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Effects of Aphidicolin on *Entamoeba histolytica* Growth and DNA Synthesis

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We have detected and characterized DNA polymerase activity in cell extracts from trophozoites of *Entamoeba histolytica* and have found that the activity of *E. histolytica* is inhibited by aphidicolin, which is a specific inhibitor of eukaryotic nuclear replicative DNA polymerases. The present study was aimed to evaluate the effect of aphidicolin on growth and DNA synthesis by this parasite. Aphidicolin blocked the growth of axenic *E. histolytica* strain HM-1: IMSS. DNA synthesis was also inhibited by aphidicolin when assayed by incorporation of [^{3}H] thymidine into the DNA. The inhibitory effect of aphidicolin on the growth of *E. histolytica* was abrogated by removal of the drug, and exposure to 3μg/ml of the drug for at least 48 hr had little effect on the viability. Synchronous growth was observed in the recovery phase after removal of aphidicolin.

Keywords : *Entamoeba histolytica*, Protozoa, Aphidicolin, Growth, DNA synthesis, DNA polymerase

INTRODUCTION

Entamoeba histolytica is responsible for human amebiasis that can include amebic dysentery and liver abscess. After infection, trophozoites proliferate in conjunction with DNA synthesis in the intestine or liver. This proliferative stage is the primary cause of the disease. Therefore, knowledge of the mechanism and its regulation of DNA synthesis during cell proliferation is necessary to understand the primary cause of this disease. Among the enzymes involved in DNA synthesis, DNA polymerases play a crucial role. We have detected and characterized DNA polymerase activity of *E. histolytica* trophozoites and have found that this enzyme is inhibited by aphidicolin [9].

Aphidicolin is a mycotoxin produced by fungi such as *Cephalosporium aphidicola* and *Nigrospora oryzae*. This tetracyclic diterpenoid is known as a specific inhibitor of nuclear replicative DNA polymerases in eukaryotic cells [14, 15]. It has been reported that aphidicolin blockes eukaryotic cells in S phase by inhibiting the replicative DNA polymerase and allows G_2, M, and G_1 cells to accumulate specifically at the G_1/S border; it does not reduce cell viability and its action is reversible [12]. Therefore, we considered it of interest to determine whether action of aphidicolin reported in other eukaryotic cells is also observed in *E. histolytica*. This report describes the effect of aphidicolin on the growth, DNA synthesis, and synchronization of *E. histolytica*.

MATERIALS AND METHODS

Trophozoites of *E. histolytica* strain HM-1: IMSS were axenically cultured in BI-S-33 medium [6]. Amoeba were harvested by centrifugation at 400 g for 5 min and washed twice with phosphate-buffered saline (PBS) and counted in a hemocytometer. For preparation of cell extract, the cell pellet was suspended in the extraction buffer, sonicated, and centrifuged in a manner similar to that previously described for *Toxoplasma gondii* [10]. After centrifugation, the supernatant fluid was collected and used for assay. The activity of DNA polymerase was measured as previously described [10]. [*methyl*-^{3}H] dTTP

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(specific activity, 50-70 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). Aphidicolin, *N*-ethylmaleimide (NEM), and 2', 3'-dideoxythymidine-5'-triphosphate (ddTTP), were used and all these chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

For experiments on the effect of aphidicolin on cell growth, duplicate cultures inoculated with $8\text{-}9 \times 10^3$ trophozoites/ml included various concentrations (0.3-3 μg/ml) of aphidicolin and were incubated for 72 hr. It was initally dissolved into dimethylsulfoxide (DMSO), then added to the culture at desired concentrations. Control cultures contained the same volume of DMSO. The cells were counted in a hemocytometer and the viability was determined by trypan blue dye exclusion.

For assay of DNA synthesis, duplicate cultures with 5×10^4 trophozoites/ml were treated with various concentrations (0.1-20 μg/ml) of aphidicolin and labeled with 5 μCi/ml [^{3}H] thymidine for 5 hr. The radioactivity incorporated into the DNA was determined after precipitation onto nitrocellulose filters. The filters then were washed and dried and the radioactivity bound to the filters was counted in a liquid scintillation counter; [*methyl*-^{3}H] thymidine (specific activity, 35 Ci/mmol) was also purchased from ICN Biomedicals.

For experiments on the reversible effects of aphidicolin, duplicate cultures with $8\text{-}9 \times 10^3$ trophozoites/ml were incubated for 1 day; then aphidicolin was added to the cultures at a concentration of 3 μg/ml and the cultures were further incubated for 1 day. For replacement of the medium, the cells were centrifuged at 400 g for 5 min; following chilling in ice, the spent medium was removed. The remaining cells were washed once with PBS, once with fresh medium, and then resuspended in fresh medium. No replacement of the medium was done in the control cultures. Both cultures were further incubated for 2 days. Cells were counted each 24 hr.

For synchronization experiments, trophozoites treated with 3 μg/ml of aphidicolin for 24 hr were once with PBS, once with drug-free medium, and resuspemded in fresh medium that was supplemented with 5 μCi [^{3}H] thymidine/ml. Trophozoites cultured in the absence of drug were used as the control. Both duplicate cultures were incubated at a density of 4×10^4 cells/ml. At 4-hr intervals, cells were counted and the amount of radioactivity incorporated into DNA was determined in 1×10^5 trophozoites as described above for DNA synthesis.

RESULTS

Effects of inhibitors on the activity of *E. histolytica* DNA polymerase

The effects of inhibitors of mammalian DNA polymerases on the activity of *E. histolytica* were investigated. As shown in Figure 1, the activity was inhibited by 66% on average by a 10-μg/ml concentration of aphidi-

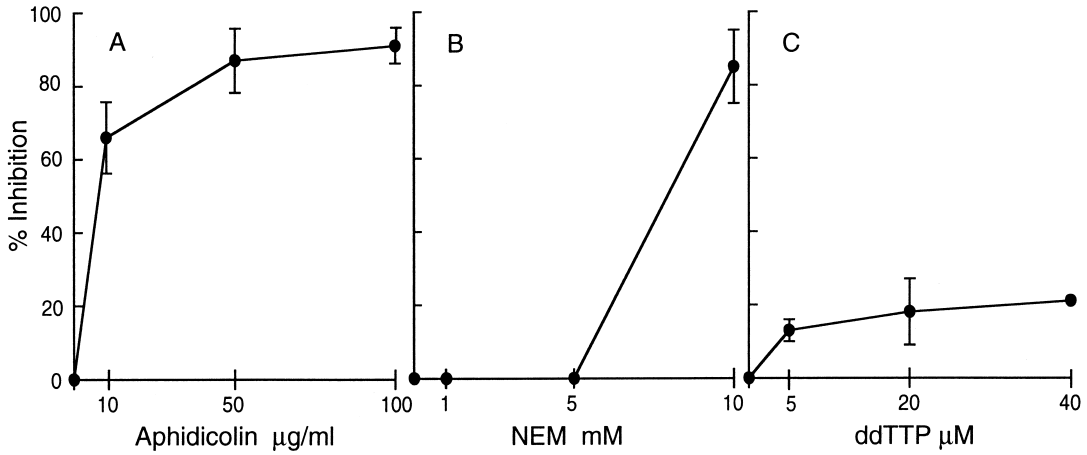

Fig. 1 Effects of inhibitors on DNA polymerase activity of *Entamoeba histolytica*. A, effect of aphidicolin. B, effect of NEM. C, effect of ddTTP. The data represent means ± S. D. of three experiments performed in duplicate.

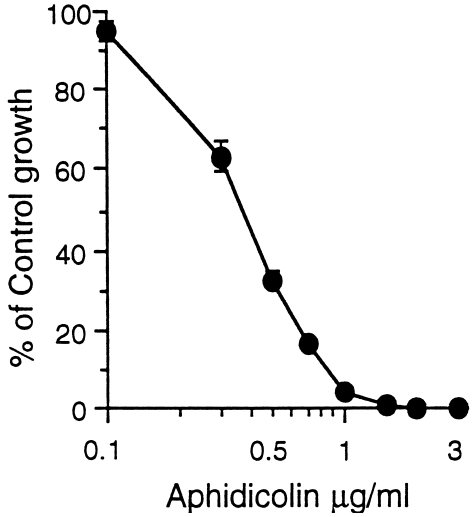

Fig. 2 Effect of aphidicolin on the growth of *Entamoeba histolytica*. Trophozoites were cultured 3 days in the presence of various concentrations of aphidicolin. The mean and range of duplicate cultures are plotted at each concentration. The control growth was measured in the absence of drug.

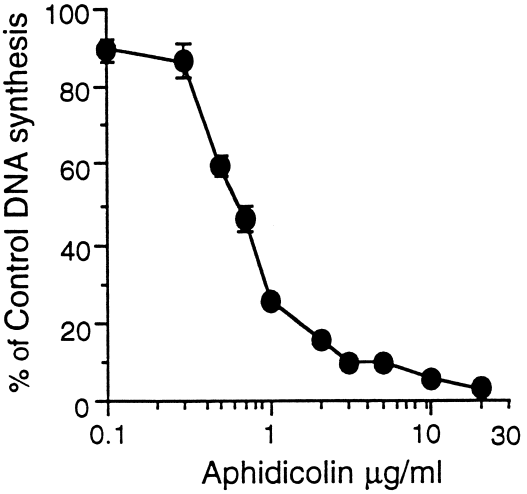

Fig. 3 Effect of aphidicolin on DNA synthesis of *E. histolytica*. Trophozoites were labeled with [^{3}H] thymidine for 5 hr in the presence of various concentrations of aphidicolin. Values are plotted as in Fig. 2. The control DNA synthesis was measured in the absence of drug.

colin and was inhibited by 91% by a concentration of 100 μg/ml. The activity was insensitive to 1 and 5 mM NEM but was inhibited markedly by 10 mM NEM. In contrast to aphidicolin and NEM, only 13-21% inhibition was induced by 5-40 μM ddTTP.

Effect of aphidicolin on the growth

The effect of various concentrations of aphidicolin on the growth of *E. histolytica* was examined. As shown in Figure 2, 0.1 μg/ml of aphidicolin had little effect on the growth, whereas 0.3, 0.5, and 1.0 μg/ml of the drug inhibited 37, 68, and 96% of control growth, respectively. More than 1.5 μg/ml of the drug inhibited the grwoth completely. Identical results were obtained from 2 other experiments.

Effect of aphidicolin on DNA synthesis

The effect of aphidicolin on DNA synthesis was examined. The radioactivity incorporated into the DNA was determined in duplicate samples for each drug concentration. Figure 3 shows that inhibition by 0.1 and 0.3 μg/ml of aphidicolin was less than 20% of the control and that with 1 μg/ml of the drug, inhibition was 74%; 20 μg/ml of the drug inhibited 97% of control DNA synthesis. The same results were obtained in 2 additional experiments.

Reversible effect of aphidicolin on growth

To determine whether the inhibitory effect of aphidicolin on growth is reversible, the spent medium of cultures treated with 3 μg/ml of aphidicolin for 24 hr was replaced with drug-free, fresh medium, and thereafter, the growth was monitored by counting the cells. The results are shown in Figure 4. Exposure of trophozoites to aphidicolin for 24 hr had little effect on their viability. After removal of the drug, an increase in the number of trophozoites was observed. The rate of growth from day 3 to day 4 was greater than that from day 2 to day 3. In contrast, the number of cells in the control was unchanged at day 3, and at day 4 few viable cells remained.

Induction of synchronous growth by aphidicolin

In order to determine whether aphidicolin induces synchronous growth of *E. histolytica*, the media of replicate cultures treated with aphidicolin for 24 hr were replaced with drug-free media containing [^{3}H] thymidine, and the subsequent growth pattern and

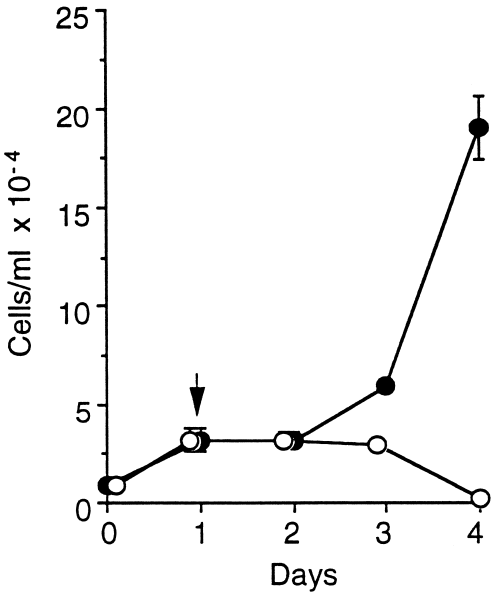

Fig. 4 Reversible effect of aphidicolin on the growth of *E. histolytica*. After exposure to aphidicolin for 24 hr, the drug was removed by replacement of the spent medium with drug-free medium. The arrow indicates the time of addition of aphidicolin. Values are plotted as in Fig. 2. Aphidicolin-removed cultures (solid circles); control cultures (open circles).

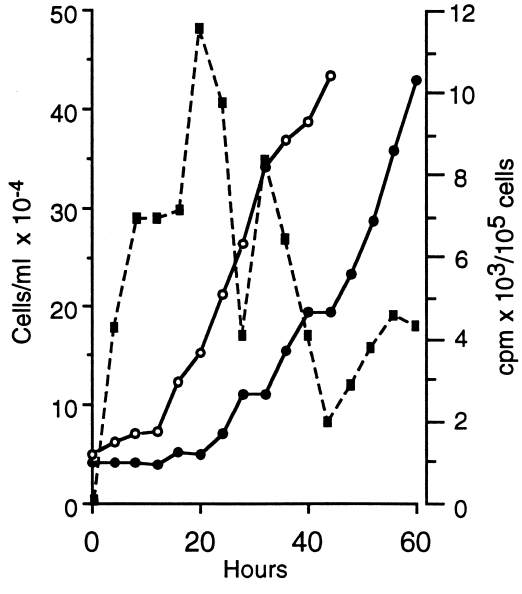

Fig. 5 Induction of synchronous growth of *E. histolytica* by aphidicolin. After removal of aphidicolin, growth was monitored at 4-hr intervals. Cell density of aphidicolin-treated cultures (solid circles); control cultures (open circles).

incorporation of radioactivity into the DNA were examined at 4-hr intervals. The results are shown in Figure 5. After removal of the drug, there was a lag of about 20 hr followed by a synchronous growth lasting 8 hr, during which cell density increased from 5.1 to 11.0×10^4/ml. This was followed by a period of no growth lasting 4 hr, and by a second cycle of synchronous growth that lasted 8 hr, during which cell density increased from 11 to 19.4×10^4/ml; there was then a second period of no growth that lasted 4 hr. Thereafter, the period of no growth was not observed. Similar results were obtained in 2 additional experiments. In contrast, there was continuous growth in the control cultures.

An increase in the incorporation of radioactivity into DNA was observed in the lag time of growth after removal of the drug; it reached a peak at 20 hr, which was the end of the lag time, and then was followed by a sharp decrease in the incorporation during which the first synchronous growth occurred. This was followed by a second increase in incorporation that corresponded to the first period of no growth, and then by a second decrease during which the second synchronous growth occurred. Thereafter the incorporaion increased again.

DISCUSSION

The results indicate that the activity of *E. histolytica* DNA polymerase was markedly inhibited by aphidicolin and NEM but not by ddTTP. Thus the *E. histolytica* enzyme is similar to those polymerases of the α family and is distinguished from polymerases β and γ. As compared with DNA polymerases of other parasitic protozoa, the *E. histolytica* enzyme is similar to aphidicolin-sensitive DNA polymerases of *P. berghei* [5] and *P. falciparum* [1, 3, 4]. In contrast to *Plasmodium* and to *E. histolytica*, there has no report on aphidicolin-sensitive DNA polymerase in other parasitic protozoa, including *Trypanosoma cruzi*, *Crithidia fasciculata*, *Leishmania mexicana*, and *T. gondii* [8].

The results indicate that aphidicolin inhibited both growth and DNA synthesis by *E.*

histolytica. Similar results have been reported for other parasitic protozoa, e.g., *P. falciparum* [4, 7] and *T. gondii* [13]. There is, however, a difference in the effective dose of the drug among these parasites. It is suggested that growth of *E. histolytica* was more resistant to aphidicolin than that of *P. falciparum* and *T. gondii* because *E. histolytica* growth was inhibited only 5% at a concentration of 0.1 μg/ml aphidicolin, whereas growth of *P. falciparum* and *T. gondii* was inhibited 93.6% and 50%, respectively. On the other hand, it is suggested that DNA synthesis of *T. gondii* was more resistant to aphidicolin than that of *P. falciparum* and *E. histolytica*. It has been reported for *P. falciparum* and *T. gondii* that higher concentrations of the drug were required to inhibit DNA synthesis as compared with growth. Similar results were obtained for *E. histolytica* in the present study. The reason for this remains unclear, but it has been suggested for *T. gondii* that the inhibition of [^{3}H] uracil incorporation into parasite DNA was measured in an acute experiment that considered only the hours immediately after treatment, whereas inhibition of growth was measured over a 3-day period.

Studies on the mechanism of action of aphidicolin have suggested that only eukaryotic replicative DNA polymerases possess a binding site for aphidicolin and that the drug most probably binds to polymerases at a site near to, or overlapping, the binding site for dCTP. Although our study indicated the presence of aphidicolin-sensitive DNA polymerase in the trophozoites of *E. histolytica*, identification and biochemical characterization of those replicative α, δ, and ε DNA polymerases in *E. histolytica* needs further study.

The results indicate that a reversible effect on growth was observed for *E. histolytica*. It is also clear from the results that exposure of 3 μg/ml drug to *E. histolytica* for at least 48 hr had little effect on its viability. The reason for the reversiblity is considered unlike that of most other inhibitors of DNA synthesis, e. g., aphidicolin does not bind directly to DNA. Moreover, it does not interfere with the synthesis of nucleic acid precursors and it does not have any detectable effect upon RNA or protein synthesis. Because aphidicolin has no effect on G_2, M, and G_1 cells, they continue their growth cycle until the end of G_1 but are unable to enter the S phase. Thus, treatment of cell populations with aphidicolin has been employed to synchronize mammalian cells [12].

The results of the present study demonstrated the occurrence of 2 periods of no growth that were not observed in the controls after removal of the drug. This was also confirmed by the incorporation of radioactivity into the DNA. Hydroxyurea and nucleotide starvation have been successfully used to synchronize *E. histolytica* growth, but the viability of trophozoites was low [2]. Orozco et al. [11] described a clone L-6 from strain HM-1: IMSS, which was made deficient in phagocytosis, that could be synchronized by colchicine, but that the wild-type strain was less sensitive to colchicine. In contrast to these inhibitors, it is suggested that aphidicolin does not affect the viability of trophozoites and also is effective on the wild-type strain HM-1: IMSS. However, a longer lag period following removal of the drug was observed before the primary burst as compared with hydroxyurea and colchicine. The reason for this observation remains unclear.

In summary, it is suggested from the present study that aphidicolin inhibits growth and DNA synthesis by *E. histolytica* through inhibition of drug-sensitive replicative polymerase in a manner similar to that seen in other eukaryotic cells, that its action is reversible, and that it is effective in inducing synchronous growth of *E. histolytica*.

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