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Combined Effects of Phytohemagglutinin and Staphylococcal Enterotoxin B on Deoxyribonucleic Acid Synthesis During Blast Transformation in Human Lymphocytes

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Three mitogenic agents, phytohemagglutinin (PHA), staphylococcal enterotoxin B (SEB), and concanavalin A (Con A) were tested for their effects on deoxyribonucleic acid (DNA) synthesis in the normal human lymphocyte. When optimal concentrations of PHA and SEB were combined, tritiated thymidine incorporation in lymphocytes derived from several donors was enhanced significantly. In the presence of graded concentrations of one of these mitogens added to fixed optimal concentrations of the other, this enhancement was shown to be additive. By contrast, when PHA or SEB were combined with Con A, the resulting thymidine incorporation was slightly lower than for either mitogen alone. An inhibition of further thymidine incorporation when puromycin was added to lymphocytes incubated with PHA and SEB suggested that the additive effect of these mitogens was due to increased enzyme synthesis. To define potential differences in mechanisms of action underlying the additive effect of SEB and PHA, the relative contribution of the de novo and salvage pathways for pyrimidine biosynthesis was tested with cytidine, a specific salvage pathway inhibitor. Cytidine (10^{-3} M) inhibited synthesis through the salvage pathway, but did not significantly alter induction of carbamyl phosphate synthetase II, the rate-limiting enzyme for the de novo pathway. An inhibition of DNA synthesis by millimolar cytidine concentrations in lymphocytes incubated with PHA or SEB, singly or in combination, suggested that pyrimidines for the observed enhancement of DNA synthesis were derived largely via the salvage pathway.

The human peripheral blood lymphocyte from an immunologically competent host is capable of responding to bacterial endotoxins (3, 13) and staphylococcal enterotoxin B (SEB; 13), in addition to a variety of plant lectins (17), by undergoing blast transformation. The initiation of lymphocyte transformation is thought to be a consequence of extensive gene activation (8) which is manifested by a predictable sequence of events including protein phosphorylation (8), ribonucleic acid (RNA) synthesis (11), deoxyribonucleic acid (DNA) synthesis, and morphological alterations (14). Despite the predictable sequence of events attending this activation, the precise regulatory steps that are altered by diverse mitogenic agents are at present unclear. The recent observation of a slightly higher incorporation of tritiated thymidine into DNA when SEB, instead of phytohemagglutinin (PHA), was utilized as the mitogenic agent (13) suggested to us that there might be alternative pathways leading to DNA synthesis, controlled perhaps by separate determinants of nuclear activity. Herein are presented data which show that two mitogenic agents, PHA and SEB, when incubated together result in an additive effect on DNA synthesis.

MATERIALS AND METHODS

Materials. Tritiated thymidine (specific activity 26 Ci/mmol), tritiated uridine (specific activity 25 Ci/mmol), and $NaH^{14}CO_3$ (specific activity 59 mCi/mmol) were obtained from Amersham Searle Corp. Cytidine, uridine, and puromycin were obtained from Sigma Chemical Co., and Eagle minimal essential medium (MEM) was from GIBCO. Ornithine carbamyltransferase (EC 2.1.3.3) for the carbamyl phosphate synthetase II (CP synthetase II) assay was prepared from beef liver by the method of Marshall and Cohen (12). PHA P was obtained from Difco;

concanavalin A (Con A) was from Pharmacia Chemicals, Uppsala, Sweden; and highly purified SEB was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.

Lymphocyte preparation. Venous blood was collected in heparinized syringes from healthy volunteers after an overnight fast. Lymphocytes were separated magnetically from whole blood after incubation with carbonyl iron, as described by Bruce (2). The resulting preparation contained one erythrocyte per lymphocyte and 5 granulocytes per 95 lymphocytes. Cultures were prepared containing 10^6 lymphocytes per 3 ml of an incubation medium which consisted of MEM with added 3.4 mM L-glutamine, 1,500 U of penicillin G, 1.5 mg of streptomycin, and 15% (by volume) autologous plasma. These cultures were incubated at 37 C in an atmosphere of air and 5% CO_2, with a controlled humidity of 80%.

Thymidine and uridine incorporation. Tritiated thymidine (2 μCi) was added to 3 ml of culture medium containing 10^6 cells. After a 4-h incubation period at 37 C, cells were centrifuged at 500 $\times$ *g* for 15 min, and the pellet was suspended in 3 ml of 0.076 M NaCl. The pellet was washed twice, resuspended, and transferred to a membrane filter (Millipore Corp.). The filter was washed twice with 5% trichloroacetic acid and once with methanol, allowed to dry, and placed into toluene containing 4 g of 2,5-diphenyloxazole per liter and 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter for liquid scintillation counting. The uridine incorporation experiments were carried out for a similar time interval with 1 μCi per 10^6 cells. The procedure was modified after incubation to include a suspension of the washed pellet in 1 ml of an aqueous RNA solution containing 0.1 mg of RNA and an addition of cold trichloroacetic acid (2 ml, 5%). The resulting precipitate was allowed to stand for 20 min at 4 C prior to the transfer to membrane filters (Millipore Corp.) and washing as described above. All incorporation studies were carried out in triplicate cultures. Maximal variability between cultures was less than 10%.

Enzyme assays. For CP synthetase II determinations, lymphocytes derived from five cultures (5 $\times$ 10^6 cells) were combined and centrifuged for 20 min at 500 $\times$ *g*. The supernatant was discarded, and 2.5 ml of a buffer containing 0.02 M tris(hydroxymethyl)aminomethane, 1 mM mercaptoethanol, and 30% glycerol (vol/vol) was added. The pellet was resuspended on a Vortex mixer and sonically treated in the cold for 2 min. The sonically treated homogenate was centrifuged for 30 min at 110,000 $\times$ *g*, and the supernatant was utilized as the enzyme source. CP synthetase II was assayed by a previously reported method (16) modified to contain in 1 ml: potassium phosphate buffer (pH 7.4), 50 μmol; adenosine triphosphate, 25 μmol; $MgSO_4$, 25 μmol; L-glutamine, 2.5 μmol; and $NaHCO_3$, 16.3 μmol, containing 10 μCi of $NaH^{14}CO_3$; dithiothreitol, 1 μmol; L-ornithine, 5 μmol; ornithine transcarbamylase, 27 U; and 110,000 $\times$ *g* supernatant, 0.5 ml. The reaction was allowed to proceed for 40 min at 37 C and stopped with trichloroacetic acid. Citrulline in the acid-soluble material was separated as described previously (16). One unit of enzyme activity is defined as one picomole of citrulline generated per minute under the conditions of the experiment.

Protein. Protein was measured in the 110,000 $\times$ *g* supernatant by the method of Lowry et al. (10).

RESULTS

To determine the optimal mitogen concentration required to produce a maximal response, tritiated thymidine incorporation was measured in lymphocytes incubated for 72 h in the presence of graded concentrations of PHA (2.5 to 750 μg/10^6 cells). The results (Fig. 1a) indicate a progressive enhancement in thymidine incorporation reaching a maximum at a PHA concentration of 25 μg. Concentrations of PHA greater than 125 μg resulted in a progressive inhibition of thymidine incorporation. The results of a similar experiment with graded concentrations of SEB (1 to 100 μg/10^6 cells) are shown in Fig. 1 on the right. A progressive increase in tritiated thymidine incorporation reaching a maximum in the presence of 10 μg of SEB was followed by a modest inhibition of incorporation at higher concentrations of this simple protein. In other experiments, Con A effected a maximal incorporation of tritiated thymidine at a concentration of 120 μg/10^6 cells.

To examine the possibility that lymphocytes might be capable of further DNA synthesis when optimal concentrations of mitogens were added, cultures were incubated for 72 h with 25 μg of PHA, 10 μg of SEB, and 120 μg of Con A, singly and in combination. Tritiated thymidine

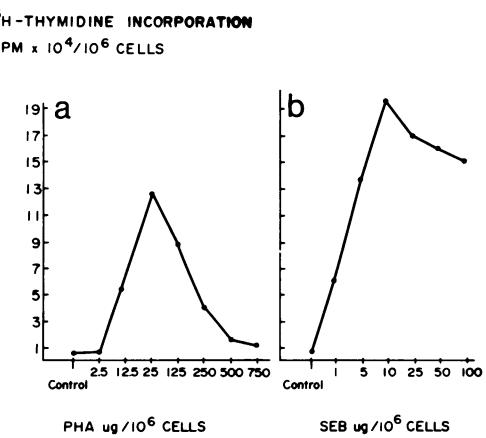

FIG. 1. *Effect of graded concentrations of PHA or SEB on tritiated thymidine incorporation. Lymphocytes from separate donors were incubated with increasing concentrations of PHA (a) or SEB (b). The incorporation of tritiated thymidine is shown on the ordinate, and the concentration of each mitogen is shown on the abscissa.*

incorporation was similar in lymphocytes incubated with optimal concentrations of PHA, SEB, or Con A (Fig. 2). When Con A was combined with either PHA or SEB, the resulting incorporation of tritiated thymidine was slightly less than that seen in the presence of either mitogen alone. By contrast, combined optimal concentrations of PHA and SEB produced an enhancement of thymidine incorporation (Fig. 2).

The reproducibility of this observation was tested in lymphocytes obtained from several donors (Table 1). The enhancement of thymidine incorporation in cultures containing both PHA and SEB was significantly greater than the incorporation seen in the presence of either mitogen alone.

To establish whether this enhancement represented an additive mitogenic effect, tritiated thymidine incorporation was measured in cultures containing graded concentrations of one mitogen added to constant optimal concentrations of the other. In the first experiment, PHA was added in increasing concentrations of 1.25 to 50 μg to cultures containing 10 μg of SEB/10^6 cells. The results indicate a slightly diminished incorporation in the presence of low concentrations of PHA, followed by a rise at 7.5 μg to reach a maximal incorporation at 25 μg/10^6 cells (Fig. 3a). The same experimental design was employed to assess the effects of increasing concentrations of SEB in the presence of a fixed optimal concentration of PHA. The results indicate a slight fall at low concentrations of SEB (2.5 to 5 μg), followed by a rise in tritiated thymidine incorporation reaching a peak at 10 μg (Fig. 3b). In both experiments, the magnitude of the additive effect diminished progressively when greater than optimal concentrations of the variable mitogen were employed (Fig. 3).

To elucidate further the nature of this additive effect, the incorporation of tritiated thymidine was examined in lymphocytes incubated with either PHA or a combination of PHA and SEB in the presence and absence of puromycin.

TABLE 1. *Effect of PHA and SEB on thymidine incorporation in lymphocytes*[a]

Mitogenic agents (μg/10^6 cells)	No.[b]	Tritiated thymidine incorporation (counts/min × 10^4/10^6 cells)	*P*
SEB (10 μg) + PHA (25 μg)	6	17.3 ± 1.3	
PHA (25 μg)	7	9.8 ± .9	<0.001
SEB (10 μg)	5	11.4 ± 2.0	<0.05

[a] Lymphocytes were incubated for 72 h in the presence of 25 μg of PHA, 10 μg of SEB, or both PHA and SEB. The results are shown as the mean ± standard error of the mean of tritiated thymidine incorporation in lymphocytes derived from separate donors. In each experiment, the results of triplicate cultures were averaged to derive a single value.

[b] Total number of experiments.

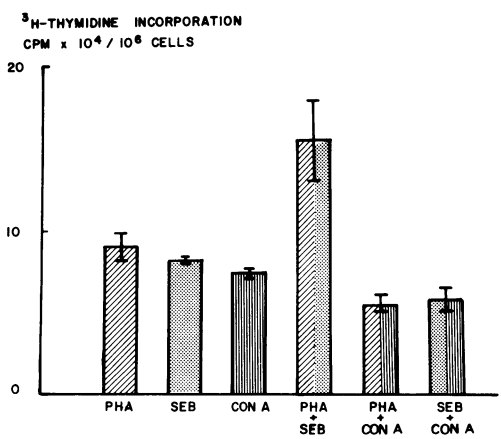

FIG. 2. *Effect of combined mitogens on DNA synthesis in the lymphocyte. Lymphocytes were incubated in the presence of PHA (25 μg), SEB (10 μg), and Con A (120 μg) singly and in combination. Tritiated thymidine incorporation is shown on the ordinate, and the specific mitogens added are shown on the abscissa. (PHA, SEB, and Con A are identified respectively by the cross-hatched, stippled, and vertically lined bars.) The results are shown as the mean and range of data obtained from two such experiments with lymphocytes derived from separate donors.*

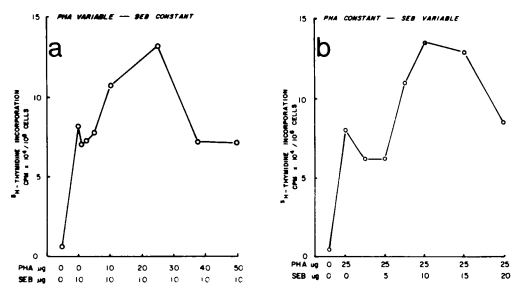

FIG. 3. *Combined effect of PHA and SEB on DNA synthesis. Lymphocytes were incubated for 72 h in the presence of optimal concentrations of SEB (10 μg) and variable concentrations of PHA (a), or optimal concentrations of PHA (25 μg) and variable concentrations of SEB (b). Tritiated thymidine incorporation is shown on the ordinate, and concentrations of PHA and SEB in micrograms per 10^6 cells are shown on the abscissa.*

All cultures exhibited a greater thymidine incorporation at 72 h than at 48 h (Fig. 4). In addition, incorporation was consistently greater in cultures containing combined mitogens. When 10^{-4} M puromycin was added at 48 h, no subsequent rise in incorporation was seen in either group, indicating a requirement for continuing synthesis of new protein for the progressive enhancement of DNA synthesis. Thymidine incorporation in puromycin-treated cultures containing combined mitogens was maintained at consistently greater levels than similarly treated cultures containing PHA alone.

Pyrimidine precursors for DNA biosynthesis can be derived from two sources: recycling of preformed pyrimidines through a "salvage pathway," or biosynthesis from L-glutamine and CO_2 through a "de novo pathway." Each pathway is controlled by a separate regulatory mechanism: cytidine inhibits induction of the rate-limiting enzyme of the salvage pathway, resulting in an inhibition of uridine incorporation into RNA (11); and uridine triphosphate inhibits CP synthetase II activity (18), the rate-limiting enzyme for the de novo pathway. CP synthetase II activity in cell-free mammalian systems is not inhibited by cytidine (18). An additive effect on DNA synthesis suggested the possibility that potential qualitative differences in the respective mechanisms of action of PHA and SEB might be reflected in a selective enhancement of either the de novo or salvage pathway for provision of the required pyrimidine precursors.

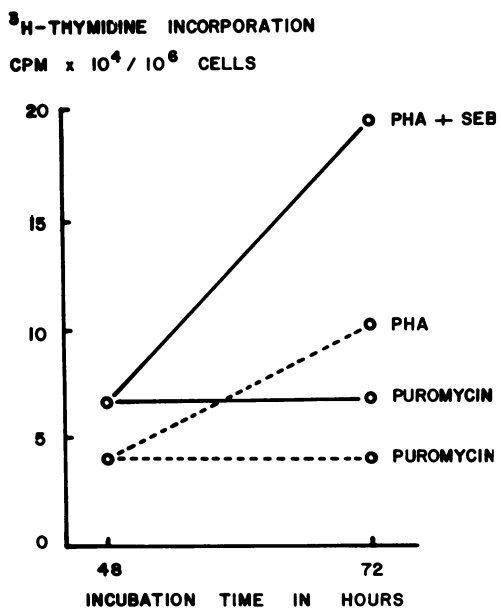

FIG. 4. *Inhibition of combined mitogen stimulation of DNA synthesis by puromycin. Lymphocyte cultures were incubated for 48 h in the presence of PHA (25 μg) or PHA (25 μg) plus SEB (10 μg). Puromycin was then added at a concentration of 10^{-4} M to half the cultures from each group, and incubation continued for another 24 h. Tritiated thymidine incorporation in counts per minute 10^4 per 10^6 cells is shown on the ordinate, and total incubation time in hours is shown on the abscissa. Lymphocytes incubated with PHA (- -O- -); cells incubated with PHA plus SEB (—O—).*

To determine whether cytidine concentrations reported to inhibit RNA synthesis might also interfere with induction of CP synthetase II activity, we first examined induction of this enzyme in lymphocytes incubated with optimal concentrations of PHA. The results (Fig. 5a) indicated a progressive rise in the specific activity of this enzyme that paralleled roughly the increase in tritiated thymidine incorporation (Fig. 5b). The rise was inhibited by 10^{-4} M puromycin, as reported by Ito and Uchino (5), and was consistent therefore with synthesis of new enzyme protein. CP synthetase II activity was then examined in 48-h cultures incubated with PHA and two concentrations of cytidine (10^{-4} and 10^{-3} M) or 10^{-3} M uridine. The results (Table 2) indicate that the highly signifi-

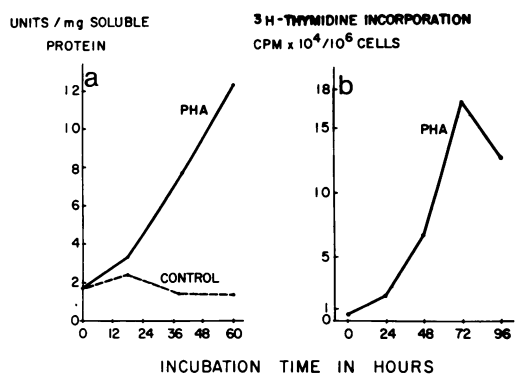

FIG. 5. *Relation of CP synthetase II induction to DNA synthesis. CP synthetase II activity was examined serially in lymphocyte cultures during incubation in the presence and absence of PHA. On the left, enzyme activity in units per milligram protein is shown on the ordinate, and incubation time in hours is shown on the abscissa. Each point represents the mean enzyme activity in duplicate pools of lymphocytes, each pool representing five cultures. Results of duplicate pools varied by less than 10%, and duplicate assays on the same pool varied by less than 5%. (a) Time course of tritiated thymidine incorporation in the presence of PHA (25 μg) per 10^6 cells. Each point represents the mean of triplicate cultures. Tritiated thymidine incorporation is shown on the ordinate and incubation time is shown on the abscissa.*

TABLE 2. *Effect of cytidine and uridine on de novo synthesis of CP synthetase II*[a]

Determination	No.[b]	CP synthetase II units per 10^6 cells	P
Control	7	1.89 ± .24	
PHA (25 μg)	9	5.43 ± .35	<0.001
PHA (25 μg) + cytidine (10^{-4} M)	6	6.73 ± 1.31	<0.01
PHA (25 μg) + cytidine (10^{-3} M)	6	4.37 ± .89	<0.025
PHA (25 μg) + uridine (10^{-3} M)	4	5.65 ± .73	<0.001

[a] Lymphocytes were incubated for 48 h in the presence of PHA and the added ribosides as indicated. CP synthetase II activity in units per 10^6 cells represents the mean ± standard error of the mean of pooled lymphocyte cultures, each pool representing five such cultures.

[b] Total number of pools.

cant threefold increase of CP synthetase II levels in PHA-treated cultures was not altered significantly by the addition of cytidine or uridine in the concentrations shown herein. By contrast, operation of the salvage pathway, as estimated by uridine incorporation into RNA, was inhibited by over 90% in the presence of 10^{-4} and 10^{-3} M cytidine (Fig. 6). These results indicated that cytidine concentrations markedly inhibitory to RNA synthesis did not inhibit the induction of CP synthetase II, and supported the utilization of cytidine as a means for determining whether a selective stimulation of the de novo or salvage pathways might underlie the additive effect of PHA and SEB on DNA synthesis. This possibility was tested by incubating lymphocytes with optimal concentrations of PHA, SEB, or a combination of both mitogens. Three concentrations of cytidine (10^{-5}, 10^{-4}, and 10^{-3} M) were added to the mitogen-containing cultures, and the incorporation of tritiated thymidine was measured at 72 h. The results (Fig. 7) indicate a progressive suppression of tritiated thymidine incorporation with increasing concentrations of cytidine in all three groups. The degree of suppression at any given concentration of cytidine was almost identical in lymphocytes incubated with either PHA or SEB. Millimolar concentrations of cytidine that had little effect on CP synthetase II induction-inhibited synthesis of DNA by 80% (Fig. 7). DNA synthesis was also inhibited by cytidine in lymphocytes incubated with a combination of these mitogens.

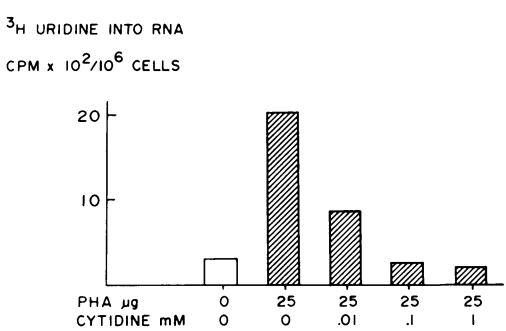

FIG. 6. *Inhibitory effect of cytidine on the salvage pathway. Lymphocytes were incubated in the presence of PHA (25 μg) and increasing concentrations of cytidine as shown on the abscissa. After a 72-h incubation period, uridine incorporation into RNA was measured and the results shown on the ordinate.*

DISCUSSION

The data shown herein represent the first demonstration of a combined action of two mitogens, PHA and SEB, resulting in an additive increase in tritiated thymidine incorporation during blast transformation in human lymphocytes. This combined mitogen effect was dependent on continuing synthesis of new protein and was qualitatively similar to thymidine incorporation in lymphocytes stimulated with PHA alone. Such an effect could result from stimulation of different cell populations or of the same cell population through actions on different regulatory mechanisms. The possibil-

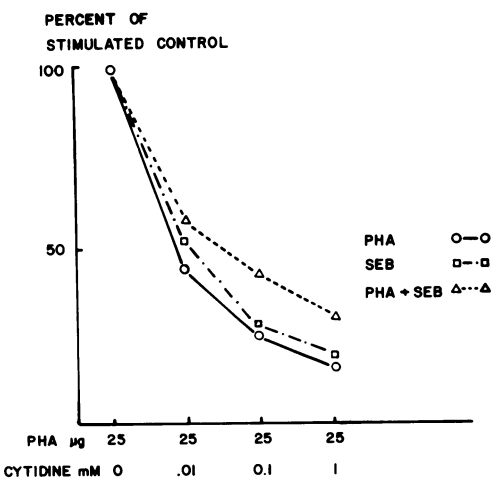

FIG. 7. *Effects on cytidine on DNA synthesis. Lymphocytes were incubated with PHA (25 μg), SEB (10 μg), or PHA (25 μg) plus SEB (10 μg) in the presence of graded concentrations of cytidine (10^{-5} to 10^{-3} M) shown on the abscissa. At 72 h, cultures were harvested, and tritiated thymidine incorporation was determined. The results are shown as a percentage of the maximal activity present in the stimulated cultures lacking cytidine, herein referred to as "control."*

ity that different subpopulations of lymphocytes might have been stimulated with PHA or SEB cannot be ruled out with certainty, since no attempt was made to identify B cells and T cells or those cells with specific immune recognition for SEB (13). Tentative evidence against stimulation of three separate populations of cells is suggested by the absence of an additive response when Con A was combined with either PHA or SEB. If we assume that PHA and SEB stimulated two separate cell populations, the lack of an additive effect when Con A was added to cultures containing either mitogen (Fig. 2) could be interpreted to mean that Con A competed with these mitogens in both cell populations. Although other alternatives exist, the data presented herein suggest that SEB and PHA may produce their additive effects on DNA synthesis in different subpopulations of lymphocytes or possibly through different mechanisms of action on the same cell population.

The separate control mechanisms regulating the de novo and salvage pathways for pyrimidine biosynthesis provided a potential site for selective actions of PHA and SEB. In PHA-stimulated human lymphocytes, the maximal incorporation of substrates into uridine through the de novo pathway based on the specific activity of the rate limiting enzyme, CP synthetase II, is about 13 pmol/min (5), whereas the rate-limiting step in the salvage pathway is capable of permitting generation of about 420 pmol/min. (11). Thus, the de novo pathway may contribute very little to the total pyrimidine requirement in the PHA-stimulated normal lymphocyte. The sensitivity of the salvage pathway function to cytidine and a requirement of the salvage pathway operation for maintenance of DNA synthesis were reported in the normal lymphocyte by Lucas (11) and in the leukemic lymphocyte by Abel et al. (1). We found that millimolar concentrations of cytidine, which failed to affect the threefold increase of CP synthetase II levels in the PHA-stimulated lymphocyte, resulted in a marked inhibition of DNA synthesis. These findings support a principal role of the salvage pathway in supplying pyrimidines required for DNA synthesis. An identical inhibitory effect of cytidine on DNA synthesis in the SEB-stimulated lymphocyte, shown herein, indicated a similar dependence on salvage pathway function. Thus, the majority of pyrimidine precursors for the enhanced DNA synthesis observed in the presence of SEB and PHA either singly or in combination were likely derived from reutilization of preformed pyrimidines and not through a selective enhancement of de novo biosynthesis.

The provision of pyrimidines through similar biosynthetic pathways in lymphocytes incubated with SEB or PHA indicated a common response to these mitogens and implied that potential differences in their effects on regulation were likely to be found in early nuclear events preceding initiation of pyrimidine biosynthesis (4, 6, 7). One event, a specific increase in non-histone nuclear proteins, has been reported to occur within minutes after lymphocyte exposure to PHA (9, 15). Whether the actions of SEB are characterized by a similar response or by a qualitatively different mechanism of action is at present unknown. Studies to resolve these questions are now in progress.

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