DNA POLYMERASES FROM NON STIMULATED AND PHYTOHEMAGGLUTININ STIMULATED NORMAL HUMAN LYMPHOCYTES

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SUMMARY: The presence and some properties of DNA polymerases isolated from normal human lymphocytes, non stimulated and stimulated by phytohemagglutinin, are described. In the non stimulated lymphocytes two cytoplasmic DNA polymerases are found, one eluting from DEAE cellulose at 0.07 M NaCl (CI_n) and the other at 0.13 M NaCl (CII_n). In the nuclear soluble fraction only one enzyme activity is found (NI_n) which does not adsorb to DEAE cellulose. In the cytoplasm of stimulated lymphocytes only one enzyme activity is detected (CI_s) which elutes from DEAE cellulose at 0.12 M NaCl. The nuclear soluble fraction contains two activities, NI_s, which does not adsorb to DEAE cellulose, and NII_s, which elutes from DEAE cellulose at 0.07 M NaCl. Some properties of the different enzymes are described which indicate that NI_n and NI_s enzymes are clearly different from the others.

The presence of several DNA dependent DNA polymerases in eukaryotic cells has been extensively reported. In general, two kinds of enzymes have been found, the large and the low molecular weight types (1). Weissbach et al. (2) have reported the presence of two DNA dependent DNA polymerases in the nuclei and only one enzymatic activity in the cytoplasm of cultured human cells. The cytoplasmic and one of the nuclear enzymes are of the large molecular weight type, whereas the other nuclear enzyme has a small molecular weight. Similar results have been reported in KB cells (3). However, the function of these enzymes in vivo is still unclear. Until conditional mutants of eukaryotic cells are available, the correlation between biochemical evidence and function must rely on circumstancial evidence. The large molecular weight enzymes appear to be correlated with the mitotic state of the cell. Thus, they have been observed to increase in regenerating liver (4), mouse L cells (5), developing rat brain (6) and embryonic muscle cells (7). On the other

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Abbreviations: PHA: phytohemagglutinin; BSA: bovine serum albumin; DTT: dithiotreitol; PCA: perchloric acid; dNTPs: deoxynucleoside triphosphates.

hand. there is no evidence on the possible role of the small molecular weight nuclear enzyme. In order to study the events related to DNA replication, a useful system should permit the transition from a resting to an active duplication state by well defined means. It has been widely reported that peripheral blood lymphocytes can be stimulated to divide by agents such as phytohemagglutinin (PHA) (8). In the case of stimulated human lymphocytes, two distinct DNA dependent DNA polymerases have been described (9). However, their subcellular distribution in the stimulated cells as well as their presence and characteristics in the normal non stimulated human lymphocytes is still unknown. In a previous report (10) we have demonstrated that nuclei isolated from non stimulated lymphocytes could incorporate dNTPs equally well as the nuclei isolated from PHA stimulated cells. We also showed that considerable amounts of DNA polymerase activity existed in the nuclei and cytoplasm of non stimulated cells.

In this paper we wish to report some characteristics of DNA dependent DNA polymerases isolated from non stimulated lymphocytes and from PHA stimulated cells.

MATERIALS AND METHODS. Lymphocyte preparation · Lymphocytes isolated from normal human peripheral blood were purified, cultured and stimulated as previously described (10). The degree of transformation by PHA was assessed by microscopic observation, demonstrating a high percentage of blast formation, and by $[^3H]$ dT incorporation into DNA.

Enzyme preparation. The preparation of the cytoplasmic extract and nuclear soluble fraction have been described (10).

Measurement of DNA polymerase activity. Unless otherwise indicated, the reaction mixture contained, in a final volume of 0.1 ml, 50 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.9 mg/ml bovine serum albumin (BSA), 5 mM dithiotreitol (DTT), 62 µg of "activated" calf thymus DNA (2), 0.1 mM each dCTP, dGTP, dATP, 0.01 mM $[^3H]$dTTP (sp. act. 1000 cpm/pmole) and 20 µl of enzyme. The reaction mixture was incubated at 37° for 45 min and the radioactivity

incorporated into a perchloric acid (PCA) insoluble fraction was determined (10).

Protein determination. It was done according to Lowry et al. (11).

Column chromatography on DEAE cellulose. An 11 x 0.9 cm column of DE52 (Whatman), was equilibrated with a buffer containing 50 mM Tris pH 7.8, 1 mM DTT, 1 mM EDTA and 10 percent glycerol (TEDG buffer). The enzymatic extract, containing 10-20 mg of protein, was applied to the column. After washing with 5 ml of TEDG buffer, a 0 to 0.3 M NaCl (in TEDG) gradient was applied. Samples of 1.2 ml were collected and enzyme activity was determined.

RESULTS. DNA polymerases from non stimulated lymphocytes. The DNA polymerase activities from the cytoplasmic extract and the soluble nuclear fraction were analyzed by DE52 column chromatography. In the cytoplasmic fraction (Fig. 1 A) the presence of two peaks of activity, designed CI_n and CII_n, were observed. The CI_n peak eluted around 0.07 M NaCl and the CII_n peak, around 0.13 M NaCl. The soluble nuclear fraction (Fig. 1 B) showed a single peak of activity, designed as NI_n, which did not adsorb to DE52.

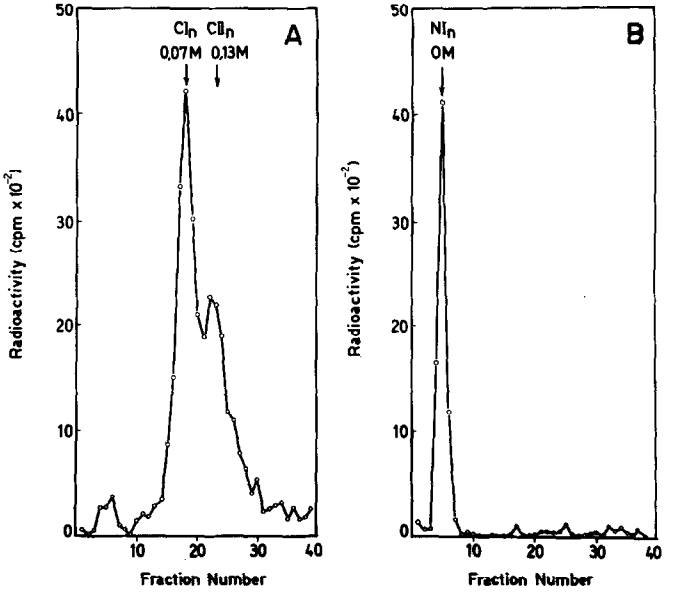

Fig. 1. DNA polymerase DNA dependent activities from non stimulated lymphocytes. The cytoplasmic (A) and nuclear soluble fractions (B) were chromatographed on DE52 cellulose. The enzyme activity was measured as described in Methods.

DNA polymerases from stimulated lymphocytes. The pattern of elution from DE52 column chromatography of the cytoplasmic and nuclear fractions is shown in Fig. 2. In the cytoplasm a single peak of activity, designed as CI_s and eluting at about 0.12 M NaCl, is observed (Fig. 2 A). In the soluble nuclear fraction two peaks of activity, designed as NI_s and NII_s, appear (Fig. 2 B). As well as in the non stimulated lymphocytes, the NI_s peak does not adsorb to DE52, while the second peak, which is larger than NI_s, elutes at about 0.07 M NaCl.

Requirements of the DNA polymerases. Some requirements of the DNA polymerase reaction by the different enzyme fractions are shown in Table I. This study was done with enzymes purified by DE52 column chromatography. From this table it can be seen that some properties from the NI enzyme from either stimulated or non stimulated cells, like its BSA requirement and salt dependence, are different from those of the other enzymatic activities. This difference is also observed in their optimum pH values. For the cytoplasmic

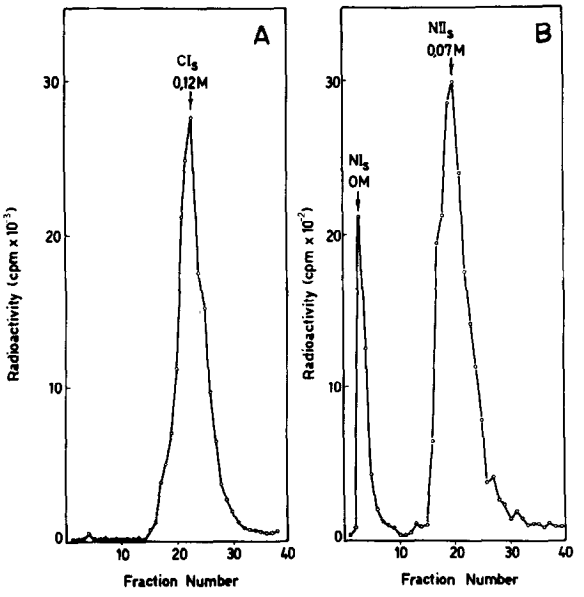

Fig. 2. DNA polymerase DNA dependent activities from stimulated lymphocytes. The cytoplasmic (A) and nuclear soluble fractions (B) were chromatographed on DE52 cellulose. The enzyme activity was measured as described in Methods.

Table I. Requirements of the DNA polymerase reaction

Conditions	Enzyme (percent of activity)					
	CI_n	CII_n	NI_n***	CI_s	NI_s***	NII_s
Complete	100	100	100	100	100	100
Omit Mg^{++}, add 0.5mM Mn^{++}	10	12	28	28	24	2
Omit BSA	37	43	86	5	92	3
Omit DTT*	36	73	66	26	55	29
KCl 0.2 M	38	33	102	13	139	32
o-phenantroline**						
1mM	36	35	68	84	74	59
2.5mM	16	17	37	59	27	29

* The 20 µl of enzyme makes the reaction mixture 0.2mM DTT.
** The inhibition by o-phenantroline was measured with 5 µg of "activated" DNA and 10mM $MgCl_2$.
***The requirements of NI_n and NI_s were tested at pH 9.
The assay for DNA polymerase activity is described in METHODS.
The enzymes used were the peak activities obtained after DEAE column chromatography.

and NII enzymes, the optimum pH is 8.0. In the case of NI enzymes the activity increases at least up to pH 9.7, however they were routinely assayed at pH 9.0 since the buffers used to obtain higher pH values were inhibitory with respect to Tris-HCl buffer. From Table I it can also be observed that the cytoplasmic and the NII enzymes have similar properties. The substrate specificity is shown in Table II. It can be seen that maximal $[^3H]$TTP incorporation is dependent of the presence of the other dNTPs for all the DNA polymerases except for NI_n and NI_s. The analysis of several DNA substrates showed that all the enzymes prefer "activated" DNA over native or heat denatured DNA. It is also seen in this Table that the reaction is sensitive to DNAse but insensitive to RNAse action. In all cases 1 mM ATP slightly affects the reaction.

DISCUSSION. Most of the DNA dependent DNA polymerase activity of the cell is detected in the cytoplasm and nucleus (2). In this paper we report two

Table II. Substrate requirements of DNA polymerases.

Conditions	Enzyme (percent of activity)					
	CI_n	CII_n	NI_n	CI_s	NI_s	NII_s
Complete	100	100	100	100	100	100
Omit "activated" DNA, plus native DNA (62 µg)	17	13	29	3	19	10
Omit "activated" DNA, plus heated DNA (62 µg)	0	0	0	0	8	0
Omit dATP,dCTP, dGTP	20	40	93	22	122	34
DNAse (10 µg/ml)	16	20	15	1	1	4
RNAse (200 µg/ml)	108	99	104	100	98	106
1mM ATP	81	78	94	125	113	79

Methods and conditions were the same as described in Table I.

main differences in the distribution of DNA polymerase activities between the non stimulated and stimulated human lymphocytes. The analysis of cytoplasmic extracts by DE52 column chromatography showed two peaks of activity in non stimulated cells and a single peak of activity in stimulated lymphocytes. Another difference is the appearance of a second peak of activity (NII_s) in the nuclei of stimulated cells. Several possibilities may be envisaged to explain the disappearance of one peak in the cytoplasm of stimulated cells. Based on the similarities between the CI_n and the NII_s enzymes it is tempting to speculate that the difference observed is due to the transport of CI_n enzyme into the nuclei. The interconversion between enzymatic species of DNA polymerases (12), which could take place when a cell begins to actively synthesize DNA could also explain the difference observed. Another possibility is that CI_n and CII_n are present in different types of lymphocytes. It has been reported that in human peripheral blood there are several types of lymphocytes (13). Since only T lymphocytes are stimulated by soluble PHA (14)

it is not unreasonable to assume that any enzymatic activity not present in T lymphocytes would be proportionally smaller at the end of 72 hrs of PHA stimulation.

Since the NII_s enzyme is only present in PHA stimulated cells, it is tempting to correlate this activity with the DNA replicating enzyme. However, this observation is difficult to reconcile with the fact that the nuclei from non stimulated lymphocytes where the NII_s enzyme is lacking, incorporate dNTPs as well as the nuclei from stimulated lymphocytes (10). Although the simplest assumption is that this incorporation is due to the NI enzyme, further work is needed to understand the nature of this discrepancy.

The NI enzymes, isolated both from stimulated and non stimulated lymphocytes, have properties clearly different from the other DNA polymerases. The fact that they incorporate a single dNTP in the absence of the other three is a common property with the terminal deoxynucleotidyl transferase, an activity which has been only isolated from calf thymus gland. However, they differ from this enzyme in their substrate specificity, optimal pH and ATP inhibition (15).

At present, work is in progress in order to understand the possible physiological role of these enzymes in DNA biosynthesis.

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