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DNA Polymerase and DNA Replication during Lymphocyte Transformation[1]

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SUMMARY

The initiation of DNA synthesis in human peripheral blood lymphocytes is coincident with the induction of DNA polymerase activity. Phytohemagglutinin was added to cultures of lymphocytes, and, at sequential times thereafter, incorporation of thymidine into DNA and various enzymatic activities in the DNA synthetic pathway were determined. An increase of 30- to 150-fold in DNA polymerase activity paralleled in time and magnitude the ability of the cells to synthesize DNA. An increase in DNase activity also paralleled DNA synthesis and the rise in polymerase. The activities of thymidine kinase and thymidine monophosphate kinase multiplied about 2- to 10-fold. In contrast, kinases such as deoxyguanosine monophosphate kinase and guanosine monophosphate kinase, which were present in greater activity, were not increased by phytohemagglutinin.

The induction of these enzyme activities by phytohemagglutinin appears to require RNA synthesis. Actinomycin D in sufficient amounts abolished the phytohemagglutinin-mediated increases in RNA and DNA synthesis, enzyme activities, and lymphocyte transformation. Smaller amounts of actinomycin prevented the induction of DNA polymerase, thymidine kinase, and thymidine monophosphate kinase, as well as the increase in RNA and DNA synthesis, but did not hinder lymphocyte transformation. Accordingly, the morphological changes characterizing lymphocyte transformation do not require DNA replication or increases in enzyme activities associated with this replication. In contrast, the stimulation of DNA synthesis is rigidly dependent on the induction of DNA polymerase.

INTRODUCTION

The initiation of DNA synthesis in nonproliferating mammalian cells has been studied almost exclusively in whole animal systems (4). Exceptions are those of stationary cell cultures infected with oncogenic viruses and those of organ cultures (4). The present study deals with the initiation of replication in cultures of human lymphocytes by PHA.[4] This system is particularly advantageous for studying the control of DNA synthesis since metabolic changes can be affected and monitored entirely *in vitro*. Furthermore, the influence of genetic factors and various disease states can be investigated.

Human peripheral blood lymphocytes are thought to be well differentiated cells at the end stage in lymphoid maturation. They seldom grow or divide *in vivo*. This quiescent behavior is usually maintained when these cells are cultured. However, the addition of PHA [an extract of the kidney bean (*Phaseolus vulgaris*)] to lymphocyte cultures causes a striking transformation: as many as 90% of the cells enlarge, synthesize DNA, divide, and assume a less differentiated appearance (21). A similar but less extensive transformation is brought about by a variety of other mitogens as well as appropriate immunological stimuli (22). Alterations in RNA (6), lipid (13), protein (12), nuclear phosphoprotein (16), and histone (24) metabolism have been reported to occur prior to the onset of DNA replication. We have started to study in detail the control mechanism for the initiation of DNA synthesis during lymphocyte transformation. This report is on the relationship of enzymes governing DNA synthesis (DNA polymerase, thymidine kinase, TMP kinase, dGMP kinase) and degradation (DNase) to DNA replication during lymphocyte transformation.

MATERIALS AND METHODS

Cell Culture and Thymidine Incorporation

Lymphocytes were isolated from the blood of healthy volunteers by the method of Bach and Hirschhorn (3). The final preparation was suspended at a concentration of 7.5×10^5 cells/ml in Eagle's minimal essential medium (Spinner modification) (Grand Island Biological Co., Grand Island, N. Y.), containing 20% fetal calf serum, 1% L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Portions

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[4]The abbreviations used are: PHA, phytohemagglutinin; DEAE, diethylaminoethyl.

(2 ml) of the cell suspension were incubated at 37° in the absence and presence of 0.05 ml of PHA-M (General Biochemicals Corp., Chagrin Falls, Ohio) in 16- x 125-mm disposable plastic culture tubes.

One hr prior to harvesting, 5.0 μCi of methylthymidine-^{3}H (Schwarz BioResearch, Inc., Orangeburg, N. Y.; 11 Ci/mmole) were added to each culture. Incorporation of labeled thymidine was terminated by adding 10 μmoles of unlabeled thymidine and placing the cultures in an ice water bath. The cells were then centrifuged for 10 min at 2500 X *g* and washed with 2.0 ml of 0.15 M potassium chloride. To the final pellet were added 0.5 ml of a solution made up of 20% (w/v) glycerol (Matheson, Coleman and Bell, East Rutherford, N. J., Spectroquality grade), 0.02 M potassium phosphate buffer (pH 7.4), 0.001 M potassium EDTA, and 0.004 M reduced glutathione. After freezing and thawing twice, the disrupted cell preparations were used for enzyme assays. The amount of radioactivity incorporated into acid-insoluble material was determined concurrently with the assay of DNA polymerase activity (1).

Enzyme Assays

DNA Polymerase Activity. The assay for DNA polymerase activity measured the incorporation of an appropriately labeled deoxynucleoside triphosphate into an acid-insoluble product (26). The reaction mixture, in a total volume of 0.3 ml, consisted of the following: 25 μmoles Tris-maleate buffer, pH 8.0; 3 μmoles magnesium chloride; 1 μmole potassium chloride; 0.3 μmole β-mercaptoethanol; 25 mμmoles each of dATP, dCTP, and dGTP; 10 mμmoles of dTTP-[α-^{32}P] (approximately 5 X 10^4 dpm/mμmole) (International Chemical Nuclear Corp., City of Industry, Calif.); 266 mμmoles "maximally activated" calf thymus DNA (19); and 0.05 ml of the lymphocyte preparation. Incubation was for 1 hr at 37°, and the reaction was stopped by adding 0.5 ml of cold 1 M perchloric acid containing 0.01 M sodium pyrophosphate. With each group of reaction mixtures, a known amount of purified sea urchin nuclear DNA polymerase (19) was assayed simultaneously. The acid-insoluble material was collected on glass fiber filters, and its radioactivity was determined by standard dual labeling techniques with liquid scintillation spectroscopy. After corrections for crossover, the radioactivity in the tritium channel represents the amount of thymidine-^{3}H which the cells had incorporated in culture. In control experiments, there was no decrease in this value during the DNA polymerase reaction. The amount of radioactivity in the ^{32}P channel represents DNA polymerase activity as measured by the incorporation of residues of TMP-[α-^{32}P] into an acid-insoluble product.

DNase Activity. DNase activity was assayed by measuring the amount of added DNA-^{3}H rendered acid soluble. The incubation mixture contained in a volume of 0.08 ml in culture tubes (6 x 50 mm) the following: 20 mμmoles of partially degraded sea urchin DNA-^{3}H, 3639 dpm/mμmole; 8 μmoles of glycine-NaOH buffer, pH 8.5; 0.66 μmole of $MgCl_2$; and 0.04 ml of lymphocyte preparation which had been cultured in the absence of radioactive precursors. After incubation for 2 hr at 37°, the reaction was stopped by cooling in an ice water bath. Then 0.1 ml of native calf thymus DNA (2 mg/ml in 0.02 M KCl) and 0.2 ml of 0.5 N perchloric acid were added to each tube. After vigorous mixing and cooling for 20 min in an ice bath, the digests were centrifuged at 8000 X *g* for 20 min. Thereafter, 250 μl of the supernatants containing acid-soluble constituents were pipetted into glass vials containing 1.5 ml of 1.5 M Hyamine chloride (Packard Instrument Co., Downer's Grove, Ill.) in methanol, followed by 17 ml of a toluene-phosphor mixture, and radioactivity was determined.

Nucleotide Kinase Activity. The rates of phosphorylations of ^{14}C-labeled nucleoside monophosphates were determined by the method of Furlong (9). Unlike the monophosphate precursor, the nucleoside diphosphates and triphosphates produced by these reactions are resistant to the action of semen phosphatase and can be tightly adsorbed onto DEAE paper. The reaction mixture for determining TMP kinase activity contained, in a volume of 0.05 ml in 6- x 50-mm culture tubes, the following: 2.15 μmoles of Tris-maleate, pH 8.0; 1.2 μmoles $MgCl_2$; 1.15 μmoles ATP; 0.8 μmole phosphoenolpyruvate; 2 μg pyruvic kinase; 3.0 mμmoles TMP-^{14}C, 25,000 dpm/mμmole; and 0.03 ml of the disrupted lymphocyte preparation. Following incubation for 1 hr at 37°, the reaction was stopped by heating at 95° for 3 min. After cooling, 40 μl of 0.4 M sodium acetate buffer (pH 4.9) and 10 μl of human semen phosphatase (18) (approximately 20 units) were added, and the mixtures were incubated an additional 5 min at 37°. The tubes were centrifuged for 10 min at 4,000 X *g*, and 50-μl aliquots of the clear supernatant were applied to DEAE paper discs, 2.2 cm in diameter (H. Reeve Angel & Co., Clifton, N. J.). The filters were washed successively in 0.004 M ammonium formate, water, and 95% ethanol. Radioactivity was determined by scintillation counting. Determinations of GMP kinase and dGMP kinase activities were carried out in a similar manner. The reaction mixtures (0.05 ml) contained 2.15 μmoles Tris-maleate, pH 8.0; 0.8 μmole $MgCl_2$; 0.64 μmole ATP; 0.8 μmole phosphoenolpyruvate; 2 μg pyruvic kinase; 2 μmoles KCl; and 3 mμmoles of either dGMP-^{14}C or GMP-^{14}C. With dGMP-^{14}C as a substrate, 0.03 ml of the lymphocyte preparation was used as an enzyme source, and incubation was for 20 min at 37°. With GMP-^{14}C, 0.01 ml of the lymphocyte preparation was used and incubation was for 15 min.

Nucleoside Kinase Activity. The reaction mixture for assay of thymidine kinase contained, in a volume of 90 μl, the following: 8 μmoles of Tris-HCl, pH 8.0; 0.9 μmole of $MgCl_2$; 1.0 μmole of ATP; 1.2 μmoles of 3-phosphoglycerate; 5 mμmoles of thymidine-^{14}C; and 50 μl of the lymphocyte preparation. Incubation was for 1 hr; the reaction was stopped by heating at 95° for 3 min. After centrifugation, portions of the supernatant were either applied to DEAE paper discs as in assays of nucleotide kinase activity or chromatographed on strips of DEAE paper by the method of Ives *et al.* (11).

Morphological Transformation. The extent of blast transformation was determined by adding 0.1 ml of Velban (Eli Lilly and Co., Indianapolis, Ind.) (0.5 μg/ml) to designated 2 ml cultures. After 2 hr, the cells were fixed in acetic

acid:methanol (1:3). Air-dried preparation was made by the method of Hungerford (10) and stained with Giemsa stain. From each culture, a total of 1000 cells were counted and classified as small lymphocytes, lymphoblasts, and cells in mitosis. Cell viability was determined by the trypan blue dye exclusion technique (23).

RESULTS

Enzyme Activity during Lymphocyte Transformation. To study the relationship of enzyme activity to DNA synthesis, we directly compared in the same cultures at various times after stimulation by PHA the ability of the cells to incorporate thymidine and the activities of enzymes associated with DNA synthesis. DNA synthesis was determined with thymidine-^{3}H and enzyme assays were carried out under optimal conditions with ^{14}C- or ^{32}P-labeled substrates. The onset and magnitude of DNA synthesis varied in detail in cultures from different individuals, but the results given here are representative. The increase in thymidine incorporation was first detectable 16 to 18 hr after the addition of PHA. The rate of thymidine incorporation thereafter increased until maximal at 70 to 90 hr.

DNA Polymerase Activity. The amount of thymidine incorporated and the DNA polymerase activity were determined on the same samples as detailed in "Materials and Methods." In nonstimulated cells, there is little DNA polymerase activity or thymidine incorporation (Chart 1). Stimulation by PHA results in a 30- to 150-fold increase in DNA polymerase activity (20). The increase occurs at about the same time as the increase in the ability of the cells to incorporate thymidine into DNA. The enhancement in DNA polymerase activity during the course of PHA stimulation is remarkably parallel to the increase in the rate of thymidine incorporation. Further, once DNA synthesis is diminished, DNA polymerase activity also decreases.

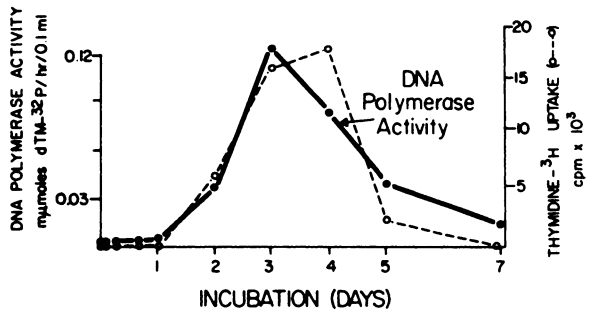

Chart 1

Charts 1 to 7. The relationship of DNA synthesis to enzyme activities during lymphocyte transformation. Lymphocytes were cultured in the presence of PHA for the times indicated. Enzyme activities were determined as detailed in "Materials and Methods" on disrupted lymphocytes that had incorporated thymidine into DNA. Thymidine incorporation is given in cpm; the value on the *ordinate* is to be multiplied by 10^3. The latter represents incorporation/0.1 ml of the final lymphocyte preparation for 1 hr prior to harvesting the cultures. Fumarase activity was determined by measuring the disappearance of fumarate by the method of Racker (25). Each *point* represents the average of a set of triplicate cultures.

DNase Activity. No DNase activity was evident in initial studies on the induction of DNA polymerase activity in PHA-stimulated lymphocytes (20). The polymerase reaction was linear for 2 hr, and the DNA product of the reaction was not degraded to acid-soluble oligonucleotides when incubation was carried out for as long as 8 hr. However, when a more sensitive assay in the present study, involving use of partially degraded radioactive DNA of high specific activity as substrate, was used, DNase activity was easily demonstrated (Chart 2). The increase in DNase activity appears to approximate the increase in polymerase. Exonuclease activities are associated with purified *Escherichia coli* DNA polymerase (26). By analogy, this DNase activity could in part reflect an exonuclease function of the lymphoblast polymerase.

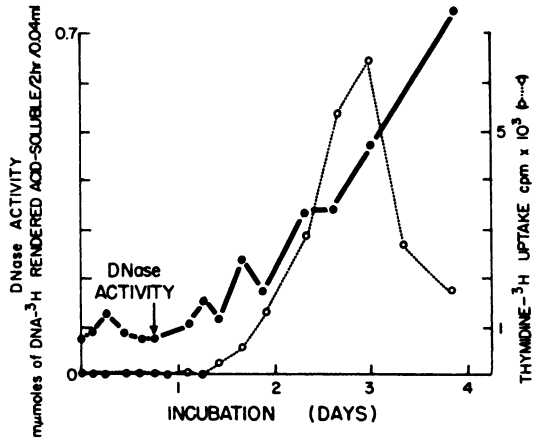

Chart 2

Thymidine Kinase and TMP Kinase Activity. Other enzyme activities functioning in the DNA synthesis are also stimulated by PHA. Thymidine kinase, an activity associated with the "salvage pathway" for DNA synthesis, was stimulated about 3-fold (Chart 3). Similarly, TMP kinase activity increased at about the same time as DNA replication (Chart 4). In cells not stimulated with PHA, the activities of these enzymes are constant or, if anything, become diminished during a similar period of culture.

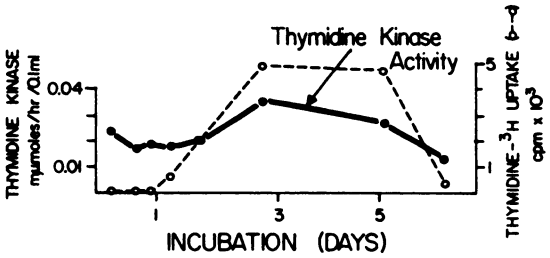

Chart 3

Deoxyguanosine and GMP Kinase. The addition of PHA to lymphocyte cultures does not appear to stimulate the activity of these kinases (Charts 5 and 6). This may be related to the high levels of these activities before PHA stimulation. The amount of dGMP kinase activity in nonstimulated lymphocytes is about 5-fold greater than the TMP

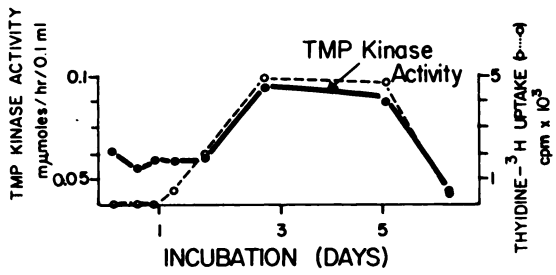

Chart 4

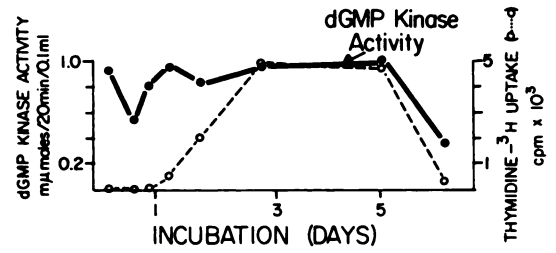

Chart 5

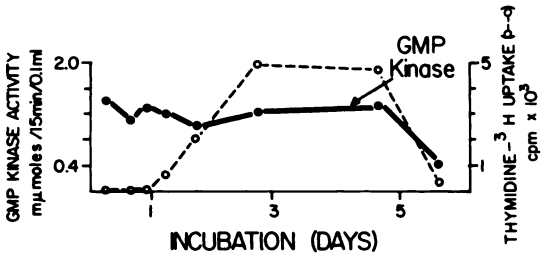

Chart 6

kinase activity and 50-fold greater than thymidine kinase activity.

Fumarase. In order to delineate the specificity of induction by PHA, we determined the activity of fumarase, an activity not directly involved in DNA synthesis (Chart 7). As shown, the amount of fumarase activity elevates gradually and reaches a maximal increase of about 50%. This increment is small compared to that of polymerase and is not temporally related or proportional to the DNA synthetic activity of these cells.

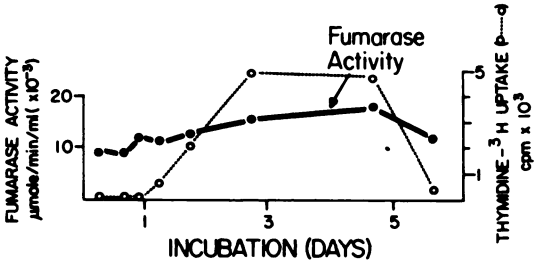

Chart 7

Prevention of Enzyme Induction by Actinomycin

Temporal relationships between RNA, protein, and DNA synthesis, and the morphological changes characterizing lymphocyte transformation are directly compared in Chart 8. Replicate cultures were incubated with PHA for the number of days indicated. One hr prior to harvesting the cultures, the appropriate radioactive precursors were added and their incorporations into an acid-insoluble precipitate were determined. As observed by others (5, 12), one of the earliest biochemical events induced by PHA is an increase in the rate of RNA synthesis. Later, and perhaps programmed by the

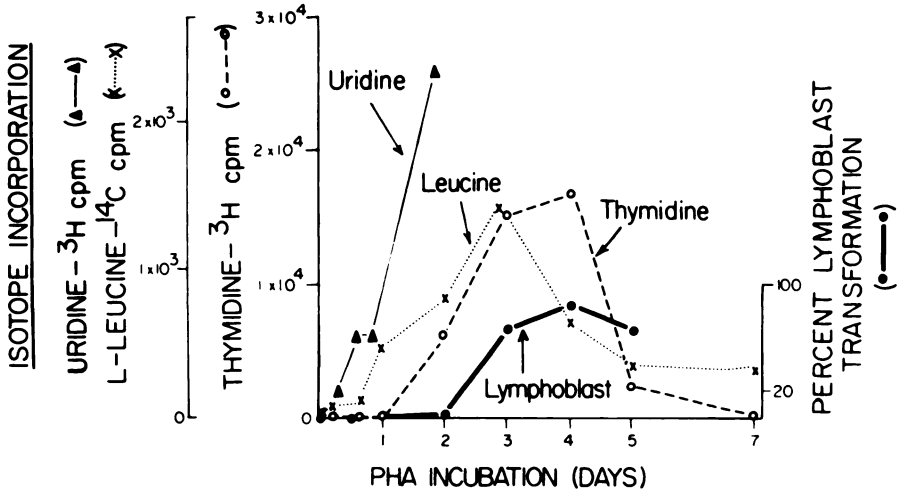

Chart 8. Alterations in lymphocyte metabolism induced by PHA. Replicate 2-ml portions of lymphocytes were incubated simultaneously with PHA for the number of days indicated. One hr prior to harvesting the cells, 4 μCi of uridine-^{3}H (specific activity, 20 Ci/mmole) or 4 μCi of L-leucine-^{14}C (specific activity, 180 mCi/mmole) or 5 μCi of thymidine-^{3}H (specific activity, 11 Ci/mmole) were added to the indicated cultures. Incorporation into acid-insoluble material was determined after precipitating the lymphocytes and washing by centrifugation with 1 N perchloric acid. The final pellet was dissolved in Hyamine hydroxide (Packard) (17). Each *point* represents the average incorporation in 3 cultures. In other cultures, the extent of blast transformation was determined as outlined in "Materials and Methods."

newly synthesized RNA, there is an increase in protein synthetic activity. The sudden rise in the rate of DNA synthesis in these cultures is usually not detected for 18 hr and may require the prior increase in RNA and protein synthesis. The complete morphological transformation of the small lymphocyte with its compact nucleus and scanty cytoplasm into a much larger lymphoblast with reticular nucleus, abundant cytoplasm, and prominent nucleoli was not observed until after DNA replication.

In order to measure the dependence of lymphocyte transformation on the synthesis of RNA, we investigated the effects of actinomycin D. PHA and graded amounts of actinomycin were added at the start of the experiment (Table 1). After 72 hr, the cultures were evaluated for the indicated metabolic and morphological changes of lymphocyte transformation. At sufficient concentrations (0.015 μg/ml or greater), the effect of actinomycin D was to prevent the PHA-mediated induction of thymidine incorporation, DNA polymerase, thymidine kinase, TMP kinase activity, uridine incorporation, and lymphoblast transformation. However, actinomycin at a concentration of 0.005 μg/ml, which abolished the increase in polymerase activity, as well as nearly all augmentation of thymidine and uridine incorporation, did not effect subsequent morphological transformation.

DISCUSSION

Human peripheral lymphocytes seldom divide *in vivo* and reportedly live for many years (8). In culture, their nonproliferative behavior is usually continued. Quantitative cytological measurements indicate that these cells contain a diploid complement of DNA; their DNA content must double prior to mitosis and cell division (6). The addition of PHA to lymphocyte cultures initiates a series of metabolic alterations resulting in DNA synthesis and the transformation of these quiescent lymphocytes into actively replicating lymphoblasts.

The present result indicates that the induction of DNA replication by PHA is accompanied by striking changes in the activities of some of the enzymes responsible for this synthesis. Nonstimulated lymphocytes contain little DNA polymerase, thymidine kinase, DNase, and TMP kinase activities. These activities are enhanced by the presence of PHA. This enhancement occurs at the same time as DNA replication and is sensitive to actinomycin D and cycloheximide (unpublished results). Since the increase in amino acid incorporation starts at a much earlier time, it is possible that these enzymes were synthesized at this earlier time in inactive forms.

dGMP kinase is an example of an enzyme activity that does not increase during lymphocyte transformation. It is present in greater activity in nonstimulated lymphocytes than TMP kinase, as if there is sufficient dGMP kinase activity to satisfy the requirements for subsequent lymphocyte transformation.

Evidence indicates that the pathway by which thymidine is incorporated into DNA is not identical to the pathway by which the cell synthesizes DNA *de novo*. In the former, thymidine is sequentially phosphorylated to TTP, one of the immediate substrates for DNA polymerase. In the latter pathway, reduction of ribonucleotides occurs at the diphosphate level (17). The increase in thymidine incorporation during lymphoblast transformation resulting from PHA occurs at the same time as the increase in thymidine kinase and DNA polymerase activity. The most direct explanation for this relationship is that both the "salvage" and *de novo* pathways are under coordinate controls. Since thymidine kinase is not on the *de novo* pathway for DNA replication and the activity is not proportional to the increase in thymidine incorporation, DNA polymerase might still limit DNA replication.

The increase in DNA polymerase, thymidine kinase, and TMP kinase activity as well as the replication of DNA by the cells could be abolished, for the most part, by the addition of as little as 0.005 μg of actinomycin per ml of culture

Table 1

Effect of actinomycin D on PHA-stimulated human lymphocytes

Multiple 2-ml portions of isolated lymphocytes from the same individual were cultured with and without PHA and actinomycin. The amount of actinomycin refers to the final concentration in the cultures. Thymidine and uridine uptake is the amount incorporated for 1 hr prior to harvesting the cultures (see legend to Chart 1). Results are averages of triplicate cultures.

Culture conditions	Thymidine-^{3}H incorporation (cpm/0.1 ml)	DNA polymerase activity (mμmole dTM-^{32}P/ hr/0.1 ml)	Thymidine kinase activity (mμmole/hr/0.1 ml)	TMP kinase activity (mμmole/hr/0.1 ml)	Uridine-^{3}H incorporation (cpm/0.1 ml)	% lymphoblasts
No PHA	11	0.002	0.032	0.158	<10	Occ.[a]
PHA	4752	0.113	0.256	0.442	3197	68
PHA + actinomycin D						
0.001 μg/ml	1070	0.010	0.034	0.095	312	77
0.005 μg/ml	238	0.005	0.030	0.032	106	57
0.01 μg/ml	62	<0.001	0.022	<0.002	<10	35
0.015 μg/ml	0	<0.001	0.015		<10	Occ.
0.02 μg/ml	0	<0.001	0.004	<0.002	<10	Occ.

[a]Cultures containing less than 1% lymphoblasts.

medium. In these experiments, actinomycin was added at the same time as PHA. After 72 hr in culture, the amount of actinomycin was sufficient to abolish 90% of the PHA-mediated increase in the rate of uridine incorporation. However, this low concentration of actinomycin did not hinder morphological transformation. These results confirm the finding of Kay *et al.* (14) that actinomycin can prevent the induction of thymidine incorporation by PHA yet allow blastogenesis to proceed and are compatible with the experiments of Salzman *et al.* (27) in which fluorodeoxyuridine prevented DNA synthesis without inhibiting blastogenesis. Our results also indicate that the induction of enzymes associated with DNA synthesis is also not a prerequisite for blastogenesis. The degree of proportionality between the amount of actinomycin added to the cultures and the decrease in DNA replication, or the activities of enzymes associated therein, is an indication of exact coordinate controls operative in regulating DNA synthesis. Since cells cultured in the presence of actinomycin and PHA are able to transform successfully, the effects of actinomycin observed here cannot be attributed only to cell death. The viability of the cells was further confirmed by their ability to exclude the dye, trypan blue (23).

Experiments with inhibitors of protein and RNA synthesis suggest that the increase in polymerase activity observed during lymphocyte transformation results from the *de novo* synthesis of the polymerase (unpublished results). The sensitivity of this induction to actinomycin is in accord with a requirement for the synthesis of new RNA. In other experiments, puromycin, at concentrations sufficient to prevent 90% of the PHA-mediated enhancement of protein synthesis, prevents the induction of the polymerase. It is possible that PHA effects the removal of an inhibitor of the polymerase; if so, this effect requires RNA and protein synthesis. However, we find no evidence for the presence of an inhibitor in unstimulated cultures (20). It is always conceivable that the enzyme is present as an inactive form and that activation requires RNA and protein synthesis. This is unlikely, since this activation is not limited to polymerase.

If our results can be generalized, the induction of replication in nondividing cells requires the synthesis or activation of a number of enzymes functioning in the DNA-synthetic pathways. Evidence supporting this concept has been obtained from a number of eukaryotic systems in which DNA replication can be induced *in vivo* (4), as well as the induction of DNA replication following viral infection (15). The induction of replication by PHA *in vitro* appears to be particularly suitable for studying the control mechansim for DNA synthesis. The magnitude of change, both as to the number of cells participating (as much as 90%) and the increased enzymatic activities associated with DNA synthesis (as much as 150-fold), facilitates detailed studies in this system.

That DNA polymerase is responsible for DNA replication in bacteria has been recently been questioned by de Lucia and Cairns (7). On the other hand, the data presented in this communication indicate a high degree of correlation between DNA polymerase activity and DNA replication in human lymphocytes. This proportionality was further evident when the induction of polymerase was diminished with actinomycin D. So far, stimulation of DNA synthesis in lymphocytes by a variety of methods results in a parallel increase in DNA polymerase activity (2). Inhibition of PHA-mediated stimulation of polymerase by a variety of methods is accompanied by a proportional decrease in DNA synthesis (unpublished results). DNA polymerase activity can only be defined as the DNA-directed polymerization of deoxynucleoside triphosphates as detailed by Kornberg (18); no other mechanism is known. We do not yet know if the enzyme responsible for this polymerization during lymphocyte transformation is similar to *E. coli* DNA polymerase.

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