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New ara-C resistant mutants of the Chinese hamster ovary cells

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SUMMARY

Mutants of Chinese hamster ovary (CHO) cell resistant to cytosine arabinoside (ara-C), an inhibitor of DNA synthesis and antitumour drug, have been isolated and characterized both biochemically and genetically. Mutants occurring spontaneously and those induced by treatment with *N*-methyl-*N'*-Nitro-*N*-nitrosoguanidine (MNG), were obtained at a frequency of $0{\cdot}24 \times 10^{-6}$ and $3{\cdot}4 \times 10^{-6}$ respectively. The mutants showed a stable ara-C resistant phenotype which was inherited as a dominant trait in genetic crosses. The wild type (CHO K-1) and the mutant (103, 002 and 003) cells showed no differences in the levels of the uptake of ara-C or of its degradation. Results of biochemical studies further excluded the involvement of deaminase, kinase and ribonucleotide reductase as the possible factor(s) in conferring drug resistance to the mutant cells. However, the wild type and mutant DNA polymerases differed in the level of the *in vitro* incorporation of specific dNMP in the presence of ara-CTP. These data suggested that the wild-type DNA polymerase which becomes error prone in the presence of ara-CTP may cause the drug sensitivity of the wild-type cells and that a change in the mutant enzyme making it resistant (or less prone) to ara-CTP induced errors in dNMP incorporation may control the drug resistance of the mutant cells.

1. INTRODUCTION

Cytosine arabinoside (ara-C) an antitumour drug is metabolized via two enzymic pathways (see Fig. 1), by phosphorylation to ara-CTP, and by deamination to ara-U (see Cohen, 1977, for a review). Since ara-CTP is an inhibitor of DNA polymerase, the isolation and characterization of ara-C resistant mutants of animal cells can provide an insight into the process of DNA replication in eukaryotes and into the mechanism of the antitumour effect of ara-C. However, in the past the ara-C resistant variants of mammalian cells showed changes in ribonucleotide reductase or in cytidine kinase (Chu & Fisher, 1965; Schreker & Urshel, 1968; Smith & Chu, 1972; Robert de Saint & Buttin, 1979). We therefore attempted to isolate additional ara-C-resistant mutants with the expectation that some of them may possibly be altered in other aspects of the drug metabolism and may provide information regarding the basic mechanism of DNA replication. In the present study an established Chinese hamster ovary cell line (CHO K-1) was used because a wealth of genetic, biochemical and cytological knowledge is available about this

cell line (Puck, 1972). Isolation and characterization of several new mutants resistant to ara-C are described in this paper. Preliminary evidence supports the involvement of an altered DNA pol (and/or associated protein) in controlling the drug resistant phenotype of the mutant cell line.

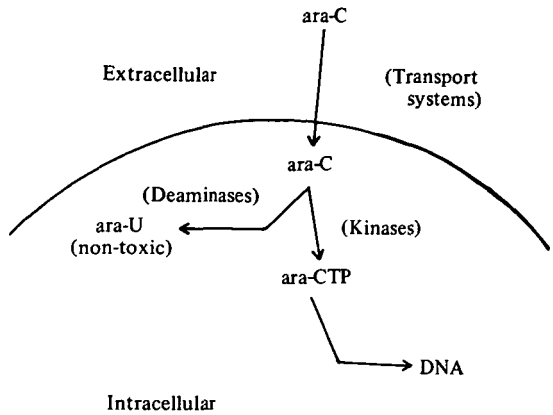


Fig. 1. The molecular mode of action of ara-C (after Cohen, 1977).

2. MATERIALS AND METHODS

(i) *Cell lines, culture, media and chemicals*

The Chinese hamster cell lines (CHO K-1 and E 36/1) obtained from the American type tissue culture collection, Rockville, Maryland, were cultivated using standard methods (also see Eagle, 1972; Puck, 1972; Jacoby & Pastan, 1970). Growth media, foetal calf serum and antibiotics were obtained from Gibco, Grand Island, New York; ara-C and all other chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri. All radioactive chemicals [e.g. [^{3}H]cytidine, [^{3}H]ara-C and [^{3}H]dNMP(s)] were purchased from NEN, Boston, Massachusets or from ICN, Irvine California. All cultures, except when specifically mentioned otherwise, were maintained in Eagle's minimal essential medium (MEM). In some experiments, Dulbecco's modified medium (DMEM) which lacked proline, was used.

(ii) *Isolation of drug-resistant mutants*

Mutants arising spontaneously or chemically induced were isolated using cloned CHO K-1 cells. To isolate spontaneously occurring mutants actively growing cells (without treatment with any mutagen) were seeded into T-75 flasks ($0{\cdot}5 \times 10^6$ or 1×10^6 cells/flask) containing MEM medium with ara-C (1 μg/ml) and incubated at 37 °C. Growing colonies were isolated after about 15–17 days of growth as the presumptive ara-C resistant mutants. Alternatively, the drug resistant mutants were induced by *N'*-methyl-*N'*-Nitro-*N*-nitrosoguanidine (MNG). The actively growing wild type cells ($0{\cdot}5 \times 10^6$ cells flask) were treated with MNG ($0{\cdot}5$ μg/ml) for 16 h and then allowed to grow for 5–6 days in MEM without MNG. After this recovery period, the surviving cells were trypsinized and seeded into flasks containing growth medium with ara-C (1–5 μg/ml). Colonies growing in medium

with ara-C (1 μg/ml) were isolated as presumptive drug-resistant mutants. All mutants were cloned and grown in minimal medium before biochemical genetic characterization.

The mutants and the parental cell lines were designated *ara-C*^{R} and *ara-C*^{S} in this paper to denote their drug-resistant and sensitive phenotypes respectively. Mutant cell lines used in the present study were 102, 103, 201, 301, 002, and 003. Of these only 103, 002, and 003 have been extensively analysed; these are representative of spontaneous (103) and induced (002 and 003) mutations obtained from different mutagenesis experiments. Mutants other than those mentioned here are resistant to many other drugs (such as ara-A and aphidicolin), these are being characterized separately (Vishwanath & Mishra, 1984, in preparation). Mutants which could not be analysed immediately were stored in glycerol medium (10% glycerol, 20% foetal calf serum and 70% minimal essential medium) at −80 °C.

(iii) *Determination of viable cell number*

This was based on the exclusion of the dye (trypan blue) by the viable cells. Cells were routinely counted by a hemacytometer after the dye treatment.

(iv) *Determination of the growth rates*

The growth rates of the wild type and mutant cell lines were compared in two different ways. In the first method, $1{\cdot}3 \times 10^5$ cells were inoculated into a series of T-25 flasks with various concentrations of ara-C (0–5 μg/ml). In the second method, actively growing cells (4×10^4) were added to a series of T-25 flasks containing drug-free medium; and ara-C (0–5 μg/ml) was added when the cell concentration reached $2{\cdot}5 \times 10^5$ cells per flask. Cell counts were made every 24 h after the addition of ara-C; duplicate hemacytometer counts were used for plotting the growth curve. The slope of individual growth curve for the wild-type (*ara-C*^{S}) and mutant (*ara-C*^{R}) cell lines were compared to reveal any differences in their growth rates. When required, culture of cells were stained with Giemsa and photographed at 100× magnification using a polaroid film.

(v) *Uptake of ara-C*

Actively growing cells ($2{\cdot}5 \times 10^6$) were incubated in MEM containing 1 μg/ml of cold ara-C and 2 μCi of [^{3}H]ara-C for 10 min under conditions of growth as described elsewhere (Kessel & Sharin, 1968). Cells were washed with HBSS several times and treated with 1 ml of tissue solubilizer before determining the radioactivity in a scintillation spectrometer (Beckman LS230).

(vi) *Enzyme assays*

The cytidine deaminase activity in CHO cell extract was assayed by the method of Steuart & Burke (1971). The cytidine kinase activity was assayed as described previously (Ives & Wang, 1971). The drug resistant (*ara-C*^{R}) mutants were examined for kinase when grown in MEM medium with or without ara-C (1 μg/ml).

The DNA Polymerase activity was assayed in the following manner: Cells were homogenized in a hypotonic buffer (10 mM-Tris-HCl pH 7·5, 1 mM-EDTA, 4 mM$MgCl_2$, 6 mM 2-mercaptoethanol and 0·025% Triton X-100) by sonication and

KCl was added to the homogenate to final conc. of 1 M and then centrifuged at 35000 rev/min in a Beckman type 65 rotor at 4 °C. The supernatant after dialysis overnight against the buffer (20 mM-Tris-HCl pH 8·1, 1 mM-EDTA, 1 mM 2-mercaptoethanol and 50 mM-KCl) was used as the source of enzyme for assay of DNA polymerase activity. The assay mixture contained 100 mM-Tris-HCl pH 8·1, 3 mM-$MgCl_2$, 1 mM-KCl, 1 mM dithiothreiotol, 250 μg activated calf thymus DNA and 100 μM each dNTP. [^{3}H]dNTP was used at 5 μCi/ml. DNA polymerase α was assayed at 37 °C for 30 min in a total volume of 200 μl.

(vii) *Effect of azacytidine*

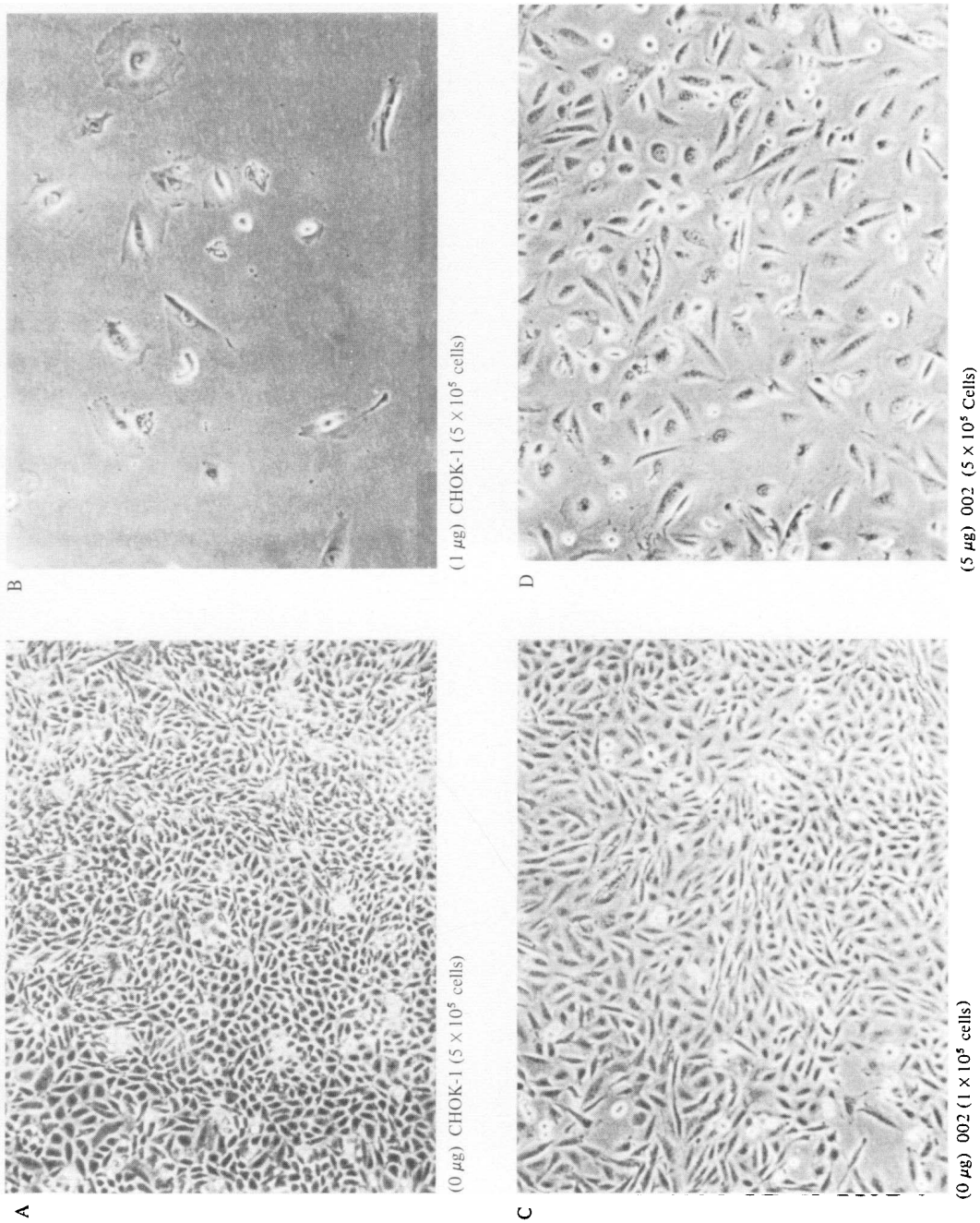
Wild-type and mutant (103, 002 and 003) cells were grown in the presence of 5′-azacytidine (0–5 μg/ml) in MEM medium for a week (with a change of media on every alternate day). The effect of azacytidine on the expression of *ara-C*^{S} locus was investigated by examining the ability of the azacytidine grown CHOK-1 cells to grow in a medium containing ara-C (1 μg/ml); likewise the effect of azacytidine on the expression of *ara-C*^{R} locus was investigated by examining the change in the growth pattern of the azacytidine-grown mutant (103, 002 and 003) cells. As a control, the effect of azacytidine on the expression of *pro*$^-$ cell was investigated by examining the ability of the azacytidine grown CHO-K1 cells (i.e. *pro*$^-$) in DMEM medium (lacking proline), colonies growing in DMEM medium were scored as *pro*$^+$.

(viii) *Genetic analysis*

The technique of polyethylene-glycol (PEG) induced cell fusion described by Davidson & Gerald (1976) was used for genetic analysis. The *pro*$^-$ *hgprt*$^+$, *ara-C*^{R} cells (103, 002 and 003) were fused with the other cell line (E36/1) carrying the opposite genetic markers *pro*$^+$, *hgprt*$^-$ and *ara-C*^{S}. The parental cell line 103, 002 and 003 could not grow in DMEM (Dulbecco's modified minimal medium) which lacked proline but could grow in MEM [containing thioguanine (TG) (100 μg/ml) or ara-C (1 μg/ml)]. The other parental cell line, E36/1 (involved in the somatic fusion) could grow in DMEM alone but not in MEM containing 6-thioguanine or ara-C. Therefore, the hybrids were selected in a DMEM+GHAT (Dulbecco's modified minimal medium, lacking proline, containing glycine, 3 μM, hypoxanthine, 100 μM, aminopterin, 0·4 μM, and thymidine, 16 μM) and then analysed for their level of resistance to different concentrations of ara-C (1–5 μg/ml). The chromosomal analysis of the parental and hybrid cell lines were carried out according to the method of Peterson, Simpson & Hukku (1979).

Plate 1

Growth of the *araC*^{s} wild-type (CHO-K1) and *araC*^{R} mutant (002) in the presence and absence of ara-C. Numbers in parenthesis below each picture indicate the ara-C concentration (μg/ml) (left), cell line (middle) and the size of cell population with which the culture was stated (right), photographs taken on the fourth day after inoculation.



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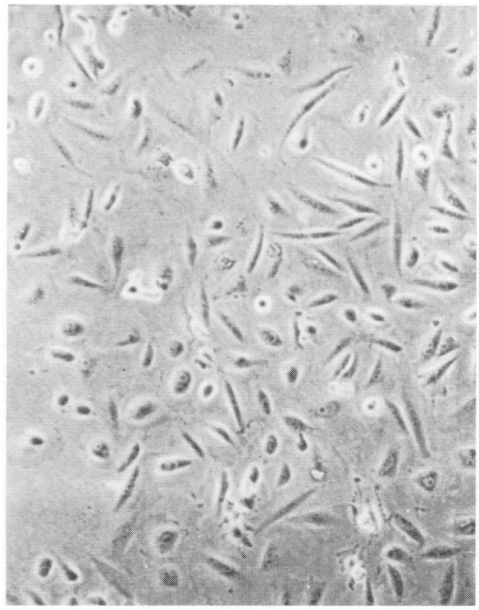
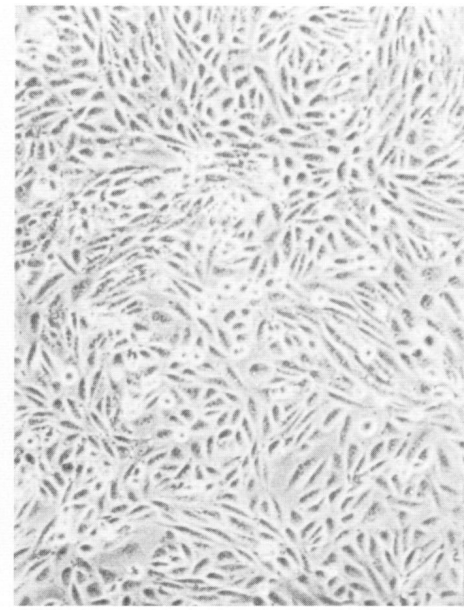
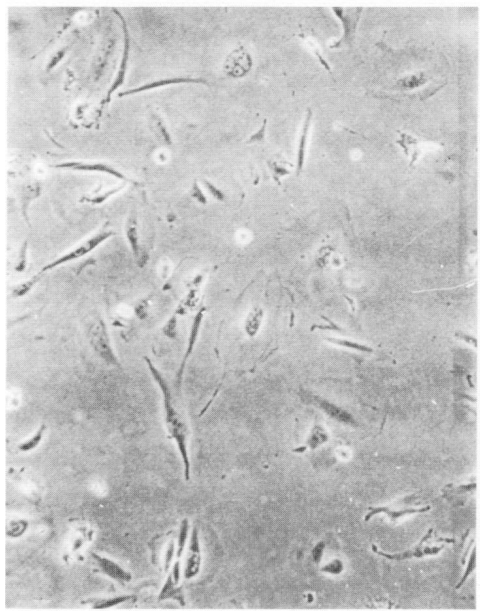


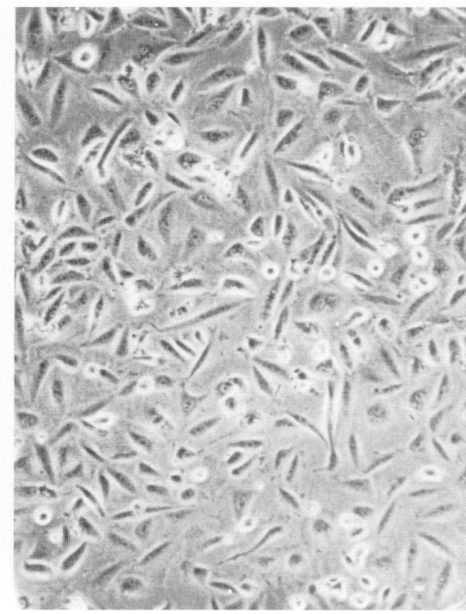
F (1 μg) 002 (2×10^5 Cells)



H (1 μg) 002 (5×10^5 Cells)



E (1 μg) 002 (1×10^5 Cells)



G (1 μg) 002 (2×10^5 Cells)

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3. RESULTS

(i) *Effect of ara-C on the growth of CHO cells and selection of the drug resistant mutants*

The lethal dose of ara-C for the growth of wild-type CHO cells was determined by seeding actively growing cells in medium containing 0–10 μg/ml of ara-C under two different growth conditions (see Materials and Methods). The effect of ara-C on the growth of the wild-type cells is shown in the Figs. 2 and 3. The wild-type cells were able to grow in medium containing 0·01 μg/ml of ara-C and the LD50 for the wild-type cells was less than 0·1 μg/ml of ara-C. The drug at a concentration of 0·2 μg/ml was found to be 100% lethal; the wild-type cells could never grow to confluency at this drug concentration. Over 40 drug-resistant mutants (occurring spontaneously or induced by MNG) were isolated by methods described in Materials and Methods. The data present in Table 1 show the frequencies of spontaneous and induced mutation were 0·24 and 3·4 per million viable cells. These mutants were found stable since they possessed their drug-resistant character even after 3 years of continuous growth in the medium without ara-C.

Table 1. *Spontaneous and induced mutation frequency of* ara-C^R *allele conferring resistance to ara-C in CHO cells*

Treatment	Expt no.	Total no. of viable cells ($\times 10^{-6}$)	No. of mutants	Mutation frequency per million viable cells plated
None	1	6·1 (4)*	1	0·24
	2	11·0 (20)*	3	
	3	4·0 (4)*	1	
MNG	1	4·2 (5)*	18	3·4
	2	4·1 (4)*	9	
	3	2·0 (4)*	8	

Mutants were isolated as described in Materials and Methods.

* Figures in parentheses indicated the number of flasks used in a particular experiment for plating viable cells. No. of viable cells was determined by hemacytometer counts of the cells capable of excluding trypan blue.

In contrast to the wild type, the mutant cell lines were able to grow in medium containing ara-C (1 μg/ml) (Fig. 2, Plate 1). The difference in the growth characteristics of the mutants and wild-type cells was shown by the changes in the nature of the slope of their growth curves (see Fig. 2). As compared to the wild type (CHO-K1) the mutant cells showed an initial lag in growth even when grown in medium without ara-C (see Fig. 2). However, the growth of the mutant (002) was much faster in medium with 1 μg/ml of ara-C than that in medium with 5 μg/ml of ara-C as seen in Fig. 2. All mutant cell-lines examined were found able to grow to confluency in medium containing 1 μg/ml of ara-C (Plate 1) and showed no cytotoxic effect of the drug. Despite their drug-resistant character, all mutants (103, 002 and 003) described here showed poor plating efficiency in ara-C (1 μg/ml) medium since only ten colonies were seen among 10^3 cells plated. In view of this

low plating efficiency, the mutants were examined for their ability to grow to confluency in the drug medium. Data presented in Plate 1 show that the drug-resistant phenotype of the mutants was cell-density dependent. A mutant culture started with 5×10^5 cells per flask became confluent in ara-C medium on the fourth day of growth whereas the cultures started with 3×10^5 cells per flask or with 1×10^5 cells per flask showed a sparse growth on the fourth day (see Plate 1) and attained confluency after 6 days or after 10 days of growth in medium containing ara-C (1 μg/ml). All mutant cultures seeded with 1×10^5–5×10^5 cells per flask reached confluency on the fourth day of growth in medium without ara-C. In contrast, no amount of the wild-type cells (10^5–10^7 cells/flask) were able to grow in a medium with ara-C (1 μg/ml).

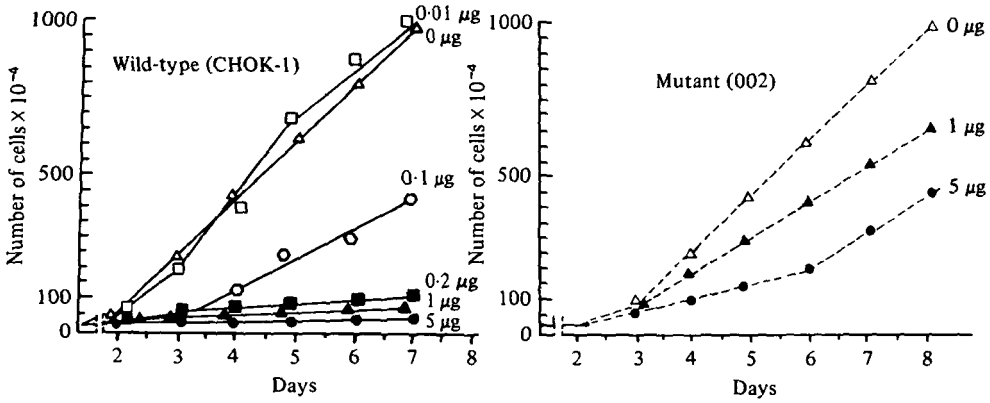

Fig. 2. The growth of wild-type (left panel) and mutant (right panel) cells in the presence or absence of ara-C. Cells were placed in a series of T-25 flasks with 0, 1·0 or 5·0 μg/ml of ara-C and growth was measured (under the second growth conditions) as described in Materials and Methods.

It is possible that the phenotype of the mutant as described above may arise from a cell-density-dependent degradation of ara-C in the growth medium. Therefore, the stability of ara-C was examined in the conditioned medium by its ability to kill the wild type; for these studies the conditioned medium was obtained as the medium in which mutant cells have been grown for 2–5 days. The conditioned drug-medium was found to kill the wild-type cells effectively (100%), although a similarly conditioned medium without ara-C (or with lower concentrations of ara-C up to 0·1 μg/ml) was found to support the growth of the wild-type cells. These results provide evidence for the stability of ara-C in the growth medium and negated the possibility of a cell-density dependent degradation of ara-C as a mechanism for the acquisition of drug-resistance by the mutant cell lines.

(ii) *Biochemical and genetic characterization of mutants*

The wild-type and mutant cells were compared for the enzymes involved in the metabolism of ara-C (see Fig. 1) and later they were analysed genetically in order to elucidate the biochemical basis of drug-resistance and its mode of inheritance.

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(iii) *Ara-C uptake*

The uptake of the radioactivity by the wild-type and mutant cells was proportional to the amount of cells and to the length of incubation up to 8 h at 37 °C. The data (presented in Table 2), showed no significant difference in the level of uptake of [H^3]ara-C by the mutants as compared to that by the wild-type cells suggesting the involvement of factors other than drug uptake in conferring the mutant drug-resistance phenotype.

Table 2. *Level of ara-C uptake, deaminase, kinase and DNA polymerase activities in the wild-type* (ara-C^S) *and mutant* (ara-C^R) *cell lines*

Cell line	Ara-C uptake* [^{3}H]ara-C (counts/min)	Deaminase† [^{3}H]uridine (counts/min)	Kinase‡ ara-C specific	Kinase‡ Cytidine activity	DNA polymerase§ specific activity
Wild type					
CHOK1	636 ± 82	260 ± 33	11·6	10·4	2·45
Mutant					
103	673 ± 65	236 ± 27	11·6	8·4	2·18
002	629 ± 68	280 ± 23	10·8	9·6	2·17
003	654 ± 62	247 ± 31	8·8	9·6	2·17

* Ara-C uptake expressed as CPM/2·6 × 10^6 cells/10 min incubation period under conditions described in Materials and Methods; the background count was 75 CPM or less.

† Activity of deaminase expressed as radioactivity (CPM) of [^{3}H]uridine formed from [^{3}H]cytidine (0·1 μCi) by 100 μg of protein obtained from the cell-free extract (Steuart & Burke, 1971). Mouse kidney cell-free extract was used to determine the conditions for the assay of cytidine deaminase since this is kidney-specific enzyme; 100 μg of mouse kidney cell-free protein yield 2937 ± 93 CPM as radioactive uridine. The background count was 123 CMP.

‡ Kinase activity expressed as nmoles of dCMP or araCMP formed by 1 μg protein/h using ara-C or cytidine as substrate.

§ Specific activity of DNA polymerase expressed as nmol of dCMP incorporated into acid insoluble product by one mg protein in 1 h incubation at 37 °C.

(iv) *Deaminase level*

Deamination of ara-C (see Fig. 1) can confer drug-resistance in the mutant cells; therefore, the level of this enzyme was compared in the extracts of the wild-type and mutant cells. A significant deaminase activity was seen in the mouse kidney cells; the radioactivity (2937 CPM) due to uridine formed by the mouse enzyme was almost 20-fold higher than the background (i.e. without enzyme) radioactivity (123 CPM). However, the CHO (wild-type and mutant) cells showed little or no deaminase activity since the radioactivity (released in the form of uridine) was only 2-fold higher than the background counts (123 CPM) (see Table 2). These data suggest that the drug-resistance of the mutants is not due to a change in the level of the deaminase. The lack of cytidine deaminase in CHO (both wild type and mutant) was not due to the presence of an inhibitor in the CHO cell-free extract; this was evident by the result of the experiments in which CHO cell extract was mixed with the mouse kidney cell extract without any decrease in the level of the mouse kidney cytidine deaminase activity. Thus the lack of the cytidine deaminase

in CHO (as seen in Table 2) can be explained on the basis of a tissue specific (expression or) non-expression of certain enzymes. This deaminase is essentially a kidney-specific enzyme; therefore, it is plausible that it may not be expressed in CHO cell line which is derived from the ovary cells. A similar lack of deaminase activity in other differentiated mammalian cells have been reported elsewhere (Hoffee, Hunt & Chiang, 1982).

(v) *Cytidine kinase and other enzyme activities*

The acquisition of ara-C resistance due to a possible kinase deficiency (see Fig. 1) was examined by determining the level of this enzyme in the wild-type and mutant cells. The level of kinase was found to be the same in the mutant and wild-type cells (see Table 2). The mutant cells showed the same level of kinase when grown in media with or without ara-C. The level of cytidine (or ara-C) kinases obtained here are comparable to the published value of these enzymes in mammalian cells (Robert de Saint & Buttin, 1979). Furthermore, all *ara-C*^{R} mutants were found resistant to ara-CMP (20 μg/ml) where as the wild-type cells (*ara-C*^{S}) were killed (100 %) by ara-CMP (2 μg/ml) added to growth medium.

Resistance to low levels of ara-C in some mammalian mutant cell lines has been shown to result from changes in the enzyme ribonucleotide reductase (Robert de Saint & Buttin, 1979). Such mutants with altered ribonucleotide reductase have been shown to possess co-resistance to hydroxyurea (Hu). Therefore, the present ara-C resistant mutants were examined for their cross-resistance to Hu. In presence of hydroxyurea, the plating efficiency of the mutant and wild-type cells were equally inhibited; 0·1 mM-Hu reduced their plating efficiencies to 70 %, whereas, 0·2 mM-Hu inhibited them by 100 %. Thus these data ruled against the possibility of an alternation in a particular subunit of ribonucleotide reductase as a factor in conferring resistance to ara-C in these mutants.

(vi) *DNA polymerase activity*

The wild-type (CHO-K1) and mutant (103, 002 and 003) cells possessed comparable specific activity of this enzyme (see Table 2). However, the data presented in Fig. 3 show that the presence of ara-CTP in *in vitro* assay equally inhibited the incorporation of all four dNMP into DNA by the mutant enzyme but not by the wild-type enzyme. In case of the wild-type enzyme, ara-CTP inhibited only the incorporation of dCMP; whereas the incorporation of other dNMP remained substantially high even at the highest ara-CTP concentration used (see Fig. 3, data shown for pyrimidine nucleotides). This difference in the inhibition of a specific dNMP incorporation by the wild-type and mutant enzyme suggests a possible change in the nature of the enzyme DNA polymerase (or of an associated protein which can influence the activity of DNA polymerase) as a result of *ara-C*^{R} mutation (see Discussion).

(vii) *Effect of azacytidine*

The *in vivo* resistance to ara-C (Boehn & Darhovsky, 1982) and the appearance of certain epigenetic mutations in the mammalian cells (Harris, 1982) have been shown to result from a change in the methylation of DNA. Therefore, the role of

methylation was investigated by examining the effect of azacytidine on the expression of the Chinese hamster *ara-C*^{R} and *ara-C*^{S} alleles (as described by Harris, 1982). First, the CHO cells were examined for their ability to grow in the presence of azacytidine. Like other Chinese hamster cell-lines (Harris, 1982), the CHO wild-type (CHO-K1) and *ara-C*^{R} mutant (103, 002, 003) cells were able to grow in the presence of azacytidine (up to 5 μg/ml). The effect of azacytidine on the

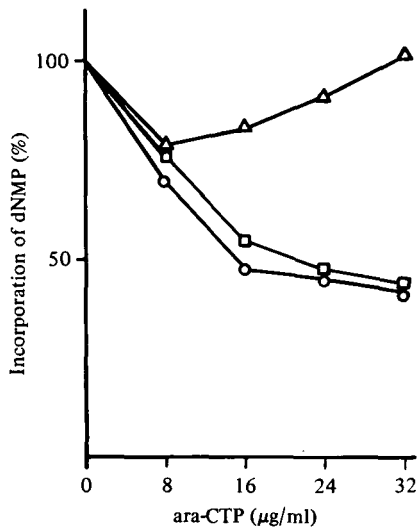

Fig. 3. Effect of ara-CTP on the dNMP incorporation by the wild-type (CHO-K1) and mutant (003) enzyme preparations. △, dTMP incorporation by the wild-type enzyme; □, dCMP incorporation by the wild-type enzyme; ○, dTMP or dCMP incorporation by the mutant enzyme.

expression of *ara-C*^{S} or *ara-C*^{R} phenotype by the CHO cells was examined in the following manner (as described in Materials and Methods); (*a*) a culture of wild-type and of mutant cells (started with 1×10^5 cells/flask) was first grown for 6 days in a MEM medium containing azacytidine (0 μg/ml, 1 μg/ml, or 5 μg/ml) and then the cells from each culture were plated (1×10^5–5×10^5 cells/flask) in medium containing ara-C (1 μg/ml); (*b*) the wild-type and mutant cells were plated (1×10^5–5×10^5 cells/flask) in medium containing ara-C (1 μg/ml) and azacytidine (0 μg/ml, 0·1 μg/ml or 1 μg/ml). Under both conditions of growth, the wild-type and mutant cells showed no adverse or favourable effects of azacytidine on their growth. The wild-type cells were killed (100%) in ara-C medium whether or not grown before or simultaneously in the presence of azacytidine. The mutant cells showed a (cell density-dependent confluent) growth in ara-C medium irrespective of their prior or simultaneous exposure to azacytidine. Thus the results of these experiments clearly rule out the possibility of methylation as the basis for drug-resistance.

Since azacytidine showed no effect on the expression of *ara-C*^{S} or *araC*^{R} alleles, therefore, its effect on the expression of another CHO gene was investigated in order to check if azacytidine has any effect on gene expression under the growth

conditions described here. In such experiments azacytidine was found to enhance the frequency of *pro*$^+$ revertants by 100-fold when the *pro*$^-$ CHO cells were treated with 5′-azacytidine (1 μg/ml). The results of these experiments showed that azacytidine was effective in the reversion of *pro*$^+$ locus even though it had no effect on the reversion of *araC*^{S} or *araC*^{R} alles of CHO.

(viii) *Genetic analysis*

The genetic nature of the mutation-conferring resistance to ara-C was examined by the analysis of somatic cell hybrids after the PEG induced fusion of the *ara-C*^{R} mutant with the wild-type (*ara-C*^{S}) cells (see Materials and Methods). All 73 hybrids examined from three somatic crosses (103 × E 36/1, 002 × E36/1, and 003 × E36/1) were found resistant to ara-C (1 μg/ml) (see Table 3). Furthermore, of the six hybrid cell lines examined, all showed a cell density-dependent resistance to ara-C (1 μg/ml). Since the hybrids showed the same level of drug-resistance as the *ara-C*^{R} parental cell lines, these data provide evidence for the dominance of the *ara-C*^{R} over the *ara-C*^{S} allele. Cytological preparations of the wild-type (*ara-C*^{S}) and mutant (*ara-C*^{R}) cells showed a normal chromosome complement, over 90 % of cells showed 21 chromosome per cell, the hybrid cells showed a chromosome complement of 42 or more. Also, all 18 hybrid progeny obtained after the (mock) hybridization of *ara-C*^{S} cell lines (i.e. CHOK-1 X E36/1) were found sensitive to ara-C (see Table 3); these data suggested that changes in chromosome numbers due to the process of hybridization cannot be considered to confer the ara-C-resistance.

4. DISCUSSION

The data presented in this paper confirm the lethal effect of ara-C on the growth of the mammalian cells (Cohen, 1977; Cohen & Plunkett, 1975) and provide evidence for the genetic basis of the ara-C resistance in CHO cells based on the facts that the frequency of *ara-C*^{R} mutation was increased significantly (14 ×) by chemical mutagenesis of the wild-type cells and that the drug-resistance was a stable phenotype which behaved as a dominant trait in somatic crosses.

The biochemical characterizations of these mutants showed that they were not defective in the uptake of ara-C or in the metabolic pathways involving phosphorylation or increased in the level of deamination of ara-C. The fact that mutants were resistant to ara-CMP further supports the idea that they are not defective for deamination or phosphorylation steps (Plunkett and others, 1974). Also, from the fact that the wild-type and mutant cells were equally sensitive to hydroxyurea suggested that at least the subunit of the enzyme ribonucleotide reductase which confers resistance to hydroxyurea has not changed as a result of mutation. This is unlike all the *ara-C*^{R} mutants described previously which were either recessive and lacked cytidine kinase activity or were semi-dominant and possessed an increased ribonucleotide reductase activity (Smith & Chu, 1972; Robert de Saint & Buttin, 1979). We have, however, found that one mutant (AC5) deficient in cytidine kinase is recessive (Mishra, unpublished results). Unlike the expression of a number of other Chinese hamster mutants (see Harris, 1982) the drug-resistance of the mutants (103, 002, 003) does not seem to be mediated by

Table 3. *Phenotypes of the hybrids and the parental cell lines*

Cell lines	No. of clones examined	Growth on medium									
		DMEM	MEM	MEM +GHAT	DMEM +GHAT	MEM +TG	MEM + ara-C (μg/ml) 0·2	0·5	1	2	5
*Hybrid											
103 × E36/1	19	+	+	+	+	−	+	+	+	−	−
002 × E36/1	28	+	+	+	+	−	+	+	+	+	+
003 × E36/1	26	+	+	+	+	−	+	+	+	−	−
CHOK-1 × E36/1	18	+	+	+	+	−	−	−	−	−	−
†Parental											
103 (*hgprt*$^+$ *pro*$^-$ *ara-C*^{R})	6	−	+	+	−	−	+	+	+	−	−
002 (*hgprt*$^+$ *pro*$^-$ *ara-C*^{R})	6	−	+	+	−	−	+	+	+	+	+
003 (*hgprt*$^+$ *pro*$^-$ *ara-C*^{R})	6	−	+	+	−	−	+	+	+	−	−
E36/1 (*hgprt*$^-$ *pro*$^+$ *ara-C*^{S})	6	+	+	−	−	+	−	−	−	−	−
CHO-K1 (*hgprt*$^+$ *pro*$^-$ *ara-C*^{S})	6	−	+	+	−	−	−	−	−	−	−

* Hybrid cells were selected in DMEM + GHAT medium after fusion of the cell lines indicated.
All media made as described in Materials and Methods.
Growth to confluency (+) or no growth (−) in the medium indicated.
† Genetic markers in parenthesis.

a change in the methylation pattern since azacytidine had effect on the expression of *ara-C*^{S} or *ara-C*^{R} alleles.

The *ara-C*^{R} mutants described herein, are also novel, in that the drug-resistance is dependent on their cell-density. The only other drug-resistance which is cell-density dependent is the resistance to iodo-acetamide by certain mammalian cells (Wright, 1982). However, these iodo-acetamide resistant cell lines have never been genetically analysed; therefore, the basis for their cell density-dependent drug-resistance remains obscure. The cell density-dependent expression of *ara-C*-resistance among the present mutants does not seem to stem from the degradation of ara-C in the growth medium since the conditioned drug media effectively killed the wild-type cells. It seems possible that drug-resistance may be mediated by the membrane involving a cell-to-cell communication among the mutant culture which would be cell-density dependent.

The data presented in Fig. 3 indicates a possible change in the properties of the mutant DNA polymerase since the mutant and wild-type enzymes were found to differ in the level of the incorporation of the various dNMP(s) in the presence of ara-CTP. These data suggest that in the presence of ara-CTP, the wild-type DNA polymerase is much more error-prone whereas the mutant enzyme is error-resistant (or much less error-prone). This difference in the properties of the wild-type and mutant DNA polymerase (or of an associated protein which can influence the activity of DNA polymerase, see Reddy & Pardee, 1980; Bell & Fridland, 1980) may account for the drug-sensitive and resistant phenotypes of the two cell lines *in vivo*. It is plausible that the fidelity of the wild-type enzyme is impaired by ara-CTP causing dNMP misincorporation and cell death. It is further possible that the mutant enzyme is so changed that its fidelity is no more impaired by ara-CTP and that the incorporation of all dNMPs is equally influenced such that the mutant cells (which may be somewhat sensitive to ara-C in the initial stage of growth) grow at a slower rate and thus manage to escape the lethal effects of the drug. The fact that all ara-C^R mutants grew slowly even in the drug-free medium with an initial period of lag (see Fig. 2), supports these ideas. Such a change in the fidelity of the wild-type polymerase induced by ara-CTP is plausible in view of the suggestion made by Topal & Baker (1982) regarding the role of modified nucleotides in controlling the fidelity of DNA replication. These findings are consistent with the previous reports suggesting the mutagenic effects of ara-nucleosides (ara-C, ara-A) in mammalian cells (Huberman & Heidelberger, 1972; Hirsch & Schooley, 1983).

The role of membrane in DNA replication and in contact inhibition of the growth of mammalian cells is very well established. It is therefore not hard to envision the role of cellular communication in the control of growth of mutants described here. However, the nature of such cell density-dependent expression of ara-C resistance in this process remains quite elusive. An extensive biochemical genetic analysis of these mutants can provide an insight into the problems of eukaryotic DNA replication and cell proliferation (including the phenomena of contact inhibition).

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