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Studies on drug resistance—II. Kinase patterns in P815 neoplasms sensitive and resistant to 1-β-D-arabinofuranosylcytosine

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RESISTANCE toward 1-β-D-arabinofuranosylcytosine (Ara-C) was attributed by Chu and Fischer,[1] Kessel *et al.*,[2] Schrecker and Urshel,[3] and Uchida and Kreis[4] to a considerable decrease of the enzymatic conversion of Ara-C into 1-β-D-arabinofuranosylcytosine-5′-diphosphate (Ara-CDP) and -triphosphate (Ara-CTP) in the resistant cell line. As reported in our previous study,[4] Ara-C deaminase and cytidine-5′-diphosphate (CDP) reductase seem to be unrelated to the development of resistance in P815 cells. However, the marked difference in respect to phosphorylation of Ara-C is not a general finding in all the reported strains resistant to Ara-C. Momparler *et al.*[5] and Bach[6] failed to find significant differences in the phosphorylation of Ara-C in Ara-C resistant and parental cell lines.

The present experiments were undertaken to better characterize the discrepancy in the occurrence of Ara-CDP and Ara-CTP in sensitive (P815) and resistant (P815/Ara-C) cells. The results indicate that the lack of Ara-CDP and of Ara-CTP in P815/Ara-C cells is due to the almost complete absence of an active Ara-C kinase.

Tritiated 1-β-D-arabinofuranosylcytosine-5′-monophosphate (^{3}H-Ara-CMP) was prepared enzymatically from tritiated 1-β-D-arabinofuranosylcytosine (^{3}H-Ara-C) and purified by paper electrophoresis or paper chromatography. ^{3}H-Ara-C (1 c/m-mole, labeled predominantly in the 5 position) was kindly supplied by the Cancer Chemotherapy National Service Center. Crude enzyme extracts were prepared as reported[4] from BDF_1 mice bearing P815 and P815/Ara-C ascites tumors 6 days after inoculation. The assay mixture for enzymatic phosphorylation of labeled Ara-C and Ara-CMP contained: 3 μmoles adenosine-5′-triphosphate (ATP), 2·8 μmoles $MgCl_2$, 2·3 μmoles 3-phosphoglyceric acid, 3·3 to 10·0 mμmoles of substrate and 100 μl of enzyme extract, all in 250 μl of 100 mM Tris-HCl buffer, pH 8·0. After incubation for 30 min at 37°, the reaction was stopped by chilling the mixture in ice to 0°. An aliquot of the mixture was used directly for paper chromatography, system A of previous publication,[4] or paper electrophoresis in 100 mM citrate–phosphate buffer, pH 3·6, at 20 V/cm for 2 hr. The radioactivity of the spots corresponding to Ara-C, Ara-CMP, Ara-CDP and Ara-CTP was measured directly with liquid scintillation techniques.[7] The evaluation of 5′-nucleotidase activities in the crude enzyme extracts was performed by following the method of Carter,[8] using radioactive ^{3}H-Ara-CMP as substrate and paper electrophoresis for the separation of the reaction products.

Purification of enzyme. Crude extracts of P815 were used for the partial purification of the fraction which phosphorylates Ara-C to Ara-C-5′-monophosphate. Gel filtration on Sephadex G-150 was performed on a column 45 × 4 cm by the method of Momparler and Fischer.[9] The column was equilibrated with 100 mM Tris HCl buffer, pH 8·0. Ten-ml fractions (1 ml/min) were collected and aliquots were used for the enzymatic assay. Chromatography on DEAE-Sephadex A-50 was performed on a column 15 × 1 cm. DEAE-Sephadex was prepared as recommended by Pharmacia, Inc. and resuspended in 0·2 M NaCl–100 mM Tris HCl, pH 8·0. The fractions from the Sephadex G-150 column with kinase activity were adjusted to 0·2 M NaCl and chromatographed on a DEAE-Sephadex A-50 column. The column was washed with 5 vol. of 0·2 M NaCl solution and the enzyme was eluted with a linear gradient of 0·2 to 1·0 M NaCl in 100 mM Tris HCl buffer. Details of the purification procedure will be given elsewhere.

The presence of Mg^{++} (10^{-5} M) and Dithiothreitol (10^{-6} M) in the elution buffers was necessary for the preservation of the enzyme activity during the purification steps.

In further experiments the enzymatic fraction containing Ara-C kinase activity obtained by Sephadex filtration and DEAE-Sephadex fractionation of crude extract from P815 cells was combined with crude extract prepared from P815/Ara-C and P815 cells. These mixtures were used in enzymatic assays with Ara-C as substrate. Protein was determined by the method of Lowry *et al.*[10] and by determining the absorbance at 280 mμ.

With crude enzyme preparations of two ascitic cell lines (P815 and P815/Ara-C), we confirmed our previous observation concerning the difference in phosphorylation of Ara-C by the solid form of these neoplasms.[4] Figure 1 shows a considerable difference of phosphorylation of Ara-C by the resistant as

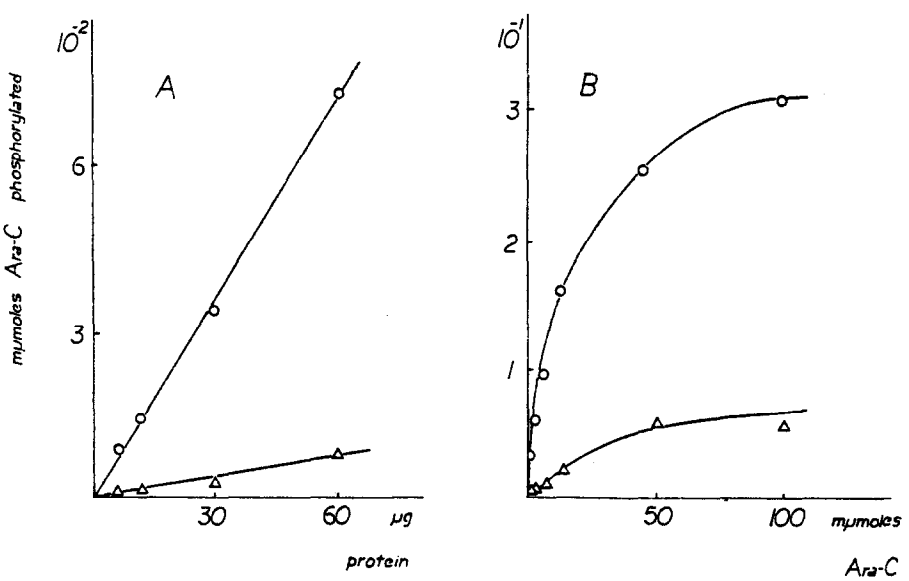

FIG. 1. Phosphorylation of Ara-C in crude enzyme preparation from P815 cells (○—○) and P815/Ara-C cells (△—△). Ordinates A and B, amount of substrate phosphorylated. Abscissa: A, protein concentration; B, substrate concentration (60 μg protein used). For procedure, see text.

compared to the sensitive cells. Crude enzyme preparations of the two strains of cells (Fig. 1A), over a wide range of protein concentration per assay, show proportional differences in the conversion of Ara-C to Ara-CMP, Ara-CDP and Ara-CTP. Furthermore, the same amounts of enzyme of the two cell lines (Fig. 1B) phosphorylate different amounts of Ara-C to proportional degrees into Ara-C-mono-, di- and triphosphates.

When Ara-CMP is used as substrate (Fig 2), the conversion of this monophosphate is equal in both lines in respect to added amounts of protein (Fig. 2A) as well as in respect to the amount of

Ara-CMP (Fig. 2B). These observations indicate that P815 and P815/Ara-C cells differ in nucleoside kinase activity, but not in the kinase activities responsible for the conversion of the monophosphate to the di- and triphosphates. This conclusion was confirmed by experiments in which purified Ara-C kinase (extracted from sensitive cells) was combined with crude enzyme preparations of resistant cells (Fig. 3). Neither purified Ara-C kinase nor crude enzyme extracts from resistant cells convert Ara-C to Ara-CDP and Ara-CTP, whereas the combination of the two does. Also, the presence of a nucleoside kinase inhibitor in P815/Ara-C cells could be excluded by this experiment. The degree of the conversion of Ara-C to Ara-CDP and Ara-CTP in the resistant crude extracts is dependent on the

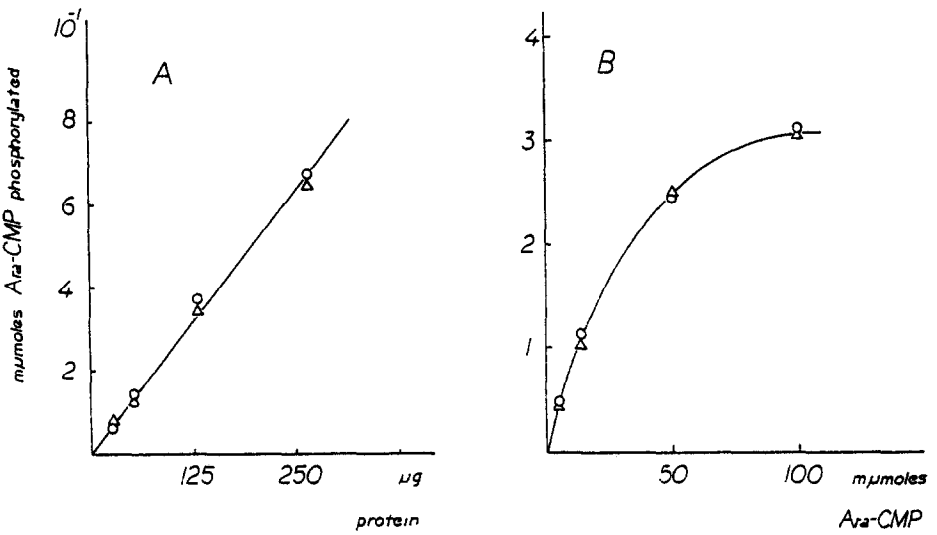

FIG. 2. Phosphorylation of Ara-CMP in crude enzyme preparation from P815 cells (○—○) and P815/Ara-C cells (△—△). Ordinates A and B, amount of substrate phosphorylated. Abscissa: A, protein concentration; B, substrate concentration (260 μg protein used). For procedure, see text.

amount of purified nucleoside kinase enzyme added (Fig. 4). It reaches an asymptotic level equal to that attained by the crude extract of sensitive cells. Further, the addition of purified nucleoside kinase to a crude enzyme preparation of sensitive cells does not increase markedly the production of Ara-C-di- and triphosphates.

When supernatants obtained from homogenates of either P815 or P815/Ara-C cells were tested for 5′-nucleotidase activities, it was found that both cell lines hydrolyzed Ara-CMP to Ara-C to about the same extent (Table 1).

These studies thus reveal that: (1) the lack of the capability of cells to convert Ara-C to Ara-CDP and Ara-CTP is due to the lack of an active nucleoside kinase; (2) the crude extracts of P815 and P815/Ara-C have the same kinase activities for the conversion of Ara-CMP to Ara-CDP and Ara-CTP; (3) neither a nucleoside kinase inhibitor nor the appearance of a 5′-nucleotidase is the cause for the development of resistance in P815 ascites cells; (4) by adding adequate amounts of purified Ara-C kinase to a crude enzyme extract of P815/Ara-C cells, equal phosphorylation *in vitro* of Ara-C to Ara-CDP and Ara-CTP is achieved with both sensitive and resistant tumor extracts.

Studies are under way in this laboratory to determine the primary cause for the lack of the nucleoside kinase activity in the resistant cells.

During the preparation of this manuscript, our attention was drawn to an abstract by Schrecker[11] in which the reduced rate of phosphorylation of Ara-C, deoxycytidine and deoxyguanosine in an Ara-C resistant subline of L1210 mouse leukemia was probably caused by a decreased synthesis of deoxycytidine kinase.

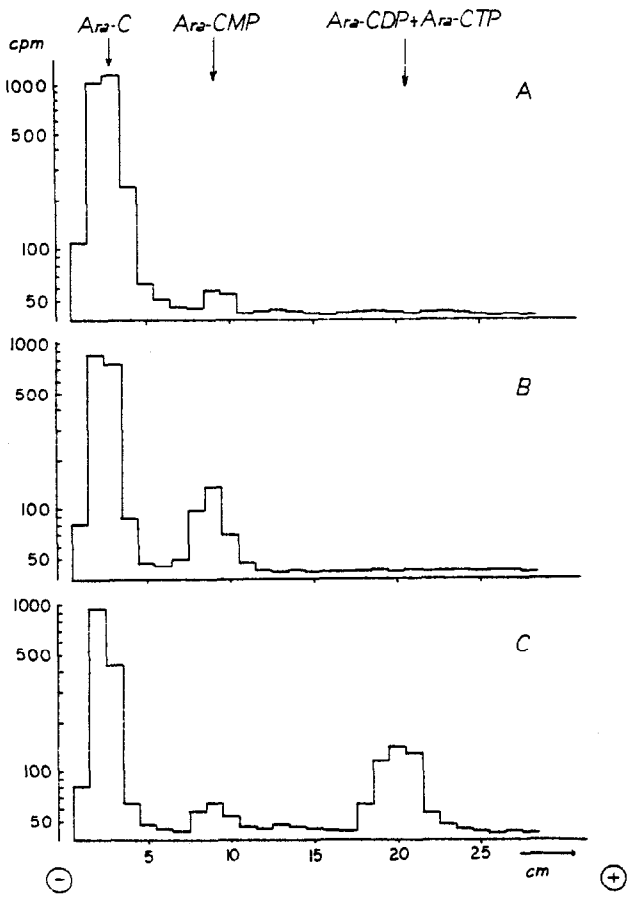

FIG. 3. Enzymatic phosphorylation of Ara-C (3·3 mμmoles substrate concentration): A, by crude extract from P815/Ara-C cells; B, by purified Ara-C kinase extracted from P815 cells; C, by a combination of both. Separation of reaction products by paper electrophoresis of an aliquot of the enzymatic assay. Ordinate, counts per minute; abscissa, distance from origin in centimeters. For conditions of electrophoresis, see text.

TABLE 1. 5′-NUCLEOTIDASE ACTIVITIES IN P815 AND P815/Ara-C CELLS

Cells	Per cent conversion of Ara-CMP to Ara-C*	
	Supernatant I	Homogenate II
P815	25·1	75·01
P815/Ara-C	27·6	76·8

* The conversion of ^{3}H-Ara-CMP to ^{3}H-Ara-C was measured in the following enzymatic assay: 10 mμmoles substrate, 0·1 ml enzyme extracts, 10^{-5} M Mg^{++} in 0·25 ml, 30 min, 37°. Supernatant I was obtained by 40,000 *g* centrifugation and supernatant II was obtained by 2000 *g* centrifugation of crude cell homogenates.

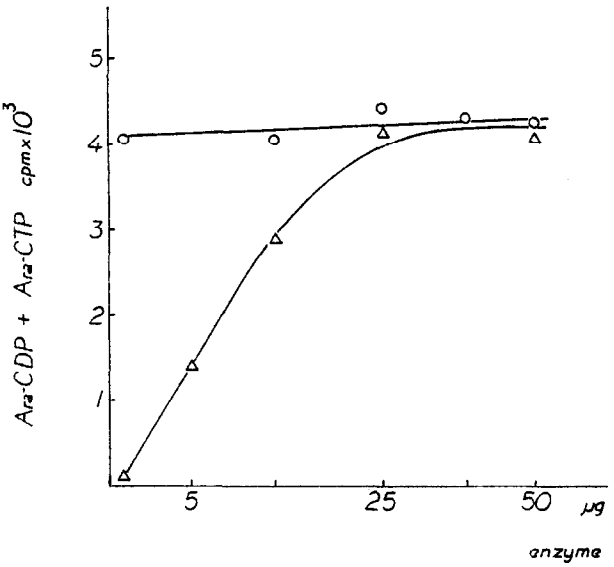

FIG. 4. Conversion of Ara-C (3·3 mμmoles substrate concentration) into Ara-CDP and Ara-CTP by equal aliquots of crude enzyme preparations from P815 and P815/Ara-C cells plus purified Ara-C kinase. Crude enzyme preparation from P815 cells plus purified Ara-C kinase from P815 cells (○—○); crude enzyme preparation from P815/Ara-C cells plus Ara-C kinase from P815 cells (△—△). Ordinate, Ara-CDP and Ara-CTP (counts per minute); abscissa, micrograms of purified Ara-C kinase added to equal amounts of the two extracts. For procedure, see text.

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