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Inhibition of Encystation of *Entamoeba invadens* by Aphidicolin

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Effects of aphidicolin, a specific inhibitor of eukaryotic nuclear replicative DNA polymerases, on the growth and encystation of a reptilian parasitic protozoan *Entamoeba invadens* were evaluated. Aphidicolin blocked the growth of axenic *E. invadens* strain IP-1 in a dose-dependent manner. Encystation induced by the glucose depletion and osmotic pressure was also inhibited by aphidicolin. The inhibition was almost complete when trophozoites were treated with the drug before being transferred to encystation medium containing the drug. The inhibitory effect of aphidicolin on *E. invadens* growth was abrogated by removal of the drug, and exposure to 3 μg/ml of the drug for at least 7 days had little effect on the viability. Encystation was also restored by removal of the drug. These results suggest that the trophozoites accumulated at G1/S border by treatment with aphidicolin cannot encyst upon induction of encystation so that they need DNA synthesis before encystation.

Keywords : *Entamoeba invadens*, Aphidicolin, Encystation, DNA polymerase

INTRODUCTION

Entamoeba histolytica, a causative protozoan parasite of human amebiasis, has two forms in the life cycle. Trophozoites are the proliferative form which is responsible for the primary cause of disease. Cysts, the infective, transmission form, are produced in the intestine, eliminated in the feces, and subsequently ingested in contaminated food or water. Despite its obvious importance for control of amebiasis, knowledge and understanding of cysts and encystation are still limited. The factors and mechanisms that trigger encystation of the parasite, in vivo, remain obscure, and also no axenic culture medium is yet available for the successful production of *E. histolytica* cysts [5]. In this respect *E. invadens*, a parasitic protozoan of reptiles, is a useful model for *E. histolytica*, because it resembles the human pathogen in morphology and life cycle [8] and encysts in vitro [5]. The links between cell division and encystation of *E. invadens* have been suggested [1, 9, 13, 16]. There was, however, no direct evidence for it. Attempts to block *E. invadens* cell division specifically with mitotic inhibitors were unsuccessful: colchicine at concentrations as high as 0.1 M had no detectable effect on growth or encystation [13].

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 [15, 17]. It has been reported that aphidicolin blocks eukaryotic cells in S phase by inhibiting the replicative DNA polymerase and allows G2, M, and G1 cells to accumulate specifically at the G1/S border; it does not reduce cell viability and its action is reversible [10]. We have recently demonstrated that aphidicolin inhibits *E. histolytica* growth and DNA synthesis and that synchronous growth of the parasite is observed in the recovery phase after removal of aphidicolin [7]. Thus aphidicolin is considered as a useful tool for elucidating the relationship between DNA synthesis and

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encystation. We considered it of interest to determine whether encystation as well as growth of *E. invadens* is affected by aphidicolin. This report describes inhibitory effects of aphidicolin on the growth and encystation of *E. invadens* and also the reversibility of its action.

MATERIALS AND METHODS

Trophozoites of *E. invadens* strain IP-1 were axenically cultured in BI-S-33 medium [3] at 26℃. For experiments on the effect of aphidicolin on cell growth, duplicate cultures inoculated with 10^4 trophozoites/ml included various concentrations (0.1 to 3 μg/ml) of aphidicolin and were incubated for 7 days. Aphidicolin was purchased from Sigma Chemical Co. (St. Louis, MO). It was initially 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 exclusion.

For experiments on the effect of aphidicolin on encystation, log-phase trophozoites (5 to 7×10^5 cells/ml) were transferred to encystation medium called 47% LG (LG is BI without glucose) [12]. Duplicate cultures included various concentrations (0.1 to 3 μg/ml) of aphidicolin as described above and were incubated for 2-3 days. The cysts and trophozoites were counted to determine the percentage of cysts and the viability was determined by trypan blue exclusion. The percentage of encystation without the drug ranged between 65% and 93%. In some experiments, trophozoites were treated with 3 μg/ml of aphidicolin for 2 days before being transferred to encystation medium with the drug at the same concentration.

For experiments on the reversible effect of aphidicolin on cell growth, duplicate cultures with 10^4 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 on ice, and the spent medium was removed. The remaining cells were washed twice with BI-S-33 medium and then resuspended in fresh medium. No replacement of the medium was done in the control cultures. Both cultures were further incubated for 5 days. Cells were counted 1, 3, and 5 days after the reincubation.

For experiments on the reversible effects of aphidicolin on encystation, duplicate cultures with 47% LG containing 5 to 7×10^5 trophozoites/ml and 3 μg/ml of aphidicolin were incubated for 2-3 days. The medium was replaced as described above except that the cells were washed with 47% LG to remove the drug and then resuspended in drug-free 47% LG. Aphidicolin was not included in the control cultures. The cysts and trophozoites were counted to determine percentage of cysts 2-3 days later.

RESULTS

The effect of various concentrations of aphidicolin on the growth of *E. invadens* was examined. As shown in Figure 1, 0.1 to 1.0 μg/ml of aphidicolin had little or slight inhibitory effect on the growth, whereas 1.5, 2, and 3 μg/ml of the drug inhibited 64, 81, and 91% of control growth, respectively. Identical results were obtained from 2 other experiments.

The results of effect of aphidicolin on encystation are shown in Figure 2. Control cultures without aphidicolin produced 93% of cysts 2 days after induction of encystation. Percentage of cysts decreased slightly by 0.1 to 0.5 μg/ml of aphidicolin, whereas it decreased to 50, 31, and 23% by 1, 2, and 3 μg/ml of the drug, respectively. Similar results were obtained in 2 additional experiments.

It has been reported that exposure of cells to aphidicolin prevents G1 cells from entering the DNA synthetic period, blocks cells in S phase, allows G2 and M cells to continue the cell cycle and to accumulate at the G1/S border. To determine the relation of cell cycle to encystation, effect of pretreatment with the drug on encystation was examined. As shown in Figure 3, encystation was almost completely inhibited when trophozoites were treated with 3 μg/ml of aphidicolin for 2 days before being transferred to encystation medium containing the drug as compared with that of cells without the pretreatment.

To determine whether the inhibitory effect of aphidicolin on the 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 at least 7 days 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 5 to day 7 was greater than those from day 2 to day 3 and from day 3 to day 5.

In order to determine whether the inhibitory effect of aphidicolin on the encystation is also reversible, the spent medium with aphidicolin was replaced with drug-free encystation medium and percentage of cysts was compared. The results are shown in Figure 5. After removal of the drug, an increase in percentage of cysts was observed and it reached 70% which was comparable to that of control.

DISCUSSION

The results indicate that aphidicolin inhibits *E. invadens* growth. We have recently found that DNA polymerase activity in cell extract from *E. invadens* trophozoites is inhibited by aphidicolin [Makioka et al. submitted]. Therefore, growth inhibition of *E. invadens* by aphidicolin is considered as due to inhibition of replicative DNA polymerases by the drug as has been demonstrated for *E. histolytica* trophozoites [6, 7]. Growth inhibition by aphidicolin has also been reported in other parasitic protozoa, e.g., *Plasmodium falciparum* [2, 4], *Trypanosoma cruzi* [14] and *Toxoplasma gondii* [11], but there is a difference in the effective dose of the drug among these parasites [7]. It is suggested that growth of *E. histolytica* is more resistant to aphidicolin than those of *P. falciparum* and *T. gondii*. Moreover, the present study suggests that the growth of *E. invadens* is more resistant to aphidicolin than that of *E. histolytica* because *E. invadens* growth was inhibited only 16% at a concentration of 1.0 μg/ml aphidicolin, whereas *E. histolytica* growth was inhibited 96% [7]. The reason for this difference remains unresolved.

The results indicate that encystation is also inhibited by aphidicolin. Several previous reports have suggested links between cell division and encystation. Balamuth (1962) noted that encystation usually followed a period of rapid vegetative growth. Myer and Morgan (1971) reported a correlation

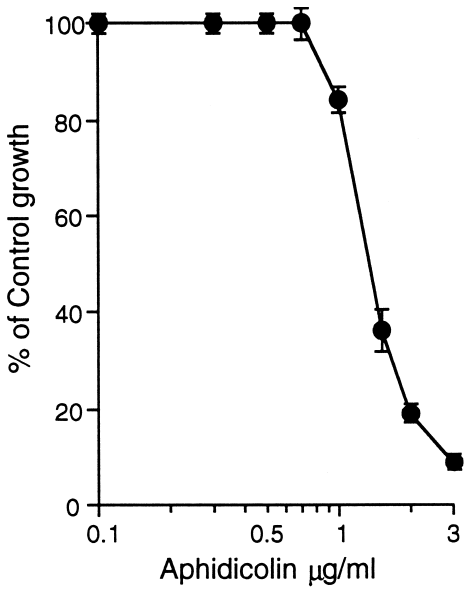

Fig. 1 Effect of aphidicolin on the growth of *Entamoeba invadens*. Trophozoites were cultured for 7 days in the presence of various concentrations of aphidicolin. The mean and range of duplicate cultures are plotted at each concentration. The control growth was measured in the absence of drug.

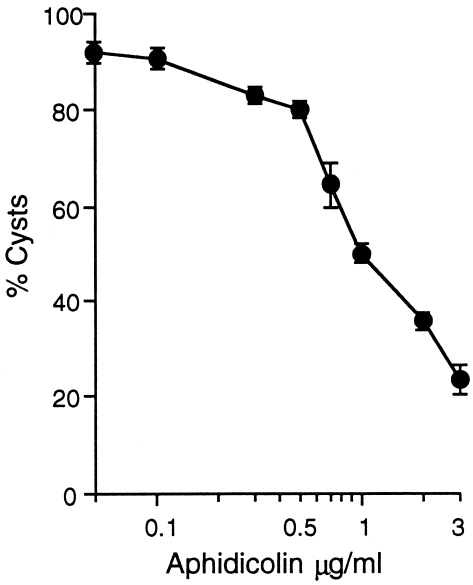

Fig. 2 Effect of aphidicolin on the encystation of *E. invadens*. Trophozoites were transfered to the encystation medium containing various concentrations of aphidicolin. The cysts and trophozoites were counted to determine percentage of cysts 2 days later.Values are plotted as in Fig. 1.

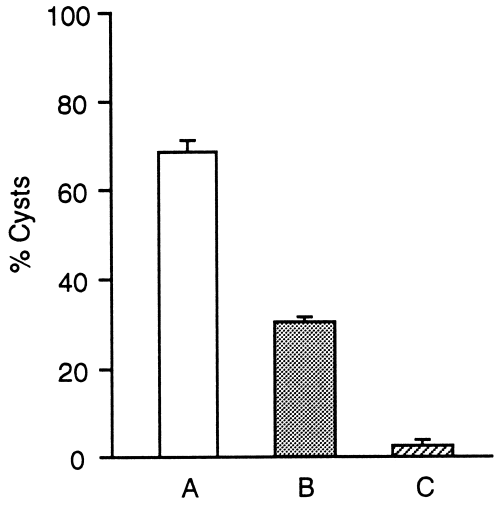

Fig. 3 Effect of pretreatment with aphidicolin on the encystation of *E. invadens*. Trophozoites were pretreated with aphidicolin before being transferred to encystation medium with the drug. Values are plotted as in Fig. 1. A, encystation in the absence of aphidicolin; B, encystation in the presence of aphidicolin but no pretreatment; C, encystation in the presence of aphidicolin following pretreatment.

between the kinetics of trophozoite multiplication and subsequent cyst formation by this species. Thepsuparungsikul et al. (1971) reported that a burst of cell division usually occurred prior to encystation after trophozoites were transferred from a rich growth medium to a suitable encystation medium. Furthermore, Sirijintakarn and Bailey (1980) reported that all encysting *E. invadens* cells divided at least once after being transferred to encystation medium. There was, however, no direct evidence for importance of DNA synthesis and/or cell division just before onset of encystation. The results of pretreatment of trophozoites with aphidicolin indicated that encystation of aphidicolin-pretreated cells was more inhibited than that of cells without the pretreatment. This can be explained that treatment with aphidicolin accumulates the cells at G1/S border and those cells can neither synthesize DNA nor divide when placed in encystation medium, whereas the cells without the pretreatment include G2 and M cells which have already synthesized DNA so that they are not affected by aphidicolin. Thus the results clearly

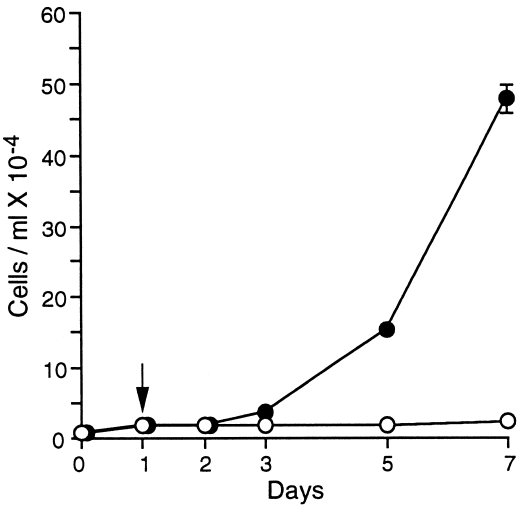

Fig. 4 Reversible effect of aphidicolin on the growth of *E. invadens*. After exposure to aphidicolin for 24 hr, the drug was removed by replacement of the spent medium with drug-free medium. The arrow indicates the time of addition of aphidicolin. Values are plotted as in Fig. 1. Aphidicolin-removed cultures (solid circles); control cultures (open circles).

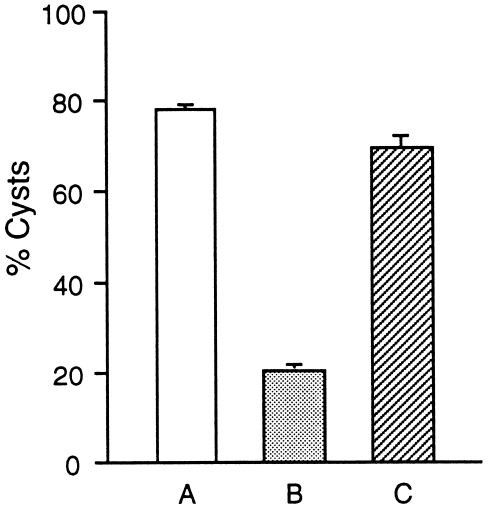

Fig. 5 Reversible effect of aphidicolin on the encystation of *E. invadens*. After exposure to aphidicolin for 24 hr, the drug was removed by replacement of the medium with drug-free encystation medium and the cultures were further incubated for 2 days. Values are plotted as in Fig. 1. A, encystation in the absence of aphidicolin; B, encystation in the presence of aphidicolin; C, aphidicolin-removed encystation.

indicate the importance of DNA synthesis before encystation. However, it remains unclear whether cell division following DNA synthesis is also necessary for encystation. In this respect, attempts to block *E. invadens* cell division specifically with mitotic inhibitors were unsuccessful: colchicine at concentrations as high as 0.1 M had no detectable effect on growth or encystation [13]. Myer and Morgan (1971) suggested that only one of the two daughter cells produced by the final cell division could encyst. On the other hand, Sirijintakarn and Bailey (1980) argued that both daughter cells encysted. The reason for necessity of the DNA synthesis before encystation and also whether or not all the daughter cells after cell division can encyst remains unresolved.

The reversible action of aphidicolin was observed for both growth and encystation of *E. invadens*. Similar results was obtained for growth of *E. histolytica* and the reason for the reversibility was considered due to no direct binding of the drug to DNA, no interference with the synthesis of nucleic acid precursors, and no detectable effect upon RNA or protein synthesis [7]. In this respect aphidicolin is a useful tool for understanding growth and encystation of *E. invadens*.

The results indicated that exposure of 3 μg/ml of aphidicolin to *E. invadens* trophozoites for at least 7 days had little effect on its viability. In this respect there is a difference in sensitivity to the drug between *E. invadens* and *E. histolytica*, because our recent study has demonstrated that *E. histolytica* trophozoites that exposed to the same dose of the drug were viable for only 2 days [7]. The reason for this difference remains unclear, but it appears that it reflects a difference in the culture doubling time between the two species.

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