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Mutagenic DNA Polymerase in Human Leukemic Cells

(tumor progression/leukemia/lymphocytes)

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ABSTRACT **Evidence is presented that DNA polymerases from human leukemic cells are mutagenic. Nucleic acid-free extracts of acute lymphoblastic leukemic cells polymerized about 10-times more dCTP using poly(dA-dT)·poly(dA-dT) as a template than did extracts from normal lymphocytes. Mutagenic DNA polymerases could perhaps play an important role in tumor progression.**

We have started to inquire whether DNA synthesis in malignant cells is less exact than in normal cells. Some of the characteristics of tumor progression (1), namely, (*a*) continuing evolution of new cell variants, (*b*) uncontrolled cellular proliferation, and (*c*) increasing number of chromosomal aberrations, suggest that as malignant cells become progressively anaplastic there is an accumulation of errors in genetic information.

For study of the fidelity of *in vitro* DNA synthesis, human lymphocytes offer several advantages. Leukemic lymphocytes of the acute "blastic" type have considerable DNA polymerase activity. DNA polymerase activity in normal lymphocytes can be increased 30- to 200-fold by stimulating them with phytohemagglutinin (2). This increase parallels the ability of these lymphocytes to incorporate thymidine into DNA, suggesting that the polymerase measured *in vitro* functions in chromosomal replication. Thus, there is sufficient polymerase activity in normal stimulated lymphocytes and their malignant counterparts to analyze and compare the exactness of DNA synthesis. The exceptionally low deoxyribonuclease activity in these cells facilitates meaningful experiments with partially purified enzymes (2). We have chosen to investigate the fidelity of DNA synthesis using peripheral blood lymphocytes rather than cells from long-term tissue culture in order to relate our findings more directly to the biology of the disease state. In these initial experiments we measured the ability of "nucleic acid-free extracts" of stimulated normal and leukemic lymphocytes to exactly copy the base sequences of the synthetic homopolymers poly(dA-dT)·poly(dA-dT) and poly(dG)·poly(dC).

METHODS

Enzymes. Lymphocytes from normal donors and from patients with acute lymphatic leukemia were purified as described (2). Leukemic lymphocytes were obtained from untreated patients with white blood cell counts greater than 40,000 cells per mm^2. Even though acute leukemic lymphocytes are probably not stimulated by phytohemagglutinin, both the normal and leukemic cells were cultured with phytohemagglutinin for 3 days (3). Cell-free extracts were obtained by disruption of the cells with repeated freeze-thawing, elimination of DNA by phase extraction (4, 5), and exhaustive dialysis against 20 mM Tris·HCl (pH 7.8) containing 4 mM 2-mercaptoethanol–1 mM EDTA–20% glycerol. Samples could be stored at −70°C with negligible loss of polymerase activity for at least 1 week. Homogeneous *Escherichia coli* DNA polymerase I was prepared by the method of Jovin *et al.* (6). Homogeneous T4 phage DNA polymerase was the generous gift of Dr. Paul Englund. Protein was determined by the method of Lowry *et al.* (7).

Polynucleotides. Poly(dG)·poly(dC) was purchased from Collaborative Research. Poly(dA-dT)·poly(dA-dT) was synthesized by the *de novo* reaction of *E. coli* polymerase I.

DNA Polymerase Activity. To measure the fidelity with which poly(dA-dT)·poly(dA-dT) is copied in lymphocyte extracts, we measured the incorporation of correct nucleotides (dATP and dTTP) and incorrect nucleotides (dGTP or dCTP) in separate assays using the same enzyme preparations. For determination of total synthesis, a standard reaction mixture was used containing in a total volume of 0.2 ml: 13.0 μM [^{3}H]dTTP (1500 dpm/pmol), 12.5 μM dATP, 20 μM dCTP, 1 mM $MgCl_2$, 100 mM Tris·HCl (pH 7.8), and 92 μM phosphorus of poly(dA-dT)·poly(dA-dT). The concentration of metal ion and template used gave maximum incorporation with both normal and leukemic extracts. Assays were initiated with 0.10 ml of enzyme and incubated at 37° for 6 hr, the maximum incubation period during which linear incorporation was observed. For measurement of dCTP incorporation, identical reaction mixtures were used except that they contained unlabeled dATP and dTTP and 20 μM [^{3}H]dCTP (28,030 dpm/pmol). Incorporation was stopped by addition of 400 μl of 0.1 N perchloric acid containing 0.05 M $Na_2P_2O_7$ (Acid Stop Solution). The mixture was incubated for 2 min; 100 μl of 0.2 M NaOH–50 mM $Na_2P_2O_7$ was added followed by 100 μl of carrier DNA (1 mg/ml of denatured calf-thymus DNA) and then 600 μl of Acid Stop Solution. To aid in washing the assays, 100 μl of unlabeled 0.05 mM carrier dCTP was also added. Samples were centrifuged at 5000 × *g* for 10 min, and the supernatant was discarded. The precipitated DNA was solubilized with 0.2 M NaOH–50 mM $Na_2P_2O_7$ and then precipitated with acid. This procedure was repeated three times. Finally, the DNA was washed onto a Whatman GF/C filter disc with aliquots of cold H_2O and 20 ml of cold Acid Stop Solution, followed by 10 ml of ethanol. This disc was then placed in a scintillation counting solution [Toluene–2,5-diphenyloxazole(PPO)] for determination of radioactivity. Determinations were done in triplicate or quadruplicate, and the results given are the average after subtraction of controls without added poly(dA-dT)·poly(dA-dT)

TABLE 1. *Incorporation of dCTP with poly(dA-dT)·poly(dA-dT)*

Enzyme source	Correct nucleotide incorporation (pmol)	Incorrect nucleotide incorporation (pmol)	Level of infidelity
Normal lymphocytes			
Donor 1	439	0.092	1/4800
2	51	0.025	1/1990
3	221	0.064	1/3460
4	335	0.056	1/5980
5	309	<0.01	<1/30,900
6	382	0.091	1/4200
Acute lymphatic leukemic			
Patent 1	53	0.11	1/480
2	108	0.43	1/250
3	189	0.35	1/540
4	58	0.069	1/850
E. coli DNA polymerase I	1843	<0.01	<1/184,300
T4 phage DNA polymerase	5509	0.48	1/11,500

template. For dGTP incorporation, conditions similar to those described above were used except for the presence of 20 μM dGTP in incorporation experiments for correct nucleotide and 20 μM [^{3}H]dGTP (26,360 dpm/pmol) in incorporation experiments for incorrect nucleotide.

In assays with homogeneous *E. coli* polymerase I, incorporation of correct and incorrect nucleotides into poly(dA-dT)·poly(dA-dT) was measured as described above except for the presence of 40.2 μM [^{3}H]dTTP (147,790 dpm/mmol), 40 μM dATP, 20 μM dCTP, 5 mM $MgCl_2$, and 219 μM phosphorus of poly(dA-dT)·poly(dA-dT). Reactions were initiated with 2.5 units of enzyme and incubated for 10 min at 37°. With T4 phage DNA polymerase, conditions were similar to those for *E. coli* except for the presence of 10 mM 2-mercaptoethanol, 10 mM ammonium sulfate, 2 mM $MgCl_2$, 100 mM Tris·HCl (pH 8.6), and correct nucleotides at concentrations of 100 μM each. The mixtures were incubated for 45 min at 30°, and each contained 10 units of enzyme.

Hydrolysis of the DNA Product. The DNA product was digested to 5′-mononucleotides by the combined action of pancreatic DNase and snake venom phosphodiesterase (8). The 5′-mononucleotides were separated by thin-layer chromatography (9).

RESULTS

Fidelity of poly(dA-dT)·poly(dA-dT) replication

Cell-free extracts were prepared from acute leukemic lymphocytes and from normal lymphocytes grown with phytohemagglutinin. The cells were disrupted by repeated freezing and thawing, and the DNA was removed by phase extraction (4, 5). For measurement of the exactness of DNA replication, experiments were performed with poly(dA-dT)·poly(dA-dT) as template in the presence of highly radioactive dGTP or dCTP and unlabeled dATP and dTTP. Both the normal and leukemic extracts catalyzed the incorporation of incorrect bases into poly(dA-dT)·poly(dA-dT) (Table 1). Replication with leukemic extracts, however, appeared to be less exact than replication with normal extracts. For dCTP incorporation, this result is indicated by an average level of infidelity about 10-fold greater for leukemic preparations (1/250–1/850) as compared to that of the normal lymphocyte enzymes (1/1990–<1/30,000). The observed differences in the exactness of poly(dA-dT)·poly(dA-dT) synthesis does not appear to be due to nonspecific factors—activators or inhibitors present in excess in either the normal or leukemic preparations. We demonstrated this hypothesis by mixing the normal and leukemic enzymes and subsequently observing that the level of infidelity of the combined preparation was intermediate to the value obtained with the individual extracts (Table 2). Preliminary experiments were also done that measured the incorporation of dGTP into the poly(dA-dT)·poly(dA-dT) template. The data we obtained so far with this nucleotide do not permit any conclusions to be drawn as to the relative levels of infidelity.

Fidelity of poly(dG)·poly(dC) replication

In experiments with poly(dG)·poly(dC) as template, dATP and dTTP were labeled with tritium while dGTP and dCTP were not labeled. The major feature of polymerization of poly(dG)·poly(dC) was that far fewer incorrect nucleotides were incorporated with either the normal or leukemic extracts as compared with polymerization with poly(dA-dT)·poly(dA-dT). The level of infidelity with dTTP and dATP by both normal and leukemic extracts was generally less than 1 in 50,000, the lower limit of detection in these assays.

Control experiments

To discount the possibility that the "errors in DNA synthesis" we observed with lymphocyte extracts were actually only a consequence of chemical contamination of either the templates or labeled nucleotide, the fidelity of homogeneous preparations of *E. coli* polymerase I and T4 phage DNA polymerase was tested with the same reagents. With poly(dA-dT)·poly(dA-dT) as template, the *E. coli* enzyme made less than 1 error in 100,000 nucleotides polymerized (Table 1). T4 phage DNA polymerase replicated the homopolymers with less precision. Polymerization of poly(dA-dT)·poly(dA-dT) appeared to have about 1 incorrect base incorporated for 10,000 correct bases polymerized, in agreement with the results of Hall and Lehman (10). Thus, contamination of reagents was not responsible for the errors that we observed with the various enzymes. This conclusion is further sup-

TABLE 2. *Fidelity of copying poly(dA-dT)·poly(dA-dT) with a mixture of normal and acute leukemic extracts*

Enzyme source*	Units of polymerase†	Level of infidelity (% total incorporation) dGTP	dCTP
Normal	110	0.025	0.049
Acute leukemic	110	0.52	0.21
Normal + acute leukemic	55 + 55	0.18	0.11

* DNA polymerase extracts. Normal, donor 1; acute leukemic, patient 2.

† One unit of enzyme is that which polymerized 1 pmol of nucleotide in 6 hr.

ported by the finding that a decrease in the specific activity of labeled dCTP and dTTP 5-fold by addition of appropriate unlabeled nucleotide led to a 4- to 6-fold decrease in the ^3H counts incorporated. In order to show that the incorrectly incorporated nucleotide was actually deoxycytidylic acid, we degraded the product of the reaction to deoxyribonucleoside 5′-monophosphates by hydrolysis with pancreatic DNase and snake venom phosphodiesterase. Subsequent chromatography identified over 80% of the incorporated ^3H label as dCMP.

In order to determine if dCTP was indeed incorporated into poly(dA-dT)·poly(dA-dT), we studied the requirements for incorporation of both correct and incorrect nucleotides (Table 3) and characterized the product of the reaction. Table 3 shows that the requirements for correct and incorrect base incorporation are similar and typical for DNA polymerases. Without an added metal cofactor (Mg^{2+}) or with heated extracts there was no detectable incorporation of either the correct or incorrect deoxynucleotides. Without the added polynucleotide templates, incorporation of incorrect nucleotides was less than 20% of the values observed with the complete reaction mixtures. Correct nucleotide incorporation in the absence of poly(dA-dT)·poly(dA-dT) was less than 0.05% of that obtained with the complete reaction mixture. Thus, any native DNA that might be a contaminant is not the template for polymerization of either correct or incorrect deoxynucleotides. As would be expected if the added poly(dA-dT)·poly(dA-dT) serves as template, equal amounts of [^3H]dATP and [α-^{32}P]dTTP were incorporated into the DNA product in each extract.

Supporting evidence that dCTP was polymerized into the added poly(dA-dT)·poly(dA-dT) template was provided by analysis of the product of the reaction by cesium chloride density gradient centrifugation (Fig. 1). The product of the reaction banded at a density expected for that of poly(dA-dT)·poly(dA-dT), indicating that both the correct nucleotide ([α-^{32}P]dTTP) and the incorrect nucleotide ([^3H]dCTP) are indeed incorporated into this polynucleotide. Neither correct nor incorrect nucleotides were incorporated into DNA in incubation mixture lacking the poly(dA-dT)·poly(dA-dT) template (Fig. 1*C*).

In order to determine the position of the incorrect bases in the polynucleotide product, we studied the relations between correct and incorrect base incorporation. Polymerization of both correct and incorrect bases were proportional to time and to protein concentration for all of the enzyme preparations. Representative data with a normal lymphocyte extract is shown in Fig. 2. Moreover, variation in protein concentration did not significantly alter the misincorporation ratio of incorrect bases into the poly(dA-dT)·poly(dA-dT) product (Fig. 2, *inset*). Since the extent of incorrect base incorporation closely paralleled incorporation of the correct bases, the observed infidelity does not appear to indicate solely the addition of a few bases to the end of polynucleotide chains. If incorporation of dCTP into poly(dA-dT)·poly(dA-dT) was merely a result of terminal labeling, the omission of correct nucleotide would not be expected to have grossly affected the extent of incorrect nucleotide incorporation. On the other hand, if the incorrect bases were polymerized into the poly(dA-dT)·poly(dA-dT) template in concert with the correct base, omission of dATP and dTTP should severely limit incorrect nucleotide incorporation. We found that dCTP incorporation without dATP and dTTP was only 0–28% of the incorporation observed in assays containing the correct nucleotides (Table 3). These correlated

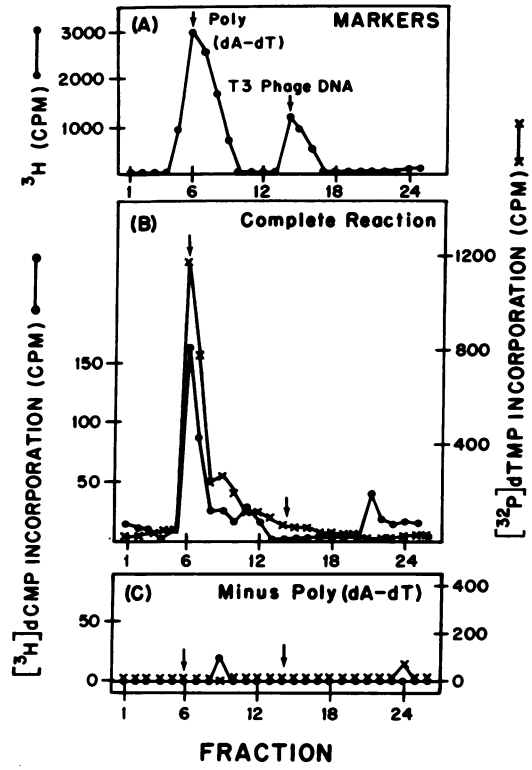

FIG. 1. Neutral cesium chloride density gradient centrifugation of the product of the reaction with DNA polymerase from patient 4. Five standard assays with [α-^{32}P]dTTP (25 dpm/pmol) and [^3H]dCTP (9300 dpm/pmol) were combined. The polynucleotide product was precipitated five times, after which it was suspended in 0.1 M Tris·HCl (pH 7.3) containing 0.1 mM EDTA and cesium chloride (final density, 1.700) in a final volume of 4.0 ml. The solution was centrifuged for 80 hr at 35,000 rpm (147,000 × *g*) at 20°. About 0.1-ml fractions were collected from the top of the tube, and the radioactivity was determined. Separate gradients contained: (*A*) marker DNA consisting of ^3H-labeled poly(dA-dT)·poly(dA-dT) and ^3H-labeled T3 phage DNA (50% G+C); (*B*) complete reaction mixture; (*C*) reaction mixture without added poly(dA-dT)·poly(dA-dT).

TABLE 3. *Reaction requirements for correct and incorrect nucleotide incorporation with poly(dA-dT)·poly(dA-dT)*

Reaction mixture	Normal (% incorporation)		Acute leukemic (% incorporation)	
	dTTP	dCTP	dTTP	dCTP
Complete	100 (330 pmol)	100 (0.06 pmol)	100 (150 pmol)	100 (0.30 pmol)
Minus enzyme	0	0	0	0
Minus poly(dA-dT)·poly(dA-dT)	<0.05	16 ± 7	<0.05	20 ± 4
Minus Mg^{++}	0	0	0	0
Minus dATP and dTTP	—	13 ± 11	—	12
Heated enzyme	0	0	0	0

Results given as mean ± SEM.

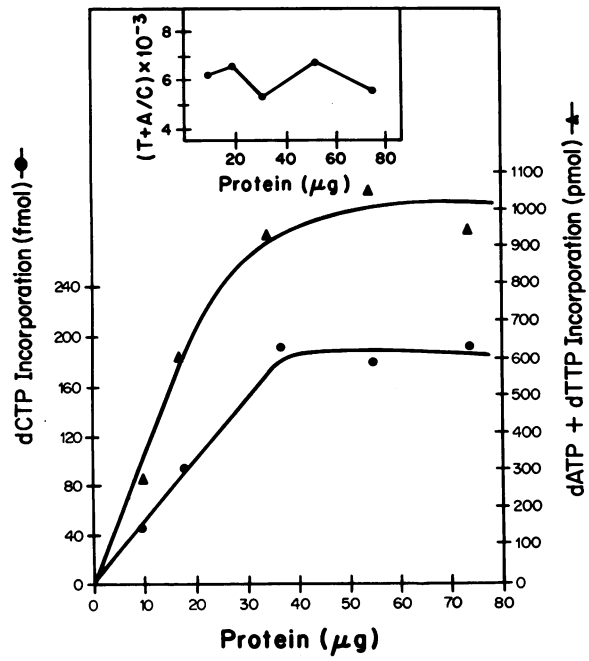

FIG. 2. Effect of protein concentration on correct and incorrect nucleotide incorporation. Normal human lymphocyte polymerase from donor 1 was used. The assay procedure was identical to that in Table 1 except that all reaction components were present in 2-fold greater quantity. *Inset* presents the ratio of correct nucleotides incorporated (dATP and dTTP) to incorrect nucleotide (dCTP) incorporated plotted against protein concentration.

results suggest that terminal labeling was not responsible for incorrect nucleotide incorporation. In another kind of experiment, the DNA product was labeled with [α-^{32}P]dTTP and [^{3}H]dCTP and hydrolyzed sequentially from the 3'-terminus with snake venom phosphodiesterase. Fig. 3 shows that the time release of correct (dTMP) and incorrect (dCMP) nucleotides closely paralleled each other, indicating that dCMP was distributed evenly throughout the entire product.

DISCUSSION

If hydrogen bonding between bases alone was responsible for the fidelity of DNA synthesis it would not be reasonable to expect differences in the exactness with which normal and leukemic polymerases copy poly(dA-dT)·poly(dA-dT). Rather, it would seem more likely that the precision of DNA synthesis would be similar for all polymerases, regardless of source. Theoretical considerations predict that precise DNA synthesis requires base-pairing specificity of greater energy than that provided by hydrogen bonding alone. For example, the ionization and spontaneous tautomerization of bases are capable of promoting transitions in one out of every hundred base pairs (11) during replication of DNA. Moreover, it has recently been demonstrated (12) that mononucleotides exhibit a degree of hydrogen bonding specificity that cannot account for the fidelity of DNA synthesis with several prokaryotic polymerases (10, 13) or for the results reported in this paper.

Two distinct mechanisms by which DNA replication enzymes could participate in guaranteeing the genetic code have been proposed. These are: (*i*) base selection during polymerization (14, 15) and (*ii*) editing the errors after polymerization by nuclease activity (15). Nuclease activity of prokaryotic DNA polymerase has a role in fidelity of DNA synthesis. *E. coli* polymerase I and T4 phage polymerase, for example, excise mismatched bases (16, 17). T4 phage containing anti-mutator genes that decrease spontaneous mutation rates have enhanced nuclease–polymerase activity (18). These data are interpreted as suggesting that the fidelity of *in vivo* replication in T4 phage is dependent on the relative rates of insertion and removal of nucleotides during polymerization. However, exonuclease activity does not appear to be invariably associated with prokaryotic DNA polymerases and has not been reported to be a prominent constituent of animal DNA polymerases. Other repair enzymes may be operative in eukaryotic cells. The data presented here do not allow us to choose between the possibilities of deficits in base selection or decreased editing of errors as an explanation of the observed increased infidelity with the leukemic extracts. Even with homogeneous enzymes this distinction may be difficult if a polymerase enzyme has both activities.

We envision that the major consequences of base pairing errors during DNA replication in leukemic cells, and perhaps in other types of cancer, would be that of promoting an increased mutation frequency. It seems probable that these random genetic changes would result in cellular alterations, some of which would have selective value for proliferation in the host. Lethal and neutral mutations, of course, would also occur. The lethal mutants will perish and, therefore, are only of minor significance. Those cells with neutral mutations will survive and be capable of undergoing subsequent genotypic changes, the nature of which will determine their importance in tumor progress. A central correlate of this hypothesis is that malignant cells, even from a single type of neoplasia, will eventually develop a wide range of variation. Many reports indicate that most tumor cell populations show marked variation with continuing proliferation (1, 19, 20).

The most direct explanations for the differences in the precision of homopolymer replication between normal and leu-

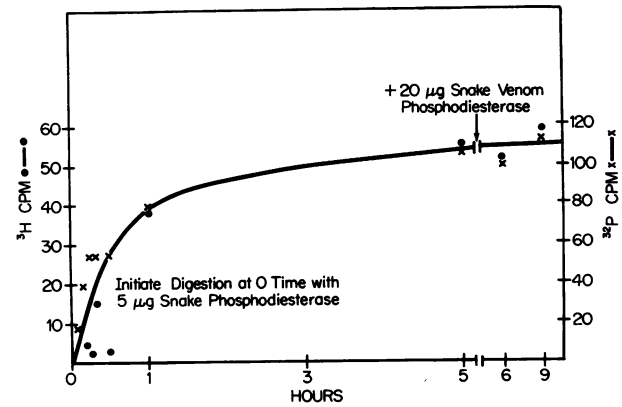

FIG. 3. Digestion of the DNA product of DNA polymerase from patient 4 with snake venom phosphodiesterase. Five standard assays with [α-^{32}P]dTTP (25 dpm/pmol) and [^{3}H]dCTP (28,030 dpm/pmol) were combined. The acid-insoluble products were washed as in *Methods*, except that after 3 successive precipitations, they were dissolved in 2.5 ml of 0.1 M Tris·HCl (pH 7.5) containing 5 mM $MgCl_2$ and 5 μg of 3'-snake venom phosphodiesterase. At intervals, 250-μl aliquots were removed, and the acid-soluble radioactivity was determined.

kemic extracts are (*i*) the presence of a viral DNA polymerase in leukemic cells or (*ii*) the presence of an altered cellular polymerase that is faulty in base selection. It is now important to determine the precision of homopolymer replication with RNA-directed DNA polymerases from animal tumor viruses. A conclusive demonstration that any cellular DNA polymerases are actually modified awaits extensive purification of these enzymes.

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