Volume 6 Number 10 1979 Nucleic Acids Research

The effect of aphidicolin on adenovirus DNA synthesis

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Received 23 May 1979

ABSTRACT

Aphidicolin inhibits adenovirus DNA replication in HeLa cells and in a cell-free,infected,nuclear extract in which viral DNA is elongated. The compound inhibits α DNA polymerase,extensively purified from HeLa cells, but has little or no effect on the β or γ DNA polymerases similarly purified. Aphidicolin does not affect thymidine uptake by cells nor does it break preformed DNA. The drug is not selective against viral DNA synthesis as it also inhibits DNA replication in uninfected cells. The inhibition by aphidicolin is reversible if the drug is removed within 18 hrs after addition to HeLa or Chinese Hamster ovary cells but the cells are irreversibly affected if the drug remains for 48 hours.

INTRODUCTION

Aphidicolin is a tetracyclic diterpenoid elaborated by the fungus Cephalosporium aphidicola. This antibiotic which is poorly soluble in water, has been shown to inhibit the growth of herpes simplex and vaccinia viruses most likely by inhibiting DNA synthesis (1). While our present studies were in progress, it was reported that aphidicolin inhibits an α DNA polymerase-like activity in extracts of regenerating rat liver, HeLa cells, and in sea urchin embryos (2,3,4). Mitosis but not meiosis of the sea urchin embryos was inhibited.

The present report demonstrates that aphidicolin inhibits HeLa cell and Chinese hamster ovary cell (CHO) growth but the effect is reversible upon washout of the drug up to 18 hours after addition. If removal of the drug is delayed for 48 hrs, cell viability is severely compromised. Aphidicolin acts directly on DNA synthesis and does not affect the entry of thymidine into the cell or the integrity of preformed DNA. Neither protein nor RNA synthesis is inhibited in drug treated cells. Extensive purification of HeLa cell α, β and γ DNA polymerases from nuclei showed that the α enzyme was markedly inhibited with little or no inhibition

of the other two enzymes. The synthesis of adenovirus DNA, both in whole cells and in a nuclear extract DNA replication system (5), was inhibited to the same extent as the α DNA polymerase; however, uninfected HeLa cell DNA synthesis was more extensively inhibited. The inhibition of α DNA polymerase by aphidicolin appears to be very rapid without any measurable lag period.

MATERIALS and METHODS

Cells and Viruses: The sources of HeLa cells (S3) and adenovirus type 2 (Ad2) have been previously described (6). The cells were grown in suspension culture in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum and 1% glutamine. When infected cells were used, 4000 purified virions per cell were added to suspension cultures at 37°. The infected-cell, soluble, nuclear extracts which continue to synthesize Ad2 DNA were prepared at 18 hrs post infection by the method of Kaplan et al (5) and were free of host cell DNA. Chinese hamster ovary cells (CHO) were obtained from Pamela Stanley of the Albert Einstein College of Medicine and were grown on alpha medium with added deoxynucleotides and 10% fetal calf serum. These cells were maintained either in monolayer or in suspension cultures.

DNA Polymerase Assays: Reaction mixtures A (0.05ml) contained 50mM Tris-HCl pH 7.9 (at 30°), 4mM dithiothreitol (DTT), 7.5mM $MgCl_2$, 0.04mM each of dATP, dGTP, and dCTP, 0.04mM {^{3}H} TTP (400-600 cpm/pmol), 20µg bovine serum albumin (BSA), and 10µg of nicked salmon sperm (NSS) DNA. Reaction mixtures B (0.05ml) were identical to reaction mixture A except that they contained 2.5mM $MnCl_2$ in place of the $MgCl_2$, and 4µg poly(rA)·oligo$(dT)_{12-18}$ replaced the NSS DNA.

For assays of column eluates, Reaction mixture B contained 100mM NaCl; both Reaction A and B were performed for 20 min at 30° and terminated by the addition of 2ml of 5% TCA, 0.1ml of 0.2M sodium pyrophosphate (NaPPi) and 100µg of denatured calf thymus DNA. TCA precipitated material was collected on glass fiber filters, washed with 1% TCA, 95% ethanol and the radioactivity was counted. 1 unit of DNA polymerase catalyzed the incorporation of 1 pmol of dTMP at 30° during a 20 min incubation. Specific activity refers to units per mg of protein. The protein was measured as described by Heil and Zillig (7).

The reaction times were varied as indicated with individual

experiments but the kinetics of incorporation were linear in each case. NaCl (100mM) was added to some reaction mixtures which used NSS and omitted from some experiments utilizing poly(rA)·oligo(dT). Each of these variations is described either in the figure legend or in the RESULTS.

Purification of HeLa Cell DNA Polymerases: The α, β and γ DNA polymerases were purified from nuclear extracts of $8x10^9$ HeLa cells, which were prepared by lysing the cells with detergent and extracting the nuclei with ammonium sulfate by a modification of methods previously described (5). The nuclei resuspended as described were further diluted to 50ml of 30mM Tris-HCl (pH 7.7, 4°), 2.5mM EDTA, 0.15M $(NH_4)_2SO_4$, 2mM DTT, 5% sucrose and incubated for 90 sec at 37° followed by 2 min at 0° before centrifugation at 40,000 rpm for 20 min in the Beckman type 40 rotor. The supernate (Step I, Table I) was diluted with 2 vols. of 50mM Tris-HCl (pH 7.7), 1mM EDTA, 2mM DTT, and 5% sucrose before the dropwise addition of 20% streptomycin sulfate to a final concentration of 1.5%. The suspension was stirred for 20 min at 0°. The precipitate was removed

TABLE I

PURIFICATION OF DNA POLYMERASES

Fractionation Procedure	Total Protein (mg)	Total Units and (%) Recovery (A)+		(B)++		Specific Activity (Units/mg) (A)	(B)
I. Nuclear Extract	492	148,000	(100)	135,000	(100)	300	275
II. Streptomycin Sulfate-Ammonium Sulfate Concentrate	264	132,000	(89)	185,000	(137)	500	700
III. Phosphocellulose							
Peak A (α*, γ, β)	8.4	48,600	(33)	10,500	(7.8)	5,780	1,250
Peak B (α, γ*, β*)	16.2	57,500	(39)	79,000	(58)	3,550	4,875
IV. DEAE Cellulose							
Peak A (α*, γ)	3.9	39,400	(27)	3,900	(3)	10,100	1,000
Peak B (α, γ)	7.5	46,500	(32)	15,800	(12)	6,200	2,100
Peak C (β)	6.2	2,900	(2)	79,000	(59)	467	12,800
V. Hydroxyapatite							
Peak 1 (γ)	0.671	-		13,800	(10.2)	-	20,600
Peak 2 (α)	0.988	41,600	(28.1)	-		42,100	-
VI. Phosphocellulose (β)	0.525	3,300	(2.2)	42,500	(31.4)	6,250	81,000
VII. DNA Cellulose (native)							
γ DNA Polymerase	0.138	-		4,800	(3.5)	-	35,000
α DNA Polymerase	0.122	7,600	(5.1)	-		62,700	-
VIII. DNA Cellulose (denatured)							
β DNA Polymerase	0.078	-		8,500	(6.3)	-	108,800

DNA Polymerase activity was determined on NSS+ or on poly(rA)·oligo(dT)++ and the units for determination of specific activity were described in METHODS. From Step III to the end of the purification, the polymerases present in a fraction are included in parenthesis. The predominant polymerase in each mixture is designated by an asterisk*.

by centrifugation in the Sorvall type SS-34 rotor at 10,000 rpm for 20 min. Solid ammonium sulfate was added to a final concentration of 75% (47.6 gms/100ml) and stirred for 30 min at 0°. The precipitated protein, pelleted at 15,000 rpm for 20 min, was dissolved in 5ml of buffer A (Tris-HCl pH 7.7, 1mM EDTA, 2mM DTT, 20% glycerol) and dialyzed against 500ml of buffer A with 0.1M KCl which was changed every 30 min for a total of 3 hrs (Step II).

The Step II product was adjusted to 5 to 6mg/ml protein with buffer A containing 0.1M KCl. The sample was loaded onto a phosphocellulose column (100ml, 2.5cm diameter) which was eluted with a 1 L linear gradient of 0.1M to 0.6M KCl in buffer A. The fractions which eluted at 0.25-0.35M KCl were more active on NSS DNA (peak A) and those eluting between 0.37-0.45M KCl were more active on poly(rA)·oligo(dT). Peak A fractions and peak B fractions were pooled separately and the enzymes were precipitated with 75% saturated $(NH_4)_2SO4$. The precipitates were resuspended in 2ml of buffer A and dialyzed against 500ml of buffer A with 50mM KCl which was changed often until the salt concentrations was reduced below 50mM (Step III, A and B).

The A and B fractions from the phosphocellulose column (Step III) were applied to separate DEAE-52 cellulose columns (5ml, 1.2cm diameter) equilibrated with buffer A containing 50mM KCl. The columns were washed with 15ml of 50mM KCl in buffer A and then eluted with 10ml of 0.2M KCl in Buffer A. One ml fractions were collected from the column. The DNA polymerases eluted in the 0.2M KCl fractions of each DEAE column were pooled separately (Step IV, A and B). However, the DNA polymerase in the pass-through and 50mM KCl fractions of both columns were pooled and combined (Step IV, C).

The DEAE cellulose peak A and B product (Step IV) were dialyzed for 4 hr against 8 changes of a 250-fold volume of buffer B (5mM potassium phosphate buffer{KPO_4} pH 7.5 at 4°, 0.1mM EDTA, 2mM DTT, 0.4M KCl, 20% glycerol) and each was applied to a hydroxyapatite column (4ml, 1.2cm diameter) equilibrated with buffer B. DNA polymerases were eluted with a 50ml linear gradient of 5 - 100mM KPO_4 in buffer B and 0.5ml fractions were collected. From each of the A and B peaks in Step IV, two distinct DNA polymerase activities were separated by elution at 17 - 28mM KPO_4 and 35 - 50mM KPO_4. The first DNA polymerase fractions (17-28mM KPO_4) from either Step IV, A or B, were most active on poly(rA)·oligo(dT) and were combined (Step V, peak 1). The latter fractions eluting at 35-50mM

KPO_4 from Step IV, A and B, were most active on NSS DNA and were combined (Step V, peak 2).

Step IV, peak C DNA polymerase which contained an N-ethylmaleimide (NEM) resistant activity on poly(rA)·oligo(dT) was applied to a second phosphocellulose column (5ml, 1.2cm diameter) equilibrated with buffer A containing 0.1M KCl and eluted with a 50ml linear gradient of 0.1-0.5M KCl in buffer A. Fractions of 0.5ml were collected. A single peak of DNA polymerase activity which was resistant to NEM on poly(rA)·oligo(dT) and also active on NSS DNA was eluted at 0.3-0.4M KCl and pooled (Step VI).

Step V, peak 1 product was dialyzed against 500ml of buffer A containing 50mM KCl which was changed often until the salt concentration was reduced to 50mM KCl. The protein was applied to a native DNA cellulose column (1.5ml, 0.7cm diameter) equilibrated with the same buffer. The column was washed with 10ml of 50mM KCl in buffer A and the DNA polymerase was eluted with 0.2M KCl in buffer A. Active fractions were pooled and dialyzed against 500ml of buffer A containing 0.2M NaCl and 50% glycerol and kept at -80°(Step VII - DNA polymerase γ).

The Step V, peak 2 product was dialyzed and applied to a native DNA cellulose column (1.5ml, 0.7cm diameter) which was washed with 50mM KCl before elution with 0.2M KCl in buffer A. The peak of activity was pooled, dialyzed and stored at -80° as above(Step VII - DNA polymerase α).

The Step VI product was diluted with buffer A to the final KCl concentration of 0.2M and applied to a denatured DNA cellulose column (1.5ml) equilibrated with buffer A containing 0.2M KCl. The column was washed with 10ml of 0.2M KCl in buffer A and DNA polymerase was eluted with 0.6M KCl in buffer A. Active fractions were pooled, dialyzed against 500ml of buffer A containing 0.2M NaCl and 50% glycerol and stored at -80° (Step VIII - DNA polymerase β).

Table I summarizes the steps and recoveries of the 3 DNA polymerases at various stages of the purification.

Table II summarizes the properties of the 3 DNA polymerases in terms of primer-template specificity, salt sensitivity and inhibition by NEM.

MATERIALS

Aphidicolin was obtained from the National Cancer Institute and dissolved in dimethyl sulfoxide (DMSO) to make a 10mM stock solution. The final concentration of DMSO in each experiment was less than 1% and

TABLE II

CHARACTERIZATION OF DNA POLYMERASE

CONDITIONS OF REACTION	DNA POLYMERASES		
	α	β	γ
NSS DNA:	30.5	0.7	2.2
+ 0.1M NaCl	1.5	6.1	0.3
+ NEM (5mM)	< 0.05	0.2	< 0.05
+ NEM (5mM), + 0.1M NaCl	< 0.05	0.4	N.D.
Poly(rA)·oligo(dT)$_{12-18}$:			
+ 0.1M NaCl	0.4	51.3	10.7
+ NEM (5mM), +0.1M NaCl	< 0.05	26.7	< 0.05

The activity of the α polymerase was measured by Reaction A, and the β and γ activities by Reaction B as described in METHODS. The conditions were varied by the addition of NaCl or N-ethylmaleimide (NEM) as shown. Activities are measured as pmol of dTMP incorporated after 20 min at 30°. In each assay, 0.5μg of α, β or γ protein were used. NEM was incubated with the enzyme in buffer for 5 min at room temperature immediately before the assay was done. N.D. indicates that this reaction was not done.

had no effect on any of the control reactions. The sodium salt of camptothecin was obtained from the same source and dissolved in water to make a $3x10^{-3}$M solution. Radioisotopes were purchased either from Schwarz-Mann or New England Nuclear. Radioactivity was determined by liquid scintillation counting in either 5 or 10ml TT-21 Scintillation fluid from Yorktown Chemicals.

RESULTS

Inhibition of HeLa and Chinese Hamster Ovary Cell Growth by

Aphidicolin: The growth of HeLa cells in the presence of various concentrations of aphidicolin was measured for a 24-hour period. Table III demonstrates that the logarithmic growth of HeLa cells was inhibited by 50% at a concentration of approximately 10^{-7}M aphidicolin and the cloning of CHO cells was inhibited by 30% at the same concentration. For experiments in which 10^4 CHO cells were plated in the continued presence of 10^{-6}M drug, there were no surviving clones.

When HeLa cells grown in the presence of 10^{-6}M aphidicolin for 18 hrs were washed free of the drug, the cells promptly resumed growth at 92% of the normal rate (4.8×10^5 in 18 hrs). When CHO cells, incubated for

TABLE III

EFFECT OF APHIDICOLIN ON GROWTH OF HeLa CELLS AND CLONING EFFICIENCY OF CHINESE HAMSTER OVARY (CHO) CELLS

Aphidicolin (M)	Number of HeLa Suspension Cells/ml ($\times 10^5$)	% Increase After 18 hrs.	Clones of CHO Cells	
			Number	%
0	5.2	100	158	100
1×10^{-8}	-	-	131	83
5×10^{-8}	4.1	63	-	-
10^{-7}	3.6	43	110	70
5×10^{-7}	2.9	19	-	-
10^{-6}	2.7	12	0	0

HeLa cells at an initial density of 2.4×10^5 cells/ml were incubated in Eagle's MEM with 5% fetal calf serum and 1% glutamine with various concentrations of aphidicolin and counted 18 hrs later.

CHO clones were cloned at concentrations of 208 cells per 60 mm plate in alpha medium containing deoxynucleotides and 10% fetal calf serum. Various concentrations of aphidicolin were included in the medium for 6 days which was the duration of the experiment. 158 clones in the control were designated as 100% (cloning efficiency = 76%).

24 hrs in the presence of 10^{-6}M aphidicolin, were washed free of the drug, 127 cells survived the reversal; whereas only 1 cell survived the reversal after 48 hrs of drug treatment. The control untreated CHO cells, produced 132 clones. Aphidicolin at 10^{-6}M had no effect on the growth of E. coli.

Effect of Aphidicolin on DNA, RNA and Protein Synthesis: Aphidicolin at a concentration of $5x10^{-5}$M was added to HeLa cells and macromolecular synthesis was measured over the subsequent 100 min. Although DNA synthesis was inhibited at the first time measured (10 min) after the addition of the drug, RNA and protein synthesis continued at rates greater than 90% of the controls for the following 90 min (Fig. 1A). Aphidicolin had no effect on the uptake of thymidine into HeLa cells as shown by measuring the intracellular concentrations of tritiated thymidine (Fig. 1B). At concentrations of aphidicolin which inhibited the incorporation of radioactive thymidine into DNA by 90% the concentrations of acid soluble tritiated thymidine and its phosphorylated derivatives were within 70% of the amounts found in cells in the absence of the inhibitor. Thus the inhibition of DNA synthesis seems to be a direct effect on the synthesis of this macromolecule rather than through an effect on thymidine transport.

The Effect of Aphidicolin on the Integrity of HeLa Cell DNA: Because a number of inhibitors of DNA synthesis act by degrading the DNA template, the size of HeLa cell DNA was measured in the presence of aphidicolin. HeLa cell DNA was labelled with{^{14}C} thymidine until sufficient radioactivity was incorporated into full size product. The remaining radioactivity was washed from the cells which were then exposed to 10^{-5}M aphidicolin for 1 hr. Another aliquot of labelled cells was treated with 10^{-5}M campothecin which is known to break HeLa cell DNA (8). A third HeLa aliquot (control) was incubated without either drug. Cells treated in this way were layered directly onto alkaline sucrose gradients in which the size of the single stranded DNA resulting from alkaline denaturation could be measured. Figure II shows that there was no degradation of prelabelled HeLa cell DNA by aphidicolin. Most of the radioactivity still sedimented as large macromolecular species and was found at the bottom of the alkaline sucrose gradients similar to cells which were not treated with drug. In contrast, the campothecin treated cells showed considerable radioactivity in regions of the gradient where small sized DNA is found (8). Thus, aphidicolin neither breaks DNA

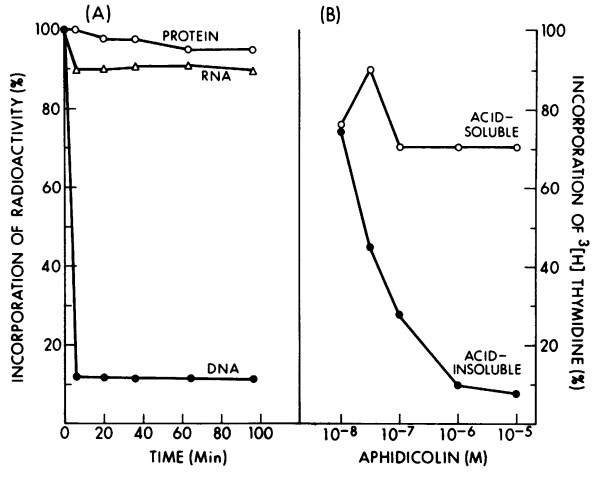

FIGURE I(A) Effect of Aphidicolin on Synthesis of DNA, RNA and Protein: HeLa cells ($3x10^5$ cells/ml) were incubated at 37° in the presence or absence of 50μM aphidicolin. At the designated intervals, 1ml aliquots were removed from each of the cultures and pulsed with either ^{14}[C]-thymidine (1μCi), ^{14}[C]-uridine (1μC) or ^{14}[C]-leucine (2μC) for 10 min at 37°. The reaction was terminated and the acid insoluble radioactivity determined as previously described (25). The rate of incorporation of radioactivity into acid-insoluble material during each time interval is represented as the midpoint of the pulse. The percent incorporation is expressed relative to controls in which incorporation of ^{14}[C]-thymidine (●), ^{14}[C]-uridine (Δ), or ^{14}[C]-leucine (0) was 700, 2,000 and 3,000 cpm/ml, respectively during a 10-min pulse without the drug.

FIGURE I(B) Effect of Aphidicolin on Thymidine Uptake: HeLa cells ($3x10^5$cells) were resuspended in 1ml MEM, and various concentrations of drug were added as previously described (9). The cells were kept at 37° for 60 min. Two μC of ^{3}(H)-thymidine (5 mCi/mmole) was added and the mixture was incubated at 37° for an additional 15 min. Cold Earle's solution was added at the end of the incubation period and the cells were washed twice with the same solution; 0.5ml of water was added, followed by 0.5ml of 20% TCA. The tubes were kept at 4° in an ice bath for 15', and centrifuged at 2000 rpm for 10'. The supernatant was transferred to scintillation vials and the precipitate was resuspended in 5% TCA, filtered through a Millipore filter and the radioactivity in the precipitate (●—●) and supernatant (0—0) was determined.

chains nor causes any damage to the DNA which would create alkaline labile bonds.

The Effect of Aphidicolin on DNA Synthesis in HeLa Cells, Adenovirus 2 Infected HeLa Cells, and Adenovirus 2 Nuclear Extracts: The dose response curve of synthesis for both HeLa cell DNA as well as adenovirus (Ad) DNA in infected HeLa cells was measured (Fig. III). HeLa cell DNA

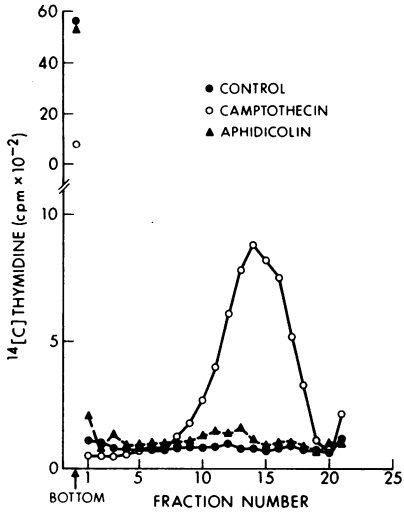

FIGURE II. Sedimentation of HeLa DNA After Treatment with Aphidicolin: HeLa cells (10ml) at a concentration of $3x10^5$ cells/ml were incubated at 37° for 3 hr with 1μCi ^{14}C}thymidine (50mCi/mmole). The labeled cells, washed with MEM containing 20μM of unlabelled thymidine, were resuspended in the original volume with MEM supplemented with 5% fetal calf serum and 1% glutamine. Two ml of this cell suspension was adjusted to 10^{-5}M aphidicolin and another aliquot was left without drug. After 60 min of incubation at 37°, the cells were centrifuged at 1,000xg for 5 min, resuspended in 0.5ml of 0.15M NaCl, layered onto an alkaline sucrose density gradient and centrifuged in an SW27.1 Beckman rotor at 24,000 RPM for 16 hrs as previously described (8). A third aliquot was treated with 10^{-5}M camptothecin. DNA sedimenting faster than 60S appeared at the bottom of the gradient.

synthesis was inhibited by 50% at concentration of $7x10^{-8}$M aphidicolin. In contrast DNA synthesis in Ad-infected HeLa cells was inhibited to the same extent only at doses of 10^{-5}M aphidicolin. At 18 hrs post infection (p.i.) incorporation of radioactive thymidine is found almost exclusively in viral DNA (10).

A subcellular DNA replicating system which efficiently elongates Ad2 DNA has recently been described from this laboratory (5). These Ad nuclear extracts which synthesize exclusively viral DNA were tested with various concentrations of aphidicolin. 50% inhibition of Ad DNA synthesis in this subcellular system was achieved at $3x10^{-6}$M aphidicolin. Thus the inhibition of Ad DNA synthesis requires higher concentrations of aphidicolin both in whole cells and in nuclear extracts than in uninfected HeLa cells.

The Effect of Aphidicolin on DNA Polymerases: The effect of

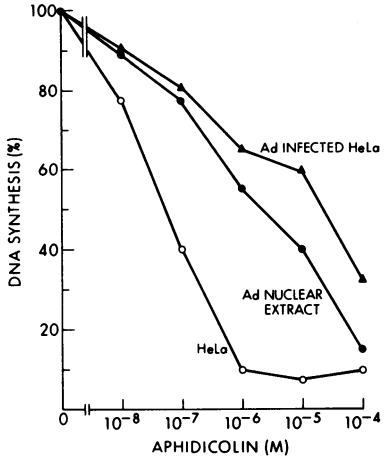

FIGURE III. Effect of Aphidicolin on DNA Synthesis in HeLa Cells, Ad2 Infected HeLa Cells and Ad2 Nuclear Extracts: DNA synthesis in uninfected and Ad2 infected HeLa cells was measured by incorporation of ^{14}C-thymidine into acid insoluble form. HeLa cells in suspension culture, at a density of $3x10^5$ cells/ml were infected and compared to a duplicate uninfected HeLa cell culture. After 18 hrs of infection, aphidicolin at the indicated concentrations plus 0.05μCi of ^{14}C-thymidine were added to one ml aliquots of infected or uninfected HeLa cells. After incubation at 37° for 1 hr, acid insoluble radioactivity was determined in each aliquot.
Ad2 nuclear extracts from HeLa cells infected for 18 hrs were prepared by extraction of nuclei with 150mM ammonium sulfate as described by Kaplan et al (5). The soluble extract was freed from the nuclear matrix and host chromosomal DNA by centrifugation at 7,000 x g for 20 min. The *in vitro* reaction mixture (52μl) contained 20mM Tris-HCl (pH 7.5), 10mM DTT, 10mM $MgCl_2$, 2.6mM ATP, 0.125mM each of dATP, dCTP, dGTP, 3μM ^{3}(H) dTTP ($3.6x10^4$ cpm/pmol) and 10μl of infected nuclear extract. Ad DNA synthesis was measured after 1 hr at 30° as previously described (5). DNA synthesis (100%)in uninfected HeLa cells was 9180 cpm; in Ad2 infected HeLa cells was 3690 cpm; in Ad2 nuclear extracts was 2 pmol.

aphidicolon on the activity of the three eukaryotic DNA polymerases (α, β and γ) and on DNA polymerases I and III from *E. coli* were measured either on nicked salmon sperm (NSS) DNA or on poly(rA)·oligo(dT) as described in MATERIALS & METHODS. α DNA polymerase assayed on NSS was inhibited 50% by drug concentrations of $3x10^{-6}$M, and greater than 90% by 10^{-4}M. The *E. coli* DNA polymerases I and III on NSS were completely resistant to inhibition at both of these concentrations. The activity of the γ DNA polymerase which is optimal on poly(rA)·oligo(dT) in the presence of 100mM NaCl also was completely resistant to the antibiotic. The β DNA

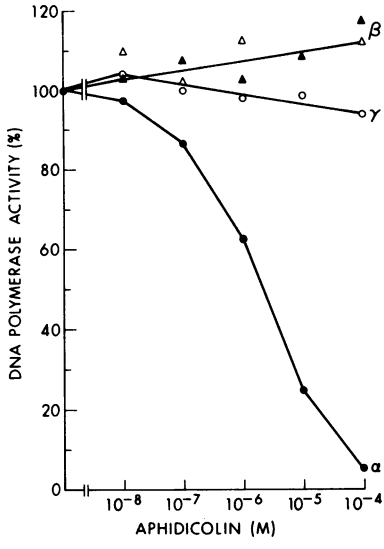

FIGURE IV. Effect of Aphidicolin on the DNA Polymerases: The α, β and γ DNA polymerases were measured for 30 min at 30° using the conditions described in the MATERIALS and METHODS. α DNA polymerase activity was measured using Method A. β DNA polymerase activity was measured using Method A either on NSS DNA (▲—▲) or Method B on poly(rA)·oligo(dT) (Δ—Δ) both in the presence of 100mM NaCl. γ DNA polymerase was measured using Method B plus 100mM NaCl. The effect of various concentrations of aphidicolin on these reactions was determined. 100% activity for each DNA polymerase was 38.6 pmol for 0.067μg α; 117 pmol for 0.87μg β on poly(rA)·oligo(dT); 22.7 pmol for 1.31μg β on NSS; 24.7 pmol for 0.471μg γ.

polymerase is unique among the 5 enzymes in that it has significant activity on both of the templates used; activity on poly(rA)·oligo(dT) and on NSS, which are optimal with the addition of 100mM NaCl, were both unaffected by aphidicolin. However, there was 30% inhibition of the β DNA polymerase on NSS by 10^{-4}M aphidicolin if the assay was done without added NaCl (final concentration approximately 8mM NaCl). Thus aphidicolin significantly inhibits only the α DNA polymerase from HeLa cells but may have a small effect on the β DNA polymerase under selected conditions.

Kinetics of Inhibition of Alpha DNA Polymerase by Aphidicolin: The kinetics of α DNA polymerase inhibition by aphidicolin was measured by the addition of several concentrations of drug to α DNA polymerase reactions already in progress. Figure V demonstrates that addition of drug 5 min after the onset of the polymerase reaction inhibits the incorporation of {^{3}H} TTP without any detectable lag whether 7.5×10^{-6} or

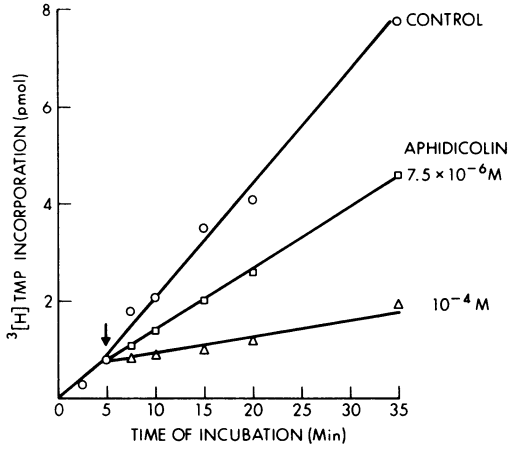

FIGURE V. Kinetics of Inhibition of α DNA Polymerase by Aphidicolin: Reaction mixtures containing α DNA polymerase were incubated for 5 min at 30° and then divided into 3 aliquots. Aphidicolin (7.5×10^{-6}M) was added to one reaction mixture; 10^{-4}M drug was added to another and DMSO (1%) which was the drug diluent was added to the control. Aliquots from each reaction were removed at various times during the subsequent 30 min and acid precipitable radioactivity measured. The reaction was done on NSS DNA as described in Method A (MATERIAL and METHODS) with 0.067μg of α DNA polymerase in each assay. The specific activity of the ^{3}(H) TTP was 7,700 cpm/pmol.

10^{-4}M aphidicolin is used.

The Effect of Aphidicolin on Enzyme Catalyzed Pyrophosphate Exchange: The α DNA polymerase catalyzed pyrophosphate (PPi) exchange with the terminal nucleotides of the growing DNA chain. (^{32}P) pyrophosphate exchange was measured by the transfer of radioactivity to the deoxynucleotide product which adsorbed to Norite (26). The effect of aphidicolin on this process was measured and is shown in Table IV. At 10^{-4}M aphidicolin, the catalysis by α DNA polymerase of ^{32}PPi transfer to nucleotide linkage was inhibited by 76%. Similar concentrations of drug did not inhibit pyrophosphate exchange catalyzed by *E. coli* DNA polymerase I.

DISCUSSION:

Aphidicolin is a potent inhibitor of eukaryotic DNA synthesis presumably by inhibiting α DNA polymerase. The mechanism of inhibition

TABLE IV

THE EFFECT OF APHIDICOLIN ON ENZYME CATALYZED PYROPHOSPHATE EXCHANGE

ENZYME	PYROPHOSPHATE EXCHANGE		
	CONTROL (pmol)	APHIDICOLIN (pmol)	INHIBITION (%)
E. coli DNA Polymerase I	23.9	24.4	0
Minus 4 dXTP's	0.8	N.D.	-
Minus Enzyme	0.1	N.D.	-
α DNA Polymerase	3.4	0.8	76
Minus 4 dXTP's	0.6	N.D.	-
Minus Enzyme	<.01	N.D.	-

Reaction mixtures (0.05ml) containing 50mM Tris HCl (pH 7.7), 4mM DTT, 7.5mM $MgCl_2$, 20µg BSA, 3µM each of dATP, dGTP, dCTP and TTP, 150µM ^{32}PPi (4500 cpm/pmol) and 1.7µg of NSS DNA were incubated 30 min at 30°. Reactions without the 4 deoxynucleotides (dXTP's) or enzyme were included as controls. The reactions were terminated by addition of 1ml TCA (10%, 0°), 100µg denatured calf thymus DNA and 0.1ml NaPPi (0.2M). Acid-insoluble material was removed by centrifugation and 0.1ml Norite suspension (26) was added to the supernate. The Norite was centrifuged, washed twice with 1% TCA, collected on GF/C filters and the radioactivity counted. Reactions with α DNA polymerase contained 129 units of enzyme and those with *E. coli* DNA polymerase contained 242 units per 50µl reaction mixture. Aphidicolin concentration was 10^{-4}M. *E. coli* DNA polymerase I was obtained from Enzo Biochemicals. N.D. indicates that reaction was not done.

does not involve any detectable breakage of the primer-template DNA, alteration of nucleoside uptake by the cell or inhibition of thymidine kinase (2). α DNA polymerase has been proposed (11-14) as the enzyme responsible for intracellular chromosome replication. This is based on

increases of activity of this enzyme during the S phase of the cell cycle and increases following partial hepatectomy which converts resting hepatocytes into replicating cells. The inhibition of HeLa and CHO DNA synthesis by aphidicolin is consistent with this formulation but genetic proof that the α DNA polymerase is the replicative polymerase is not yet available.

Adenovirus DNA synthesis is also inhibited by aphidicolin but concentrations greater than those needed for inhibiting HeLa DNA synthesis are required. The amount of aphidicolin needed to inhibit intracellular Ad DNA synthesis is similar to that needed to inhibit both Ad DNA elongation in nuclear extracts and the isolated α DNA polymerase. The discrepancy in the concentrations of drug needed in the uninfected and infected cell are not readily explainable. It is possible that the transport or concentrating ability of the infected cell is not as efficient as the uninfected cell or some basic difference in the mechanism of Ad and HeLa DNA synthesis may explain this observation. The Ad replication complex has been shown to contain considerable amounts of both α and γ DNA polymerase activity (15-19). The amount of β DNA polymerase in these complexes is very small and perhaps a contaminant. Recent experiments in this and other laboratories have shown that dideoxythymidine triphosphate, a TTP analog, inhibits the γ DNA polymerase more readily than the other eukaryotic polymerases and is also a potent inhibitor of adenovirus but not host DNA synthesis (20-22). The mechanism of this selective inhibition is not readily apparent. If the results of studies on isolated DNA polymerases can be applied to physiologic replication complexes, the results would suggest that there is a need for both the α and γ DNA polymerases for the replication of Ad DNA. The mechanism of Ad DNA replication includes a strikingly asymetric synthesis for each of the strands of this duplex DNA (23). The first strand replicates by a displacement reaction (Type I) in which a single strand is displaced by an asymetrically growing replication fork from an origin at either end of the linear DNA. The displaced single strand is subsequently replicated from an origin at the opposite end of the molecule (Type II) so that each strand replicates continuously from a 5' end. If one polymerase were responsible for the displacement reaction and another for the replication on the single strand, the need for two polymerases might be explained. In the Ad nuclear extract system which does not initiate new strands but elongates both Type I and II molecules (27), a

specific inhibitor of one polymerase might be expected to decrease replication by no more than 50%. However, the inhibition by either aphidicolin or dideoxythymidine triphosphate can reach 90% which suggests that both enzymes are coordinately needed in a single complex for the replication of both Type I and Type II DNA. This two polymerase model perhaps explains the discrepancy in the concentration needed for the inhibition of HeLa and Ad DNA synthesis. A previous report (1) on the inhibition of herpes simplex and vaccinia viruses by concentrations of 10^{-5} to 10^{-6}M aphidicolin noted that adenovirus cytopathic effect was not inhibited by the drug. Our present results suggest that Ad growth should be inhibited by aphidicolin at 10^{-5}M as viral DNA synthesis is 50% inhibited at this concentration. The concordance of Ad DNA inhibition by similar concentrations of drug both in whole cells and in the nuclear extracts extends the fidelity of the nuclear extract system.

The results in this manuscript demonstrate that the effects of aphidicolin are reversible in whole cells and Ohashi et al (2) have shown that the α DNA polymerase activity can be restored upon dialysis of the enzyme to free it of the drug. The irreversible effect on cell viability which becomes manifest after 24 hours of drug treatment is not readily explained. However, the unbalanced synthesis occasioned by the near normal synthesis of RNA and protein may explain this loss of viability as the cells grow in the absence of DNA synthesis.

An interaction of aphidicolin with the α DNA polymerase is suggested by the observation that the inhibition seems to be determined by the nature of the polymerase rather than the primer-template DNA. The structure of aphidicolin shows very few regions that are coplanar, which is a prerequisite for a DNA intercalating agent. The small amount of inhibition of the β DNA polymerase on NSS DNA in the absence of added NaCl may be explained either on the basis of some α DNA polymerase contaminant or by a configurational change of the β enzyme which makes it resistant to drug at 100mM NaCl.

α DNA polymerase catalyzed two reactions with DNA. In addition to the incorporation of nucleotides into DNA, this enzyme fraction catalyzed an exchange reaction between PPi and deoxynucleotide triphosphates. Since aphidicolin blocked both of these reactions catalyzed by α DNA polymerase, it suggests that the drug acts on this polymerase at a step prior to translocation. In contrast, neither polymerization or pyrophosphate exchange catalyzed by E. coli DNA polymerase I was

inhibited by drug. These observations add further evidence that the specificity of inhibition by aphidicolin is a result of drug interaction with the enzyme rather than with the DNA.

We recently have detected an additional DNA polymerase activity in HeLa cell nuclei which is inhibited by aphidicolin. This activity co-purified with the α enzyme through Step IV (Table I) but could then be separated on a hydroxyapatite column. The new enzymatic activity, like α, is only detected on nicked salmon sperm DNA; however, unlike the α DNA polymerase it is enhanced 1.5 times by 100mM NaCl. This other aphidicolin-sensitive activity bears some resemblance to one of the three components of the alpha-like DNA polymerase activity described by Matsukage et al (24). Thus, conclusions as to the physiologic significance of α DNA polymerase in either HeLa cell or Ad DNA replication based on aphidicolin inhibition must await a clarification of the relationship between the two aphidicolin sensitive DNA polymerase activities and the isolation of drug resistant mutants in these enzymes.

ACKNOWLEDGEMENTS: We are grateful to Jerard Hurwitz for helpful advice and critical reading of the manuscript. This work was supported by Public Health Service Grants CA-11512, CA-15714, and CA-23187 from the National Cancer Institute; and Grants NP89K, VC 201 and CH86 from the American Cancer Society. M. L. was the recipient of support from a National Institutes of Health Training grant MB-9602, CA-09060; J. I. from the Cancer Research Center (National Cancer Institute P30 CA-13330; MSH and SBH are recipients of Irma T. Hirschl Trust Career Scientist Awards. The data in this paper are from a thesis to be submitted by M. L. in partial fulfillment for the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

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