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EFFECT OF OXYGENATED STEROLS ON 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE AND DNA SYNTHESIS IN PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES

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SUMMARY :

Fifteen oxygenated sterols at the concentration of 25 µg/ml were tested on DNA synthesis of phytohemagglutinin stimulated human lymphocytes. In a cholesterol containing medium, the inhibitory effect was strictly dependent of the side chain structure of the sterol and only due to an hydroxylation at position 25. Three oxygenated sterols, which slightly inhibited DNA synthesis, strongly suppressed the peak of 3-hydroxy-3-methylglutaryl CoA reductase activity that normally precedes DNA synthesis. The 25-hydroxycholesterol suppressed the reductase activity even at 5 µg/ml, but was active on DNA synthesis only at 25 µg/ml ; at this concentration, the later the 25-hydroxycholesterol was added, the weaker the inhibition of DNA synthesis was. Hence the sterol synthesis related to the early increase of 3-hydroxy-3-methylglutaryl CoA reductase activity is probably not essential to the cellular division. Several hypothesis on the mechanism of action of the 25-hydroxycholesterol are discussed.

1. INTRODUCTION :

Stimulation of human peripheral lymphocytes by mitogenic lectins induces early phenomena among which are observed a temporary increase of the membrane fluidity within 30 min. of the lectin binding to the cell surface receptors (1), and an enhanced phospholipid synthesis (2) followed by sterol synthesis which reaches a maximum between 15 and 20 hours (2,3). This sterol synthesis occurs even when the lymphocytes are cultured in a lipid-rich medium (3). It is important to determine whether this synthesis is necessary to the blastic transformation, that is if its suppression involves an inhibition of DNA synthesis.

Oxygenated sterols are known to be effective suppressors of 3-hydroxy-3-methylglutaryl CoA reductase (EC 1.1.1.34) activity and of sterol synthesis. Such is the case for 7-ketocholesterol and cholesterol derivatives hydroxylated in the positions 7α, 15α, 15β, 20α, 22α, 22β and 25 which have been tested in primary cultures of mouse liver cells and L-cell cultures (4,5,6) and, for some of them, in human cultured fibroblasts (7) and hepatoma cultured cells (8). 25-hydroxycholesterol and 25-hydroxy or 1,25-dihydroxy-

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cholecalciferol showed the same inhibitory activity on guinea pig leukemic cells (9). The ability of some oxygenated sterols (7α and 7β-hydroxy, 7-keto and 25-hydroxycholesterol to inhibit sterol synthesis correlated well with their capacity to inhibit growth of L-cells in culture (10,11). Growth inhibition was reversed by the addition of cholesterol or mevalonate, and was due to inhibition of DNA synthesis and not of RNA nor protein synthesis (12). After phytohemagglutinin (PHA) stimulation, DNA synthesis was likewise suppressed by 25-hydroxycholesterol in mouse lymphocytes (13) or by 20α-hydroxycholesterol in human lymphocytes (2).

The present report deals with the effect of a great number of oxygenated sterols on the blastic transformation induced by PHA. The effect of some of them has been tested on the activity of 3-hydroxy-3-methylglutaryl CoA reductase. It has been found that, although most of them were inhibitors of the reductase, only the 25-hydroxylated derivatives were active in the blastic transformation.

2. MATERIALS AND METHODS :

2.1 Materials : Bacto-phytohemagglutinin-P, DIFCO ; [methyle-^{3}H]-thymidine (25 Ci/mole), C.E.A. ; 7α-, 20α-, 25-hydroxycholesterol[1] and 7-ketocholesterol, Steraloids ; 25-hydroxycholecalciferol, Roussel, France ; 24-25-dihydroxycholecalciferol, Hoffmann-Laroche, Switzerland ; other oxygenated sterols[1], generous gift from Professor Ourisson, Strasbourg, France ; micro-test II plates, Falcon ; ethanol, Merck ; 2-5 diphenyloxazole (PPO), Koch-Light Laboratories.

2.2 Methods : Collection and separation of human peripheral lymphocytes were achieved as previously described (14). Blastic transformation was measured by [^{3}H]-thymidine incorporation into DNA according to the method in (14). Cells were cultured in microplates : each well contained 10^5 lymphocytes in 100 µl of culture medium (RPMI 1640, 40 mM Hepes pH 7,2, gentamicine 80 µg/ml) supplemented with 30 % of heat inactivated human AB serum. PHA was added to

[1] The abbreviations used are :

7β-hydroxy-22,23-dehydrobrassicasterol : (24 R)-24-methylcholest-5,22 diene-3β, 7β-diol ; 7α-hydroxydesmosterol : cholest-5, 24-diene-3β, 7α-diol ; 22 (R)-hydroxydesmosterol : cholest-5,24-diene-3β, 22 R-diol ; 7-ketodesmosterol : 3β-hydroxycholest-5,24-diene-7-one ; 7β-hydroxycholesterol : cholest-5-ene-3β, 7β-diol ; 7α-hydroxycholesterol : cholest-5-ene-3β, 7α-diol ; 7 ketocholesterol : 3β-hydroxycholest-5-ene-7-one ; 20α-hydroxycholesterol : cholest-5-ene-3β, 20α-diol ; 25-hydroxycholecalciferol : 9,10-seco-5,7,10(19)-cholestatriene-3β, 25-diol ; 24, 25-dihydroxycholecalciferol : 9,10-seco-5,7,10(19)-cholestatriene-3β, 24, 25-triol ; 20-22-dehydro-25-hydroxycholesterol : cholest-5, 20(22)-diene-3β, 25-diol ; 23-dehydro-25-hydroxycholesterol : cholest-5, 23-diene-3β, 25-diol ; 25 hydroxy-7-ketocholesterol : 7-oxocholest-5-ene-3β, 25-diol ; 25 hydroxy-7-ketocholesteryl acetate : 7-oxocholest-5-ene-3β, 25-diol 3-acetate ; 25-hydroxycholesterol : cholest-5-ene-3β, 25-diol.

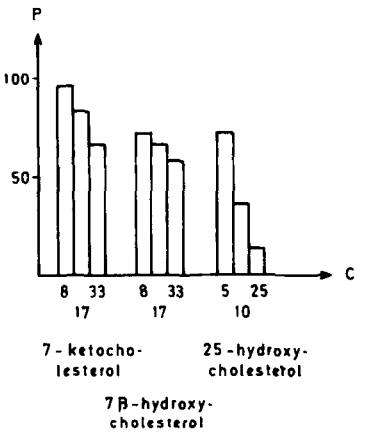

FIGURE 1 : Effect of various concentrations of oxygenated sterols on DNA synthesis of PHA stimulated human lymphocytes.
P = percentage of blastic transformation (see methods)
C = concentration of sterol = µg/ml.

each well in 50 µl of culture medium at various concentrations (75, 150, 300 or 450 µg/ml). The oxygenated sterol was dissolved in ethanol and added to the medium to a final concentration of 0.5 % of ethanol. The cultures were placed at 37°C in a humid atmosphere for 72 hours and 24 hours before the end of the incubation, 0,2 µCi of [^{3}H]-thymidine was added to each well. The cells were harvested on a multiple automatic sample harvester (MASH II) and the radioactivity counted in a scintillation fluid with a Packard liquid scintillation spectrometer Model 3320. Triplicate or quadruplicate assays of the samples were made. Cell viability was checked by the trypan blue exclusion test after 3 days of culture. In the results, blastic transformation in presence of oxygenated sterol is expressed as the ratio (X100) of dpm of [^{3}H]-thymidine incorporation to the dpm incorporated by control cells at the optimal concentration of PHA.

To determine the 3-hydroxy-3-methylglutaryl CoA reductase activity, the cells were cultured in plastic flasks at a concentration of 4.10^6 cells per ml of culture medium supplemented with 20 % of heat-inactivated human AB serum and 150 µg/ml of PHA. The oxygenated sterol was dissolved in ethanol and added to the culture medium (final concentration of sterol : 5 or 25 µg/ml, of ethanol : 0.5 %). The flasks were placed at 37°C and 3-hydroxy-3-methylglutaryl CoA reductase activity was assayed as previously described (15) after different times of culture.

3. RESULTS

3.1. Effect of oxygenated sterols on DNA synthesis :

In order to determine the optimal conditions for inhibition of DNA synthesis, 3 oxygenated sterols were tested at different concentrations (5 to 33 µg/ml) (Fig. 1). But the inhibitory effect regularly increased with the concentration, slowly for the 7-keto and 7α -hydroxycholesterol and rapidly for the 25-hydroxycholesterol. At the concentration of 25 µg/ml,

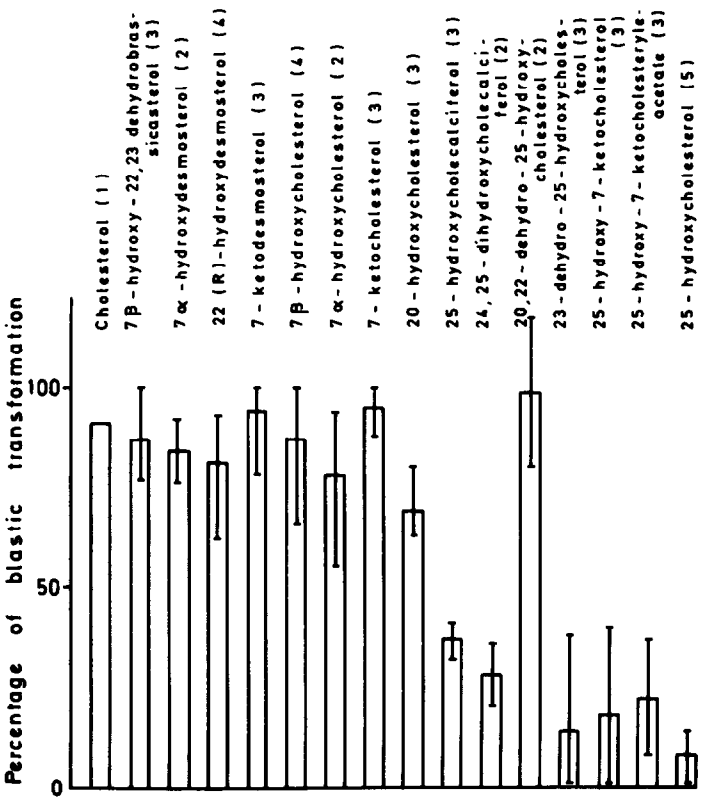

FIGURE 2 : Effect of cholesterol and fifteen oxygenated sterols on DNA synthesis of PHA stimulated human lymphocytes. The number of experiments is indicated in brackets and the minimal and maximal values on the schema. Sterol concentration = 25 μg/ml.

25-hydroxycholesterol suppressed DNA synthesis nearly completely and this concentration was chosen for testing all the other sterols.

The effects of fifteen oxygenated sterols on DNA synthesis, after stimulation by the optimal dose of PHA, are listed on figure 2. The inhibition was no more than 20% for the 4 sterols derived from 22,23 dehydrobrassicasterol or desmosterol. Among oxygenated derivatives of cholesterol (cholesterol itself had no effect), 7-ketocholesterol did not suppress DNA synthesis at all whereas 7α - and 7β -hydroxycholesterol were somewhat more inhibitory (20%). Presence of a hydroxyl function at position 20 increased the effect a little more (30%). The hydroxylated analogs of cholecalciferol were effective inhibitors (63% for the 25-hydroxy and 70% for the 24,25-dihydroxycalciferol). But the most potent ones were the 25-hydroxylated derivatives of cholesterol (80 to 90%). However, a

TABLE I

Effect of 25-hydroxycholesterol 5 µg/ml on DNA synthesis of PHA stimulated human lymphocytes cultured in different mediums.

CULTURE MEDIUM : RPMI - 1640 +	PERCENTAGE OF BLASTIC TRANSFORMATION.
20% AB-serum	72
10% AB-serum	12
5% AB-serum	0.4
20% lipoprotein-free AB-serum	0

great specificity of the side chain structure was evident since the 20,22-dehydro derivative did not suppress DNA synthesis whereas the 23-dehydro-derivative did so strongly.

The toxicity of all the sterols for the lymphocytes was also checked at the concentration of 25 µg/ml. After 3 days of culture in the presence or the absence of sterol, the number of viable cells remained the same in both cases.

Some oxygenated sterols are known to inhibit cell growth in a lipid-free medium but not in a serum-enriched medium (10,11). It can be seen in TABLE I that 25-hydroxycholesterol, which is nearly inefficient on DNA synthesis at the concentration of 5 µg/ml, becomes inhibitory when the percentage of serum decreases in the medium, and completely suppresses blastic transformation in a lipoprotein-free medium.

3.2. *Effect of oxygenated sterols on 3 hydroxy-3 methylglutaryl coenzyme A reductase activity* :

Lymphocytes were cultured in the presence of PHA and an oxygenated sterol at the concentration of 5 and 25 µg/ml. Four sterols were chosen according to their ability to inhibit or not DNA synthesis : 25-hydroxycholesterol, 25-hydroxycholecalciferol, 7α-hydroxycholesterol and 22(R)-hydroxydesmosterol (cf. Fig. 2). 3-hydroxy-3-methylglutaryl CoA reductase activities were measured 18 hr, 22 hr and 28 hr after PHA stimulation i.e. at the maximal enzyme activity of the control (3). Results are shown in figure 3.

In the absence of sterol, the reductase activity was maximal at 18 hr, and then decreased. At the concentration of 5 µg/ml, only 25-hydroxy-

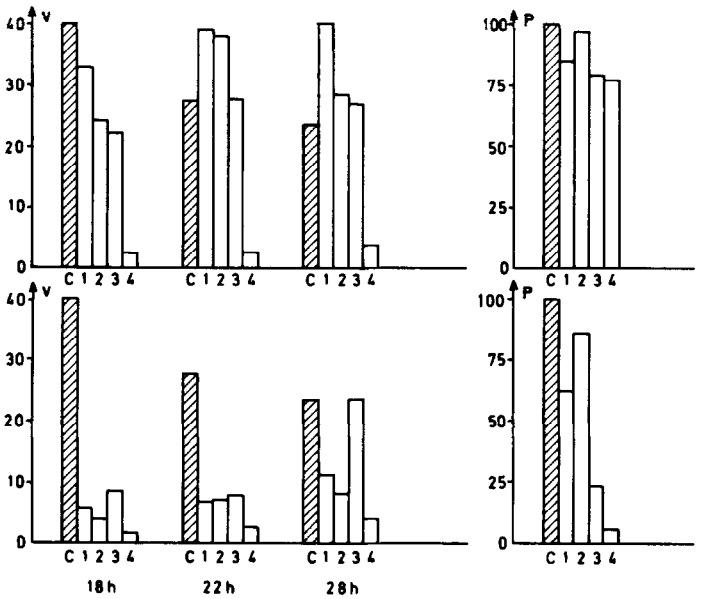

FIGURE 3 : Effect of some oxygenated sterols on 3-hydroxy-3-methylglutaryl CoA reductase activity after different times of PHA stimulation and on DNA synthesis.
Sterol concentration : 5 µg/ml in 3 a, 25 µg/ml in 3 b.
C = control ; 1 = 22(R)-hydroxydesmosterol ; 2 = 7α-hydroxycholesterol ; 3 = 25-hydroxycholecalciferol. V = 3-hydroxy-3-methylglutaryl CoA reductase activity = pmoles/min/mg protein. P = percentage of blastic transformation.

cholesterol strongly suppressed at any time 3-hydroxy-3-methylglutaryl CoA reductase activity. For the other sterols (except for the 25-hydroxycholecalciferol), the maximum of enzyme activity was delayed : it reached the same maximal value as the control at 22 hr instead of 18 hr. Nevertheless none of the 4 sterols tested inhibited DNA synthesis more than 25 %. At the concentration of 25 µg/ml, the 4 sterols depressed 3-hydroxy-3-methylglutaryl CoA reductase levels, particularly at 18 hr and 22 hr. 25-hydroxycholesterol was the most active.

However, no correlation was observed between the depression of the enzyme activity and the inhibition of DNA synthesis.

3.3. *Effect of addition of 25-hydroxycholesterol at different times after PHA stimulation* :

Figure 4 represents the effect of 25-hydroxycholesterol (25 µg/ml) on 3-hydroxy-3-methylglutaryl CoA reductase activity and on

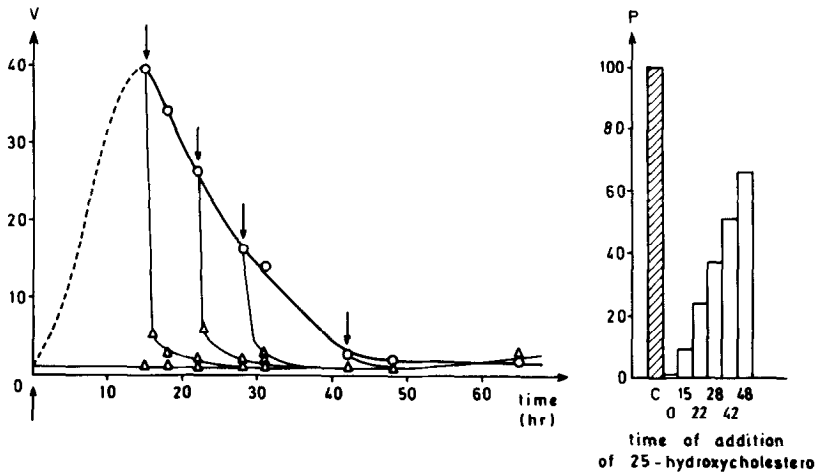

FIGURE 4 : Effect of 25-hydroxycholesterol (25 µg/ml) added after different times of PHA stimulation on 3-hydroxy-3-methylglutaryl CoA reductase activity and DNA synthesis. V = 3-hydroxy-3-methylglutaryl CoA reductase activity = pmoles/min/mg protein. P = percentage of blastic transformation. O = control ; Δ = in presence of 25-hydroxycholesterol 25 µg/ml. The arrows indicate the time of addition of the sterol after PHA.

DNA synthesis when the sterol was added at different intervals after stimulation. When 3-hydroxy-3-methylglutaryl CoA reductase activity was high (15 or 22 hours after PHA stimulation), it was reduced by more than 75% one hour after the addition of the sterol and reached its basal value 5 hours later.

DNA synthesis was totally inhibited if 25-hydroxycholesterol was added at the same time as PHA, or 1 to 5 hours later. When 3-hydroxy-3-methylglutaryl CoA reductase activity has reached its maximum, the later the sterol is added the weaker the inhibition synthesis is. But even after 44 or 48 hours of PHA stimulation, although the reductase activity has fallen again to its basal value, the addition of sterol still slightly impairs the incorporation.

4. DISCUSSION :

Some oxygenated sterols were known to inhibit cell culture growth (10,11) and 2 of them have been shown to inhibit lymphoblastic transformation : 25-hydroxycholesterol on mouse lymphocyte (13) and 20α-hydroxycholesterol on human lymphocyte (2). But this is the first time that such a great number of oxygenated sterols has been tested on blastic transformation of human peripheral lymphocytes. The results show that the inhibi-

tory effect on DNA synthesis is closely related to the sterol structure. The most important structural requirements were the presence of an hydroxyl function at position 25 and a specific conformation of the side chain : for instance, the presence of a 20-22 double bond suppressed the inhibition.

On the other hand, structural changes in the polycyclic nucleus only slightly reduced the inhibitory effect, since 25-hydroxycholecalciferol was nearly as active as the 25-hydroxycholesterol. The acetylation of the 3 β-hydroxyl function did not hinder the inhibition, but it could be possible that the identity in inhibitory activity is due to the enzymatic hydrolysis of the ester. Contrary to the observations on fibroblast cultures (10), the 7 -oxygenated derivatives of cholesterol showed no effect in our model using human lymphocytes.

Several mechanisms can be put forward to explain the action of these oxygenated sterols : First, they could prevent the binding of PHA and early events that follow this fixation. But the inhibitory effect of 25-hydroxycholesterol was the same when the sterol was added 5 hours after PHA.

Secondly, cytotoxicity of the sterols could be involved. Indeed, at the concentration of 25 µg/ml, 22 (R)-hydroxydesmosterol, 7-ketodesmosterol, 7β-hydroxycholesterol and 23-dehydro-25-hydroxycholesterol were cytotoxic for hepatoma cells in culture but not for normal cultured fibroblasts, even at 80 µg/ml (16,17). None of the tested sterols showed cytotoxicity towards the normal PHA stimulated lymphocyte at the concentration used.

Thirdly, they could act by suppressing 3-hydroxy-3-methylglutaryl CoA reductase activity and so inhibit sterol synthesis. A relation was effectively observed between the time of addition of 25 hydroxycholesterol at 25 µg/ml and the DNA synthesis : the later the 3-hydroxy-3-methylglutaryl CoA reductase activity was suppressed, the higher the DNA synthesis (Fig. 4). But if we consider the results of figure 3, we observe that, at the concentration of 25 µg/ml, 7α-hydroxycholesterol and 22(R)-hydroxydesmosterol strongly reduced the reductase activity after 18 or 22 hours of PHA stimulation but weakly affected the DNA synthesis. Moreover, the same effects were observed with 25-hydroxycholesterol at the concentration of 5 µg/ml, although the enzyme activity was more reduced than with the two precedent sterols and similar to the activity measured at 25 µg/ml. So the hypothesis of a "threshold" of 3-hydroxy-3-methylglutaryl CoA reductase activity below which DNA synthesis would be inhibited seems to be unlikely.

Hence, it seems that the sterol synthesis related to 3-hydroxy-3-methylglutaryl CoA reductase activity, which precedes DNA synthesis of PHA stimulated lymphocytes, is probably not essential to the cellular division if the cells are cultured in a cholesterol containing medium (20% serum) similar to a physiological medium.

So an additional mechanism that we intend to study must be evoked to explain the inhibitory effect of 25-hydroxylated sterols. These sterols might be incorporated into the membrane where they would induce some perturbations : changes in permeability and fluidity, specific modification of the hydrophobic microenvironment of the reductase leading to some changes in its activation energy (and hence its kinetic parameters) as suggested by Sabine (18) and Mitropoulos (19) for liver reductase of rats fed with a high cholesterol diet. Another possibility is that the 25-hydroxycholesterol might bind to a specific cytoplasmic protein, as suggested by the accurate structural specificity of the "active" sterols. Such a protein has been characterized for 25-hydroxycholesterol in L-cells (20) : the sterol protein complex might independently modify the reductase activity (that could explain the strong and rapid effect of this sterol on the enzyme) and the DNA synthesis.

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