Complete Replacement of Membrane Cholesterol with 4,4',14'-Trimethyl Sterols in a Human T Cell Line Defective in Lanosterol Demethylation*

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A3.01 is a hypoxanthine/aminopterin/thymidine-sensitive, human immunodeficiency virus-susceptible, human T cell line derived by Folks et al. (Folks, T., Benn, S., Rabson, A., Theodore, T., Hoggan, M. D., Martin, M., Lightfoote, M., and Selig, K. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4539–4543) following exposure of CEM cells to 8-azaguanine. In the present study, it is shown that A3.01 also contains a heretofore unrecognized mutation in cholesterol biosynthesis. A3.01 cells grown in the presence of 10% fetal bovine serum (FBS) contain primarily cholesterol in their membranes, but based on [14C]acetate labeling, synthesis only lanosterol and 24,25-dihydrolanosterol. Reduction in the amount of FBS provided resulted in decreased cellular levels of cholesterol with corresponding increases in the two 4,4',14'-trimethyl sterols. In A3.01 cells cultured in 1% FBS medium, lanosterol and 24,25-dihydrolanosterol accounted for 7 and 45%, respectively, of total cellular sterols. Following dilution of the 1% FBS-grown cells into serum-free media, the level of membrane cholesterol gradually declined, such that after three passages it became virtually undetectable, whereas the proportions of lanosterol and 24,25-dihydrolanosterol rose to 25 and 75%, respectively. Even after eight passages in the serum-free media, A3.01 cells displayed a complete absence of cholesterol with no obvious effect on cell growth. Membranes isolated from A3.01 cells grown in the presence or absence of 10 μg/ml of cholesterol displayed similar phospholipid:sterol ratios, but membranes from the unsupplemented cells contained only ~5% as much cholesterol as the supplemented cell membranes. Finally, A3.01 cells grown in the absence of cholesterol were extremely resistant to the cytotoxic effects of amphotericin B, whereas cells cultured in the combined presence of 1% FCS and 10 μg/ml of cholesterol were sensitive to the drug. Collectively, these results demonstrate that 4,4',14-trimethyl sterols can effectively replace cholesterol in a human T cell line, indicating that not all mammalian cells have a requirement for cholesterol, per se. The A3.01 T cell line should prove useful in defining the role of cholesterol in membrane fusion and human immunodeficiency virus-mediated syncitia formation and cytotoxic effects.

Sterols are a major component of nearly all eukaryotic cell plasma membranes. In mammalian cells, the predominant sterol is cholesterol, and numerous studies have demonstrated that cholesterol biosynthesis is essential for sustaining cellular proliferation and function (1–5). However, it is not yet clear to what extent cholesterol, per se, is required for mammalian cell growth, since other non-sterol isoprenoids derived from early stages of the cholesterol biosynthetic pathway also appear to be essential for mammalian cell proliferation (6). Previous investigators have attempted to define the sterol specificity of mammalian cells by testing various sterols as growth promoters for cell lines deficient in cholesterol biosynthesis (4–7). Such studies have resulted in two general concepts: 1) compared with cholesterol, plant and fungal sterols containing side chain alkylations and/or unsaturations support mammalian cell growth poorly or not at all and 2) lanosterol, an intermediate in the cholesterol biosynthetic pathway, is totally ineffective as a mammalian cell sterol supplement. The latter concept is based primarily upon the inability of mammalian cell mutants blocked in lanosterol demethylation to survive in the absence of exogenous cholesterol (8–11).

The relative ineffectiveness of lanosterol and other C-14 alkylated sterols in modulating various physical properties of model membranes has been well documented (12–15). However, lanosterol has nevertheless been shown to satisfy the "bulk" sterol requirements of Mycoplasma pneumoniae (16, 17) and Saccharomyces cerevisiae strain GL7 (18, 19). Such findings suggest that whereas protrusion of the C-4 and C-14 methyl groups from the sterol α-face may interfere with sterol-phospholipid interactions (20), the resultant effects on bilayer physical properties are not greater than can be compensated for by accompanying membrane lipid changes (17). Accordingly, it seems likely that in mammalian cells, too, lanosterol can satisfy at least a portion of the bulk sterol requirement. Indeed, in the Chinese hamster ovary 14a-methyl demethylase mutant AR45 isolated by Chen et al. (11), 40% of membrane cholesterol was replaced with lanosterol. Thus, the sterol requirement of mammalian cells may actually be less stringent than is generally believed.

We have recently been examining several human T cell lines for possible changes in lipid metabolism and/or composition following infection with the human immunodeficiency virus (HIV). During the course of such studies, it was unexpectedly found that A3.01, a previously described T lymphoid cell line (21), was defective in cholesterol biosynthesis. As is

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1The abbreviations used are: HIV, human immunodeficiency virus; GLC, gas-liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; PBS, phosphate-buffered saline; HAT, hypoxanthine/aminopterin/thymidine; FBS, fetal bovine serum.
described in this paper, A3.01 cells, as well as two T cell lines derived from it, are unable to convert lanosterol to cholesterol, and when grown in a serum-free medium, virtually all of their membrane cholesterol is replaced with lanosterol and 24,25-dihydrolanosterol. The ability of the cell lines to survive and proliferate under such conditions demonstrates that all mammalian cells have an absolute requirement for cholesterol, and at least in this particular T cell lineage, 4’,14-trimethyl sterols can satisfy the cellular sterol requirement.

MATERIALS AND METHODS

Cell Lines and Culture Media—The human T cell lines used are described in Table I. The CEM (22) and MOLT-4 (23) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Both the A3.01 and 8E5 cell lines have been described previously (21, 24). The 8E5L cell line was derived from the 8E5 cell line and contains the integrated HIV provirus but does not express any HIV proteins. The CEM.AGl.OU1 cell line (25) was obtained from Dr. P. Cresswell, Duke University. With the exception of the CEM.AGl.OU1 cell line, all of the T cell lines were maintained in RPMI 1640 supplemented with 1% iron-supplemented, fetal bovine serum (Clone. Logan City, UT). CEM.AGl.OU1 cell lines were grown in the same medium additionally supplemented with 2 mM glutamine and 10 mM HEPES buffer. Cells were cultured at 37°C in a humidified incubator containing 6% CO₂, 94% air and were routinely sub-cultured (1:5) every 4 days.

Several of the T cell lines were grown in a serum-free medium (RPMI/BHTS) consisting of RPMI 1640, 0.1% essentially fatty acid-free bovine serum albumin (Sigma), 10 mM HEPES (pH 7.2) and a premixed supplement containing insulin, transferrin, and selenium (11884, Sigma). In specific experiments, the serum-free medium was supplemented with 1% bovine serum albumin and either cholesterol or lanosterol (5 μg/ml). Sterols were added from 10 mg/ml of EtOH stock solutions.

Lipid Analyses—Cell membranes were collected by centrifugation and washed twice with PBS prior to being extracted with chloroform/methanol (26). Total cellular lipids recovered were further separated by thin-layer chromatography on silica gel 60 TLC plates (EM Science) developed with a solvent system containing diethyl ether/benzene/EtOH/acetic acid (40:50:2:0:2) (27). In this TLC system, cellular phospholipids remain at the origin, and cholesterol and lanosterol are well separated from each other as well as from other neutral lipids. Separated lipids were visualized either by spraying with a 0.1% solution of 8-anilino-1-naphthalenesulfonate followed by illumination with long wave UV light (28) or by spraying with 50% H₂SO₄, followed by heating at 110°C. With the latter detection method, cholesterol appeared as a brownish-yellow spot, whereas lanosterol reacted to give a pink spot. Sterols localized with 8-azaguanine (29) and 1-naphthalenesulfonate were subsequently eluted with diethyl ether for further analysis by gas-liquid chromatography (GLC) and GLC-mass spectrometry. GLC was carried out using a Varian 300 gas chromatograph (Houston, TX) equipped with a flame ionization detector interfaced to a Varian integrator. Sterol analyses were run isothermally at 280°C using a 6-ft column packed with 3% SP2250 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA). Separated sterols were tentatively identified based on their retention times as compared with known standards. Definitive identification of cholesterol, cholest-7-en-3β-ol, lanosterol, and 24,25-dihydrolanosterol was achieved by GC-mass spectrometry performed by the GC-mass spectrometry facility located at North Carolina State University. Fragmentation patterns of the identified sterols were identical with those reported in the literature (29).

In order to assay endogenous sterol synthesis, cells were labeled for varying periods of time with [1-¹⁴C]acetate (65 mCi/mmol, Du Pont-New England Nuclear). In general, 50-ml cultures were labeled with 10 μCi of [¹⁴C]acetate for 24 or 48 h to uniformly label cellular lipids. For some experiments, the cells were “pulsed” with [¹⁴C]acetate for 24 h, after which time the cells were washed, resuspended in growth media, and incubated for an additional 24 h to “chase” the label into final sterol products. Following extraction and TLC as described above, radiolabeled lipids were localized by autoradiography and visualized with 50% H₂SO₄. The [¹⁴C]-labeled lipids were quantified using a linear radioactivity scanner (Raytest).

Isolation and Analysis of Cell Membranes—A3.01 cells were harvested by centrifugation, washed twice with PBS and resuspended in a minimal volume of PBS. The cell suspension was sonicated for 10 s using a Biosonik III sonicator (Browall Scientific) fitted with a microtip probe. Unbroken cells and nuclei were pelleted by a 10-min centrifugation at 250 g. Cellular membranes were subsequently pelleted by a 3-h centrifugation at 105,000 × g. In one experiment, plasma membranes were isolated from 8E5L cells by the procedure of Maeda et al. (30). 8E5L cells were harvested, washed, and disrupted using a Polytron homogenizer (Brinkmann Instruments). The resultant homogenate was layered onto a cushion of 41% sucrose and centrifuged at 95,000 × g for 1 h. Membranes comprising the white interfacial band were collected, diluted with homogenization buffer, and pelleted by centrifugation at 105,000 × g for 30 min. Membrane pellets were resuspended in PBS-CHCl₃-CH₃OH (0.8:1:0.2:0.6 v/v), and 100 μg of cholesterol was added as an internal standard. Following the addition of 1 volume each of distilled H₂O and CHCl₃, cellular lipids were recovered in the lower (CHCl₃) layer. Aliquots were removed for quantitation of phospholipids (31) and membrane sterols (see above).

Chemicals—Amphoteracin B, cholesterol, cholestane, and lanosterol were purchased from Sigma. The lanosterol was recrystallized twice from EtOH prior to use, and based on GLC analysis, contained ~40% of 24,25-dihydrolanosterol.

RESULTS

Identification of Human T Cell Lines Defective in Cholesterol Biosynthesis—The human T cell lines described in Table I were initially selected to determine whether infection with HIV altered host cell lipid metabolism. Accordingly, the T cell lines were cultured in a low (1%) serum medium to maximize endogenous lipid biosynthesis and thus enhance the incorporation of [¹⁴C]acetate into cellular lipids. In the initial studies, the cells were incubated in the presence of [¹⁴C]acetate for 24 h (pulse), followed by washing and reincubation for an additional 24-h period (chase). Aliquots were removed from the cultures immediately following the pulse and chase periods, and total cellular lipids were separated by TLC. Autoradiography was used to localize endogenously synthesized sterols, whereas total cell-associated sterols were visualized by spraying the TLC plate with 50% H₂SO₄, followed by heating at 110°C. The results obtained are shown in Fig. 1. Of the five T cell lines examined, MOLT-4 appeared to be the most efficient in cholesterol synthesis. Following both the 24-h pulse and subsequent 24-h chase periods, [¹⁴C]cholesterol accounted for >98% of total radiolabeled sterols, a finding consistent with it being the only sterol detected with 50% H₂SO₄. Compared with MOLT-4, the CEM cell line appeared to be somewhat less efficient in synthesizing cholesterol. Following the pulse incubation, [¹⁴C]cholesterol was the major (62%) radiolabeled sterol detected, but significant amounts of sterol intermediates having TLC mobilities corresponding to

### Table I

<table>
<thead>
<tr>
<th>T cell line</th>
<th>Relevant characteristics</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>Acute T cell leukemia isolate</td>
<td>22</td>
</tr>
<tr>
<td>A3.01</td>
<td>HAT-sensitive derivative of CEM obtained by treatment with 8-azaguanine</td>
<td>21</td>
</tr>
<tr>
<td>8E5</td>
<td>× MOLT-4; contains a single, integrated copy of defective HIV provirus</td>
<td>24</td>
</tr>
<tr>
<td>8E5L</td>
<td>Derived derivative of 8E5; retains HIV layer but does not express HIV proteins</td>
<td>25</td>
</tr>
<tr>
<td>CEM.AGl.OU1</td>
<td>HAT-sensitive derivative of CEM obtained by treatment with 8-azaguanine</td>
<td>23</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>Acute T cell leukemia isolate</td>
<td>22</td>
</tr>
</tbody>
</table>

1. T. M. Folks, unpublished results.
were collected by centrifugation, washed, and resuspended in the same medium. One-half of each pulse-labeled culture was used for lipid analyses, whereas the remaining halves were incubated for an additional 24 h to chase the label. Total cellular lipids were extracted, separated by TLC, and visualized with $\text{H}_2\text{SO}_4$. Radiolabeled lipids were detected by autoradiography and quantitated with a linear radioactivity detector.

Fig. 1. Sterol biosynthesis in human T cell lines. The indicated human T cell lines (∼1 × 10⁷) were grown in 10 ml of 1% FBS/RPMI 1640 containing 1 μCi/ml of [1-14C]acetate. After 24 h, cells were collected by centrifugation, washed, and resuspended in the same medium. One-half of each pulse-labeled culture was used for lipid analyses, whereas the remaining halves were incubated for an additional 24 h to chase the label. Total cellular lipids were extracted, separated by TLC, and visualized with $\text{H}_2\text{SO}_4$. Radiolabeled lipids were detected by autoradiography and quantitated with a linear radioactivity detector.

An additional finding evident from Fig. 1 was that the remaining three T cell lines, A3.01, 8E5, and 8E5L, were defective in cholesterol biosynthesis. Visualization of cellular sterols with 50% $\text{H}_2\text{SO}_4$ demonstrated the presence of both $\text{C}_{27}$ and $\text{C}_{30}$ sterols in all three cell lines in roughly equal proportions. However, based on autoradiographic analyses, the subsequent chase period, levels of 14C-labeled sterol intermediates declined as they were converted to [14C]cholesterol, which increased to 81% of the total. The contrasting levels of sterol intermediates in the CEM and MOLT-4 cell lines suggest that one or more later stages in the cholesterol biosynthetic pathway occur at a reduced rate in the former cell line.

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The results of the TLC analyses were subsequently confirmed by GLC and GLC-mass spectrometry analyses. As shown in Fig. 2 and Table II, the nonsaponifiable lipids recovered from MOLT-4 cells grown in the presence of 1% FBS contained only cholesterol, whereas similarly grown CEM cells additionally contained small amounts of sterols yielding GLC retention times and GLC-mass spectrometry fragmentation patterns corresponding to cholest-7-en-3β-ol (10 min), 24,25-dihydrolanosterol (12 min) and lanosterol (14 min). Of particular interest was the finding that A3.01 cells, and the two cell lines derived from it, are defective in lanosterol demethylation. Further, the cholesterol that was detected in these cells was primarily derived from the 1% FBS present in the growth medium.

Collectively, the findings shown in Figs. 1 and 2 demonstrate that treatment of CEM cells with 8-azaguanine to derive the hypoxanthine/aminopterin/thymidine (HAT)-sensitive A3.01 cell line (21) simultaneously led to an unexpected mutation in cholesterol biosynthesis. Furthermore, the secondary mutation persists in two cell lines derived from A3.01. In order to determine if 8-azaguanine routinely induces mutations in cholesterol biosynthesis, sterol synthesis was assayed in CEM.AGl.OU1 cells, a HAT-sensitive cell line which, like A3.01, was derived following exposure of the CEM cell line to 8-azaguanine (25). Following a 48-h labeling with [14C]acetate, CEM.AGl.OU1 cells were found to display a sterol biosynthetic pattern that was identical with that observed for CEM cells (data not shown). Thus CEM.AGl.OU1 cells were found not to be defective in lanosterol demethylation, indicating that the mutation that arose in A3.01 cells was serendipitous and not a routine effect of 8-azaguanine exposure.

Fig. 2. Sterol profiles of human T cell lines. Human T cell lines were cultured in either 1% FBS/RPMI 1640 or RPMI/BHIT (0% FBS) to a final density of 2 × 10⁶ cells/ml. Following extraction of total cellular lipids, nonsaponifiable lipids were analyzed by GLC. DHL, dihydrolanosterol.
Sterol Structure and Function in Human T Cells

TABLE II

Sterol compositions of human T cell lines

Human T cell lines were cultured in RPMI 1640 containing either 1-10% FBS or RPMI/BHTS containing 0-10 μg/ml of cholesterol. After 5 days, lipids were extracted from harvested cultures, and nonsaponifiable lipids were analyzed by GLC.

<table>
<thead>
<tr>
<th>T cell line</th>
<th>Medium supplement</th>
<th>% total cell sterols</th>
<th>14C Lanthosterol</th>
<th>14C Cholesterol</th>
<th>14C 24,25-Dihydrolanosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3.01</td>
<td>10 µg/ml</td>
<td>99</td>
<td>0</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>88</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 µg/ml</td>
<td>48</td>
<td>7</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>8E5</td>
<td>10 µg/ml</td>
<td>97</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>54</td>
<td>4</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 µg/ml</td>
<td>53</td>
<td>4</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>8E5L</td>
<td>10 µg/ml</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MOLT-4</td>
<td>1 µg/ml</td>
<td>94</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>CEM</td>
<td>1 µg/ml</td>
<td>94</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A3.01</td>
<td>10 µg/ml</td>
<td>55</td>
<td>9</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>14</td>
<td>32</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 µg/ml</td>
<td>9</td>
<td>35</td>
<td>56</td>
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<tr>
<td></td>
<td>0.5 µg/ml</td>
<td>2</td>
<td>37</td>
<td>61</td>
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<tr>
<td></td>
<td>0 µg/ml</td>
<td>Trace</td>
<td>22</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>CEM</td>
<td>10 µg/ml</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 µg/ml</td>
<td>87</td>
<td>Trace</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* FBS (%).
* Cholesterol (µg/ml).
* Cells contained 12% cholest-7-en-3β-ol.

Fig. 3. Effect of cholesterol on growth of A3.01 cells in serum-free media. A3.01 cells grown in RPMI/BHTS were diluted to a density of 2 × 10^6 cells/ml and either supplemented with 10 µg/ml of cholesterol or left unsupplemented. At the indicated times, aliquots from both culture conditions were stained with trypan blue, and viable cells were enumerated using a hemocytometer.

shown in Fig. 3, similar growth curves were obtained for A3.01 cells grown in the absence and presence of exogenous cholesterol (10 µg/ml). In three separate experiments, cell doubling times in the presence and absence of cholesterol were 35 (± 4) h and 39 (± 1) h, respectively.

To determine if their respective patterns of sterol synthesis persisted during growth in the serum-free medium, the CEM and A3.01 cell lines were grown in the presence (+) or absence (-) of cholesterol (10 µg/ml). When cultures reached a density of 1 × 10^6 cells/ml, cells were pulsed with [1-14C]acetate (1 µCi/ml) for 24 h. Total cellular lipids were extracted, separated by TLC, and detected by H2SO4 and autoradiography.

Fig. 4. Sterol biosynthesis in human T cell lines cultured in serum-free media. CEM and A3.01 cells were grown in RPMI/BHTS in the presence (+) or absence (-) of cholesterol (10 µg/ml). When cultures reached a density of 1 × 10^6 cells/ml, cells were pulsed with [1-14C]acetate (1 µCi/ml) for 24 h. Total cellular lipids were extracted, separated by TLC, and detected by H2SO4 and autoradiography.

(2%), and lanosterol (3%). Supplementing CEM cells with 10 µg/ml of cholesterol resulted in decreased total endogenous sterol synthesis and a corresponding loss of the three precursor sterols from the mass analyses (Fig. 4 and Table II). By comparison, A3.01 cells grown in the absence of cholesterol contained predominantly 24,25-dihydrolanosterol (77%) and lanosterol (22%), with only a trace amount (1%) of cholesterol. Supplementation of A3.01 cells with varying levels of cholesterol resulted in a dose-dependent incorporation of cholesterol and a corresponding decrease in both lanosterol and 24,25-dihydrolanosterol. However, even at the highest dose of cholesterol added (10 µg/ml), the 4,4',14-trimethyl sterols accounted for nearly one-half of total cellular sterols. The inability of A3.01 cells to completely replace the precursor sterols with cholesterol may reflect sterol insolubility in the serum-free medium, since higher levels of cholesterol enrichment could be obtained by supplementing A3.01 cells with 5-10% FBS (Table II).

We next determined whether the anomalous sterol composition of A3.01 cells persisted even after continued passage in serum-free media. A3.01 cells cultured in 1% FBS (passage number 0) were diluted (1:5) into serum-free medium and subsequently subcultured (1:5) eight times. At each passage, cells were analyzed for their total sterol composition by GLC. As shown in Fig. 5, following dilution into serum-free media, the cholesterol content of A3.01 cells declined such that by passage number 3 cholesterol was virtually undetectable in the cells. The decline in cholesterol was once again offset by increased levels of lanosterol and 24,25-dihydrolanosterol.
During the entire time period (~40 days) shown in Fig. 5, we did not detect a change in the growth rate of the A3.01 cells. Thus, in marked contrast to the animal cell sterol mutants described previously (8-11), in A3.01 cells, the inability to demethylate lanosterol results in the near total replacement of cellular cholesterol with 24,25-dihydrolanosterol and lanosterol, with no obvious effects on growth. It should be noted that A3.01 cells have been maintained in the serum-free medium for >1 year, during which time the proportion of cholesterol increased to 15% by 12 months. This gradual increase in cholesterol content may be reflective of the emergence of revertants that eventually overgrow the cholesterol-deficient mutants (see "Discussion").

Membrane Sterol Composition—Given the relative scarcity of 4,4',14-trimethyl sterols in mammalian cell membranes (20) and considering their reported inability to properly modulate membrane physical properties (12-15), it was deemed necessary to determine if the trimethyl precursor sterols that accumulated were, in fact, present in cell membranes and not merely associated with intracellular lipid droplets. Therefore, two approaches were taken. First, total cellular membranes were isolated from A3.01 cells grown in the serum-free medium for >1 year, during which time the proportion of cholesterol increased to 15% by 7 months and to 85% by 12 months. This gradual increase in cholesterol content may be reflective of the emergence of revertants that eventually overgrow the cholesterol-deficient mutants (see "Discussion").

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Table III
Membrane lipid composition of T cells grown in various media

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>nmoles</th>
<th>PPL sterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No sterol</td>
<td>12</td>
<td>270</td>
</tr>
<tr>
<td>10 µg/ml cholesterol</td>
<td>329</td>
<td>288</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No sterol</td>
<td>10</td>
<td>204</td>
</tr>
<tr>
<td>10 µg/ml cholesterol</td>
<td>167</td>
<td>174</td>
</tr>
<tr>
<td>Experiment III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No sterol</td>
<td>18</td>
<td>247</td>
</tr>
<tr>
<td>0.25% FBS</td>
<td>140</td>
<td>275</td>
</tr>
<tr>
<td>10% FBS</td>
<td>355</td>
<td>33</td>
</tr>
</tbody>
</table>

In experiments I and II, A3.01 cells were cultured in RPMI/BHITS containing 0 or 10 µg/ml of cholesterol until cultures reached a density of 2 x 10⁶ cells/ml. Harvested cells were washed and resuspended, and unbroken cells and nuclei were removed by slow speed centrifugation. Membranes were pelleted by centrifugation at 105,000 x g. In Experiment III, 8E5L cells were cultured in RPMI/BHITS containing 0, 0.25, or 10% FBS to a final density of 2 x 10⁶ cells/ml. Harvested cells were washed and resuspended with a Polytron homogenizer. The homogenate was overlaid onto 41% sucrose followed by centrifugation at 95,000 x g. The white interfacial band was collected and diluted with buffer, and membranes were pelleted by centrifugation at 105,000 x g. Membrane lipids were extracted and subsequently analyzed for sterols and total phospholipids. DHL, dihydrolanosterol; PPL, phospholipid.
A3.01 cells were considerably more resistant to the drug. The greater susceptibility of the former cell lines could not simply be attributed to their cholesterol contents, because under conditions wherein A3.01 contained similar or even higher amounts of cholesterol, the latter continued to be much more resistant to amphotericin B. Similar differences in polyene sensitivity were observed after a 3-h exposure (data not shown). In an attempt to identify the cellular parameter(s) that could best be correlated with sensitivity to the drug, the cell viability data obtained for the three cell lines were collectively analyzed as a function of: 1) the mass of cholesterol/10^7 cells (r = -0.24), 2) the mass of total sterol/10^7 cells (r = 0.02), and 3) percent of total cellular sterol as cholesterol (r = -0.86). As indicated by the correlation coefficients, susceptibility to amphotericin B could be correlated with the proportion of cellular sterol as cholesterol but not with the absolute amounts of either cholesterol or total cellular sterols. Two additional points should be mentioned. First, the results obtained with 8E5L cells grown in the absence or presence of varying amounts of FBS were virtually identical with those shown in Table IV (data not shown). Second, the cholesterol-proficient A3.01 cells that grew out after prolonged (~12 months) culture in serum-free media were completely killed by 3 μg/ml of amphotericin B (data not shown). Thus, the marked resistance of cholesterol-deprived A3.01 and 8E5L cells to amphotericin B is most likely due to the inability of 4,4',14-trimethyl sterols to form intramembranous, water-permeable complexes with the polyene antibiotic.

**DISCUSSION**

The results described in this study demonstrate that the A3.01 human T cell line, which has previously been used in studies pertaining to the physiology of HIV infection (21, 24, 34), is totally defective in cholesterol biosynthesis. As a result, A3.01 cells synthesize, and accumulate within their membranes, large amounts of lanosterol and 24,25-dihydrolanosterol. The ability of A3.01 to continue growing despite a virtual absence of membrane cholesterol distinguishes these cells from all other previously described mammalian cell sterol mutants. The unique sterol composition of A3.01 cells was also observed in two additional cell lines derived from it: 8E5, which expresses most of the HIV proteins as a result of an integrated, replication-deficient, HIV provirus (24); and 8E5L, a derivative of 8E5 that retains the integrated HIV provirus but does not express any virus proteins. Thus the sterol defect in the A3.01 lineage constitutes a mutation that (in the presence of serum) is stable and has persisted through many generations of subculture and the selection of two subclones.

Although the precise biochemical defect in the A3.01 lineage remains to be determined, their sterol content is consistent with a block in lanosterol demethylaation, most likely at the level of the 14α-methyl demethylase (11). A3.01 cells were derived from the CEM human T cell line following exposure of the latter to 8-azaguanine, followed by a subsequent selection for cells susceptible to the cytopathic effects (CPE) of HIV (21). However, neither treatment consistently selects for cholesterol biosynthetic mutants, as shown by the lack of a similar sterol defect in CEM.AGL.OU1 cells, another HAT-sensitive, HIV-susceptible cell line derived from CEM (25).

Thus, the simultaneous introduction of mutations resulting in HAT sensitivity, HIV susceptibility, and impaired cholesterol synthesis appears to have been a serendipitous event stemming from the mutagenic effects of 8-azaguanine.

The finding that cholesterol, per se, was not required for growth of A3.01 cells was unexpected, based on previous studies with other lanosterol demethylaation mutants (8, 9, 11). There are several possible explanations for this apparent discrepancy. First, in the other lanosterol demethylaation mutants, the levels of 4,4',14-trimethyl precursor sterols synthesized may not have been sufficient to satisfy the cells' sterol requirements. For example, even though lanosterol accounted for >65% of the sterols synthesized in the Chinese hamster ovary sterol mutants isolated by Chang and co-workers (8, 9), following cholesterol deprivation, there was little to no mass accumulation of lanosterol. Thus, what has been interpreted as being a specific requirement for cholesterol may simply reflect an insufficient level of total sterol synthesis. Support for this possibility is provided by the studies of Chen et al. (11) wherein AR45 Chinese hamster ovary cells deprived of exogenous cholesterol accumulated lanosterol and dihydrolanosterol and, according to the authors, survived for an unspecified period of time in a chemically defined medium. Apparently, in both A3.01 and AR45 cells, 4,4',14-trimethyl sterols can substitute for at least some, if not all, of the cellular cholesterol.

A second possibility is that as a result of their exposure to 8-azaguanine, A3.01 cells acquired an unidentified mutation that minimizes a specific need for cholesterol. In *M. pneumoniae*, *S. cerevisiae*, and mouse LM cells, sterols are required...
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as a bulk membrane component to regulate bilayer physical properties and as an essential growth factor with possible involvement in gene expression (7, 16–19, 35–38). The former function can be satisfied by a wide variety of sterols, in some cases with compensatory membrane fatty acid changes (7, 17, 39). By contrast, the latter requirement is considerably more specific and can only be satisfied by the particular sterol that normally predominates in each organism. Based on the studies of Rujanavech and Silbert with LM cells (7), it seems likely that most mammalian cells require small amounts of cholesterol for the second, i.e. nonbulk membrane, function. Thus, the outgrowth of A3.01 cells capable of synthesizing cholesterol following prolonged (7–12 months) incubation in serum-free media may indicate that a small amount of cholesterol is essential for survival. Alternatively, the ability of A3.01 revertants to convert lanosterol to cholesterol may provide them with a slight growth advantage that, although not detectable in short term growth studies (Fig. 3), nevertheless enables them to overgrow the cholesterol-deficient mutants over an extended period of time. Such would be consistent with Bloch’s proposal (20) that cholesterol’s greater competence for modulating membrane properties provided the selective basis for the evolution of organisms that synthesize cholesterol rather than lanosterol.

A third possibility is that the sterol requirement of lymphoid cells is less stringent than has been observed in other cell types. Numerous studies have shown that de novo sterol synthesis is a prerequisite to DNA synthesis in mitogen-stimulated lymphocytes (1, 5), but the specificity of the sterol requirement was not determined. In a previous report, it was shown that the antifungal agent, ketoconazole, inhibited both cholesterol and DNA synthesis in mitogen-stimulated human and mouse lymphocytes (40). However, the inhibition of DNA synthesis was abrogated by the addition of cholesterol-depleted human serum, even though cholesterol synthesis was not restored and the cells continued to synthesize 4,4’,14-trimethyl precursor sterols (40). It is therefore possible that in order to proliferate, lymphoid cells do not require cholesterol synthesis, per se. Rather, they may require an overall sterol biosynthetic pathway to ensure an adequate supply of other, non-sterol, isoprenoid molecules involved in cell growth (6). Consistent with this possibility are our findings that neither exogenous cholesterol nor lanosterol were able to restore the growth of A3.01 cells when endogenous sterol synthesis was inhibited by 25-hydroxycholesterol.3

Compared with similarly grown CEM and MOLT-4 cells, A3.01 cells cultured in serum-free media were shown to be extremely resistant to the cytotoxic effects of amphotericin B. This was not unexpected, since the lanosterol demethylation mutants described by Chen et al. (11) were isolated on the basis of being amphotericin B-resistant. Polyene antibiotics such as amphotericin B and filipin are thought to induce cell lysis by forming stoichiometric complexes with cholesterol in the plasma membrane, resulting in the formation of ion-permeable pores (31). In order to form such a complex with polyene antibiotics, sterols must possess a 3β-hydroxyl moiety and a planar ring system (32). While alkylations to the sterol side chain do not prevent complex formation with the polyenes (32), it is not known whether methyl groups at C-4 or C-14 interfere. Based on the fact that amphotericin B resistance in mammalian cells (11) and in yeast (41) has been associated with impaired lanosterol demethylation, it would appear that 4,4’,14-trimethyl sterols do not similarly associate with polyene antibiotics. Alternatively, sterols such as lanosterol and dihydrolanosterol may interact with the polyenes, but in a fashion that is distinct from cholesterol and which does not result in the formation of transmembrane channels. In the present study, the amphotericin B sensitivity of A3.01 cells could best be correlated with the proportion of cholesterol in their membranes, rather than with their absolute cholesterol content. This finding suggests that lanosterol and dihydrolanosterol compete with cholesterol for binding to the antibiotic, but that only the latter forms pores leading to cell lysis.

In summary, the present study demonstrates that three human T cell lines derived following exposure of the CEM cell line to 8-azaguanine possess an unexpected mutation in cholesterol synthesis. The mutation, which most likely inhibits lanosterol demethylation, results in the complete replacement of membrane cholesterol with lanosterol and dihydrolanosterol. The unique sterol composition of A3.01, coupled with its additional property of being HAT-sensitive, should make this cell line a useful model system for further characterizing the role of membrane sterols in cellular growth and cell-to-cell fusion. In addition, the A3.01 and 8E5 cell lines provide a means for determining whether cholesterol is involved in HIV infection and assembly, HIV-induced syncitia formation, and HIV-mediated CPE and resultant T cell death.

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REFERENCES


T. M. Buttke, unpublished observations.
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