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Relationship Between Sterol Synthesis and DNA Synthesis in Phytohemagglutinin-Stimulated Mouse Lymphocytes*

(cholesterol/hydroxymethylglutaryl coenzyme A reductase/ inhibitory cholesterol derivatives/blastogenesis)

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ABSTRACT **Incubation of peripheral blood or isolated lymphocytes of C57L/J mice with phytohemagglutinin stimulated the incorporation of thymidine into DNA of lymphocytes as they transformed into large lymphoblasts. DNA synthesis began after about 24 hr of incubation and reached a peak at 48 hours. The *de-novo* synthesis of sterols from acetate was stimulated much earlier, at 4 hr of incubation, and the rate reached a maximum at 24 hr, approximately at the time DNA synthesis began. Rates of incorporation of radioactivity from [^{14}C]acetate into fatty acids and into CO_2 by phytohemagglutinin-treated blood were not significantly different from control values. Phytohemagglutinin stimulation of sterol synthesis could be abolished by the addition of certain oxygenated derivatives of cholesterol (e.g., 25-hydroxycholesterol and 20α-hydroxycholesterol) which specifically depress the activity of the regulatory enzyme in the sterol synthesis pathway, 3-hydroxy-3-methylglutaryl CoA reductase [mevalonate: NADP$^+$ oxidoreductase (CoA acylating); EC 1.1.1.34]. This treatment also abolished DNA synthesis and blastogenesis which otherwise followed the peak of sterol synthesis. Furthermore, DNA synthesis was repressed only if the inhibitor was added early enough to prevent sterol synthesis from reaching its usual maximum. When the compound was added after the rate of sterol synthesis had reached its maximum, DNA synthesis was not affected. These findings suggest that the synthesis of cholesterol is an essential prerequisite for successful initiation and completion of the cell cycle in lymphocytes after phytohemagglutinin activation.**

The utilization of nonspecific mitogens such as phytohemagglutinin (PHA) to initiate the proliferative cell cycle of lymphocytes has been of great value in studies of the mechanism of cell activation. Many of the events that occur after PHA addition to lymphocyte cultures have been extensively studied and well documented (for reviews see refs. 1 and 2). The sequence of events is rapid; immediately after the addition of PHA to lymphocytes the molecules bind to cell receptors and, in a few minutes, increases occur in the concentrations of cyclic AMP and cyclic GMP, and in phospholipid synthesis (1–3). Hours later RNA, proteins, and DNA are sequentially synthesized preceding cell division. In this respect the lymphocytes response to mitogens resembles a synchronized cell system and it can be utilized to study mechanisms for the transformation of cells from the nonproliferating to proliferating state.

Abbreviations: PHA, phytohemagglutinin; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

* During the course of this work Liljeqvist et al. (12) reported that sterol synthesis from acetate was stimulated in PHA-treated human lymphocytes.

To determine whether or not a markedly elevated rate of sterol synthesis in leukemic cells (4, 5) is associated with an alteration in growth, we investigated sterol synthesis during blastogenesis of normal lymphocytes. The results of our studies with PHA-stimulated lymphocytes showed that a peak in the rate of sterol synthesis precedes the so-called S-phase of DNA synthesis in the cell cycle. Treatment of the stimulated lymphocytes with some oxygenated derivatives of cholesterol that appear to be specific inhibitors of sterol synthesis (6–8, 11) prevented both the increase in sterol synthesis and the increase in DNA synthesis that are associated with blastogenesis. DNA synthesis was not affected when the inhibitor was added after the cycle in sterol synthesis had reached its maximum. These observations of an apparent relationship between sterol synthesis and DNA synthesis are described in this report.

MATERIALS AND METHODS

Analysis of Lipid, CO_2 Production and 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase Activity in Whole Blood and Isolated Lymphocytes. Female C57L/J strain mice, aged 3–4 months old, were supplied by the Production Department of the Jackson Laboratory. They were decapitated following asphyxiation in CO_2 and blood was pooled from several animals in sterile test tubes that contained sodium heparin (30 USP units of heparin per ml of blood; Sigma). Aliquots (0.5 ml) of the heparinized blood were pipetted into sterile 24 ml Erlenmeyer flasks containing 5 ml of RPMI 1640 medium (Grand Island Biological Co.) with or without PHA (Difco, PHA-M 0.5 mg/ml of final concentration). The flasks were sealed with stoppers and incubated in a shaking water bath at 37° for various lengths of time. Two hours before the end of incubation 50 μl of RPMI medium containing 25 μCi of [1-^{14}C]acetate (58.5 Ci/mol, New England Nuclear) was added to the incubation mixture and the flasks were sealed with stoppers fitted with plastic cups (Kontes). The procedures for analysis of radioactive CO_2, fatty acids, and sterol fractions of the samples were described previously (4, 5).

In some experiments isolated lymphocytes were used in place of whole blood. The procedure used for isolation of lymphocytes has been described (4, 9). Approximately 7 ml of heparinized blood pooled from eight animals was passed through a column containing an equal volume of 0.3 mm glass beads to remove the "sticky" population of platelets. The blood was then mixed with 7 ml of RPMI medium containing Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)

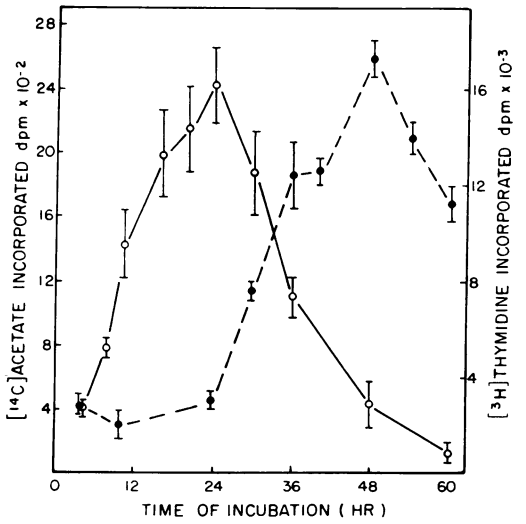

FIG. 1. Time course of PHA stimulation of the syntheses of sterol and DNA. PHA was added at the beginning of the experiment and radioactive acetate or thymidine was added 2 hr before the end of the experiments. In experiments concerned with sterol synthesis (O——O) 0.5 ml of blood was cultured in a total volume of 5 ml. In those concerned with DNA synthesis (●——●) 0.05 ml of blood was cultured in a total volume of 0.5 ml. Triplicate samples were analyzed; points and range represent mean ± SEM. Data for control cultures without PHA are not shown; however, both sterol and DNA radioactivity counts were very low and were linear over the entire incubation period.

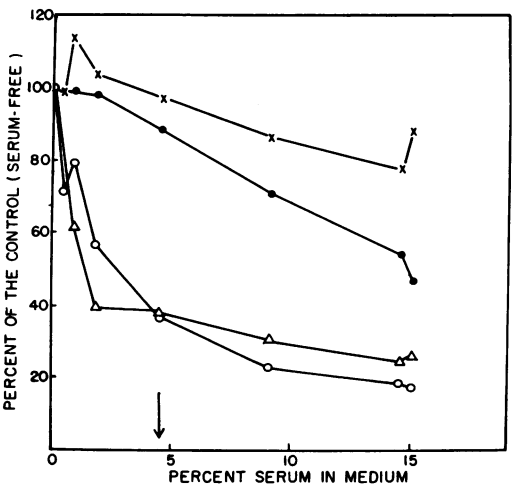

FIG. 2. Effect of homologous serum on acetate metabolism and DNA synthesis in PHA-stimulated cells. Serum was collected from blood by centrifugation. Blood cells were washed with RPMI medium and then suspended in the medium containing PHA and various concentrations of serum. The arrow indicates the final concentration of serum routinely used in blood cultures as in experiments shown in Fig. 1. Cultures used for investigation of metabolism were incubated with PHA for 20 hr and labeling with [^{14}C]acetate was carried out for the last 2 hr. Cultures used for measurements of DNA synthesis were incubated with PHA for 48 hr and labeling with [^{3}H]thymidine was carried out for the last 2 hr. Assays for sterol synthesis (O), fatty acid synthesis (△), CO_2 production (×), and DNA synthesis (●) were performed as described in the *text*.

buffer (pH 7.0), 25 mM and EDTA, 1.35 mM. This mixture was layered over a 10 ml Ficoll-Hypaque gradient and centrifuged at 500 × *g* for 25 min at 12°. [Ficoll was obtained from Sigma, and Hypaque (sodium diatrizoate) was purchased from Winthrop Laboratory, New York, N.Y.] Under these conditions the erythrocytes sedimented to the bottom, the lymphocytes appeared in an interphase layer, and the platelets occurred between the supernatant plasma and interphase layer. The lymphocyte layer was withdrawn and washed twice in 0.9% NaCl, centrifuging each time at 250 × *g* for 15 min. The pelleted cells were then resuspended in RPMI medium with or without PHA and used in experiments similar to those carried out with whole blood. HMG-CoA reductase activity [mevalonate:NADP$^+$ oxidoreductase (CoA-acylating); EC 1.1.1.34] in isolated lymphocytes was assayed essentially as described previously (4, 6).

PHA Stimulation of Lymphoblastic Transformation of Blood Lymphocytes. The micro-culture of whole blood or of isolated lymphocytes was carried out as previously described (10). Briefly, 0.05 ml of heparinized blood or of a suspension of isolated lymphocytes was mixed with 0.5 ml of RPMI 1640 medium with or without PHA (0.5 mg/ml). After 46 hr of incubation at 37° in a 5% CO_2-balanced air incubator, 1 μCi (0.5 mmol) of [^{3}H]thymidine (New England Nuclear) was added to the culture, and the mixture was incubated for an additional 2 hr. Incorporation of [^{3}H]thymidine into DNA was measured by the radioactivity in the perchloric-acid-insoluble fraction of the cells.

Steroids. Procedures for the purification of cholesterol and other sterols were similar to those previously reported (5–7). A sterol (0.5 mg) was mixed with 2 ml of RPMI medium containing 5% crystallized bovine serum albumin (Pentex) and the mixture was sonicated for 30 sec to obtain a homogenous suspension. The freshly prepared sterol suspensions were diluted with RPMI medium for incubation with cultures so that the final concentration of bovine serum albumin in the medium was 0.015%. This amount of albumin was added to the medium of control cultures.

RESULTS

Following treatment of the cells with PHA the rate of sterol synthesis began to increase by 4 hr, reached a maximum at 24 hr, and then declined to the basal rate by 48 hr (Fig. 1). In the same experiments rates at which fatty acids and CO_2 were produced from acetate showed little difference between PHA-treated and control cultures, i.e., both rates decreased steadily over the entire incubation period (not shown). Under similar conditions increased incorporation of thymidine into DNA was first detected 24 hr after the addition of PHA, the time at which the rate of sterol synthesis was the highest. The rate of DNA synthesis increased steadily thereafter until the 48th hr and then declined.

In the experiments indicated in Fig. 1, the incubation mixture contained homologous serum at a final concentration of 5%. Removal of serum from the blood cells by centrifugation and washing with fresh medium increased the sterol and fatty acid synthesis in both PHA-treated and untreated cells. Increases in sterol and fatty acid synthesis induced by treatment with PHA or by the removal of serum appeared to be additive. Fig. 2 shows the extent to which rates of sterol and fatty acid synthesis in PHA-stimulated cultures were diminished by increasing concentrations of homologous serum. In contrast, the production of CO_2 was not significantly affected by the amount of serum in the culture, and the rate of

TABLE 1. *Activity of HMG-CoA reductase and conversion of acetate to sterols, fatty acids, and CO_2 by isolated lymphocytes*

	N	PHA	25-hydroxy-cholesterol	HMG-CoA reductase, nmol mevalonate/ mg of microsomal protein per hr	Sterols, dpm × 10^{-3}	Fatty acids, dpm × 10^{-4}	CO_2, dpm × 10^{-4}
Exp. 1	4	−	−	0.08* ± 0.04			
	5	+	−	2.8 ± 2.5			
Exp. 2	3	−	−		0.1 ± 0	58 ± 3	53 ± 7
	3	+	−		82 ± 14	80 ± 1	32 ± 2
Exp. 3	1	−	−		1.0	29	3
	2	+	−		79	53	10
	3	+	+		0.3 ± 0.1	29 ± 4	4 ± 1

For Exp. 1 each flask contained lymphocytes isolated from 12 ml of fresh blood. The flasks were incubated with or without PHA for 20 hr. The procedure for the enzyme assay was previously described (4, 6). Microsome protein per flask was about 0.18 mg. The reaction mixture contained 34 nmol of DL-hydroxymethyl[3-^{14}C]glutaryl-CoA (0.2 μCi), 0.5 μmol of glucose 6-phosphate, 0.5 units of glucose-6-phosphate dehydrogenase, 0.5 μmol of $NADP^+$, 3 μmol of EDTA, 7.5 μmol of NaCl, 2.5 μmol of 2-mercaptoethanol, 17.5 μmol of potassium phosphate buffer (pH 6.8), and lymphocyte microsomes in a total volume of 0.25 ml. Incubation time was 60 min at 37° in a N_2 atmosphere. For Exps. 2 and 3 each flask contained lymphocytes isolated from approximately 1 ml of fresh blood. Total proteins per flask were 0.8 mg in Exp. 2 and 0.4 mg in Exp. 3. Lymphocytes suspended in 5 ml of RPMI medium were incubated with or without PHA or 25-hydroxycholesterol (1 μg/ml) for 19 hr and then labeled with [^{14}C]acetate for an additional 2 hr.

* Below lower limit of confidence in assay.

thymidine incorporation into DNA declined slowly with increasing serum concentration so that at a serum concentration of 15% the rate was 50% of that obtained in the absence of serum.

The results of studies with isolated lymphocytes shown in Table 1 provide evidence that the changes in the rate of sterol synthesis observed in PHA-treated cultures of blood cells are due to stimulation of the lymphocytes therein. HMG-CoA reductase activity in unstimulated lymphocyte preparations was below the limit of accurate measurement. However, 20 hr after stimulation with PHA the enzyme activity was elevated to levels similar to those found in cultures of mouse liver cells or fibroblasts (6). The rate of sterol synthesis in isolated lymphocytes incubated for 18 hr with PHA was vastly (at least 80-fold) greater than that in control lymphocytes. Moreover, 25-hydroxycholesterol (cholest-5-ene-3β,25-diol), added simultaneously with the PHA, fully counteracted the stimulatory effect of the mitogen upon the rate of sterol synthesis. The inhibitory sterol also counteracted the moderate increases in fatty acid synthesis and CO_2 production that resulted from treatment with PHA.†

TABLE 2. *25-Hydroxycholesterol reduces transformation of mouse peripheral lymphocytes by PHA*

Sample	N	Cell viability (%)	Small lymphocytes, % of total, mean ± SEM	Large lymphocytes (blasts), % of total, mean ± SEM
Control	7	95	87.6 ± 1.6	12.4 ± 1.6
+ PHA	8	91	56.2 ± 3.4	48.8 ± 3.4
+ PHA + 25-hydroxy-cholesterol (0.5 μg/ml)	9	93	73.9 ± 3.6	26.1 ± 3.6

Samples contained 0.05 ml of blood in 0.5 ml of RPMI medium with or without PHA or 25-hydroxycholesterol. They were incubated for 48 hr at 37°. At the end of incubation the blood was pipetted into hematocrit capillary tubes and centrifuged for 5 min in a micro-capillary centrifuge (model MB, International Equipment Co.). The capillary tube was then broken at the interphase of the buffy coats and the culture medium, and the lymphocyte layer was removed. Lymphocyte viability was checked in portions of these samples by trypan blue exclusion tests. Brushed slides of the preparation were stained with Giemsa stain and the percentage of large lymphoblasts was determined after examining 150 to 200 cells per slide.

The effects of graded concentrations of 25-hydroxycholesterol upon PHA-stimulated cultures of blood cells are shown in Fig. 3. The concentration of the steroid required to inhibit sterol synthesis totally was 0.75 μg/ml, whereas DNA synthesis was inhibited by 80% when the concentration of 25-hydroxycholesterol reached 1 μg/ml. The utilization of acetate for fatty acid synthesis and CO_2 production was not significantly affected over the range of steroid concentrations tested.

Fig. 4 demonstrates that the incorporation of thymidine into DNA in the PHA-stimulated cultures of either whole blood or serum-free blood cells was inhibited by 25-hydroxycholesterol or 20α-hydroxycholesterol (cholest-5-ene-3β,20α-diol) at concentrations required to inhibit sterol synthesis. Similar results were observed when 7-ketocholesterol or 7β-hydroxycholesterol were used, although approximately 2- to 4-fold higher dosages were required to obtain the same extent

†**Note Added in Proof.** The following observations provide further evidence that stimulation of sterol synthesis by PHA is a result of the blastogenic transformation of lymphocytes per se rather than a direct activation of HMG-CoA reductase: first, addition of 0.1 M *N*-acetyl-D-galactosamine, which competes with the PHA-binding site on lymphocytes, abolished the lectin's ability to stimulate the sterol synthesis; second, addition of PHA over a concentration range from 2 to 200 μg protein/ml to a reaction mixture containing microsomes (1 mg protein/ml) from cultured L-cells did not affect the level of HMG-CoA reductase activity.

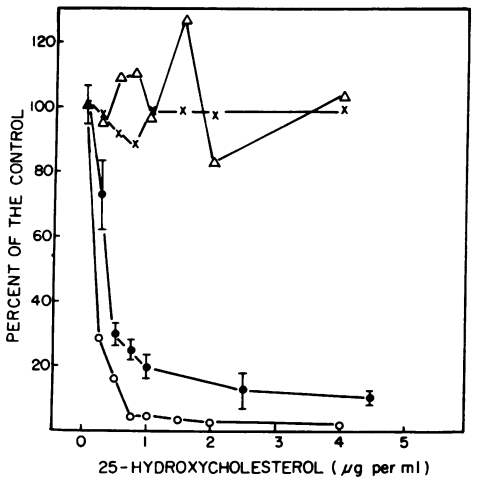

FIG. 3. Effects of various concentrations of 25-hydroxycholesterol on acetate metabolism and DNA synthesis in PHA-stimulated cultures of mouse blood cells. Procedures were the same as those in Fig. 2 except that no serum was present in the medium and 25-hydroxycholesterol was added to the cultures at the beginning of the incubation period. Measurements of sterol synthesis (○), fatty acid synthesis (△), CO_2 production (×), and DNA synthesis (●) were carried out as described in the *text*. Values for DNA synthesis represent means ± SEM from triplicate samples.

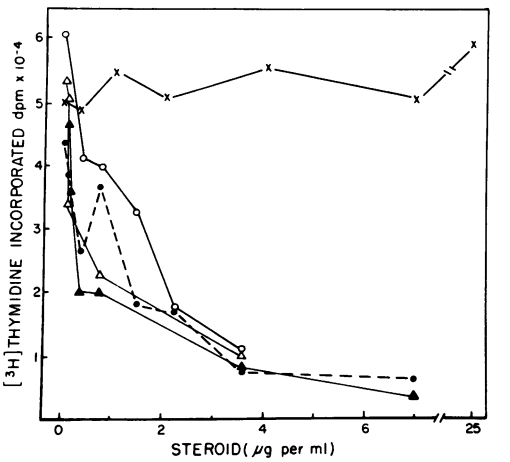

FIG. 4. Effects of various concentrations of 25-hydroxycholesterol, 20α-hydroxycholesterol, and cholesterol on the DNA synthesis of PHA-stimulated cultures of whole blood or blood cells. Procedures were the same as those used to determine DNA synthesis in Fig. 3, except that serum was not removed in some cultures. Incubation mixtures contained 25-hydroxycholesterol in serum-free blood cell cultures (▲——▲); 25-hydroxycholesterol in whole blood cultures (△——△); 20α-hydroxycholesterol in serum-free blood cell cultures (●——●); 20α-hydroxycholesterol in whole blood cultures (○——○); or cholesterol in whole blood cultures (×——×).

of inhibition as that shown in Fig. 4. Cholesterol, at concentrations as high as 25 μg/ml, did not affect DNA synthesis.

25-Hydroxycholesterol effectively inhibited PHA-induced sterol synthesis when it was added to the culture several hours after the addition of PHA (Fig. 5). Even when the sterol was added as late as 14 hr after the addition of PHA (6 hr before termination of the experiment) sterol synthesis was inhibited by 68%. In the same experiments 25-hydroxycholesterol treatment did not affect rates of fatty acid and CO_2 production. DNA synthesis was inhibited only if 25-hydroxycholesterol was added early enough (within 15 hr after the addition of PHA) to produce a significant reduction in the sterol synthesis. No inhibition of DNA synthesis occurred when 20 hr elapsed between the addition of PHA and 25-hydroxycholesterol. Since the concentration of 25-hydroxycholesterol used in these experiments was 0.5 μg/ml, inhibition of DNA synthesis was not complete even when the sterol was added at the beginning of the incubation period. Incubation of 25-hydroxycholesterol at the same concentration (0.5 μg/ml) in the PHA-stimulated lymphocytes reduced the number of transformed cells by approximately one half, in agreement with the extent to which thymidine incorporation was inhibited. The viabilities of lymphocytes in cultures treated with inhibitor and in the controls was not significantly different, being over 90% in all cases (Table 2).

DISCUSSION

These results suggest that a period of enhanced sterol synthesis is a prerequisite for the subsequent phase of DNA synthesis and for the completion of the mitotic cycle of lymphocyte transformation. The conclusion is based upon the following observations: (*a*) A temporal sequence of sterol synthesis and DNA synthesis followed PHA activation of lymphocytes, and (*b*) certain oxygenated derivatives of cholesterol inhibited both sterol and DNA synthesis if they were added to the culture before the rate of sterol synthesis had reached its maximum; when the inhibitor was added after the rate of sterol synthesis had reached its maximum, neither sterol nor DNA synthesis was affected. In interpreting the effects of inhibitors of sterol synthesis, we assume that the sterols act specifically to depress the activity of HMG-CoA reductase and, as a consequence, the rate of sterol synthesis. Evidence for this is provided by our previous studies with L

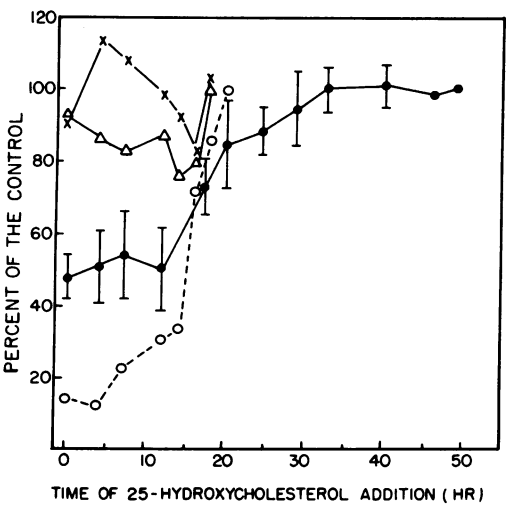

FIG. 5. Effects of 25-hydroxycholesterol on acetate metabolism and DNA synthesis as a function of elapsed time following the addition of PHA to mouse blood cells. Acetate metabolism to sterol (○——○), fatty acids (△——△), and CO_2 (×——×) was determined 20 hr after the addition of PHA. DNA synthesis (●——●) was determined after 48 hr of incubation with PHA. PHA was added to all cultures at zero time and 25-hydroxycholesterol was added at the time indicated on the abscissa to give a final concentration of 0.5 μg/ml. Values for DNA synthesis represent means ± SEM from six separate experiments.

cells and mouse fetal liver cells in culture. Incubation of the inhibitors with these cells for 6 hr did not alter rates of protein, RNA, or DNA synthesis, did not affect rates of fatty acid or CO_2 production from acetate (6, 7), and did not alter intracellular concentrations of cyclic AMP (11). The rate of sterol synthesis from acetate and the level of HMG-CoA reductase activity were, however, almost completely depressed under similar conditions. Prolonged incubation of L cells with the inhibitors results in the depletion of cellular sterols and in arrest of cell growth. However, normal growth is restored when mevalonate, the product of the reaction catalyzed by HMG-CoA reductase, or appropriate sterols are added to the culture, indicating that the inhibitors are not toxic to the cells except through their effect upon sterol synthesis (8). In the present studies, trypan blue exclusion tests showed that the number of viable small lymphocytes in the culture was not significantly reduced after exposure to 25-hydroxycholesterol for as long as 48 hr, indicating that the inhibitor was not toxic to these cells. Furthermore, when lymphoctyes were pre-incubated with 25-hydroxycholesterol (1 μg/ml) for 25 hr and the inhibitor was then removed by washings, the addition of PHA induced the synthesis of DNA after the usual time lag. DNA synthesis in the pretreated cells was somewhat less than in untreated controls (60%) but was considerably greater than when the inhibitor remained in the culture (20%). The fact that the inhibitory sterols reduced only slightly or not at all the metabolism of acetate to fatty acid and CO_2 in lymphocytes and blood cultures is further evidence that they did not produce general cell damage and is in accord with their specific effect upon HMG-CoA reductase.

Presumably the requirement for sterol synthesis in cells undergoing blastotransformation is related either to the need to generate additional membrane during the process leading to cell division or to an alteration in the composition of the membrane during the cell cycle. The time lapse between PHA addition and the induction of sterol synthesis is longer than that required for the induction of phospholipid synthesis (1, 3), suggesting that these two classes of membrane lipids are produced in sequential order rather than simultaneously. Possibly inhibition of sterol synthesis aborts the formation of new membrane even when the stimulated lymphocytes are cultured in the presence of serum (which contains about 1.5 mg of cholesterol per ml). Abolition of DNA synthesis and blastotransformation by inhibitors of sterol synthesis may thus be an indication that membrane formation or an alteration in the sterol composition of existing membranes is essentially involved in the division of mammalian cells.

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