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Immunology

Cholesterol is a critical cellular component for T-lymphocyte cytotoxicity

(sterol synthesis/inhibitory cholesterol derivatives)

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ABSTRACT **Preincubation of cytolytic T lymphocytes (CTLs) generated in secondary C57BL/6 anti-DBA/2 mixed leukocyte cultures with an inhibitor of cellular cholesterol synthesis (25-OH-cholesterol) for 24 hr strongly depressed the cytolytic activity as determined in a 3-hr ^{51}Cr assay. The effect of the inhibitor was reversed by the simultaneous addition of cholesterol or of mevalonic acid during the preincubation period (mevalonate is the product of the regulatory enzyme in the sterol synthesis pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (NADP) [mevalonate:$NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34]). Because, under the same culture conditions, inhibition of DNA synthesis had no effect on CTL activity, the experiments suggest that the effect of 25-OH-cholesterol is related to its inhibitory effect on sterol synthesis, resulting in decreased levels of membrane-bound cholesterol, rather than to inhibition of cellular proliferation.**

The most prominent feature of immunocompetent cells is that they exert most of their biological functions through the interactions of their plasma membrane with cellular, particulate, or soluble components of their environment. Of particular interest in this context is the specific interaction of cytolytic T lymphocytes (CTLs) with target cells carrying relevant membrane-associated antigens (1–3). In the last decade, many studies have been devoted to the understanding of the mechanism of CTL-mediated target-cell lysis (4–7). Although the actual mechanism remains largely unknown, it has been shown that the lytic process can be divided into three steps: (*i*) binding of the effector cells to target cells; (*ii*) lethal damage to the target cells; and (*iii*) detachment of the effector cells from the target cells which subsequently disintegrate. It is generally accepted that the specificity of the lytic interaction is related to the existence of specific receptors on the plasma membrane of the effector cells that are complementary to antigenic determinants on the plasma membrane of the target cells.

Little is known about the possible role of lipids in the plasma membrane of CTLs or target cells in the lytic mechanism. However, it was recently reported by Schlager *et al.* (8, 9) that tumor cells under attack by complement-dependent cytotoxic antibody display a burst of lipid synthesis and release considerable amounts of triglycerides and cholesterol esters. Cholesterol represents a particularly specific component of plasma membranes of mammalian cells. It modulates their fluidity over a wide temperature range, due to its interference with the cooperative interactions of phospholipids (10–12) resulting in an "intermediary gel state" (13). It now appears that the fluidity of the plasma membrane may be critical for many, if not all, membrane functions. It influences the mobility of cell surface receptors and thus their ability to transmit transmembrane signals (14, 15). Membrane fluidity is also involved in the regulation of membrane-bound enzymes (16) and is critical for the formation of membrane vesicles during pinocytosis (17). A recent report by Berke *et al.* (18) indicates that pretreatment of CTLs or target cells with liposomes may inhibit CTL–target cell interaction (18).

The concentration of cholesterol in the plasma membrane of mammalian cells can be decreased by blocking the *de novo* synthesis of cholesterol with certain oxygenated derivatives of cholesterol, such as 25-OH-cholesterol (19, 20). Using this approach it was found that decreased sterol synthesis in L cells resulted in increased flux of K^+ and Na^+ (16), decreased endocytosis (17), and cessation of cell proliferation (21). It has also been shown that phytohemagglutinin-stimulated lymphocytes exhibited a pronounced phase of sterol synthesis between 18 and 24 hr prior to entering DNA synthesis (22, 23). The *de novo* synthesis of cholesterol and DNA synthesis, were completely suppressed by 25-OH-cholesterol, even in the presence of serum that contained cholesterol bound to lipoprotein.

In this report we demonstrate that CTLs generated in mixed leukocyte culture (MLC) lose their cytolytic activity when preincubated for 24 hr with an inhibitor of sterol synthesis, 25-OH-cholesterol. Inhibition of sterol synthesis also prevents the induction of CTLs in secondary MLC, and this effect is independent of DNA synthesis. In both instances, the effect of 25-OH-cholesterol was reversed by the addition of cholesterol or mevalonic acid (MVA).

EXPERIMENTAL PROCEDURES

Mice. Two- to 4-month-old C57BL/6 and DBA/2 mice were supplied by the mouse colony maintained at the Swiss Institute for Experimental Cancer Research (Epalinges s/Lausanne, Switzerland). Original breeding pairs (C57BL/6J, DBA/2J) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cytolytic T Lymphocytes. CTLs were generated in secondary MLC essentially as described (24). Briefly, 2.5×10^7 responding cells from C57BL/6 mice injected intraperitoneally 2–3 months previously with 3×10^7 P-815-X2 mastocytoma cells (DBA/2 origin, *H-2*^{d}) (P-815 cells) were mixed with equal numbers of irradiated [1000 rads (1 rad = 1×10^{-2} Gy)] stimulating DBA/2 spleen cells. The cells were placed in 20 ml of Dulbecco's modified Eagle's medium containing 50 μM 2-mercaptoethanol and 2% fetal bovine serum in 30-ml tissue culture flasks kept upright and incubated for 5 days in a CO_2 incubator at 37°C. For inhibition studies, 2×10^6 of these secondary MLC cells in 1 ml of medium containing 2% fetal bovine serum were placed into 16-mm wells of Costar tissue culture plates (Costar, Cambridge, MA).

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Abbreviations: CTL, cytolytic T lymphocytes; MLC, mixed leukocyte culture; P-815 cells, P-815-X2 mastocytoma cells (DBA/2 origin, *H-2*^{d}); LU, lytic unit; MVA, mevalonic acid; Ara-C, cytosine-β-D-arabinofuranoside hydrochloride (cytosine arabinoside).

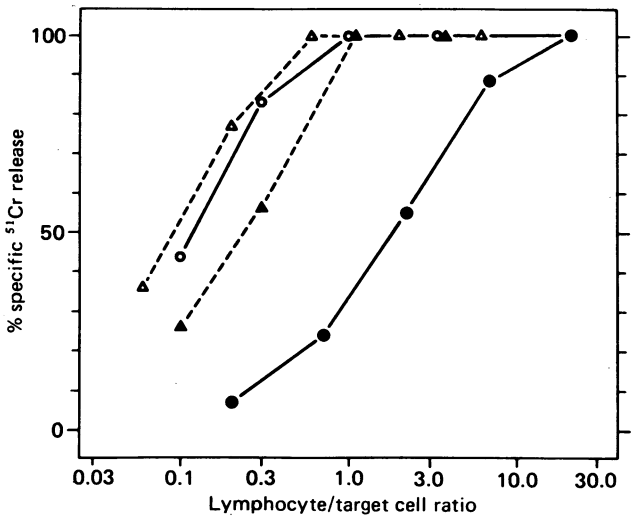

FIG. 1. Cytolytic activity of secondary MLC populations after preincubation with inhibitor (25-OH-cholesterol) or with inhibitor and cholesterol. Cells recovered from C57BL/6 anti-DBA/2 secondary MLC at the peak of the CTL response (day 5) were incubated for 24 hr either alone (control) (○), with 25-OH-cholesterol (2 μg/ml) (●), with 25-OH-cholesterol (2 μg/ml) and cholesterol (100 μg/ml) (▲), or with cholesterol alone (100 μg/ml) (△). At the end of the incubation period, viable cell recoveries were determined and cytolytic activities were assayed.

Restimulation of Long-Term MLC Populations. Primary MLC (set up as described above, but containing normal instead of immune C57BL/6 responding cells) were incubated for 11–12 days. These long-term primary MLC cells were washed, and 2×10^6 cells were placed into 16-mm diameter wells of Costar tissue culture plates in 1 ml of medium. For stimulation, either 4×10^6 irradiated (1000 rads) DBA/2 spleen cells or secondary MLC supernatant fluids (25) at 50% final concentration were included in each well, and the cultures were incubated for 24 hr in the presence or absence of 25-OH-cholesterol.

Table 1. Incubation of secondary CTL populations with 25-OH-cholesterol for 24 hr decreases cytolytic activity

Incubated with	Conc., μg/ml	% cells recovered	LU/10^6 cells	LU/ culture
	Experiment 1			
—		42	833	683
25-OH-Cholesterol	2	27	55	30
25-OH-Cholesterol	2			
+ cholesterol	100	45	416	374
Cholesterol	100	25	1111	555
Ara-C	10	20	666	266
Ara-C	10			
+ 25-OH-cholesterol	2	16	55	18
	Experiment 2			
—		43	416	357
25-OH-Cholesterol	0.1	50	333	333
25-OH-Cholesterol	0.3	35	357	249
25-OH-Cholesterol	1.0	26	87	45
25-OH-Cholesterol	3.0	30	20	12
25-OH-Cholesterol	10.0	28	<1	<1
25-OH-Cholesterol	1			
+ MVA	1000	50	227	227
MVA	1000	33	500	330

Cells recovered from C57BL/6 anti-DBA/2 secondary MLC at the peak of the CTL response (day 5) were incubated for 24 hr either alone or with various concentrations of inhibitor (25-OH-cholesterol), with inhibitor and cholesterol, with cholesterol alone, with cytosine-β-D-arabinofuranoside hydrochloride (Ara-C) alone, with Ara-C and inhibitor, with inhibitor and MVA, or with MVA alone.

Assay for Cytolytic Activity. The ^{51}Cr release assay of Brunner *et al.* (26) was modified as described (24) and performed in round-bottomed wells of microtiter plates (Greiner, Nürtingen, Germany). The formula used to calculate the percentage of specific ^{51}Cr release and the calculation of lytic units (LU) from dose-response curves have been described (24, 27); 1 LU corresponds to the number of lymphoid cells required to lyse 50% of 1×10^4 target cells within 3 hr.

Steroids. Procedures for the purification of cholesterol and 25-OH-cholesterol were similar to those reported (20, 22). A stock solution (200 μg/ml) of 25-OH-cholesterol was prepared by dissolving 2 mg of the compound in 0.2 ml of ethanol and adding 10 ml of modified medium [Dulbecco's modification of Eagle's medium containing 5% crystallized bovine serum albumin (Pentex, 5× recrystallized)]. A stock solution (2 mg/ml) of cholesterol was freshly prepared for each experiment by dissolving 20 mg of recrystallized cholesterol in 1 ml of ethanol and adding 9 ml of modified medium.

Analysis of CO_2 Production, Fatty Acid Synthesis, and Sterol Synthesis. Three replicate experimental or control cultures, each from a Costar 16-mm well, were pooled after gentle resuspension of the lymphocytes and transfer of them to a 25-ml erlenmeyer flask. The total volume of medium was adjusted to 5 ml, 5 μCi of [1-^{14}C]acetate (58.5 Ci/mmol, New England Nuclear) was added, and the flasks were tightly sealed with rubber stoppers fitted with plastic cups (Kontes) and incubated for 2 hr at 37°C in a shaking water bath (70 rpm). The cell suspensions were then acidified with 0.15 ml of 6 M H_2SO_4; the liberated CO_2 was trapped in the plastic cup containing 0.3 ml of Hyamine hydroxide (Calbiochem) and the remaining mixture was made alkaline and saponified by autoclaving for 1 hr. Labeled fatty acids and sterols (precipitated by digitonin from the nonsaponifiable fraction) were determined as described (19, 20, 22, 28, 29), and the radioactivity in each fraction was corrected to total numbers of viable cells as determined by the trypan blue exclusion method.

RESULTS

CTLs Incubated for 24 hr with 25-OH-Cholesterol Lose Their Lytic Activity. Secondary MLC populations collected at the peak of the CTL response (day 5) were preincubated for 24 hr in the presence of 25-OH-cholesterol (2 μg/ml), and their cytolytic activity was measured in a short-term (3 hr) ^{51}Cr release assay. As shown in a typical experiment presented in Fig. 1 and Table 1 (Exp. 1), the secondary MLC population preincubated for 24 hr in the presence of the inhibitor showed a greatly reduced cytolytic activity compared to the control population preincubated in the absence of inhibitor. Expressed in terms of LU per culture, the activity decreased to 4% of control. In a second, similar experiment (Exp. 2, Table 1), the secondary MLC populations were preincubated with various concentrations of 25-OH-cholesterol. The results confirmed those of Exp. 1, demonstrating a decrease of cytolytic activity to 13% at 1 μg/ml, to 3% at 3 μg/ml, and to <0.3% at 10 μg/ml. Cell recoveries were not significantly different, indicating no cytotoxic effect of the inhibitor on MLC cells during the 24 hr of incubation. Inclusion of the inhibitor in control cultures during the 3-hr ^{51}Cr release assay had no effect on CTL activities (results not shown). Exp. 1 (Table 1) also demonstrates that prevention of DNA synthesis by cytosine-β-D-arabinofuranoside hydrochloride (Ara-C) during the preincubation period (24 hr) had little effect on the activity of the CTL population.

Addition of Cholesterol or MVA Reverses the Effect of 25-OH-Cholesterol on CTL Activity. The results presented in Fig. 1 and Table 1 demonstrate that preincubation of secondary MLC cells with 25-OH-cholesterol together with exogenous cholesterol prevented the effect of the inhibitor, leading to less than 50% reduction in CTL activity. Similarly, the addition of MVA reversed the inhibition by 25-OH-cholesterol, from a decrease to 4% to a decrease to 64% (Table 1, Exp. 2).

Generation of CTLs Is Inhibited by 25-OH-Cholesterol. It had been shown that highly active CTLs are induced within 24 hr by the addition of either allogeneic cells or secondary MLC supernatant fluids to long-term primary MLC populations (2, 25, 30). In both situations, CTL generation could occur independently of DNA synthesis and led to a size transition from small lymphocytes to larger cells.

Cultures were set up by using both methods of restimulation in the presence or absence of 25-OH-cholesterol, incubated for 24 hr, washed, and then tested for CTL activity. As shown in Fig. 2, the induction of CTLs by stimulation with allogeneic spleen cells or by the addition of secondary MLC supernatant fluid for 24 hr was completely abolished in the presence of the inhibitor. Comparison with control cultures that had not been restimulated showed that the residual CTL activity was also strongly decreased by the presence of the inhibitor during the induction phase.

In two subsequent experiments, the inhibitory effect of 25-OH-cholesterol on CTL induction was confirmed (Table 2). In the first experiment, the addition of the sterol at a concentration of 1 μg/ml decreased the induction of lytic activity to <2%. In the second experiment, various concentrations of inhibitor were added, leading to dose-dependent inhibition of CTL induction, with significant reduction of activity even with the lowest dose tested (0.1 μg/ml); the residual CTL activity present before the induction was also decreased to very low levels.

Inhibitory Effect of 25-OH-Cholesterol on Induction of CTL Is Independent of DNA Synthesis. Fig. 2 also demonstrates that the addition of Ara-C at a concentration of 10 μg/ml, which inhibited [^{3}H]dThd incorporation to background levels (data not shown), to long-term secondary MLC during the 24-hr restimulation phase did not inhibit CTL generation in the control cultures. This confirmed that the 24-hr CTL generation could occur independently of DNA synthesis. Moreover, the results show that, under the conditions of the test,

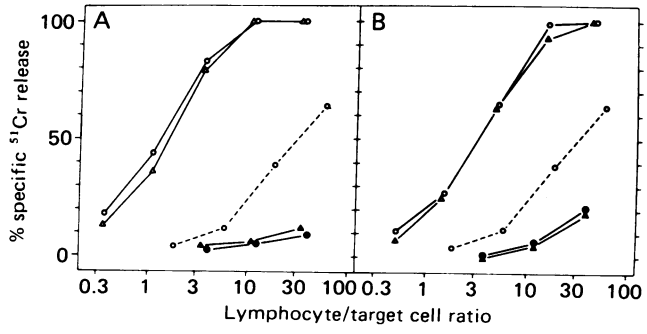

FIG. 2. Effect of inhibitor (25-OH-cholesterol) on induction of CTLs. Cells recovered from C57BL/6 anti-DBA/2 long-term primary MLC (days 11–12) were restimulated with irradiated allogeneic (DBA/2) spleen cells (*A*) or with secondary MLC supernatants (*B*), alone (control) (○), in the presence of Ara-C (10 μg/ml) (△), in the presence of 25-OH-cholesterol (2 μg/ml) (●), or in the presence of 25-OH-cholesterol (2 μg/ml) and Ara-C (10 μg/ml) (▲). Additional cultures were left unstimulated (○- - ○). After 24 hr of incubation, viable cell recoveries were determined, and cytolytic activities were assayed.

the inhibitory effect of 25-OH-cholesterol on CTL induction was not related to an inhibitory effect on DNA synthesis.

Addition of Cholesterol or MVA Reverses the Inhibitory Effect of 25-OH-Cholesterol on CTL Induction. As shown in Table 2 (Exp. 1), the addition of cholesterol at 200 μg/ml to cultures containing 25-OH-cholesterol at 1 μg/ml during the 24-hr stimulation of long-term primary MLC cells reversed the effect of the inhibitor, leading to a reduction of CTL activity to 14% instead of to <2% as observed in the presence of inhibitor alone. In the presence of 25-OH-cholesterol at 2 μg/ml, the reversing effect of 200 μg/ml cholesterol at 200 μg/ml was somewhat diminished. When MVA at 1 mg/ml was added to the cultures together with 25-OH-cholesterol at 1 μg/ml, inhibition of CTL induction was also effectively reversed, inhibition to 38% instead of to 1% in the presence of inhibitor alone (Table 2, Exp. 2).

In parallel experiments we analyzed cytotoxicity, sterol synthesis, CO_2 production, and fatty acid synthesis in secondary CTLs during the induction phase. Table 3 demonstrates that the effect of 25-OH-cholesterol on cytotoxicity is directly correlated with its impact on *de novo* synthesis of cholesterol in MLC cells. After restimulation, CO_2 production approximately doubled and fatty acid synthesis increased about 8-fold, whereas sterol synthesis increased 20-fold and cytotoxicity increased 10-fold. Addition of 25-OH-cholesterol decreased cytotoxicity from 111 to <1 LU/10^6 cells, and sterol synthesis decreased to <1000 dpm/10^6 cells. At the same time, fatty acid synthesis remained either unchanged in the case of stimulation by MLC supernatant fluid or was reduced only by half in the case of stimulation by allogeneic cells. The CO_2 production remained unaffected by the presence of the inhibitor. These experiments demonstrate the specificity of the inhibitor on *de novo* sterol synthesis. With secondary MLC at day 5, as described above, the same correlation between inhibition of sterol synthesis and depression of cytotoxicity by 25-OH-cholesterol was found (data not shown).

DISCUSSION

Incubation of CTL-containing lymphocyte populations with 25-OH-cholesterol, a specific inhibitor of sterol synthesis (19, 20, 31), results in a strong depression of their cytolytic activity. Several lines of evidence are provided which suggest that the effect of 25-OH-cholesterol on CTL-mediated lysis is related to its inhibitory effect on *de novo* sterol synthesis, resulting in decreased levels of membrane-bound cholesterol: (*i*) inhibition is dependent upon preincubation of the cells with 25-OH-cholesterol, whereas inclusion of the compound during the assay has no effect on CTL activity; (*ii*) sterol synthesis is severely inhibited in CTL preincubated with 25-OH-cholesterol (Table 3); (*iii*) the effect of the inhibitor on CTL activity is counteracted by the simultaneous addition of cholesterol or MVA during the preincubation period; (*iv*) under identical culture conditions, inhibition of DNA synthesis by Ara-C has no effect on the CTL populations tested and thus it is unlikely that the inhibitory effect of 25-OH-cholesterol observed in our study is related to its known inhibition of cell proliferation (21, 22, 31); (*v*) preliminary results indicate that other inhibitors of sterol synthesis such as 7-ketocholesterol and 20-α-OH-cholesterol (19, 31) have a similar inhibitory effect on CTL activity although they required 2- to 5-fold higher concentrations.

25-OH-cholesterol has an inhibitory effect not only on the lytic potential of active CTL but also on the induction of lytic activity in long-term MLC populations by specific or nonspecific stimulation. As shown before (25, 30, 32), the lytic activity of long-term MLC populations is low but increases manyfold within 24 hr after specific restimulation with allogeneic cells

Table 2. Induction of CTLs in long-term MLC is inhibited by 25-OH-cholesterol but is independent of DNA synthesis and is reversed by cholesterol and by MVA

Long-term MLC cells	Addition	Conc., μg/ml	% cells recovered	LU/10^6 cells	LU/culture
		Experiment 1			
AC	—		32	87	56
AC	25-OH-Cholesterol	1	46	<1.0	<1.0
AC	25-OH-Cholesterol	2	37	<1.0	<1.0
AC	Cholesterol	200	38	36	27
AC	Cholesterol + 25-OH-cholesterol	200 1	44	9	8
AC	Cholesterol + 25-OH-cholesterol	200 2	44	5	4
AC	MVA	1000	42	95	80
AC	MVA + 25-OH-cholesterol	1000 1	46	6	6
AC	MVA + 25-OH-cholesterol	1000 2	41	4	3
—	—		97	<1.0	1.5
—	25-OH-Cholesterol	1	77	1.0	1.5
—	25-OH-Cholesterol	2	87	<1.0	<1.0
		Experiment 2			
AC	—		29	238	138
AC	25-OH-Cholesterol	0.1	51	91	93
AC	25-OH-Cholesterol	0.3	36	71	79
AC	25-OH-Cholesterol	1.0	56	1.4	1.6
AC	25-OH-Cholesterol	3.0	44	<1.0	<1.0
AC	25-OH-Cholesterol	10.0	50	<1.0	<1.0
AC	25-OH-Cholesterol + MVA	1.0 1000	56	35	39
AC	MVA	1000	54	357	385
—	—		63	15	19

Cells recovered from C57BL/6 anti-DBA/2 long-term primary MLC (days 11–12) were restimulated with irradiated allogeneic (DBA/2) spleen cells (AC) alone or in the presence of various concentrations of inhibitor (25-OH-cholesterol), of inhibitor and cholesterol, of MVA, or of inhibitor and MVA. Additional cultures were left unstimulated and were incubated in the presence or absence of inhibitor. After 24 hr of incubation, viable cell recoveries were determined and cytolytic activities were assayed.

or stimulation with cell-free supernatant fluids containing nonspecific factors. This increase in activity is accompanied by an enlargement of effector cells and is independent of DNA synthesis (refs. 30 and 32; this report). The addition of 25-OH-cholesterol during the 24-hr period of specific or nonspecific restimulation not only prevents the induction of high levels of lytic activity but also results in a strong depression of the residual activity exhibited by the long-term MLC populations. The finding that the effect of 25-OH-cholesterol is counteracted by simultaneous addition of cholesterol or MVA strongly suggests that it is directly related to inhibition of sterol synthesis, resulting in a decreased cellular concentration of cholesterol in the surface membranes. The inhibition of induction of CTL activity by 25-OH-cholesterol cannot be explained by an effect of the inhibitor on the stimulating cells because similar results are obtained when cell-free supernatant fluids are used for induction of lytic activity in long-term MLC populations. This effect of 25-OH-cholesterol is not due to inhibition of cell proliferation because induction of CTL activity in long-term MLC populations within 24 hr does not require DNA synthesis (ref. 33; Fig. 2).

The experiments suggest that the lipid composition of the

Table 3. Sterol synthesis, fatty acid synthesis, CO_2 production, and cytotoxicity 24 hr after induction of secondary CTLs

Culture	$^{14}CO_2$ production, dpm/10^6 cells	Fatty acid synthesis, dpm/10^6 cells	Sterol synthesis, dpm/10^6 cells	LU/10^6 cells	Recovery of cells, %
Unstimulated control	14,556 ± 772	5,166 ± 313	1,215 ± 298	11	66
+ allogeneic cells	23,960 ± 5,430	43,687 ± 9,260	23,582 ± 6,033	111	47
+ MLC supernatant	12,821 ± 3,140	16,602 ± 2,151	8,778 ± 1,334	57	61
+ allogeneic cells + 25-OH-cholesterol	19,780 ± 8,047	23,247 ± 5,669	609 ± 54	<1	44
+MLC supernatant + 25-OH-cholesterol	11,675 ± 2,046	16,821 ± 1,848	157 ± 34	5	42

Cells recovered from C57BL/6 anti-DBA/2 long-term primary MLC were restimulated with irradiated allogeneic spleen cells (DBA/2) or MLC supernatant alone or in the presence of 25-OH-cholesterol at 2 μg/ml. After 24 hr of incubation, part of the cells were used in a 3-hr ^{51}Cr release assay on P-815 (DBA/2) target cells to determine the cytolytic activity and the remainder of the cells were used to determine $^{14}CO_2$ production, fatty acid synthesis, and sterol synthesis. The values represent means ± SEM from three cultures.

surface membrane of CTLs and their precursors is of primary importance during both induction and effector phases. It is now well established that the concentration of membrane-bound cholesterol decreases when cells are cultured in the presence of 25-OH-cholesterol (20, 31). All the secondary effects of this inhibitor on cell growth (21, 22), ion flux (16), and pinocytosis (17) are readily understandable in terms of a change in lipid membrane composition—i.e., a decrease in cholesterol/phospholipid ratio (for discussion, see refs. 31, 34, and 35). In our study, determination of the membrane cholesterol/phospholipid ratio in the effector populations used would not be conclusive because the cultures contain large populations of cells other than CTLs. In view of the well-established correlation between sterol synthesis and membrane cholesterol concentration (20), however, it is logical to infer that membrane-bound cholesterol in CTLs preincubated with 25-OH-cholesterol is decreased, resulting in an increased membrane fluidity at 37°C (13, 34, 35). Such a change may impair the ability of CTLs to bind to target cells due to a modified mobility of receptors, an alteration of the formation of microvilli (36), or impairment of other, as yet undefined, membrane characteristics. Whether changes in CTL membrane cholesterol concentrations affect binding of the effector to the target cell or steps subsequent to it cannot be ascertained at the present time.

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