The presence of terminal deoxynucleotidyl transferase in the N-methyl-N-nitrosourea induced leukaemia in BDF1 mice and its effect on the accuracy of the DNA polymerases.

Roy Saffhill and Leena Chaudhuri*

Paterson Laboratories, Christie Hospital and Holt Radium Institute,
Manchester M20 9BX, UK

Received 3 December 1975

ABSTRACT

DNA polymerases have been prepared from leukaemic and normal spleens and their fidelity in copying a polyd(AT).polyd(AT) template assessed. The leukaemic cytoplasmic DNA polymerases were less accurate than the controls whereas no difference in accuracy was observed in the nuclear DNA polymerases. The preparations of leukaemic cytoplasmic DNA polymerase also contained the enzyme terminal deoxynucleotidyl transferase. When this enzyme was removed by further purification the accuracy of the cytoplasmic DNA polymerases increased to that of the controls.

INTRODUCTION

The DNA polymerases in crude extracts of human acute lymphoblastic leukaemic cells have been reported to be error-prone¹. Such cells also contain the enzyme terminal deoxynucleotidyl transferase², an enzyme which could act as a mutagen³ and, if present in DNA polymerase preparations, affect the apparent accuracy of the copying of a DNA template⁴. DNA polymerising enzymes must play an important role in maintaining the accuracy of DNA synthesis and the possible relevance of this to carcinogenesis has been shown by the lowering of the accuracy of DNA polymerases on treatment in vitro with a carcinogen⁵. That there may be a connection between errors in DNA synthesis and the carcinogenic process has been indicated by these and other recent reports. Certain carcinogen modified DNA bases (e.g. 6-O-methylguanine⁶ and bases damaged by ionising radiation⁷) have been shown to be capable of mis-coding and at least one of these, 6-O-alkylguanine, is excised faster from the DNA of a non-target tissue than from the DNA of the target tissue^{8,9}.

We have been investigating the accuracy of DNA synthesis in leukaemic cells from BDF1 mice¹⁰, after a single dose of N-methyl-N-nitrosourea (MNU) which induces a high incidence of leukaemias. The

*Visiting Scientist from Indian Institute of Experimental Medicine,
4 Raja S.C. Mullick Road, Calcutta 32, India.

accuracy of the DNA polymerases isolated from the leukaemic cells has been measured along with the presence of the enzyme terminal deoxynucleotidyl transferase.

MATERIALS AND METHODS

Animals: BDF1 mice (8 - 10 weeks old) were injected with MNU dissolved (2mg/ml) in ice-cold physiological saline and administered as a single-dose of 50mg/kg by injection into one of the lateral tail veins. The animals were killed when suspected of having leukaemia and the spleens removed from the animals.

Chemicals: Methylnitrosourea was prepared in these laboratories and was stored at -20° . Bovine serum albumin (BSA) was purchased from the Sigma Chemical Co., Ltd., London; polyd(AT).polyd(AT) and the deoxyribonucleoside 5'-triphosphates were obtained from the Boehringer Corporation (London) Ltd.; oligod(pT)₄ and oligod(pA)₁₂₋₁₈ were purchased from P-L Biochemicals Inc., Milwaukee, U.S.A.; radioactive materials were obtained from the Radiochemical Centre, Amersham.

Preparation of DNA Polymerases from Mouse Spleen: The DNA polymerases were prepared by a modification of the method of McCaffrey *et al*². One leukaemic spleen (weight 1- 2g), or 20 spleens from untreated 12 week old mice in the case of controls, was homogenised in 10ml buffer A (0.05M Tris/HCl pH 7.7, 0.001 M EDTA and 0.001M mercaptoethanol) using an all glass Dounce homogeniser. 0.05ml Triton X-100 was added and mixed and the whole centrifuged at 15,000g for 10 min. The turbid supernatant was centrifuged at 144,000g for 60min and the clear supernatant kept. 1 M acetic acid was added dropwise until the pH had fallen to 5.0 and the mixture was stirred at 0° for 15 min. The precipitate was centrifuged down at 15,000g for 10 min and the supernatant discarded. The precipitate was extracted with 5ml buffer B (0.05 M Tris/HCl pH 7.7, 0.001 M EDTA, 0.001 M mercaptoethanol, and 20% v/v glycerol) in a glass homogeniser and then centrifuged at 15,000g for 10min. The supernatant was applied to a phosphocellulose P-11 column (1cm x 15cm), equilibrated with buffer B, washed with buffer B containing 1mg/ml BSA until the E_{280} of eluate had stabilised and then eluted with a linear gradient of KCl (0 to 1M) in buffer B containing 1mg/ml BSA (total gradient volume was 60ml) collecting 1ml fractions. The fractions were assayed for DNA polymerase using a polyd(AT).polyd(AT) template and for terminal deoxynucleotidyl transferase.

Fractions containing cytoplasmic and nuclear DNA polymerase activities were pooled separately and the accuracy of the enzymes determined.

Assay for DNA Polymerase Activity: Each assay had a total volume of 100 μ l and contained the following: 50 μ l of the enzyme fraction, 1.5 nmole each of dATP and ^3H -dTTP (1.33 μ Ci/nmole), 8 μ l of polyd(AT).polyd(AT) $E^{260} = 5$ and was 0.05M in Tris/HCl pH 8.0, 0.006 M in MgCl_2 and 0.001 M in mercaptoethanol. The mixture was incubated at 37 $^\circ$ for 1 hour. The reaction was stopped by cooling in ice, adding 100 μ g carrier DNA and precipitated by the addition of 3ml ice-cold 5% w/v trichloroacetic acid containing 5% v/v saturated $\text{Na}_4\text{P}_2\text{O}_7$. After standing in ice for 30min the mixture was filtered through a glass fibre filter GF/C (presoaked in 1 M HCl with 1% w/v $\text{Na}_4\text{P}_2\text{O}_7$), washed with the 5% trichloroacetic acid mixture, then with ethanol, dried at 90 $^\circ$, and counted in a liquid scintillation system.

Assay for Terminal Deoxynucleotidyl Transferase: Each assay had a total volume of 250 μ l and contained the following: 50 μ l of enzyme fraction, 30nmole ^3H -dATP (0.083 μ Ci/nmole), 15 μ l oligod(pT) $_4$ $E^{260} = 2$ and was 0.05M in cacodylate buffer pH 6.8, 0.005 M in MgCl_2 and 0.001 M in mercaptoethanol. In assays with oligod(pA) $_{12-18}$ as initiator the dATP was replaced by dGTP. The mixture was incubated for 1 hour at 37 $^\circ$ and the acid insoluble radioactivity determined as described in the DNA polymerase assay above.

Assay of Wrong Base Incorporation: The method of concurrent assays was used. Two sets of assays were carried out in triplicate. The two assays were identical in every respect apart from the deoxyribonucleoside 5'-triphosphate which was labelled with radioactivity. The assays had a total volume of 250 μ l and contained the following: 100 μ l of the pooled enzyme fractions, 3 nmole each of dATP and dTTP, 2nmole dGTP, 50 μ l polyd(AT).polyd(AT) $E^{260} = 5$ and was 0.05 M in Tris/HCl pH 8.0, 0.005 M in MgCl_2 and 0.001 M in mercaptoethanol. Assays for the measurement of total synthesis contained ^3H -dTTP (0.33 μ Ci/nmole) and assays for the measurement of wrong base incorporation contained ^3H -dGTP (12.5 μ Ci/nmole). Incubation was at 37 $^\circ$ for 2 hours. Blanks containing either no enzyme or no template were carried out at the same time. Acid insoluble radioactivity was determined as described for the DNA polymerase assay above. In these assays it was necessary to wash the GF/C filters extensively. The wrong base incorporation is expressed as G as a percentage of the total bases (A + T = 2T) incorporated.

Sephadex G-200 Chromatography: The pooled fractions from the leukaemic cytoplasmic DNA polymerases were combined, dialysed against 1 litre buffer C (0.1 M potassium phosphate pH 7.2 and 0.001 mercaptoethanol) containing 20% w/v polyethyleneglycol in order to concentrate the enzymes and then dialysed against two changes of 1 litre buffer C. The concentrated enzyme solution was applied to a Sephadex G-200 column (2cm x 25cm) equilibrated in buffer C and eluted with the same buffer. Fractions of 4ml were collected and assayed for DNA polymerase and terminal deoxynucleotidyl transferase activities. The combined cytoplasmic DNA polymerases from normal spleens were treated in a similar manner.

RESULTS

DNA Polymerases from the Phosphocellulose P-11 Column

Elution of the phosphocellulose P-11 column with a linear gradient yielded two peaks of DNA polymerase, as measured with a polyd(AT).polyd(AT) template, (see figure 1) eluting at 0.27M KCl and 0.48M KCl. These were assigned to the cytoplasmic and nuclear DNA polymerases respectively as they elute in positions similar to those found by other workers². The relative amounts of enzyme activity in the two peaks differed from sample

Figure 1. Elution of DNA polymerases (leukaemic sample 3) from phosphocellulose P-11 column.

O DNA polymerase activity

X Terminal deoxynucleotidyl transferase activity.

to sample but the activities always eluted in the same position. Assays for terminal deoxynucleotidyl transferase were also carried out. Four of the five leukaemic samples showed a peak of activity (see figure 1 and table I) eluting at 0.29 M KCl, almost coincidental with the cytoplasmic DNA polymerase. Also, there is evidence of a second, smaller peak of activity at 0.22M KCl. A similar peak of terminal deoxynucleotidyl transferase activity has recently been reported from murine thymocytes¹¹. The two control samples and one of the leukaemic samples showed no detectable terminal deoxynucleotidyl transferase activity. The four leukaemic samples were also capable of polymerising dGTP with an oligod-(pA)₁₂₋₁₈ initiator.

The terminal deoxynucleotidyl transferase activity detected in these assays is not a true measure of the amount of enzyme present, nor does the fact that the enzyme is not detectable necessarily mean that the enzyme is absent in a particular sample since glycerol, BSA and KCl, all present in the elution buffer, inhibit the enzyme and make the detection of small amounts of enzyme activity difficult.

The accuracy of both the cytoplasmic and nuclear DNA

TABLE I

ACCURACY OF CYTOPLASMIC AND NUCLEAR DNA POLYMERASES

Sample	Terminal Addition Enzyme	dG Incorporation			
		Cytoplasmic		Nuclear	
		%	1 wrong base in	%	1 wrong base in
1	+	0.12	833	0.014	7,143
2	+	0.14	714	0.010	10,000
3	+	0.97	103	0.012	8,333
4	-	0.02	5,000	0.010	10,000
5	+	0.21	476	0.015	6,667
Control 1	-	0.030	3,333	0.021	4,762
Control 2	-	0.013	7,692	0.014	7,143

+ controls were the spleens of 20 untreated 3 month old mice.

polymerases was measured using a polyd(AT).polyd(AT) template and measuring the incorporation of dGTP into newly synthesised DNA-like material. Four out of the five leukaemic cytoplasmic DNA polymerases showed an elevated level of wrong base incorporation (G) when compared with controls (see Table I). With the other leukaemic cytoplasmic DNA polymerase and all of the leukaemic nuclear DNA polymerases there was no detectable difference from the controls.

DNA Polymerases from the Sephadex G-200 Column

The Sephadex G-200 column separated the cytoplasmic DNA polymerase and the terminal deoxynucleotidyl transferase (see figure 2). The DNA polymerase eluted at 1.2 bed volumes (measured with blue dextran) and the terminal transferase at 2.5 bed volumes. Fractions containing DNA polymerase and terminal deoxynucleotidyl transferase activities were pooled separately. The activity of the terminal transferase was greatly enhanced (compared to that before Sephadex G-200 chromatography) as the procedure had removed the inhibitory glycerol, BSA and KCl. The cytoplasmic DNA polymerase from the control samples showed no detectable terminal deoxynucleotidyl transferase activity when treated in the same manner.

Figure 2. Chromatography of combined leukaemic cytoplasmic DNA polymerases on Sephadex G-200.

O DNA polymerase activity
X Terminal deoxynucleotidyl transferase activity.

The accuracy of the cytoplasmic DNA polymerase was assessed before and after the Sephadex G-200 chromatography (see Table II). The Sephadex G-200 chromatography decreased the error frequency of the leukaemic cytoplasmic DNA polymerase to the same level as the controls whilst the accuracy of the control cytoplasmic DNA polymerase was unchanged.

DISCUSSION

The results presented here show that the infidelity of the cytoplasmic DNA polymerase observed after phosphocellulose chromatography is not a property of the DNA polymerase but arises from the presence of the enzyme terminal deoxynucleotidyl transferase, which elutes from the column almost coincidentally with the cytoplasmic DNA polymerase. Sephadex G-200 chromatography separates the DNA polymerase and the terminal addition enzyme. After this separation the accuracy of the DNA polymerase has risen to that of the controls and of the nuclear DNA polymerase.

Previously Springgate and Loeb¹ have reported the presence of an error-prone DNA polymerase in crude extracts from human leukaemic lymphoblasts. Srivastava⁴, using more purified DNA polymerases concluded that there was no difference in the accuracy of the DNA polymerases from leukaemic and normal cells and proposed that the error-proneness observed by Springgate and Loeb was due to the presence of either terminal deoxy-nucleotidyl transferase, which has been shown to be present in acute leukaemic lymphoblasts², or to another unknown enzyme. The present results clarify the position; the infidelity observed for the leukaemic DNA polymerase after phosphocellulose chromatography is due to the presence

TABLE II

ACCURACY OF POOLED LEUKAEMIC CYTOPLASMIC DNA POLYMERASE BEFORE
AND AFTER SEPHADEX G-200 CHROMATOGRAPHY

	dG Incorporation	
	%	1 wrong base in
Before G-200 Chromatography	0.24	417
After G-200 Chromatography	0.018	5,556

of terminal deoxynucleotidyl transferase. Experiments involving the addition of terminal addition enzyme to purified DNA polymerases show that even small amounts of terminal addition enzyme can lower the accuracy of the DNA polymerase¹².

Terminal deoxynucleotidyl transferase is found in large quantities in the thymus¹³ and is believed to be specific for thymic or pre-thymic cells. The function of the enzyme is unknown although a role in immunological diversity has been proposed³. The enzyme has also been found to be present in large amounts in acute lymphoblastic leukaemia² and some other leukaemias^{14,15,16} which could have a T-cell component. The present finding that the enzyme is present in the MNU induced leukaemic cells in BDF1 mice is consistent with the finding of Dexter et al¹⁰ who have shown that this leukaemia is also of T-cell origin.

ACKNOWLEDGEMENTS

We wish to thank Miss Anne Cogley for skilled technical assistance. This work was supported by grants from the Cancer Research Campaign, the Medical Research Council and the Leukaemia Research Fund. One of us (L.C) was supported by the British Council under the Exchange Scientist Programme. We thank Dr T.M. Dexter for helpful advice and suggestions.

REFERENCES

1. Springgate, C.F. and Loeb, L.A. (1973) *Proc.Natl.Acad.Sci.* **70**, 245 - 249.
2. McCaffrey, R., Smoler, D.F. and Baltimore, D. (1973) *Proc.Natl.Acad.Sci.* **70**, 521 - 525.
3. Baltimore, D. (1974) *Nature* **248**, 409 - 411.
4. Srivastava, B.I.S. (1974) *Biochem.J.* **141**, 585 - 587.
5. Saffhill, R. (1974) *Biochem.Biophys.Res.Comm.* **61**, 802 - 808.
6. Gerchman, L.L. and Ludlum, D.B. (1973) *Biochim.Biophys.Acta.* **308**, 310 - 316.
7. Saffhill, R. (1974) *Biochim.Biophys.Acta.* **349**, 23 - 31.
8. Goth, R. and Rajewsky, M.F. (1974) *Proc.Natl.Acad.Sci.* **71**, 639 - 643.
9. Margison, G.P. and Kleihues, P. (1975) *Biochem.J.* **148**, 521 - 525.
10. Dexter, T.M., Schofield, R., Lajtha, L.G. and Moore, M. (1974) *Br.J.Cancer.* **30**, 325 - 331.
11. Kung, P.C., Silverstone, A.E., McCaffrey, R.P. and Baltimore, D. (1975) *J.Exp.Med.* **141**, 855 - 865.
12. Saffhill, R. (1975) unpublished results.
13. Chang, L.M.S. (1971) *Biochem.Biophys.Res.Comm.* **44**, 124 - 130.
14. Coleman, M.S., Hutton, J.J., de Simone, P. and Bollum, F.J. (1974) *Proc.Natl.Acad.Sci.* **71**, 4404 - 4408.
15. Sarin, P.S. and Gallo, R.C. (1974) *J.Biol.Chem.* **249**, 8051 - 8053.
16. McCaffrey, R., Harrison, T.A., Parkman, R. and Baltimore, D. (1975) *New Eng.J.Med.* **292**, 775 - 780.