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Selective toxicity of purine deoxynucleosides for human lymphocyte growth and function

(immune deficiency/cell growth/antibody response)

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ABSTRACT **A role for the enzymes adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) and purine-nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) in the functional maturation of lymphoid cells has been revealed by the association of inherited deficiencies of these enzymes and profound immune deficiency. Previous studies have suggested that the selective toxicity for lymphocytes may be mediated by the accumulation of toxic deoxynucleoside metabolites, likely through the action of specific kinases enriched in lymphoid cells. In order to study possible mechanisms whereby lymphocyte function may be impaired in these disorders, we have studied the effect of nucleosides and their deoxy analogues on both T and B lymphocyte growth and function. In the presence of deoxyguanosine, there was marked inhibition of T lymphoblast growth, phytohemagglutinin-induced cell proliferation, and T suppressor cell activity. T helper cell activity and the differentiation of B cells to an antibody-secreting stage were unaffected. Deoxyadenosine was much less inhibitory, but in the presence of an inhibitor of adenosine deaminase, its effects on lymphocyte growth and function were markedly potentiated. The addition of deoxycytidine prevented deoxyadenosine toxicity in all assays, whereas it only interfered with deoxyguanosine effects on T lymphoblast growth. These studies provide some initial understanding for the selective loss of specific lymphocyte functions in individuals with inborn errors of purine metabolism.**

The association of disorders of purine nucleoside catabolism with severe immunologic dysfunction provides compelling evidence for the role of these metabolic pathways in the biochemical control of lymphocyte function. Since the original descriptions of combined B and T cell abnormalities in children with a deficiency of the enzyme adenosine deaminase (Ado deaminase; adenosine aminohydrolase, EC 3.5.4.4) (1) and apparently selective T cell incompetence in a child with purine nucleoside phosphorylase (Puo phosphorylase; purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) deficiency (2), attention has been directed to the molecular mechanisms whereby lymphocyte differentiation and function are impaired. A number of hypotheses have been proposed to relate the lymphocyte abnormalities to the biochemical alterations, particularly the accumulation of toxic metabolites. The recent reports of elevated dATP levels in erythrocytes from Ado deaminase-deficient patients (3, 4) and elevated urinary and erythrocyte levels of deoxyinosine (dIno) and deoxyguanosine (dGuo) in Puo phosphorylase deficiency (5, 6), have focused interest on deoxynucleosides as the potential mediators of lymphocyte dysfunction.

Some support for this theory has been derived from the observation that deoxyadenosine (dAdo) is a more potent inhibitor of the normal T lymphocyte blastogenic response to phytohemagglutinin (PHA) than adenosine, particularly in the presence of the Ado deaminase inhibitor, *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA) (7, 8). Inosine, which is elevated in the serum and urine of patients with Puo phosphorylase deficiency, has little capacity to inhibit normal PHA responses (9). dGuo and, to a lesser degree, dIno were capable of inhibiting growth of a human B cell line, whereas inosine and guanosine were relatively ineffective (7). In addition, deoxynucleoside kinases are largely confined to lymphoid cells (7). Since these enzymes are capable of phosphorylating deoxynucleosides (10), the accumulation of increased intracellular quantities of deoxynucleoside triphosphates may lead to an inhibition of cell growth through their inhibition of ribonucleotide reductase (11).

In the present studies we have compared the effects of both nucleosides and 2′-deoxy compounds on lymphocyte growth as well as on specific T and B cell functions. These data confirm the toxicity of dAdo in the presence of EHNA for T lymphocytes and provide evidence for the relatively selective toxicity of dGuo on T cell growth and function, whereas certain aspects of B cell growth and function were generally unaffected.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBL) or tonsillar lymphocytes were obtained after Ficoll–hypaque gradient centrifugation (12). B and T lymphocytes were obtained by rosette depletion on density gradients (13, 14). Two human T cell lines, Jurkat and Molt-3, were used in these studies as well as several human B cell lines established from normal individuals (15). The cell lines were carried as continuous cultures in medium (RPMI 1640) supplemented with 20% fetal calf serum.

Lymphocyte Studies. Growth studies using the T and B cell lines were carried out in the following way: $2.5–5.0 \times 10^5$ viable cells (assessed by trypan blue dye exclusion) were incubated for 68 hr at 37°C in 5% CO_2 in 12 × 75 mm plastic tubes (Falcon) containing 0.5 ml of RPMI 1640 supplemented with 1–10% heat-inactivated (56°C for 60 min) fetal calf serum. The cells were then washed two or three times in medium and incubated for a further 4 hr in the presence of 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) or a mixture of [^{14}C]leucine, [^{14}C]threonine, and [^{14}C]valine. DNA or protein synthesis was assayed by harvesting the cells with an automated harvester (Skatron, Flow Laboratories, Rockville, MD) on fiber glass filters, drying, and measuring radioactivity in a Beckman liquid scintillation counter. Nucleosides were added at the initiation of the cultures.

PBL were stimulated by the addition of 10 μg of purified PHA per ml (Burroughs–Wellcome, Beckenham, England) to

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Abbreviations: Ado deaminase, adenosine deaminase; Puo phosphorylase, purine-nucleoside phosphorylase; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; PFC, plaque-forming cells; EHNA, *erythro*-9-[3-(2-hydroxynonyl)]adenine.

3×10^5 PBL in 0.5 ml of RPMI supplemented with 10% heat-inactivated AB serum. After 68 hr of incubation, cells were washed and labeled for 4 hr with [^{3}H]thymidine or with [^{14}C]leucine, [^{14}C]threonine, and [^{14}C]valine and DNA or protein synthesis was measured as above. Nucleosides were added at the initiation of cultures.

In vitro induction and measurement of ovalbumin-specific human plaque-forming cells (PFC) were done as described (13, 14). Briefly, tubes containing 2×10^6 B lymphocytes and 1×10^6 T lymphocytes (from tonsils) were cultured in 10 ml of RPMI supplemented with 10% heat-inactivated AB serum, 50 μM 2-mercaptoethanol, and several concentrations of ovalbumin. After 5 days the cells were harvested and washed and the PFC response was assayed. Various concentrations of the drugs were added at the initiation of the culture.

Reagents. Nucleosides, deoxynucleosides, and ovalbumin were obtained from Sigma. Stock solutions were made up in phosphate-buffered saline (pH 7.2) and filtered through a Millipore filter. EHNA was kindly provided by G. Elion, Burroughs-Wellcome (Research Triangle Park, NC).

RESULTS

Cell Proliferation. The effects of adenosine, inosine, guanosine, and their deoxyderivatives on the proliferative capacity of T cell lines are illustrated in Fig. 1*A*. Inosine and adenosine were inhibitory only at the highest concentrations tested; dIno, dAdo, and guanosine were inhibitory to roughly the same degree. At all concentrations tested, dGuo was the most inhibitory to both DNA and protein synthesis, with 50% inhibition at 0.02 mM dGuo. There were essentially no differences between the two T cell lines tested or the effects of the drug on either DNA or protein synthesis (data not shown). In cultures with 1% fetal calf serum as opposed to 10%, the dose–response curves were virtually identical except for a slightly increased susceptibility to the drug effects. This may reflect residual enzyme activity (Puo phosphorylase and Ado deaminase) in the 10% fetal calf serum. At drug concentrations below 0.3–0.6 mM, cell viability was greater than 80–90%, except with dGuo, where more than 50% of the cells were nonviable at this concentration (0.6 mM).

The PHA-induced proliferation of normal peripheral blood T cells was, in general, less susceptible to the drug effects (Fig. 1*B*). Indeed, inosine regularly enhanced the response, as previously reported (9). dIno was inhibitory only at concentrations greater than 1.0 mM, and there was only moderate inhibition of DNA and protein synthesis between 0.3 and 0.6 mM dAdo, adenosine, and guanosine. dGuo remained the most potent inhibitor of the response, with 50% inhibition at approximately 0.1 mM dGuo. At concentrations less than 1.0 mM, cell viability was greater then 85% in all groups and controls.

In contrast, the B cell lines were unaffected by inosine, dIno, adenosine, or dAdo. Two of the B cell lines were relatively resistant to the effects of guanosine or dGuo, with 50% inhibition of thymidine uptake at 1.25 mM. One B cell line was more sensitive to inhibition of DNA or protein synthesis (50% inhibition at 0.15 mM) (data not shown).

Reversal of dAdo Toxicity. In the presence of 7.5 μM EHNA, 80 μM dAdo inhibited T lymphoblast or PHA-induced DNA synthesis (Fig. 2). At these concentrations of dAdo or EHNA alone, there was no impairment of thymidine uptake. Thus, the toxicity of dAdo (or adenosine) was markedly potentiated by the addition of EHNA, likely due to its inhibition of Ado deaminase. Addition of 50–100 μM deoxycytidine (dCyd) to the incubation mixtures reversed the toxicity of dAdo plus EHNA (Fig. 2) or the toxicity of higher concentrations of dAdo alone. dCyd alone was always slightly inhibitory.

Results with the B lymphoblast lines were similar except that higher concentrations of dAdo were required for inhibition of [^{3}H]thymidine uptake in the presence of EHNA.

Reversal of dGuo Toxicity. dCyd was effective in reversing the toxicity of dGuo on T lymphoblast growth (Fig. 3); shifting the concentration of dGuo resulting in 50% inhibition from 0.06 to 0.25 mM. The increase in DNA and protein synthesis was paralleled by an increase in cell viability. In contrast, there was no effect on the PHA-induced proliferative response. Indeed, at all concentrations of dGuo, dCyd was somewhat inhibitory. Similarly, there was no effect of dCyd on dGuo toxicity for B lymphoblast cell line growth, confirming the findings of Carson *et al.* (7).

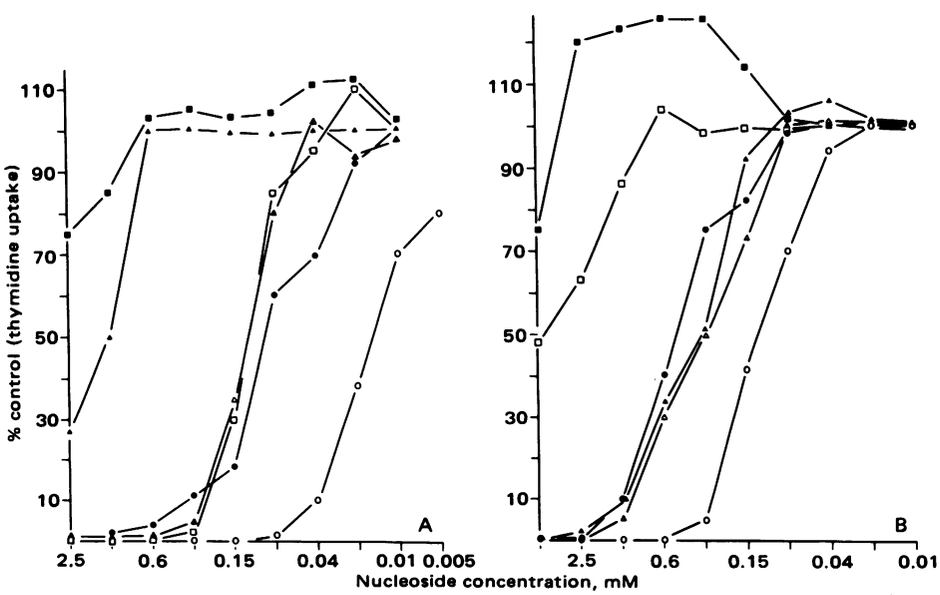

FIG. 1. Effect of nucleosides and deoxynucleosides on [^{3}H]thymidine incorporation. (*A*) T cell line (Molt-3); (*B*) PHA-stimulated PBL. Cells were cultured in the presence of the indicated nucleoside concentration. Results are expressed as the % of untreated control [^{3}H]thymidine uptake; each point represents the mean of a minimum of three separate experiments carried out in triplicate. SD in all experiments never exceeded 10%. Control values for [^{3}H]thymidine incorporation for Molt-3 ranged from 120,000 to 210,000 cpm; for PHA-induced proliferation of PBL, from 45,000 to 95,000 cpm. ■, Inosine; ▲, adenosine; ●, guanosine; □, dIno; △, dAdo; ○, dGuo.

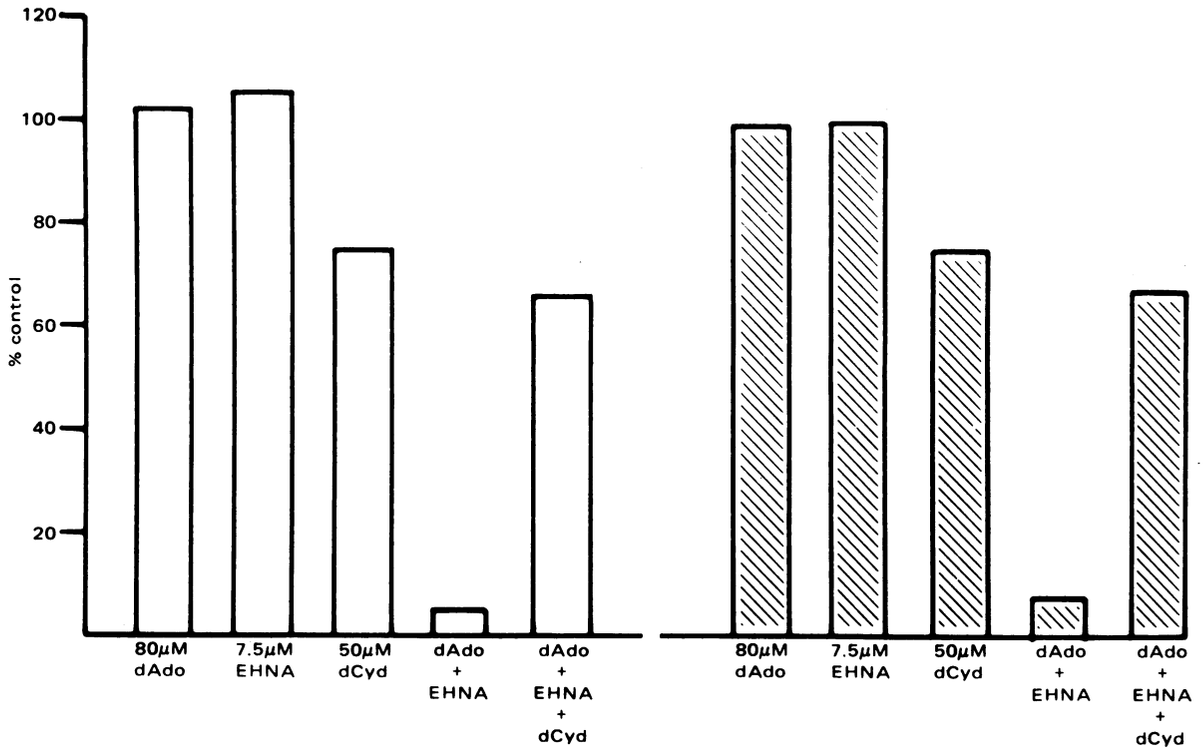

FIG. 2. Potentiation of dAdo toxicity in the presence of EHNA and reversal by dCyd. T lymphoblasts (*Left*) or PHA-stimulated PBL (*Right*) were cultured with combinations of dAdo, EHNA, and dCyd, and uptake of [^{3}H]thymidine was determined. EHNA was added to the cultures 15 min prior to addition of nucleosides. Each point represents the mean of three separate experiments; SD did not exceed 8%.

PFC Response. The generation of an ovalbumin-specific PFC response requires the cooperation of both B and T cells, particularly the balance between T helper and T suppressor cell signals (9, 13, 14). In contrast to the antigen-induced activation of T helper cells, the differentiation of B cells to an antibody-secreting stage and the activation of antigen-specific T suppressor cells require proliferation (16–18). We examined the effects of dGuo on the cellular components of the PFC response. As shown in Fig. 4, the untreated control cells generated a typical bell-shaped antigen dose dependent PFC response (13), with suppression of the PFC response at higher antigen con-

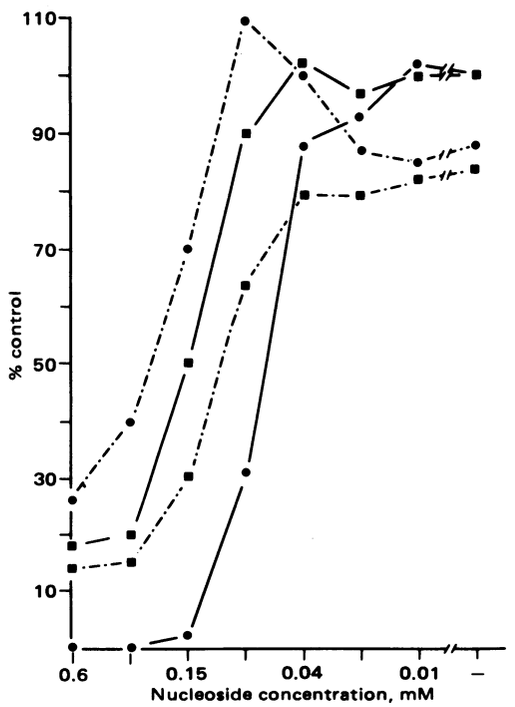

FIG. 3. Reversal of dGuo toxicity by dCyd. T lymphoblasts (●) or PHA-stimulated PBL (■) were incubated with different concentrations of dGuo (on abcissa; —, no dGuo) in the presence (- - -) or absence (——) of 100 μM dCyd. Results are expressed as the % control [^{3}H]thymidine uptake; each point represents the mean of at least two experiments carried out in triplicate.

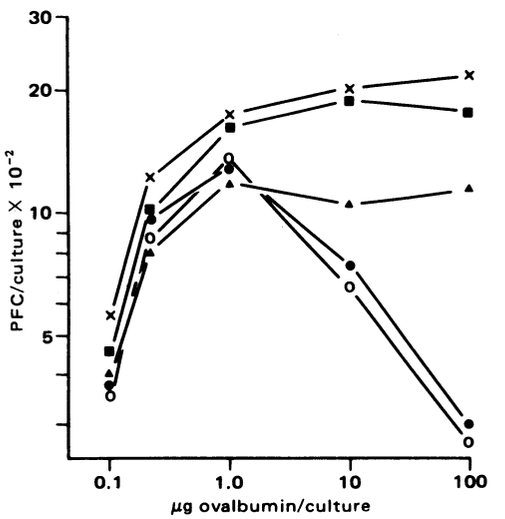

FIG. 4. Effect of dGuo on the PFC response. Cultures containing 2 × 10^6 B lymphocytes and 1 × 10^6 T lymphocytes, obtained from tonsil, were incubated for 5 days in the presence of different concentrations of dGuo and increasing concentrations of ovalbumin. Results are expressed as the mean PFC/culture carried out in triplicate. Two additional experiments gave similar results. O, Untreated control cultures; ▲, cultures containing 250 μM dGuo; ×, 25 μM dGuo; ■, 2.5 μM dGuo; ●, 1.0 μM dGuo.

centrations. After addition of 2.5–250 μM dGuo there was no inhibition of the PFC response at the higher antigen concentrations, suggesting that in the presence of dGuo, suppressor cell effects were not manifested. On the other hand, B cell and the nonproliferative dependent T helper cell effects were refractory to these concentrations of dGuo, which were inhibitory to both T cell growth and PHA-induced proliferation (Fig. 1). The addition of 2.5–25 μM guanosine did not effect the expression of suppressor cell activity, whereas 250 μM guanosine had only a marginal effect.

DISCUSSION

A number of different mechanisms have been suggested to explain the immunodeficiencies associated with the absence of purine salvage pathway enzymes. Adenosine-induced pyrimidine starvation (19), hypoxanthine deficiency (20), adenosine-mediated elevation in cyclic AMP concentrations (21), and dAdo accumulation (7, 8) have been proposed to explain the association of Ado deaminase deficiency and combined T and B lymphocyte dysfunction. dGuo has been suggested as the toxic metabolite in Puo phosphorylase deficiency, perhaps through its phosphorylation to dGTP, which acts as a potent inhibitor of mammalian ribonucleotide reductase (6). Whatever the mechanism, one must account for the preferential toxicity of these metabolites to selectively impair distinct lymphocyte subpopulations at discrete stages of functional maturation (9, 22).

Because of current interest in nucleoside accumulation and reported potency of deoxynucleosides in inhibiting lymphocyte growth, we have evaluated the effects of these compounds on lymphocyte growth and function. Distinct susceptibility profiles, typical for the different cell types and functions studied, were obtained. We have demonstrated that T lymphoblasts from continuous cell lines are most sensitive to dGuo, mitogen-induced lymphoblasts are somewhat less susceptible, and most B lymphoblasts are relatively resistant. Variable degrees of sensitivity were seen with the other nucleosides tested. One B cell line was inhibited by dGuo (and guanosine) to a degree comparable to PHA-induced T cell proliferation.

To further assess the effect of dGuo on distinct lymphocyte functions and to circumvent the use of malignant cell lines, we carried out experiments in an *in vitro* system for the measurement of specific T-cell-dependent antibody production. We previously established that this assay involves activation of B lymphocytes, T helper cells, and T suppressor lymphocytes (13, 14). Only the maturation of B lymphocytes to an antibody-secreting stage and the expression of T suppressor cell effects are proliferative-dependent events (16, 17). In the normal B and T cell mixtures, the PFC response peaks at an optimal antigen concentration and falls off at higher concentrations, secondary to the induction of suppressor cells (14). In the presence of 2.5–250 μM dGuo, there was a failure of expression of these T suppressor cell effects. T helper cell activity and the maturation of B cells were equivalent to untreated controls. These data re-emphasize the selective toxicity of dGuo for T-cell-dependent events that require proliferation for their expression, whereas B cells and B cell growth or proliferation are relatively spared. The results also suggest that the small T cell population responsible for suppression may be more sensitive to dGuo than the majority of T cells since, at low concentrations of dGuo, mitogen-induced proliferation was unaffected. Furthermore, these results may partially explain the laboratory findings in patients with Puo phosphorylase deficiency and associated dGuo accumulation. We have shown that the early steps in T cell differentiation and T helper cell activity (and, to some degree, T regulator activity) were intact in these patients, as was B cell function (9). Thus, Puo phosphorylase deficiency leads to a selective deficiency of (proliferative-dependent) T cell function, lymphopenia, but normal *in vitro* and *in vivo* antibody production (2, 9, 23, 24).

On its own, dAdo was only moderately toxic to T lymphoblast proliferation. In the presence of EHNA, an inhibitor of Ado deaminase, the toxicity of dAdo to T cell proliferation was markedly potentiated. In the presence of EHNA, dAdo was mildly toxic to B lymphoblast proliferation, but only at concentrations greater then 0.6 mM. In preliminary studies, the addition of dAdo plus EHNA did not abolish the PFC response. It would thus appear that the addition of dAdo and EHNA does not mimic the clinical and laboratory experience with Ado deaminase deficiency and combined immune deficiency (22). There may be several explanations for the differences observed in the patients and our studies. One explanation may be that dAdo accumulation alone does not represent the precise mechanism. Alternatively, Ado deaminase deficiency is frequently associated with a profound lymphopenia. Early stages of lymphocyte differentiation may therefore be more susceptible to dAdo toxicity, whereas at the stage of B lymphocytes or E-rosetting T cells, lymphocytes may be relatively insensitive. Finally, it must be recalled that Ado deaminase deficiency is indeed a heterogeneous disease; not all patients have combined deficiency since B lymphocytes and B cell functions have been described in certain patients (1, 22, 25).

dCyd was effective in preventing the toxicity of dAdo (plus EHNA) in all assays. Reversal of dGuo toxicity by dCyd was only observed with T lymphoblast growth. As previously reported (7), there was no interference with its effects on B lymphoblast proliferation and, surprisingly, no reversal of its effects on PHA-induced proliferation. dCyd may work through competitive inhibition of dCyd kinase, thereby preventing the direct phosphorylation of dGuo (and dAdo) to dGTP (and dATP). This could explain the reversal of dGuo and dAdo toxicity for T lymphoblasts since there is substantial dCyd kinase activity in thymus tissue (7, 10) and the T lymphoblasts resemble thymocytes in a number of ways (15). It is also possible that dCyd acts by inhibiting the transport of other deoxynucleosides into lymphoid cells. The failure of dCyd to reverse the toxicity of dGuo for PHA-induced proliferation may suggest that PHA-activated cells have altered K_M values for dCyd and dGuo when compared to resting cells or T lymphoblasts or that dGuo toxicity is not mediated through dGTP accumulation in normal PBL.

These data confirm and extend observations of many groups which have associated nucleoside accumulation with impairment of lymphocyte function. The mechanisms whereby these compounds, particularly the deoxynucleoside derivatives, inhibit lymphocyte growth are unclear. Phosphorylation to their respective triphosphates has been demonstrated (7, 10, 26), and these deoxynucleoside triphosphates may act by inhibiting ribonucleotide reductase (11). Carson *et al.* (7) have shown that the enzyme (5) responsible for phosphorylation of dGuo is enriched in the thymus and, to a lesser degree, in PBL. The selective toxicity of dGuo for dividing T cells may be explained by the selective accumulation of dGTP in T lymphocytes mediated by dGuo kinase. Further studies of the tissue distribution of specific kinase activities will be required before complete understanding of these biochemical-lymphotoxic correlations is achieved. Finally, because dCyd can, to a degree, reverse the toxicity of deoxynucleoside triphosphate accumulation, it may be of value in the treatment of these life-threatening deficiencies.

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1. Giblett, E. L., Anderson, J. E., Cohen, F., Pollara, B. & Meuwissen, H. J. (1972) *Lancet* ii, 1067–1069.
2. Giblett, E. L., Ammann, A. J., Wara, D. W., Sandman, R. & Diamond, L. K. (1975) *Lancet* i, 1010–1013.
3. Cohen, A., Hirschorn, R., Horowitz, S. D., Rubinstein, A., Polmar, S. H., Hong, R. & Martin, D. W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 472–476.
4. Coleman, M. S., Donofrio, J., Hutton, J. J., Hahn, L., Daoud, A., Lampkin, B. & Dyminski, J. (1978) *J. Biol. Chem.* **253**, 1619–1626.
5. Cohen, A., Doyle, D., Martin, D. W. & Ammann, A. J. (1976) *N. Eng. J. Med.* **295**, 1449–1454.
6. Cohen, A., Gudas, L. J., Ammann, A. J., Staal, G. E. J. & Martin, D. W. (1978) *J. Clin. Invest.* **61**, 1405–1409.
7. Carson, D. A., Kaye, J. & Seegmiller, J. E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5677–5681.
8. Simmonds, H. A., Panayi, G. S. & Corrigall, V. (1978) *Lancet* i, 60–63.
9. Gelfand, E. W., Dosch, H-M., Biggar, W. D. & Fox, I. H. (1978) *J. Clin. Invest.* **61**, 1071–1080.
10. Krenitsky, T. A., Tuttle, J. V., Koszalka, G. W., Chen, I. S., Beacham, L. M., Rideout, J. L. & Elion, G. B. (1976) *J. Biol. Chem.* **251**, 4055–4061.
11. Moore, E. C. & Hurlbert, R. B. (1966) *J. Biol. Chem.* **241**, 4802–4809.
12. Boyum, A. (1974) *Tissue Antigens* **4**, 269–274.
13. Dosch, H-M. & Gelfand, E. W. (1977) *J. Immunol.* **118**, 302–308.
14. Shore, A., Dosch, H-M. & Gelfand, E. W. (1978) *Nature (London)* **274**, 586–587.
15. Chechik, B. E., Pyke, K. W. & Gelfand, E. W. (1976) *Int. J. Cancer* **18**, 551–556.
16. Dosch, H-M., Lee, J. W. N., Gelfand, E. W. & Falk, J. A. (1978) *Clin. Exp. Immunol.* **34**, 260–267.
17. Dosch, H-M. & Gelfand, E. W. (1979) in *In Vitro Induction and Measurement of Antibody Synthesis in Man*, eds. Fauci, A. S. & Ballieux, R. E. (Academic, New York), in press.
18. Siegal, F. P. & Siegal, M. (1977) *J. Immunol.* **118**, 642–647.
19. Green, H. & Chan, T. S. (1973) *Science* **182**, 836–837.
20. Benke, P. J. & Dittman, D. (1976) *Pediatr. Res.* **10**, 642–646.
21. Wolberg, G., Zimmerman, T. P., Hiemstra, K., Winston, M. & Chu, L. C. (1975) *Science* **187**, 957–959.
22. Gelfand, E. W., Dosch, H-M. & Shore, A. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1465.
23. Stoop, J. W., Zegers, B. J. M., Hendricks, G. F. M., Siegenbeck van Henkelom, L. H., Staal, G. E. J., deBree, P. K., Wadman, S. K. & Ballieux, R. E. (1977) *N. Engl. J. Med.* **296**, 651–655.
24. Hamet, M., Griscelli, C., Cartier, P., Ballay, J., deBruyn, C. & Hosli, P. (1977) *Adv. Exp. Biol. Med.* **76A**, 477–480.
25. Meuwissen, H. J., Pickering, R. J., Moore, E. C. & Pollara, B. (1975) in *Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency*, eds. Meuwissen, H. J., Pickering, R. J., Pollara, B. & Parker, I. H. (Academic, New York), pp. 73–83.
26. Tattersal, M. H., Ganeshaguru, K. & Hoffbrand, A. V. (1975) *Biochem. Pharmacol.* **24**, 1495–1498.