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Regulation of Cholesterol Synthesis in L1210 Murine Leukemia Cells by Dietary Cholesterol*

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Abstract—*Studies were undertaken to measure cholesterol synthesis by L1210 murine leukemia cells and to determine if there is modulation of the rate of cholesterol synthesis by the content of cholesterol in the diets of the host animals. When the animals were fed a cholesterol-free diet for* 6 weeks, *the L1210 leukemia cells synthesized digitonin-precipitable sterols from* [2-^{14}C] *acetate at rates that were* 2–3 *fold greater than cells from animals fed a* 5% *cholesterol diet. This difference was also evident after only* 2 weeks *of feeding. The total and free cholesterol content of the cell-free ascites fluid of the animals fed the* 5% *cholesterol diet was higher than the ascites fluid of the animals fed the cholesterol-free diet. However, there were no differences in the cholesterol content of the L1210 cells from the two diet groups. These results suggest that there is dietary regulation of the rate of cholesterol synthesis by L1210 leukemia cells.*

INTRODUCTION

EVERY normal mammalian cell studied except the mature erythrocyte has the ability to synthesize cholesterol, though the rate and control of production varies widely between tissues and species [1]. Dietary feedback regulation of this synthesis has been shown in the normal livers of all vertebrate species so far studied [2]. Many tissues other than the liver show dietary control. However, many neoplastic tissues lack dietary regulation of this process. Based on this observation, it has been suggested that there is a link between abnormal control of cholesterol synthesis and carcinogenesis [3, 4]. The present study was designed to investigate *in vivo* whether there is *de novo* cholesterol synthesis in the L1210 lymphoid leukemia and establish whether the negative feedback system which controls the rate of synthesis is intact.

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MATERIALS AND METHODS

Male DBA/2j mice initially weighing 14–16 g (Sprague–Dawley, Madison, WI) were fed a 5%cholesterol or a cholesterol-free diet (Teklad Test Diets, Madison, WI). The composition of the diets is shown in Table 1. Food and water were provided *ad libitum*. The animals were fed the diet for either 1 or 5 weeks prior to the intraperitoneal injection of 1×10^5 L1210 tumor cells and for 1 additional week during growth of the tumor. One week after injection of the tumor cells, the mice were sacrificed by cervical dislocation and the cells were harvested. The sources, method of tumor transplantation, cell harvest, handling, and enumeration have been previously described [5]. The viability of the cells was determined in each experiment prior to incubation and it averaged 85.2% ± 1.6 (mean ± S.E.M.). Studies of acetate incorporation were carried out on pooled samples. This experimental design was chosen rather than the study of cells from individual mice for the following reasons: (a) sufficient cells for only two metabolic incubations (L1210 cells) or less than one determination (thymus lym-

Table 1. Composition of experimental diets

Ingredient	Cholesterol-free diet content (g/kg)	5% Cholesterol diet
Cholesterol	0	50.0
Sucrose	499.99	449.99
Casein, high protein	200.0	200.0
Corn starch	150.0	150.0
Non-nutritive fiber (cellulose)	50.0	50.0
Corn oil	50.0	50.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
DL-Methionine	3.0	3.0
Choline bitartrate	2.0	2.0
Ethoxyquin*	0.01	0.01

*Antioxidant

phocytes) could be obtained from each mouse; (b) separate enumeration and handling of cells from 20 different mice would prolong the time from harvesting to incubation with isotope and might adversely affect the results.

Normal lymphocytes from the thymus were obtained from animals on commercial chow (Teklad Mills, Winfield, IA) not injected with L1210 cells by dissecting out the thymus with capsule intact. The tissue was then gently pushed through a 200 mesh screen into phosphate buffered saline (PBS) containing 1 mg/ml glucose. Large tissue fragments were sedimented and the cells in the supernatant were then further dispersed by aspirating them repeatedly through a syringe and needle. The cells were washed three times with 15 ml of PBS and sedimented at 350 ***g*** before counting.

Incubations to measure acetate incorporation were carried out in Erlenmeyer flasks containing 5×10^7 L1210 cells in Krebs–Ringer phosphate buffer (124 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$ and 16 mM Na_2HPO_4), 40 μCi of [2-^{14}C]acetate (58 mCi/mmole, 0.5 mM) (Amersham Searle Corp., Arlington Heights, IL) and 1% albumin (fatty acid free bovine albumin fraction V, Miles Laboratories, Elkhart, IN) in a total volume of 5 ml for 30, 60, 90 and 120 min. For the studies which compared the rate of synthesis of digitonin-precipitable sterols by L1210 cells and normal lymphocytes, the cells were incubated in Eagle minimum essential medium with glutamine (Grand Island Biological Corporation, Grand Island, NY) containing 20% fetal calf serum, [2-^{14}C]acetate (20 μCi, 58 mCi/mmole 0.5 mM) in a total volume of 5 ml. The cells were preincubated for 4 hr at 37°C, then the isotope was added during a final 2 hr of incubation. All the sealed flasks were equilibrated with 5% CO_2 – 95% air and incubations in triplicate were carried out at 37°C in an Eberbach shaker bath at 88 strokes/min. The incubations were terminated by pouring the contents of the flasks into 20 ml of ice cold PBS and then sedimenting the cells (3000 ***g***) for 5 min. After being washed twice with 20 ml cold PBS, the cells were dispersed in 20 ml $CHCl_3$:CH_3OH (2:1, v/v) and extracted for 2 hr at 20°C [6]. The lipid extracts were saponified in 0.4 N KOH in 91% ethanol for 1 hr at 56°C and the sterols were precipitated with digitonin [7]. Aliquots were taken from the total lipid, non-saponifiable, saponifiable and digitonin-precipitable fractions and counted in a Beckman LS-3133T scintillation spectrometer.

The cholesterol content of cells and cell-free ascites fluid was determined on cells from animals fed the experimental diets for a total of 6 weeks. Cells and ascites fluid from animals were collected individually and separated by centrifugation at 3000 ***g*** for 5 min at 0°C. The ascites fluid was decanted and the cells were washed three times with 20 ml of ice-cold PBS. The ascitic fluid was centrifuged two additional times at 3000 ***g*** for 10 min at 0°C to insure removal of cells. Analysis of cholesterol content was measured using an enzymatic method based upon cholesterol oxidase (EC 1.1.3.6) and cholesterol ester hydrolase (EC 3.1.1.13) [8] (Boehringer Mannheim Corporation, Indianapolis, IN).

RESULTS

L1210 cells utilized acetate as a labeled substrate for synthesis of digitonin-precipitable sterols. For example, cells from animals fed a cholesterol-free diet synthesized sterols at a rate 161.7 ± 5.7 pmole/hr/10^8 cells. In initial experiments the rate of synthesis was shown to be constant from 30–120 min of *in vitro* incubation. The process demonstrated saturation kinetics at 0.4–0.5 mM acetate; therefore, all further experiments were carried out 0.5 mM. In separate experiments the rate of synthesis of L1210 cells was compared to that of normal lymphocytes. The L1210 cells incorporated acetate into digitonin precipitable material at a rate 28,364.2 ± 5228.3 dis/min/hr per 10^8 cells (mean and S.E.M. of three separate determinations) compared to 615.7 ± 30.5 dis/min/hr per 10^8 cells by normal lymphocytes.

The rate of synthesis of digitonin precipitable sterols by L1210 cells on the 5% cholesterol or cholesterol-free diets is shown in Fig. 1. The cells from the animals maintained on the cholesterol-free diet for 6 weeks synthesized more digitonin precipitable material than the cells from the animals fed the 5% cholesterol diet. This 6-week experiment was repeated and the results were identical. The difference in synthetic rate was evident after only 2 weeks of feeding (Fig. 1). To eliminate the possibility that these differences might be due to differences in acetyl CoA pool size in the two types of cells, the synthesis of saponifiable lipids from labeled acetate was examined. The L1210 cells from animals fed the 5% cholesterol diet had rates that were not significantly different, at most incubation times, as compared to animals fed the cholesterol-free diet (Fig. 2).

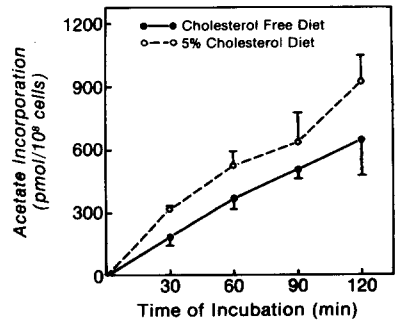

Fig. 2. Acetate incorporation into saponifiable lipids by L1210 murine leukemia cells from mice fed a 5% cholesterol or cholesterol-free diet. 5×10^7 L1210 cells, 40 μCi [2-^{14}C] acetate (58 mCi/mmole, 0.5 mM), 1% albumin and Krebs Ringer phosphate buffer were in the incubation flasks which contained a total volume of 5 ml. Incubation was carried out at 37°C in a shaker bath for 30, 60, 90 and 120 min. Each point represents triplicate determinations on pooled cells from 9–10 mice. Shown is the value for L1210 cells from animals fed the experimental diet for 6 weeks. In each case, L1210 cells were transplanted 1 week prior to sacrifice. At 30 min, the values are significantly different ($P < 0.05$) and at 60, 90 and 120 min there is no significant difference in the values for cells from the two diet groups.

It is important to note that the experiments shown in Figs. 1 and 2 were carried out on pooled samples. The triplicate determinations at each of the four time points for the cells from animals fed the cholesterol-free diet were drawn from one pool. The same is true of the data shown for the cells from animals fed the

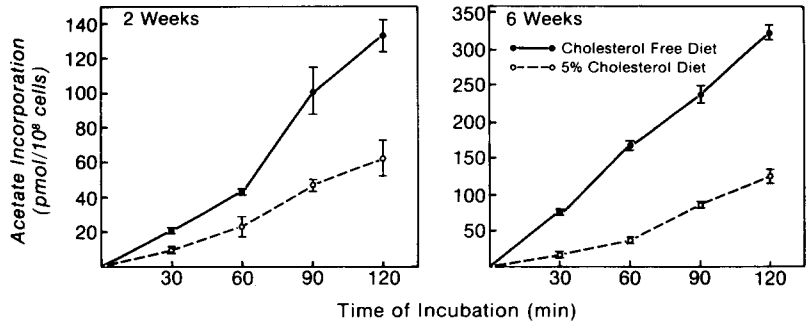

Fig. 1. Acetate incorporation into digitonin-precipitable sterols by L1210 murine leukemia cells from mice fed a 5% cholesterol or cholesterol-free diet. 5×10^7 L1210 cells, 40 μCi [2-^{14}C]acetate (58 mCi/mmole, 0.5 mM), 1% albumin and Krebs Ringer phosphate buffer were in the incubation flasks which contained a total volume of 5 ml. Incubation was carried out at 37°C in a shaker bath for 30, 60, 90 and 120 min. Each point represents triplicate determinations (6 week, 5% cholesterol diet, 60 min value is a duplicate) on pooled cells obtained from 5–10 mice. Shown are the values for L1210 cells from animals fed the experimental diets for 2 and 6 weeks. In each case, the L1210 cells were transplanted 1 week prior to sacrifice. At 2 weeks the 30, 60, 90 and 120 min values are significantly different at the $P < 0.01$, < 0.05, < 0.02, < 0.01 levels. At 6 weeks all values are different at the $P < 0.001$ level.

5% cholesterol diet. Therefore, the statistical comparison is of the variation between random samples from a pool at each time point. With such a design it is not possible to make inferences regarding mouse to mouse experimental variation. For this reason the cells from such pools were studied in three separate experiments (once after 2 weeks of feeding and twice after 6 weeks of feeding) and in all three studies the cells from the animals fed the cholesterol-free diet incorporated acetate into digitonin-precipitable sterols at a significantly higher rate than cells from animals fed the 5% cholesterol diet.

The effect of cholesterol in the diet on the cholesterol content of the ascites fluid bathing the tumor cells is shown in Table 2. There was a significant difference between the total and free cholesterol values in the ascites fluid of the two diet groups ($P<0.05$). However, there was no difference in the proportions of free and esterified cholesterol in the two groups. Table 2 also shows data from the analysis of cholesterol content of the tumor cells. There was no significant difference in total or free cholesterol content of the cells from animals fed the two diets.

The animals fed the 5% cholesterol diet gained 0.247 g per day during the 6-week diet period. This weight gain was similar to that of the mice maintained on the cholesterol-free diet which was 0.276 g per day. There was no significant difference at the $P<0.05$ level between the weight gain of the two diet groups at any point in time. In addition, the animals in each group consumed approximately the same amount of food.

DISCUSSION

The present studies were carried out to examine cholesterol synthesis and its dietary regulation *in vivo* using an animal tumor which is used extensively in the pre-clinical screening of anti-neoplastic drugs. The results suggest that L1210 cells modulate their rate of synthesis of digitonin-precipitable materials in response to dietary cholesterol. Sterol synthesis was appreciably higher in cells from animals fed the cholesterol-free diet as compared to animals fed the 5% cholesterol diet. This difference was evident after only 2 weeks of feeding. However, such a difference in incorporation of acetate by L1210 cells from animals on the cholesterol-free and 5% cholesterol diet could result from differences in intracellular acetate pool size and not be associated with any real differences in rate of synthesis of digitonin-precipitable sterols. To rule out this possibility, we examined the rate

Table 2. Cholesterol content of ascites fluid and L1210 cells from animals fed a 5% cholesterol or cholesterol-free diet

Specimen	Cholesterol	
	Total	Free
Ascites fluid* (μg/ml)		
5% cholesterol diet	891.6 ± 62.2†	507.4 ± 47.1‡
cholesterol-free diet	683.2 ± 30.8	385.0 ± 20.6
L1210 Cells (μg/10^8 cells)		
5% cholesterol diet	408.0 ± 40.1§	401.6 ± 31.9¶
cholesterol-free diet	424.1 ± 35.3	422.7 ± 29.2

*Cell-free.

†Significantly different from ascites fluid from the cholesterol-free diet animals ($P<0.02$).

‡Significantly different from ascites fluid from the cholesterol-free diet animals ($P<0.05$).

§Not significantly different from cells of animals fed the cholesterol-free diet ($0.7<P<0.8$).

¶Not significantly different from cells of animals fed the cholesterol-free diet ($0.6<P<0.7$).

Mice were fed the experimental diets for 5 weeks. L1210 cells (1×10^5) were injected i.p. and the diets were continued for 1 additional week during tumor growth. The animals were then sacrificed and the ascites fluid containing the L1210 cells was harvested and the cells separated from cell-free ascites fluid by centrifugation. Cholesterol content was determined as described in text. Values shown are the mean and S.E.M. of determinations on samples from 6 animals.

of synthesis of saponifiable lipids by cells from animals on the two diets. Any changes in intracellular acetyl CoA pool size resulting from the experimental diets would affect the synthesis of digitonin-precipitable and saponifiable fractions in a similar manner. However, in our study, the rate of synthesis of saponifiable lipids was equal to or higher in the cells from animals ingesting the 5% cholesterol diet even though the rate of synthesis of digitonin-precipitable material was higher in the cells from animals on the cholesterol-free diet. This observation makes it unlikely that diet-induced alterations of intracellular common precursor pools is the cause of the differences in rate of synthesis of digitonin-precipitable material.

Many neoplasms have shown a loss of feedback control of cholesterol biosynthesis when the regulation is studied using experimental diets. Results of this kind have been obtained using mouse hepatoma BW 7756 [9], minimum deviation hepatoma [2], guinea pig L_2C leukemia [3], precancerous liver in rodent and trout [10, 11] and rat leukemia [12]. On the basis of these studies, it has been suggested that the deletion of feedback regulation of cholesterol synthesis is a metabolic characteristic of neoplasia [2]. However, hepatoma cells in tissue culture can regulate their rate of cholesterol synthesis in response to exogenous cholesterol-rich lipoproteins [13]. Similarly, human leukemic blast cells [14, 15] and renal carcinoma [16] modulate their cholesterol synthesis in short term tissue culture when exposed to exogenous cholesterol *in vitro*. Furthermore, Beirne and Watson have demonstrated that tissue culture hepatoma cells express regulation *in vitro* when exposed to lipoproteins but show no dietary regulation *in vivo* [17]. Recently, partial dietary control of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [mevalonate:$NADP^+$ oxidoreductase (CoA acylating), EC 1.1.1.34] activity in rat hepatocellular carcinomas has been demonstrated by feeding 5% cholesterol or 2% cholestyramine diets [18]. The present study is consistent with the conclusion that there is *in vivo* regulation of cholesterol biosynthesis of a tumor cell by varying the amount of cholesterol contained in the diet of the host.

Following the 6 week feeding period the cell-free ascites fluid of the mice on the cholesterol-free diet contained 23.4% less cholesterol than that from the animals on the 5% cholesterol diet. It seems likely that the changes in the rate of sterol synthesis are mediated by changes in the cholesterol of the ascites fluid which may in turn be a result of equilibrium with the cholesterol contained in plasma lipoproteins. The ability of the L1210 to modulate its rate of cholesterol synthesis suggests that these cells may contain plasma membrane receptors of high specificity such as are found in human fibroblasts and lymphocytes [19, 20]. In contrast to the remarkable change in cholesterol content of the ascites fluid, the L1210 cells themselves contained the same amounts of cholesterol regardless of diet. Although it is known that the cholesterol content of the erythrocytes of some rodents [21] and liver cells [22] can be altered by dietary means, to our knowledge, there have been no such alterations of the cholesterol content of leukocytes. This lack of a change in cholesterol content does not preclude the possibility of normal control by a receptor-mediated process. If the receptor theory of the regulation of cholesterol synthesis as postulated for the human fibroblast and more recently for the lymphocyte [20, 23] applies to the L1210 cell, exposure of the cell to extracellular cholesterol may result in regulation of the number of receptors on the cell surface. This effect may have little to do with the amount of detectable cholesterol contained within the whole cell when the cholesterol content of the ascites fluid changes from 890 to 680 μg/ml.

The presence of regulation of cholesterol synthesis at the concentrations of cholesterol contained in the ascites fluid would not be expected in the L1210 cell based on work with the human fibroblast [24]. In fibroblast studies, HMG CoA reductase activity became maximally inhibited at cholesterol concentrations above 50 μg/ml [24]. Likewise, in the normal human leukocyte, the induction of HMG CoA reductase *in vitro* does not occur until the cholesterol content of the medium is reduced to 210 μg/ml [25]. In contrast, the L1210 cell showed feedback control when the extracellular cholesterol content changed from 680 to 890 μg/ml. In the fibroblast and human leukocyte both levels of cholesterol would have maximally suppressed the HMG CoA reductase based on *in vitro* tissue culture studies. This suggests that the L1210 cholesterol receptor sensor is set at a different level. Alternatively, the major lipoprotein of the ascites fluid may not be low-density lipoprotein. The lipoprotein which regulates cholesterol production may be a molecule or complex the content of which is not accurately reflected by measuring the cholesterol content.

Studies of regulation in various rat tissues have disclosed that control of sterol synthesis by some tissues may be mediated by high density lipoproteins or chylomicron remnants rather than low-density lipoproteins [26].

The mechanism for the control of cholesterol synthesis in the human fibroblast involves a series of steps initiated by the binding of low density lipoproteins to surface receptors [19]. A reduction of cholesterol synthesis depends upon a suppression of HMG CoA reductase by unesterified cholesterol present within the cell [19]. The decrease in HMG CoA reductase is accompanied by an increase in cholesterol ester formation, both resulting from binding of low-density lipoproteins [27]. The reciprocal changes suggest that the accumulation of cholesterol esters within the cell may be an associated event that is necessary for suppression of HMG CoA reductase. However, regulation occurs in the L1210 cell even though there is almost no cholesterol ester within the cell. Based on this observation, cholesteryl ester synthesis does not appear to be an obligatory step in the regulatory process of the L1210 leukemia cell.

The L1210 cell synthesizes cholesterol at a rate that is about 50-fold greater than the normal mouse lymphocyte obtained from the thymus. The elevated rate may be necessary to provide sterols for new membrane synthesis by this rapidly dividing cell. High rates of sterol synthesis have also been noted in cells from leukemia AKR/J mice [28], guinea pig L_2C leukemia lymphocytes [29], human leukemia blast cells [15], simian virus-transformed human fibroblast cell line [30], mouse hepatoma [31], and precancerous liver from rats or trout treated with carcinogens [10, 11]. It has been established that the rate limiting enzyme for the synthesis of cholesterol is HMG CoA reductase [2, 32]. The elevated rates of synthesis by the L1210 cells are likely due to high levels of enzyme activity although no enzyme assays were carried out in the present study. The high rate of synthesis of digitonin-precipitable sterols by the L1210 leukemia cells compared to normal lymphocytes is not due to an absence of feedback regulation.

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