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INCREASE IN dATP POOL IN APHIDICOLIN-RESISTANT MUTANTS OF MOUSE FM3A CELLS

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SUMMARY

Mutants that were resistant to aphidicolin were isolated from mutagenized mouse FM3A cells at a frequency of about 10^{-6}. Resistance to aphidicolin in these mutants was not due to an effect on [^{3}H]thymidine incorporation into DNA, DNA synthesis in permeabilized cells, or DNA polymerase α.

All the mutants showed a greatly increased dATP pool and decreased ability to incorporate [^{3}H]deoxycytidine into DNA. They also showed cross-resistance to both 1-β-D-arabinofuranosyladenine and 1-β-D-arabinofuranosylcytosine.

These results indicate that an enzyme involved in production of dATP or its requlation is altered in these mutants. It is suggested that dATP competes with aphidicolin at its killing site or that dATP reverses the effect of aphidicolin by some unknown mechanism *in vivo*.

INTRODUCTION

Aphidicolin (1) inhibits DNA synthesis *in vivo* in eucaryotic cells and the growth of Herpes simplex and Vaccinia viruses (2-4). Of the three DNA polymerases α, β, and γ, only DNA polymerase α is sensitive to aphidicolin (3,4). α-Polymerase may be involved in DNA replication and also in DNA repair synthesis and viral DNA replication, since all these processes are inhibited by aphidicolin (3-7). However, the site of action of aphidicolin in the cell is unknown. Genetic and cell biological studies are required to determine the true target of aphidicolin *in vivo*. This paper reports the isolation and characterization of aphidicolin-resistant mutants of mouse FM3A cells.

MATERIALS AND METHODS

Cell culture. The parental cell line used was a subclone, F28-7, isolated from FM3A cells, which were established from a spontaneous mammary carcinoma in a C3H/He mouse (8). The cells were grown in floating culture at 37°C in

Abbreviations: Ac^r, aphidicolin-resistance; Ara-Ade, 1-β-D-arabinofuranosyladenine; Ara-Cyt, 1-β-D-arabinofuranosylcytosine.

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Eagle's MEM supplemented with 0.1% Bactopeptone (Difco) and 2% fetal calf serum (GIBCO) as previously described (9). Agar-medium consists of MEM supplemented with 0.1% Bactopeptone, 10% fetal calf serum, and 0.5% purified agar (Dufco).

Isolation of mutants. A cell suspension at 1 x 10^5 cells/ml was treated with 1μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (Sigma) for 3 h. The cells were cultured for 2 days in fresh medium to allow expression of mutated genes. Mutants resistant to aphidicolin (kindly supplied by Dr. B. Hesp of Imperial Chemical Industries, Ltd., U.K.) were isolated in two ways. One way was to culture mutagenized cells in medium containing 0.2μg/ml of aphidicolin, pick up the cells that grew and clone them on agar-medium. The other way was to seed mutagenized cells on agar-medium containing 0.2μg/ml of aphidicolin and isolate the colonies that appeared.

Incorporation of labeled nucleosides. Inocula of 1 x 10^5 cells were seeded into 1 ml of medium in a glass tube and cultured overnight. [Methyl-^{3}H]thymidine (42Ci/mmol), [6-^{3}H]2'-deoxyadenosine (25Ci/mmol), [8-^{3}H]2'-deoxyguanosine (5.7Ci/mmol), [5-^{3}H]2'-deoxycytidine (22Ci/mmol), [2-^{3}H]-adenosine (25Ci/mmol), [5,6-^{3}H]cytidine (49Ci/mmol), or [^{3}H]amino acid mixture (Radiochemical Centre, Amersham) was added to the culture at 5μCi/ml. After incubation for 60 min, 1 ml of cold 10% trichloroacetic acid was added to each tube and the precipitate was transferred to a glass fiber filter (Whatman GF/C). Filters were washed with 20 ml of cold 5% trichloroacetic acid and their radioactivity was counted in toluene-based scintillator.

Assay of DNA synthesis. Lysolecithin-mediated permeabilization of cells was carried out as described by Miller et al. (10). Assay of DNA synthesis in permeabilized cells was carried out essentially as described by Miller et al. except that the concentration of ATP was increased to 3mM, and $CaCl_2$ and phosphoenolpyruvate were omitted from the reaction mixture. The reaction mixture (50μl) was mixed with 1 ml of 5% cold trichloroacetic acid. Then 10mM sodium pyrophosphate was added and the precipitate was washed three times with 2 ml of the same solution by centrifugation. The precipitate was solubilized in 1 ml of tissue solubilizer (Soluene 350, Packard) and its radioactivity was counted in toluene-based scintillator.

DNA polymerase α was extracted from exponentially growing cells and fractionated on a DEAE-cellulose (Whatman DE-52) or a phosphocellulose (Whatman P-11) column as described previously (11). DNA polymerase α was measured using calf thymus activated DNA as template (11).

Determination of dNTP pools. Intracellular dNTPs were extracted from exponentially growing cells at a density of about 5 x 10^5 cells per ml with ice-cold 60% methanol. The extract was processed and analyzed as described by Skoog (12) and Lindberg and Skoog (13).

RESULTS

Isolation of aphidicolin-resistant mutants. Seven aphidicolin-resistant clones (Ac^r) were isolated from separate samples of mutagenized cells by culture in medium containing 0.2μg/ml aphidicolin. Mutants appeared with a frequency in the order of 10^{-6} when mutagenized cells were seeded on agar medium containing 0.2μg/ml aphidicolin. The phenotypes of the resistant clones were the same whether the mutants were isolated in liquid medium or in

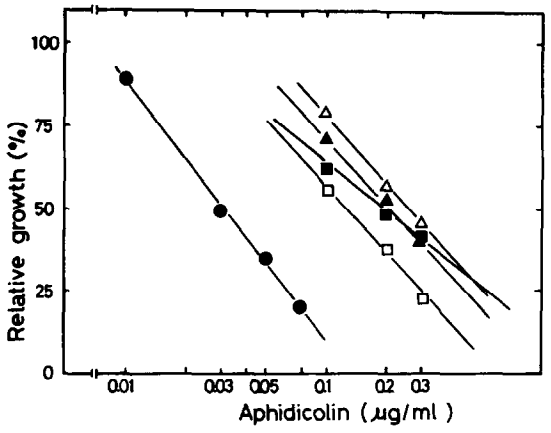

Fig. 1. Effect of aphidicolin on growth of wild-type F28-7 (●) and the Ac^r mutants FSac2 (▲), FSac7 (△), FSac3 (■) and FSac6 (□). Cells were seeded at 1 x 10^4 cells/ml and cell titers were measured after culture for 3 days in the presence of various concentrations of aphidicolin.

agar-medium. The mutants were 5-10 times more resistant to aphidicolin than the parent line F28-7, judging from the concentration of aphidicolin required for 50% inhibition of growth. Figure 1 shows results on four typical clones, FSac2, FSac3, FSac6, and FSac7, of the seven Ac^r clones isolated. The reason for choice of these four clones is explained later. Aphidicolin-resistance in all the Ac^r clones was stable for over 6 months in culture in the absence of aphidicolin and the phenotype was shown to be genetically semi-dominant by cell-cell hybridization experiments (not shown). Thus, the resistance to aphidicolin is caused by a structural alteration of some polypeptide.

Effect of aphidicolin on DNA synthesis in vivo and in vitro. Incorporation of [^{3}H]thymidine into acid-insoluble macromolecules in the mutants was not more resistant to aphidicolin than that in F28-7 (Fig. 2A). This somewhat unexpected result indicates that the factors involved in bulk DNA synthesis in vivo are not altered in Ac^r mutants. Next, the effect of aphidicolin on DNA synthesis in permeabilized cells was examined (Fig. 2B). DNA synthesis in all the Ac^r mutants showed similar sensitivity to aphidicolin to that of F28-7.

DNA polymerase α was extracted from F28-7, FSac3, and FSac7 cells and fractionated on a DEAE-cellulose or phosphocellulose column. The elution

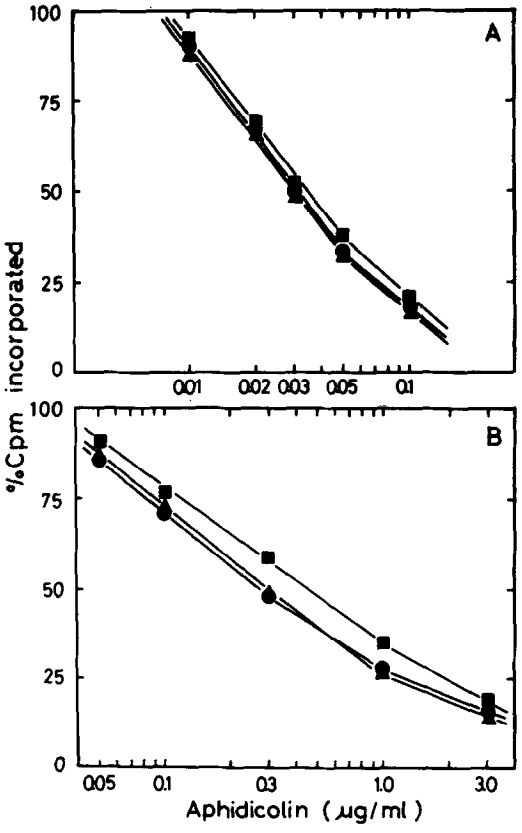

Fig. 2. Effect of aphidicolin on the incorporation of [^{3}H]thymidine into acid-insoluble materials of cells (A) and on DNA synthesis in permeabilized cells (B). Assay procedures are described in Materials and Methods. Symbols: ●, wild-type F28-7; ▲, FSac2; ■, FSac3.

profiles of the polymerase from the two mutants were essentially the same as that of the polymerase from F28-7. Three peaks of activity of polymerase α (14) were obtained on DEAE-cellulose chromatography of extracts of FSac3, FSac7 and F28-7, but the sensitivities of the polymerase α fractions of the two Ac^r mutants to aphidicolin were similar to that of polymerase α from F28-7 (data not shown). These data indicate that the mutated site causing resistance to aphidicolin in the Ac^r mutants measured by incorporation of labeled thymidine or TTP into DNA is not in polymerase α or in the DNA synthetic machinery. Nevertheless, Ac^r mutants were capable of growth, although at a reduced rate, in the presence of aphidicolin where the growth of F28-7 cells was completely inhibited (data not shown).

Table 1. Sensitivity of aphidicolin-resistant mutants to nucleosides and nucleoside analogues.

Cell line	Cell growth (GD_{50}*) in the presence of					
	Ara-Ade (μM)	Ara-Cyt (μM)	Azido-Cyd (μM)	Thd (mM)	dAdo (mM)	dGuo (mM)
F28-7	1 (1)**	0.13 (1)	10 (1)	0.45 (1)	0.12 (1)	0.45 (1)
FSac2	14 (14)	3.2 (25)	170 (17)	21 (47)	0.45 (2.8)	0.49 (1.1)
FSac7	22 (22)	1.7 (13)	130 (13)	7.5 (17)	0.5 (4.2)	1 (2.2)
FSac3	1.5 (1.5)	0.45 (3.5)	52 (5.2)	1.2 (2.7)	0.16 (1.3)	0.32 (7.1)
FSac6	3.5 (3.5)	0.44 (3.4)	42 (4.2)	1 (2.2)	0.17 (1.4)	0.8 (1.8)

Growth of cells was determined as described in the legend to Fig. 1.
* The concentration reducing the growth rate to 50% of that of control cells.
** Relative resistance.

Sensitivity of Ac^r mutants to nucleosides and nucleoside analogues. It is conceivable that aphidicolin acts as a nucleoside analogue in vivo, as implied from in vitro studies on α-polymerase (15). Thus, the sensitivities of Ac^r mutants to various nucleosides and nucleoside analogues were examined (Table 1). All the mutants showed cross-resistance to Ara-Ade, Ara-Cyt and 2'-deoxy-2'-azidocytidine. On the basis of the extent of this cross-resistance, the seven mutants isolated could be classified as three groups: FSac2 and FSac7 showed high cross-resistance, FSac3 and FSac6 showed lower cross-resistance, and the rest (not shown) showed intermediate cross-resistance. The highly cross-resistant mutants were also resistant to thymidine, and slightly resistant to deoxyadenosine. These results strongly suggest that in these mutants the metabolic pathway of deoxynucleosides is altered.

Incorporation of [^{3}H]nucleosides into macromolecules. The labeling efficiency of DNA with exogenous [^{3}H]deoxynucleosides is affected by the size of the dNTP pool and the ability of cells to utilize deoxynucleosides. Therefore, incorporation of various [^{3}H]nucleosides into acid-insoluble macromolecules in the mutants was examined. Table 2 shows that the abilities to take up or metabolize deoxycytidine and deoxyadenosine are altered in the

Table 2. Incorporation of [^{3}H]nucleosides and [^{3}H]amino acid mixture into macromolecules in aphidicolin-resistant mutants.

Cell line	Cpm x 10^{-3}/1x10^5 cells						
	Thd	dCyd	dAdo	dGuo	Ado	Urd	a.a. mix.
F28-7	59.6	20.4	77.5	33.4	104.4	76.4	8.7
FSac2	35.8	1.4	24.8	33.8	130.6	98.4	8.6
FSac7	30.4	1.0	27.1	30.9	60.3	63.5	14.0
FSac3	54.6	3.0	34.2	27.8	126.5	66.7	13.6
FSac6	58.3	4.2	17.0	35.8	93.8	92.5	13.3

The incorporation of labeled materials into the acid-precipitable fraction was determined as described in Materials and Methods.

Ac^r mutants. The incorporations of other [^{3}H]nucleosides, including cytidine (not shown), were not significantly altered.

Determination of dNTP pools. The pool sizes of dNTPs in Ac^r mutants were determined. Table 3 shows that the dATP pool was greatly increased in all the Ac^r mutants examined. Changes in the sizes of other nucleotide pools were also observed, but they were less than that of the dATP pool. Thus, the changes in the other nucleotide pools may be influenced secondarily by the great increase in the dATP pool. The decreased incorporation of [^{3}H]-deoxycytidine in the Ac^r mutants (Table 2) without concomitant change in the level of the dCTP pool (Table 3) must be due to decreased ability to trans-

Table 3. Deoxynucleoside triphosphate pools in aphidicolin-resistant mutants.

Cell line	dATP	dGTP	dCTP	dTTP
	(pmoles/1 x 10^6 cells)			
F28-7	130	7.6	118	52
FSac2	2,080	4.6	36	76
FSac7	1,900	13	56	88
FSac3	3,300	26	86	108
FSac6	2,700	22	86	102

The dNTP pools were determined as described in Materials and Methods.

port or phosphorylate deoxycytidine. This could account for their resistance to Ara-Cyt. The cross-resistance to Ara-Ade could also be explained in part by dilution of Ara-ATP with an increased amount of dATP in the cell.

DISCUSSION

The distinct phenotypes of Ac^r mutants are the marked increase in the dATP pool size. Cross-resistances to Ara-Ade, Ara-Cyt, and azidocytidine, and the decreased utilization of deoxycytidine are of another interest. The latter change alone cannot give resistance to aphidicolin, since mutants that lack deoxycytidine kinase are as sensitive to aphidicolin as the wild type cell line (unpublished data). However, it is not known why the marked increase in the dATP pool is coupled with decreased ability to utilize deoxycytidine in all the Ac^r mutants that were isolated with the frequency of a single mutation. Coordinated regulation may operate in increase in the dATP pool and decrease in the uptake of deoxycytidine. Despite the similarities in the phenotypes, the mutation sites in FSac2 and FSac7 must differ from those in FSac3 and FSac6, since FSac2 and FSac7 are clearly resistant to thymidine and deoxyadenosine (Table 1), whereas FSac3 and FSac6 are not. However, the enzyme responsible for increase in dATP in the Ac^r mutants is not yet identified. ADP reductase is one of the most probable candidates, but other possibilities cannot be disregarded.

Mutants isolated by resistance to drugs or metabolite analogues, e.g., amethopterin, hydroxyurea, Ara-Cyt and deoxyadenosine, are known to arise by three main types of mechanism (16-22): overproduction of a target enzyme, alteration of the enzyme itself, and dilution of analogues by increased synthesis of substrates that compete with the analogues or their metabolites. In the Ac^r mutants the resistance seems to be caused by the third type of mechanism.

Aphidicolin was shown to inhibit DNA polymerase α by competing with dCTP (Ki value, 0.5μg/ml), but not with the other three dNTPs (15). It should be mentioned in this connection that inhibition of growth of wild type cells by aphidicolin could not be reversed by the addition of deoxycytidine (our un-

published results). The concentrations of aphidicolin that reduced the growth rate of FM3A cells by 50% and that inhibited [^{3}H]thymidine incorporation into DNA *in vivo* by 50% are both 0.03μg/ml. The concentration for 50% inhibition of DNA synthesis in permeabilized cells is 0.3μg/ml. In contrast, a much higher concentration of aphidicolin (6μg/ml) is required to inhibit purified α-polymerase activity by 50%. In the Ac^r mutants, DNA synthesis measured by incorporation of labeled thymidine or TTP into DNA was as sensitive to aphidicolin as that in the wild type line. Nevertheless, Ac^r mutants were capable of growth in the presence of aphidicolin where the growth of wild type cells was completely inhibited. It is also worth mentioning that inhibition of α-polymerase by aphidicolin was not reversed by addition of dATP with other nucleotides (data not shown). This difference in the sensitivities of α-polymerase to aphidicolin *in vivo* and *in vitro*, and also the apparently similar sensitivities of DNA synthesis to aphidicolin in the wild type line and the Ac^r mutants suggest that aphidicolin may have another target besides α-polymerase. The present study strongly indicates that dATP alone or in combination with other nucleotides competes with aphidicolin at its killing site or reverses the effect of aphidicolin by some unknown mechanism.

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REFERENCES

1. Brundet, K. M., Dalziel, W., Hesp, B., Jarvis, J. A. J. and Neidle, S. (1972) J. Chem. Soc. D. Chem. Commun. 1027-1028.
2. Bucknall, R. A., Moores, H., Simms, R. and Hesp, B. (1973) Antimicrob. Ag. Chemother. 4, 294-298.
3. Ohashi, M., Taguchi, T. and Ikegami, S. (1978) Biochem. Biophys. Res. Commun. 82, 1084-1090.
4. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature (London) 275, 458-460.
5. Hanaoka, F., Kato, H., Ikegami, S., Ohashi, M. and Yamada, M. (1979) Biochem. Biophys. Res. Commun. 87, 575-580.
6. Pedrali-Noy, G. and Spadari, S. (1979) Biochem. Biophys. Res. Commun. 88, 1194-1202.
7. Berger, N. A., Kurohara, K. K., Petzold, S. J. and Sikorski, G. W. (1979) Biochem. Biophys. Res. Commun. 89, 218-225.

8. Nakano, N. (1966) Tohoku J. Exp. Med. 88, 69-84.
9. Koyama, H. and Ono, T. (1976) J. Cell. Physiol. 88, 49-56.
10. Miller, M. R., Castellot, J. J. and Pardee, A. B. (1978) Biochemistry 17, 1073-1080.
11. Kozu, T., Aida, M., Ikawa, T. and Seno, T. (1978) Eur. J. Cancer 14, 1065-1076.
12. Skoog, L. (1970) Eur. J. Biochem. 17, 202-208.
13. Lindberg, V. and Skoog, L. (1970) Anal. Biochem. 34, 152-160.
14. Matsukage, A., Sivarajan, M. and Wilson, S. H. (1976) Biochemistry 15, 5305-5314.
15. Oguro, M., Suzuki-Hori, C., Nagano, H., Mano, Y. and Ikegami, S. (1979) Eur. J. Biochem. 97, 603-607.
16. Albrecht, A. M., Biedler, J. L. and Hutchison, D. J. (1972) Cancer Res. 32, 1539-1546.
17. Meuth, M. and Green, H. (1974) Cell 3, 367-374.
18. Flintoff, W., Davidson, S. V. and Siminovitch, L. (1976) Somat. Cell Genet. 2, 245-261.
19. Chang, S. and Littlefield, J. (1976) Cell 7, 391-396.
20. Lewis, W. and Wright, J. A.(1978) J. Cell. Physiol. 97, 73-86.
21. Robert de Saint Vincent, B. and Buttin, G. (1979) Somat. Cell Genet. 5, 67-82.
22. Lewis, W. and Wright, J. A. (1979) Somat. Cell Genet. 5, 83-96.