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Immunology

Cholesterol synthesis in polyclonally activated cytotoxic lymphocytes and its requirement for differentiation and proliferation

(T cells/plasma membrane/25-hydroxycholesterol/compactin/3-hydroxy-3-methylglutaryl-CoA reductase)

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ABSTRACT **The kinetics of sterol synthesis and DNA synthesis in polyclonally activated, concanavalin A-stimulated spleen cell cultures were analyzed. Inhibition of DNA synthesis by 1-β-D-arabinofuranosylcytosine (Ara-C) did not abrogate the formation of cytotoxic effector cells. However, inhibition of sterol synthesis by 25-hydroxycholesterol inhibited formation of cytotoxic effector cells as well as cellular proliferation. The inhibition of cytotoxicity correlated well with the dose of 25-hydroxycholesterol administered and was dependent on the time of administration. The agent had to be present when sterol synthesis occurred normally during the time lapse before DNA synthesis began. Compactin had the same effect as 25-hydroxycholesterol. The effects of inhibition of sterol biosynthesis on cytotoxicity could be counteracted by addition of cholesterol-containing liposomes. Based on these experiments, the links between proliferation and differentiation in lymphocytes are discussed.**

Lymphocytes stimulated by mitogens such as concanavalin A (Con A) simulate to some degree a synchronized cell population during the first proliferative cycle after stimulation (1–3). Many metabolic phases have been demonstrated to occur during this activation, among them being a distinct cycle of sterol synthesis (4–6), a cycle of cAMP synthesis (7), and ultimately the S phase of DNA synthesis (4, 5). Recently the important observation was made that one or more subpopulations of lectin-stimulated splenic lymphocytes differentiate into cytotoxic effector cells. This cytotoxicity appeared to be polyclonal because its detection required a ligand, such as phytohemagglutinin (PHA) (8). MacDonald and Lees (9) subsequently showed that these cells differentiated into cytotoxic lymphocytes, even if DNA synthesis was completely inhibited.

In this paper we demonstrate, using specific inhibitors of sterol biosynthesis, that the polyclonal induction of naive, splenic lymphocytes into differentiated cytotoxic lymphocytes calls for the synthesis of cholesterol as an absolute requirement, whether DNA synthesis is inhibited or not. We will use this experimental model system to discuss some aspects of the relationship of proliferation and differentiation in immunocompetent cell populations. Our experiments result in the hypothesis that proliferation is not a prerequisite for differentiation but rather appears to be controlled by the latter in normal tissues (as opposed to uncontrolled proliferation in transformed cells) and merely serves to amplify the product of differentiation.

MATERIALS AND METHODS

Chemicals. Bactophytohemagglutinin M (PHA-M) was purchased from Difco. The hydrochloride salt of 1-β-D-arabinofuranosylcytosine (Ara-C) was purchased from Calbiochem, Con A from Miles, and α-methyl D-mannoside from Sigma. Sterols were purchased from Steraloids (Wilton, NH), recrystallized from methanol three times (10), dissolved in absolute ethanol, and added to Dulbecco's modified Eagle's medium (DME medium) containing 5% bovine serum albumin (Pentex, Miles) (11).

Compactin (ML-236B), an allosteric inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (NADPH) [HMG-CoA reductase; mevalonate:NAPD$^+$ oxide reductase (CoA-acylating), EC 1.1.1.34] isolated from *Penicillium citrinum*, was obtained from A. A. Kandutsch, who received it as a gift from Akira Endo, Tokyo Noko University, Tokyo, Japan. A stock solution of 10 mg in 1 ml of absolute ethanol containing 1% NaOH was diluted appropriately with DME medium prior to addition to the cell cultures.

Filipin U-5956 (no. 8393-DEG-11-8), a polyene isolated from *Streptomyces fillipineasis*, was obtained as a gift from Joseph E. Grady (Upjohn). It was unpacked and dissolved in the dark in absolute ethanol and then further diluted in phosphate-buffered saline before it was added to the cultures.

Spleen Cell Cultures. Spleens of C57BL/6J mice (The Jackson Laboratory) were aseptically removed and placed into DME medium. Cell suspensions were prepared with a Dounce tissue homogenizer as described (12) and transferred into flat-bottomed 16-mm wells (COSTAR) in 2 ml of DME medium supplemented with 5% (vol/vol) fetal bovine serum, 50 μM 2-mercaptoethanol, additional amino acids (12), and Con A at 2 μg/ml. Each culture well contained 5–7 × 10^6 cells. Cultures were maintained in humidified 5% CO_2/95% air. Prior to testing for cytolytic activity, the cells were washed once in DME medium containing 50 mM α-methyl D-mannoside and 5% fetal bovine serum and then were incubated in the same medium for 30 min before being used for the cytotoxic assay (8, 9).

Target Cells. P815 mastocytoma cells (EG & G Mason Research Institute, Worcester, MA) were maintained in DME medium supplemented with 5% fetal bovine serum and were labeled prior to use as targets with 100 μCi of $Na_2{}^{51}CrO_4$ (200–500 Ci/g of Cr, New England Nuclear; 1 Ci = 3.7 × 10^{10} becquerels) for 45 min as described (11).

Assay for Cytolytic Activity. The cytolytic activity of Con A-stimulated spleen cells was assayed as described by the method of MacDonald and Lees (9). Briefly, varying numbers of effector cells were mixed with 10^4 radiolabeled P815 cells in 0.2 ml of

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Abbreviations: Ara-C, 1-β-D-arabinofuranosylcytosine; PHA, phytohemagglutinin; PHA-M, bactophytohemagglutinin M: DME medium, Dulbecco's modified Eagle's medium; Con A, concanavalin A; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase (NADPH); MLR, mixed lymphocyte reaction; CPR, cholesterol/phospholipid ratio.

DME medium (with 5% fetal bovine serum and amino acids) containing 50 mM α-methyl D-mannoside and 2.5 μl of PHA-M per ml. The assay was performed in round-bottomed microtiter plates (Flow Laboratories, McLean, VA), and the radioactivity released in the supernatant was assayed in a Nuclear Chicago well-type γ spectrophotometer. The specific release percentage was calculated by using the formula of Cerottini *et al.* (12). Lytic units per 10^6 cells were calculated from the dose–response curves, 1 unit corresponding to the number of lymphocytes required to lyse 50% of the target cells within 3 hr.

Analysis of Sterol Synthesis. Three to six replicate samples of the Con A-stimulated lymphocytes were pooled from the COSTAR culture wells, and the number of viable cells were determined by the trypan blue dye-exclusion test. Cells were gently suspended in 5 ml of DME medium containing 25 μCi of [1-^{14}C]acetate (59.7 Ci/mol; Amersham) in 25-ml Erlenmeyer flasks and incubated for 1 hr in a 37°C shaking water bath. Labeled sterols, precipitated by digitonin, from the nonsaponifiable fraction were determined as described (10).

Analysis of DNA Synthesis. Replicate samples were removed from three COSTAR culture wells, and the viable, dye-excluding cells therein were determined. The cells were transferred in 1-ml aliquots to tubes containing 1 μCi of [5-*methyl*-^{3}H]thymidine (specific activity, 20 Ci/mmol; New England Nuclear) and were incubated for 1 hr in a 37°C shaking water bath. Incorporation of [^{3}H]thymidine was measured in the perchloric acid-insoluble fraction of the cells as described (2).

Preparation of Liposomes. To prepare cholesterol-containing liposomes, L-α-phosphatidylcholine (L-α-lecithin) from egg yolk (Sigma) was mixed with pure, recrystallized cholesterol in chloroform in a cholesterol/phospholipid molar ratio (CPR) of 0.5. The solvent was evaporated under nitrogen with a rotary evaporator apparatus. Then a "swelling solution" (0.29 M glucose/0.05 M phosphate, pH 7.4) was added and allowed to stand 2–3 hr at room temperature under nitrogen. An aliquot was examined by electron microscope to ascertain the morphological features of the liposomes, and the remaining mixture was stored at 4°C under nitrogen for not longer than 24 hr prior to use. Liposomes were added to the cells to give a phospholipid concentration of 15 × μM.

RESULTS

Kinetics of Incorporation of [^{14}C]Acetate and [^{3}H]Thymidine into Con A-Stimulated Cells. The relationship between cholesterol synthesis ([^{14}C]acetate incorporation into sterol fraction) and DNA synthesis ([^{3}H]thymidine incorporation into DNA) in polyclonally activated lymphocytes is depicted in Fig. 1. The rate of sterol synthesis began to rise rapidly within 4 hr after the initiation of the cultures, reaching a maximum at ≈20 hr, and then declined steadily to a low rate by 48 hr. Increase of incorporation of thymidine into DNA showed a delay in onset of ≈24–36 hr after addition of Con A. After reaching a peak at 48 hr, DNA synthesis declined rapidly to basal levels by 72 hr. Nonstimulated lymphocytes maintained a low rate of sterol and DNA synthesis during the entire 48-hr culture period (data not shown). These observations are similar to our findings in a previous study with blood lymphocytes stimulated by PHA (4).

Lysis of P815 Target Cells by Polyclonally Activated Cytolytic Lymphocytes in the Absence of DNA Synthesis. Cells were cultured for 48 hr with Con A in the presence or absence of the DNA synthesis inhibitor Ara-C and then were assayed for cytolytic activity against P815 mastocytoma cells in the presence of PHA-M. Cytolytic titers of control cells were indistinguishable from the titers of those cells whose DNA synthesis had been blocked (Fig. 2).

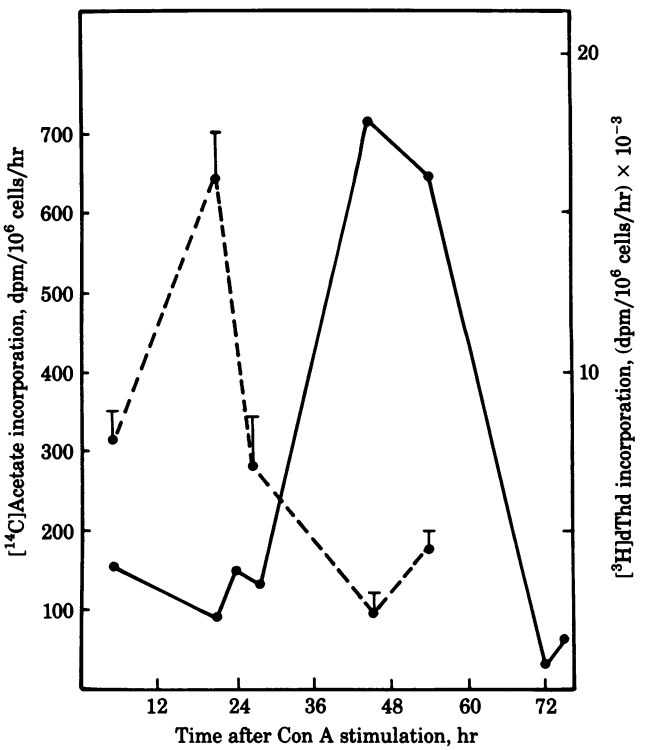

FIG. 1. Relationship of sterol synthesis (---) and DNA synthesis (——) in Con A-stimulated lymphocytes from spleen cell suspensions. Synthesis of cholesterol was measured by determination of [^{14}C]acetate incorporation into the digitonin-precipitable material from the nonsaponifiable lipid fraction. DNA synthesis was determined by the amount of [^{3}H]thymidine incorporated into the acid-insoluble fraction after perchloric acid precipitation. (For similar previous experiments, refer to refs. 5, 6, and 13.)

In the same experiment, we exposed cultures to the sterol synthesis inhibitor 25-hydroxycholesterol at the initiation of stimulation, using 1 μg/ml to inhibit the *de novo* synthesis of cholesterol. It is evident (Fig. 2) that sterol synthesis is required for the generation of Con A-induced cytolytic lymphocytes independent of its requirement for DNA synthesis because the lytic activity of cells cultured in the presence of 25-hydroxycholesterol was suppressed, whether or not Ara-C was present.

The Effect of Varying Concentrations of 25-Hydroxycholesterol upon the Induction of Con A-Induced Cytolytic Activity. The effects of increasing doses of 25-hydroxycholesterol upon the cytotoxic titers generated by polyclonally activated lymphocytes are shown in Fig. 3. The minimal concentrations of 25-hydroxycholesterol required to inhibit sterol synthesis by at least 50% in these cultures appears to lie between 0.1 μg/ml and 0.5 μg/ml, comparable to results obtained in whole-blood leukocyte cultures (4) and in allogeneic mixed lymphocyte cultures (11, 14). The inhibition of cytotoxicity titers by various doses of 25-hydroxycholesterol correlates well (correlation coefficients, 0.81–0.87) with the inhibition of sterol synthesis by these inhibitor doses. To eliminate the possibility of any direct action of 25-hydroxycholesterol on the membrane, we added, in separate experiments, 50 μg/ml for 1 hr prior to the cytolytic assay and left it in the test medium for the entire 3-hr test. Such high doses of 25-hydroxycholesterol had no effect at all upon the cytotoxic titers, excluding a direct toxic action on the cytotoxic effector mechanisms of the lymphocytes under the culture conditions used in this study (control, 5.0 lytic units per 10^6 cells; 25-hydroxycholesterol present during the test, 4.8 lytic units per 10^6 cells).

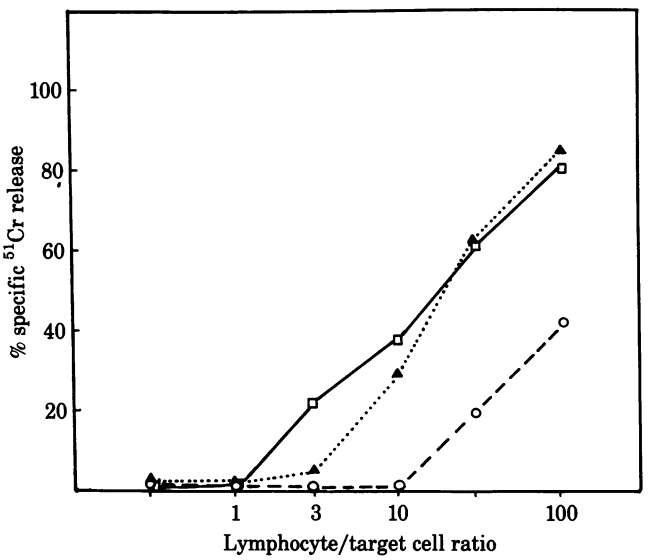

FIG. 2. Cytotoxic titers in polyclonally activated lymphocytes stimulated by Con A. The titers were determined 48 hr after culture initiation. ▲, Untreated control cultures; □, lymphocytes cultured in the presence of Ara-C (5 μg/ml); ○, cultures containing 25-hydroxycholesterol (1 μg/ml). The additives were present during the entire 48-hr culture period, after which viable cell recoveries and cytotoxic activities were determined.

Effects of Inhibition of Sterol Synthesis on the Generation of Cytotoxic Effector Cells. If the cells were allowed to proceed through the first 30 hr of the sterol synthesis cycle and 25-hydroxycholesterol was added at later times, little or no effect was observed on the cytotoxic titers, whereas when the inhibitor was added prior to the peak of sterol synthesis, effective inhibition of cytotoxic titers was obtained. Fig. 4 depicts these experiments in an inverse plot.

Table 1 summarizes the analysis of cytotoxic activity, sterol synthesis, and DNA synthesis in spleen cell cultures under various experimental conditions. Sterol synthesis was measured at 24 hr after stimulation, whereas DNA synthesis and cytolytic activity were determined at 48 hr. In cultures treated with Ara-C, DNA synthesis was abolished, yet sterol synthesis and cytolytic activity were unaffected. Sterol synthesis and DNA synthesis as well as cytotoxic titers were effectively depressed in cultures treated with 25-hydroxycholesterol. Compactin (ML326B) was found to interfere with sterol synthesis by direct, reversible, allosteric inhibition of HMG-CoA reductase (15). This mechanism is entirely different from that of 25-hydroxycholesterol, which is still not fully understood (for review, see ref. 16), but both Compactin and 25-hydroxycholesterol have identical physiological effects, (i.e., inhibition of cytotoxicity by two different mechanisms of inactivation of HMG-CoA reductase).

Abrogation of Cytotoxicity by Formation of Polyene–Cholesterol Complexes in the Effector Cell Membrane. To address the question of whether interactions of cholesterol with other membrane constituents (proteins or phospholipids, or both) are required in the effector cell membrane for the expression of a differentiated function (i.e., cytotoxicity), we exposed polyclonally activated T cells to filipin (5 μg/ml) 30 min before and during the cytotoxicity test. This polyene, which binds specifically to cholesterol (17, 18), abolishes cytotoxicity if lymphocytes encounter it shortly before the cytotoxic assay or if it

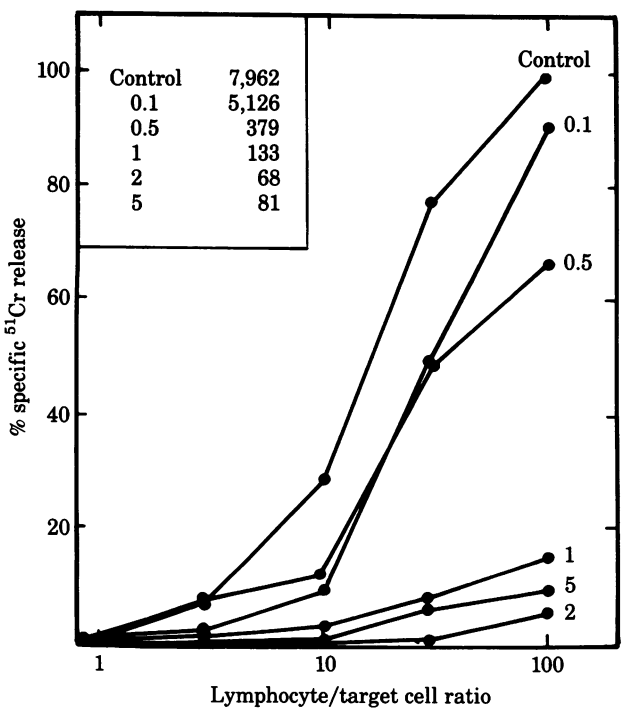

FIG. 3. Dose dependence of the inhibition of cytotoxicity by 25-hydroxycholesterol. The various doses of the oxygenated sterol are indicated in μg/ml to the right of each titration curve. Cytolytic activities were determined after 48 hr of culture. (*Inset*) Sterol synthesis in parallel cultures (cpm per culture) measured at 24 hr after culture initiation. (At doses of 1 μg/ml and higher, both sterol synthesis and cytotoxic titers are essentially completely depressed.)

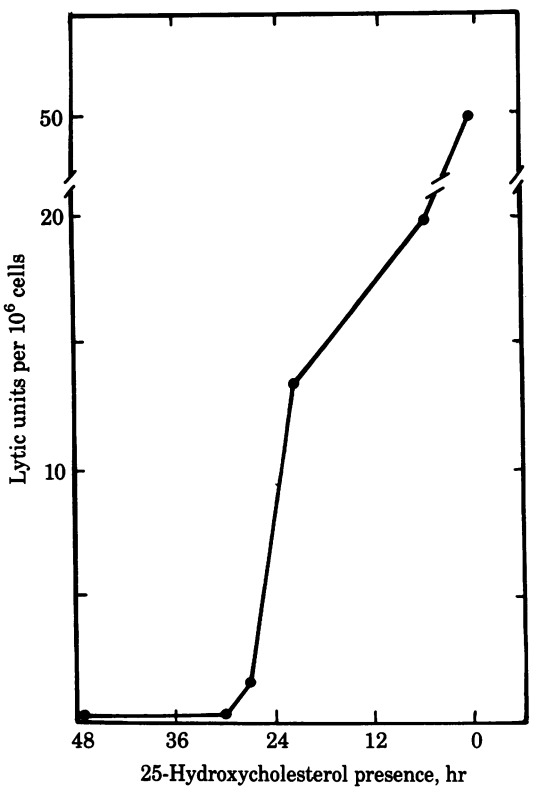

FIG. 4. Time requirements for presence of 25-hydroxycholesterol in the culture medium to inhibit cytotoxic titers in Con A-stimulated lymphocyte cultures. As long as the oxygenated sterol is present 24 hr or longer before harvest at 48 hr, the cytotoxic activity is inhibited. Presence of the sterol for less than 20 hr prior to harvesting does not affect cytotoxicity significantly. Lytic units per 10^6 viable lymphocytes were determined as described.

Table 1. Effects of 25-hydroxycholesterol, Ara-C, Compactin, or filipin on various activities of polyclonally activated T cells

Treatment	Activities per 1 × 10^6 viable T cells: Lytic units	Sterol synthesis,* dpm/hr	DNA synthesis,† dpm/hr	Recovery,‡ %
Control	7.1 ± 1.0	747 ± 43	75,975 ± 12,002	71.1
25-Hydroxycholesterol	<1.0	36 ± 13	6,351 ± 1,130	58.6
Ara-C	5.26	643 ± 18	1,639 ± 104	59.0
Compactin	<1.0	199 ± 29	ND	74.8
Filipin	1.08	761 ± 24	ND	ND
Unstimulated control	<1.0	30 ± 517	9,035 ± 557	64.1

Control cells were untreated; 25-hydroxycholesterol (2 μg/ml), Ara-C (5 μg/ml), and Compactin (5 μg/ml) were added 18 hr after stimulation and left in the culture media until termination of the culture period. Filipin (5 μg/ml) was added 30 min prior to each respective assay. The cytotoxic assay was performed 48 hr after stimulation. ND, not determined.

* Incorporation of [^{14}C]acetate into sterol fraction, mean ± SEM, analyzed at 24 hr after stimulation.

† Incorporation of [^{3}H]dThd into DNA, mean ± SEM, analyzed at 48 hr after stimulation.

‡ Percentage of viable cells recovered from original input.

is present during the assay (Table 1), suggesting that the previous observations made on the necessity for sterol synthesis probably reflect the need for the end product of the pathway, cholesterol, in the membrane. Filipin present in the test had no effect upon release of chromium from target cells.

Recovery of Cytotoxicity in the Presence of Cholesterol-Containing Liposomes. To corroborate the hypothesis that the suppression of cytotoxicity by 25-hydroxycholesterol is due to an insufficient availability of cholesterol in the plasma membrane of the cells (rather than due to the inhibition of the biosynthesis of the minor products of HMG-CoA reductase, such as dolichol, ubiquinone, or isopentenyl adenosine), we exposed the lymphocytes to 25-hydroxycholesterol for 30 hr, and liposomes (CPR, 0.5) were added 2 hr prior to the assay for cytotoxicity. Before addition to the target cells, the effectors were washed twice to remove the liposomes. Fig. 5 shows that these liposomes reverse, although not completely, the inhibitory effect of 25-hydroxycholesterol.

We also tried to prevent the effect of the oxygenated sterol by adding cholesterol simply as a suspension in 5% bovine serum albumin to the cultures simultaneously with the inhibitor. A partial recovery was observed in only 1 out of 13 experiments (results not shown).

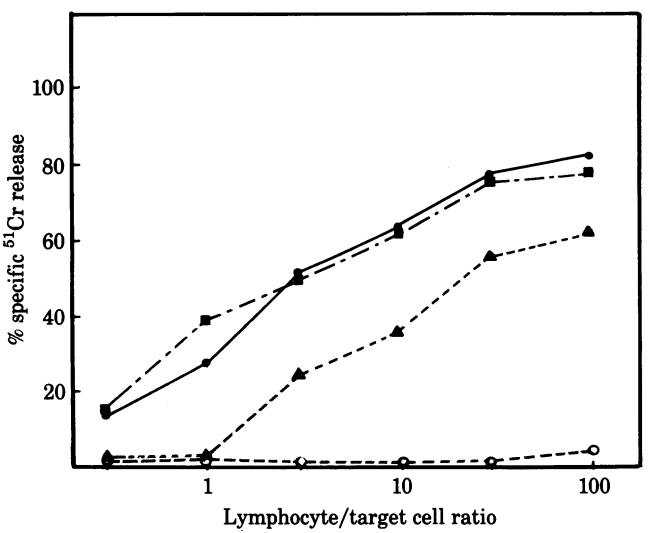

FIG. 5. Reversal of the inhibitory effect of 25-hydroxycholesterol by addition of cholesterol-containing liposomes (CPR, 0.5). 25-Hydroxycholesterol (0.5 μg/ml) was added 18 hr after culture initiation; then 28 hr later, the liposomes were added (i.e., 2 hr prior to the assay for cytotoxicity). ●, Control; ○, 25-hydroxycholesterol only; ■, liposomes only; ▲, 25-hydroxycholesterol and liposomes.

DISCUSSION

Certain biochemical steps appear to be necessary for both the proliferative cycle and the differentiation step leading from a noncytotoxic lymphocyte to a cytotoxic effector cell. One such distinct metabolic requirement appears to be the *de novo* synthesis of cholesterol. It has been shown (4–6) that DNA synthesis does not proceed in PHA-stimulated leukocytes or in leukemic lymphocytes if the preceding cycle of sterol synthesis is inhibited. In the present study, we provide evidence that there is also an absolute requirement for sterol synthesis in Con A-stimulated lymphocytes for differentiation into cytotoxic effector cells. This requirement for sterol synthesis appears to be distinct from the one for DNA synthesis because, in lymphocytes whose DNA synthesis is suppressed by Ara-C, the differentiation into cytotoxic effector cells is unaffected as long as they are allowed to proceed through a normal cycle of sterol synthesis.

Inhibition of sterol synthesis and cytotoxic titers exhibit identical kinetics with respect to increasing doses of 25-hydroxycholesterol (Fig. 3). Similar dose–response curves have been observed in tissue culture cell lines (19–22), mouse liver cells (23), mouse lymphatic leukemias (13), PHA-stimulated mouse lymphocytes (4), and mixed lymphocyte reactions (14).

It may be argued on the basis of the studies of Streuli *et al.* (24) that 25-hydroxycholesterol may act by inserting itself into the membrane lipid bilayer of the effector cells. Under the conditions used in our experiments, it appears unlikely that the oxygenated sterol is exerting its effect by this mechanism because we did not observe any suppressive effect by preexposing the effector lymphocytes to high doses (50 μg/ml) of 25-hydroxycholesterol prior to and during the assay for cytotoxicity.

To extend the results obtained with 25-hydroxycholesterol, we also used compactin (MB-326B), a competitive inhibitor of HMG-CoA reductase (15). Exposure of the lymphocytes to this inhibitor depressed both their sterol synthesis and their cytotoxic titers, similar to their exposure to 25-hydroxycholesterol. This lends support to the conclusion that the inhibition of HMG-CoA reductase *per se* leads to abrogation of the cytotoxic function of the lymphocytes rather than some unknown coincidental toxic effect of 25-hydroxycholesterol or compactin.

Because cholesterol is not the only end product of HMG-CoA reductase, it seemed appropriate to ask the question whether

the observed detrimental effect on the cytotoxicity of T cells is due to a lack of supply of cholesterol *per se* or, alternatively, to the fact that other metabolites of mevalonate (25), such as dolichol, coenzyme Q, or isopentenyladenosine, become limiting when cells were treated with reagents that inhibit HMG-CoA reductase. This question is addressed by the use of cholesterol-containing liposomes, whose addition to the cells only 2 hr before the test restores cytotoxicity lost due to inhibition of sterol synthesis. These experiments actually suggest that, in cells whose sterol synthesis is suppressed, many or all biochemical steps involved in the formation of cytotoxic effector cells progress unperturbed, but that the differentiated function cannot be expressed due to a lack of cholesterol in specific domains of the plasma membrane. However, when cholesterol alone was added in 5% bovine serum albumin for the entire culture period, it was not able to restore cytotoxicity, indicating that cholesterol present in the culture medium in micellar form cannot be easily inserted as a functional entity into the cell plasma membranes, even if their sterol biosynthesis is inhibited. This particular finding in Con A-activated lymphocytes is in agreement with the results of Dabrowski *et al.* (26) in human lymphocytes but contrasts to our previously reported findings in MLR (11) where partial recovery was observed when cholesterol was added directly to the culture medium at 0.1–0.2 mg/ml.

These results are further supported by the finding that filipin, which binds specifically to membrane-associated cholesterol (16, 17, 27, 28) and prevents the sterol from interacting with membrane lipids and proteins, abrogates the cytotoxic capacity of effector cells within minutes of its addition to the cells. Thus, it appears logical to hypothesize that membrane-bound cholesterol has an as yet unknown critical function in cytotoxic lymphocytes and that an inadequate availability of the molecule in the cell membrane results in the loss of the cytotoxicity of the cells as also proposed by Dabrowski *et al.* (26). This report corroborates these findings with mouse splenic lymphocytes and extends our previous findings in secondary mixed lymphocyte cultures (11). Thus, some more general conclusions regarding at least the reticulo-endothelial system can be proposed.

It is generally believed that, in many differentiating systems, the primary driving force for differentiation from a noncommitted state to a functionally specialized state of mammalian cells is proliferation and that the cells become more differentiated with every cell cycle. Our experiments result in the conclusion that differentiation and proliferation are distinct events in lymphocytes. Both processes obviously share some biochemical steps. Our present results identify a complete cycle of sterol synthesis as a shared prerequisite for (*i*) the differentiation into a cytotoxic effector cell and (*ii*) the initiation of DNA synthesis, which in itself is not a requirement for differentiation. How many specific steps are related to the differentiation event *per se* rather than to the proliferation cycle remains unknown.

The question may be raised whether the initial stimulus to differentiate and proliferate involves a common signal or two separate, distinct signals for each process. Our study does not conclusively answer this question because in this system we cannot activate proliferation alone, keeping differentiation suppressed. However, we favor as a working hypothesis the concept that the initial activation results from a common signal induced by the specific binding of Con A to the lymphocyte surface. After an initial common sequence of cellular activation, the metabolic cascade may split into two pathways—one pertinent to differentiation and the other to proliferation. Our results suggest that such a hypothetical split occurs between the peak of cholesterol synthesis and of DNA synthesis in lymphocytes. Alternatively, the activation signal may initiate differentiation, which in turn calls for proliferation in order to amplify the differentiation product. This model would suggest that differentiation has the highest priority among the cellular activities and that proliferation is controlled by it. The hypothesis also would imply that biochemical errors or defects during the process of differentiation could be one source of uncontrolled proliferation such as that encountered in malignant tissues.

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