Vol. 110, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1983 Pages 82-87

INHIBITION OF STEROL AND DNA SYNTHESIS IN PERIPHERAL BLOOD LYMPHOCYTES BY AY9944

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Received November 23, 1982

Summary: The effect of the inhibitor of cholesterol synthesis AY9944 on DNA synthesis was tested in phytohemagglutinin stimulated lymphocytes incubated in delipidated serum. AY9944 caused a decrease in cholesterol synthesis from [^{14}C] acetate and an accummulation of [^{14}C] 7-dehydrocholesterol. This inhibition of cholesterol synthesis resulted in an inhibition of DNA synthesis in a dose related manner. Inhibition could be partly reversed by added lipoprotein. Inhibition of DNA synthesis occurred if AY9944 was added at the early stages of blast transformation but not when the response was well established. These findings suggest that some endogenous cholesterol synthesis may be required for the complete phytohemagglutinin stimulation of lymphocytes.

Introduction: Stimulation of lymphocytes with mitogenic lectins result in a linked sequence of changes resulting in blast transformation, DNA synthesis and mitosis (1). One of the early changes in lymphocyte metabolism is an increase in the rate of endogenous sterol biosynthesis and in the activity of the rate-limiting enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase). Inhibition of this enzyme activity by 25-hydroxycholesterol results in inhibition of DNA synthesis (2), not as a result of an inhibition of the initiation of blast transformation but as a result of inhibition of later events (3,4). It is still unclear which of the end-products of HMGCoA reductase is required for cell proliferation. In fibroblasts and smooth muscle cells, non-sterol products have been shown to be required for DNA synthesis, however a requirement for some endogenous cholesterol synthesis cannot be ruled out. In an attempt to clarify the situation we have used an inhibitor of the final step of cholesterol synthesis AY9944 to

0006-291X/83/010082-06$01.50/0

separate the requirement for endogenous cholesterol from a requirement for other products of HMGCoA reductase. It has been found that inhibition of cholesterol synthesis reduces mitogenic stimulation of lymphocytes by PHA when the cells are incubated in cholesterol reduced media. This block can be only partially relieved by addition of saturating levels of lipoprotein to the media. We conclude that some endogenous cholesterol synthesis is necessary for PHA stimulation.

Materials and Methods:

Materials

Radiochemicals ([methyl-^{3}H]-thymidine, 68 Ci/mmole, [2-^{14}C] sodium acetate, 60 mCi/mmole, and [1α,2α(n)^{3}H] cholesterol 58 Ci/mmole were obtained from Amersham, Australia. AY9944 was a generous gift from Dr. J.R. Sabine, Waite Institute, University of Adelaide, Australia. Other materials were supplied by (material:supplier): Ficoll-Hypaque: Pharmacia; cell culture media: Gibco; plasticware: Nuclon Denmark.

Cell culture

Human peripheral blood lymphocytes were isolated on a Ficoll-Hypaque gradient. Lymphocyte activation was monitored by the incorporation of [^{3}H]-thymidine into acid precipitable material. Cells were incubated in 16 mm multidish trays (10^{5} cells per well) in 100 µl of culture media (RPMI1640 supplemented with 10% lipoprotein deficient foetal calf serum. Quadruplicate cultures were labelled for 1 h with 0.5 µCi [^{3}H]-thymidine. In experiments with [^{14}C] acetate, 2 x 10^{7} cells were incubated in 200 ml flat-bottom flasks with 20 ml of cell-culture media containing 10 µg/ml PHA and 2 µCi/ml [^{14}C] acetate.

Preparation of a lipoprotein fraction

Serum was prepared from fresh clotted human blood. The serum was made density 1.25 with KBr and centrifuged at 150,000 xg for 48 hr in the 30 ml tubes in a Beckman 50.2 rotor. The top 5 ml of serum was removed, and termed the lipoprotein rich fraction, the lower 10 ml was removed and termed delipidated serum. Cholesterol measurements were 1350 µg/ml for the lipoprotein fraction and 50 µg/ml for the delipidated serum.

Sterol synthesis

Sterols were extracted by the method of Anderson and Dietschy (5). After treatment of the digitonide precipitate with pyridine, the free sterols, including authentic carriers were separated on T.L.C. Δ^{7}-dehydrocholesterol and cholesterol were separated on argenated silica gels developed using chloroform/diethyl ether/acetic acid (97:25:0.5). Recovery of the sterols was monitored using [^{3}H] cholesterol added before extraction.

Results

3.1 Dose response of PBL to AY9944

Inhibition of cholesterol synthesis with increasing dose of AY9944 and the effect of these concentrations of AY9944 on [^{3}H]thymidine incorporation

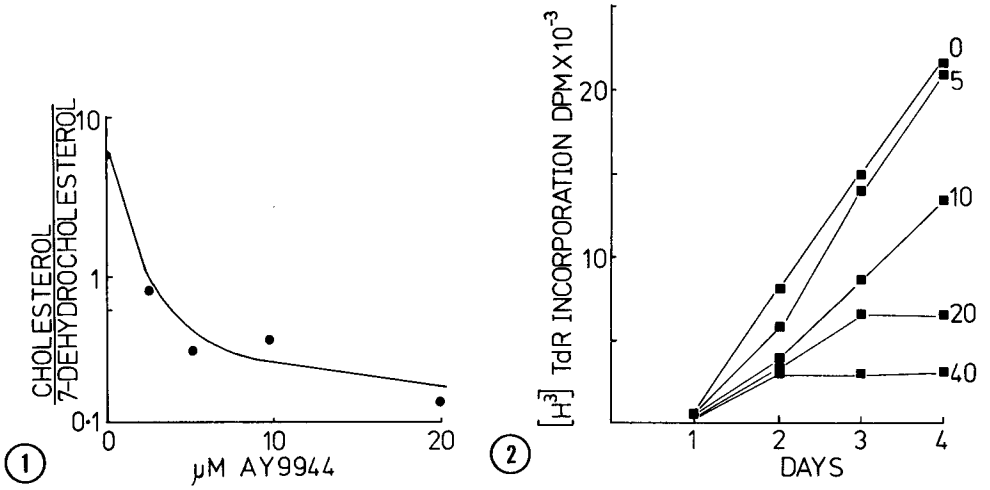

Figure 1. Effect of increasing concentrations of AY9944 on sterol synthesis.

2 x 10^7 peripheral blood lymphocytes were incubated with 2 μCi/ml [^{14}C] acetate for 12-36 h after stimulation with 10 μg/ml phytohemagglutinin. AY9944 at the concentrations shown (μM) was added at time zero. Sterols were precipitated with digitonin and after treatment with pyridine, [^{14}C] Δ^7-dehydrocholesterol and [^{14}C] cholesterol were separated by T.L.C.

Figure 2. Effect of increasing concentrations of AY9944 on incorporation of [^{3}H] thymidine.

10^5 peripheral blood lymphocytes were incubated in 100 μl of media containing 10 μg/ml phytohemagglutinin and AY9944. Cells were pulse labelled with 0.5 μCi/100 μl [^{3}H] thymidine. Points represent means of quadruplicate wells. Standard deviation was less than 10% of the mean.

are shown in Fig. 1 & 2 respectively. The ratio of [^{14}C] labelled 7-dehydrocholesterol to [^{14}C] labelled cholesterol was found to be 1:5 in control incubations. Addition of upwards of 7 μM AY9944 decrease this ratio to 7:1. Over the concentration range 2 μM-20 μM, incorporation of ^{14}C acetate into total sterols was slightly increased. Above 20 μM, there was a marked decrease in incorporation indicating a possible cytotoxic effect. In subsequent experiments a dosage of 15 mM was used. The small amount of cholesterol synthesis which appears to be uninhibited by AY9944 is presumably synthesised by the alternative pathway via desmosterol. This 35 fold reduction in cholesterol synthesis using AY9944 would confirm the finding of Dvorkik *et al.* (6) that this pathway is of prime importance. The effect of these concentrations of

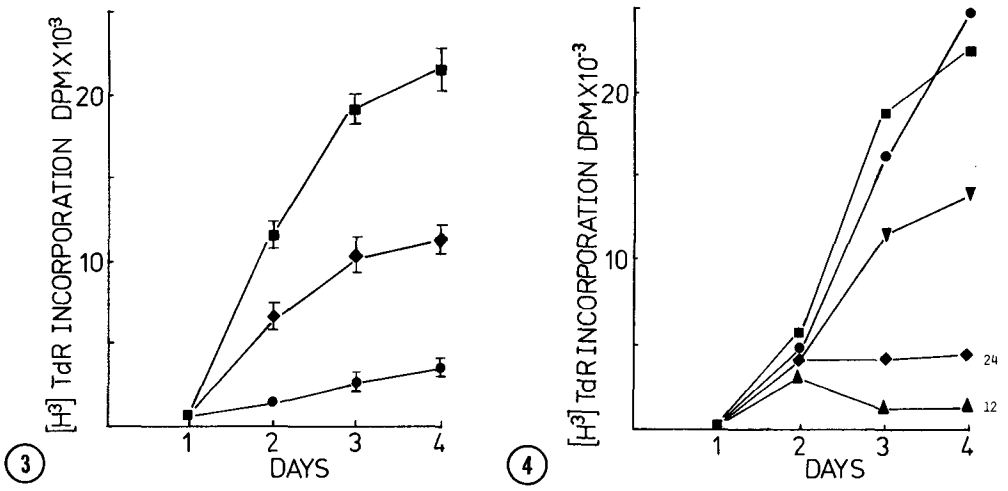

Figure 3. Reversal of inhibition of [^{3}H] thymidine incorporation by AY9944 with lipoproteins.

10^5 peripheral blood lymphocytes were incubated with medium containing 10 µg/ml phytohemagglutinin and delipidated serum (cholesterol content of the medium 5 µg/ml) (■), 10% delipidated serum + 15 µM AY9944 (●), 10% delipidated serum + 15 µM AY9944 + lipoprotein rich fraction (cholesterol content of the medium 85 µg/ml) (◆). Cells were pulse labelled with 0.5 µCi [^{3}H] thymidine. Points represent mean ± S.D. of quadruplicate determinations.

Figure 4. Time-Course of inhibition of [^{3}H] thymidine incorporation by AY9944.

10^5 peripheral blood lymphocytes were incubated with 10 µg/ml phytohemagglutinin. 15 µM AY9944 was added 12 h (▲), 24 h (◆), 36 h (▼) or 60 h (■) after phytohemagglutinin. (●) no AY9944. Points represent mean of quadruplicate determinations. Standard deviation was less than 10% of the mean.

AY9944 are shown in Fig.2. Increasing concentrations of AY9944 result in an inhibition of [^{3}H] thymidine incorporation into DNA. Cell viability assessed by trypan blue exclusion remained constant at 80-90% throughout the incubation. Viability decreased markedly with concentrations of AY9944 above 20 µM.

3.2 Reversal of inhibition with lipoprotein

Lymphocytes were cultured with PHA and 15 µM AY9944 in delipidated serum. In some samples lipoprotein was added to a cholesterol concentration of 95 µg/ml. The results of these experiments are shown in Fig.3. Inhibition by AY9944 was reversed by the addition of the lipoprotein but only to approximately 30% of the control. Reversal of the inhibition was not increased further with additional lipoprotein.

3.3 Time course of inhibition

The timing of the addition of the inhibitor appeared to be critical (Fig.4). Addition of the inhibitor at time zero 12 h and 24 h after PHA severely inhibited stimulation. Addition of AY9944 36 h after PHA had an intermediate effect and no inhibition was evident when the inhibitor was added 60 h after PHA. Inhibition by the AY9944 added at the earlier times could be partially reversed with added lipoproteins (data not shown).

Discussion: Resting lymphocytes can be activated by mitogenic lectins to enter the cell cycle, undergo blast transformation and proliferate. An early event in this sequence is the increase sterol synthesis. Chen and colleagues (2) first noted that inhibition of HMGCoA reductase also inhibited DNA synthesis and concluded that sterol synthesis may be required for DNA synthesis and for cell division. But it would appear from further work with inhibitors of HMGCoA reductase, that lymphocytes (3), like synchronized BHK21 cells (7) and cultured monkey smooth muscle cells (8), have a requirement for mevalonate for DNA synthesis. Enzyme activity does not appear to be required for initial blast transformation (3). This study was designed to indicate if there was a specific requirement for cholesterol synthesis in lymphocyte activation. The results presented here would suggest that there is a requirement for some endogenous cholesterol synthesis. AY9944 inhibited the activation of lymphocytes by PHA, an inhibition that could only partly be overcome with an excess of exogenous cholesterol. Although possibly cytotoxic at higher concentrations, at the concentrations used for these experiments the effect of AY9944 would appear to be through an inhibition of Δ^7 dehydrogenase rather than by a cytotoxic effect. Three points support this view: the continued high rate of sterol synthesis in the presence of less than 20 μM AY9944; the maintenance of a high cell viability; and the reduced inhibition at the late times of the response. One would then conclude that the inhibitor effect is through a reduction in the supply of endogenous cholesterol to the cell. Since this block could not be overcome with

exogenous cholesterol, the endogenous cholesterol synthesis may represent a more fundamental role in the growth and division of the lymphocyte. Possibly this endogenous synthesis is required for a coordinated synthesis of new membranes. The reduced inhibition by AY9944 when the inhibitor was added at times 60 h after PHA would support this concept.

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