APHIDICOLIN: A SPECIFIC INHIBITOR OF DNA SYNTHESIS IN SYNCHRONOUS MASTOCYTOMA P-815 CELLS

Atsushi ICHIKAWA*, Manabu NEGISHI*, Kenkichi TOMITA* and Susumu IKEGAMI**

*Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan
**Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku Tokyo 113, Japan

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Abstract—The influence of aphidicolin on cell multiplication and DNA synthesis was examined using synchronous mouse mastocytoma P-815 cells. Aphidicolin was cytotoxic specifically to the cells of the S phase of the cell cycle. This cytotoxicity was reversed by appropriately washing the drug-treated cells, but not by the addition of deoxyribonucleosides. Aphidicolin, a potent inhibitor of DNA synthesis, selectively inhibited the activity of partially purified DNA polymerase α from the nucleus and the cytosol of mastocytoma cells, but did not affect the activity of DNA polymerase β. Furthermore, aphidicolin had no effect on the synthesis of RNA and protein, and produced no changes in cell size at least for one generation.

Aphidicolin, a tetracyclic diterpenoid antibiotic produced by the mold Cephalosporium aphidicola (1), has been reported to inhibit growth of herpes simplex virus both in rabbit eye and in tissue culture (2), DNA synthesis in cultured human embryonic lung cells (2), and mitotic cell division of sea urchin embryos (3, 4). Aphidicolin also inhibits not only DNA replication in HeLa cells but UV-induced DNA repair in hydroxyurea-arabinosyl cytosine treated cells (5). Furthermore, aphidicolin is known to suppress selectively the activity of DNA polymerase α, which is assumed to be the replication enzyme in DNA synthesis (6), but not that of DNA polymerase β or γ obtained from sea urchin blastulae (3) and rat liver cytosol (7).

Since these observations indicate that interference of aphidicolin with DNA synthesis is responsible for its cytotoxic effect, we examined the effect of the drug on the cell division cycle using synchronous mammalian cells, mouse mastocytoma P-815 cells, in culture, and our results are reported herein.

MATERIALS AND METHODS

Cell growth and viability test: Mouse mastocytoma P-815 cells (8) were provided by Dr. M. Potter, National Cancer Institute, NIH, Bethesda, Maryland, U.S.A., and maintained in suspension culture in Fischer-Sartorelli’s medium supplemented with 5% fetal calf serum at 37.5°C in a 5% CO₂-containing humidified atmosphere. Stock cultures were diluted every 1 or 2 days with the fresh medium to maintain the exponential growth at the

* To whom correspondence should be addressed.
cell density of 0.8 to $4.5 \times 10^5$ cells/ml. Under these conditions, the population doubling time was approximately 9 to 10 hr. Total cell number and cell size were determined with a model Z Coulter Counter and Coulter Channalyzer (Coulter Electronics, Hialeah, Florida, U.S.A.). Cell viability was determined by the method of Kaltenbach et al. (9) with a hematocytometer after suspending cells in 0.2% nigrosin in Earl's balanced salt solution.

Preparation of synchronous cells: Suspensions (100 ml) of exponentially growing cells, which reached a cell density of 1.8 to $2.0 \times 10^5$ cells/ml, were synchronized for growth and DNA synthesis by the addition of colcemid dissolved in 0.01% dimethyl sulfoxide (DMSO) to a final concentration of 0.06 µg/ml, followed by incubation for 5 hr at 37.5°C. To initiate the synchronous DNA synthesis, colcemid was removed by washing the cells with freshly prepared conditioned medium (the supernatant fraction of identical cell suspension incubated without colcemid), and centrifuged at 110 x g for 3 min at 37°C. Synchronous cells were distributed into three flasks and resuspended in the fresh conditioned medium at the cell density of $1.5 \times 10^5$ cells/ml. Aphidicolin dissolved in 0.1% DMSO was added to one flask to a final concentration of 2 µg/ml, and the vehicle alone was added to the control flask. The medium in a third flask was used as the conditioned medium after centrifugation. After incubating at 37.5°C in a CO₂-incubator for various time, 6 ml aliquots were withdrawn from each flask, and 1.5 ml of each suspension were incubated with [³H]thymidine (0.2 µCi) for 30 min at 37.5°C to measure DNA synthesis. The residual suspension (4.5 ml) were immediately centrifuged at 110 x g for 3 min at 37°C to remove aphidicolin. Cell pellets were resuspended in 4.5 ml of the freshly prepared conditioned medium, and distributed 1.5-ml each into three siliconized glass tubes (1 x 10 cm). The first tube was used to determine DNA synthesis in the absence of aphidicolin by incubating with [³H]thymidine (0.2 µCi) for 30 min at 37.5°C, the second one to measure cell growth by reincubating for another 15 hr at 37.5°C, and the third one to estimate the cell number at the onset of reincubation of the second tube.

Measurement of mitotic index: An aliquot of cell suspension was mixed with an equal volume of a fixing mixture, ethanol: acetic acid: water (5:2:3, by volume), centrifuged, and resuspended in a small volume of 0.025% crystal violet in 1% acetic acid. The percentage of the number of cells in metaphase and anaphase (mitotic index) was determined with a hematocytometer.

Assay of synthesis of DNA, RNA and protein: Syntheses of DNA, RNA and protein were measured by incorporating [³H]thymidine, [³H]uridine and [³H]leucine, respectively, into acid-insoluble fractions during a 30-min pulse incubation at 37.5°C. After incubation, cells were homogenized in 5% trichloroacetic acid (TCA), and centrifuged at 3,000 x g for 15 min. Acid insoluble precipitates were dissolved in 0.4 ml of tissue solubilizer NCS (Amersham Co., Arlington Heights, Ill., U.S.A.), and counted for radioactivity.

Extraction of DNA polymerase: All steps were performed at 0-4°C. Cells were swollen in 5 volumes of a hypotonic buffer, 0.05 M Tris-HCl (pH 8.0) containing 4 mM MgCl₂, 1 mM EDTA and 6 mM β-mercaptoethanol for 7 min. Swollen cells were homogenized 10 to 12 strokes in a tight fitting Dounce homogenizer, and examined for cell dis-
ruption by phase microscopy. After the nuclei were sedimented by centrifugation at 800 × g for 5 min, the supernatant fraction was further centrifuged at 105,000 × g for 60 min to obtain the cytosol fraction.

DNA polymerase was extracted from nuclear pellets by suspending in 5 volumes of 1 M sucrose containing 4 mM MgCl₂, 1 mM EDTA, 6 mM β-mercaptoethanol and 0.1% Triton X-100 at 4°C for 60 min (10). A partial purification of DNA polymerases in the nuclear extract and the cytosol was performed on a column (1 x 5 cm) of DE-32 (Sigma) equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 4 mM MgCl₂, 6 mM β-mercaptoethanol and 50 mM KCl. The column was eluted with a 200 ml linear KCl gradient of 50 mM to 500 mM in the same buffer. DNA polymerase β was not retained by the column, whereas polymerase α was eluted with 200 mM KCl. The specific activities of these DNA polymerase preparations were 28 units/mg and 58 units/mg for nuclear polymerases α and β, respectively, and 103 units/mg and 18 units/mg for cytosolic polymerases α and β, respectively. A unit was defined as 1 nmol of deoxyribonucleoside triphosphate incorporated into an acid-insoluble fraction in 20 min at 37°C. Protein was measured by the method of Lowry et al. (11) using bovine serum albumin as the standard.

**DNA polymerase assay:** DNA polymerase activity was measured by a slightly modified method of Hershey et al. (12). The standard incubation mixture (0.3 ml) contained 53.3 µmol of Tris-HCl (pH 8.4), 5.3 µmol of MgCl₂, 2 µmol of β-mercaptoethanol, 125 µg of activated calf thymus DNA, 0.1 µmol each of dATP, dCTP, dGTP and [³H]dTTP (41.7 µCi/µmol) and enzyme. The incorporation of [³H]dTTP into DNA fraction was linear for at least 1 hr at 37°C. After incubation at 37°C for 15 min, the reaction was stopped by chilling in ice, and mixing with 0.05 ml of 5% bovine serum albumin and 3 ml of 0.8 M perchloric acid containing 0.01 M sodium pyrophosphate. After washing three times with 0.4 M perchloric acid containing 0.005 M sodium pyrophosphate, the precipitate was dissolved in 0.5 ml of NCS, and counted for radioactivity in 10 ml of a toluene scintillant [4 g of 2,5-diphenyl-oxazole (PPO) in 1 liter of toluene-Triton X-100 (2:1, v/v)].

**RESULTS**

**Effect of aphidicolin on growth of mastocytoma P-815 cells:** As shown in Fig. 1, the continuous presence of aphidicolin, in a concentration range from 0.08 to 2 µg/ml, progressively inhibited the multiplication of exponentially growing mastocytoma P-815 cells. The potency of aphidicolin required to inhibit the growth of this cell line was similar to that reported for the inhibition of mitotic cell division of sea urchin embryos (3).

Mastocytoma P-815 cells maintained in suspension culture were found to have the following mean duration time for various phases of cell cycle: G₁, 2.0 hr; S, 4.5 hr; G₂, 1.5 hr; and M, 1.5 hr (bottom in Fig. 2). Aphidicolin (2 µg/ml) added at 0-time (M phase) to the synchronous cells almost completely suppressed the replication in the next cell cycle, which began 9 to 10 hr later in the control cells (Fig. 2). Aphidicolin added at various stages of the cell cycle prior to mid-S phase (~7 hr) also inhibited the next replication. On the other hand, the drug added at late S and G₂ phases (7 to 9 hr) was not inhibitory, and the cells
grew as normally as control cells and entered M phase about 24 hr later.

The cytotoxic effect of aphidicolin was reversible. When the cells were exposed to the drug during various phases of cell cycle, washed and resuspended in the fresh medium, they began to multiply almost as normally as untreated cells, doubling the cell number in about 15 hr. However, when the mid-S cells were washed of the aphidicolin cytotoxicity, the result was less satisfactory as compared with the preservation of cells in other phases.

**Fig. 1.** Inhibition of growth of mastocytoma P-815 cells by aphidicolin. Suspensions of logarithmically growing mastocytoma P-815 cells (1.2×10⁵ cells/ml, 30 ml) were incubated with various amounts of aphidicolin at 37.5°C for 30 hr. The cell density in control flask (0 µg of aphidicolin) was 6.8×10⁵ cells/ml after 30 hr incubation, and this value was taken as 100%.

**Fig. 2.** Effect of aphidicolin on growth of synchronous mastocytoma P-815 cells. Suspension of mastocytoma P-815 cells (1.8-2.0×10⁵ cells/ml, 100 ml/flask) were synchronized for growth by colcemid treatment. At time 0, colcemid was removed by washing, and synchronous cells were cultured with (●, aphidicolin-cells) or without (○, control cells) aphidicolin (2 µg/ml) for up to 18 hr at 37.5°C. (Time scale and duration of various phases of cell cycle are indicated at the bottom of the figure.) At each indicated time, aliquots of cell suspensions were withdrawn from each flask, washed, and resuspended in the fresh medium. Aphidicolin-cells were then incubated without the drug (⋯Δ⋯), and control cells incubated with the drug (⋯●⋯) for another 15 hr. (Time scale reincubation experiments is shown at the top of the figure.) Cell number was determined as described in Materials and Methods.
Furthermore, the addition of any one of deoxyribonucleosides to high concentrations (about $10^{-4}$ M) or their various combinations did not preserve the cells from the cytotoxicity of aphidicolin (data not shown), indicating that the drug did not affect the biosynthesis of deoxyribonucleotides.

**Effect of aphidicolin on DNA synthesis:** Aphidicolin almost completely inhibited the incorporation of [3H]thymidine into DNA fraction during S period of cell cycle (Fig. 3). The inhibitory effect of aphidicolin on DNA synthesis was also reversible. Removal of aphidicolin by washing the drug-treated cells during all phases of cell cycle partially restored DNA synthesis. This preservation was more evident with late S and G2 cells. When the

![Fig. 3. Effect of aphidicolin on DNA synthesis in synchronous mastocytoma P-815 cells. Synchronous mastocytoma cells were incubated with (▲) or without (○) aphidicolin for up to 10 hr as in Fig. 2. At intervals, aliquots were withdrawn, and incubated with [3H]thymidine at 37°C for 30 min to measure DNA synthesis. Another aliquot of aphidicolin-treated cells was washed, and resuspended in the fresh medium prior to the incubation with [3H]thymidine (○).](image)

**TABLE 1. Effect of aphidicolin on DNA, RNA and protein synthesis in mastocytoma P-815 cells**

| Aphidicolin concentration (μg ml⁻¹) | [3H]Thymidine incorporation (%) | [3H]Uridine incorporation (%) | [3H]Leucine incorporation (%) |
|-----------------------------------|---------------------------------|-------------------------------|-----------------------------|
| 0 (control)                       | 100*                            | 100*                          | 100*                        |
| 0.08                              | 49                              | 87                            | 118                         |
| 0.4                               | 12                              | 89                            | 109                         |
| 2.0                               | 3.9                             | 89                            | 98                          |
| 10.0                              | 1.4                             | 120                           | 110                         |

Mastocytoma P-815 cells (3.64 x 10⁶ cells) were incubated for 1 hr at 37.5°C in 1 ml of PBS containing [3H]thymidine (0.2 μCi, 40 Ci mmol⁻¹), [3H]uridine (0.2 μCi, 20 Ci mmol⁻¹) or [3H]leucine (0.5 μCi, 58 Ci mmol⁻¹) with or without various amounts of aphidicolin. The radioactivity in the acid-insoluble fraction was counted as described in the text. Aphidicolin dissolved in DMSO was added to the incubation mixture to give a final concentration of DMSO as 0.1% (v/v), which did not affect DNA, RNA and protein synthesis in mastocytoma cells.

*Radioactivities incorporated in control (aphidicolin = 0 μg) experiments were: 22,886 cpm with [3H]thymidine, 3,306 cpm with [3H]uridine and 104,289 cpm with [3H]leucine, respectively.*
preserved cells were continuously incubated for another 15 hr at 37.5°C, the DNA synthesis was restored to a near control level.

Table 1 also shows that aphidicolin specifically inhibited the incorporation of [3H] thymidine into acid-insoluble DNA fraction of mastocytoma P-815 cells, but did not affect precursor incorporations into RNA and protein fractions.

**Effect of aphidicolin on cell size:** As shown in Fig. 4, the cell size was smallest during G1 phase, and gradually increased during S and G2 phases. G2 cells were of maximum size of 15.8 μ and diameter, representing the average sized mastocytoma P-815 cells. Exposure of the synchronous cells to aphidicolin produced no evident changes in cell size during the first generation. However, the treated cells began to swell at about 10 hr of exposure, and became markedly distorted about 20 hr later, indicating the cytotoxicity of aphidicolin.

**Effect of aphidicolin on DNA polymerase:** Table 2 shows a selective inhibition by aphidicolin of DNA polymerase α, but not β. Apparently DNA polymerase α from cytosol

![Fig. 4. Effect of aphidicolin on cell size of synchronous mastocytoma P-815 cells.](image)

**TABLE 2. Effect of aphidicolin on activities of DNA polymerases from nuclear and cytosolic fractions of mastocytoma P-815 cells**

| Aphidicolin concentration (μg ml⁻¹) | DNA polymerase activity (%) | Nuclear | Cytosolic |
|------------------------------------|-----------------------------|---------|-----------|
|                                    | α              | β      | α        | β        |
| 0 (control)                        | 100*           | 100*   | 100*     | 100*     |
| 0.5                                | 40             | 95     | 28       | 97       |
| 2                                  | 10             | 102    | 5        | 93       |
| 10                                 | 3              | 99     | 2        | 99       |

DNA polymerases α and β were partially purified from the nucleus and cytosol of mastocytoma P-815 cells and assayed as described in the text.

*100% activity: 2,860 cpm of the radioactivity incorporated from [3H]dTTP with nuclear DNA polymerase α (35.8 μg protein per assay); 2,508 cpm with nuclear DNA polymerase β (29.6 μg protein per assay); 2,010 cpm with cytosolic DNA polymerase α (10.5 μg protein per assay); and 2,008 cpm with cytosolic DNA polymerase β (82.5 μg protein per assay) in the control experiments.
was more susceptible to the inhibitory effect of aphidicolin than the nuclear enzyme.

DISCUSSION

The results of our studies on synchronous mouse mastocytoma P-815 cells have shown that aphidicolin is specifically cytotoxic to the S phase cells, as expected from its potent inhibition of DNA synthesis in this cell line and in cultured human embryonic lung cells (2). The cytotoxicity of aphidicolin was reversed by washing the treated cells of the drug (Fig. 2), but not prevented by the addition of deoxyribonucleosides. In this respect, aphidicolin differs from arabinonucleosides, since the inhibition of DNA synthesis by low concentrations of arabinonucleosides is usually reversed by washing, and prevented also by deoxyribonucleosides (13–15). Apparently, aphidicolin does not act by preventing the biosynthesis of deoxyribonucleotides. A similar conclusion was also drawn by Ikegami et al. (3), because the mitotic cell division of sea urchin embryos, which was inhibited by aphidicolin, was not affected by inhibitors of deoxyribonucleotide biosynthesis such as hydroxyurea and 5-fluorouracil.

The present data on partially purified DNA polymerases from mastocytoma P-815 cells (Table 1), and the results reported by other investigators on DNA polymerases from sea urchin embryos (3), HeLa cells (5) and regenerating rat liver (7), indicate that aphidicolin is a selective inhibitor of DNA polymerase α. Of the three distinct DNA polymerases, DNA polymerase α is thought to be utilized for DNA replication in eukaryotic cells, because this enzyme activity is predominant in growing cells and is elevated during DNA replication phase of the cell cycle (6, 16). On the other hand, DNA polymerase β, a minor fraction of the total DNA polymerase activities in growing cells, is assumed to play a role in DNA repair synthesis. However, the role of these polymerases in DNA synthesis seems to be complicated. Berger et al. (17) suggested that DNA polymerase α might be involved both in DNA replication and repair, since aphidicolin, a selective inhibitor of DNA polymerase α, inhibited both processes in normal human lymphocytes. Hanaoka et al. (5) also made similar observations on HeLa cells. Reversibility of the cytotoxicity of aphidicolin and its inhibition of DNA synthesis may make it unsuitable for clinical application. However, aphidicolin, a selective and reversible inhibitor of DNA polymerase α, should certainly prove to be a useful tool for studies on the mechanism of DNA replication.

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