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ADAPTATION OF A CHOLESTEROL DEFICIENT HUMAN T CELL LINE TO GROWTH WITH LANOSTEROL

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SUMMARY: A3.01 is a human T cell line previously shown to be defective in cholesterol biosynthesis. Following passage into serum-free medium, A3.01 cells displayed a gradual decline in growth rate which correlated with a depletion of cellular cholesterol content and an accumulation of lanosterol and 24,25-dihydrolanosterol. At the point when cholesterol became undetectable, the growth rate of serum-deprived cells was only one-tenth of the rate observed for serum-supplemented cells. The addition of low density lipoproteins (LDL) restored cellular cholesterol content and resulted in a 7-fold higher growth rate, confirming that cholesterol-deprivation was responsible for the slower growth in the absence of serum. Following prolonged culture in serum-free medium, A3.01 cells underwent a phenotypic change such that the cells achieved a growth rate which was ~65% of either LDL-supplemented or cholesterol-proficient cells. This apparent adaptation was not attributable to changes in either fatty acid or sterol composition. These results demonstrate that while cholesterol is preferred, this lymphoid cell line can adapt to the use of lanosterol to satisfy its membrane sterol requirement.

Cholesterol is a major component of mammalian cell plasma membranes, wherein it functions to regulate both bilayer fluidity and solute permeability (1). As a general rule, cholesterol is essential for mammalian cell viability and growth, and cells defective in cholesterol biosynthesis die within 1-2 generations following removal of an exogenous supply (2-5). Cholesterol is the end-product of a complex biosynthetic pathway in which the first sterol intermediate, lanosterol, is successively demethylated at C14 and C4, followed by various double-bond rearrangements to ultimately yield the cholest-5-en-3ß-ol molecule (6). Based primarily upon molecular models of cholesterol and lanosterol, Bloch proposed that the presence of methyl substituents at C14, and to a lesser extent C4, would diminish van der Waals interactions between the sterol molecule and adjacent phospholipid acyl chains (7). Accordingly,

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the cholesterol biosynthetic pathway should result in the transformation of a marginally competent sterol, lanosterol, to cholesterol, a more finely-tailored molecule capable of interacting optimally with membrane fatty acids (7,8). Subsequent studies with model membranes and the sterol-requiring prokaryote, *Mycoplasma capricolum* provided considerable support for the hypothesis. In both systems, lanosterol was considerably less effective than cholesterol (8-13). Efforts to perform similar studies with mammalian cells have been hampered by the greater stringency of such cells for the structural features of cholesterol. While some latitude in the numbers and positions of double bonds in the sterol molecule is permissible, alkyl substituents either on the steroid nucleus or in the isooctyl side chain are generally not tolerated (3,14). The growth characteristics of mammalian cells containing equivalent levels of lanosterol versus cholesterol have, therefore, not been compared.

A3.01 is a human T cell line recently shown to be defective in a late stage of cholesterol biosynthesis (15). Whereas A3.01 cells cultured in serum-supplemented medium contain primarily cholesterol in their membranes, following culture in serum-free medium the cholesterol is completely replaced by lanosterol and 24,25-dihydrolanosterol (15). In the previous study, no significant difference in growth was observed when the serum-free medium was supplemented with cholesterol (15), but an in-depth study of the relationship between sterol content and cell growth was not performed. In the present studies a more sensitive assay of cell growth was used to compare the growth of A3.01 cells enriched in either cholesterol or 4,4',14-trimethyl sterols. The results obtained identify cholesterol as the optimal sterol for A3.01 growth, thus providing further support for the notion that metabolic removal of the C4 and C14 methyl groups of lanosterol results in a sterol structure optimally effective for membrane function.

MATERIALS AND METHODS

Cell culture A3.01, a hypoxanthine/aminopterin/thymidine (HAT)-sensitive (16), cholesterol-deficient (15), derivative of the human CCRF-CEM $CD4^+$ T cell line (17), was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. A3.01.C7 is a cholesterol-synthesizing spontaneous variant of A3.01 isolated as part of the present study. A3.01.C7 accumulates only 4,14-desmethyl sterols and, like the CCRF-CEM cell line (15), accumulates cholest-7-en-3ß-ol in addition to cholesterol. The A3.01.C7 cell line resembles A3.01 with regard to CD4 expression and sensitivity to HAT. The T cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (10% FBS/RPMI). Experiments involving serum-deprivation were done by diluting the cells into a serum-free medium consisting of RPMI 1640 supplemented with bovine serum albumin, insulin, transferrin and selenium (15). Cells were cultured at 37°C in a humidified incubator containing 6% CO_2/94% air. Cultures were routinely passed 1:5 as needed to maintain cell densities between 0.4-2 X 10^6 cells per ml.

Growth assay Cell growth was measured using the tetrazolium compound, MTS (18). Immediately following dilution of cultures with fresh serum-free medium, 12 aliquots (0.2 ml) from each culture condition were transferred to a 96-well, flat-bottomed, tissue culture plate. At that time, and at daily intervals thereafter, 10 μl of a solution containing 6 Mm MTS

(Promega) and 0.33 Mm PMS (Aldrich) were added to triplicate sets of culture wells, and 5 hours later the amount of reduced formazan produced was assayed at 570 nm (690 nm reference filter) using an Anthos model 2001 ELISA plate reader.

Lipid analyses Total cellular sterols and phospholipid fatty acid compositions were assayed by gas-liquid chromatography (GLC) as described previously (15,19). Endogenous sterol synthesis was measured by labeling cells with [1-^{14}C]acetate (55 mCi/mmol; Du Pont-New England Nuclear), followed by thin-layer chromatography of radiolabeled lipids (15).

Chemicals Human LDL and HAT were purchased from Sigma. The sources of all medium components and other biochemicals have been described (15,20).

RESULTS

A3.01 cells maintained in the presence of 10% FBS were diluted 1:5 into serum-free medium, followed by subsequent passage whenever the culture achieved a density of ~1X10^6 cells/ml. At each passage, and at daily intervals thereafter, cell growth was measured based on the cells' ability to reduce MTS (18) in order to generate "growth curves" (Fig. 1A). The slopes of the growth curves (i.e., growth rates) were plotted as a function of the number of passages in serum-free media (Fig. 1B) to better visualize the relationship between serum-deprivation and A3.01 growth. Cells recovered from each passage were also analyzed for their total sterol composition. After inoculation into serum-free medium, A3.01 displayed a gradual growth retardation up to passage 3, after which time the growth rate remained constant for two additional passages at a level that was ~10% of serum-supplemented cells. Between passages 1-4, the decline in growth rate coincided with a decreased proportion of cholesterol, suggesting a direct relationship between growth rate and membrane sterol content.

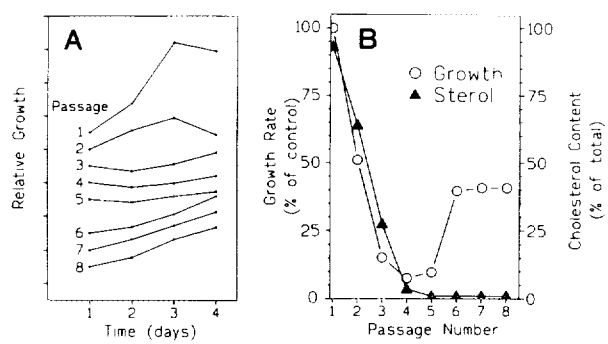

Figure 1. Correlation between growth rate and cholesterol composition of A3.01 cells. **A.** A3.01 cells cultured in 10% FBS/RPMI were diluted 1:5 into serum-free medium (passage 1), and at daily intervals thereafter, triplicate aliquots were assayed for cell growth using MTS. At each passage, an aliquot was diluted 1:5 into fresh serum-free medium, followed by repeated measurements of cell growth. **B.** For each of the growth curves, a growth rate was derived based on linear regression slope determinations for the linear portion of each curve. The resultant growth rates are expressed as the percent of the growth rate of control (serum-supplemented) cells. At each passage, an aliquot was removed for GLC analyses of cellular sterol content.

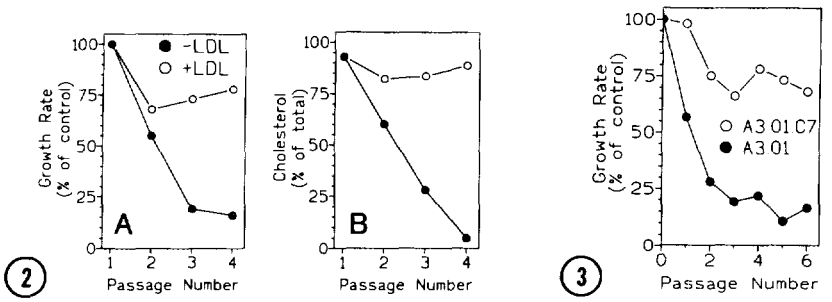

Figure 2. Effect of LDL supplementation on A3.01 growth and cholesterol composition. A3.01 cells cultured in 10% FBS/RPMI were repeatedly passaged 1:5 into serum-free medium either lacking or containing human LDL (10 μg/ml, based on protein). **A.** Growth rates based on linear regression slope determinations for the linear portion of each growth curve. **B.** Cholesterol content of cells grown in the absence or presence of LDL.

Figure 3. Comparative growth responses of A3.01 and A3.01.C7 in serum-free medium. A3.01 and A3.01.C7 cells cultured in 10% FBS/RPMI were diluted 1:5 into serum-free medium (passage 1), followed by repeated passage in serum-free medium. At daily intervals following each passage, growth was assayed using MTS.

Two approaches were taken to account for possible effects of serum starvation on growth that were unrelated to cholesterol deprivation. First, the serum-free medium was additionally supplemented with human LDL as a source of exogenous cholesterol. In this case, A3.01 cells inoculated into the cholesterol-supplemented medium displayed an initial growth retardation, but between the second and fourth passages, A3.01 cells grown in the LDL-supplemented medium maintained a growth rate which was only 25% less than that displayed by serum-supplemented cells (Fig. 2A). The cholesterol content of LDL-supplemented cells plateaued at a similar level (~85%), further demonstrating a relationship between growth rate and cholesterol content (Fig. 2B). The second approach was to compare the growth rates of serum-deprived A3.01 cells with that of A3.01.C7, a cholesterol-synthesizing revertant. As shown in Fig. 3, A3.01.C7 displayed an initial decrease in growth, but then maintained a growth rate which was ~70% of serum-supplemented cells, and thus identical to the growth rate of A3.01 cells in LDL-supplemented, serum-free medium. Thus, provision of cholesterol, whether by exogenous supplementation or endogenous synthesis, resulted in a significant increase in growth rate as compared to cells containing only lanosterol and 24,25-dihydrolanosterol.

As shown in Fig. 1, by the sixth passage, A3.01 growth in the serum-free medium improved significantly, achieving a rate that was ~40% of serum-supplemented cells, and ~65% of the rates observed for A3.01.07 or LDL-supplemented A3.01 in serum-free media. However, despite this marked improvement in growth, cellular cholesterol remained virtually undetectable up to passage 8, suggestive of an adaptation mechanism other than a regained ability to synthesize 4,14-desmethyl sterols. One possibility was that "adapted" cells had altered

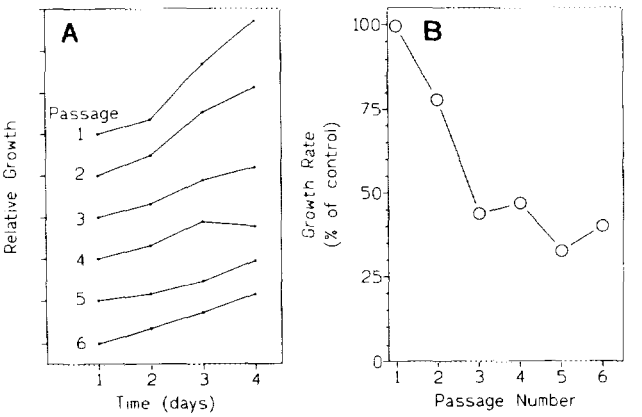

Figure 4. Persistence of A3.01 adaptation to cholesterol-free growth. A3.01 cells were cultured in serum-free medium for 8 passages, followed by 4 passages in 10% FBS/RPMI. After the 4th passage, the cells were re-inoculated into serum-free medium, aliquots were removed for MTS measurements (**A**) and growth rates were determined (**B**).

their membrane fatty acids to compensate for the differing effects of the precursor sterols on membrane properties. Therefore, cells recovered from each passage were assayed for changes in phospholipid fatty acid content. While the expected replacement of serum-derived polyenoic fatty acids (e.g., linoleic and arachidonic acids) with endogenously-synthesized palmitoleic and oleic acids was observed between passages 1-3, no significant fatty acid changes occurred between passages 4-6, during which time the growth rate increased 4-fold (data not shown). Further studies were done to determine whether the adaptation of A3.01 cells to cholesterol-free growth was a stable phenotype. A3.01 cells grown for 8 passages in serum-free medium were re-introduced into medium containing 10% FBS for 4 passages, followed by re-inoculation into serum-free medium. As shown in Fig. 4, re-inoculation into serum-free medium resulted in slightly retarded growth, but in contrast to the situation observed with naive cells (Fig. 1), the growth rate of the adapted cells did not fall below the level observed prior to resupplementing with serum. Thus, the adaptation of A3.01 to cholesterol-free growth persisted even in the absence of selective pressure.

DISCUSSION

The results obtained in this study demonstrate a marked difference in the growth-promoting competencies of cholesterol and lanosterol for the A3.01 human T cell line, and thus agree with the disparate effects of these two sterols in prokaryotic (11,13) and model membranes (8-12). Of the two sterols, cholesterol is clearly the optimum. Following transfer from serum-supplemented to serum-free media, A3.01 cells displayed a passage-dependent decline in growth

rate which coincided with a depletion of cellular cholesterol. At the point when cholesterol became undetectable, the growth rate was only one-tenth of that observed in the presence of serum. Supplementing the serum-free medium with human LDL provided a source of exogenous cholesterol, and by so doing, we could enhance A3.01 growth in the medium roughly 7-fold. An identical level of growth enhancement was observed when the cholesterol-proficient, A3.01.C7 clone was cultured in serum-free medium. The similarity in growth rates for A3.01.C7 and LDL-supplemented A3.01 cells in serum-free medium, strongly suggests that replacement of the 4,4',14-trimethy sterols with cholesterol is directly responsible for the improved growth. Based on the correlation between cell growth and the proportion of cholesterol in cell membranes, the growth enhancing property of cholesterol appears to be related to the lipid's role as a modulator of bulk membrane properties, rather than as essential "growth factor" (8). Thus, the incompetency of lanosterol in preserving bilayer fluidity and permeability of model and prokaryotic membranes carries over to mammalian cell membranes as well.

The ability of A3.01 cells to grow, albeit more slowly, with lanosterol and 24,25-dihydrolanosterol as their only membrane sterols, enabled the detection of an apparently novel adaptation to cholesterol-free growth. The stability of the adaptation suggests a genetic change that was not merely due to a recovery of cholesterol synthesis. Given lanosterol's impotency in regulating membrane fluidity and permeability, it seemed reasonable to expect adaptation to involve compensatory changes in membrane fatty acid composition. However, no significant fatty acid changes were observed. This finding contrasts with results obtained with cholesterol-depleted LM cells (14), but agrees with studies involving *M. capricolum* (13) and cultured insect cells (21). Based on our previous studies (15), a change in the sterol to phospholipid ratio can also be discounted.

Prolonged culture of A3.01 in serum-free medium infrequently led to the emergence of revertants proficient in cholesterol biosynthesis. Such revertants have never been detected in A3.01 populations maintained continuously in serum-supplemented media, indicating that the mutational defect preventing A3.01 cells from demethylating lanosterol is not especially prone to spontaneous reversion. Rather, it seems likely that selective pressure stemming from the limited capability of unadapted A3.01 cells to grow in a cholesterol-free environment leads to the appearance of cholesterol-proficient variants which have a significant growth advantage over cells containing only 4,4',14-trimethyl sterols. These findings emphasize the relative superiority of cholesterol in modulating the physical properties of mammalian cell membranes. Furthermore, they are consistent with the notion that the biosynthetic transformation of lanosterol to cholesterol is driven by selective pressure for a sterol optimally capable of interacting with other membrane components.

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