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DIFFERENTIAL MODULATION OF 1-β-D-ARABINOFURANOSYLCYTOSINE METABOLISM BY HYDROXYUREA IN HUMAN LEUKEMIC CELL LINES

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Abstract—The ability of hydroxyurea (HU) to modulate 1-β-D-arabinofuranosylcytosine (Ara-C) metabolism was investigated in human leukemic cell lines. Exposure of HL-60 cells to 1 mM HU enhanced the accumulation of Ara-CTP up to 2.5-fold, whereas HU did not have significant effects on Ara-C metabolism in CEM cells. In addition, two adenine nucleosides, deoxyadenosine (dAdo) and 9-β-D-arabinofuranosyladenine (Ara-A), which are known to be activated by deoxycytidine (dCyd) kinase as Ara-C, were more effectively phosphorylated after the addition of HU only in HL-60 cells. However, the changes of intracellular dCTP and TTP pools induced by HU, i.e. decrease in dCTP and increase in TTP, were the same in both cell lines. Finally, dCyd production under normal culture conditions was at least 3- to 4-fold higher in HL-60 cells and was inhibited significantly by HU administration. These results suggest that the modulation of Ara-C metabolism by HU occurs at the level of dCyd kinase through the regulation of *de novo* dCyd generation.

1-β-D-Arabinofuranosylcytosine (Ara-C) is one of the most effective drugs currently used in the treatment of human acute leukemia [1]. To exert its anti-tumor effect, Ara-C requires phosphorylation to Ara-CTP [2], which directly inhibits DNA polymerase or DNA chain elongation after incorporation into DNA [3, 4]. Therefore, the intracellular metabolism and retention of Ara-CTP play a crucial role in the response of leukemic cells to the drug.

Hydroxyurea (HU), an inhibitor of ribonucleotide reductase [5], is reported to enhance the activity of Ara-C both *in vivo* [6] and *in vitro* [7–9]. The biochemical basis for this synergism, presumably through the increased formation of Ara-CTP, is the subject of several earlier studies [7–9]. However, the exact mechanism of enhanced activation of Ara-C to Ara-CTP by HU still remains undetermined.

In the present study, we describe the difference in the regulation of Ara-C and Ara-CTP metabolism by HU between the human T lymphoblastoid cell line, CEM, and the human promyelocytic leukemia line, HL-60. Our data suggest that it is due to differential rates of *de novo* production of deoxycytidine (dCyd), which acts competitively with Ara-C at the level of dCyd kinase, the initial and rate-limiting step of Ara-C phosphorylation [10].

MATERIALS AND METHODS

Cell lines. Two human leukemic cell lines, the T lymphoblastoid CEM [11] and the promyelocytic HL-60 [12], were mainly analyzed in the present study. Other cell lines included were: three malignant T lymphoblastoid cell lines, Molt 3, Molt 4 and JM; three malignant B cell lines, Raji, Dauji and RPMI-8866; a histiocytic lymphoma, U937; a non-T, non-B lymphoblastoid cell line, HPB-NULL; and a normal B cell line (LCL-KIN) established in the Kitazato Lab. Institute after infection of lymphocytes with Epstein–Barr virus. The origin and antigenic membrane characteristics of most of the lines have been reviewed [13], except for RPMI-8866 [14] and HPB-NULL [15].

All cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) (regular medium). The cultures were kept at 37° in a humidified atmosphere of 95% air/5% CO_2 and were checked for mycoplasma by the method of McGarrity and Carson [16].

Measurement of Ara-CTP, dATP and Ara-ATP production. Logarithmically growing cells were incubated with Ara-C, deoxyadenosine (dAdo) or 9-β-D-arabinofuranosyladenine (Ara-A) at a density of 5×10^5/ml for various intervals (30–180 min). For determination of dATP and Ara-ATP levels, the cells were preincubated with 10 μM deoxycoformycin (dCF) (provided by the Yamasa Shoyu Co.) for 30 min to inhibit adenosine deaminase (ADA) [17]. Then, the cells were extracted with 0.4 M perchloric acid after three washings by cold phosphate-buffered saline (pH 7.4) and neutralized by Alamine/Freone [17]. Nucleotides were separated and quantified by high performance liquid chromatography (HPLC) on a Partisil 10-SAX anion exchange column eluted with 0.5 M KCl/0.25 M KH_2PO_4, pH 3.4 (buffer A) for dATP and Ara-ATP, and buffer A/water (4:1) for Ara-CTP at 1.0 ml/min.

Determination of intracellular dCTP and TTP pools. For the measurements of dCTP and TTP,

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ribonucleotides in cell extracts were destroyed by treatment with sodium periodate as described by Garrett and Santi [18]. The samples were separated on a Partisil 10-SAX column by using 0.4 M ammonium phosphate (pH 3.25)/acetonitrile (10:1) at a flow rate of 2.0 ml/min.

Selection of mutants deficient in dCyd kinase. HL-60 cells (2×10^7) were mutagenized by incubation with ethyl methanesulfonate (200 μg/ml) for 5 hr. Surviving cells were expanded to allow phenotype expression and were grown with gradually increasing concentrations of Ara-C (10 nM to 10 μM). After 3 months, an Ara-C resistant population emerged. The drug-resistant cells were cloned by limiting dilution under non-selective conditions. The activity of dCyd kinase was measured with [^{14}C]dCyd as the substrate by the method described earlier [17]. Protein concentration was estimated by the method of Lowry *et al.* [19].

Determination of dCyd excretion. For the study of dCyd excretion, cells were resuspended in the medium supplemented with dialyzed fetal bovine serum at a density of 3–4 $\times 10^6$/ml. After incubation at 37° for 4 hr, the cells were spun down and the supernatant fractions were deproteinized followed by neutralization. They were analyzed by HPLC on a C_{18} μ-Bondapak column eluted with 1.0% acetonitrile either directly or after the treatment with SEP-PAK C_{18} as reported [20].

RESULTS

Effects of HU treatment on Ara-C and Ara-CTP metabolism. The time course of Ara-CTP accumulation at 10 μM Ara-C with or without HU was first investigated in HL-60 and CEM cells. The cells were preincubated with 1 mM HU for 2 hr followed by further incubation with Ara-C for various intervals. As illustrated in Fig. 1, Ara-CTP generation in HL-60 cells was augmented significantly at each incubation time ($P < 0.01$, paired *t*-test), whereas no significant effect was observed in CEM cells. This was also true at lower concentrations of Ara-C (1 and 0.2 μM) (Table 1).

Table 1. Effect of hydroxyurea on Ara-CTP generation*

Ara-C (μM)	HU (1 mM)	Ara-CTP (pmol/10^6 cells) HL-60	CEM
10	−	104.1 ± 12.5†‡	298.7 ± 11.3
	+	246.5 ± 13.7	340.8 ± 28.9
1	−	39.3 ± 2.2‡	78.4 ± 15.9
	+	96.4 ± 14.0	81.7 ± 10.9
0.2	−	8.8 ± 1.1‡	19.3§
	+	19.3 ± 1.3	21.3

* The cells were incubated with or without HU for 2 hr followed by incubation with Ara-C for 2 hr and were extracted.
† Mean ± SD of five experiments.
‡ The difference between the values with and without HU treatment was significant ($P < 0.01$, paired *t* test).
§ Mean of two experiments.

Next, we examined the effects of HU on nine leukemic cell lines to clarify whether HU-induced alteration in Ara-C metabolism is a phenomenon specific for HL-60. For comparing the effects of HU among different cell lines more clearly, we introduced the index of Ara-CTP production (details in the legend of Fig. 2). As seen in Fig. 2, a significant

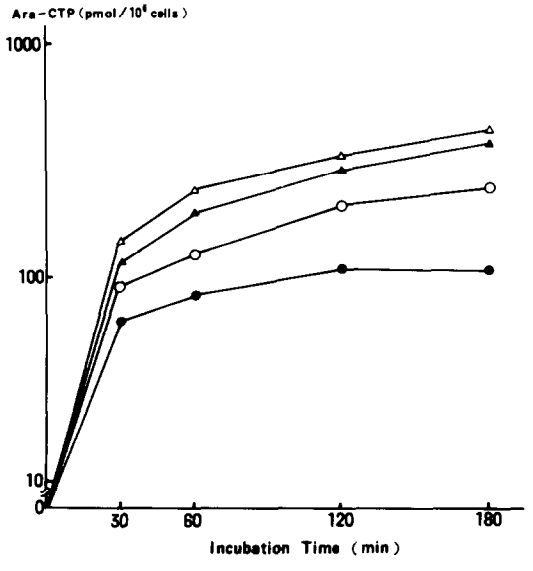

Fig. 1. Effect of hydroxyurea on Ara-CTP generation in HL-60 and CEM cells. The cells were preincubated either with or without HU (1 mM) for 2 hr followed by further incubation with Ara-C (10 μM). At various time points thereafter, the cells were extracted. Ara-CTP was determined by HPLC. Key: (●) HL-60; (○) HL-60 + HU; (▲) CEM; and (△) CEM + HU.

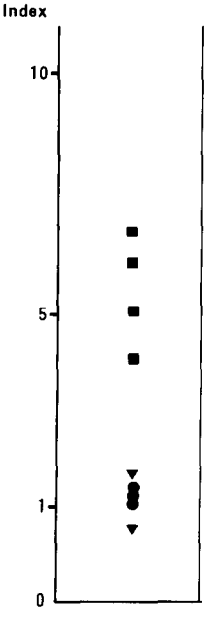

Fig. 2. Effect of hydroxyurea on Ara-CTP production in various human leukemic cell lines. The effects of HU were evaluated exactly as described in the legend of Fig. 1, and the cell extraction was carried out at 2 hr. The index was calculated as follows: Index = Ara-CTP with HU ÷ Ara-CTP without HU. Key: (■) B cell lines; (●) T cell lines; and (▼) others.

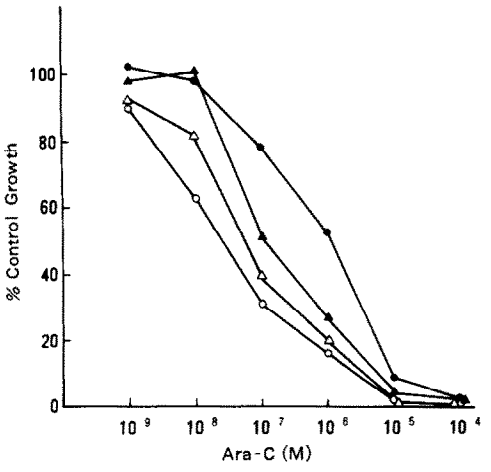

Fig. 3. Effect of hydroxyurea on Ara-C toxicity. The cells were preincubated either with or without 1 mM HU for 2 hr followed by a further 2-hr incubation with various concentrations of Ara-C (10^{-9} to 10^{-4} M). Cells were washed twice and seeded at a density of 1×10^5/ml. After 72 hr, viable cells were determined by trypan blue dye exclusion and were expressed as percent growth of untreated controls. Values represent the mean of four experiments. Key: (●) HL-60; (○) HL-60 + HU; (▲) CEM; and (△) CEM + HU.

increase of Ara-CTP accumulation was observed in the four cell lines showing B cell phenotypes (index = 5.47 ± 1.01). On the other hand, three T cell lines and U937 were, like CEM, unresponsive to HU treatment in terms of Ara-CTP metabolism (index = 1.05 ± 0.16).

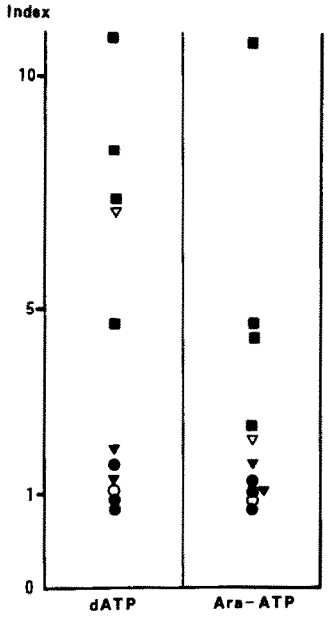

Fig. 4. Effect of hydroxyurea on dATP and Ara-ATP production. The cells were incubated with dAdo (10 μM) and Ara-A (100 μM) plus 10 μM deoxycoformycin for 2 hr after incubation either with or without HU (1 mM) and then extracted. The production index was estimated as described earlier. Key: (■) B cell lines; (▽) HL-60; (●) T cell lines including CEM (○); and others (▼).

Effects of HU on Ara-C toxicity. To evaluate whether the biochemical modulation described above has any correlationship with biological activity, we determined the effect of HU on Ara-C-induced cytotoxicity (Fig. 3; see the legend for the details of the method). In HL-60 cells, the addition of 1 mM HU increased Ara-C toxicity about 50-fold as calculated from an approximate IC_{50}, whereas it did not make a significant difference in CEM cells.

Effects of HU on dAdo and Ara-A phosphorylation. Both dAdo and Ara-A are effectively phosphorylated into their corresponding triphosphates in intact cells under the inhibition of ADA by 10 μM dCF. The initial step of phosphorylation in these adenine nucleosides is known to be mediated mainly by dCyd kinase [21, 22]. When we investigated the effect of HU on dATP formation with 10 μM dAdo, significant enhancement was observed in B cell lines including HL-60, but not in T cell lines including U937 (the index of B cells vs T cells: 7.74 ± 2.07 vs 1.01 ± 0.16) ($P < 0.001$). This was also the case in Ara-ATP generation (100 μM Ara-A), i.e. the index was 4.87 ± 2.09 and 0.95 ± 0.11 for B cells and T cells respectively ($P < 0.01$) (Fig. 4). HL-60 cells, in spite of a promyelocytic leukemic origin, are regarded as representative of B cells hereafter, since their biochemical response to HU treatment is quite comparable to that of B cells.

Changes in dCTP and TTP pools after HU treatment. Since HU is known to modulate intracellular deoxynucleotide pools through its interaction with ribonucleotide reductase, changes occurring in dCTP and TTP pools after HU treatment have been measured [23, 24]. Table 2 shows similar time-dependent reduction of dCTP levels in HL-60 and CEM, although it was more prominent in the latter. In contrast, the pool size of TTP started to expand during a 1-hr incubation with HU and returned to almost initial levels after 4 hr. Again, the responses were indistinguishable between the two cell types.

dCyd excretion. We measured the excretion of dCyd into the culture medium to evaluate the production of the nucleoside, since the excretion of purine/pyrimidine bases or nucleosides is considered to be reflective of *de novo* synthesis [20, 25, 26]. As shown in Table 3, HL-60 cells generated 28.9 pmol dCyd/hr/10^7 cells [27, 28]. CEM excreted dCyd at less than one-third that rate. We selected two clones deficient in dCyd kinase, Ara-CR1 and Ara-CR2,

Table 2. Effect of hydroxyurea (1 mM) on dCTP and TTP pools

Cell line	Time (hr)	dCTP (pmol/10^6 cells)	TTP (pmol/10^6 cells)
HL-60	0	4.7 ± 0.6* (8)	17.5 ± 2.9 (6)
	1	3.7 ± 0.5 (6)	23.5 ± 2.5 (4)
	2	2.4 ± 0.5 (6)	22.8 ± 2.7 (4)
	4	2.0 ± 0.4 (6)	13.4 ± 1.6 (4)
CEM	0	5.7 ± 0.8 (8)	22.5 ± 2.1 (6)
	1	2.9 ± 0.8 (8)	32.9 ± 2.7 (4)
	2	2.0 ± 0.5 (6)	32.3 ± 3.9 (4)
	4	1.1 ± 0.2 (6)	23.0 ± 3.1 (4)

* Mean ± SD; values in parentheses show the number of experiments.

Table 3. Effect of hydroxyurea on deoxycytidine excretion

Cell line	Deoxycytidine excretion (pmol/hr/10^7 cells)	
	Hydroxyurea*	
	(−)	(+)
HL-60 wild type	28.9 ± 2.4†	<10.0
HL-60 Ara-CR1	36.0 ± 3.2	<10.0
HL-60 Ara-CR2	42.0 ± 2.1	11.3 ± 0.5
CEM wild type	<10.0	Not done

* The concentration of hydroxyurea was 1 mM.
† Mean ± SD of more than three experiments.

from wild type HL-60 cells after mutagenesis with ethyl methanesulfonate to abrogate the rephosphorylation of dCyd. The activity of dCyd kinase in wild type HL-60 cells was 0.09 ± 0.01 nmol/min/mg protein, whereas these variants contained less than 1% of wild-type activity. Mutational loss of the enzyme allowed the cells to increase dCyd excretion by 30–50%. Furthermore, HU inhibited dCyd production by at least 60% in both wild-type and in dCyd kinase deficient variants. The effects of HU were unable to be evaluated in CEM, since the excretion even without HU was below 10 pmol/hr/10^7 cells, the lower limit of our assay system.

DISCUSSION

The biochemical basis for the synergistic interaction of Ara-C and HU has been investigated in several studies. For example, Walsh *et al.* [7] demonstrated that HU (0.1 to 10 mM) is capable of increasing the level of Ara-CTP in L1210. Such augmentation of the Ara-C nucleotide pool following administration of HU has also been reported in SB cells, a human B lymphocyte line transformed by the Epstein–Barr virus [8], and in HL-60, a promyelocytic leukemic cell line [9]. On the other hand, according to Abe *et al.* [29], Molt 4 does not show any significant response to HU treatment. In an effort to extend these studies by using several leukemic cell lines, we have concluded that the expansion of the Ara-CTP pool by HU was rather specific to cells of the B-cell phenotype.

The possible mechanism underlying the effects of HU is believed to be mediated through the activation of dCyd kinase, which is responsible for the first, and rate-limiting step converting Ara-C to its triphosphate. We have shown that the phosphorylation of dAdo and Ara-A was enhanced by HU mainly in phenotypically B cells. It is noteworthy that dCyd kinase is the principal enzyme that phosphorylates these adenine nucleosides. Therefore, our present results may serve to support the previous hypothesis on the crucial role of dCyd kinase [7–9].

dCyd kinase in intact cells is known to be subject to regulation by various compounds. dCTP interferes with dCyd kinase activity by feedback inhibition [30], and TTP activates the enzyme either directly or through the depletion of dCTP [31]. Since HU is a potent inhibitor of ribonucleotide reductase, the sole enzyme responsible for *de novo* synthesis of deoxyribonucleotides, the drug was expected to induce alterations in the dNTP pool size. Actually, HU treatment profoundly depleted the cells of dATP and dGTP and led to the initial expansion of TTP [23, 24]. However, controversial results have been reported concerning the modulation of dCTP pools. In this paper, we have proven that HU causes a decrease in dCTP pools and an increase in TTP pools that are the same in HL-60 and CEM cells. Considering the different responses of the two cell types to HU, these results argue against the possibility that changes of pyrimidine dNTP levels play a major role in regulating dCyd kinase during HU exposure.

Carson and coworkers [20, 28] have postulated the critical role of *de novo* production for dCyd on the toxicities of several nucleosides. Two kinds of enzymes mainly participate in the generation of deoxynucleosides including dCyd; one is ribonucleotide reductase and the other is cytosolic deoxy-5′-nucleotidase [28]. Since deoxy-5′-nucleotidase activity is known to be higher in B lymphoblasts than in T lymphoblasts, the production of dCyd is speculated to be accelerated in the former cells [32]. Indeed, our results revealed about 3-fold higher synthetic rates of the nucleoside in HL-60 cells, which is in agreement with the report by Iizasa *et al.* [26]. Thus, under normal culture conditions, the accumulation of dCyd competes with the phosphorylation of Ara-C at the level of dCyd kinase due to the higher affinity of the enzyme for dCyd than for Ara-C [10, 33]. This situation may be changed by HU administration. The endogenous production of dCyd decreased drastically in HL-60 cells at a velocity comparable to that of the T cell line. This results in the functional activation of dCyd kinase. The final outcome of these sequential events is a significant increase in Ara-CTP formation.

In the present paper, we have described the modulation of Ara-C and Ara-CTP metabolism by HU that occurs in cells mainly of B-cell origin. As the biochemical basis of this phenomenon, we suggest a critical role of endogenous dCyd production through deoxy-5′-nucleotidase. Finally, although we have not measured the activity of deoxy-5′-nucleotidase in the current study, we want to emphasize that the evaluation of the enzyme may offer a reasonable prediction of the efficiency of combining HU with Ara-C in the treatment of certain leukemias.

REFERENCES

1. B. Clarkson, M. D. Dowling, T. S. Gee, I. B. Cunningham and J. H. Burchenal, *Cancer, N.Y.* **36**, 775 (1975).

2. M. Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **11**, 423 (1962).
3. F. L. Graham and G. F. Whitemore, *Cancer Res.* **30**, 2636 (1970).
4. D. W. Kufe, P. P. Major, E. M. Egan and G. P. Beardsley, *J. biol. Chem.* **255**, 8997 (1980).
5. I. H. Krakoff, N. C. Brown and P. Reichard, *Cancer Res.* **28**, 1559 (1968).
6. F. M. Schabel Jr., W. R. Laster Jr. and M. W. Trader, *Proc. Am. Ass. Cancer Res.* **12**, 67 (1971).
7. C. T. Walsh, R. W. Craig and P. P. Agarwal, *Cancer Res.* **40**, 3286 (1980).
8. J. A. Streifel and S. B. Howell, *Proc. natn. Acad. Sci. U.S.A.* **78**, 5132 (1981).
9. F. Rauscher III and E. Cadman, *Cancer Res.* **43**, 2688 (1983).
10. P. G. W. Plagemann, R. Marz and R. M. Wohlhueter, *Cancer Res.* **38**, 978 (1978).
11. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, *Cancer, N.Y.* **18**, 522 (1965).
12. S. T. Collins, R. C. Gallo and R. E. Gallagher, *Nature, Lond.* **270**, 347 (1977).
13. J. Minowada, G. Janossy, M. F. Greaves, T. Tsubota, B. I. S. Srivastava, S. Morikawa and E. Tatsumi, *J. natn. Cancer Inst.* **60**, 1269 (1978).
14. M. A. Pellegrino, S. Ferrone and A. N. Theofilopoulos, *J. Immun.* **115**, 1065 (1975).
15. T. Konishi, J. Minowada, E. S. Henderson and T. Ohnuma, *Gann* **75**, 275 (1984).
16. G. J. McGarrity and D. A. Carson, *Expl Cell Res.* **139**, 199 (1982).
17. M. Kubota, N. Kamatani, P. E. Daddona and D. A. Carson, *Cancer Res.* **43**, 2606 (1983).
18. C. Garrett and D. V. Santi, *Analyt. Biochem.* **99**, 268 (1979).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 266 (1951).
20. M. Kubota, C. J. Carrera, D. B. Wasson and D. A. Carson, *Biochim. biophys. Acta* **804**, 37 (1984).
21. D. A. Carson, D. B. Wasson, J. Kaye, B. Ullman, D. W. Martin Jr., R. K. Robins and J. A. Montgomery, *Proc. natn. Acad. Sci. U.S.A.* 77, 6865 (1980).
22. M. S. Hershfield, J. E. Fetter, M. C. Small, A. S. Bagnara, S. R. Williams, B. Ullman, D. W. Martin Jr., D. B. Wasson and D. A. Carson, *J. biol. Chem.* **257**, 6380 (1982).
23. L. Skoog and B. Nordenskjold, *Eur. J. Biochem.* **19**, 81 (1971).
24. L. Skoog and G. Bjursell, *J. biol. Chem.* **249**, 6434 (1974).
25. R. C. Willis, A. H. Kaufman and J. E. Seegmiller, *J. biol. Chem.* **259**, 4157 (1984).
26. T. Iizasa, M. Kubota and D. A. Carson, *J. Immun.* **131**, 1776 (1983).
27. S. Eriksson, S. Skog, B. Tribukait and K. Jaderberg, *Expl Cell Res.* **155**, 129 (1984).
28. D. A. Carson, T. Iizasa, S. Seto, C. J. Carrera, M. Kubota, E. H. Willis, D. B. Wasson and O. Kajander, *Ann. N.Y. Acad. Sci.* **451**, 34 (1985).
29. I. Abe, S. Saito, K. Hori, M. Suzuki and H. Sato, *Cancer Res.* **42**, 2846 (1982).
30. D. H. Ives and J. P. Durham, *J. biol. Chem.* **245**, 2285 (1970).
31. M. S. Blumenreich, T. C. Chou, M. Andreeff, K. Vale, B. D. Clarkson and C. W. Young, *Cancer Res.* **44**, 825 (1984).
32. D. A. Carson, J. Kaye and D. B. Wasson, *J. Immun.* **126**, 348 (1981).
33. P. P. Major, E. M. Eagan, G. P. Beardsley, M. Minden and D. W. Kufe, *Proc. natn. Acad. Sci. U.S.A.* **78**, 3235 (1981).