*INDUCTION OF DNA POLYMERASE IN HUMAN LYMPHOCYTES BY PHYTOHEMAGGLUTININ**

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The lymphocytes of human peripheral blood have been the subject of recent immunologic studies. Although these cells seldom grow or divide *in vitro*, the addition of phytohemagglutinin (PHA), an extract of the kidney bean (*Phaseolus vulgaris*), to the culture medium initiates a striking transformation: 90 per cent of the cells enlarge, synthesize DNA, and divide.[1] Smaller percentages of cells show similar responses to many other mitogens, including appropriate immunologic stimuli.[2] The effects of PHA have been extensively monitored by the morphologic transformation of the cells (blastogenesis) and by labeling the DNA of the cell upon the addition of radioactive thymidine to the culture. Changing patterns of RNA[3–5] and protein[5, 6] metabolism as well as histone acetylation[5] have also been well documented. The mitogenic effect of PHA has been extensively utilized for studies of human chromosomes.

This communication is the first report that the DNA polymerase activity of human lymphocytes increases 30- to 100-fold when these cells are cultured in the presence of phytohemagglutinin. The increase in DNA polymerase activity as measured in disrupted cell preparations is correlated both quantitatively and temporally with the ability of these cells to incorporate thymidine into DNA.

Materials and Methods.—[α-P^{32}]-labeled thymidine triphosphate was purchased from the International Chemical and Nuclear Corporation, City of Industry, California. H^3- and C^{14}-labeled deoxynucleotides were purchased from Schwarz BioResearch, Inc., and unlabeled nucleotides from the California Foundation for Biochemical Research. Calf thymus DNA, *E. coli* DNA, crystalline pancreatic DNase, and micrococcal nuclease were acquired from Worthington Biochemical Corporation. Sea urchin nuclear DNA polymerase was purified about 300-fold from *S. purpuratus* embryos,[7] and *E. coli* DNA polymerase was fraction IX in the procedure of Richardson *et al.*[8] Poly dAT was made by the *de novo* reaction catalyzed by *E. coli* DNA polymerase.[9] "Activated" calf thymus DNA was made by digesting the DNA with minute amounts of pancreatic DNase.[10] The course of the reaction was monitored with sea urchin nuclear DNA polymerase. Maximum activation was achieved when about 2% of the DNA was rendered acid-soluble. The $s_{20,w}$ of the "activated" DNA was 2.4, as determined by boundary centrifugation on a Spinco analytical ultracentrifuge, and the increase in absorbancy at 260 mμ was 21% upon heating. "Activated" dAT was made in a similar manner, except that at a maximum activation 6% was acid-soluble. DNA was heat-denatured by keeping a solution of DNA (1 mg/ml in 0.01 *M* KCl) at 100°C for 10 min and then immediately cooling in an ice-water bath. Actinomycin D was a gift of Merck Sharp and Dohme, Rahway, New Jersey. Phytohemagglutinin M (1 mg protein/ml) was obtained from General Biochemicals, Chagrin Falls, Ohio (lot 685, 291) and Difco Laboratories, Detroit, Michigan (lot 513, 461).

Lymphocytes were isolated from human peripheral blood by the method of Bach and Hirschhorn.[11] The lymphocytes were suspended at a concentration of 7.5×10^5 cells/ml in MEM Spinner medium supplemented with 20% fetal calf serum, penicillin, streptomycin, and glutamine. Aliquots of 2 ml were incubated with 0.05 ml of phytohemagglutinin in 15-ml Corex tubes at 37°C for 3 days. These conditions resulted in maximal incorporation of thymidine into DNA, as well as maximum levels of DNA polymerase

activity. The cells were collected and washed in 2 ml of 0.15 *M* KCl at 0°C by centrifugation at 2500 × *g* for 10 min. To the final pellet was added 1 ml of a solution made up of 20% (w/v) glycerol (Matheson, Coleman and Bell, Spectroquality grade), 0.02 *M* potassium phosphate buffer (pH 7.4), 0.001 *M* potassium versenate, and 0.004 *M* reduced glutathione. The suspension was sonicated at 0°C until 99% of the cells were disrupted. Sonication was performed three times for 30 sec at a setting of 4 on a Branson Sonifier, model S-75, equipped with a microtip. The final preparation was stored in this medium at −40°C with no detectable loss of DNA polymerase activity up to 2 months. Large amounts of enzyme were prepared by combining many cultures of 2 or 4 ml each.

Assay of DNA polymerase: The assay measures the incorporation of H^3-, C^{14}-, or P^{32}-labeled deoxynucleoside triphosphates into an acid-insoluble product. The standard reaction mixture, contained in a total volume of 0.3 ml, is the following: 25 μmoles tris-(hydroxymethyl)aminomethane-maleate (tris-maleate) buffer, pH 8.0; 3 μmoles $MgCl_2$; 1 μmole KCl; 0.3 μmole β-mercaptoethanol; 25 mμmoles each of dATP, dCTP, and dGTP, and 10 mμmoles of [α-P^{32}]-dTTP (about 5 × 10^4 dpm/mμmole); 266 mμmoles of "activated" calf thymus DNA; and 0.025–0.10 ml of the lymphocyte preparation. Incubation was for 1 hr at 37°C and the reaction was stopped with 0.5 ml of cold 1 *N* perchloric acid containing 0.01 *M* sodium pyrophosphate. After 20 min at 0°C, the precipitate was collected on glass-fiber filters[12] or by a modification of the centrifugation method of Mazia and Hinegardner.[13] In the latter, the DNA is repetitively solubilized in 0.2 *N* NaOH and precipitated with acid. The final pellet is dissolved in 1 ml of hyamine-OH (Packard Instrument Co.) and after addition of a liquid scintillation solution, the radioactivity is determined. In the filtration method 0.02–0.1% of the unincorporated radioactivity remains on the filter, whereas the centrifugation method reduces nonspecific adsorption to less than 0.003%. In both procedures the amount of deoxynucleotide incorporated into acid-insoluble material is the same. All assays were performed in triplicate and processed by the filtration method, unless stated otherwise.

Other methods: The relative rate of protein synthesis was determined by adding L-leucine-C^{14} (spec. act., 180 mc/mM, 2.0 μc/ml of culture) 1 hr before harvesting the cells. After sonication, the radioactivity incorporated into a 0.2-ml aliquot of the culture was determined by precipitating the protein with 1 ml of 1 *N* perchloric acid and washing by centrifugation as in the polymerase assay. The incorporation of thymidine into DNA was determined with thymidine-methyl-H^3 (Schwarz BioResearch, Inc., 11 c/mmole, 2.5 μc/ml of culture) added 1 hr prior to terminating the culture. Just before the cells were harvested, 0.1 ml of 0.1 *M* unlabeled thymidine was added; and after sonication, 0.1 ml of denatured calf thymus DNA (1 mg/ml) was added as a carrier. The radioactivity incorporated into acid-insoluble material was determined by the centrifugation procedure. Protein was determined by the method of Lowry *et al.*[14] Phosphate was determined by the method of Ames and Dubin.[15]

Results.—A direct comparison of the amount of DNA polymerase activity in human lymphocytes which have been cultured in the presence and absence of phytohemagglutinin (PHA) is given in Table 1. In all, 16 such comparisons have been made, and without exception the level of DNA polymerase activity in the cells cultured in the presence of PHA has been at least 30-fold greater than that in identical cultures maintained in the absence of PHA. The DNA polymerase activity per milligram of protein of the PHA-treated lymphocytes is as great or greater than that of most other rapidly proliferating animal tissues.

(1) *DNA polymerase activity:* (a) *Requirements:* Table 2 shows that the requirements for DNA polymerase activity are similar to those reported in other DNA polymerase systems. The presence of Mg^{++}, DNA, and the four deoxynucleoside triphosphates is required for maximal incorporation. In the

TABLE 1. *Induction of DNA polymerase activity by phytohemagglutinin.*

Experiment	dTMP^{32} Incorporated ($\mu\mu$moles/hr)	
	Control	PHA-treated
1	2	109
2	1	74
3	0	74
4	4	193
5	1	148

In each experiment, lymphocytes from the same individual were grown for three days in the absence (control) or the presence of PHA. DNA polymerase activity was assayed as given in *Methods* with 0.1 ml of the final preparation from control and PHA-treated lymphocyte preparations. The incorporation into acid-insoluble material was determined by the centrifugation method.

absence of dGTP, dCTP, and dATP, incorporation of residues of dTMP^{32} is reduced to 18 per cent of the complete reaction mixture. In other experiments in which dCTP-C^{14}, dATP-H^3, and dGTP-C^{14} were added in the absence of the other three deoxynucleotides, incorporation was reduced to 28, 6, and 18 per cent, respectively, from that obtained in the complete reaction mixture. The lack of absolute dependence on the presence of four deoxynucleotides is consistent with results reported for other crude extracts from animal tissues and may be due to the presence of "terminal" deoxynucleotidyl transferase enzymes.[16] It is also shown that the product of the reaction is sensitive to degradation by DNase, but not by RNase.

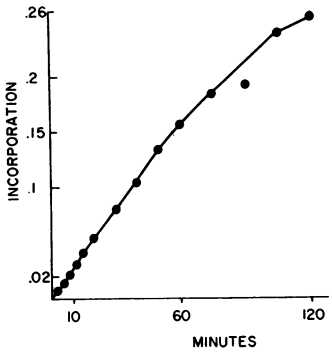
(b) *Kinetics:* As is shown in Figure 1*a*, the rate of incorporation is linear for nearly two hours. After incubation for four hours, a time at which there is little further incorporation, each component of the reaction was added back individually. The greatest stimulation was elicited by adding the enzyme. This suggests that the termination of the reaction results, for the most part, from the

TABLE 2. *Requirements for DNA polymerase activity.*

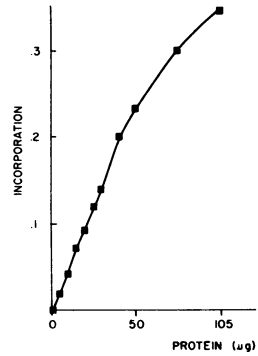
Reaction mixture	dTMP^{32} incorporated ($\mu\mu$moles/hr)
Complete	104
Minus DNA	0
Minus Enzyme	0
Minus $MgCl_2$	0
Minus dATP	34
Minus dCTP	30
Minus dGTP	25
Minus dGTP, dCTP	17
Minus dGTP, dCTP, dATP	19
Plus actinomycin (5 μg)	100
Complete + 20 μg RNase*	107
Complete + 20 μg DNase*	2

The complete system for the reaction is described in the text. The enzyme preparation was from PHA-stimulated lymphocytes which had been extensively dialyzed at 0°C against 2000 volumes of 20% (w/w) glycerol, 0.02 *M tris*-maleate buffer (pH 7.8), 0.001 *M* potassium versenate, 0.01 *M* β-mercaptoethanol. Unless otherwise indicated, each reaction mixture contained 0.05 ml of the dialyzed lymphocyte preparation (53 μg of protein). All determinations were performed in triplicate and the acid-insoluble precipitate was washed by the centrifugation method.

* In these tubes, after incubation the reaction was terminated by heating at 60°C for 10 min. After cooling, 0.02 ml of either DNase (1 mg/ml) or RNase (1 mg/ml) was added and the tubes were incubated an additional 30 min at 37°C. The reaction was then stopped as in the standard DNA polymerase assay.



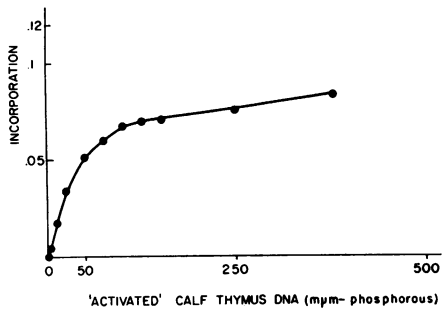
(a)



(b)

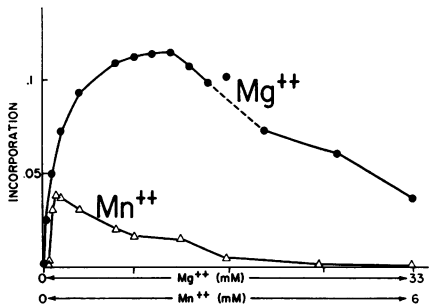
FIG. 1.—(*a*) Time course of the DNA polymerase reaction. The standard assay was used with 26 μg of the lymphocyte preparation which had been cultured for 3 days in the presence of PHA. Incorporation in (*a*), (*b*), (*c*), and (*d*) is expressed as mμmoles of residues of $dTMP^{32}$/hr.

(*b*) DNA polymerase activity as a function of enzyme concentration. The concentration of the PHA-treated lymphocyte preparation is expressed as micrograms of protein added to each tube. Enzyme dilutions were made in the same solution used for sonication of the lymphocytes.



(c)

(*c*) Activity as a function of DNA concentration. The concentration of DNA is given in mμmoles of DNA-phosphorus per reaction mixture. The DNA was diluted in 0.02 *M* KCl.



(d)

(*d*) Activity as a function of $MgCl_2$ and $MnSO_4$ concentration.

destruction of the enzyme during incubation. After prolonged incubation for as long as 20 hours, there was no detectable destruction of the product of the reaction. The extremely low level of DNase activity in these lymphocyte preparations has been confirmed by measuring the rate of hydrolysis of labeled DNA into acid-soluble products.

(c) *Optimal conditions:* The pH optimum in *tris*-maleate buffer is 8.0. The incorporation is linear over a range of protein concentrations from 5 to 50 μg which corresponds to 7×10^3 to 7×10^4 cells (Fig. 1*b*). The DNA polymerase activity as a function of the amount of added primer is shown in Figure 1*c*. Even though the lymphocyte preparations contain some DNA (0.73 μg/10^6 cells), there is no detectable activity in the absence of added DNA. Figure 1*d* shows

the DNA polymerase activity as functions of added Mg^{++} and Mn^{++}. In the absence of these divalent cations there is no activity. Maximal incorporation is observed at Mg^{++} concentration of 8–10 mM, and considerable activity occurs at an Mn^{++} concentration of 0.2 mM.

(d) *Effect of various treatments on priming:* A limited digestion of calf thymus DNA with trace amounts of pancreatic DNase, an endonuclease that cleaves phosphodiester bonds of DNA to form 3′-hydroxyl and 5′-phosphoryl termini ("activated" DNA), causes a marked stimulation in the effectiveness of the DNA as a primer (Table 3). Digestion to a similar extent with micrococcal nuclease, an endonuclease that splits DNA to produce 3′-phosphoryl and 5′-hydroxyl groups, abolishes all priming activity. Less extensive but similar effects have been reported in several DNA polymerizing systems[12, 17–19] and show that the nature of the terminal group has marked effects on the ability

TABLE 3. *Primer requirement.*

Primer		Pretreatment	dTMP^{32} incorporation ($\mu\mu$moles/hr)
Calf thymus DNA	(1)	Activated with pancreatic DNase	87
	(2)	Activated and then heat denatured	30
	(1)	None (native)	4
	(2)	Heat denatured	6
	(1)	Digested with micrococcal nuclease	0
	(2)	Digested and then heat denatured	0
E. coli DNA	(1)	None (native)	9
	(2)	Heat denatured	5
Cl. perfringens DNA	(1)	None (native)	10
	(2)	Heat denatured	8
Poly dAT	(1)	None (native)	45
	(2)	Activated with pancreatic DNase	78

DNA polymerase activity was determined, as given in *Methods*, by the procedure of washing the acid-insoluble material by centrifugation. Each assay contained 52.7 μg of protein from PHA-stimulated lymphocytes, 266 mμmoles of DNA, or poly dAT having an absorbance at 260 mμ of 1.0. dGTP and dCTP were omitted from the poly dAT-primed reaction mixture. Pretreatment of calf thymus DNA and poly dAT with pancreatic DNase is described in *Methods*. Calf thymus DNA was digested with micrococcal nuclease until 4% was rendered acid-soluble.[21]

of DNA to act as a primer. It is apparent that both native and heat-denatured DNA have limited priming ability; with different DNA's varying preferences were exhibited. The effectiveness of the exact helical structure of poly dAT as a primer and the reduction in the priming activity of "activated" DNA by heat denaturation confirm that the enzyme is capable of utilizing double-stranded DNA as a primer. This is in contrast to calf thymus DNA polymerase which requires single-stranded DNA as a primer.[20]

(2) *Increase in DNA polymerase activity in lymphocytes:* Figure 2 shows the results of concomitant assays of DNA polymerase activity and of the ability of cells to incorporate leucine into protein and thymidine into DNA at various times after the addition of PHA to these cultures. The increase in DNA polymerase activity is not immediate, and only after two days is it evident. The increase and maximal stimulation of polymerase activity is closely paralleled by the increase in the ability of the cells to incorporate labeled thymidine into DNA.

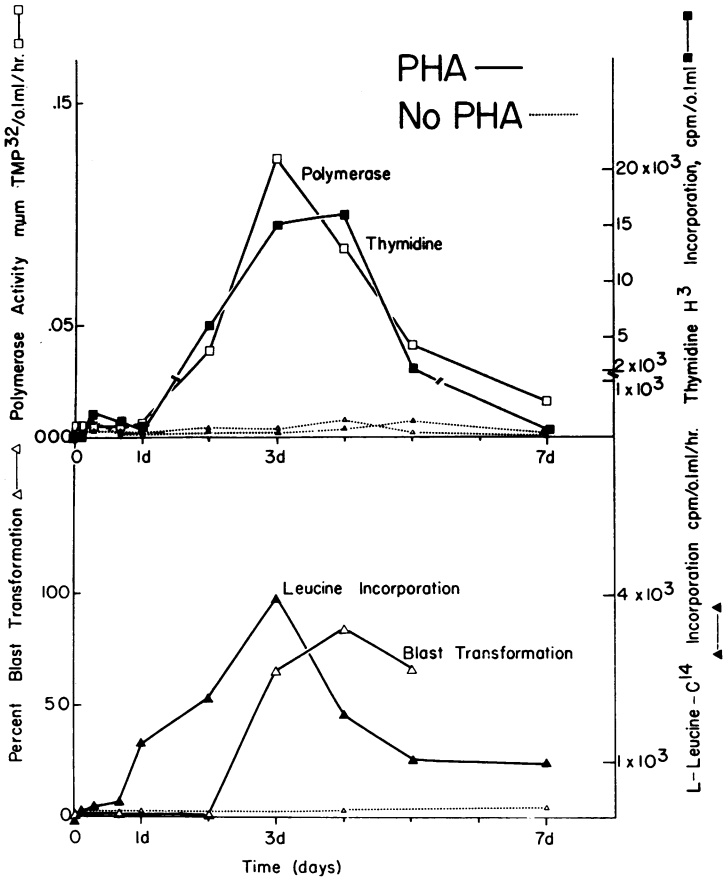


FIG. 2.—Alterations in lymphocyte metabolism induced by phytohemagglutinin. All results shown were obtained from aliquots of lymphocytes from the same individual which were cultured simultaneously in the presence and absence of PHA. Thymidine-H^3 was added 1 hr prior to harvesting the cells, and incorporation represents the averages of quadruplicate determinations. On the same cultures, DNA polymerase activity was determined with the standard reaction mixture. At three days the percent standard deviation for thymidine incorporation and DNA polymerase activity was 19.7 and 6.9, respectively. Velban was added 2 hr prior to harvesting cells for microscopic examination. In other cultures the rate of protein synthesis was monitored by adding *L*-leucine-H^3 1 hr prior to harvesting the cells (see *Methods*).

The enhanced incorporation of labeled leucine into protein occurs prior to the increase in polymerase activity and may include the augmented synthesis of this enzyme. The observed lymphoblast transformation occurs after the increase in polymerase activity.

It could be argued that the increase in polymerase activity is brought about by a phytohemagglutinin-induced loss of an inhibitor. No evidence in support of an inhibitor hypothesis was obtained in experiments directed at detecting the presence of an inhibitor in uncultured preparations of lymphocytes as well as in preparations cultured in the absence of phytohemagglutinin. There is no

change in activity after the cells have been washed or after the disrupted preparations of untreated lymphocytes have been dialyzed. When graded amounts of untreated lymphocytes are added to PHA-stimulated lymphocytes, there is no diminution of polymerase activity. Even the addition of the unstimulated lymphocytes to purified sea urchin nuclear DNA polymerase and *E. coli* polymerase does not diminish their activities.

Discussion.—The correlation between the incorporation of thymidine into these lymphocyte cultures and the DNA polymerase activity *in vitro* is remarkably close. However, the conditions for DNA synthesis, when thymidine is supplied exogenously, differ sharply from the conditions used to assay DNA polymerase activity. In the cultures, DNA synthesis was determined by measuring the incorporation of thymidine, whereas in the polymerase assay thymidine triphosphate was the labeled precursor. There is considerable evidence in the literature to indicate 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 thymidine triphosphate; in the latter, the ribonucleoside diphosphates are reduced to the corresponding deoxyribonucleotides.[22] The correlation observed here means either that both pathways are under stringent coordinate controls or that DNA polymerase activity is limiting in unstimulated cells. Experiments are being conducted to distinguish between these alternatives.

It is possible that the stringent requirement of this DNA polymerase activity for DNA primers with 3′-hydroxyl termini may be representative of the conditions during actual chromosomal replication. The parallel between the synthesis of DNA and the amount of DNA polymerase activity indicates that such a polymerase may indeed be involved in DNA replication. A similar requirement for 3′-hydroxyl ends has been noted for other DNA polymerase activities,[12, 17–19] even though such a requirement does not prevent *E. coli* DNA polymerase from replicating single-stranded circular DNA.[23] In eucaryotic tissues there is considerable evidence to indicate that replication is initiated at multiple chromosomal sites.[24, 25] There is not sufficient evidence to establish whether this initiation of replication involves prior breaks in the DNA molecule and whether these breaks generate 3′-hydroxyl termini.

Human peripheral lymphocytes are well-differentiated cells and have been classified as the end stage of lymphoid maturation. *In vivo*, they do not appear to replicate or synthesize DNA,[1] they synthesize little protein,[26] and they may have a life span of many years.[27] Without external stimuli their nonproliferative behavior is continued in culture. As shown, the addition of PHA to such a culture markedly alters this behavior and the cells commence to replicate. Although the increase in DNA polymerase activity is not the earliest effect of PHA on these cells, yet this specific alteration can serve as an index characteristic of the transformation of these cells into a replicating population. Since DNA polymerase activity is determined *in vitro*, it is independent of fluctuations in the concentrations of metabolites that affect the incorporation of labeled precursors into whole cells. The increase in polymerase activity may be essential for replication of lymphocytes and so may be relevant for the expression of their immunologic properties.

Summary.—Human lymphocytes cultured in the presence of phytohemagglutinin exhibit a marked stimulation of DNA polymerase activity. This increase in activity parallels the ability of the cells to incorporate thymidine into DNA. Native DNA which has been maximally "activated" with pancreatic deoxyribonuclease is the most effectively utilized primer for the PHA-induced polymerase activity.

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