for only 6 hr to this concentration of methotrexate significantly decreased subsequent blast formation and the effect was increased as the length of exposure increased. Exposure for 24 hr caused extensive though incomplete inhibition of subsequent blast formation.

In an experiment similar except that methotrexate addition was made at the same time as PHA, the effect of the 24-hr exposure to methotrexate was much less (Fig. 6), and the decrease in blast production was significant (50 per cent inhibition) only on day 3. Exposure of cells to methotrexate for 6 or 12 hr gave essentially the same results as exposure for 24 hr.

Reversal of methotrexate inhibition of cell division by thymidine, hypoxanthine and purine nucleosides. The effect of methotrexate on transformation of lymphocytes and replication of the transformed cells is presumably due to metabolic blocks at thymidylate synthetase and possibly at steps in purine synthesis that involve tetrahydrofolate derivatives. To obtain evidence whether this is the case, the effect of thymidine, hypoxanthine and purine nucleosides on the inhibitory effects of methotrexate was examined. Thymidine partially reversed the inhibitory effect of 50 nM methotrexate, the inhibition decreasing as the concentration of thymidine was increased from 5 to 50 μM. Methotrexate inhibition of blast proliferation was 77 per cent in the absence of thymidine and 17 per cent in the presence of 50 μM thymidine. However, 50 μM

Table 2. Effect of antifolates on mitosin, maximum number of blasts, and the incorporation of thymidine and deoxyuridine into DNA, in PHA-stimulated human lymphocytes

Inhibitors	Drug concn (nM) for 50 per cent decrease*			
	Mitotic index	Blast No.	dUdr	dThd
BW 58-283b	11	13	0.9	65,000
BW 60-205d	159	130	67	60,000
BW 57-43	238	168	118	75,000
Pyrimethamine	814	1,190	405	80,000
SRI 4736	124,000	152,000	173,000	70,000
SRI 4754	84	56	76	†
SRI 4782	63	81	22	35,000
SK 29,861	36	36	22	280,000
SK 29,836	17	25	39	‡
SK 29,758	37	17	32	250,000
Methotrexate	12	19	17	§

* Results for the effect on mitotic index and blast number were calculated from data obtained in three experiments each with lymphocytes from a different donor. Results for the effect on deoxyuridine incorporation were from data on lymphocytes from two to five donors; results on thymidine incorporation from data on lymphocytes from two donors.

† Thirty per cent inhibition at 5,000,000 nM.

‡ Ten per cent inhibition at 500,000 nM.

§ No inhibition at 500,000 nM.

Table 3. Effect of antifolates on the growth of four lines of human lymphoblasts

Antifolate	Concn for 50 per cent inhibition of growth (nM)			
	CCRF-CEM	L33-6-1	E34	HMW9
BW 60-205d	350	279	165	131
BW 57-43	583	169	130	157
Pyrimethamine	4500	1540	1120	1500
SRI 4782	177	142	188	90
SK 29,758	38	31	30	19
Methotrexate	11	11	18	12

hypoxanthine, adenosine, deoxyadenosine or deoxyguanosine caused no significant reversal of the effect of methotrexate either alone or in the presence of 50 μM thymidine.

Effects of other antifolates on PHA-stimulated lymphocytes. The antifolates listed in Table 1, comprising representatives of the diamino derivatives of pyrimidines, pyridopyrimidines, triazines, deazapteridines and quinazolines, were also tested for their effects on the transformation of PHA-stimulated lymphocytes. From the results in Table 2 it may be seen that under these conditions in which the antifolates were continuously present in the medium, a similar ID_{50} was found for the effect of a particular antifolate on mitotic index, blast number and deoxyuridine incorporation, whereas thymidine incorporation was inhibited only at much higher concentrations. SRI 4736 is exceptional in regard to the latter but this is related to the low inhibitory effects of this compound.

Effects of antifolates on established lymphoblastic cell lines. Table 3 shows the concentrations of six of these antifolates required to cause a 50 per cent decrease in the growth of four extablished human lymphoblastic cell lines. For any particular antifolate, growth of the four lines was inhibited by essentially the same concentration of that antifolate.

Relative inhibitory effect of the antifolates on mammalian dihydrofolate reductase. In order to relate the effects of the antifolates on lymphoblast proliferation to their inhibitory action on dihydrofolate reductase, it was desirable to determine the inhibition effects of the compounds on lymphoblast dihydrofolate reductase. However, since isolation of enough purified lymphoblast enzyme for such a study proved to be a major undertaking, we have instead examined inhibition of reduction from a more accessible mammalian source: bovine liver. The results are presented in Table 4.

DISCUSSION

Methotrexate continuously present in cultures of stimulated lymphocytes suppresses mitosis, blast formation and deoxyuridine incorporation into DNA, all three parameters showing very similar sensitivity to methotrexate (Table 2). This is in contrast to the results of Rozenszajn and Radnay [25], who reported that mitosis is 1000 times more sensitive than transformation (blast formation). However, these authors measured both mitosis and blast counts at 72 hr, a time at which blast formation had scarcely commenced in our experiments, and this may account for the discrepancy.

Exposure of lymphocytes to 50 nM methotrexate during induction (first 24 hr with PHA), with subsequent removal of the methotrexate, caused little decrease in subsequent blast formation (Fig. 6), but similar exposure for 24 hr between days 3 and 4 after activation caused marked inhibition of blastogenesis (Fig. 5). Similarly, 50 mM methotrexate throughout the proliferative phase (day 3 on) caused almost the same inhibition of blastogenesis as 50 mM methotrexate throughout both the induction and proliferative phases (day 0 on, Fig. 4). Thus, methotrexate exerts

Table 4. Concentration of antifolates to produce 50 per cent inhibition of beef liver dihydrofolate reductase

Inhibitor	Antifolate concn (nM)	Immunosuppressive/ enzymatic*
BW 58-283b	4.8	2.9
BW 60-205d	5.7	23
BW 57-43	13	13
Pyrimethamine	30	40
SRI 4736	1280	118
SRI 4754	260	0.22
SRI 4782	26	3.1
SK 29,861	9.7	3.7
SK 29,836	6.5	3.9
SK 29,758	4.3	4.0
Methotrexate	6.5	2.9

* Ratio of the concentration required to decrease the blast count by 50 per cent (Table 3) to the concentration for 50 per cent reductase inhibition (column 1).

its effect almost exclusively during the proliferation phase (days 3–6) when the cells are synthesizing DNA. Whether methotrexate was present during the induction or proliferative phase, removal of the antifolate partially released the cells from the arrest of proliferation that occurs as long as the drug is present. The basis for this release is uncertain, since methotrexate would be expected to bind tightly to all dihydrofolate reductase in the cell. Recovery may be due either to loss of methotrexate from the cells during washing or subsequent incubation, or to synthesis of more dihydrofolate reductase after removal of methotrexate from the medium.

Relation of immunosuppressive effects of antifolates to inhibition of dihydrofolate reductase. When the ID_{50} for blast proliferation of PHA-stimulated lymphocytes is compared with the I_{50} for a mammalian dihydrofolate reductase, the ratio of the two concentrations is very similar for six out of the eleven drugs (Table 4), suggesting that for most of the antifolates the immunosuppressive effects are directly related to the ability to inhibit the reductase. The four inhibitors that have less effect on blast proliferation than predicted from reductase inhibition are probably poorly transported into the cells. SRI 4754 is of interest because its immunosuppressive effect appeared to be an order of magnitude greater than expected from its inhibition of the enzyme.

Since deoxyuridine incorporation into DNA and blast proliferation are similarly affected by methotrexate, blockade of thymidylate synthesis *de novo* is probably the major consequence of the inhibition of dihydrofolate reductase by methotrexate. The increased incorporation of labeled, exogenous thymidine into DNA in methotrexate-treated cells supports this view, since it probably reflects a decreased contribution of unlabeled endogenous thymidylate to the thymidylate pool. It is also supported by the reversal by thymidine of the inhibition of mitosis and blastogenesis by methotrexate.

Hypoxanthine added alone or in addition to thymidine at any time during the culture did not reverse methotrexate inhibition of blast proliferation. This and the similar ineffectiveness of adenosine, deoxyadenosine, deoxyguanosine or combination of these nucleosides, are surprising, since there is evidence that *de novo* synthesis of purine nucleotides occurs in PHA-stimulated human lymphocytes [26]. Moreover, Hryniuk [27] has reported a transient purineless state produced by 1 μM methotrexate in a murine lymphoblastic cell line. Both adenosine and hypoxanthine can be utilized by human lymphoblastic cell lines [28,29] so that these compounds should overcome methotrexate inhibition when *de novo* purine synthesis is limiting. Despite this evidence, methotrexate does not appear to act by interrupting *de novo* purine synthesis in our experiments. This is perhaps true only at the low concentration of methotrexate employed (50 nM). The decreased activation of [^{3}H]thymidine incorporation into DNA obtained with 500 μM methotrexate may be due to some inhibition of purine deoxyribonucleotide synthesis at the latter concentration of methotrexate.

The hypothesis that only thymidylate synthetase and not purine biosynthesis is blocked at concentrations of antifolates near the ID_{50} is supported by the data in Table 3. There it may be seen that the lymphoblastic line HMW9, which is a mutant of E34 deficient in hypoxanthine-guanine phosphoribosyl transferase and therefore unable to synthesize purine nucleotides by the major salvage pathway, is no more sensitive to antifolates than the parent line.

Immunosuppression with methotrexate in vivo. It is of interest to compare the concentration of methotrexate causing a 50 per cent decrease in blast formation by PHA-stimulated lymphocytes *in vitro* with concentrations likely to be attained in patients given methotrexate for immunosuppression. Neiman *et al.* [5] have adopted as the dose schedule for patients who have received marrow transplantation 15 mg/m^2 on the day after marrow infusion and 10 mg/m^2 on days 3, 6, 11 and weekly thereafter to day 100 after transplantation. From data on plasma concentrations of methotrexate after administration to human subjects [30–34], it appears probable that the dose schedule employed by Nieman *et al.* would maintain the plasma methotrexate concentration at a level which would be predicted sufficient to suppress blast formation during much of the treatment period.

It is also of interest to consider the predicted degree of immunosuppression during methotrexate therapy for leukemia. Earlier reports suggested that leukemic cells are relatively insensitive to methotrexate compared with PHA-stimulated lymphocytes *in vitro*. Thus, whereas our results show that 50 nM methotrexate abolishes proliferation in the latter, it has been reported that 2.2 μM methotrexate caused only 45–65 per cent inhibition of deoxyuridine incorporation into DNA of leukemic marrow cells [35]. Hoffbrand *et al.* [36] reported that the incorporation of deoxyuridine into DNA by bone marrow cells from normal subjects and from patients with acute myeloid leukemia required 1 μM methotrexate, but in marrow cells from most patients with acute lymphoblastic leukemia or from patients with methotrexate-resistant leukemia the inhibition was incomplete at this concentration of methotrexate. On the basis of these data it would seem probable that concentrations of methotrexate adequate to suppress the proliferation of leukemic cells would also effectively suppress immune responses. However, our results with an established human lymphoblastic cell line (CCRF-CEM) derived from the peripheral lymphocytes of a leukemic patient suggest that the sensitivity of (nonresistant) leukemic cells may be quite similar to the sensitivity of the PHA-stimulated lymphoblasts (compare Tables 2 and 3). Indeed all four established lymphoblastic cell lines were quite similar to each other and to the PHA-activated lymphocytes in their sensitivities to each of the six antifolates tested. This seems to be more consistent with the observation that, although increased susceptibility to infection has been reported in patients treated with methotrexate [37], it is usually a problem only in connection with advanced toxicity.

The data also suggest that, when methotrexate is used for immunosuppression after bone marrow transplantation, the effect of methotrexate on bone marrow repopulation is likely to be almost as severe as that on T-lymphocyte transformation. With such a small differential it is likely that scheduling of the drug is of critical importance, particularly during the first week after transplantation, when most of the

marrow repopulation occurs. Whether results *in vitro* suggest that the differential is equally small for other S-phase inhibitors is under investigation.

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REFERENCES

1. D. E. Uphoff, *Proc. Soc. exp. Biol. Med.* **99,** 651 (1958).
2. G. W. Santos and A. H. Owens, *Nature, Lond.* **210,** 139 (1966).
3. R. Storb, R. B. Epstein, T. C. Graham and E. D. Thomas, *Transplantation* **9,** 240 (1970).
4. H. J. Kolb, R. Storb, T. C. Graham, H. Kolb and E. D. Thomas, *Transplantation* **16,** 17 (1973).
5. P. Neiman, F. D. Thomas, C. D. Buckner, R. Storb, A. Fefer, H. Glucksberg and K. G. Lerner, *A. Rev. Med.* **25,** 179 (1974).
6. R. Storb, C. D. Buckner, A. Fefer, R. A. Clift, P. A. Neiman, H. Glucksberg, K. G. Lerner and E. D. Thomas, *Transplantn Proc.* **6,** 355 (1974).
7. G. W. Santos, L. L. Sensenbrenner, P. J. Burke, G. M. Mullins, P. N. Anderson, P. J. Tutschka, H. G. Braine, T. E. Davis, R. L. Humphrey, M. D. Abeloff, W. B. Bias, D. S. Borgaonkar and R. E. Slavin, *Transplantn Proc.* **6,** 345 (1974).
8. G. Mathé and L. Schwarzenberg, *Transplantn Proc.* **6,** 335 (1974).
9. G. Janossy and M. F. Greaves, *Clin. exp. Immun.* **9,** 483 (1971).
10. F. Daguillard, *Proc. Seventh Ann. Leucocyte Culture Conf.* (Ed. F. Daguillard), p. 571. Academic Press, New York (1972).
11. M. F. Greaves and G. Janossy, *Transplantn Rev.* **11,** 87 (1972).
12. M. F. Greaves, G. Janossy and M. Doenhoff, *Nature, Lond.* **248,** 698 (1974).
13. J. F. A. P. Miller, K. T. Brunner, J. Sprent, P. J. Russell and G. F. Mitchell, *Transplantn Rev.* **3,** 915 (1971).
14. G. Möller, *Transplantn Proc.* **3,** 15 (1971).
15. P. Goldstein, H. Blomgren, E. A. Svedmyr and M. Wigzell, *Transplantn Proc.* **5,** 1441 (1973).
16. H. Cantor, *Prog. Biophys. molec. Biol.* **25,** 93 (1972).
17. S. A. Eisen, H. J. Wedner and C. W. Parker, *Immun. Commun.* **1,** 571 (1972).
18. M. F. Greaves and G. Brown, *J. Immun.* **112,** 420 (1974).
19. H. B. Dickler and H. G. Kunkel, *J. exp. Med.* **136,** 191 (1972).
20. Z. Bentwich, S. D. Douglas, F. P. Siegal and H. G. Kunkel, *Clin. Immun. Immunopath.* **1,** 511 (1973).
21. H. J. Phillips and J. E. Terryberry, *Expl Cell Res.* **13,** 341 (1957).
22. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, *Cancer, N.Y.* **18,** 522 (1965).
23. D. L. Peterson, J. M. Gleisner and R. L. Blakley, *Biochemistry,* **14,** 5261 (1975).
24. R. L. Blakley, *Nature, Lond.* **188,** 71 (1960).
25. L. A. Rozenszajn and J. Radnay, *Blood* **43,** 401 (1974).
26. J. Schwarzmeier and K. Moser, *Verh. dt. Ges. inn. Med.* **77,** 398 (1971).
27. W. M. Hryniuk, *Cancer Res.* **32,** 1506 (1972).
28. T. Fields and L. Brox, *Can. J. Biochem.* **52,** 441 (1974).
29. A. Fridland, *Cancer Res.* **34,** 1883 (1974).
30. M. V. Freeman, *J. Pharmac. exp. Ther.* **120,** 1 (1957).
31. M. V. Freeman, *J. Pharmac. exp. Ther.* **122,** 154 (1958).
32. L. L. Anderson, G. J. Collins, Y. Ojima and R. D. Sullivan, *Cancer Res.* **30,** 1344 (1970).
33. J. M. Kinkade, Jr., W. R. Vogler and P. G. Dayton, *Biochem. Med.* **10,** 337 (1974).
34. J. R. Bertino, *Cancer Res.* **23,** 1286 (1963).
35. T. F. Necheles, A. Maniatis and D. M. Allen, *Clin. Res.* **16,** 310 (1968).
36. A. V. Hoffbrand, E. Tripp, D. Catovsky and K. C. Das, *Br. J. Heaemat.* **25,** 497 (1973).
37. L. Delmonte and T. H. Jukes, *Pharmac. Rev.* **14,** 91 (1962).