Biochemical Pharmacology, Vol. 30, No. 19, pp. 2651–2656, 1981.
Printed in Great Britain.

0006-2952/81/192651-06 $02.00/0

EFFECTS OF FORMYCIN B ON HUMAN LYMPHOCYTE DEOXYRIBONUCLEIC ACID SYNTHESIS

OPTIMIZATION OF CELL CULTURE CONDITIONS*

MORTON J. COWAN,† DANIEL CASHMAN and ARTHUR J. AMMANN
Division of Pediatric Immunology, University of California, San Francisco, San Francisco, CA 94143, U.S.A.

(*Received* 30 *October* 1980; *accepted* 25 *February* 1981)

Abstract—We evaluated the effect of formycin B, an inhibitor of purine nucleoside phosphorylase (PNP), on DNA synthesis in phytohemagglutinin (PHA)-stimulated human peripheral blood lymphocytes using various culture conditions. We found that the dose–response curve for formycin B inhibition of lymphocyte PNP activity was similar to that for the inhibition of DNA synthesis when formycin B was added to the cultures at the time of maximum DNA synthesis. Under these conditions formycin B inhibited protein synthesis less than DNA synthesis. We also evaluated the effects of substrates of PNP added at the time of maximum DNA synthesis; deoxyguanosine was inhibitory, whereas guanosine enhanced thymidine incorporation into DNA. To assess the potential use of formycin B in exploring lymphocyte toxicity in PNP deficiency, we evaluated its effects on DNA synthesis in combination with other nucleosides. Deoxyguanosine and formycin B, when used together, were not synergistic in their combined effects on DNA synthesis. Further, deoxycytidine did not prevent the inhibition by formycin B of DNA synthesis. Our results suggest a relationship between formycin B inhibition of PNP and DNA synthesis in PHA-stimulated lymphocytes. The role of formycin B in a human cell model of PNP deficiency, however, is limited by its effects on other enzyme systems and its relatively high K_i for PNP. Its potential, if any, for exploration of lymphocyte toxicity in PNP deficiency remains to be defined.

Deficiencies of two sequential enzymes of the purine catabolic pathway, adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), result in distinct immunodeficiency diseases [1, 2]. ADA deficiency usually causes a severe immunodeficiency disease affecting both T lymphocyte and B lymphocyte mediated immunity, while PNP deficiency almost exclusively affects T cell immunity.

The recognition of the relative specificities of these enzyme deficiencies for the immune system has resulted in a great deal of interest in various inhibitors of ADA and PNP as potential pharmacotherapeutic agents [3, 4] and, also, as probes for determining the mechanisms of the toxicity to the immune system in the enzyme-deficient state. For example, deoxycoformycin, an inhibitor of ADA, has been effective in temporarily treating a T cell leukemia [3]. Moreover, it has been suggested that inhibition of erythrocyte PNP, which may be responsible for degrading potential nucleoside or nucleoside analogues before they reach their target cells, could enhance their efficacy [4]. Finally, cell models of ADA deficiency have been developed using potent inhibitors of ADA, including deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) [5, 6]. From these models there is evidence which suggests that one mechanism for selective toxicity to the immune system in ADA deficiency is an accumulation of deoxyATP in lymphocytes which inhibits ribonucleotide reductase [7, 8].

We have been interested in inhibitors of PNP as probes for understanding the role of PNP in normal immune regulation and the mechanism for the selective T cell abnormality in PNP deficiency. It has been postulated that a build-up of lymphocyte deoxyGTP in PNP deficiency, which also inhibits ribonucleotide reductase, can account for the toxicity to the immune system. Evidence from a mouse tumor cell line, S49, supports this explanation [8]. S49 cells are extremely sensitive to deoxyguanosine and accumulate high concentrations of deoxyGTP. Deoxycytidine protects these cells from deoxyguanosine toxicity, supporting the mechanism of deoxyGTP inhibition of ribonucleotide reductase. Mutant cells that lack deoxyguanosine kinase are resistant to the toxic effects of deoxyguanosine and do not accumulate deoxyGTP. Although inhibition of ribonucleotide reductase is one explanation for the toxicity to the immune system, it alone is not sufficient to explain the distinct effects on immune function that are found in ADA and PNP deficiency.

A non-malignant human cell model of PNP deficiency would be valuable for exploring the possible mechanisms for selective toxicity to the immune system since tumor cells and virus-transformed cells by their very natures have an altered regulation of

* This research was supported by the Pediatric Clinical Research Center (PCRC) (NIH 5M01RR000-79-16) and an American Heart Association Grant-in-Aid (79824). M.J.C. is a Clinical Associate Physician in the PCRC.

† Author to whom all correspondence should be addressed: Morton J. Cowan, M.D., Department of Pediatrics, M 679, University of California, San Francisco, San Francisco, CA 94143, U.S.A.

DNA synthesis. Formycin B, a competitive inhibitor of PNP [9], has been used in both human and murine stimulated lymphocyte culture systems [10, 11]. Formycin B is also known to inhibit poly(ADA-ribose) polymerase and xanthine oxidase [12], and it has been suggested that the effects on the lymphocyte culture systems are due to inhibition of these enzymes [11].

In our study we have altered the culture conditions under which formycin B is used. We have found that under these culture conditions the effect of formycin B on phytohemagglutinin-induced human lymphocyte DNA synthesis closely parallels its inhibition of PNP.

MATERIALS AND METHODS

Materials. Formycin B, deoxyguanosine, guanosine, inosine, purified PNP and xanthine oxidase were obtained from the Sigma Chemical Co., St. Louis, MO.

Lymphocyte separation and stimulation. Heparinized peripheral blood was obtained from normal young adult volunteers. Peripheral blood lymphocytes (PBL) were separated on Hypaque-Ficoll, and microcultures using 2500 cells per culture were established with phytohemagglutinin (PHA) as descibed previously [13]. Briefly, PBL were placed in culture medium consisting of RPMI-1640 and 15% pooled heat-inactivated human plasma. Six replicate cultures of 2500 cells each were placed in round bottom microculture wells (Linbro Scientific, Inc.) with either 0 or 5 μg/ml of PHA. To measure the rate of DNA synthesis, [^{3}H]-thymidine (New England Nuclear Corp., Boston, MA; 6.7 Ci/mmole) was added on day 6 of culture for a final concentration of 4 μCi/ml. The cultures were harvested on day 7 and the incorporation of thymidine into DNA was determined using an automated cell culture harvester. To measure the rate of protein synthesis, [^{3}H]-leucine (New England Nuclear Corp.; 5 Ci/mmole) was added on day 7 of culture and harvested 6 hr later. Purine nucleosides and formycin B were added in various concentrations and at various times as described in Results. Preliminary experiments showed that addition of nucleosides or formycin B did not alter the dose of PHA (5 μg/ml) at which maximum DNA synthesis occurred, the kinetics of maximum lymphocyte response, or the transport of label into the cell.

Cell counting. The cell counting experiments were done as described previously [13]. Briefly, 24–36 replicate aliquots of 2500 cells were placed into microculture wells. The number of aliquots depended upon whether the cells were under resting or stimulated conditions. Replicate cultures were harvested by washing each well with phosphate-buffered saline three times and combining the washes into a single centrifuge tube. Using a Pasteur pipette, the resulting cell suspension was vigorously mixed to break up clumps of cells. With this procedure, the amount of clumping was always less than 5 per cent and did not vary with the culture conditions. Cells were counted with eosin Y in a hemocytometer using phase optics.

Enzyme determination. For the experiments in which the effects of formycin B on PNP from extracts of T lymphocytes were measured, PNP activity was determined using a modification of the method of Kalckar [14]. Briefly, PBL were isolated over Hypaque-Ficoll and added to a 3% suspension of sheep erythrocytes in 40% fetal calf serum. After a short incubation at 4°, the suspension was centrifuged over Hypaque-Ficoll, and the pelleted (sheep erythrocyte-rosetting) T lymphocytes were collected, the erythrocytes were lysed with ammonium chloride. The T lymphocytes (more than 95% sheep erythrocyte-rosetting) were then resuspended in 50 mM KPO_4 buffer (pH 7.4) and lysed by freezing and thawing four times. Protein determination of the extract was made using the Biorad Protein Determination Kit (Coomassie Blue) with bovine serum albumin standard. For determining PNP activity with inosine as the substrate, the reaction mixture consisted of 1 mM inosine, 0.175 units xanthine oxidase and the lymphocyte extract (8–10 μg protein). When deoxyguanosine was the substrate, the reaction mixture also contained guanine deaminase (GDA) prepared from the liver of a patient who had died with PNP deficiency (manuscript in preparation). When determining the inhibitory effect of formycin B on PNP activity, different concentrations of formycin B were added to the reaction mixture, which contained a concentration of substrate that was approximately equal to K_m. Preliminary experiments showed that, at the concentrations of formycin B which were used, GDA and xanthine oxidase were not rate limiting.

RESULTS

Formycin B inhibition of T lymphocyte PNP activity. The inhibitory effect of formycin B on PNP activity in extracts from human T lymphocytes (E-rosetting) using deoxyguanosine as the substrate is shown in Fig. 1. Doses of formycin B of 1000 μM or more also completely inhibited PNP activity when inosine and guanosine were the substrates at concentrations approximately equal to their respective K_m values (data not shown).

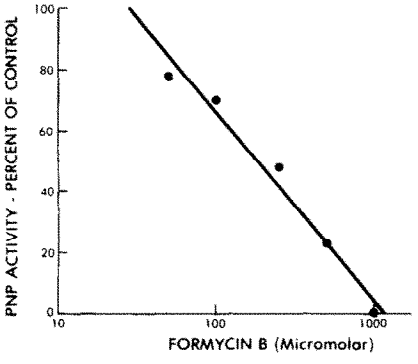

Fig. 1. Dose–response curve of formycin B inhibition of T lymphocyte PNP activity. Various doses of formycin B were added to a reaction mixture containing 99 μM deoxyguanosine. The activity is expressed as a percentage of that seen with PHA alone.

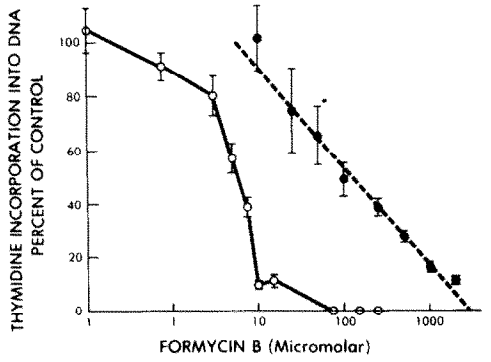

Fig. 2. Dose–response curves of formycin B inhibition of DNA synthesis in PHA-stimulated PBL. Results are expressed as a percentage of thymidine incorporated into DNA with PHA alone. Key: (○——○) formycin B added on day 0 of culture; (●——●) formycin B added on day 6. Each point is the mean ± S.E.M. of three to ten controls from six to eight separate experiments.

Effect of formycin B on PHA-stimulated lymphocytes. When formycin B was added on the initial day of culture (day 0) along with PHA in the microculture system, we found that as little as 10 μM formycin B resulted in a 90 per cent inhibition of the lymphocyte response to PHA, as measured by thymidine incorporation into DNA (Fig. 2). Concentrations of 75 μM formycin B and higher completely inhibited the lymphocyte response to PHA. This dose–response curve was very different from that seen for inhibition of PNP activity (Fig. 1), suggesting that the mechanism of inhibition of DNA synthesis was also different.

To explore the effects of different culture conditions on the kinetics of the inhibition of DNA synthesis by formycin B, we added 10 μM formycin B to the PHA-stimulated PBL on different days during the 7-day culture period (Fig. 3). When formycin B was added on culture day 3, there was still significant inhibition of DNA synthesis measured of day 7. When it was added on days 4, 5 and 6, however,

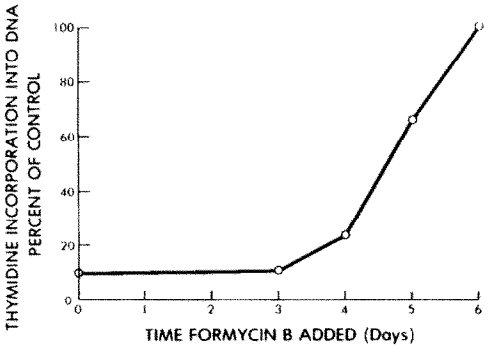

Fig. 3. Kinetics of formycin B inhibition of DNA synthesis. Formycin B (10 μM) was added to PHA-stimulated PBL on different days following stimulation of DNA synthesis. At the end of the culture period (day 7), thymidine incorporation into DNA was measured. Results are expressed as a percentage of thymidine incorporated into DNA with PHA alone. Each point is the average using PBL from two normal individuals.

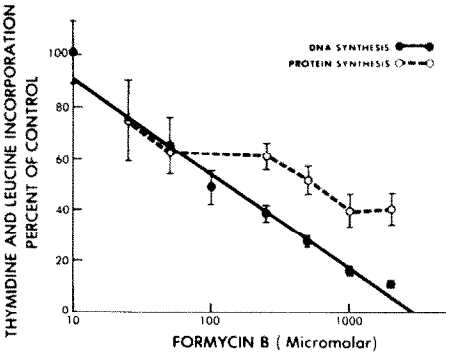

Fig. 4. Effect of formycin B on DNA and protein synthesis in PHA-stimulated PBL. Results are expressed as percentages of thymidine and leucine incorporated into DNA and protein, respectively, with PHA alone. Each point is the mean ± S.E.M. of three to five experiments.

there was an increasingly smaller effect. A dose–response curve of formycin B inhibition of DNA synthesis in PHA-stimulated lymphocytes when the formycin B was added on day 6 of culture (maximum time of DNA synthesis) is also shown in Fig. 2. There was approximately a 1 log shift of the dose–response curve to the right compared to formycin B added on day 0. Whereas 75 μM formycin B added on day 0 completely inhibited DNA synthesis, when it was added on day 6 there was only 50 per cent inhibition. Formycin B had no detectable effect on DNA synthesis in unstimulated cultures.

Effect of formycin B on protein synthesis. A dose–response curve of formycin B added at the time of maximum DNA synthesis (days 6–7) and leucine incorporation into protein is shown in Fig. 4. When expressed as a percentage of control cultures (PHA alone), formycin B inhibited protein synthesis, but to a lesser extent than DNA synthesis. Formycin B at a concentration of 2000 μM resulted in approximately 90 per cent inhibition of PHA-stimulated DNA synthesis, but only inhibited protein synthesis by about 60 per cent. When added to unstimulated cultures, formycin B had no effect on protein synthesis in our assay system, suggesting that formycin B under these conditions was not directly toxic to protein synthesis.

Cell counting experiments. To determine whether the inhibition of DNA synthesis by formycin B was due to cell destruction, formycin B was added on day 6 (T = 0) to the PHA-stimulated microcultures, and the cultures were harvested after 6 hr. Cell were counted at T = 0 and T = 6 hr and parallel cultures were processed for [^{3}H]thymidine incorporation into DNA. The results of three experiments using cells from five donors are shown in Table 1, and are expressed as the per cent change in cell number on day 6 and as DNA synthesis per cell from T = 0 to T = 6 hr. When PHA alone (0 formycin B) was in the culture, there was a significant increase in cell number over this 6-hr culture period (165 ± 77 per cent). When either 500 or 2000 μM formycin B was present, there was no significant difference in this increase (134 ± 102 per cent and 128 ± 104 per cent respectively; $P > 0.5$). There was no effect of formycin B on cell number or viability (eosin Y dye

Table 1. Change in cell number and DNA synthesis of PHA-stimulated lymphocytes exposed to formycin B for 6 hr on day 6 of culture

Formycin B (μM)	Viable cell number (% change)	P*	DNA synthesis per viable lymphocyte (cpm/cell)	P*
0	165 ± 77†		0.680 ± 0.150†	
500	134 ± 102	> 0.5	0.422 ± 0.230	< 0.05
2000	128 ± 104	> 0.5	0.174 ± 0.088	< 0.001

* One-way analysis of variance: 0 vs 500 μM and 0 vs 2000 μM.
† Mean ± S.D.

exclusion) of unstimulated cultures or on cell viability when compared to the control cultures with PHA alone. When the corresponding cultures were evaluated for thymidine incorporation into DNA and expressed as cpm per viable cell, there was significant inhibition of DNA synthesis per cell for both 500 and 2000 μM formycin B compared to cultures with PHA alone ($P < 0.05$ and < 0.001 respectively). Under these culture conditions concentrations of formycin B that significantly inhibited PNP, and DNA synthesis, per cell did not result in loss of cell viability or number of cells.

Effects of nucleoside substrates of PNP on DNA synthesis. It is currently believed that, of the four substrates of PNP, deoxyguanosine and/or guanosine are responsible for the toxicity to the immune system. Experiments were performed to determine the effects of guanosine and deoxyguanosine on DNA synthesis in PHA-stimulated PBL. Various concentrations of these substrates were added to cultures either on day 0 or day 6, and thymidine incorporation into DNA was measured on day 7 (Fig. 5). When the nucleosides were added on day 0, deoxyguanosine was a more potent inhibitor of DNA synthesis than guanosine at concentrations of 75–250 μM. When the nucleosides were added on day 6 of culture, the inhibitory effect of as much as 500 μM deoxyguanosine was only 20 per cent. Doses of guanosine up to 500 μM were not inhibitory at all, and instead resulted in an enhancement of thymidine incorporation into DNA. Thus, the relative difference between the effects on DNA synthesis of these two nucleosides persisted when they were added on day 6; however, the toxicity of each was significantly less under these culture conditions.

Effects of formycin B and deoxyguanosine on DNA synthesis. If lymphocyte toxicity in PNP deficiency results from an accumulation of deoxyguanosine and deoxyGTP, there should be an additive or synergistic effect of formycin B and deoxyguanosine in our cell model, similar to that found using EHNA and deoxyadenosine. To explore this possibility, a total of five experiments using PBL from eight donors was performed. On day 6 of culture, concentrations of formycin B of 100, 1000 and 2000 μM were evaluated for their effects on thymidine incorporation into PHA-stimulated lymphocytes, with and without 10, 75, 150 and 500 μM deoxyguanosine. An example of two experiments using two donors is shown in Table 2. In none of the experiments was there consistent evidence for synergistic effects of deoxyguanosine and formycin B.

Evaluation of the effects of deoxycytidine on formycin B inhibition of cell division and protein synthesis in PHA-stimulated lymphocytes. One proposed mechanism for inhibition of DNA synthesis in PNP deficient lymphocytes is the inhibition of ribonucleotide reductase by deoxyGTP. As in ADA-inhibited cell models, this inhibition might be partially reversed or prevented by providing the cells

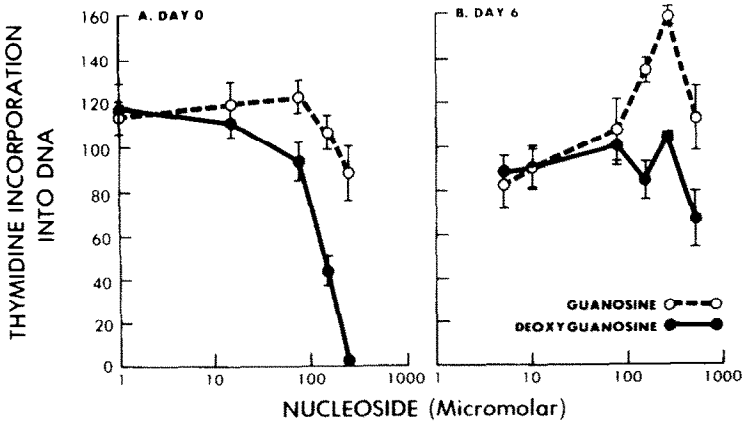

Fig. 5. Effects of guanosine and deoxyguanosine on DNA synthesis in PHA-stimulated PBL. Results are expressed as a percentage of thymidine incorporated into DNA with PHA alone. Panel A: nucleosides added on day 0 of culture; panel B: nucleosides added on day 6. Each point is the mean ± S.E.M. of three to five experiments.

Table 2. Effects of formycin B (FB) and deoxyguanosine (DG) on PHA-stimulated lymphocyte DNA synthesis

Culture condition*	Thymidine incorporation into DNA (cpm)
Experiment 1	
PHA	18,795
PHA + 100 μM FB	6,430
PHA + 500 μM DG	11,596
PHA + 100 μM FB + 500 μM DG	5,845 (3,962)†
Experiment 2	
PHA	13,212
PHA + 1000 μM FB	1,673
PHA + 150 μm DG	6,986
PHA + 1000 μM FB + 150 μM DG	1,774 (910)†

* PHA was added on day 0 of culture while all other substances were added on day 6.

† Numbers in parentheses equal predicted cpm if effects of FB and DG were additive.

with deoxycytidine. The possible protective effect of deoxycytidine on formycin B-inhibited lymphocytes was evaluated using cell counting and measuring the incorporation of leucine into protein. A preliminary experiment revealed that 250 μM deoxycytidine inhibited the incorporation of thymidine into DNA in PHA-stimulated lymphocytes by as much as 80 per cent, while there was no effect on leucine incorporation into protein or a decrease in the number of viable cells compared to cultures with PHA alone (data not shown). In a subsequent study, the results of which are shown in Table 3, PHA-stimulated cultures with 500 μM formycin B had approximately half the number of cells as control cultures (PHA alone) after 24 hr exposure. In cultures that had 500 μM deoxycytidine in addition to 500 μM formycin B, there was no significant increase in cell number. Parallel cultures of PHA-stimulated PBL inhibited by formycin B and evaluated for leucine incorporation into protein showed no significant improvement when deoxycytidine was present. Under these conditions we could not demonstrate a beneficial effect of deoxycytidine on formycin B-inhibited lymphocytes.

DISCUSSION

Formycin B is a competive inhibitor of erythrocyte PNP with a K_i value of 100 μM for inosine [9]. It is also an inhibitor of PNP in intact erythrocytes [9] and murine spleen cells [10]. Studies in our laboratory indicate that formycin B also inhibits PNP in intact human PBL. We have found that in extracts of human T lymphocytes the K_i of formycin B with deoxyguanosine as the substrate is 68 μM; the K_m for deoxyguanosine in these extracts is 60 μM (manuscript in preparation). Formycin B is not specific for PNP. It has been found to inhibit poly(ADP-ribose) polymerase [12] and xanthine oxidase [15], although not sufficiently to interfere with the xanthine oxidase coupled assay used in this study. These features of formycin B limit its efficacy in cell models of PNP deficiency in which conventional culture conditions are used [10, 11]. In this report we have explored different culture conditions in an effort to optimize the value of formycin B as an inhibitor of PNP and DNA synthesis in a human cell model system.

In most experimental cell models of ADA and PNP deficiency, nucleosides and inhibitors have been added on the initial day of culture (day 0) and DNA synthesis has been measured after 48–72 hr. Willemot *et al.* [10], using murine spleen cells found that the doses of formycin B which inhibited DNA synthesis by as much as 70–80 per cent inhibited PNP activity only by 30 per cent. In studies by Osborne *et al.* [11] using EBV infected cells, leukemic cell

Table 3. Effect of deoxycytidine (DC) and formycin B (FB) on PHA-stimulated lymphocytes

Culture condition*	Cells per well	
	Control No. 1	Control No. 2
Resting	736	722
PHA	45,417 (7,000)†	50,625 (7,321)
PHA + 500 μM FB	26,458 (5,448)	17,500 (4,287)
PHA + 500 μM FB + 500 μM DC	27,708 (6,303)	20,833 (4,732)

* PHA was added on day 0 of culture whereas all other substances were added on day 6.

† Numbers in parentheses are stimulated minus resting cpm of leucine incorporation into protein; mean of six replicate cultures.

lines and PHA-stimulated PBL under conventional culture conditions, the doses of formycin B were insufficient to inhibit PNP significantly. When we added as little as 10 μM formycin B on day 0, using a microculture system that we have found to be very sensitive to defects in lymphocyte response to PHA [13], there was nearly complete inhibition of DNA and protein synthesis 6–7 days later, when maximum DNA synthesis normally occurs in this system. This dose of formycin B had little effect on human T lymphocyte PNP activity, indicating that inhibition of other enzymes, such as poly(ADP-ribose) synthetase, was more important. Muller *et al.* [12], using mouse leukemia cells, found that 40 μM formycin B inhibited DNA synthesis and synthesis of poly(ADP-ribose) by 60 per cent. However, when we added 10–40 μM formycin B to PHA-stimulated PBL on culture day 6 (during maximum DNA synthesis), there was minimal inhibition on DNA synthesis, suggesting that under these conditions inhibition of enzymes such as poly(ADP-ribose) synthetase has less effect on DNA synthesis. Recent studies that support this conclusion suggest that poly(ADP-ribose) synthesis is important for normal DNA repair mechanisms associated with early stages of differentiation, and that under certain conditions synthetase activity does not correlate with DNA synthesis [16, 17].

By adding formycin B at the time of maximum DNA synthesis, we found that the dose-dependent inhibition of DNA synthesis by formycin B was quite similar to its inhibition of extracted T lymphocyte PNP activity (Figs. 1 and 2). Furthermore, there was significantly less inhibition of protein synthesis under these conditions. Moreover, we could not detect an effect of formycin B on thymidine incorporation into DNA or leucine incorporation into protein in unstimulated lymphocytes, and we found that PNP activity per cell was 100 per cent higher in PHA-stimulated PBL than in resting cells just preceding the time of maximum DNA synthesis (data not shown). Finally, the cell counting and viability experiments indicate that the decreased incorporation of thymidine into DNA in the presence of formycin B was due to decreased DNA synthesis per cell rather than to an absolute loss of viable cells. These results are compatible with, although they do not prove an association between, formycin B inhibition of PNP and inhibition of PHA-stimulated lymphocyte DNA synthesis. The effects on protein synthesis under these culture conditions appear to be indirect and could be explained by interference with guanine nucleotide metabolism [18].

The inhibition by Formycin B of DNA synthesis in our culture system is not comparable to the clinical disorder of PNP deficiency. Heterozygotes for the disease with approximately 50 per cent PNP activity have normal immunity whereas in our culture system formycin B inhibition of PNP by 50 per cent resulted in significant inhibition of lymphocyte response to PHA. This limits the use of formycin B in a cell model of PNP deficiency and suggests that even under these conditions the effect of formycin B on DNA synthesis is due to its effect on other enzymes in addition to PNP.

We were unable to demonstrate a synergism between formycin B and deoxyguanosine using a range of concentrations, and deoxycytidine did not prevent the toxicity of formycin B. These findings suggest that under these culture conditions formycin B inhibition of DNA synthesis is not mediated through deoxyGTP inhibition of ribonucleotide reductase. In other experiments (data not shown), we determined that formycin B did not inhibit deoxyguanosine kinase or interfere with deoxyguanosine and deoxycytidine transport. The significance of the inhibition of PNP by formycin B with respect to lymphocyte DNA synthesis may be determined by additional studies of nucleoside and nucleotide pools (i.e. deoxyguanosine, deoxyGTP and GTP) in these cells.

REFERENCES

1. E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet* **1**, 1067 (1972).
2. E. R. Giblett, A. J. Ammann, D. W. Wara, R. Sandman and L. K. Diamond, *Lancet* **1**, 1010 (1975).
3. H. G. Prentice, K. Ganeshaguru, K. Bradstock, A. H. Goldstone, J. F. Smyth, B. Worke, G. Janossy and A. V. Hoffbrand, *Lancet* **2**, 170 (1980).
4. J. D. Stoekler, C. Cambor and R. E. Parks, Jr., *Biochemistry* **19**, 102 (1980).
5. I. H. Fox, E. C. Keystone, D. D. Gladman, M. Moore and D. Cane, *Immun. Commun.* **4**, 419 (1975).
6. T. Hovi, J. Smyth, A. Allison and S. Williams, *Clin. exp. Immun.* **23**, 395 (1976).
7. B. Ullman, L. Gudas, A. Cohen and D. W. Martin, Jr., *Cell* **14**, 365 (1978).
8. L. J. Gudas, B. Ullman, A. Cohen and D. W. Martin, Jr., *Cell* **14**, 531 (1978).
9. M. R. Sheen, B. K. Kim and R. E. Parks, Jr., *Molec. Pharmac.* **4**, 293 (1968).
10. J. Willemot, R. Martineau, C. Des Rosiers, S. Kelly, J. Letourneau and M. Lalanne. *Life Sci.* **25**. 1215 (1979).
11. W. R. Osborne, J. L. Sullivan and C. R. Scott, *Immun. Commun.* **9**, 257 (1980).
12. W. E. G. Muller, R. Steffen, A. Maidhof, M. Lachmann, R. Zahn and H. Umezawa, *Cancer Res.* **35**, 3673 (1975).
13. M. J. Cowan, P. Fujiwara and A. J. Ammann, *Clin. Immun. Immunopath.* **17**, 595 (1980).
14. H. M. Kalckar, *J. biol. Chem.* **167**, 429 (1947).
15. M. R. Sheen, H. F. Martin and R. E. Parks, Jr., *Molec. Pharmac.* **6**, 255 (1969).
16. N. A. Berger, S. J. Petzold and S. J. Berger, *Biochim. biophys. Acta* **564**, 90 (1979).
17. K. Morioka, K. Tanaka and T. Ono, *Biochem. biophys. Res. Commun.* **94**, 592 (1980).
18. C. G. Kurland, *Biophys. J.* **22**, 373 (1978).