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APHIDICOLIN RESISTANT MUTANT OF WHICH DNA POLYMERASE α IS INDUCED BY THIS DRUG

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SUMMARY

Mutants resistant to aphidicolin, a specific inhibitor of DNA polymerase α of eukaryotic cells, were selected from cultured FM3A cells, derived from mouse mammary carcinoma. One of them, designated as Aph 212, grew in the presence of 1 μg/ml of the drug, which did not permit wild type cells to grow. The resistance of Aph 212 cells to aphidicolin seems to be due to the increment of the activity of DNA polymerase α when Aph 212 cells were cultivated in the presence of the drug.

INTRODUCTION

Eukaryotic cells contain at least three species of DNA polymerase, named α, β and γ (1). At present, however, their functional roles in DNA replication and/or in repair replication were not completely understood (1). The isolation of mutants which are related to DNA polymerases would facilitate to search for the roles of DNA polymerases.

Recently, it was reported that aphidicolin, a tetracyclic diterpenoid antibiotic, inhibits DNA replication *in vivo* (2,3,5-8) and is a specific inhibitor of DNA polymerase α of eukaryotic cells *in vitro* (3,4,7,8). We tried to isolate aphidicolin resistant mutants from cultured FM3A cells, derived from mouse mammary carcinoma (9) and obtained several resistant mutants. We report here that one of them, desiganted as Aph 212, showed the increased activity of DNA polymerase α when cultivated in the presence of the drug.

MATERIALS AND METHODS

Chemicals: Deoxyribonucleoside triphosphates and poly(A) were purchased from Boehringer-Mannheim-Yamanouchi (Tokyo) and oligo(dT)$_{12-18}$ from PL-Bio-

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chemicals. Tritiated dTTP (53Ci/mmol), [^{3}H]thymidine (27Ci/mmol), [^{3}H]uridine (5Ci/mmol) and [^{3}H]leucine (53Ci/mmol) were obtained from The Radiochemical Centre, Amersham. Aphidicolin was obtained from the culture of the fungus, *Harziella entomophia*, as described elsewhere (5).

Cell culture and mutant selection: FM3A cells, cell line derived from mouse mammary carcinoma (9), were cultured in suspension in RPMI 1640 containing 10% calf serum at 33°C. Aphidicolin resistant mutants were selected as follows: Logarithmically growing cells at 33°C were mutagenized with 0.25 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine for 16 h (dose of 20% survival) and incubated for 4 days as expression time. The cells remained were inoculated in soft agar plates (10) containing 0.3 μg/ml aphidicolin and incubated at 33°C for about two weeks. The colonies developed were cultured in a liquid medium containing 0.3 μg/ml for 6 days, 0.6 μg/ml for 6 days and 1 μg/ml for 12 days, successively. The cells which were able to grow in the presence of 1 μg/ml aphidicolin were isolated as aphidicolin resistant mutants.

Measurement of rate of DNA, RNA and protein synthesis: Cells were pulse-labeled with [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine (each 1 μCi/ml) for 30 min to measure DNA, RNA or protein synthesis, respectively. After pulse-labeling period, cells were centrifuged and acid insoluble materials were collected on Whatman glass fiber filter (type GF/C). The radioactivities were counted by liquid scintillation spectrometer with toluene based scintillation fluid.

Assay of DNA polymerases: Crude enzyme extract was prepared as follws: Cells were suspended in 1 ml of hypotonic buffer A (10 mM Tris-HCl, pH7.5-1 mM EDTA-4 mM $MgCl_2$-6 mM 2-mercaptoethanol-0.025% Triton X-100). They were homogenized with a teflon pestle glass Potter Elvehjem homogenizer by 15 strokes and then 3 M KCl was added to the homogenate to make a final concentration of 1 M. After standing at 4°C for 1 h, it was centrifuged at 105,000 g for 1 h. The supernatant was dialyzed against dialyzing buffer (20 mM Tris-HCl, pH8.1-1 mM EDTA-1 mM 2-mercaptoethanol-50 mM KCl). The dialysate was used for enzyme source. The DNA polymerase α was assayed in 50 mM Tris-HCl (pH7.5), 2 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 250 μg/ml activated calf thymus DNA, 100 μM dGTP, dATP, dCTP and 10 μM[^{3}H]dTTP (10 μCi/ml, 1Ci/mmol). The assay for DNA polymerase β was essentially the same as the assay for DNA polymerase α except that 13 mM N-ethylmaleimide was present. The value of DNA polymerase β assay was subtracted from the value of DNA polymerase α assay when the activity of DNA polymerase α was calculated. DNA polymerase γ was assayed according to Knopf et al. (11). The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 50 mM potasium phosphate (pH 8.5), 0.5 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 50 μg/ml poly(A):oligo(dT)$_{12-18}$ (5:1 w/w), 10 μM[^{3}H]dTTP (5 μCi/ml, 0.5Ci/mmol). Each reaction was carried out at 33°C for 30 min. Acid insoluble materials were collected and the radioactivities were counted as described above.

RESULTS

Effect of aphidicolin on cell growth and DNA synthesis: Several aphidicolin resistant mutants were selected from FM3A cells by the procedures described in MATERIALS AND METHODS. One of these mutants, designated as Aph 212, was able to grow in the presence of 1 μg/ml aphidicolin, which did not permit wild type cells to grow. The resistance of Aph 212 cells to aphidicolin was stable at least for 4 months' culture. As shown in Fig. 1, the rate of growth was a little lower in the presence of the drug than in the absence of it. The doubling time of Aph 212

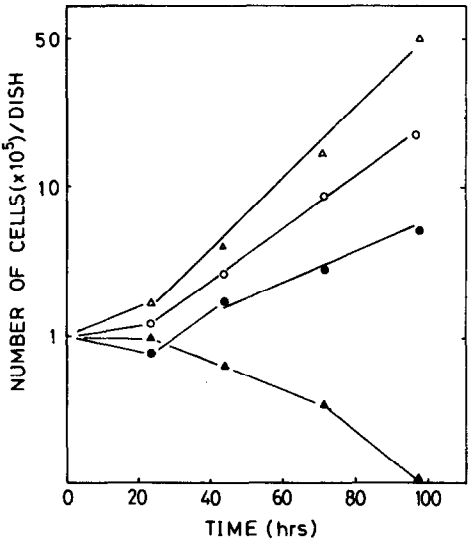

Fig. 1. *Growth curve of Aph 212 cells and wild type cells in the presence or absence of aphidicolin.* Cells were inoculated into 30 mm Petri dishes with 2 ml of RPMI 1640 containing 10% calf serum and cultured in 5% CO_2-95% air at 33°C. At appropriate times, the number of cells was counted. The dead cells detected by dye-exclusion test using erythrosin were deducted. The doubling times of Aph 212 cell in the presence (●), in the absence (○) of the drug and wild type cells were 30, 24 and 18 h, respectively. Wild type cells in the presence (▲), absence (△) of the drug (1 μg/ml).

cells at 33°C is 24 h in the absence of the drug and 30 h in the presence of it. The doubling time of untreated wild type cells was 18 h at 33°C. When aphidicolin at a concentration of 1 μg/ml was added to the culture of wild type cells, DNA synthesis rapidly decreased, while RNA and protein synthesis did not (Table 1). Though Aph 212 cells were able to grow in the presence of 1 μg/ml aphidicolin, DNA synthesis rapidly decreased by the drug to the same level as wild type cells did (Table 1). Since Aph 212 cells were able to grow in the presence of aphidicolin , DNA synthesis must be recovered by further incubation with the drug. The long lag time in the growth curve of Aph 212 cells in the presence of the drug suggests that some molecular events leading the cells to recover from the inhibition of DNA synthesis occurred in the cell (Fig. 1). The recovery of the activity of DNA synthesis was not attributed to the decomposistion of the drug by the cultivation of Aph 212 cells, because the medium of 4 day-culture in the presence of aphidicolin was able to inhibit DNA synthesis of Aph 212 cells to the same extent as the original concentration (Table 1).

Table 1. Inhibition of DNA synthesis of Aph 212 cells and wild type cells by aphidicolin.

Cells	Aphidicolin (1 μg/ml)	The synthesis of DNA	RNA	protein
Wild type	-	15776(100%)	11820(100%)	9272(100%)
	+	2450(16%)	15246(129%)	10664(115%)
Aph 212	-	7366(100%)	8236(100%)	2882(100%)
	+	447(6%)	7089(86%)	2941(102%)
	+*	213(3%)	——	——

Each value represents the radioactivity (counts/min) incorporated into macromolecules as described in Materials AND METHODS (mean of triplicate experiments).
+* represents that Aph 212 cells were treated with the medium from the culture which was incubated for 4 days with 1 μg/ml aphidicolin.

Sensitivity of DNA polymerase α of Aph 212 cells to aphidicolin: The sensitivity of DNA polymerase α of Aph 212 cells to aphidicolin was examined. As shown in Fig. 2, the sensitivity was almost the same as that of wild type cells. DNA polymerase α of Aph 212 cells preincubated with aphidicolin for 4 days also showed the same sensitivity. This result agreed with the data that the rapid

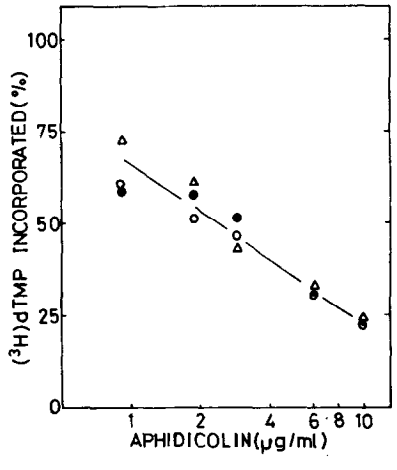

Fig. 2. *Sensitivity of DNA polymerase α of Aph 212 cells to aphidicolin.* Wild type cells (△), Aph 212 cells cultured for 4 days without (○) and with (●) aphidicolin (1 μg/ml) were prepared for the assay for DNA polymerase α as described in MATERIALS AND METHODS. Aphidicolin was added to the assay mixture with final concentrations indicated in abscissa. Values were expressed by percent of the value without aphidicolin.

Table 2. Increase in the activity of DNA polymerase α of Aph 212 cells cultured with aphidicolin.

Cells	The activities of DNA polymerases		
	α	β	γ
Wild type	7.38 ± 0.99 (103%)	0.57 ± 0.11 (100%)	0.34 ± 0.01 (72%)
Aph 212 without Aph	7.16 ± 0.39 (100%)	0.56 ± 0.03 (100%)	0.47 ± 0.03 (100%)
Aph 212 with Aph	16.74 ± 0.38 (234%)	0.79 ± 0.01 (141%)	0.68 ± 0.02 (144%)

The activities of DNA polymerases were expressed by the [^{3}H]dTMP incorpolated (pmol/min/mg protein) at 33°C (mean ± s.e.).
Aph 212 cells were incubated for 4 days with or without 1 μg/ml aphidicolin and wild type cells were also incubated for 4 days without aphidicolin.

decrease in DNA synthesis occurred by the addition of this drug. It was concluded that the recovery from the inhibition of DNA synthesis and cell growth was not attributed to the resistance of DNA polymerase α itself.

Induction of the activity of DNA polymerase α in the presence of aphidicolin: Since aphidicolin was a specific inhibitor of DNA polymerase α *in vitro* (3,4,7, 8) and the sensitivity of Aph 212 cells to the drug did not decrease (Fig. 2), the most possible explanation would be given in that the increase in the activity of DNA polymerase α occurred in the culture of Aph 212 cells in the presence of aphidicolin. In order to ascertain this possibility, the activities of DNA polymerases were assayed in 4 day-culture of Aph 212 cells in the presence or absence of aphidicolin (Table 2). As expected, the specific activity of DNA polymerase α increased 2.3 folds by 4 day-culture in the presence of aphidicolin, whereas the sensitivity of DNA polymerase α of Aph 212 cells did not change by 4 day-culture in the presence of this drug (Fig. 2). The activities of DNA polymerase β and γ of 4 day-culture in the presence of the drug were not so higher than those in the absence of the drug.

DISCUSSION

The activity of DNA polymerase α of aphidicolin resistant mutant, Aph 212, increased by 2.3 folds when cultured for 4 days in the presence of aphidicolin at a concentration of 1 μg/ml. The dose inhibited DNA syntheses of wild type

cells and Aph 212 cells, but could not inhibit growth of Aph 212 cells cultivated in the presence of aphidicolin. The result suggested that aphidicolin should react to DNA polymerase α not only *in vitro* but also *in vivo* to inhibit DNA synthesis and that DNA polymerase α should play an important role in DNA replication.

Up to date, it has been reported that there were several drug resistant mutants which had the increased activities of the target enzymes (12-14). For example, aminopterin resistant mutants reported by Littlefield showed the increase in the activities of folate reductases (12). The mutants, however, had the increased activities in the culture without aminopterin. In this point, the property of aminopterin resistant mutant was different from that of Aph 212 cells which had not the increased activity of DNA polymerase α when they were cultivated in the absence of aphidicolin. One of the mutants (Ama 102) resistant to α-amanitin, a specific inhibitor of RNA polymerase II, looks similar to our Aph 212 cells. In Ama 102 cells, the activity of RNA polymerase II increased when the cells were cultivated in the presence of α-amanitin (14). However, the enzyme showed resistance to α-amanitin, the fact being different from our case.

The mechanism of increase in the activity of DNA polymerase α in Aph 212 cells is not clear as yet. In the case of bacterial histidine biosynthesis, for example, Goldberger presented autogenous regulation hypothesis that the enzyme may act as its own repressor (15). Considering that the amount of DNA polymerase α may be regulated by the same mechanism as in bacterial histidine biosynthesis and that aphidicolin may change the structure of DNA polymerase α, the resistance to aphidicolin of Aph 212 cells may be explained in this manner. To ascertain the hypothesis, the increase in the *de novo* enzyme synthesis instead of the increase in the activity should be confirmed.

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REFERENCES

1. Weissbach, A. (1977) Annu. Rev. Biochem., 46, 25-47.
2. Bucknall, R. A., Moores, H., Simms, R. and Hesp, B. (1973) Antimicrob. Ag. Chemother., 4, 294-298.
3. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature, 275, 458-460.
4. Ohashi, M., Taguchi, T. and Ikegami, S. (1978) Biochem. Biophys. Res. Commun., 82, 1084-1090.
5. Ikegami, S., Kawada, K., Kimura, Y. and Suzuki, A. (1979) Agric. Biol. Chem., 43, 161-166.
6. Hanaoka, F., Kato, H., Ikegami, S., Ohashi, M. and Yamada, M. (1979) Biochem. Biophys. Res. Commun., 87, 575-580.
7. Pedrall-Noi, G. and Spadari, S. (1979) Biochem. Biophys. Res. Commun., 88, 1194-1202.
8. Wist, E. and Prydz, H. (1979) Nucleic Acid Res., 6, 1583-1590.
9. Nakano, N. (1966) *Tohoku* J. Exptl. Med., 88, 69-84.
10. Nakano, M. M., Sekiguchi, T. and Yamada, M. (1978) Somatic Cell Genet., 4, 169-178.
11. Knof, K. W., Yamada, M. and Weissbach, A. (1976) Biochemistry, 15, 4540-4548.
12. Littlefield, J. W. (1968) Proc. Natl. Acad. Sci. USA, 62, 88-95.
13. Chan, V. L., Whitmore, G. F. and Siminovitch, L. (1972) Proc. Natl. Acad. Sci. USA, 69, 3119-3123.
14. Somers, D. G., Pearson, M. L. and Ingles, C. J. (1975) Nature, 253, 372-374.
15. Goldberger, R. F. (1974) Science, 183, 810-816.