Isolation of 2'-Deoxy-2'-Azidocytidine-Resistant Mutants Deficient in Deoxycytidine Kinase in Mouse FM3A Cells

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ABSTRACT. We isolated several mutants that are highly resistant to 2'-deoxy-2'-azidocytidine by culturing mutagenized mouse FM3A cells in a medium containing 10^{-4} M of this analogue. The mutants incorporated deoxycytidine into acid-insoluble materials at a reduced rate, but incorporated cytidine normally. All the mutants isolated also were resistant to 1- β -D-arabinofuranosylcytosine. The activity of deoxycytidine kinase in the crude extracts of these mutants was reduced to about 10% that of the FM3A cells. Our results indicate that 2'-deoxy-2'-azidocytidine is recognized as deoxyribonucleoside by deoxycytidine kinase in contrast to reports that phosphate forms of this nucleoside analogue are recognized as ribonucleotide by the ribonucleotide reductase from calf thymus and *E. coli* and by *E. coli* primase.

A nucleoside analogue, 2'-deoxy-2'-azidocytidine (Cz, or azidocytidine)* inhibits the growth of cultured mammalian cells by interfering with DNA replication (9). Isolation and characterization of mutants resistant to Cz in mammalian cells should be of great value for the study of inhibitory mechanisms *in vivo*. Resistance to any nucleoside analogue can be produced by more than one mechanisms. Among the Cz-resistant mutants isolated in our laboratory, we found some that were stable and highly resistant to Cz. We here report the characterization of these mutants.

A subclone, F28-7, isolated from FM3A cells (7), was used as a wild type cell line in this study. Cells were grown in a floating culture at 37°C in Eagle's minimum essential medium supplemented with 2% fetal calf serum and 0.1% Bactopeptone as described previously (1). Cells were mutagenized with 0.6 μ g/ml of N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Co.) for 3 h then cultured for 2 days to stabilize mutated phenotypes. These cells then were cultured for 10 days in a medium containing 5×10^{-4} M Cz (Boehringer, Mannheim GmbH), after which they were transferred to fresh medium containing 1×10^{-3} M Cz and cultured for another week. Cells that grew under this selection condition were cloned on agar medium (0.5% agar). Fig. 1 shows the growth in the presence or absence of Cz of the wild type FM3A and its two Cz-resistant mutants (FScz2 and FScz3) as representatives of ten mutant clones isolated. From the concentration of Cz that reduced the growth rate of cells to 50%, we judged the two resistant clones to be about 100-times more resistant to Cz than the wild type FM3A. This phenotype has been stable for over a year.

* Abbreviations: Cz and azidocytidine, 2'-deoxy-2'-azidocytidine; CzDP, 5'-diphosphate of Cz; CzTP, 5'-triphosphate of Cz; araC, 1- β -D-arabinofuranosylcytosine.

Fig. 1. Effect of azidocytidine on the growth of wild type FM3A and the mutant FScz2 and FScz3 cells. Cells were inoculated at a density of 1×10^5 cells/ml. After three days of culture, viable cells were counted. ●, FM3A cells; ○, FScz2 cells; △, FScz3 cells.

Fig. 2. Effect of 1-β-D-arabinofuranosylcytosine on the growth of wild type FM3A and the mutant FScz2 and FScz3 cells. Growth was determined as described in the legend to Fig. 1. ●, FM3A cells; ○, FScz2 cells; △, FScz3 cells.

To check for any alterations in the transport or phosphorylating steps of the nucleosides or in the polymerases in the mutants, we measured the ability of the cells to incorporate [^3H]cytidine and [^3H]deoxycytidine (Radiochemical Centre, Amersham) into an acid-insoluble fraction. The mutants, FScz2 and FScz3, both showed reduction in their ability to incorporate [^3H]deoxycytidine into acid-insoluble macromolecules as compared with wild type FM3A. By contrast, the incorporation of [^3H]cytidine was normal (data not shown). We then examined the cross resistance to a deoxycytidine analogue, 1- β -D-arabinofuranosylcytosine (araC), because mutants concerning utilization of deoxycytidine have been reported to undergo alterations in their sensitivity to araC (3, 6). The mutants also showed more than a 100-times higher resistance to araC than the wild type FM3A (Fig. 2). These results indicate that the mutants may have defects in the transport of deoxycytidine or alterations either in the metabolic pathway of deoxycytidine or in the DNA polymerizing machinery. Mutants highly resistant to araC are reported to be deficient in deoxycytidine kinase (3). Table 1 shows the activities of deoxycytidine kinase and cytidine kinase of the crude extracts obtained from the two resistant mutants, FScz2 and FScz3. The activity of deoxycytidine kinase in FScz2 and FScz3 cells decreased markedly to 9% and 14% of the activity of the FM3A cells, but the activity of cytidine kinase was unaltered in the two mutants. The actual activities of deoxycytidine kinase in the mutants should be much less than those shown in Table 1 because the assay method for deoxycytidine kinase used can not exclude the deoxyuridine monophosphate produced by the action of the pyrimidine nucleoside deaminase and deoxyuridine kinase present in the crude extracts. Thus, there is strong evidence that the resistant phenotype was caused by the defect in deoxycytidine kinase. To determine whether the Cz-resistance is genetically dominant or recessive, the mutant FScz3 cells were hybridized with FS5oua1 cells, which carry a recessive trait of temperature-sensitivity for growth, the dominant trait of resistance to ouabain, as well as the wild type trait of Cz-sensitivity (unpublished). Cell-hybridization was carried out with polyethylene glycol 1540 (Wako Pure Chemical Industries, Japan) as described previously (1). The hybrid cells (FScz3 $\times$ FS5oua1) grew at a non-permissive temperature, 39.5°C, on agar medium containing 2 mM ouabain. The hybrid

TABLE 1. ACTIVITIES OF DEOXYCYTIDINE KINASE AND CYTIDINE KINASE IN WILD TYPE FM3A AND ITS TWO AZIDOCYTIDINE-RESISTANT MUTANTS.

Cell line	Deoxycytidine kinase		Cytidine kinase	
	Activity ^{a)}	(%)	Activity ^{a)}	(%)
FM3A	0.07 ^{b)}	100	1.41 ^{b)}	100
FScz2	0.006	9	1.59	113
FScz3	0.01	14	0.93	66

Cells were grown in 100 ml of the medium to a density of 1×10^6 cells/ml. After harvest, the cells were washed once with ice-cold phosphate-buffered saline then disrupted by repeated freezing and thawing (3 times), after which they were suspended in 5 ml of a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 5 mM 2-mercaptoethanol. The supernatant, obtained by centrifugation of the cell homogenate at $105,000 \times g$ for 90 min, was dialyzed against buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol and 50% (v/v) glycerol, and used for the enzyme assay and to determine the protein content. Assay of the kinases was according to Chu and Fischer (2). Protein was determined by the method of Lowry *et al.* (5). a) Activity is given as the number of pmoles dCMP or CMP formed per mg protein per 15 min at 30°C. b) A separate assay gave similar results.

clones that were isolated could not grow in the medium containing 10^{-5} M araC, although FScz3 cells grew normally under the same condition (data not shown). Our results show that the araC-resistance is a recessive character. Hence, we concluded that Cz-resistance was produced by the deficiency in deoxycytidine kinase.

The results of this study show that Cz, an analogue of both ribo- and deoxynucleoside (see ref. 8), is recognized by deoxycytidine kinase, but not by cytidine kinase at the step of nucleoside phosphorylation in mouse FM3A cells. CzDP has been reported to be an inhibitor of calf thymus ribonucleotide reductase competitive with CDP (4) and to inactivate *E. coli* ribonucleotide reductase (10). In addition, CzTP is a substrate of primase in *E. coli* (8). Thus, our present results are evidence that a chemically modified sugar moiety of a nucleoside does not always act strictly as an analogue of ribonucleoside or deoxyribonucleoside *in vivo*. Thus, a modified sugar moiety of nucleosides other than Cz also may be recognized as a deoxyribonucleoside analogue by one enzyme and as a ribonucleoside analogue by another in a cell.

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