Elevated Sterol Synthesis in Lymphocytic Leukemia Cells from Two Inbred Strains of Mice

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SUMMARY

Cell suspensions of normal and leukemic mouse lymphocytes from spleens, thymuses, and mesenteric lymph nodes actively incorporate acetate into cellular lipids. The de novo synthesis of digitonin-precipitable sterols in leukemic cells is tenfold greater than that of normal cells and is associated with a correspondingly increased activity of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase. In contrast, synthesis of fatty acids is only slightly enhanced and production of CO₂ is not affected because of leukemogenesis.

INTRODUCTION

Sterol synthesis in bone marrow, leukocytes, and circulating blood cells has been the subject of several investigations (2, 3, 17, 27). In vitro studies showed that leukocytes and reticulocytes accounted for most of the cholesterol synthesis of rabbit blood. Because this synthetic activity was inhibited after prefeeding the animals with cholesterol, these blood cells must possess a feedback system for regulating cholesterol synthesis similar to that of the liver (23, 27). In liver, alterations in the rate of sterol synthesis involved changes in the activity of the rate-limiting enzyme of the pathway, HMG-CoA² reductase (EC 1.1.1.34) (11, 15, 23, 25). This regulatory system appears to be deleted in hepatomas (10, 21, 24), and a similar loss of feedback control of sterol synthesis has been reported for the transplantable leukemia L₂C cells of guinea pigs (22).

Since leukemia develops spontaneously in several inbred strains of mice, we studied lipid synthesis of leukemic cells from 2 leukemia-prone strains rather than use transplantable leukemias. Over 90% of AKR/J mice develop lymphoid leukemia between 7 and 9 months of age (19, 26). Strain HRS/J is unique in that over one-half of the hairless (hr/hr) mice suffer from lymphoid transplantable leukemias. Over 90% of AKR/J mice were from about 7 to 9 months. Unless otherwise indicated, mice were given Old Guilford chow and water ad libitum.

Leukemia was diagnosed by clinical inspection, i.e., palpation for lymphadenopathy and splenomegaly, and verified grossly and by histological examination. Tissues were fixed in 10% neutral formalin, embedded in paraffin, cut at 6 to 8 nm, and stained with hematoxylin-eosin.

Preparation of Cell Suspensions and Chemical Analysis.

The mice were killed by cervical dislocation, and spleens, thymuses, and mesenteric lymph nodes were dissected. The organs were weighed and washed quickly in Waymouth's chemically defined Medium MB752/1 (28) modified by supplementation with trace metals (13) and additional amounts of leucine (120 µg/ml), isoleucine (120 µg/ml), and serine (64 µg/ml).

The tissues were finely teased with scissors and tweezers in 5 ml of medium. The suspensions were allowed to stand for 5 min so that the stromal connective tissue could settle. The supernatants containing the lymphoid cells were carefully pipetted into a centrifuge tube and the cells were collected by centrifugation at 800 x g for 5 min. Brush slides of cells were prepared from some samples and stained with May-Grunwald-Giemsa for differential cell counts. Twice washed packed cells were resuspended in a 20-ml volume of medium per ml of cells. Cell viability was determined during experiments by the trypan blue exclusion method (4). Aliquots (2 ml) of the final cell preparation were pipetted into sterile 50-ml Erlenmeyer flasks to which were added 10 µCi (5 µmoles) of sodium acetate-1-¹⁴C (New England Nuclear, Boston, Mass.). The flasks were sealed with septum stoppers fitted with plastic cups (Kontes, Vineland, N. J.) and incubated in a shaking water bath (Dubnoff) for 4 hr at 37°. Following incubation the cell suspensions were acidified with 0.15 ml of 12 N H₂SO₄; the released CO₂ was trapped in a plastic cup containing 0.3 ml of Hyamine-10X (Packard...
Instrument Co., Downers Grove, Ill.) after gentle shaking of the flasks at room temperature for 15 min. The cups containing the Hyamine were then placed in vials containing 4.7 ml of toluene and 1 aliquot was taken for $^{14}$C analysis. The remaining mixture was made alkaline by the addition of 0.5 ml of 90% KOH and saponified by autoclaving at 15 lb of pressure for 1 hr. Labeled fatty acids and sterols were determined by a previously described method (11); the protein content of the cell preparations was measured by the Lowry method (16) using bovine albumin (Pentex, Miles Laboratories, Kankakee, Ill.) as a standard.

**HMG-CoA Reductase Activity.** Enzyme activity was determined in unwashed microsomes from whole spleen, isolated as described previously (11), following incubation with DL-HMG-CoA-3-$^{14}$C and other required components of the reaction mixture. The mixture was acidified with HCl; then mevalonic acid-5-$^3$H (42,000 dpm; 0.008 µg) was added and mevalonolactone extracted. Cold mevalonolactone (0.3 mg) was added and the extract was chromatographed on a Brinkman silica gel plate (Brinkman Instruments, Inc., New York, N. Y.) using acetone:benzene (1:1) as the developing solvent. After visualization, by spraying with rhodamine B, mevalonolactone in a 1-cm-wide band was scraped into a scintillation vial. Ethanol (0.5 ml) and 15 ml of toluene scintillation fluid were introduced and $^3$H and $^{14}$C were assayed.

**RESULTS**

Following a 24-hr incubation period about 70% of the lymphoid cells remained viable independent of the organ of origin or whether they were normal or leukemic (Chart 1). The calculated regression lines for normal and leukemic cells do not differ significantly, although a few preparations from leukemic organs contained considerable numbers of trypan blue-positive cells. Various nucleated cell types were present in the following proportions. Normal thymus contained about 92% lymphoid cells and 8% granulocytes, macrophages, erythroid precursors, and some unidentified cells; leukemic thymus consisted of 95% lymphoid cells and 5% other types. In both normal and leukemic lymph nodes, 95 to 96% were lymphoid cells; they were mainly parablasts from leukemic nodes. Normal spleens contained about 73% lymphoid and 27% other cell types, whereas leukemic spleens had 89% lymphoid cells. The standard error for these counts varied from 2 to 5%.

The ability of cells suspended in modified Waymouth’s Medium MB752/1 to metabolize acetate was better than that of cells in Krebs-Ringer solution, which is ordinarily used for metabolic studies with liver slices. In fact, after a 4-hr incubation period, both normal and leukemic cells incorporated 10 times more radioactivity when suspended in Waymouth’s Medium MB752/1 than in Krebs-Ringer solution.

The rate of acetate incorporation to sterols, fatty acids, and CO$_2$ was found to be virtually linear for incubation periods up to 6 hr (e.g., the sterol synthesis in Chart 2); a standard 4-hr incubation period was used in all subsequent experiments. Linearity with cellular protein concentration was limited to a narrow range of 3 to 8 mg/flask; acetate metabolism was reduced at higher and lower dilutions of cells. Rates of sterol synthesis in duplicate samples rarely varied by more than 10%. The relatively large variation in values for cells from leukemic mice in different experiments may reflect a wide range in the degree of leukemia development among mice of the same age.

The rate of splenic sterol synthesis was similar in normal mice of 3 genetically different strains of various ages (Table 1). In contrast, a 10-fold or greater increase in sterol synthesis occurred in lymphatic cells from the spleen, lymph nodes, and thymus of leukemic AKR/J mice (Table 2), whereas fatty acid synthesis was only moderately (2- to 3-fold) elevated, and CO$_2$ production
from acetate via the tricarboxylic acid cycle remained the same as in normal cells (Table 2). A similar elevation of sterol synthesis was also observed in leukemic cells of hairless mice of strain HRS/J. Thus, the data obtained from normal and leukemic HRS/J mice was essentially the same as those from AKR/J mice.

HMG-CoA reductase activity of splenic microsomes from leukemic AKR/J mice was 2- to 10-fold greater than that from normal AKR/J mice; hence, the enzyme activity paralleled the increase in sterol synthesis (Table 3). Similarly, the elevation of HMG-CoA reductase activity correlated well with the degree of leukemic splenomegaly as determined by gross and histopathological examination. The linear relationship between enzyme-specific activities and the spleen weights is presented in Chart 3.

Table 2
Comparison of acetate metabolism in normal and leukemic cells of AKR/J mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Normal Age (wk)</th>
<th>dpm/mg protein/4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>550 ± 78 (11)°</td>
<td>935 ± 3450 (10)</td>
</tr>
<tr>
<td>Spleen</td>
<td>563 ± 68 (12)</td>
<td>8491 ± 2044 (10)</td>
</tr>
<tr>
<td>Thymus</td>
<td>478 ± 114 (11)</td>
<td>5028 ± 1209 (9)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate numbers of experiments.

Table 3
HMG-CoA reductase activity in the spleens of normal and leukemic AKR/J mice

Reaction mixtures contained 40 nmol of D-[1-14C]-HMG-CoA (0.17 µCi); 1 µmol of glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, 0.5 µmole of NADP+, 6 µmole of EDTA, 15 µmole of NaCl, 5 µmole of 2-mercaptoethanol, 35 µmole of potassium phosphate buffer (pH 6.8), and spleen microsomes in a total volume of 0.5 ml. Incubation time was 45 min at 37° in an N2 atmosphere. Levels of enzyme activity were estimated from plots of rate against 4 levels of enzyme (0.1 to 1.5 mg of microsomal protein per flask) obtained from pooled spleens from 1 to 3 leukemic mice or 10 normal controls.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Normal</th>
<th>Leukemic</th>
<th>Leukemic</th>
<th>Leukemic</th>
<th>Leukemic</th>
<th>Leukemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>560 ± 109 (10)</td>
<td>638 ± 158 (8)</td>
<td>1.1</td>
<td></td>
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<tr>
<td>Spleen</td>
<td>620 ± 52 (11)</td>
<td>914 ± 137 (9)</td>
<td>1.5</td>
<td></td>
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<tr>
<td>Thymus</td>
<td>574 ± 121 (10)</td>
<td>723 ± 141 (9)</td>
<td>1.3</td>
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</tr>
</tbody>
</table>

* Mean ± S.E.
* Numbers in parentheses indicate numbers of experiments.

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Sterol Synthesis in Lymphocytic Leukemia Cells

Consistent with its function as the regulatory enzyme for de novo sterol synthesis, HMG-CoA reductase activity was elevated in leukemic spleens. Not only the specific activity of the enzyme increased in leukemic spleens but their weights were 3 to 10 times larger than normal, so that the total enzyme activity of leukemic spleens was as high as 100 times of the normal. Also, the concentration of the enzyme activity correlated well with the degree of splenomegaly. Thus, the extent of splenic enlargement as well as the amount of enzyme activity may serve as a good index for the stage of leukemia progression in AKR/J mice.

The basis for the high rate of sterol synthesis in leukemic cells is not known except that elevated sterol synthesis may be a common feature of cancer. Howard and Kritchevsky (9) reported an elevated rate of cholesterol synthesis in a simian virus 40-transformed human diploid fibroblast cell line compared to the untransformed line. These viral transformed cells were capable of unlimited proliferation and appeared to be tumorigenic (9).

The synthesis of cholesterol in liver is known to be regulated either by a system that involves inhibition of HMG-CoA reductase or by repression by an end product of the pathway (10, 11, 15, 21-23, 25). This regulatory mechanism seems to be deleted in hepatomas resulting in an elevated rate of sterol synthesis. Whether or not cholesterol feedback inhibition of sterol synthesis occurs in lymphatic cells cannot conclusively be established by our results. Since normal lymphatic cells possess a low sterol synthetic rate, its inhibition was difficult to show. However, the cholesterol feedback mechanism did appear to be present in spleen cells from mice with spontaneous leukemia, although the inhibited values from leukemic cells were still 5 to 6 times higher than those of normal controls. This is in contrast to the reported loss of feedback regulation in transplantable leukemic L2C cells (22). Obviously, results obtained merely from transplantable tumors must be interpreted with caution. For ex-

**DISCUSSION**

Cell preparations from spleens, lymph nodes, and thymuses of 3 different strains of mice remained viable in modified Waymouth's Medium MB752/1 for up to 12 hr and readily converted acetate into sterols, fatty acids, and CO₂. Although sterol synthesis in normal lymphatic organs was relatively low, its rate in leukemic tissues was comparable to that in normal liver. In leukemic cells, sterol synthesis was greatly increased, whereas the rates of fatty acid synthesis and CO₂ production from acetate in the same cells were only moderately, if at all, affected. Preliminary data from circulating leukemic lymphocytes reveal a similar elevation of sterol synthesis (H. W. Chen and H. J. Heiniger, unpublished results).

Effect of cholesterol feeding on sterol synthesis in spleen cells of AKR/J mice

Cholesterol (Schwarz/Mann, Orangeburg, N. Y.) was suspended in ether and mixed with powdered chow. The control mice were fed the powdered chow.

<table>
<thead>
<tr>
<th></th>
<th>dpm/mg protein/4 hr after</th>
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<tr>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>Normal</td>
<td>597 ± 83 (6)*</td>
</tr>
<tr>
<td>Normal, fed 2% cholesterol in chow</td>
<td>332 ± 13 (5)</td>
</tr>
<tr>
<td>Leukemic</td>
<td>6210 ± 574 (4)</td>
</tr>
<tr>
<td>Leukemic, fed 2% cholesterol in chow</td>
<td>7049 ± 3096 (5)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate numbers of experiments.
ample, Gore and Popjak (5) found that both mouse Ehrlich ascites carcinoma and rat ascites tumor (RD3) lost the ability to convert acetate to fatty acids and cholesterol. Horton et al. (7) reported a normal cholesterol uptake in a chemically induced hyperplastic liver node and primary hepatoma, whereas defective uptake systems in transplantable hepatomas were shown by other studies (6, 8, 23). Although we could not accurately estimate the proportion of normal cells in a leukemic spleen, the observed inhibition is unlikely to be entirely due to diminished sterol synthesis in the residual normal cells. This is indicated by the fact that the sterol synthetic rate of normal spleen cells was far lower than the reduction in sterol synthesis of leukemic cells induced by dietary cholesterol.

Free and esterified cholesterol constitutes one-fourth to one-fifth of total lipids in mammalian plasma membranes (14, 20); except for liver, placenta, and some endocrine glands, which are capable of catabolizing cholesterol to bile acids or to steroid hormones, the utilization of cholesterol by cells and tissues may essentially be limited to membrane synthesis. An accelerated sterol synthesis in tumor cells or tumor tissues may, therefore, reflect their rapid growth and division, i.e., processes which require cholesterol as major cell structural elements. This view is supported by our unpublished observation that in mouse L-cells the synthesis of sterol from acetate is rapidly inhibited in confluent nondividing cell culture, but the production of fatty acids and CO₂ continues for a longer period. There are, however, other possible explanations for high sterol synthesis in tumor cells, e.g., tumor cell membranes may have a higher cholesterol content than normal cell membranes (12), or they may store cholesterol inside the cells as lipid droplets (1).

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REFERENCES

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