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Oxysterol Sensitive and Resistant Lymphoid Cells: Correlation with Regulation of Cellular Nucleic Acid Binding Protein mRNA

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Oxygenated derivatives of cholesterol inhibit cholesterol synthesis, prevent lymphoid cell growth, and evoke cell death. We have employed a novel selection method to isolate M10 cells, a line of oxysterol-resistant cells, from the sensitive clone CEM C7. Concentrations of the potent sterol 25-hydroxycholesterol that occupy the oxysterol binding protein cause cell death in CEM C7, but not in M10 cells. Both cell lines have similar amounts of the oxysterol binding protein with similar affinities for oxysterol. However, in neither line are the levels of oxysterol binding protein mRNA affected by 1 μM 25-hydroxycholesterol. Furthermore, both cells express the cellular nucleic acid binding protein (CNBP), a 7 zinc finger, DNA-binding protein of unknown function, regulated by oxysterols. The levels of CNBP mRNA are significantly reduced by 25-hydroxycholesterol in the sensitive CEM C7 cells, in which the dose response and time course are consistent with occupancy of the oxysterol binding protein by oxysterol and with subsequent cell kill. However, in the resistant M10 cells, CNBP mRNA levels are unaffected by these concentrations of the 25-hydroxycholesterol. Our results suggest a role for CNBP in oxysterol-induced regulation of cell viability and growth.

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INTRODUCTION

During recent years there has been increasing interest in elucidating the role of oxygenated sterols as potent modulators of cellular functions, including regulation of genes involved in cholesterol metabolism and cell replication [1–4]. One of the more dramatic effects of oxysterols is inhibition of cell growth. The first demonstration of this in lymphoid cells used mitogen-stimulated mouse lymphocytes, in which it was shown that reduction in cholesterol synthesis was followed by proportionate reduction in DNA synthesis [5]. In the intervening years, considerable information has been gained about the regulation of the cholesterol pathway, but the mechanism by which oxysterols cause cell lysis remains obscure. One popular theory has been that by blocking cholesterol synthesis, the sterols deplete replicating cells of cholesterol for membranes, with eventual loss of membrane integrity [6]. Recently, as an alternative proposal, it has been suggested that oxysterol-induced lymphoid cell death is an example of apoptosis, a form of programmed cell death [7, 8]. In this process, hypothetical genes are thought to be regulated so as to cause cell death in characteristic ways [9–11].

We have been employing clones of the human lymphoid cell line CEM to study oxysterol-evoked lymphocytolysis. These cells contain the oxysterol binding protein (OBP), are growth-inhibited and after a delay of about 24 h, killed by 25-hydroxycholesterol. There is a correlation between the lethal dose of sterol which kills 50% of the cells [12] and its affinity for the oxysterol-specific, intracellular oxysterol binding protein [2, 13]. In this report we describe the isolation from CEM C7 cells of the oxysterol-resistant M10 line. We then compare these cells for their expression of two genes, OBP and the recently discovered cellular nucleic acid binding protein (CNBP). CNBP was isolated

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Abbreviations: BSA, bovine serum albumin; CNBP, cellular nucleic acid binding protein; dpm, disintegrations per minute; FBS, fetal bovine serum; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; kb, kilobase; kDa, kilodalton; LDL, low density lipoprotein; OBP, oxysterol binding protein; PBS, phosphate buffered saline; SDS, sodium dodecyl/lauryl sulfate.

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from a screen for proteins capable of binding DNA sequences thought to be important for the regulation of genes in the cholesterol synthesis pathway [14, 15]. CNBP is a 19 kDa protein containing 7 zinc fingers and is structurally related to genes involved in yeast sporulation [16]. As suspected when originally described [14] and later confirmed (A. J. Lusis, personal communication), CNBP does not seem to be involved directly in the pathways for cholesterol homeostasis. We find that OBP mRNA is expressed constitutively and that OBP expression and oxysterol binding are the same in both the sensitive and resistant cells. However, CNBP mRNA levels are diminished in CEM C7 cells by concentrations of 25-hydroxycholesterol consistent with those that occupy OBP. The suppression of CNBP mRNA occurs at the onset of the loss of cell viability in the sensitive cell line, whereas in the resistant M10 cells grown in oxysterol CNBP levels remain unchanged.

EXPERIMENTAL

Materials

We obtained 25-hydroxycholesterol (5-cholestene-3β,25-diol), Dulbecco's phosphate buffered saline (PBS) and bovine serum albumin (BSA) from Sigma (St Louis, MO); [^{3}H]25-hydroxycholesterol from New England Nuclear (Boston, MA); RPMI 1640 culture medium from Fisher (Houston, TX). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Kansas City, MO). All the reagents used for RNA extraction were molecular biology grade. Formamide was purchased from Gibco/BRL Life Technologies (Grand Island, NY), and guanidine thiocyanate was from Fluka Chemical (Rankonkoma, NY). Formaldehyde was obtained from J. T. Baker Chemical (Phillipsburg, NJ), and electrophoresis grade agarose was from IBI (New Haven, CT). The blotting membranes were from Millipore (Bedford, MA). The DNA labeling kit was purchased from Amersham (Arlington Heights, IL) and the ^{32}P-labeled dCTP (deoxycytidine 5′-triphosphate) from ICN Radiochemicals (Cleveland, OH). Probes, rabbit OBP cDNA, and human CNBP cDNA were kindly provided by Drs P. A. Dawson (University of Texas Southwestern Medical Center, Dallas, TX) and A. J. Lusis (University of California, Los Angeles, CA), respectively.

Cell culture

CEM C7 cells were selected by cloning the CEM line established originally from a patient with acute lymphoblastic leukemia [17]; M10 cells were obtained by mutagenesis of CEM C7 cells and selection as described below. Cells were cultured in RPMI 1640 medium, pH 7.4, with 5–10% heat-inactivated whole or delipidated [18] FBS at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All experiments were performed with cells maintained in logarithmic phase growth.

Isolation of 25-hydroxycholesterol-resistant cells

CEM C7 cells were grown with the mutagen ICI 191 (1 μg/ml) in RPMI 1640 medium + 10% FBS for 16 h and subsequently allowed to recover by growth in fresh medium without mutagen for 7 days. These cells were subdivided and cultured in 10^{-7}, 10^{-6}, and 10^{-5} M 25-hydroxycholesterol for 7 days before culturing in fresh medium without oxysterol. This treatment had previously been shown to kill ~70% of the cells and provide optimal mutagenesis for studies on the glucocorticoid receptor [19]. For selection against 25-hydroxycholesterol an adaptation of the "penicillin disc" technique, commonly used in microbiology, was devised. Various numbers of CEM C7 cells were mixed uniformly in 0.54% agarose with growth medium and poured into 100 mm tissue culture dishes over fibroblast feeder layers (Armor cells from American Type Culture Collection, Rockville, MD). After the gel had formed, a glass fiber filter disc (Whatman) was saturated in an ethanolic solution of 1.24 mM 25-hydroxycholesterol, air dried to the point of dampness, and placed on the surface of the gel in the center of the dish. As the culture was incubated, a galaxy of cells grew uniformly in the gel except for a "cleared" ring surrounding the disc. Microscopic examination of this cleared ring showed a gradient of cells across it: no cells could be seen next to the disc, and gradually increasing numbers of, first, individual cells, then colonies appeared as one moved toward the perimeter of the "cleared" zone. The colonies (and cells within them) gradually increased in size with distance from the disc until they were indistinguishable from the general galaxy of colonies covering most of the plate. Preliminary studies showed that the ring diameter depended on the concentration of 25-hydroxycholesterol used to saturate the disc and on the number of cells used to seed the plate. M10 cells were isolated from CEM C7 cells seeded at 10^4 cells/dish by this procedure. A group of colonies growing near the perimeter of the cleared ring were transferred from the agarose by Pasteur pipette and cultured in RPMI 1640 + 5% whole FBS. This was supplemented with 100 to 500 nM 25-hydroxycholesterol for several weeks, after which the cells were tested and found to be resistant to at least 1.3 μM 25-hydroxycholesterol. This cell line was designated "M10".

Assays of cell cultures treated with 25-hydroxycholesterol

Cell growth. Cells which had been cultured in RPMI 1640 + 5% whole FBS were washed once with PBS and resuspended in RPMI 1640 + 5–10% delipidated serum for 24 h. Aliquots of 2.5 mg/ml 25-hydroxycholesterol in 5% BSA + medium were added so as to give different final concentrations of the oxysterol. Ethanol alone was added (<1%) in control cultures. Cells were routinely counted by Coulter Counter. For growth studies, a hemacytometer was used to determine the number of viable cells by trypan blue dye exclusion [20].

Oxysterol binding protein. To determine 25-hydroxycholesterol/OBP binding characteristics of CEM C7 and M10 cells, a modification of the methods of Kandustch and Thompson were followed [21, 22]. Cells in logarithmic growth were concentrated to 3×10^6 cells/ml in serum-free RPMI 1640 medium, tricine buffered to pH 7.4. Concentrations from 5×10^{-9} to 2×10^{-7} M of [^{3}H]25-hydroxycholesterol $\pm$ 100 $\times$ unlabeled oxysterol were evaporated to dryness by gaseous N_2 in microtubes before the addition of 1 ml of cells. After 40 min at 37°C, the cells were washed with cold PBS and resuspended in 500 μl buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA). A cytosolic fraction was prepared after a -70°C freeze/thaw by ultracentrifugation (90,000 *g*, 4°C, 45 min) and 350 μl layered on top of 5–20% sucrose gradients in 10 mM Tris–HCl, 1.5 mM EDTA, 0.3 M KCl. The remainder of the cytosol was used to determine the actual concentration of sterol and the protein concentration of the cytosol. Radiolabeled [^{14}C]BSA was used as a marker in the gradients which were centrifuged at 150,000 *g* for 17 h at 4°C in an SW 50.1 rotor in a Beckman L8-70M ultracentrifuge. Gradient aliquots were suspended in an aqueous scintillation cocktail and counted in a Beckman Scintillation Counter LS5801. Counts as disintegrations per minute (dpm) were normalized to 1 mg protein/ml cytosol and Scatchard analysis of the data was carried out by use of the computer software program Ligand.

Oxysterol binding protein and CNBP mRNA. Total RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform method [23]. The RNA samples were denatured by adding 37% formaldehyde in 10 $\times$ SSC (1 $\times$ SSC = 150 mM NaCl, 15 mM sodium citrate) and incubating at 60°C for 15 min. For dot blot assays, serial dilutions of these samples were applied to Immobilon-N membranes under vacuum using a BioRad dot blotting apparatus. For Northern blot analysis, 20 μg of total RNA was subjected to electrophoresis on a 1% agarose, 3% formaldehyde gel and electroblotted on Immobilon-N membranes. The filters were treated as recommended by the membrane's manufacturer (Millipore). Hybridization was carried out for 18 h at 42°C with a ^{32}P-labeled random-primed 0.5 kilobase (kb) EcoR 1 CNBP cDNA fragment or a 1.5 kb EcoR 1 rabbit OBP cDNA fragment with specific activities of 1×10^8 to 1×10^9 dpm per μg. Autoradiography was performed using intensifying screens for 1–2 days. In order to normalize the signals obtained from the dot blot assays, the filters were reprobed with β-actin cDNA after removal of radioactivity by boiling for 15 min in a 0.1% SDS, 5 mM EDTA solution. Northern blot normalization was accomplished by using the ethidium bromide stained ribosomal RNA that was transferred on the filter. The relative amounts of mRNA on the filters were determined by image analysis using an Image Analyser (Biological Visions Inc., San Mateo, CA). X-ray film has a limited linear response range. We therefore checked to be sure that we were looking within this range. At the exposure times we used for quantitation, we found that all dilutions of RNA employed gave signals that plotted linearly. The concentration of RNA used for the Northern blots were also within this response range.

RESULTS

Isolation of oxysterol-resistant human lymphoid cells

We have recently demonstrated that CEM C7 cells, as well as two glucocorticoid-resistant derivative clones, are killed by 25-hydroxycholesterol in concentrations that occupy OBP sites [12]. This fact provided a convenient way to select a resistant subline. The parental clone CEM C7 was treated with the mutagen ICI 191, allowed to recover, and the mutagenized cells that appeared to be resistant to cell kill by 25-hydroxycholesterol were selected by the modified "penicillin disc" technique as described in the Experimental section. The plate of cells growing in agarose shown in Fig. 1(A) demonstrates this method. The zone without cells, surrounding the filter disc from which 25-hydroxycholesterol was diffusing, can be seen clearly. Mutagenized cells could sometimes grow in the cleared zone. Without mutagenesis, resistant cells growing within the cleared ring near the source of oxysterol were rare; we have seen none in several such experiments. When purified cholesterol rather than 25-hydroxycholesterol was used in the disc, no effect on growth was seen (data not shown). Figure 1(B) shows that at a constant concentration of oxysterol, the cleared area bears a log-linear relationship with the number of cells plated. A line of cells designated M10 was isolated by pooling several colonies growing within the cleared zone on a single plate. M10 cells were able to proliferate without pause in 1.3 μM 25-hydroxycholesterol in medium containing 5% FBS, a lethal oxysterol concentration for CEM C7 cells. The phenotype of M10 cells was stable; after growth in medium lacking 25-hydroxycholesterol for several weeks, they were still resistant when rechallenged. Figure 2(A) demonstrates the time course of cell kill of CEM C7 cells, and the resistance of M10 cells to culture in 1 μM 25-hydroxycholesterol when grown in medium containing 5% FBS. We noted that significant loss of sensitive CEM C7 cells did not occur for 24 h in oxysterol, with progressive cell death following thereafter. The M10 cells, however, exhibited significant resistance to 25-hydroxycholesterol, growing in the oxysterol as well as untreated control cells.

To avoid the confounding effects of sterols in whole serum, we carried out further experiments on cells grown in medium supplemented with 5% delipidated FBS. We first verified that M10 cells still showed resistance to 25-hydroxycholesterol under these conditions. As expected, when serum cholesterol was removed, the oxysterol showed greater potency toward the sensitive CEM C7 cells (20% cell kill after 24 h treatment). M10 cells still demonstrated resistance, but under these conditions concentrations could be reached

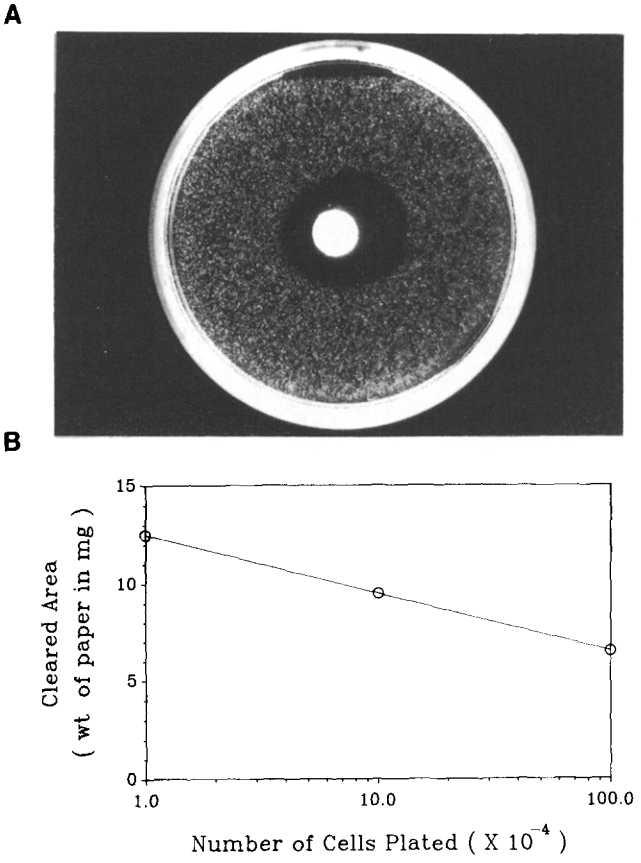

Fig. 1. Inhibition by 25-hydroxycholesterol of CEM C7 cells growing suspended in agarose. (A) Microcolonies created by 10^5 cells seeded in agarose gel in a 60 mm tissue culture dish form the lawn of white particles. A cleared area surrounding the filter paper disc, saturated with 1.24 mM 25-hydroxycholesterol, and placed atop the agarose gel, is clearly seen. To allow for quantification of the inhibition, this area has been delineated by an outline drawn on the bottom of the dish. (B) The relationship between the cell number plated and the area cleared of cells by diffusion of 25-hydroxycholesterol is shown. Cells were plated in soft agarose gel at 10^4, 10^5 or 10^6 per 60 mm tissue culture dish. A disk of filter paper saturated with 1.24 mM 25-hydroxycholesterol was dried and placed in the center of the surface of the agarose. After incubation for approx. 2 weeks to allow cell growth and cell inhibition, the area cleared of colonies was defined by visual inspection and marked by pen on the bottom of the dish. A tracing of this cleared area was made on paper and the paper weighed. Results of two or three dishes were averaged and plotted as shown. Actual weights of the papers were: 10^4 cells plated = 14.2 and 10.6 mg; 10^5 cells = 8.4 and 7.7 mg; 10^6 cells = 5.5, 7.0 and 6.6 mg.

which could cause their lysis. A comparison of the dose–response curves of the two cell lines allowed a quantitative estimate of the degree of resistance of the M10 cells to be made. The data in Fig. 2(B) show that the concentration of 25-hydroxycholesterol required to achieve 50% kill of M10 cells is approx. 17 times greater than that for CEM C7 cells. Thus CEM C7 and M10 cells may serve as a valuable system in which to compare the effects of molecules suspected of playing a role in oxysterol regulation of human lymphoid cell growth and viability. In this paper we examine correlations between two known macromolecules: OBP, proposed as an important regulator in cholesterol metabolism and cell growth; and CNBP, a gene whose function remains unknown.

Properties of the oxysterol binding protein and levels of oxysterol binding protein mRNA in sensitive and resistant CEM cells

CEM C7 cells contain an OBP with properties similar to the classic form of the protein [Fig. 3(A)]. We have shown that concentrations of 25-hydroxycholesterol which bind to this protein correspond to those that kill the cells [12]. M10 cells also contain OBP with the same sedimentation characteristics [12]. We examined the quantity of OBP in M10 cells, and its affinity for 25-hydroxycholesterol, by competitive

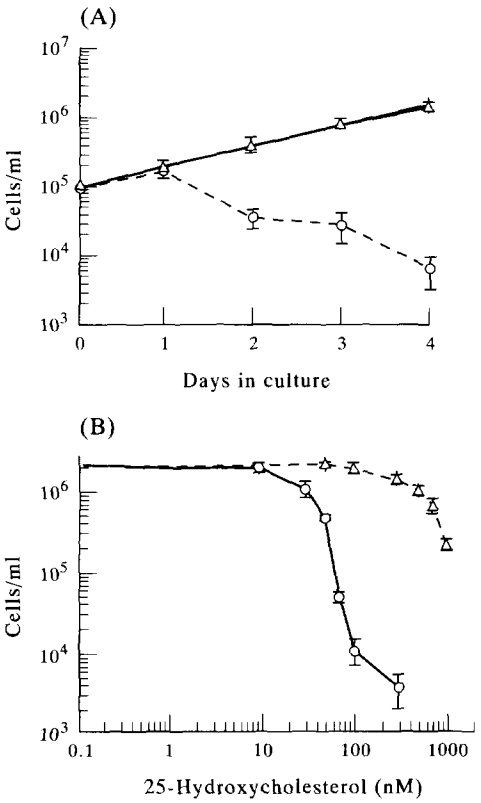

Fig. 2. Resistance of M10 cells and sensitivity of CEM C7 cells to 25-hydroxycholesterol treatment. (A) Cells in logarithmic growth, cultured in RPMI 1640 medium with 5% whole FBS, were exposed to either ethanol vehicle only (+) or 1 μM 25-hydroxycholesterol (M10 = open triangles, CEM C7 = open circles); ethanol <1%. Initial cultures for both cell types were 10^5 cells/ml. Error bars indicate the mean ± standard deviation ($\delta - 1$) for at least three determinations. (B) The dose–response of M10 and CEM C7 cells to 25-hydroxycholesterol treatment when cultured in 5% delipidated serum is shown. M10 cells (open triangles) and CEM C7 cells (open squares) in logarithmic growth, cultured in RPMI 1640 medium with 5% delipidated FBS, were exposed to increasing concentrations of ethanolic 25-hydroxycholesterol stock solution diluted in RPMI medium + 5% BSA. Initial cultures for both cell types were 10^5 cells/ml; final cell count on day 4 of controls was $2.1 \pm 0.2 \times 10^6$ cells/ml. Hemacytometer counts using trypan blue dye exclusion to distinguish viable cells were conducted on day 4; error bars indicate the mean with standard deviation for three determinations.

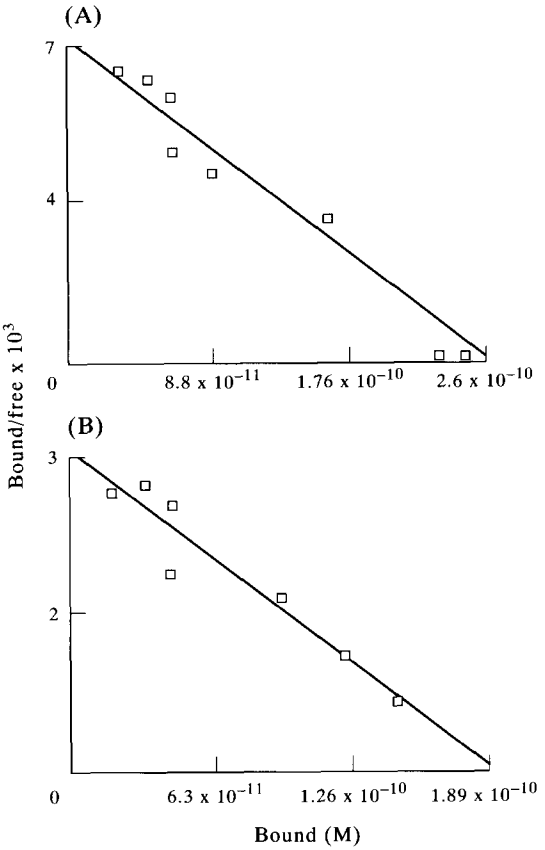

Fig. 3. OBP binding characteristics of CEM C7 and M10 cells determined by competitive binding assays on velocity sedimentation gradients. Cells were grown in RPMI 1640 medium + 5% FBS and briefly transferred to serum-free RPMI medium, tricine buffered pH 7.4, for labeling with [^{3}H]25-hydroxycholesterol as described in Experimental. Scatchard analysis of the data from four separate determinations was carried out by use of the computer software program Ligand. (A) Data from CEM C7 cells are shown, $r = 0.961$ and (B) from M10 cells, $r = 0.944$.

binding assays [Fig. 3(B)]. Scatchard analysis of the data showed that it could be fitted to a curve consistent with a single binding site, such as has been demonstrated in human liver and rodent cells and tissues [24, 25]. Neither property appeared to differ significantly between M10 and CEM C7 cells.

We next examined the cells for the expression and regulation of OBP mRNA. Figure 4 shows the results of such analyses. In Fig. 4(A), Northern blot data show that both CEM C7 and M10 cells express a single, 4.6 kb message for OBP. Dot blots were used to quantify the OBP message levels. The data in Fig. 4(B) show that OBP mRNA levels are not significantly altered in either cell line after 1, 7, or 24 h exposure to 300 nM 25-hydroxycholesterol.

Effect of 25-hydroxycholesterol on cellular nucleic acid binding protein mRNA levels

We also examined the effects of 25-hydroxycholesterol on CNBP mRNA levels in both CEM C7 and M10 cells. Logarithmically growing cells of both lines showed a single 1.5 kb mRNA as determined by Northern blotting [Fig. 5(A)] consistent with the original description of the mRNA for this protein in HepG2 cells [14]. Treatment by the oxysterol for 1 or 7 h produced no change in CNBP mRNA levels. But after 24 h there was a highly significant, 50% decrease in CNBP mRNA levels in the CEM C7 cells [Fig. 5(B)]. This effect continued for up to 48 h (data not shown). The basal level of CNBP mRNA in M10 cells was indistinguishable from that of CEM C7 cells,

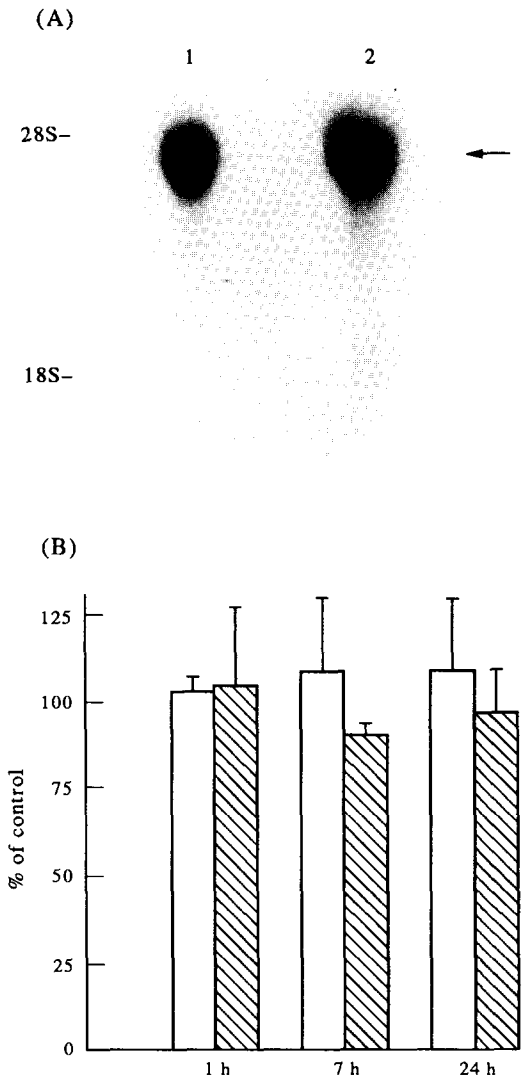

Fig. 4. Expression of OBP mRNA in CEM C7 and M10 cells. (A) Northern blot analysis of OBP mRNA in control conditions. Cells were cultured in RPMI 1640 with 5% delipidated serum and harvested 24 h later. 20 μg of total RNA were electrophoresed, transferred to an Immobilon-N membrane and hybridized with a rabbit OBP probe. The OBP message (arrow) is shown in CEM C7 cells (lane 1) and M10 cells (lane 2). RNA size was estimated by 18 and 28S rRNAs. (B) OBP mRNA levels after treatment with 25-hydroxycholesterol. Cells were cultured as above and treated with either vehicle only (control) or 300 nM 25-hydroxycholesterol. CEM C7 cells (open bars) and M10 cells (hatched bars) were harvested for RNA extraction at 1, 7, and 24 h later and analyzed by dot blotting as described in Experimental. The signals were normalized by reprobing the filters with β-actin cDNA. Error bars indicate the mean ± standard deviation of 2–6 determinations; P for 1, 7, 24 h were not significantly different at the 0.05 level when values for the control and treated cells were compared by a paired Student's t-test.

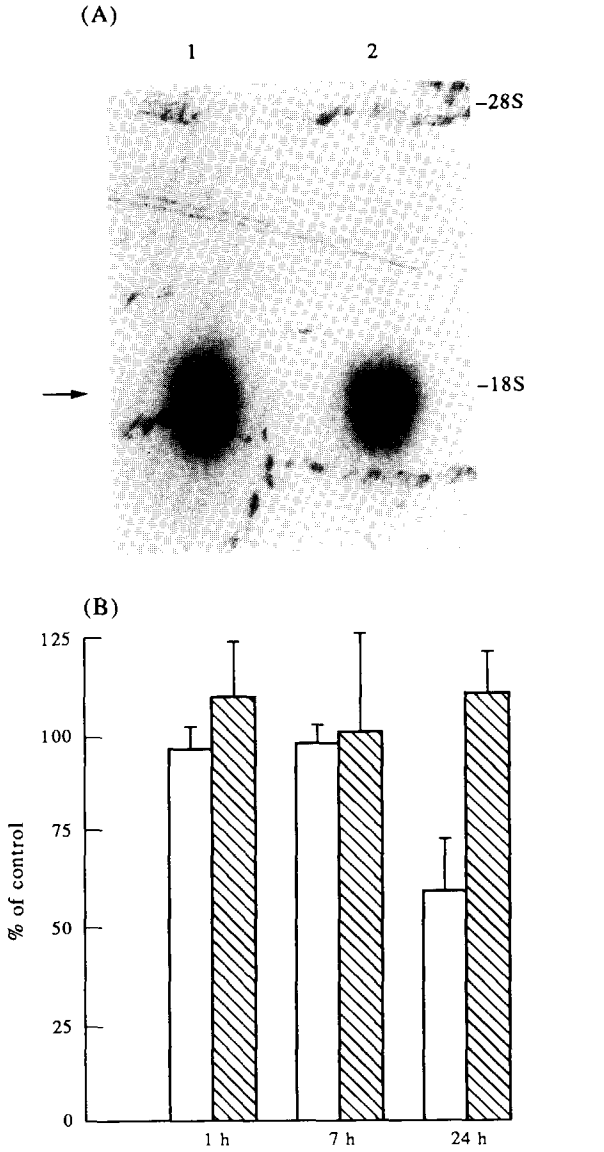

Fig. 5. Differential regulation of CNBP mRNA in CEM C7 and M10 cells. In A and B conditions were the same as in Fig. 4 with the exception that a human CNBP probe was used. (B) Error bars indicate the mean ± standard deviation of 2–6 determinations; for CEM C7 cells P at 24 h ≤ 0.05 when values for the control and treated cells were compared by a paired Student's t-test.

but it was unaffected by exposure to the oxysterol [Fig. 5(B)].

Dose–response experiments were performed in the two cell lines in order to define the concentrations of oxysterol required to lower CNBP mRNA levels. Figure 6(A) shows a Northern blot of RNA from cells treated with 1 μM 25-hydroxycholesterol for 24 h, compared with vehicle-only treated cells, and probed with CNBP cDNA. A single, unambiguous signal of about 1.5 kb was seen, with clear reduction after the steroid treatment. For a full dose response, serially diluted dot blot technique was employed [Fig. 6(B)]. Concentrations of 25-hydroxycholesterol from 30 nM to 1 μM applied to CEM C7 cells caused decreased CNBP mRNA levels by 24 h (open bars). Between 1 and 10 nM the sterol did not affect the message levels to a statistically significant extent, although there were numerical reductions in the values obtained for the mRNA. There was no effect of any sterol concentration tested on the β-actin signal used to normalize the dot blots. In M10 cells [Fig. 6(B), hatched bars], the basal level of CNBP mRNA was similar to that of clone CEM C7 cells, but none of the oxysterol concentrations used significantly decreased the levels of this message.

DISCUSSION

Oxysterols are powerful inhibitors of sterol synthesis and cell growth in a variety of cultured cells, including lymphocytes [3, 26–28]. The strong inhibition of the growth of CEM C7 cells by 25-hydroxycholesterol allowed the development of a simple selection technique for resistant cells. Bacterial geneticists often select resistant clones by placing a concentrated source of a poorly soluble substance on a "lawn" of freshly plated bacteria. As the toxic agent diffuses from the source, a cleared ring develops, with only resistant clones able to grow within it. The quantitative level of resistance is often related to the proximity of the clones to the drug source. We adapted this method to our lymphoid cells, taking advantage of the fact that these cells can grow suspended in soft agarose. We developed conditions that permitted growth of a three-dimensional "galaxy" of cells, with a cleared ring around the source of slowly diffusing oxysterol. Sterol, cell concentrations and time of growth were all optimized. The M10 cells described here are the first example of oxysterol-resistant cells isolated by this method. This pair of closely related sterol-sensitive and sterol-resistant cell lines, M10 and its parent CEM-C7, provide interesting possibilities for contributions to both the basic mechanisms of oxysterol regulation of cell growth and cholesterol synthesis as well as for pharmacologic studies of oxysterols as antimalignant agents [29, 30].

Since oxysterols elicit cellular responses without the LDL/LDL receptor system [1, 2, 31], a major question is how they do so. The cytoplasmic protein OBP fulfills the pharmacologic definition of a cellular receptor in that it is present in limited amounts and had high affinity for its ligands, and thus OBP has been proposed as the specific receptor for oxysterols [2]. The affinities of a large number of oxysterols for OBP correlate well with their potencies for regulation of HMG CoA reductase and other relevant genes in mouse L cells [26, 32]. However, proof that OBP transduces oxysterol signals to regulate cholesterol synthesis is still lacking, and the fact that overexpression of OBP in CHO cells leads to its localization in what appears to be the Golgi apparatus [32] has confounded the issue.

Our studies demonstrate that the human CD4+, acute lymphoblastic leukemic cell line CEM C7 contains an OBP with properties similar to those reported for the protein from other mammalian sources. We

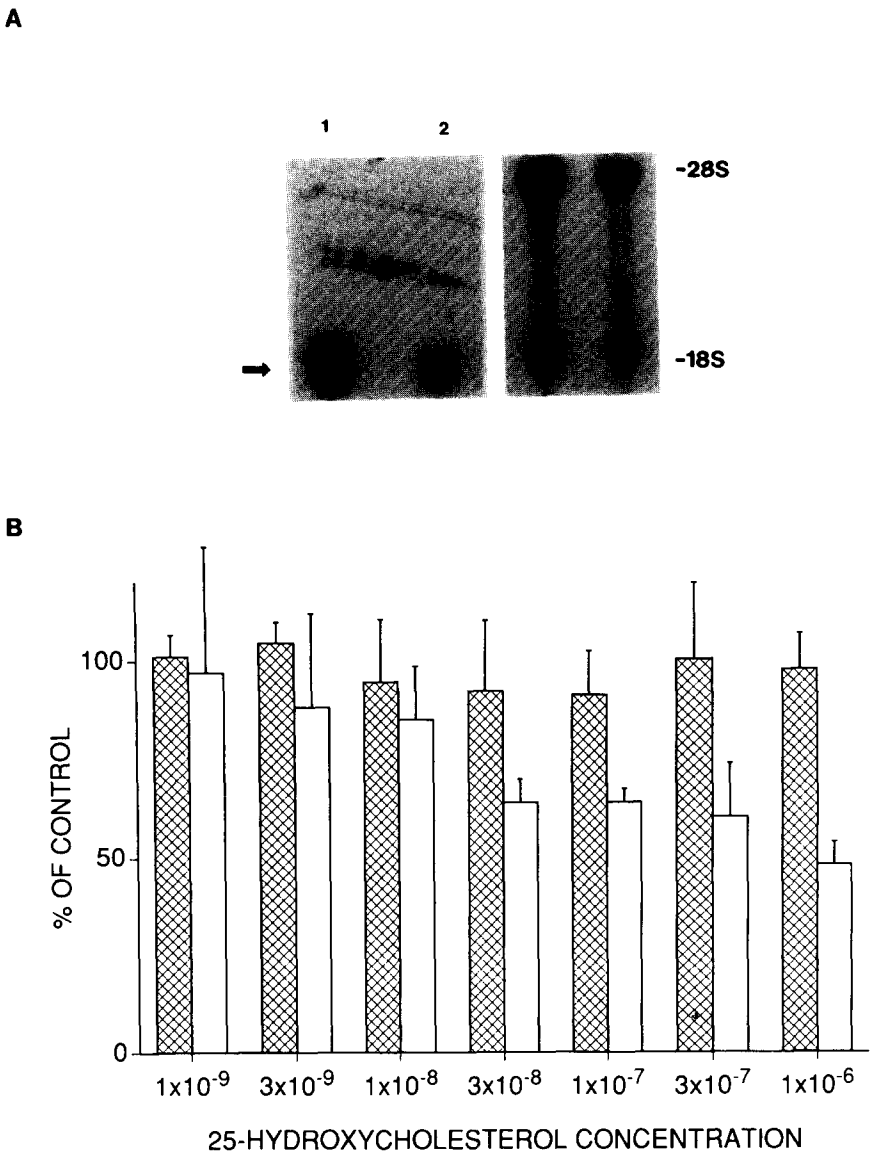

Fig. 6. Effect of various concentrations of 25-hydroxycholesterol on the level of CNBP mRNA in CEM C7 and M10 cells. (A) Northern blot analysis of CNBP mRNA levels after treatment with 25-hydroxycholesterol. Cells were cultured in RPMI 1640 medium with 5% delipidated serum and treated with either vehicle only (lane 1) or 1 μM 25-hydroxycholesterol (lane 2). Total RNA was extracted and analyzed 24 h after treatment. 20 μg of total RNA were electrophoresed, transferred to Immobilon-N membrane and hybridized with a human CNBP cDNA. (A) Shows ethidium bromide stained ribosomal RNA transferred to the filter as an indication that equal amounts of RNA were loaded and transferred in each lane. (B) Cells were cultured as above and exposed to either vehicle only (control) or to different concentrations of the oxysterol. After 24 h treatment, total RNA was extracted and analyzed by dot blotting as described in Experimental. Double hatched bars represent M10 and open bars CEM-C7 cells. Error bars indicate the mean $\pm$ standard deviation for at least 3 determinations. Values for the control and treated cells were significantly different at the 0.05 level for concentrations $\geqslant 3 \times 10^{-8}$ M 25-hydroxycholesterol when compared by a paired Student's t-test.

report elsewhere that OBP occupancy correlates with cell kill by 25-hydroxycholesterol in these cells [12]. OBP has a sequence suggesting that it can interact with other proteins [25, 34], and its effect on gene regulation while remaining a cytoplasmic protein may therefore be determined by such interactions, for example by sequestration of DNA-binding transcription factors. Examples of control through sequestration are known [35].

Comparison of OBP in the sensitive and resistant lines showed no significant differences in behavior of the protein on velocity sedimentation gradients, number of sites per cell, and affinity for 25-hydroxycholesterol. A single species of OBP mRNA was expressed equally in the two cell lines and was not affected by adding oxysterol to the cultures. While these tests are certainly not exhaustive, we tentatively conclude that the OBP is the same in the two cell lines and that it is not regulated by oxysterols in these cells. Other lines of oxysterol-resistant cells from other species have shown similar results [36]. A single line of CHO cells with reduced oxysterol binding activity associated with resistance to 25-hydroxycholesterol has been produced [37].

On the other hand, CNBP in the sensitive and resistant cells responds differentially. Although the mRNA size and basal level of CNBP were identical in both, in the oxysterol-resistant M10 cells this basal level remained unchanged when challenged with 25-hydroxycholesterol, whereas the CNBP mRNA level was reduced significantly in the sensitive clone. This reduction was observed 24 h after treatment, the time point where the sensitive CEM C7 cells were still alive. The concentrations of oxysterol effective in causing the reduction of CNBP are consistent with those which should occupy OBP. Though it is not statistically significant at this time, at the lower concentrations there clearly is a trend towards reduction of CNBP mRNA starting at 3×10^{-9} M 25-hydroxycholesterol. The time course of reduction of CNBP mRNA is also consistent with it or its product playing a role in the growth regulation of CEM cells by oxysterols. After 4 days treatment, all those cultures whose cells showed some reduction of CNBP at 24 h also show decreased numbers of viable cells. That there is not an exact proportion between the early amount of CNBP mRNA decrease and the later loss of viability is not surprising; it suggests a continuing, amplified process. Why CNBP was induced by oxysterol in HepG2 cells [14] and suppressed in CEM C7 cells we do not know. Perhaps this represents fundamentally different regulation in liver-derived vs lymphoid cells. In our results, it appears that CNBP expression correlates with the inhibition of cell growth by oxysterols, raising the possibility that CNBP may play a role in oxysterol regulation of cell growth processes. The cell system described here should provide the means to investigate this possibility.

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