# INDUCTION OF POLYNUCLEOTIDE LIGASE IN HUMAN LYMPHOCYTES STIMULATED BY PHYTOHEMOAGGLUTININ

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## SUMMARY

The treatment of human lymphocytes with phytohemoagglutinin causes the appearance of the activity of polynucleotide ligase, rising at least 50 fold from levels below the background. This increase takes place in the fourth or fifth day after treatment, and is delayed by one day approximately with respect to the rise of DNA synthesis rate; the activity of two other enzymes of DNA metabolism, DNA polymerase and a DNase acting on single-stranded DNA, increases in parallel with the DNA synthesis rate.

## INTRODUCTION

The treatment of human leukocytes with phytohemoagglutinin (PHA) leads to the transformation of the small lymphocytes into large cells that synthesize actively DNA and multiply in culture, whereas no modifications are observed in untreated cultures (1). The rate of thymidine incorporation rises more than one hundred-fold over the control levels, reaching its maximum in the third or fourth day of incubation. Loeb et al. (2) and Rabinowitz et al. (3)have shown that the

DNA polymerase levels of cultured lymphocytes rise in parallel (2)or slightly before (3) the rise in DNA synthesis rate, as detected by thymidine pulses. We have extended similar observations to two other enzymes of DNA metabolism. In this paper we report the data for a DNase specific for single-stranded DNA and for polynucleotide ligase, as well as for DNA polymerase and the rate of DNA synthesis. We have observed that also polynucleotide ligase activity rises by about 100-fold over the control levels, but its rise is delayed by one day with respect to the rise of DNA synthesis and of DNA polymerase. The DNase activity increases instead in parallel with DNA synthesis and DNA polymerase.

## MATERIALS AND METHODS

### Cultures

To 300 ml of peripheral blood from a healthy male, haeparin (sodium salt, Fluka Corp., Switzerland) was added to a final concentration of 10 mg/ml, and the blood was divided into 30 ml aliquots from which the white cells were separated by spontaneous sedimentation at room temperature for about two hours. 10 ml cultures containing approximately $2 \times 10^6$ white cells/ml in Eagle's medium supplemented with 10% calf serum and 10% of autologous plasma, were incubated at 37°C. The appropriate cultures were treated with 0.01 ml/ml of phytohemoagglutinin (Difco). At the indicated times the cells were either assayed for DNA synthesis rate or collected by centrifugation, washed twice with cold balanced saline solution (Hanks BSS) and once with 0.35 M sucrose, containing 0.05 M Tris-HCl pH 7.5, 0.025 M KCl, 6 mM $MgCl_2$, 5mM $\beta$-mercaptoethanol, 1 mM reduced glutathione, and stored at -20°C.

### DNA synthesis

DNA synthesis was followed by $H^3$-thymidine incorporation into acid-precipitable material. $H^3$-methyl-thymidine (Radiochemical Centre Amersham, 20 Ci/mmole) was added at a concentration of 10 $\mu$Ci/ml one hour before harvesting the cells; the suspension was then diluted into 20 ml of cold Hanks supplemented with 5 $\mu$g/ml of sodium azide

and the cells were centrifuged, washed with Hanks and frozen at -20°C. 24 hours later the cells were thawed and lysed by incubation in 1 ml of 0.2 N NaOH, 0.01 M EDTA at 37°C for 45 minutes. The macromolecules were precipitated with 0.5 ml of cold 7% PCA and 2 ml of cold $H_2O$, collected by centrifugation, washed twice by resuspension in 0.2 ml of 2 N NaOH and finally collected by acid precipitation on Whatman GF/C glass filter; after washing and drying, the radioactivity remaining on filter was measured.

Cell extracts

Cell extracts were prepared by diluting approximately 0.2 g of thawed cells in 0.8 ml of 0.05 M $NaHCO_3$ pH 9,5 mM EDTA; the cells were homogenized by pipetting them for 5 minutes through a 0.5 ml pipette. After centrifugation for 10 minutes at 17,000 x g, the supernatant was collected and dialyzed against 0.05 M phosphate buffer pH 7.5, 2.5 mM EDTA and 6 mM $\beta$-mercaptoethanol. If a precipitate was observed, it was eliminated by centrifugation at 17,000 x g. The protein content of the extracts varied from 1 to 3 mg/ml.

Enzymic assays

Within three days from the extraction the following enzymic assays were performed: DNA polymerase according to Gold and Helleiner (4), using single-stranded calf thymus DNA as template; polynucleotide ligase as described by Spadari et al.(5) using the interrupted poly(dT)-poly(dA) pair bound to cellulose; denatured DNA DNase as follows: 0.25 ml incubation mixtures containing 40 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 6 mM $\beta$mercaptoethanol, 0.02 mM heat-denatured $^3$H-DNA from Bacillus subtilis (specific radioactivity $2.3 \times 10^7$ cpm/$\mu$mole) were incubated at 37°C for 30 minutes; the reaction was stopped by adding 0.2 ml of calf thymus DNA (660 $\mu$g/ml) and 0.5 ml of cold 10% TCA. After centrifugation 0.5 ml of the supernatant were added to 20 ml of dioxane scintillation fluid in a counting vial. One unit of DNase is the amount of enzyme able to solubilize 10 nmoles of $^3$H-DNA in the assay conditions.

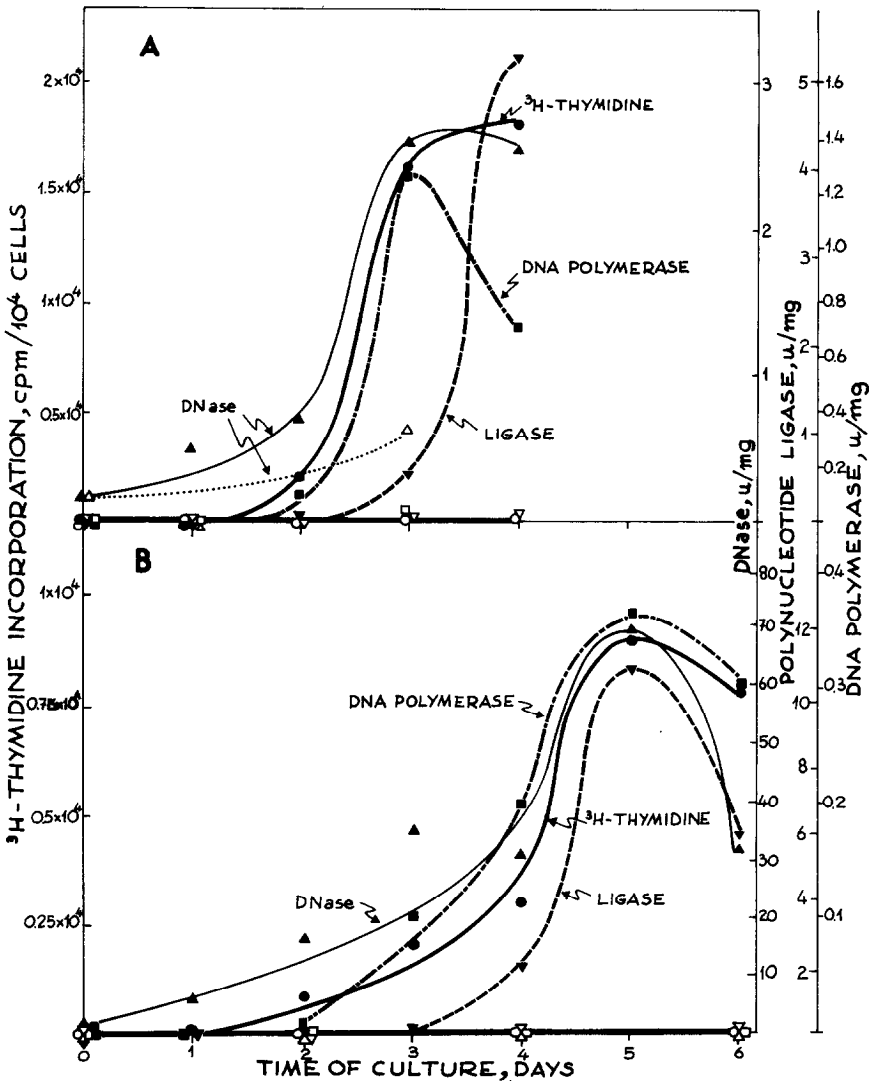


Fig. 1 - Activity of some enzymes of DNA metabolism in human lymphocytes after PHA stimulation.

Cultures of human leukocytes were treated with PHA and the cells were assayed at the indicated times for DNA synthesis rate and for the levels of polynucleotide ligase, of DNA polymerase and of a DNase acting on denatured DNA by the procedure described in the text. The empty symbols correspond to the unstimulated controls. A and B correspond to two experiments performed with blood from two different patients.

## RESULTS AND DISCUSSION

Fig. 1 (A and B) reports the results of experiments performed with blood samples from two different subjects. Thymidine incorporation was followed by 1 hour pulses; from the level of 50 cpm/$10^6$ cells it rises more than 300-fold at the fourth (A) or the fifth (B) day after stimulation, whereas no significant change takes place in the controls without PHA. DNA polymerase is at a level very close to (A) or lower than (B) the background and it rises at least one hundred-fold reaching a maximum in the third (A) or in the fifth (B) day. Again, no significant change took place in the controls. A nucleolytic activity relatively specific for single-stranded DNA (in the sense that with native DNA its velocity is reduced to one fifth) is observed also in non-stimulated cultures; after stimulation, the activity is increased by a factor of 15 (A) or 50 (B), reaching a maximum in the third (A) or in the fifth (B) day.

Polynucleotide ligase from levels below background rises by a factor of at least 50 (A) or 100 (B) in the fourth (A) or in the fifth (B) day. No ligase activity over the background is observed in the control.

The data for DNA polymerase are in agreement essentially with those of Loeb et al., (2) insofar as the rise in activity paralleles the rise in DNA synthesis. The nucleolytic activity on single-stranded DNA (most probably corresponding to the DNase III described in rabbit bone marrow by Lindahl et al. (6)) is also enhanced during the stimulation, and it seems to parallel the other two phenomena. The ligase activity is instead atypical since it rises later than the two other enzymes and than the rate of DNA synthesis.

The data reported are qualitatively very reproducible for the three enzymes studied; a certain variability is observed in the time at which the maximum of thymidine incorporation rate is reached, ranging between the third and the fifth day; the latter occurrence (such as reported in B) is rather rare, patterns like those of A being more frequent.

The relatively strict parallelism of DNA polymerase level with DNA synthesis rate is suggestive of a function of the former in the latter, as inferred also by the cited authors (2, 3). Less obvious is the reason why the amount of a nucleolytic enzyme should parallel DNA synthesis: it is worth recalling that also in bacteria the level of an enzyme very similar to DNase III, namely the exonuclease I of E. coli, reaches its maximum during the logarithmic phase of growth (7).

Ligase is implicated in the current models of DNA replication and its involvement in that process has been satisfactorily proven in phage $T_4$ (8) and in E. coli (9). The delay in its rise with respect to DNA synthesis is somewhat puzzling; of course it is possible (and likely) that the enzyme is present at the early times of increase of DNA synthesis but below the threshold of sensitivity of our assay; nonetheless, it remains to explain why it should not parallel the other possibly involved enzymes; perhaps it has an important function also in processes of DNA repair taking place at later times. Clearer indications of the function of the enzyme may conceivably be acquired by extending our study to other related enzymes and to longer stimulation times.

The observations of Loeb et al.(2), of Rabinowitz et al. (3) and ours are in any case also of a certain practical interest: who is interested in sampling the levels or the quality of enzymes acting on DNA in human subjects affected by inhereted diseases possibly involving DNA metabolism (10), can find an easily obtainable source of enzymes in stimulated cultures of leukocytes.

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