

# CHARACTERIZATION OF LYMPHOCYTE TRANSFORMATION INDUCED BY ZINC IONS

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## ABSTRACT

Lymphocyte cultures from all normal human adults are stimulated by zinc ions to increase DNA and RNA synthesis and undergo blast transformation. Optimal stimulation occurs at 0.1 mM  $Zn^{++}$ . Examination of the effects of other divalent cations reveals that 0.01 mM  $Hg^{++}$  also stimulates lymphocyte DNA synthesis.  $Ca^{++}$  and  $Mg^{++}$  do not affect DNA synthesis in this culture system, while  $Mn^{++}$ ,  $Co^{++}$ ,  $Cd^{++}$ ,  $Cu^{++}$ , and  $Ni^{++}$  at concentrations of  $10^{-7}$ - $10^{-3}$  M are inhibitory. DNA and RNA synthesis and blast transformation begin to increase after cultures are incubated for 2-3 days with  $Zn^{++}$  and these processes reach a maximum rate after 6 days. The increase in  $Zn^{++}$ -stimulated lymphocyte DNA synthesis is prevented by rendering cells incapable of DNA-dependent RNA synthesis with actinomycin D or by blocking protein synthesis with cycloheximide or puromycin.  $Zn^{++}$ -stimulated DNA synthesis is also partially inhibited by 5'-AMP and chloramphenicol.  $Zn^{++}$  must be present for the entire 6-day culture period to produce maximum stimulation of DNA synthesis. In contrast to its ability to independently stimulate DNA synthesis, 0.1 mM  $Zn^{++}$  inhibits DNA synthesis in phytohemagglutinin-stimulated lymphocytes and L1210 lymphoblasts.

## INTRODUCTION

A number of agents are known to stimulate resting lymphocytes to initiate DNA synthesis. These include plant lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) and bacterial extracts such as tuberculin-purified protein derivative and staphylococcal filtrates (1). Recently it has been shown that zinc ions can also stimulate DNA synthesis in cultured lymphocytes (2). In contrast to the plant and bacterial extracts,  $Zn^{++}$  is required as an essential metal by living organisms (3, 4). It may be involved in the physiologic initiation of DNA synthesis (5) and is required for DNA synthesis in cultured chick

embryo cells, mouse 3T3 cells (6), and PHA-stimulated lymphocytes (7). It was therefore of interest to determine the specificity and characteristics of  $Zn^{++}$  stimulation of lymphocyte DNA synthesis.

## MATERIALS AND METHODS

### *Materials*

Metals of reagent grade were obtained from Fisher Scientific Co., Fair Lawn, N. J. In these experiments the chloride salts were used although previous studies demonstrated that  $Zn^{++}$  stimulation occurs with

several different anions (2). 10-mM stock solutions of each metal were prepared in 1 mM HCl. Deionized distilled water was used for further dilutions. Solutions were sterilized by filtration through MF-Millipore filters, 0.45- $\mu$ m mean pore size, Millipore Corp., Bedford, Mass.

The PHA used in these experiments is the erythroagglutinating PHA (8) prepared from PHA-P, Difco Laboratories, Detroit, Mich. (9) and was added to lymphocyte cultures at a concentration of 15  $\mu$ g/ml.

Actinomycin D was obtained from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. Puromycin, chloramphenicol, cycloheximide, and 5'-AMP were obtained from Sigma Chemical Co., St. Louis, Mo. Dextran (average mol wt 100-200,000) and Ficoll (approximate mol wt 400,000) were also purchased from Sigma Chemical Co. Hypaque brand of sodium diatrizoate, 50% wt/vol, was purchased from Winthrop Laboratories, Div. Sterling Drug Inc., New York. Lymphocyte cultures were performed in screw-capped polystyrene tissue culture tubes, 16  $\times$  125 mm, Falcon Plastics, Div. Bioquest, Oxnard, California. Tissue culture medium 199 with Earle's salts (M-199), L-glutamine, penicillin, and streptomycin were all purchased from Grand Island Biological Company, Grand Island, N. Y. Nylon fibers were obtained from Leuko-Pak leukocyte filters, Fenwal Laboratories, Morton Grove, Ill. [5- $^3$ H]Uridine (sp act = 28 Ci/mmol) and [methyl- $^3$ H]thymidine (sp act = 2.0 Ci/mmol) were purchased from New England Nuclear, Boston, Mass.

### *Lymphocyte Preparation*

Human lymphocytes are isolated from defibrinated blood by isopycnic centrifugation on Ficoll-Hypaque gradients (10), cell number is determined in a hemacytometer, and differential counts are determined by supravital staining with neutral red and Janus green (11) and also in Wright's-stained, fixed films (12). This procedure yields a cell preparation composed of greater than 90% lymphocytes. To further purify lymphocytes, cells from the Ficoll-Hypaque gradient are applied to nylon fiber columns and eluted with M-199 (13). This additional purification step results in a preparation containing 100% lymphocytes.

Cultures are performed with  $2 \times 10^6$  lymphocytes/2 ml of M-199. The medium is supplemented with 10% autologous serum, 4 mM L-glutamine, penicillin 50 U/ml, and streptomycin 50  $\mu$ g/ml. Stimulating or inhibiting agents are added at desired times and cultures are incubated at 37°C in 5% CO<sub>2</sub>, 95% air, and high humidity.

L1210 lymphoblasts are maintained by weekly passage in DBA/2J mice, purchased from the Jackson Laboratory, Bar Harbor, Maine. The cells are passed by intravenous injection of a suspension containing

$4 \times 10^4$  leukemic cells prepared from the spleens of leukemic mice inoculated with the cells during the preceding week.<sup>1</sup> Monodisperse suspensions of L1210 spleen cells are cultured in M-199 at  $2-3 \times 10^6$  cells/ml, using the same incubation conditions as indicated for human lymphocytes except that serum, L-glutamine, and antibiotics are not added for these short term experiments.

### *Nucleic Acid Synthesis*

DNA synthesis is measured as incorporation of [ $^3$ H]thymidine into cold 5% TCA-insoluble polymers by adding 3.0  $\mu$ Ci [methyl- $^3$ H]thymidine (sp act = 2 Ci/mmol) to each 2-ml culture. Incubation is continued for 4 h with human lymphocytes and for 1 h with L1210 mouse leukemic lymphocytes. RNA synthesis is determined in a similar fashion by measuring incorporation of [ $^3$ H]uridine into cold 5% TCA-insoluble polymers. 10  $\mu$ Ci [5- $^3$ H]uridine (sp act = 28 Ci/mmol) are added to each 2-ml culture and incubation continued for 4 h. Cultures are terminated by washing with cold 0.9% NaCl, then with cold 5% TCA. They are sonicated, the TCA-insoluble precipitate dissolved in 0.5 ml NCS (Amersham/Searle Corp., Arlington Heights, Ill.), and radioactivity is counted in 10-ml Bray's solution (14) in a Packard model 3380 liquid scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.). All experimental points were determined in triplicate cultures.

## RESULTS

### *Concentration and Specificity of Cation Effect on Lymphocyte DNA Synthesis*

To determine the optimal concentration of Zn<sup>++</sup> needed to stimulate lymphocyte DNA synthesis, lymphocyte cultures were incubated with varying concentrations of Zn<sup>++</sup>, and DNA synthesis was measured on the 3rd and 6th days of culture. The change in DNA synthesis which occurs in response to incubating lymphocytes for 6 days with increasing concentrations of Zn<sup>++</sup> is shown in Fig. 1. These results are presented as the ratio of [ $^3$ H]thymidine incorporation by metal-treated cells compared to untreated cells. Thus, a culture which shows neither stimulation nor inhibition of DNA synthesis compared to control cultures has a ratio of one. The maximum increase in lymphocyte DNA synthesis occurs at 0.1 mM Zn<sup>++</sup>. As demonstrated in Fig. 1, stimu-

<sup>1</sup>Edelstein, M., T. Vietti, and F. Valeriotte. 1974. Schedule dependent synergism for the combination of 1- $\beta$ -D-arabinofuranosylcytosine and Daunorubicin. *Cancer Res.* 34. In press.

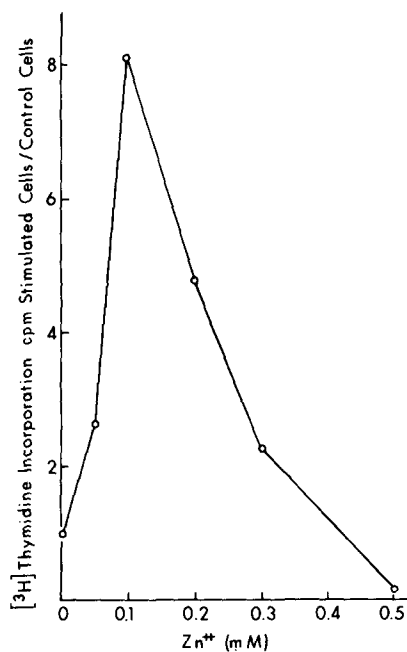


FIGURE 1 Effect of  $Zn^{++}$  concentration on DNA synthesis in cultured lymphocytes from normal adult human donors. DNA synthesis measured on 6th day of culture. Average of 34 donors.

lation of DNA synthesis also occurs at 0.05 mM  $Zn^{++}$  and at 0.2 and 0.3 mM  $Zn^{++}$  but not as effectively as at 0.1 mM  $Zn^{++}$ . At higher concentrations, the amount of  $Zn^{++}$ -stimulated DNA synthesis decreases, and by 0.5 mM  $Zn^{++}$  the rate of lymphocyte DNA synthesis is less than that which occurs in untreated controls.

All preparations of lymphocytes from normal donors respond to 0.1 mM  $Zn^{++}$  with an increase in rate of DNA synthesis. Fig. 2 illustrates the magnitude of this response in individual donors. Although  $Zn^{++}$ -treated lymphocytes always show an increased rate of DNA synthesis, there is a large individual variation in the magnitude of the response which also varies when lymphocytes are prepared from the same donor on different occasions. The increase in DNA synthesis ranges from 2 to 31 times that observed in controls with an average of an eightfold increase for all cultures examined and a median increase of 4.8.

Other divalent cations in concentrations from  $10^{-7}$  to  $10^{-8}$  M were incubated with lymphocyte cultures and the rate of DNA synthesis was determined after 3 and 6 days. As indicated in Fig. 3, lymphocyte DNA synthesis is affected in one of three ways by the different cations. It is either stimulated, unchanged, or inhibited. The first

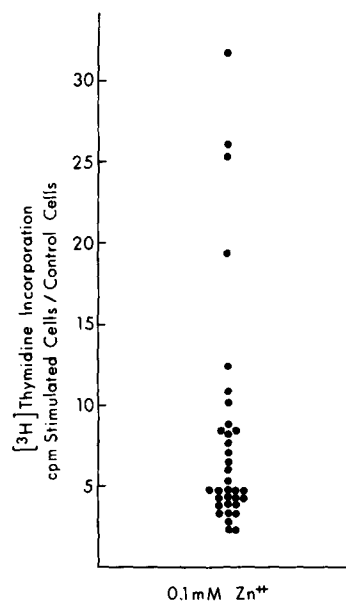


FIGURE 2 DNA synthesis in cultured lymphocytes from normal adult human donors incubated with 0.1 mM  $Zn^{++}$ . DNA synthesis measured on 6th day of culture.

type of response, characterized by stimulation of DNA synthesis, occurs with  $Hg^{++}$  as well as with  $Zn^{++}$ . The magnitude of the response to  $Hg^{++}$  is similar to that elicited by  $Zn^{++}$ . The optimal concentration of  $Hg^{++}$  ions to produce maximal stimulation of DNA synthesis is 0.01 mM, which is 10-fold less than the optimal stimulating concentration of  $Zn^{++}$ . No change in the rate of DNA synthesis occurs in lymphocytes incubated with additional  $Ca^{++}$  or  $Mg^{++}$  over the concentration range tested. This observation is expected, since the medium already contains 1.8 mM  $Ca^{++}$  and 0.8 mM  $Mg^{++}$ .

Lymphocytes incubated with the divalent metal ions  $Mn^{++}$ ,  $Co^{++}$ ,  $Ni^{++}$ ,  $Cu^{++}$ , and  $Cd^{++}$  all show the third type of response, a decrease in the rate of DNA synthesis compared to control untreated cells. These metal ions were evaluated at multiple concentrations extending as low as  $10^{-7}$  M with the result that all produced a decrease in the rate of DNA synthesis.

#### Cation Inhibition of Lymphocyte DNA Synthesis

The decrease in lymphocyte DNA synthesis brought about by incubation with  $Mn^{++}$ ,  $Co^{++}$ ,  $Ni^{++}$ ,  $Cu^{++}$ , and  $Cd^{++}$  suggests that all these

metals are producing toxic effects in the cells. The mechanism of this toxicity may be different for each metal examined. Metal ion toxicity could be the result of competition with a physiologic

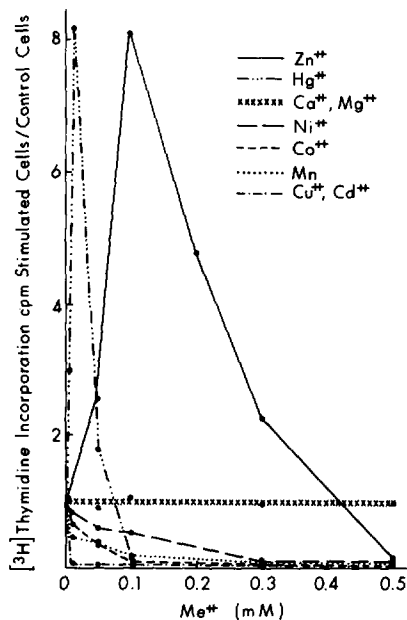


FIGURE 3 Concentration effect of divalent metal ions ( $Me^{++}$ ) on DNA synthesis in cultured lymphocytes from normal adult human donors. DNA synthesis measured on 6th day of culture. The points (●) describing the curve for each metal ion are indicated for the concentration range from 0.01 to 0.5 mM. Each point is an average of triplicate cultures from at least three donors. The effects of metal concentrations from 0.1 to 5.0  $\mu M$  are not indicated on the graph; however, no stimulation of DNA synthesis occurred at these lower concentrations.

metal. In addition, each of the metals can coordinate to a variety of ligands in the cells and medium, altering macromolecular structure and function. These possible modes of toxicity should apply to zinc as well as to the other ions investigated and probably account for the inhibition of lymphocyte DNA synthesis observed at  $Zn^{++}$  concentrations of 0.5 mM and above.

Since 0.5 mM  $Zn^{++}$  drastically inhibits the rate of lymphocyte DNA synthesis, the possibility was considered that lower concentrations of  $Zn^{++}$  might also inhibit DNA synthesis, but that this effect might be masked by the simultaneous stimulation of DNA synthesis. To examine this possibility, PHA was used to stimulate lymphocyte DNA synthesis, and the effect of  $Zn^{++}$  on this process was evaluated (Table I). The concentration of 0.1 mM  $Zn^{++}$ , which independently produces optimal stimulation of lymphocyte DNA synthesis, inhibits PHA stimulation of DNA synthesis by 33%. The effect of a 2-h exposure to  $Zn^{++}$  on spontaneous DNA synthesis in L1210 leukemic lymphocytes was also evaluated. Increasing concentrations of zinc caused progressive inhibition of DNA synthesis in these L1210 cells with 0.1 mM  $Zn^{++}$  inhibiting DNA synthesis by 33% after only 2-h incubation. An overlap of  $Zn^{++}$  stimulation and inhibition is clearly illustrated by these results where the concentration of  $Zn^{++}$  which produces optimal stimulation of DNA synthesis in resting lymphocytes also inhibits DNA synthesis in PHA-stimulated cells and in L1210 leukemic lymphocytes. This finding suggests that the increase in lymphocyte DNA synthesis stimulated by  $Zn^{++}$  may be reduced by  $Zn^{++}$  toxicity

TABLE I

Effect of  $Zn^{++}$  on  $[^3H]$ Thymidine Incorporation by PHA-Stimulated Human Lymphocytes and by Mouse L1210 Lymphoblasts

	$[^3H]$ Thymidine incorporation cpm/ $10^6$ lymphocytes			
	Human lymphocytes		L1210 lymphoblasts	
	Exp. 1	Exp. 2		
Control	3,030	1,679	Control	28,661
15 $\mu g/ml$ PHA	30,224	37,295	0.05 mM $Zn^{++}$	23,265
15 $\mu g/ml$ PHA + 0.1 mM $Zn^{++}$	19,887	24,153	0.1 mM $Zn^{++}$	19,241
15 $\mu g/ml$ PHA + 0.3 mM $Zn^{++}$	5,469	19,875	0.3 mM $Zn^{++}$	6,721
15 $\mu g/ml$ PHA + 0.5 mM $Zn^{++}$	190	236	0.5 mM $Zn^{++}$	2,284

$[^3H]$ Thymidine incorporation was measured during a 4-h period in human lymphocytes after 3-days incubation with PHA and  $Zn^{++}$  and during a 1-h period in L1210 lymphoblasts after 2-h incubation with  $Zn^{++}$ .

and that the observed increase may be the net result of these processes.

### The $Zn^{++}$ Responsive Cells

The cell suspensions prepared on Ficoll-Hypaque gradients contain greater than 90% lymphocytes. The other leukocytes present are neutrophils, basophils, and monocytes. The increase in DNA synthesis stimulated by  $Zn^{++}$  could be occurring in the lymphocytes or in the other non-lymphocytic cells. It is also possible that the effect of  $Zn^{++}$  on the lymphocytes is mediated through a primary effect of  $Zn^{++}$  on the other cells. This problem was investigated by examining  $Zn^{++}$  stimulation in cultures of pure lymphocytes obtained by passing Ficoll-Hypaque gradient lymphocytes through nylon fiber columns. Ficoll-Hypaque and nylon column lymphocytes from each donor were cultured simultaneously at the same lymphocyte concentration and under identical conditions.

0.1 mM  $Zn^{++}$  stimulates DNA synthesis in lymphocyte cultures prepared both from Ficoll-Hypaque gradients and from nylon columns (Table II). The spontaneous rate of DNA synthesis in control, unstimulated lymphocytes prepared by nylon column is the same or less than that observed in unstimulated Ficoll-Hypaque gradient cells. These values are indicated for exp. 1, and this observation has been previously reported (13). In these experiments, the rate of DNA syn-

TABLE II  
Effect of Lymphocyte Preparation on  $Zn^{++}$  Stimulation

[ $^3H$ ]Thymidine incorporation cpm/10 <sup>6</sup> lymphocytes				
Exp.	Day	Control*		
		Unstimulated	Ficoll-Hypaque purified	Nylon column purified
			0.1 mM $Zn^{++}$	0.1 mM $Zn^{++}$
1	3	322 (267)	1,149	1,484
	6	2,059 (1,404)	8,698	8,366
2	3	377	882	1,027
	6	825	7,890	8,633
	9	1,395	10,939	17,844

\* Control cultures performed with lymphocytes from Ficoll-Hypaque gradient. Numbers in parentheses are additional controls performed on nylon column-purified lymphocytes.

thesis which occurs in response to  $Zn^{++}$  stimulation is the same or greater in nylon column-purified lymphocytes than in those from the Ficoll-Hypaque gradient. These studies indicate that the DNA synthesis stimulated by  $Zn^{++}$  in lymphocyte-enriched cultures does in fact occur in lymphocytes. Other nonlymphoid cells are not required as mediators of the  $Zn^{++}$  response.

### Kinetics of $Zn^{++}$ -Stimulated Nucleic Acid Synthesis

The kinetics of  $Zn^{++}$  stimulation were examined at the optimal concentration of 0.1 mM  $Zn^{++}$ . Fig. 4 a shows the increase in rate of DNA synthesis in  $Zn^{++}$ -stimulated cultures over a 10-day period. There is no difference in the rate of DNA synthesis in control and  $Zn^{++}$ -stimulated cells initially or during the first 2 days of culture. After 3 days, the rate of DNA synthesis in  $Zn^{++}$ -stimu-

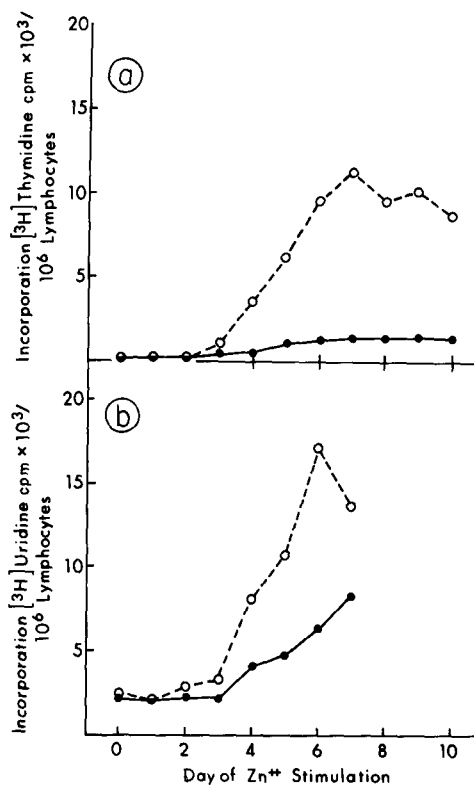


FIGURE 4 Effect of  $Zn^{++}$  on lymphocyte DNA (a) and RNA (b) synthesis at daily intervals after addition of 0.1 mM  $Zn^{++}$  to cultures. --○--, 0.1 mM  $Zn^{++}$ ; ●—●, control. DNA synthesis, average of four experiments. RNA synthesis, average of three experiments.

lated cultures increases above control levels and continues to rise until a maximum is reached on the 6th or 7th day of culture. The increased rate of DNA synthesis in  $Zn^{++}$ -stimulated cultures is maintained for several more days and then begins to decline.

$Zn^{++}$ -stimulated lymphocytes show a definite increase in the rate of RNA synthesis compared to control cells (Fig. 4 b). Unstimulated lymphocytes maintain a steady rate of RNA synthesis for 3 days in culture, and then the rate begins to increase spontaneously.  $Zn^{++}$ -stimulated lymphocytes usually show an increase in rate of RNA synthesis on the 2nd or 3rd day of culture and then the rate continues to increase more rapidly than controls. In a series of cultures this increase in RNA synthesis appears to just precede or coincide with the increase in DNA synthesis.

The increase in rate of DNA synthesis observed in  $Zn^{++}$ -stimulated lymphocytes is slow in comparison to the increase in rate induced by the plant mitogen PHA (Table III). All of the PHA-stimulated cultures show a marked increase in DNA synthesis which is evident on the 3rd day of culture. The  $Zn^{++}$ -stimulated cultures show a small increase in the rate of DNA synthesis on the 3rd day which continues to increase until a maximum is reached near day 6. After 6 days, the rate of DNA synthesis in  $Zn^{++}$ -stimulated cultures reaches 20-50% of the rate observed in

PHA-stimulated cultures.  $Zn^{++}$  stimulation and PHA stimulation are not additive; in fact,  $Zn^{++}$  inhibits PHA stimulation, as is indicated in Table I and expts. 2 and 3 in Table III.

#### Requirements for $Zn^{++}$ Stimulation of DNA Synthesis

Studies were performed to determine how long  $Zn^{++}$  must be present in the cultures to stimulate the maximum increase in DNA synthesis. Lymphocyte cultures were stimulated with 0.1 mM  $Zn^{++}$  and the rate of DNA synthesis was determined 6 days later. On each day after starting the cultures, the medium was aspirated from a series of culture tubes and replaced by fresh medium containing 10% autologous serum. After the medium exchange,  $Zn^{++}$  was added to restore the concentration to 0.1 mM in half of the cultures. All cultures were then incubated for the remainder of the 6-day period. In Fig. 5 the amount of DNA synthesis occurring in the cultures in which medium was exchanged is expressed as a percent of the DNA synthesis occurring in lymphocytes incubated undisturbed for the entire 6

TABLE III  
Lymphocyte Stimulation by PHA and  $Zn^{++}$

Exp.	Stimulating agent	Incorporation [ <sup>3</sup> H]thymidine cpm/10 <sup>6</sup> lymphocytes	
		Day 3	Day 6
1	Control	122	964
	0.1 mM $Zn^{++}$	1,344	10,376
	15 $\mu$ g/ml PHA	14,871	21,433
2	Control	227	2,134
	0.1 mM $Zn^{++}$	838	8,354
	15 $\mu$ g/ml PHA	21,941	15,314
	0.1 mM $Zn^{++}$ + 15 $\mu$ g/ml PHA	11,534	
3	Control	352	3,030
	0.1 mM $Zn^{++}$	1,615	12,015
	15 $\mu$ g/ml PHA	30,826	30,906
	0.1 mM $Zn^{++}$ + 15 $\mu$ g/ml PHA	19,938	

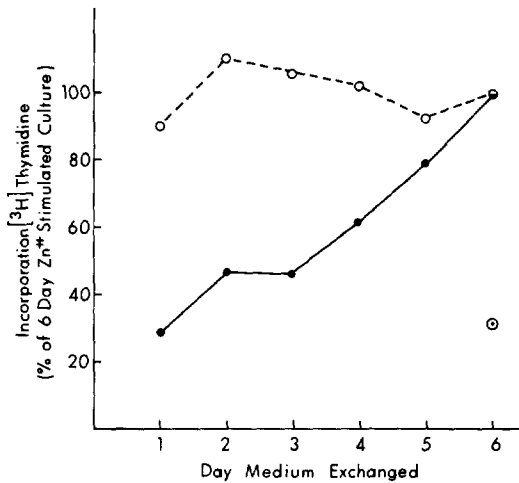


FIGURE 5 Effect of removing and replacing  $Zn^{++}$  in medium used to culture normal adult lymphocytes. DNA synthesis measured on 6th day of culture.  $\odot$  unstimulated control,  $\bullet$ — $\bullet$  medium containing 0.1 mM  $Zn^{++}$  replaced at indicated time by new medium with no added  $Zn^{++}$ ,  $\circ$ — $\circ$  medium containing 0.1 mM  $Zn^{++}$  replaced by new medium and  $Zn^{++}$  concentration restored to 0.1 mM. DNA synthesis expressed as percent of that occurring in lymphocytes incubated for 6 days in the presence of 0.1 mM  $Zn^{++}$  without exchange of medium.

days in the presence of 0.1 mM Zn<sup>++</sup>. Cultures stimulated by 0.1 mM Zn<sup>++</sup> for 6 days without medium exchange demonstrated a threefold increase in the rate of DNA synthesis compared to control unstimulated cultures. Cultures in which the medium was exchanged followed by zinc replacement showed incorporation of [<sup>3</sup>H]thymidine that ranged from 90 to 110% of that which occurred in the unexchanged, Zn<sup>++</sup>-stimulated cultures. This observation indicates that the procedures involved in exchanging the medium did not significantly affect the cells or their ability to respond to Zn<sup>++</sup>.

Cultures from which the Zn<sup>++</sup> was removed showed less stimulation of [<sup>3</sup>H]thymidine incorporation than cultures which contained Zn<sup>++</sup> for the entire 6 days. Lymphocytes which had Zn<sup>++</sup> washed out after 1 day of culture demonstrated essentially the same rate of [<sup>3</sup>H]thymidine incorporation as unstimulated controls. The rate of [<sup>3</sup>H]thymidine incorporation on the 6th day of culture increased with each additional day of incubation in the presence of 0.1 mM Zn<sup>++</sup>. These exchange studies suggest that the maximal response of [<sup>3</sup>H]thymidine incorporation on the 6th day of culture occurs only if Zn<sup>++</sup> is present for the duration of the culture. However, they do not eliminate the possibility that Zn<sup>++</sup> is required in a permissive fashion on the 6th day for maximal DNA synthesis to occur. If this was the case, then removing Zn<sup>++</sup> before the 6th day would diminish the response to Zn<sup>++</sup> stimulation.

The following experiment was performed to determine if Zn<sup>++</sup> stimulation was simply a requirement for the metal during the latter part of the culture. Zn<sup>++</sup> was added to a group of unstimulated cultures on each day after the cultures were started. DNA synthesis was determined 6 days after the cultures were initiated. Thus, all cultures had Zn<sup>++</sup> present when the rate of DNA synthesis was determined but for a variable period preceding this measurement. Lymphocytes which were incubated with Zn<sup>++</sup> from the beginning of the culture demonstrated the maximum rate of [<sup>3</sup>H]thymidine incorporation (Fig. 6). Shorter incubation periods with Zn<sup>++</sup> produce progressively less incorporation of [<sup>3</sup>H]thymidine. This study indicates that the response to Zn<sup>++</sup> is not simply dependent on the presence of Zn<sup>++</sup> during the later days of the culture, and confirms the observation that the maximal response to

Zn<sup>++</sup>, requires the presence of Zn<sup>++</sup> for the entire culture period.

To determine if RNA synthesis is required for Zn<sup>++</sup> stimulation, actinomycin D was employed to block DNA-dependent RNA synthesis. Actinomycin D, 5 µg/ml, was added at daily intervals to Zn<sup>++</sup>-stimulated cultures and the effect on RNA synthesis determined during the next 4 h. Cultures with actinomycin D added at corresponding times were maintained for the remainder of the 6-day incubation period and then the effect on DNA synthesis was determined. Actinomycin D, 5 µg/ml, inhibits [<sup>3</sup>H]uridine incorporation in Zn<sup>++</sup>-stimulated cells (Table IV), and addition of this inhibitor on any of the first 5 days of cultures also prevents the increase in DNA synthesis which occurs on day 6 (Table V). When actinomycin D was added to the cultures on the 6th day, 30 min before a 4-h pulse with [<sup>3</sup>H]thymi-

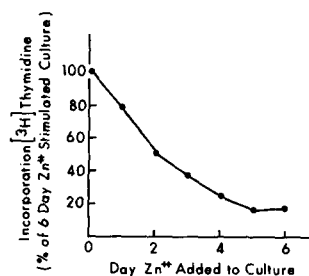


FIGURE 6 Effect of time of addition of Zn<sup>++</sup> on DNA synthesis in cultured lymphocytes from normal human adults. 0.1 mM Zn<sup>++</sup> added at indicated day after initiation of culture. DNA synthesis measured in all cultures on 6th day and expressed as percent of synthesis occurring in lymphocytes incubated with 0.1 mM Zn<sup>++</sup> for entire 6 days.

TABLE IV  
Inhibition of Zn<sup>++</sup>-Stimulated [<sup>3</sup>H]Uridine Incorporation

	Incorporation [ <sup>3</sup> H]uridine cpm/10 <sup>6</sup> lymphocytes	
	Day 3	Day 6
Control	1,678	4,403
0.1 mM Zn <sup>++</sup>	2,165	15,937
0.1 mM Zn <sup>++</sup> + 5 µg/ml actinomycin D*	785	1,543

\* Actinomycin D added 30 min before determination of RNA synthesis.

TABLE V  
Inhibition of  $Zn^{++}$ -Stimulated [ $^3H$ ]Thymidine Incorporation\*

	Day inhibitor added	Incorporation [ $^3H$ ]thymidine cpm/ $10^6$ lymphocytes	Percent inhibition
Control	—	1,220	
0.1 mM $Zn^{++}$	—	10,050	
0.1 mM $Zn^{++}$ + 5 $\mu$ g/ml actinomycin D	0	162	98
0.1 mM $Zn^{++}$ + 5 $\mu$ g/ml actinomycin D	6	2,500	75
Control	—	2,980	
0.1 mM $Zn^{++}$	—	6,743	
0.1 mM $Zn^{++}$ + 5 $\mu$ g/ml cycloheximide	0	187	97
0.1 mM $Zn^{++}$ + 5 $\mu$ g/ml cycloheximide	6	994	85
0.1 mM $Zn^{++}$ + 75 $\mu$ g/ml puromycin	0	309	95
0.1 mM $Zn^{++}$ + 75 $\mu$ g/ml puromycin	6	1,152	83
Control		1,899	
0.1 mM $Zn^{++}$		10,122	
0.1 mM $Zn^{++}$ + 10 $\mu$ g/ml chloramphenicol	0	8,368	17
Control		1,305	
0.1 mM $Zn^{++}$		8,366	
0.1 mM $Zn^{++}$ + 0.5 mM 5'-AMP	0	3,547	57
0.1 mM $Zn^{++}$ + 0.5 mM 5'-AMP	6	6,230	26

\* [ $^3H$ ]Thymidine incorporation measured on 6th day of culture in all cases. Inhibitors on day 6 were added 30 min before determination of DNA synthesis.

dine,  $Zn^{++}$ -stimulated DNA synthesis was inhibited by 75%.

Inhibitors of protein synthesis were included in the  $Zn^{++}$ -stimulated cultures and the effects on DNA synthesis determined. The presence of cycloheximide, 5  $\mu$ g/ml, for the duration of the culture inhibits  $Zn^{++}$ -stimulated DNA synthesis by 97%. Similarly the presence of puromycin, 75  $\mu$ g/ml, for the entire culture period inhibits DNA synthesis by 95%. Cycloheximide and puromycin inhibit DNA synthesis by 85 and 83%, respectively, even when added on the 6th day of culture, 30 min before a 4-h pulse with [ $^3H$ ]thymidine.

Chloramphenicol inhibits protein synthesis in prokaryotes and in the mitochondria of eukaryotes (15). The presence of 10  $\mu$ g/ml of chloramphenicol in the lymphocyte cultures inhibits  $Zn^{++}$  stimulation of DNA synthesis by 17%, suggesting a requirement for mitochondrial protein synthesis in responding cells. The amount of inhibition is not increased by raising the concentration of chloramphenicol to 50  $\mu$ g/ml. Nucleotides inhibit the increase in lymphocyte DNA synthesis after PHA stimulation (16). Incubating cells in the

presence of 5'-AMP inhibits the response to  $Zn^{++}$  stimulation by 57%. Addition of 5'-AMP to cultures on the 6th day inhibits  $Zn^{++}$ -stimulated DNA synthesis by 26%.

#### *Morphologic Transformation in $Zn^{++}$ -Stimulated Lymphocytes*

$Zn^{++}$ -stimulated lymphocytes undergo a morphologic transformation. At the start of the cultures the cells have the typical appearance of small lymphocytes with a condensed heterochromatic nucleus and scant cytoplasm. Cells in the  $Zn^{++}$ -stimulated cultures demonstrate an enlarged cytoplasm and nucleus, and blast cells develop which are similar to those described in PHA-stimulated lymphocyte cultures (17). The morphologic changes of blast transformation occur in unstimulated controls, but less than 5% of cells show this spontaneous transformation at any time during a 10-day culture period. An increase in the percent of blasts in  $Zn^{++}$ -stimulated cultures becomes apparent after 3 days in culture, coincides with the increase in DNA and RNA synthesis,



and continues to increase until the 6th or 7th day. By the 6th day,  $Zn^{++}$ -stimulated cultures attained an average blast transformation of 21% with a range of 11–44% for seven different experiments. The same percentage of cells appear as blasts in  $Zn^{++}$ -stimulated cultures on the following 2–3 days.

#### DISCUSSION

These studies demonstrate that  $Zn^{++}$  stimulates lymphocyte DNA and RNA synthesis and blast transformation. They confirm the report by Rühl et al. (2) that lymphocyte preparations from all normal donors respond to  $Zn^{++}$  stimulation with an increased rate of DNA synthesis and blast transformation. Schöpf et al. have also demonstrated that  $Hg^{++}$  stimulates the morphologic changes of blast transformation and mitosis in cultured human lymphocytes (18). The kinetics of the increase in DNA synthesis and blast transformation observed in the present study are similar to those previously reported for  $Zn^{++}$  stimulation. Rühl et al. (2) observed a 75-fold increase of DNA synthesis in  $Zn^{++}$ -stimulated cultures compared to controls, whereas an eightfold increase was observed in the present series. The reason for this difference is unknown. In our experiments,  $Zn^{++}$  stimulation of DNA synthesis was observed in cultures containing 100% lymphocytes, confirming that it is the lymphocyte that responds to  $Zn^{++}$  stimulation and that this response does not require the presence of accessory nonlymphoid cells.

The response to  $Zn^{++}$  is not due to the change in cation concentration produced by adding the metals to lymphocyte cultures, since among the cations evaluated in this series, only  $Zn^{++}$  and  $Hg^{++}$  stimulate lymphocyte DNA synthesis.  $Ca^{++}$  and  $Mg^{++}$  did not affect DNA synthesis in this system although previous studies have demonstrated that  $Ca^{++}$  and  $Mg^{++}$  are required for cell viability and also must be present for lymphocytes to carry out DNA synthesis (19).

Lymphocytes rendered incapable of DNA synthesis by incubation with actinomycin D do not demonstrate  $Zn^{++}$ -stimulated DNA synthesis. Cells incubated with the protein synthesis inhibitor cycloheximide or puromycin also fail to demonstrate  $Zn^{++}$ -stimulated DNA synthesis. These inhibitors of protein and RNA synthesis partially inhibit DNA synthesis in  $Zn^{++}$ -stimulated cells even when added after a 6-day period of incubation, but just before DNA synthesis is determined.

Inhibitors added at this time are not expected to inhibit the stimulation process leading to DNA synthesis but rather should affect the processes directly involved in DNA synthesis. The inhibition of DNA synthesis which occurs when actinomycin D is added at the end of the cultures indicates a requirement for RNA synthesis during DNA synthesis. This finding is consistent with the demonstration that initiation of a DNA strand requires the synthesis of a RNA primer, which is then covalently extended into a growing DNA chain. This phenomenon has been demonstrated in bacteria (20), and a covalent association of RNA with nascent DNA has also been demonstrated in lymphocytes (21), suggesting the occurrence of a similar process in eukaryotes. The inhibition of DNA synthesis which occurs when puromycin or cycloheximide is added at the end of cultures suggests a requirement for protein synthesis during DNA synthesis, and such a requirement has previously been demonstrated in eukaryotes (22, 23).

PHA stimulates a rapid increase in the rate of lymphocyte DNA synthesis which approaches a maximum after 72 h in culture (1). 60–90% of the cells in PHA-stimulated cultures undergo blast transformation (24). Antigen-stimulated lymphocyte cultures demonstrate a slower increase in rate of DNA synthesis reaching a maximum after 6 days (25), at which time 5–30% of cells in culture show blast transformation (26).  $Zn^{++}$ -stimulated lymphocytes reach a maximum rate of DNA synthesis after 6–7 days, at which time 11–44% of cells are found to undergo blast transformation. The kinetics of  $Zn^{++}$  stimulation of lymphocyte DNA synthesis are similar to antigen stimulation in time and magnitude of response. Lymphocyte stimulation by a particular antigen requires previous sensitization of the donor to the antigen (27, 28) whereas  $Zn^{++}$  stimulation does not require previous sensitization. In this regard,  $Zn^{++}$  resembles PHA in that lymphocytes from all normal donors respond to these agents.

When the plant mitogens PHA or Con A bind to the lymphocyte membrane, a series of macromolecular synthetic events are initiated which lead to the synthesis of DNA (1). In contrast to the immediate burst of RNA synthesis (29) and RNA polymerase activity (30) observed in PHA-stimulated cells, 2–3 days are required before an increase in the rate of RNA synthesis is observed in  $Zn^{++}$ -stimulated cells. An earlier change in RNA synthesis could occur if  $Zn^{++}$  stimulates a qualitative

change in the classes of RNA synthesized before the quantitative increase is apparent. If the increase in RNA synthesis observed after 2-days incubation with  $Zn^{++}$  is the first alteration in RNA metabolism that occurs, then  $Zn^{++}$  stimulation may offer a slow motion example in which to evaluate the events between mitogen stimulation and RNA synthesis.

These experiments demonstrate that  $Zn^{++}$  must be present for the entire culture period to produce optimal stimulation of lymphocyte DNA synthesis. The gradual increase in rate of DNA synthesis may be accounted for by several mechanisms.  $Zn^{++}$  may be stimulating a clone or group of cells which promptly initiate DNA synthesis, undergo mitosis, and continue to proliferate until the number of dividing cells is sufficient to produce a measurable increase in the synthetic rate of the entire culture. Such a response has been found in antigen-stimulated cultures where single cells begin to divide 48 h after addition of antigen and then continue to proliferate with an average generation time of 8–13 h (26). Alternatively,  $Zn^{++}$  stimulation may continuously recruit new cells into the S phase of the cell cycle but require variable periods to recruit different cells. This type of variable initiation period has been demonstrated in lymphocytes responding to PHA (31). The requirement for  $Zn^{++}$  to be present for the entire culture period to produce the maximum increase in DNA synthesis suggests that the latter mechanism is occurring in  $Zn^{++}$ -stimulated cultures.

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