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DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS AND ADENOSINE TOXICITY

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Abstract—The toxic effect of adenosine on the metabolism of malignant lymphoid cells has been studied in relation to the activity of intracellular adenosine deaminase. Exposure *in vitro* of L1210 and L5178Y cells for 48 hr to adenosine demonstrated that concentrations above 10^{-5} M inhibited cell division, the toxic effect being inversely proportional to intracellular adenosine deaminase levels. Measurement of the deoxyribonucleoside triphosphate pools in cells exposed to adenosine resulted in a 22 per cent reduction in the pyrimidine deoxyribonucleoside triphosphates. Adenosine-mediated growth inhibition was markedly enhanced by coformycin, a potent inhibitor of adenosine deaminase.

The recent identification of an inborn error of metabolism characterized by severe combined immunodeficiency and an absence of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) (ADA) activity [1–3] has focussed attention on the toxicity of adenosine to lymphocytes, and the protective role that ADA may serve in preventing potential toxicity from this nucleoside or its phosphorylated derivatives. The specific association of ADA activity with lymphocyte metabolism is further supported by studies indicating that ADA levels are particularly high in tissues of the reticulo endothelial system [4–7]. Hall [8] demonstrated an increase in sheep lymphocyte ADA activity after antigenic stimulation *in vivo*, while changes in enzyme activity have also been reported after stimulation of human lymphocytes *in vitro* by lectin mitogens [9–11]. The role of ADA in lymphocyte metabolism remains unproven, but a protective function is supported by studies showing that adenosine, the substrate for ADA, inhibits DNA and protein synthesis in human peripheral blood lymphocytes stimulated *in vitro* by phytohaem-agglutinin [10, 12].

With regard to malignant lymphocytes, although ADA activity was found to be subnormal in lymphocytes from patients with chronic lymphatic leukaemia [13], we have recently demonstrated a mean 35-fold increase in ADA activity in the lymphoblasts of patients with acute lymphocytic leukaemia [14].

The study of lymphoid cell lines in continuous suspension tissue culture provides a useful model for examining the biochemical events associated with exposure of malignant lymphocytes to adenosine. In the present paper, we have measured pools of deoxyribonucleoside triphosphates resulting from exposure of L5178Y cells to adenosine, shown that adenosine toxicity is related inversely to the cellular ADA, and demonstrated that adenosine toxicity is potentiated by coformycin, a potent inhibitor of ADA [15–17].

MATERIALS AND METHODS

Reagent chemicals were obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex, or from BDH, Poole, analytical reagent grades being used where available. Purine and pyrimidine nucleosides were obtained from the Boehringer Corp. Ltd. (London), and isotopically labeled materials from the Radiochemical Centre, Amersham. Coformycin was a generous gift from Professor Umezawa (Institute of Microbial Chemistry, Tokyo).

L5178Y cells were grown in Fischer's medium, and L1210 cells in RPMI-1640 medium, supplemented with 10% horse serum (Wellcome). All cultures contained, in addition, benzyl penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Cultures were initiated at a concentration of 5×10^4 cells/ml and frequent sampling for cell counting confirmed that control cultures remained in logarithmic growth for a period exceeding 48 hr. Cell counts were performed in duplicate in either a Neubauer or Fuchs–Rosenthal haemacytometer. Adenosine, coformycin, uridine or inosine solutions were prepared immediately prior to use and sterilized by filtration through a Millipore filter (0.22 μm).

ADA was assayed spectrophotometrically using a double beam Cary 16 spectrophotometer [14]. The sample cuvette (1 cm path length) contained 1.0 ml adenosine (0.2 mM aqueous), $(2 - x)$ ml 0.15 M phosphate buffer, pH 7.1, equilibrated at 30°, and the reaction started by the addition of $\times$ ml (in the range 0.01 to 0.2 ml) extract, making a total volume of 3.0 ml. The reference cuvette (1 cm) contained 1.0 ml of 0.1 mM adenosine and 2.0 ml of 0.15 M phosphate buffer, pH 7.1. The reaction was followed at 265 nm and was linear for at least 5 min. One unit of adenosine deaminase activity is defined as the amount of enzyme in 10^7 cells which produces a decrease in optical density of 0.010/min under the conditions described.

Deoxynucleoside triphosphates were measured according to the method of Solter and Handschumacher [18], as modified by Tattersall and Harrap [19].

RESULTS

Two separate strains each of L5178Y and L1210 cell lines were cultured, characterized by differences in ADA content; we have adopted suffixes "H" and "L" to designate these inter-strain differences in

Table 1. ADA activities of L5178Y and L1210 cell lines growing logarithmically in suspension culture, and the reduction in growth rate after a 48-hr exposure to adenosine (AR)

Cell type		ADA activity* Mean	ADA activity* Range	% Reduction in growth rate relative to controls† (extracellular AR 10^{-4} M)
L1210	(L)	21.3	20.7–22.0	36
	(H)	35.5	35.0–36.1	25
L5178Y	(L)	8.0	7.7–8.4	68
	(H)	45.0	43.8–46.0	12

* Units/10^7 cells: these figures are the means of duplicate assays from three separate experiments on each cell type.

† These figures are the means of triplicate cultures in two separate experiments. Overall scatter, ⅄ ± 5 per cent.

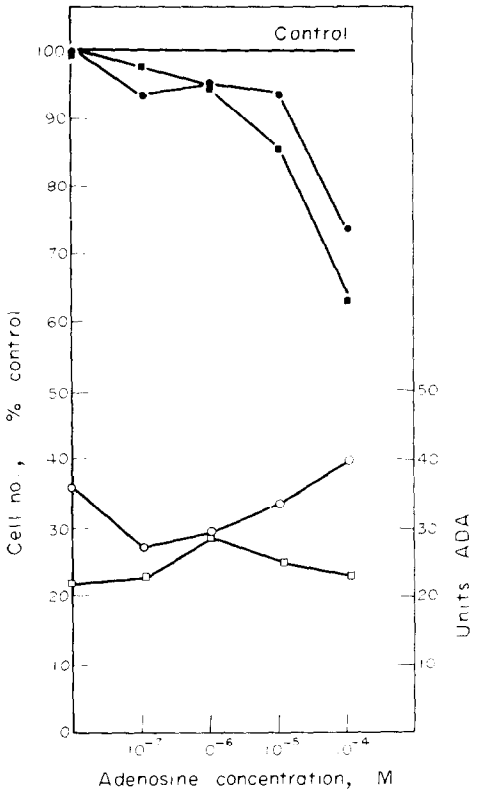

Fig. 1. Effects of extracellular adenosine on the growth and ADA activity of two strains of L1210 cells [L1210 (L) and L1210 (H)] which differ in ADA content. Cells in logarithmic growth were exposed for 48 hr to adenosine at the concentrations shown. Triplicate cultures were incubated for each adenosine concentration and duplicate ADA assays performed on each culture. The results are the means from two separate experiments. Overall scatter: ADA activity, ⅄ ± 5 per cent; cell number, ⅄ ± 7 per cent. Key: (●——●) cell number L1210 (H); (■——■) cell number L1210 (L); (○——○) ADA activity L1210 (H); and (□——□) ADA activity L1210 (L).

enzyme levels, which are compared in Table 1. The growth rates of high (H) and low (L) ADA strains of each line were identical. Table 1 also lists the per cent reduction in cell number for each cell type at an initial extracellular adenosine concentration of 10^{-4} M.

The effects of extracellular adenosine on growth rate and ADA activity are further compared in Figs. 1 (L1210 cells) and 2 (L5178Y cells). It can be seen that adenosine toxicity is inversely proportional to the cellular ADA activity, being less toxic to lines possessing relatively high levels of ADA activity [L1210 (H), L5178Y (H)] and more toxic to those possessing lower levels [L1210 (L), L5178Y (L)].

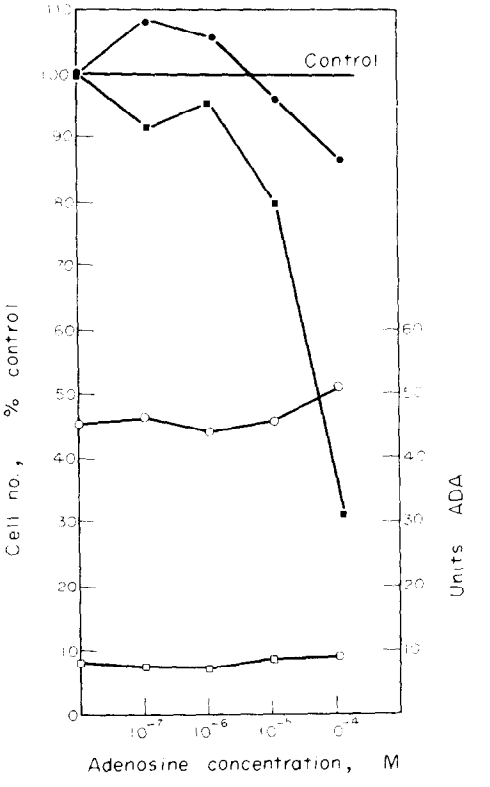

Fig. 2. Effects of extracellular adenosine on the growth and ADA activity of two strains of L5178Y cells [L5178Y (L) and L5178Y (H)] which differ in ADA content. Cells in logarithmic growth were exposed for 48 hr to adenosine at the concentrations shown. Triplicate cultures were incubated for each adenosine concentration and duplicate ADA assays performed on each culture. The results are the means from two separate experiments. Overall scatter: ADA activity, ⅄ ± 5 per cent; cell number, ⅄ ± 7 per cent. Key: (●——●) cell number L5178Y (H); (■——■) cell number L5178Y (L); (○——○) ADA activity L5178Y (H); and (□——□) ADA activity L5178Y (L).

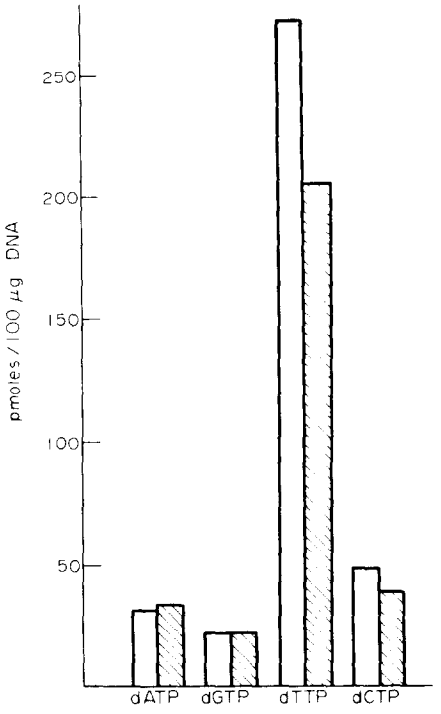

Fig. 3. Deoxyribonucleoside triphosphate pools of L5178Y (H) cells exposed for 48 hr to 10^{-4} M adenosine. The values represent the means of duplicate assays from three separate experiments. Overall scatter, ⅄ ± 5 per cent. Open columns: control; hatched columns: + adenosine.

Exposure to adenosine seemed to be without significant effect on the activity of ADA in any of the four cell types investigated. Other experiments were carried out in which each cell type was exposed to inosine, the product of the ADA-catalyzed reaction. This nucleoside, at all concentrations up to and including 10^{-4} M, was without effect on cell growth rate.

Green and Chan [20] demonstrated pyrimidine deficiency in lymphoid cells subjected to adenosine toxicity. They measured ribonucleotide pools, and we have explored further their hypothesis, that adenosine toxicity is mediated by pyrimidine deprivation, by measuring the deoxyribonucleoside triphosphate (dNTP) pools in L5178Y (H) cells after their exposure to adenosine. This cell strain compares closely, in terms of ADA activity, with lymphoblasts of patients with acute lymphocytic leukaemia [14]. The results are shown in Fig. 3. The purine dNTP pools were scarcely changed; dTTP and dCTP levels were suppressed by 24 and 19 per cent, respectively, though not sufficiently to restrict DNA synthesis. In agreement with the studies of Green and Chan [20], we have been able to protect both L5178Y strains from adenosine toxicity with uridine.

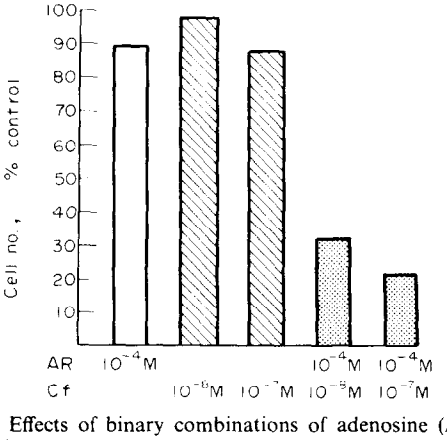

Fig. 4. Effects of binary combinations of adenosine (AR) and coformycin (Cf) on the growth of L5178Y (H) cells. Logarithmically growing cells were exposed for 48 hr to either the single agents or the combinations at the concentrations shown. The results are the means of four experiments. Overall scatter, ⅄ ± 8 per cent.

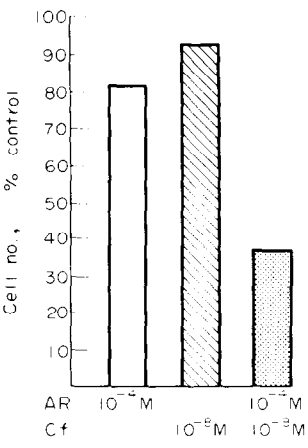

Fig. 5. Effect of a binary combination of adenosine (AR) and coformycin (Cf) on the growth of L1210 (H) cells. Logarithmically growing cells were exposed for 48 hr to either single agents or the combination at the concentrations shown. The results are the means of three experiments. Overall scatter, ⅄ ± 5 per cent.

Results of experiments designed to enhance the growth-inhibitory effects of adenosine by simultaneous inhibition of adenosine catabolism are summarized in Figs. 4 and 5. It can be seen that the growth-retarding effects of binary combinations of coformycin and adenosine are greater than those elicited by either component alone [both in L1210 (H) and L5178Y (H) cells].

DISCUSSION

The work described in this paper confirms that adenosine is toxic to cultured lymphoid cells. The hypothesis that ADA fulfills a protective role in relation to adenosine toxicity is supported by the finding that the effects of the nucleoside are inversely proportional to intracellular ADA activity. Hirschhorn *et al.* [12] reported a 50 per cent inhibition of PHA-induced thymidine incorporation into human lymphocytes by concentrations of adenosine greater than 1×10^{-4} M, but ADA levels were not reported. Likewise, ADA activity was not measured in the studies of Green and Chan [20] who found that exposure of L5178Y cells to 2×10^{-5} M adenosine resulted in virtual depletion of pyrimidine ribose di- and triphosphates. However, our measurements of deoxyribonucleoside triphosphate pools in L5178Y (H) cells exposed to

10^{-4} M adenosine clearly show that adenosine-induced pyrimidine deficiency does not extend to the level of the deoxyribonucleoside triphosphates. Accordingly, the metabolic consequences of exposing these cells to adenosine must lie elsewhere than at the level of DNA synthesis.

Inhibition of ADA activity is of more than theoretical interest, since the genetic deletion of this enzyme in man results in severe combined immunodeficiency disease [1–3], and inhibition of lymphocyte ADA activity may induce a specific antilymphocytic effect of therapeutic use for immunosuppression and the treatment of lymphoid malignancies [10]. The availability of coformycin, a recently characterized inhibitor of ADA [15–17], permitted us to explore the effects of ADA inhibition and the possibility of enhancing the effects of adenosine. The particular advantage of this compound is that it is a tight-binding inhibitor of ADA, with a K_i of 1.2×10^{-10} M [15]. Coformycin clearly enhanced the growth-retarding effects of adenosine in both L1210 (H) and L5178Y (H) cells.

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REFERENCES

1. J. Dissing and B. Knudsen, *Lancet* **2**, 1316 (1972).
2. E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet* **2**, 1067 (1972).
3. J. Yount, P. Nichols, H. D. Ocho, S. P. Hammar, C. R. Scott, S.-H. Chen, E. R. Giblett and R. J. Wedgwood, *J. Paediat* **84**, 173 (1974).
4. J. M. Barnes, *Br. J. exp. Path.* **21**, 264 (1940).
5. T. G. Brady and C. I. O'Donovan, *Comp. Biochem. Physiol.* **14**, 101 (1965).
6. E. J. Conway and R. Cook, *Biochem. J.* **33**, 479 (1939).
7. Y. H. Edwards, D. A. Hopkinson and H. Harris, *Ann. hum. Genet.* **35**, 207 (1971).
8. J. G. Hall, *Aust. J. exp. Biol. med. Sci.* **41**, 93 (1963).
9. R. Hirschhorn, in *Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency* (Eds. H. J. Meuwissen, R. J. Pickering, B. Pollara and I. H. Porter), p. 121. Academic Press, New York (1975).
10. T. Hovi, J. F. Smyth, A. C. Allison and S. C. Williams, *Clin. exp. Immun.* **23**, 395 (1976).
11. H. Ochs, in *Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency* (Eds. H. J. Meuwissen, R. J. Pickering, B. Pollara and I. H. Porter), p. 190. Academic Press, New York (1975).
12. R. Hirschhorn, J. Grossman and G. Weissman, *Proc. Soc. exp. Biol. Med.* **133**, 1361 (1970).
13. E. M. Scholar and P. Calabresi, *Cancer Res.* **33**, 94 (1973).
14. J. F. Smyth and K. R. Harrap, *Br. J. Cancer* **31**, 544 (1975).
15. S. Cha, R. P. Agarwal and R. W. Parks, Jr., *Biochem. Pharmac.* **24**, 2187 (1975).
16. H. Nakamura, G. Koyama, Y. Iitaka, M. Ohno, N. Yagisawa, S. Kondo, K. Maeda and H. Umezawa, *J. Am. chem. Soc.* **96**, 4327 (1974).
17. T. Sawa, Y. Fukagawa, I. Homma, T. Takeuchi and H. Umezawa, *J. Antibiot. [A] Tokyo* **20**, 227 (1967).
18. A. W. Solter and R. E. Handschumacher, *Biochim. biophys. Acta* **174**, 585 (1969).
19. M. H. N. Tattersall and K. R. Harrap, *Cancer Res.* **33**, 3086 (1973).
20. H. Green and T. Chan, *Science, N.Y.* **182**, 836 (1973).