NO INHIBITION OF ENDOGENOUS DNA POLYMERASE BY SYNTHESIS OF POLY (ADP–RIBOSE) IN NUCLEI FROM LYMPHOID CELLS

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1. Introduction

The nuclei of eukaryotic cells contain an enzyme (poly ADP-ribose polymerase) which converts NAD into poly ADP-ribose with the elimination of nicotinamide [1–4]. The chromatin-bound enzyme is absolutely dependent on DNA [1,5,6] and the product is covalently attached to chromosomal proteins [5,6]. Because of the close association of both enzyme and polymer with chromatin, there has been much speculation about the role of poly ADP-ribose as a regulator molecule. Most of the existing work on the polymer and its synthesis *in vitro* has been summarized by Shall [7].

Burzio and Koide recently showed that pre-incubation of rat liver nuclei or chromatin with NAD led to an inhibition of the ability of the nuclei or chromatin to incorporate [^{3}H] TTP into DNA in a DNA polymerase assay using the endogenous DNA as template [8]. They showed that this inhibition appeared to be caused by ADP-ribosylation of nuclear proteins, especially histones [9] and they suggested that poly ADP-ribose might act *in vivo* to inhibit DNA synthesis. Hilz and Kittler made similar observations with a variety of cell lines but expressed doubt as to the validity of this suggestion [10]. They showed that there was no correlation between the activity of the DNA polymerase using an endogenous template and the ability of the cells to synthesize DNA *in vivo*, and hence effects on such a system were of dubious relevance. In this paper we add further weight to the doubts cast by Hilz and Kittler [10]. Using nuclei from lymphoid cells, we have studied an endogenous DNA polymerase system, and we show that it has some relationship to DNA synthesis *in vivo*. The activity of this polymerase is not decreased by pre-incubation of the nuclei with NAD.

Abbreviations:

PHA = Phytohaemagglutinin;
Poly ADP-ribose = Poly adenosine diphosphate ribose.

2. Materials and methods

2.1. *Cell lines*

Mouse L5178Y lymphoma cells were grown in Fischer's medium plus 10% foetal calf serum as described in earlier work [11]. Pig lymphocytes were generously provided by Drs. A.Cooke, J.Kay and Miss T.Ahern. They were obtained from peripheral pig blood, which was defibrinated by immediate whisking. Most of the erythrocytes were removed by sedimentation under gravity at 37° in the presence of 1% dextran. The supernatant was poured into a column of cotton wool for 10 min to remove phagocytic cells. The lymphocytes and contaminating erythrocytes were then centrifuged out of the plasma, and subsequently incubated in Eagle's medium plus 15% autologous serum at a concentration of 2×10^6 lymphocytes/ml. For stimulation, phytohaemagglutinin-Q1 (PHA) was added at 3 μg/ml.

2.2. *Poly ADP-ribose polymerase assay*

Nuclei were prepared by the method of Takakusu et al. [12] and resuspended at a concentration of about 10^6 nuclei in 0.1–0.3 ml of 81 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 3.3 mM KF, 50 mM KCl, 3.3 mM dithiothreitol. The reaction was started by adding 1.7 μCi/ml [^{3}H] NAD (1.13 Ci/mmole, 1.5 μM) and placing the suspension at 25°. At various times the reaction was stopped with 5%

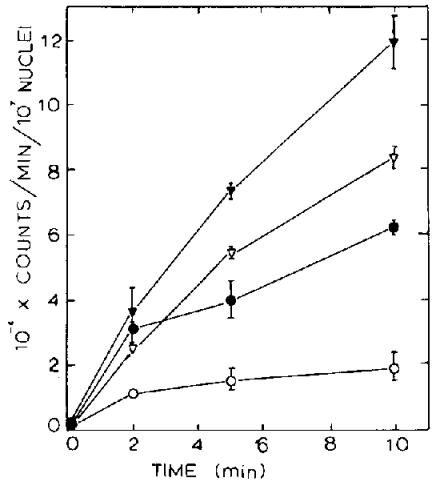

Fig. 1. Uptake of NAD by isolated nuclei. Nuclei from rat liver (▽–▽–▽), L5178Y (▼–▼–▼), unstimulated (o–o–o) or 48 hr-stimulated (●–●–●) pig lymphocytes were incubated with [^{3}H] NAD for various time periods. The reaction was stopped with 5% trichloracetic acid. Error bars indicate variations between triplicates.

trichloracetic acid and after 30 min the acid-precipitable radioactivity was measured by filtration on glass-fibre discs.

2.3. *DNA polymerase assay*

After incubation of the nuclei in the above buffer for 10 min at 25° with or without (unlabelled) NAD, they were spun down and resuspended in 0.2 ml of 60 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 5 mM mercaptoethanol, 5 mM ATP. dATP, dCTP, dGTP, if present, were at 0.5 mM and [^{3}H] TTP at 16 μCi/ml (10 Ci/mmole, 1.6 μM) [13].

After 30 min incubation at 37° 2 ml 5% trichloracetic acid and 0.2 ml carrier calf thymus DNA (2 mg/ml) were added. The precipitates were spun down, washed three times with 5% trichloracetic acid, once with methanol, dried and dissolved in 0.2 ml hyamine at 60°. The radioactivity was measured in a Beckman liquid scintillation counter. All assays were done in triplicate.

3. Results and discussion

Mouse lymphoma L5178Y is a highly anaplastic cell line which grows rapidly in culture with a generation time of about 12 hr. Pig lymphocytes, like human lymphocytes, do not normally synthesize DNA nor do they divide. Addition of PHA stimulates pig lymphocytes to synthesize RNA, protein and subsequently DNA [14]. Synthesis of the latter commences about 24 hr after stimulation.

3.1. *Poly ADP-ribose polymerase*

PHA-stimulated pig lymphocytes and mouse L5178Y lymphoma cells had an active poly ADP-ribose polymerase system (fig.1). The enzyme activity, expressed as amount of NAD incorporated per minute per nucleus, was of the same order as that in rat liver (fig.1). The level in unstimulated lymphocytes was in general about one third of that in 48 hr-stimulated lymphocytes taken from the same animal, although there was some variability in measured levels of incorporation in different experiments.

3.2. *Lack of inhibition of DNA polymerase by pre-incubation with NAD*

Burzio and Koide [8] found that incubation of rat liver nuclei with NAD caused an inhibition of subsequent incorporation of TTP in a DNA polymerase assay using endogenous DNA as template. We have confirmed this observation. Table 1 shows the results of a similar experiment with lymphoid cell nuclei. The third column (△) for each cell line shows that there was no significant inhibition of TTP incorporation by preincubation of the nuclei with NAD. With lymphocytes, no inhibition was observed at 4 or 10 mM NAD. With the lymphoma there was no inhibition at 4 mM, possibly a 15% inhibition with 10 mM NAD.

3.3. *Relationship of DNA polymerase to DNA synthesis*

In the liver systems used by Burzio and Koide [8] and Hilz and Kittler [10], the levels of DNA polymerase in neonatal and adult liver were very similar [10] as were those in normal and regenerating liver [8] despite the fact that the rates of DNA synthesis *in vivo* were very different. In contrast, in pig lymphocytes some correlation between DNA polymerase levels and DNA synthesis rates was obtained. Resting lymphocytes had a very low basal level of DNA synthesis. On stimulation with PHA, DNA

Table 1
Effect of NAD on DNA polymerase in isolated nuclei.

Conc. of NAD (mM)	Unstimulated lymphocytes			48 hr-stimulated lymphocytes			L5178Y		
	−	+	Δ	−	+	Δ	−	+	Δ
0	237	507	270	719	2350	1630	1440	7854	6414
4	227	563	336	774	2572	1799	1452	8148	6796
10	199	535	336	807	2657	1850	1640	6284	4644

Nuclei were incubated at 25° with the indicated concentration of NAD for 10 min, spun down and incubated at 37° for 30 min in the DNA polymerase assay mixture containing [^{3}H] TTP with (+) or without (−) the other three nucleotide triphosphates. The figures given are the counts per minute per 10^6 nuclei. The third column for each cell line (Δ) gives the difference between the (+) and (−) columns.

synthesis commenced after about 24 hr and in our hands reached a maximum level, as judged by incorporation of [^{3}H] thymidine, at about 48 hr (unpublished observations). Correspondingly, the level of DNA polymerase in isolated nuclei at 48 hr in three separate experiments was four to five times greater than that in nuclei from unstimulated cells (see table 1). There was, however, a significant basal level in unstimulated cells although the DNA synthesis rate *in vivo* was very low indeed.

With the mouse lymphoma cells a different type of experiment was carried out. In this experiment it was shown that the DNA made *in vitro* by the isolated nuclei was attached to the growing DNA strands being made *in vivo* before isolation of the nuclei. The experiment was similar to that performed by Lynch et al. [13] on regenerating rat liver. Cells were grown in the heavy thymidine analogue, bromodeoxyuridine (BU), nuclei isolated and incubated with [^{3}H] TTP. The density shift of the ^{3}H-label, seen on centrifugation of the DNA in alkaline cesium chloride (fig.2d) shows that the incorporated TTP is attached to the BU-substituted segment of DNA. A similar shift was seen with DNA synthesis *in vivo* (fig.2b) but no shift was seen if the cells were not first incubated with bromodeoxyuridine (compare figs.2a,c), i.e. the incorporation in isolated nuclei represents elongation of growing strands.

In rat liver, poly ADP-ribose production inhibited DNA polymerase [9,10] in an assay which did *not*

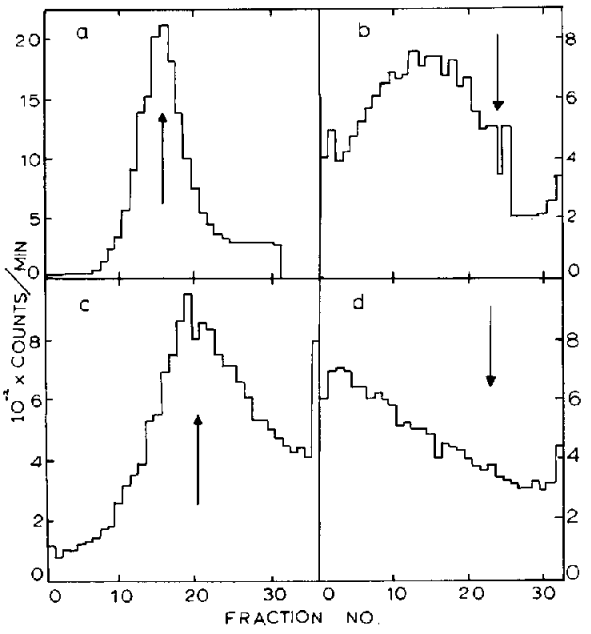

Fig. 2. Attachment of DNA made *in vitro* to that made previously *in vivo* [13]. L5178Y cells were incubated with or without 20 μM bromodeoxyuridine, 10 μM deoxycytidine. After washing out the bromodeoxyuridine, a part of the culture was pulse-labelled for 2½ min with [^{3}H] thymidine and nuclei were subsequently prepared. From the rest of the culture, nuclei were isolated and incubated with [^{3}H] TTP for 10 min in the complete DNA polymerase system. All nuclei were lysed in 1 ml 0.1 M NaOH, 0.01 M EDTA and sheared through a Pasteur pipette and a 26 G hypodermic needle. 2.4 ml of water and about 4.9 g CsCl were added to give a refractive index of about 1.4065. The alkaline CsCl solutions were centrifuged at 42,000 rpm for 45 hr in a 50 Ti rotor in a Beckman L2 centrifuge, and fractions collected on Whatman grade 17 paper strips and counted for radioactivity [15]. DNA labelled *in vivo* (a,b) or in isolated nuclei (c,d). Incubated *in vivo* without (a,c) or with (b,d) bromodeoxyuridine. Centrifugation is from right to left. Arrows show the position of [^{14}C] T4 DNA marker.

reflect DNA synthesis *in vivo* [10]. It remains to be seen whether this inhibition would still be seen in a more meaningful system such as that used by Lynch et al. with regenerating rat liver [13]. In both the lymphocytes and the lymphoma cells used in our experiments, endogenous nuclear DNA polymerase activity as measured by our assay did bear some relationship to DNA synthesis *in vivo*. In neither the normal nor the cancer cells did preincubation with NAD inhibit the incorporation of TTP al-

though the nuclei had active poly ADP-ribose polymerases. Thus if poly ADP-ribose production does in some cases inhibit DNA polymerase activity, this is not a general phenomenon.

Even with the more meaningful DNA polymerase assays used by ourselves and especially the carefully tested system of Lynch et al. [13], it is still uncertain whether extrapolations of effects on these systems can be made to DNA synthesis *in vivo*. Even in the best nuclear systems studied, the rate of polymerisation was only about 1–2% of the rate of DNA synthesis *in vivo* and furthermore it ceased fairly soon after commencement of incorporation. It can be calculated that only a few tens of nucleotides are synthesized on each growing strand. Such *in vitro* systems may therefore be of limited use in the study of important control mechanisms of *in vitro* DNA synthesis.

Finally, Hilz and Kittler [10] showed that the activity of poly ADP–ribose polymerase in the nuclei of liver from rats of various ages was proportional to the amount of DNA in the nuclei. They suggested that poly ADP-ribose may have a structural role in the nucleus. Preliminary observations in the pig lymphocyte system and also during the growth cycle of L cells (unpublished observations of P.Stone), show that the activity of the enzyme is not in fact proportional to the DNA content of these cells under different conditions. A similar conclusion can be drawn from the results obtained by Haines et al. [16] with different classes of rat liver nuclei isolated by zonal centrifugation. Hence the relationship, if any, of poly ADP-ribose and its polymerase to DNA synthesis remains to be elucidated.

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