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APHIDICOLIN INHIBITS EUKARYOTIC DNA REPLICATION AND REPAIR --- IMPLICATIONS FOR INVOLVEMENT OF DNA POLYMERASE α IN BOTH PROCESSES

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SUMMARY: Normal human lymphocytes were stimulated with phytohemagglutinin or treated with UV radiation or N-methyl-N'-nitro-N-nitroso guanidine then rendered permeable to exogenously supplied nucleotides and used to measure the replicative and repair modes of DNA synthesis. Aphidicolin, N-ethyl maleimide and several other compounds inhibited both DNA replication and repair. Since aphidicolin and N-ethyl maleimide are selective inhibitors of DNA polymerase α, these findings suggest that DNA polymerase α is involved in both the replicative and repair modes of DNA synthesis.

INTRODUCTION

Aphidicolin is a tetracyclic diterpinoid that has recently been shown to inhibit eukaryotic DNA polymerase α but not DNA polymerases β or γ (1,2). This differential inhibition suggests that this compound may be used to determine which of the eukaryotic polymerases are involved in the processes of DNA replication and repair. The present study examines the effect of aphidicolin and other inhibitors on these processes in human lymphocytes. Peripheral blood lymphocytes prepared from normal donors were selected for this study because these cells can be obtained in a resting, intermitotic, G_0 state and then be stimulated to conduct either DNA repair or replication (3). Treatment of lymphocytes with ultraviolet irradiation or N-methyl-N'-nitro-N-nitroso guanidine was used to induce DNA repair synthesis. Treatment with phytohemagglutinin was used to stimulate replicative DNA synthesis. Using several different criteria, including alkaline cesium chloride gradients of BrdUMP labelled DNA, we have previously demonstrated that treatment of resting lymphocytes with UV irradiation or N-methyl-N'-nitro-N-nitroso guanidine causes DNA repair synthesis

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while treatment with phytohemagglutinin causes replicative DNA synthesis (3). The actual measurements of DNA synthesis and the effects of the inhibitors were determined in cells made permeable to exogenously supplied compounds (3, 4). Working with permeable cells allows us to supply all the reaction components and inhibitors in well defined concentrations. By using this technique we also bypass the problems associated with pool sizes and/or transport of the reaction components or inhibitors (3-6).

METHODS

Lymphocytes were prepared from the peripheral blood of normal human donors (3). Cells were cultured at 3 x 10^5/ml in alpha modified Eagles medium supplemented with 10% fetal calf serum, 25 mM HEPES, pH 7.2. For mitogen stimulation, leukoagglutinating phytohemagglutinin was added to cultures at a final concentration of 1.75 µg/ml and cultures were incubated at 37°C. For DNA repair studies, freshly prepared lymphocytes were suspended in phosphate buffered saline and spread in a thin layer in open plastic petrie dishes then UV irradiated with a total dose of 50J/m^2. Another method to induce DNA repair was to treat cells with N-methyl-N'-nitro-N-nitroso guanidine at a final dose of 20 µg/ml. After the DNA damaging treatment, cells were collected by centrifugation and incubated in complete growth medium at 37°C for 3 hr.

To measure DNA synthesis, cells were counted, collected by centrifugation for 10 min, 1000 x g, at 4°C, then rendered permeable to exogenously supplied compounds by a 15 min incubation at 4°C in a hypotonic buffer composed of 0.01 M Tris/HCl, pH 7.8, 1 mM EDTA, 30 mM 2-mercaptoethanol, and 4 mM $MgCl_2$. The cells were centrifuged again and resuspended at 2 x 10^7 cells/ml in the same buffer. The reaction mixture for DNA synthesis contained 0.1 M HEPES, pH 7.8, 0.02 mM $MgCl_2$, 0.21 mM NaCl, 15 mM ATP, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, and 0.79 µM [Me-^{3}H] dTTP (Sp Act 140 x 10^6 dpm/nmol) (New England Nuclear). The reactions were performed by combining 50 µl aliquots containing 1 x 10^6 cells with 25 µl of the DNA synthesis mixture in an ice water bath. Inhibitors were added to reaction tubes to the final concentrations indicated in the Figures and Tables. Aphidicolin was dissolved in DMSO before addition to reaction tubes This resulted in a final concentration of 3% DMSO in the reactions. Control tubes for aphidicolin reactions were also adjusted to a final concentration of 3% DMSO. Reactions were started by transferring tubes to a 37°C water bath and stopped at the indicated times by addition of an excess of cold 10% trichloroacetic acid, 2% sodium pyrophosphate. Trichloroacetic acid precipitates were prepared for scintillation counting on GF/C filter discs as previously described (3-5).

RESULTS

In previous studies we have shown that the DNA synthesis measured in human lymphocytes on the third day after PHA stimulation represents the replicative mode of DNA synthesis (3). We have also demonstrated that the DNA synthesis

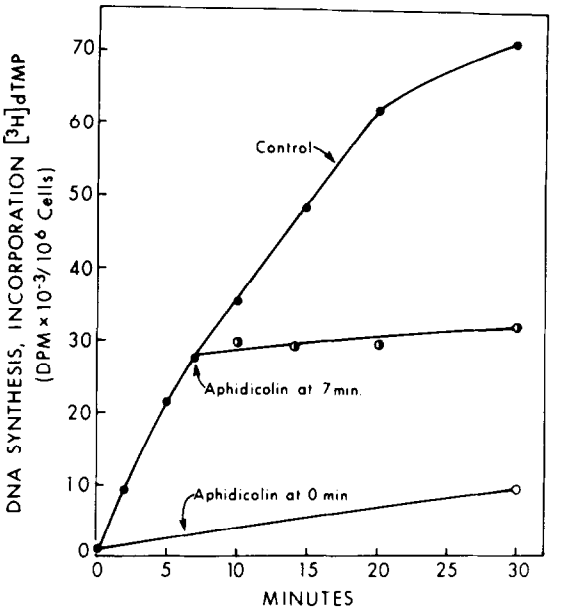

FIGURE 1: Peripheral blood lymphocytes were stimulated with phytohemagglutinin and incubated at 37°C as described in Methods. On the third day in culture the cells were counted, collected by centrifugation and rendered permeable to exogenously supplied compounds as described in Methods. Reactions were performed by combining 50 µl aliquots containing 1 x 10^6 cells with 25 µl of the DNA synthesis mix described in Methods. Aphidicolin dissolved in DMSO was added to the reaction tubes to a final concentration of 100 µg/ml and 3% DMSO. All control tubes were also adjusted to a final concentration of 3% DMSO. Reactions were started by transferring tubes to a 37°C water bath and stopped at the indicated times by addition of an excess of cold 10% trichloroacetic acid, 2% sodium pyrophosphate. TCA precipitates were prepared for scintillation counting as described. All points are the mean values of assays performed in triplicate. Control, untreated cells (●—●); 100 µg/ml aphidicolin added before start of reaction (before transfer from ice bath to 37°C) (O—O); 100 µg/ml aphidicolin added after permeable cells incubated with reaction mix at 37°C for 7 min (●—●).

measured in freshly prepared human lymphocytes 3 hrs after treatment of the cells with UV irradiation or N-methyl-N'-nitro-N-nitroso guanidine represents the repair mode of DNA synthesis (3). Fig 1 shows that addition of aphidicolin to permeable cells produces rapid inhibition of DNA synthesis. In this study, lymphocytes were stimulated with phytohemagglutinin, then 3 days later the cells were permeablized and supplied with all four deoxynucleoside triphosphates, ATP, Mg^{++}, and [^{3}H] dTTP to measure replicative DNA synthesis (3-5). When aphidicolin was included from the beginning of the reaction, replicative DNA synthe-

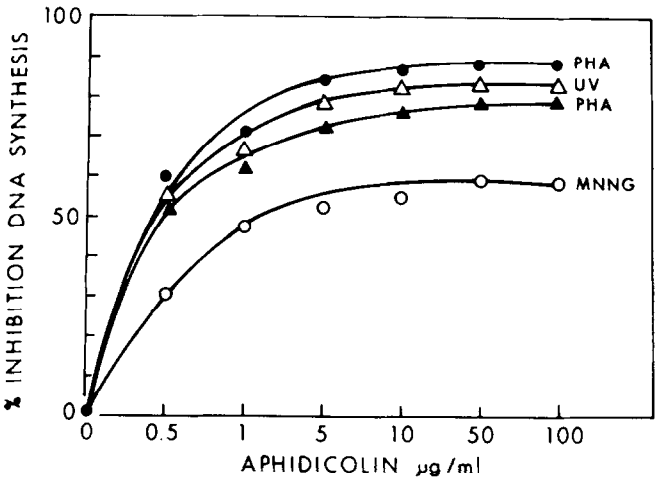

FIGURE 2: Effect of aphidicolin on replicative and repair DNA synthesis in permeabilized human lymphocytes. Peripheral blood lymphocytes prepared from normal human donors and UV irradiated with a total dose of 50J/m^2 or treated with N-methyl-N'-nitro-N-nitroso guanidine at a final dose of 20 µg/ml.After the DNA damaging treatment the cells were incubated in complete growth medium at 37°C for 3 hrs. They were then made permeable to exogenously supplied nucleotides and DNA synthesis was measured during a 30 min incubation at 37°C as described in Methods. PHA stimulated lymphocytes from two different donors were also permeabilized and incubated in the DNA synthesis system. Aphidicolin was added at the indicated concentrations while the components were in an ice water bath. When all components were combined the reactions were started by transferring tubes to a 37°C water bath where they were incubated for 30 min. Incorporation of radioactivity from [^{3}H] dTTP into acid precipitable material was determined as outlined in Methods. Percent inhibition was calculated relative to the DNA synthesis performed by cells in the absence of aphidicolin. Cells from donor 1, repair DNA synthesis induced by N-methyl-N'-nitro-N-nitroso guanidine (O——O), replicative DNA synthesis stimulated by PHA (●——●). Cells from donor 2, repair DNA synthesis induced by UV irradiation (Δ——Δ), replicative DNA synthesis stimulated by PHA (▲——▲). The values for [^{3}H] dTMP incorporation in the absence of aphidicolin for each of the different conditions were as follows. Donor 1, N-methyl-N'-nitro-N-nitroso gaunidine induced DNA synthesis 10,900 dpm/10^6 cells, donor 1 PHA induced DNA synthesis 77,801 dpm/10^6 cells; donor 2, UV irradiation induced DNA synthesis 13,600 dpm/10^6 cells, donor 2 PHA induced DNA synthesis 50,500 dpm/10^6 cells.

sis was inhibited by 86%. When aphidicolin was added to the cells 7 min after the reaction was started, the inhibitory effect on DNA synthesis occurred immediately. Addition of aphidicolin to cells conducting DNA repair synthesis resulted in the same type of rapid inhibition. Thus, under conditions of our assay, aphidicolin enters cells rapidly and exerts a prompt inhibitory effect on DNA synthesis.

Fig 2 shows the dose response curves for aphidicolin inhibition of DNA synthesis in the lymphocytes from two different donors. Aphidicolin was found

to inhibit both the replicative and repair synthesis of DNA in a dose-dependent fashion. However, even at very high concentrations of 100 μg/ml aphidicolin was unable to inhibit completely either type of DNA synthesis. Inhibition of replicative DNA synthesis asymptotically approached 80 and 90% in the cells from the two different donors. Inhibition of the DNA repair synthesis induced by treating cells with N-methyl-N'-nitro-N-nitroso guanidine reached a maximum of 60%. Inhibition of the DNA repair synthesis induced by UV irradiation reached an 80% maximum. Thus, aphidicolin inhibited both the replicative and repair types of DNA synthesis in eukaryotic cells, but in each case the inhibition was only partial.

Table I shows the effects of several other inhibitors on the processes of DNA replication and repair measured in permeable human lymphocytes. Cytembena, which has been previously shown to inhibit replicative DNA synthesis in mouse L cells (6), was also found to be an effective inhibitor of DNA replication and repair in human lymphocytes. Cytosine arabinoside triphosphate caused similar degrees of inhibition of both processes. Phosphonoacetic acid, which has been shown to inhibit all three eukaryotic DNA polymerases (7), showed slightly greater inhibition of replication than repair. Pyridoxal phosphate, which presumably inhibits DNA synthesis by its ability to form a Schiff base with the enzymes involved in DNA synthesis (8), also showed somewhat greater inhibition of replication than repair. Similarly, daunorubicin and adriamycin showed greater inhibition of replication than repair. These latter two compounds inhibit DNA synthesis by complexing with the DNA template (9) and this interaction apparently has greater inhibitory effects on replication than on repair. Studies in permeable cells have previously shown that ATP is required for maximum measurements of replicative DNA synthesis (5). This requirement is specific for ATP in that it cannot be filled by other ribonucleotides or by higher quantities of deoxynucleotides. The present study shows that both replication and repair were reduced when ATP was deleted from the incubation system. Both aphidicolin and N-ethylmaleimide each inhibited DNA replication and repair to a similar degree.

TABLE I

Inhibition of Different Types of DNA Synthesis in Human Lymphocytes

		DNA Repair in UV Irradiation Cells	DNA Replication in PHA Stimulated Cells
		% Inhibition	% Inhibition
Cytembena	1 mM	58	63
Cytosine arabinoside triphosphate	1 mM	54	65
	0.1 mM	28	22
Phosphonoacetic acid	1 mM	70	87
	0.1 mM	63	79
Pyridoxal PO_4	10 mM	82	99
	1 mM	73	92
Daunorubicin	0.1 mM	71	95
	0.05mM	61	88
	0.01mM	7	38
Adriamycin	0.1 mM	50	90
Minus ATP		59	86
Aphidicolin	100 μg/ml	67	76
N-ethylmaleimide	1 mM	66	75

Lymphocytes were prepared from peripheral blood then UV irradiated with 50J/m^2 or stimulated with PHA as described in Fig 1. 3 hr after UV irradiation or 3 days after PHA stimulation the cells were permeabilized and incubated with DNA synthesis reaction mix containing [^{3}H] dTTP. The inhibitors were combined with the reaction components in tubes that were held in an ice water bath. The reactions were started by transferring tubes to a 37°C water bath where they were incubated for 30 min. The reactions to test the effect of N-ethylmaleimide were conducted in cells that were permeabilized with the mercaptoethanol containing buffer described in Methods, then, just before they were used in the reaction mix they were centrifuged and resuspended in the same buffer without mercaptoethanol. The reactions to measure DNA synthesis in the absence of ATP were conducted with the DNA synthesis reaction mix that contained all of the components listed in the legend to Fig 1 except that ATP was completely omitted. All reactions were performed in triplicate, similar results were obtained with cells prepared from two different donors. Percent inhibition is calculated relative to the [^{3}H] dTMP incorporated into acid precipitable material by reactions that were conducted in the absence of any inhibitors. Values for the uninhibited reactions were 6,018 dpm/10^6 cells for the UV irradiated cells and 42,400 dpm/10^6 cells for the PHA stimulated cells.

DISCUSSION

These studies demonstrate that aphidicolin inhibits the processes of DNA replication and repair in normal human lymphocytes. It should be noted that

aphidicolin is not a general inhibitor of all nucleic acid synthesis since under similar conditions this compound did not inhibit the synthesis of RNA or poly(adenosine diphosphoribose). In addition, cells exposed to aphidicolin and then washed to remove the drug resumed DNA synthesis at their control rate. These observations, coupled with the earlier demonstration that aphidicolin is a selective inhibitor of DNA polymerase α (2), suggests that DNA polymerase α is involved in both DNA replication and repair. Involvement of DNA polymerase α in DNA replication has been extensively reviewed (10-12). While the amount of DNA polymerase α is usually slight in nonreplicating cells, the enzyme is in fact present in resting lymphocytes and thus it can be involved in the repair processes measured in these cells (13). The fact that aphidicolin could not completely inhibit DNA replication and repair could be due to the involvement of one of the other DNA polymerases β or γ. This latter suggestion is supported by the recent demonstration that DNA polymerase β accounts for part of the DNA synthesis that occurs in replication and repair in L929 cells (14).

Aphidicolin and N-ethylmaleimide each inhibited DNA replication and repair to a similar degree. Some of the other inhibitors showed greater differences in their abilities to inhibit DNA replication and repair, but no agent was capable of inhibiting one process and not the other. In addition, concentrations of agents which effectively inhibited one process were always capable of inhibiting the other process. While the permeable cell system is a complex multicomponent system,the present fingings suggest that similar enzymes or enzymes with similar susceptibilities are involved in eukaryotic DNA replication and repair. Since aphidicolin and N-ethylmaleimide inhibited DNA synthesis to the same degree in both replication and repair and since both aphidicolin and N-ethylmaleimide have been shown to inhibit DNA polymerase α but not DNA polymerase β (2,10-12) it is likely that DNA polymerase α is responsible for much of the DNA synthesis in both replication and repair. The residual replicative and repair synthesis that occurs in aphidicolin and N-ethylmaleimide treated cells suggests that other DNA polymerases such as β and/or γ are involved in each of these processes.

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REFERENCES

1. Dalziel, W., Hesp, B., and Stevenson, K.M. (1973) J. Chem. Soc. Perkin Trans. I, 2841-2852.
2. Ikegami, S., Taguchi, T., and Ohashi, M. (1978) Nature 275, 458-460.
3. Berger, N.A., Sikorski, G.W., Petzold, S.J., and Kurohara, K.K. (1979) J. Clin. Invest. In Press.
4. Berger, N.A. (1978) Methods in Cell Biol. 20, 325-340.
5. Berger, N.A., and Johnson, E.S. (1976) Biochim. Biophys. Acta 425, 1-17.
6. Berger, N.A., and Weber, G. (1977) J. Natl. Cancer Inst. 58, 1167-1169.
7. Allaudeen, H.S., and Bertino, J.R. (1978) Biochim. Biophys. Acta 520, 490-497.
8. Berger, N.A., Weber, G., and Kaichi, A.S. (1978) Biochim. Biophys. Acta 519, 87-104.
9. Kim, J.H., Gelbord, A.S., Djordjevic, B., Kim, S.H., and Perez, A.G. (1968) Cancer Res. 28, 2437-2442.
10. Loeb, L. (1974) The Enzymes 10, 173-209.
11. Weissbach, A. (1975) Cell 5, 101-108.
12. Bollum, F.J. (1975) Progress in Nucl. Acid Res. and Molec. Biol. 15, 109-144.
13. Bertazzoni, U., Stefanini, M., Noy, G.P., Giulotto, E., Nuzzo, F., Falaschi, A., and Spadari, S. (1976) Proc. Natl. Acad. Sci. USA 73, 785-789.
14. Butt, T.R., Wood, W.M., McKay, E.L., and Adams, R.L.P. (1978) Biochem. J. 173, 309-314.