5′-Nucleotidase activity and arachidonate metabolism in doxorubicin sensitive and resistant P388 cells

A. Ramu[1], D. Glaubiger[2], P. Soprey[4], G.H. Reaman[4] & N. Feuerstein[3]

[1]*Department of Radiation and Clinical Oncology, Hadassah University Hospital, Jerusalem, Israel.* [2]*Radiation Oncology Branch.* [3]*Laboratory of Pathophysiology, National Cancer Institute, Bethesda, Maryland, and* [4]*Division of Hematology/Oncology, Children's Hospital National Medical Center, Washington, D.C. USA.*

Summary 5′-nucleotidase activity, arachidonate metabolism and adenosine uptake were measured in P388 murine leukaemia cells and in a subline resistant to doxorubicin. These membranes related activities were found to be increased in the doxorubicin resistant cell line, compared to the drug sensitive cells. It is suggested that these differences do not play a role in the mechanism of resistance to doxorubicin. Rather they reflect alterations in plasma membrane composition and structure between these cell lines.

This study also suggests that the use of decreased 5′-nucleotidase activity as a marker of certain leukaemias should be reviewed with caution. An increase in cell enzyme activity in treated patients may not necessarily indicate a shift toward normal behaviour of these cells, but rather a selection of certain cell subpopulations.

Uusitalo & Karnovsky (1977) have shown that the activity of 5′-nucleotidase of different populations of mouse lymphocytes may vary considerably. Raz *et al.* (1978) measured the activity of this enzyme in Moloney virus-induced lymphoma, methylcholanthrane-induced lymphoma and their normal parental mouse lymphocytes. They found a marked reduction in the specific activity of 5′-nucleotidase in the lymphoma cells compared to normal lymphocytes. Similar differences in 5′nucleotidase activity were found by Petitou *et al.* (1978) between human normal donors and leukaemic patients. They have also found a marked decrease in the cell membrane lipid structural order of leukaemic cells compared to normal lymphocytes. These findings suggest a possible relationship between the two phenomena.

We have recently reported that the membrane lipid structural order of doxorubicin-resistant P388 cells is higher than that measured in the parent doxorubicin-sensitive P388 cell line (Ramu *et al.*, 1983*a*). This change is apparently the result of the difference in membrane lipid composition (Ramu *et al.*, 1983*b*). Therefore, we have studied whether the increase in membrane lipid structural order, found in doxorubicin-resistant P388 cells is also associated with an increase in the cell 5′-nucleotidase activity.

As changes in cell membrane lipid composition and structural order may affect cell membrane-associated activities, we have also compared in these cell lines the metabolism of arachidonate and the uptake of adenosine.

Correspondence: A. Ramu, Department of Radiation and Clinical Oncology, Hadassah University Hospital, P.O. Box 12000, Jerusalem 91120, Israel.

Materials and methods

P388 murine leukemia cells (Dawe & Potter, 1957) and a subline resistant to doxorubicin (Johnson *et al.*, 1976) were grown in suspension culture in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 10% heat-inactivated foetal bovine serum (Grand Island Biological Co.), $10\,\mu M$ 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.), $50\,u\,ml^{-1}$ penicillin base and $50\,\mu g\,ml^{-1}$ streptomycin base (both from Grand Island Biological Co.). Cell densities were measured using a Coulter Counter (ZB₁; Coulter Electronics Ltd., Harpenden, Herts, England). Cells were transferred to fresh medium every 4 days to sustain exponential growth. Initial cell densities were $10^5\,cells\,ml^{-1}$ and after 4 days, their density reached $1–2\times10^6\,cells\,ml^{-1}$. In all studies where doxorubicin-sensitive and -resistant P388 cells were compared, measurements were performed with cells harvested on the fourth day of growth.

The sensitivity of both cell lines to doxorubicin was assessed as follows: cells were cultured in the presence of various drug concentrations and the slope of the log cell density *versus* time plot was calculated by linear regression analysis. The growth rate at each drug concentration was expressed as the percentage of the control growth rate. Dose effect curves were thus produced and were used to determine the concentration of drug effective in inhibiting the growth rate by 50% (ED_{50}). The doxorubicin ED_{50} for drug-sensitive and drug-resistant P388 cells was 2 to $4\times10^{-8}\,M$ and $1–2\times10^{-6}\,M$ respectively. No change in drug sensitivity of either cell line was observed during 3 years of continuous *in vitro* culture. Similarly, the sensitivity of both cell lines to indomethacin and nitro-

benzylthioinosine (both from Sigma Chemical Co.), in the presence and absence of doxoribicin, was also measured.

5′-nucleotidase assay

Cells were harvested by centrifugation (700 g for 10 min), washed × 3 in Tris-buffered saline (TBS, Tris-buffer 10 mM, pH 7.8, NaCl 0.9%) and adjusted to 4–6 × 10⁶ cells ml⁻¹. Enzyme activity was assayed by a modification of a previously described method (Reaman et al., 1979). The reaction was carried out in a plastic tube (2040 Falcon, Div. Becton, Dickinson & Co., Oxnard, Ca.), and the reaction mixture contained 10 μmol β-glycerophosphate (Sigma Chemical Co.), 10 μmol MgCl₂ and 10⁵ cells. The mixture was preincubated for 15 min at 37°C and then substrate was added: Adenosine 5′-monophosphate (AMP, Sigma Chemical Co.) 10 nmol with (U-¹⁴C)Adenosine 5′-monophosphate (¹⁴C-AMP, Amersham Searle, Park Ridge, Ill.). The volume of the reaction mixture was adjusted to 0.5 ml, mixed thoroughly, and the tubes were then incubated at 37°C for 30 min. The reaction was stopped by adding 100 μl of 0.25 M ZnSO₄. Unreacted AMP was precipitated by adding 100 μl of 0.25 M Ba(OH)₂. The final volume was adjusted to 1.0 ml and the tubes kept cold for 1 h and then centrifuged at 1200 g for 15 min. Supernatant (0.5 ml) was aspirated into a vial containing 5 ml scintillation fluid (ScintiVerse, Fisher Scientific Instruments, Pittsburgh, Pa.) and counted in a scintillation counter. Controls consisted of samples which contained neither cells nor ZnSO₄ and Ba(OH)₂, samples with ZnSO₄ and Ba(OH)₂, and samples with cells held in boiling water for 10 min. Normally between 5 and 20% of the AMP substrate was hydrolysed and under these conditions, >95% of the cells were viable. Substrate hydrolysis was directly proportional to the cell concentration. Radioactivity present in the supernatant is proportional to the 5′nucleotidase activity of the sample. β-glycerophosphate was included in the assay to insure its specificity for 5′nucleotidase activity (Belfield & Goldberg, 1968).

Assay for arachidonate metabolites

Washed cells from both lines were incubated in RPMI 1640 medium (without serum) at 37°C. Cell density was 2 × 10⁶ ml⁻¹. After 3 h, the samples were centrifuged at 700 g for 10 min and the supernatants were separated and frozen at −20°C until assayed. Prostaglandins in the supernatants were determined by direct radioimmunoassay (Granstrom & Kindhal, 1976). The radiolabelled prostaglandins and the antibodies for 6-keto-PGF₁α and for thromboxane B₂ were purchased from New England Nuclear (Boston, Ma). The antibody for PGE₂ was purchased from Accurate Chemicals and Scientific Co., (New York, NY).

Adenosine uptake

Uptake of [2-³H] Adenosine (Amersham Searle) was determined in both cell lines. Cell suspensions containing 5 × 10⁴ cells in 0.25 ml medium were incubated on a shaker at 37°C. After 10 min of preincubation [³H]-adenosine was added. The incubation was terminated after 15 min by centrifugation for 2 min in a microfuge (model 152 Beckman Instruments, Inc., Palo Alto, Ca.). The cells were washed × 3 with cold PBS and then solubilized in 0.1 ml of detergent (Nonidet P40, Shell Oil Co., UK). Radioactivity was determined after the addition of 5 ml scintillation fluid. In some experiments, an inhibitor of adenosine transport: p-nitrobenzyladenosine (PBTA, obtained from Chemical Synthesis branch, National Cancer Institute, Bethesda, Md) was added at a concentration of 10⁻⁶ M.

Results

5′-nucleotidase activity

The activity of 5′nucleotidase did not change in cells from either line as the cells were passaged in vitro, between transfers 23 and 90 (Table I). The activity of this enzyme was 10 times higher in doxorubicin resistant cells than in the drug sensitive

Table I 5′-nucleotidase activity in doxorubicin-sensitive and -resistant P388 cells

	nmol AMP hydrolyzed by 10⁶ cells h⁻¹ mean ± s.d. (no. experiments)	
	control	+ 1% non idet P40
Doxorubicin-sensitive P388 cells	1.11 ± 0.58 (n = 12)	3.51 ± 1.49 (n = 4)
Doxorubicin-resistant P388 cells	14.63 ± 5.61 (n = 8)	39.87 ± 5.91 (n = 4)

P388 cells. Cells that were incubated for 24 h in media containing $20\,\mu M$ of adenosine or AMP, showed the same level of enzyme activity as untreated cells. These levels of enzyme activity were also found in cells of both lines freshly collected from ascites fluid withdrawn from tumour-bearing CDF_1 mice.

In the presence of the detergent Nonident P40 (1%) the enzyme activity increased 3-fold in both cell lines and the relative difference in activity between the lines was maintained.

Metabolism of arachidonate

The spontaneous release of PGE_2, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 from drug sensitive and resistant cells is shown in Table II. The doxorubicin resistant P388 cells exhibited higher release of all the arachidonate metabolites assayed. The most conspicious difference observed was in the release of PGE_2 (6-fold).

In order to examine a possible correlation between inhibition of growth by doxorubicin and the metabolism of arachidonic acid, cells of both lines were exposed to the drug in concentrations which inhibit the cell growth rate by 50%. Under these conditions, the release of PGE_2 from drug-sensitive cells was reduced to 52.2% of that obtained from these cells incubated without doxorubicin. In doxorubicin-resistant cells, the drug inhibited the release of PGE_2 by only 18.5%.

Indomethacin, at the highest non-inhibitory concentration $(3 \times 10^{-5}\,M)$, did not affect the sensitivity of either cell line to doxorubicin.

Adenosine uptake

The initial rate of adenosine uptake by doxorubicin-sensitive and -resistant P388 cells, is shown in Table III. This uptake was largely suppressed by p-nitrobenzyladenosine, a blocker of adenosine transport. The uptake of adenosine in doxorubicin-resistant cells is considerably higher than that measured in drug-sensitive P388 cells.

Nitrobenzylthioinosine, another blocker of adenosine transport (Lauzon & Paterson, 1977), at the highest non-inhibitory concentration $(3 \times 10^{-6}\,M)$, did not affect the sensitivity of either cell line to doxorubicin.

Discussion

The present experiments have shown that the parent P388 cell line, a methylcholanthrene-induced lymphoid neoplasm in DBA/2 mouse (Dawe & Potter, 1957), have measurable 5'nucleotidase activity. This activity is much lower than that measured in normal DBA/2 mouse lymphocytes (Uusitalo & Karnovsky, 1977). The activity of 5'-nucleotidase measured in the doxorubicin-resistant

Table II The spontaneous release of arachidonate metabolites from doxorubicin-sensitive and -resistant P388 cells

	pg of metabolite 10^6 cells $3\,h^{-1}$ mean $\pm$ s.d. (4 experiments)		
	PGE_2	6-keto-$PGF_{1\alpha}$	thromboxane B_2
Doxorubicin-sensitive P388 cells	46.1 ± 15.8	100 ± 10.2	2.2 ± 0.3
Doxorubicin-resistant P388 cells	296.2 ± 22.8	200 ± 21.4	4.6 ± 0.4

Table III Adenosine uptake by doxorubicin-sensitive and -resistant P388 cells

	pmol adenosine 10^6 cells h^{-1} mean $\pm$ s.d. (n = 3)	
	control	+ nitrobenzyladenosine
Doxorubicin-sensitive P388 cells	4.56 ± 0.80	1.21 ± 0.04
Doxorubicin-resistant P388 cells	14.84 ± 2.68	1.28 ± 0.36

subline, selected by the drug from the parent P388 cell population, was significantly higher than that measured in the parent cell line and was not significantly different from the activity reported for normal DBA/2 mouse lymphocytes.

The activity of this enzyme in either cell line was not affected by prior incubation of the cells in the presence of adenosine or AMP.

The increased activity of 5'-nucleotidase measured in doxorubicin-resistant cells, compared to drug-sensitive cells, may represent an increase in the amount of enzyme protein or an increase in its specific activity as a result of some change in the protein structure or its relation to other constituents of the cell membrane. After solubilization with a detergent, an increase in 5'-nucleotidase activity was measured. However, similar increase in enzyme activity was obtained in both cell lines. A detergent may on one hand increase membrane-bound enzyme activity by increasing its exposure (Solomonson et al., 1976) and on the other hand, may disrupt some enhancing effect of the lipid/protein interaction (Englehard et al., 1976). Therefore this negative result does not support either possibility.

Although this study gives further support for the suggested relationship between the level of activity of 5'-nucleotidase, a membrane-bound enzyme, and the degree of structural order of the cell membrane lipid domain, the explanation for this relationship remains obscure.

The activity of 5'nucleotidase measured in human T- and B-lymphoma cell lines (Carson et al., 1979) was much lower than that reported for the corresponding normal human T- and B-lymphocytes (Thompson et al., 1979; Rowe et al., 1979). It was suggested that the activity level of this enzyme is related to the degree of cell maturity (Poplack et al., 1981). Decreased 5'-nucleotidase activity was reported in most chronic lymphatic leukaemias (Lopez et al., 1973; Marique & Hildebrand, 1973; Quagliata et al., 1974; Kramers et al., 1976), in lymphocytes of immunodeficient patients with hypogammaglobulinemia (Johnson et al., 1977; Edwards et al., 1978; Webster et al., 1978), T cell lymphoblastic leukaemias (Reaman et al., 1979) and B cell acute lymphoblastic leukaemias (Reaman et al., 1981). It was suggested that the activity of this enzyme may be used as a new biological marker of certain diseases (Koya et al., 1981). Our data clearly point toward the possibility that following drug treatment and the emergence of drug-resistant

cell populations, the activity of 5'-nucleotidase in the remaining tumour cells may change considerably and an increase in the enzyme activity in the remaining cells may not necessarily indicate a shift toward higher differentiation.

Doxorubicin-resistant P388 cells release more prostaglandin PGE_2, thromboxane and prostacyclin than drug-sensitive P388 cells. The differences in arachidonate metabolism may reflect change in the availability of arachidonic acid in these cells as a part of the changes in cell lipid composition (Ramu et al., 1983b). However, in preliminary experiments where arachidonic acid was added in excess to the incubation medium, the differences in arachidonate metabolism were maintained. This indicates that the change in arachidonate metabolism is caused by changes in the activity of certain enzymes involved in arachidonate metabolism rather than a difference in the availability of arachidonic acid.

At equicytostatic concentrations of doxorubicin (ED_{50}), the release of PGE_2 from drug-resistant P388 cells was significantly less influenced by the presence of doxorubicin than its release from drug-sensitive cells. Therefore, we suggest that the inhibition of release of PGE_2 by the drug is unrelated to its cytostatic effect. Furthermore as indomethacin did not change the sensitivity of either cell line to doxorubicin, we suggest that the increase in arachidonate metabolism is not the cause of doxorubicin resistance but rather reflects changes in the membrane structure of these cells. Changes in other membrane related activities were found in drug resistant cells as: glycosidase (Bosmann & Kessel, 1970), membrane glycoproteins (Beck et al., 1979), high macromolecular lipid (Taylor et al., 1981) and carrier-mediated uptake of methotrexate (Kessel et al., 1965; Herman et al., 1979). In the present study we have also noted that these cell lines differ in another carrier-mediated uptake system of the cell membrane, namely the uptake of adenosine. The rate of adenosine transport was significantly higher in doxorubicin-resistant cells than in drug-sensitive cells. In both cell lines the uptake could be blocked by a specific adenoside transport blocker, nitrobenzyladenosine. The finding that nitrobenzylthioinosine, another potent inhibitor of nucleosine transport (Lauzon et al., 1977), did not change the sensitivity of either cell line to doxorubicin, suggests that the increase in adenosine uptakes is not the cause of drug resistance, but rather another reflection of the change in membrane structure.

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