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Isolation and Characterization of Cells Resistant to ML236B (Compactin) with Increased Levels of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase*

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ML236B is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (EC 1.1.1.34), the major regulatory enzyme in cholesterol biosynthesis. This compound inhibits cell growth when present in the culture medium of CHO-K1 cells at a concentration as low as 0.1 μg/ml. Addition of the product of the HMG-CoA reductase reaction, mevalonate, to the culture medium prevents the cytotoxic effects of ML236B at a concentration of inhibitor as high as 50 μg/ml. Using a stepwise selection procedure, we have obtained two variant cell lines which are resistant to the presence of 8 μg/ml of ML236B in the culture medium. The rates of cholesterol synthesis and the cholesterol levels in the variant cell lines, grown in the presence of ML236B, are similar to those of the parental CHO-K1 cell line grown in the absence of inhibitor. Assays of HMG-CoA reductase activity from extracts of variant cells, grown in the presence of inhibitor, reveal that the variant cell lines have an approximately 40-fold higher HMG-CoA reductase activity than does the parental CHO-K1 cell line grown in the absence of inhibitor. However, when the variant cell lines are grown without ML236B in the culture medium, the HMG-CoA reductase activity returns to the parental CHO-K1 level within 5 days, but the resistant phenotype is stable for up to 9 months. We conclude that the variant cell lines are able to overcome the cytotoxic effects of ML236B by a mechanism which leads to overaccumulation of HMG-CoA reductase which in turn permits normal mevalonate metabolism and cholesterol synthesis to take place.

HMG-CoA reductase,[1] which catalyzes the conversion of HMG-CoA to mevalonate, has long been established as a key regulatory enzyme in the cholesterogenic pathway (1). More recent studies have emphasized the role of this enzyme in the pathway of other mevalonate-derived products, such as ubiquinone, dolichol, and isopentenyladenine (2-8). It has become increasingly clear that this enzyme is central to both cell growth and division (9-14).

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[1] The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MVA, mevalonate.

Although a number of physiological stimuli (*i.e.* low density lipoprotein, hormones, cholesterol) have been shown to modulate the activity of HMG-CoA reductase (1), less information has been obtained on the actual regulatory mechanisms involved. Several mechanisms have been proposed to account for these observations. The two most frequently suggested are regulation by changes in the rates of synthesis and degradation of the enzyme (1, 15, 16) and rapid conversion of inactive to active enzyme via a phosphorylation-dephosphorylation mechanism (17-20). In no case, however, has the molecular basis for these types of regulation been firmly established.

A primary goal of our research is to study the mechanisms whereby HMG-CoA reductase is regulated at the molecular level. This study would be aided greatly by a cell system in which the amount of HMG-CoA reductase represented a significant per cent of total cellular protein. Such a system would facilitate the isolation of HMG-CoA reductase, the production of antibodies to the enzyme and the generation of cDNA complimentary to HMG-CoA reductase mRNA. This cDNA would be a sensitive probe with which to assay the effects of HMG-CoA reductase modulators at the transcriptional and translational levels. Unfortunately, the biological systems studied so far suggest that HMG-CoA reductase constitutes a particularly low per cent of total cellular protein. Based on reported purifications from rat liver (21, 22), HMG-CoA reductase accounts for approximately 0.04% of total microsomal protein.

The discovery by Endo and co-workers of a fungal metabolite that is a competitive inhibitor of HMG-CoA reductase has provided a new tool for the study of this enzyme and its regulatory role in cholesterol biosynthesis (23). This compound produced by *Penicillium citrinum* has been designated ML236B by Endo *et al.* (23). Compactin, a compound identical in structure with ML236B, has been isolated from cultures of *Penicillium brevicompactum* by Brown *et al.* (24). ML236B has been shown to inhibit HMG-CoA reductase activity at nanomolar concentrations in a number of systems (23, 25-28). This inhibition appears to be specific for HMG-CoA reductase since rates of fatty acid biosynthesis and mevalonate conversion to sterols are not affected at concentrations required for inhibition of HMG-CoA reductase (25-27). Unlike the oxygenated sterols that inhibit sterol synthesis *in vivo* (29, 30), ML236B is a potent competitive inhibitor of HMG-CoA reductase both *in vivo* and *in vitro*.

Studies have shown that cells challenged with potent competitive inhibitors of essential enzymes are able to develop resistance to the inhibitor by overproduction of the target enzyme (31-35). These studies suggest that ML236B would be a suitable compound with which to select cell lines that produce elevated levels of HMG-CoA reductase. In support of this idea, Brown *et al.* (28) have shown that acute treatment

of human fibroblasts in culture with ML236B results in elevated levels of HMG-CoA reductase.

In this paper, we describe the selection of two variant cell lines which are resistant to the presence of 8 μg/ml of ML236B in the culture medium. These cell lines were derived from a CHO-K1 cell line via a stepwise selection procedure. As predicted, these cell lines display elevated levels of HMG-CoA reductase activity. We report the phenotype of these cell lines with regard to HMG-CoA reductase activity levels, cholesterol synthesis, and membrane sterol content.

EXPERIMENTAL PROCEDURES

Materials—[1,2-^{14}C]Acetic acid, sodium salt (56.5 mCi/mmol), ^3H_2O (25.0 mCi/ml), [1,2-^{3}H]cholesterol (44 Ci/mmol), [4-^{14}C]cholesterol (54.0 mCi/mmol), DL-3-hydroxy-3-methyl [3-^{14}C]glutaryl coenzyme A (26.2 mCi/mmol), and [5-^{3}H]mevalanolactone (2 to 10 Ci/mmol) were obtained from New England Nuclear Corp. DL-3-Hydroxy-3-methylglutaryl coenzyme A was purchased from P-L Biochemicals. Dolichol, coenzyme Q_{10}, mevalonic acid lactone, and *Escherichia coli* alkaline phosphatase (30 to 60 units/mg) and N^6-[Δ^2-isopentenyl]-adenine were purchased from Sigma. All other chemicals were of highest quality from commercial sources.

Experimental Methods—ML236B was used in the lactone form for all experiments unless otherwise noted. Stock ethanolic solutions were stored frozen or at 4 °C. ML236B was converted to the salt form by treatment with 0.1 N NaOH at 55 °C for 45 min. After adjusting the pH to 8.0, ML236B-salt was stored in liquid nitrogen.

Cell Growth—Chinese hamster ovary (CHO-K1) cells were obtained from the American Type Culture Collection (ATCC CCL-61). Cells were maintained on Minimal Essential Medium (Flow Laboratories) supplemented with nonessential amino acids and 5% (v/v) delipidated fetal calf serum. Delipidated fetal calf serum was prepared by the method of Rothblat *et al.* (36) and shown to be essentially free of sterols and 90 to 95% free of fatty acids. Cells were grown as monolayers in tissue culture flasks in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. Variant cell lines were maintained in the same manner, but with ML236B (8 μg/ml) added to the media.

Growth Curves—Cells were inoculated into multiwell plates at 2.5 × 10^4 cells/well (2 cm^2) in 1.0 ml of growth medium and incubated overnight at 37 °C. A zero time sample was removed to determine the initial inoculum size. The growth medium was then removed from the remaining wells by aspiration and replaced with 1 ml of medium containing the indicated supplement. At the indicated times throughout the growth curves, the cells were washed with 3 ml of cold phosphate-buffered saline and total protein determined by the method of Lowry *et al.* (37).

Extract Preparation and Assay of HMG-CoA Reductase—Subconfluent cells grown as described above were removed from a 75-cm^2 tissue culture flask with 0.25% trypsin or by scraping with a rubber policeman, 24 h after feeding. Cells (3 to 5 × 10^6 cells/flask) were collected in 5.0 ml of medium and pelleted by centrifugation. The cells were washed twice in 50 mM Tris-HCl, pH 7.4, and 0.15 M NaCl. Cell extracts were prepared essentially according to the procedure of Brown *et al.* (38). The final cell pellet was frozen in dry ice/ethanol and then resuspended in approximately 0.3 ml of a buffer containing 50 mM potassium phosphate, pH 7.4; 5 mM dithiothreitol; 5 mM EDTA; and 0.25% Kyro EOB. The extract was briefly sonicated in a bath sonicator to disperse any particulate matter. Aliquots (10 to 30 μg of protein) of the extract were taken for protein and HMG-CoA reductase assay.

HMG-CoA reductase activity was determined by the method of Shapiro *et al.* (39). After 20 min preincubation at 37 °C, the assay was initiated by addition of substrate. Inclusion of alkaline phosphatase in the preincubation essentially according to the procedure of Brown *et al.* (42) did not result in increased HMG-CoA reductase activity and we assume the enzyme is fully active. All values are reported as the average of duplicate or triplicate samples.

Incorporation of [^{14}C]Acetate and ^3H_2O into Total Cellular Cholesterol and Fatty Acids by CHO-K1 and Variant Cells—On day 0, 10^6 cells were seeded into 75 cm^2 flasks containing 10 ml of medium. CHO-K1 cells received ML236B-free medium; whereas, variant cells received medium containing 8 μg/ml of ML236B. On day 1, all cells were refed with their respective medium. On day 2, 1 h prior to the incubation period with radioactive precursors, the cells received the appropriate incubation medium, plus or minus ML236B. After 1 h at 37 °C, either [1,2-^{14}C]acetate (5 μCi) to a final concentration of 1 mM or 10 mCi of ^3H_2O was added to the medium and the incubation continued for 1 h or 6 h, respectively.

The incubation period was terminated by aspirating the media, washing the monolayers three times with cold buffer (50 mM Tris-HCl, 0.14 M NaCl, pH 7.4) and dissolving the cells with 2 ml of 0.2 N KOH for 30 min at 37 °C. The cell suspension was transferred to a glass tube and the flask rinsed with 1 ml of water. The rinse and cells were combined and duplicate aliquots removed for protein determination by the method of Lowry *et al.* (37). To each tube was added 3 ml of 4 N KOH, 6 ml of methanol, and 10^5 cpm of [1,2-^{3}H]cholesterol (44 Ci/nmol) or 10^4 cpm of [^{14}C]cholesterol (54.0 mCi/nmol) as an internal standard. The samples were saponified at 80 °C for 1 h, cooled to room temperature, and extracted three times with 20 ml of petroleum ether. The extracts for each sample were pooled and evaporated under a stream of nitrogen. Cholesterol (1 mg) was added to each tube, followed by 2 ml of acetone:ethanol (1:1), 50 μl of a 10% sodium acetate solution and 2 ml of a 5 mg/ml of digitonin, 50% ethanol solution. Digitonin precipitation occurred overnight at room temperature. The precipitate was pelleted at 400 × *g*, washed twice with acetone:ethanol (1:3), resuspended in 1 ml of methanol and counted in a liquid scintillation counter.

The rate of fatty acid synthesis was determined by acidifying the remaining aqueous phase to pH 1 with concentrated HCl and extracting three times with 20 ml of petroleum ether. The extracts for each sample were pooled, evaporated under a stream of nitrogen, resuspended in 1 ml of petroleum ether, and counted in a liquid scintillation counter.

Lipid Analysis—Cells grown as described above were harvested either by treating with 0.25% trypsin or by scraping the flasks with a rubber policeman. The cell suspension, approximately 1 to 1.5 × 10^7 cells from one 150-cm^2 flask, were pelleted in 5.0 ml of medium and washed twice in phosphate-buffered saline. An aliquot of the final cell suspension was removed for protein determination by the method of Lowry *et al.* (39). The remaining cell material was then extracted twice with a 2:1 mixture of chloroform:methanol according to the procedure of Bligh and Dyer (40). This extract, referred to as the total lipid fraction, was used for sterol determinations by the fluorimetric assay of Solow and Freeman (41). Alternatively, sterols were separated by silicic acid chromatography and quantified by gas chromatography (6-foot 10% OV17 Supelco column) using coprostanol as an internal standard.

RESULTS

Effect of Mevalonate and Products of Mevalonate Metabolism on Growth of CHO-K1 Cells in the Presence of ML236B—ML236B, present in the culture medium at concentrations less than 0.02 μg/ml, has been shown to block the conversion of acetate to cholesterol in cultured cells by inhibiting HMG-CoA reductase and consequently blocking mevalonic acid production (26). Since the experiments described below involve the use of much higher levels of ML236B (1 to 8 μg/ml) it was important to establish that cytotoxicity resulted solely from an inhibition of mevalonic acid synthesis and not other metabolic steps. The ability of exogenous mevalonate to prevent the cytotoxic effects of ML236B would support HMG-CoA reductase as the primary site of inhibition.

We have examined the ability of mevalonate and a number of mevalonic acid-derived products to prevent death of the parental cell line in the presence of 8 or 50 μg/ml of ML236B. At 8 μg/ml of ML236B, cell death was prevented in the CHO-K1 cell line by the addition of mevalonate (data not shown) or cholesterol to the culture medium (Fig. 1*a*). Addition of dolichol and ubiquinone had no effect when added in the presence or absence of cholesterol. When ML236B was present in the culture medium at 50 μg/ml, only mevalonate was capable of preventing the cytotoxic effects of ML236B (Fig. 1*b*). Neither cholesterol alone, nor cholesterol with added dolichol, ubiquinone, and isopentenyladenine was effective in preventing cell death. These data suggest that at 8 μg/ml, ML236B inhibition of cell growth is primarily due to a lack of cholesterol. Other essential products of mevalonate metabolism, such as ubiquinone and dolichol, are not growth-limiting. At 50 μg/ml of ML236B, however, additions of cholesterol are

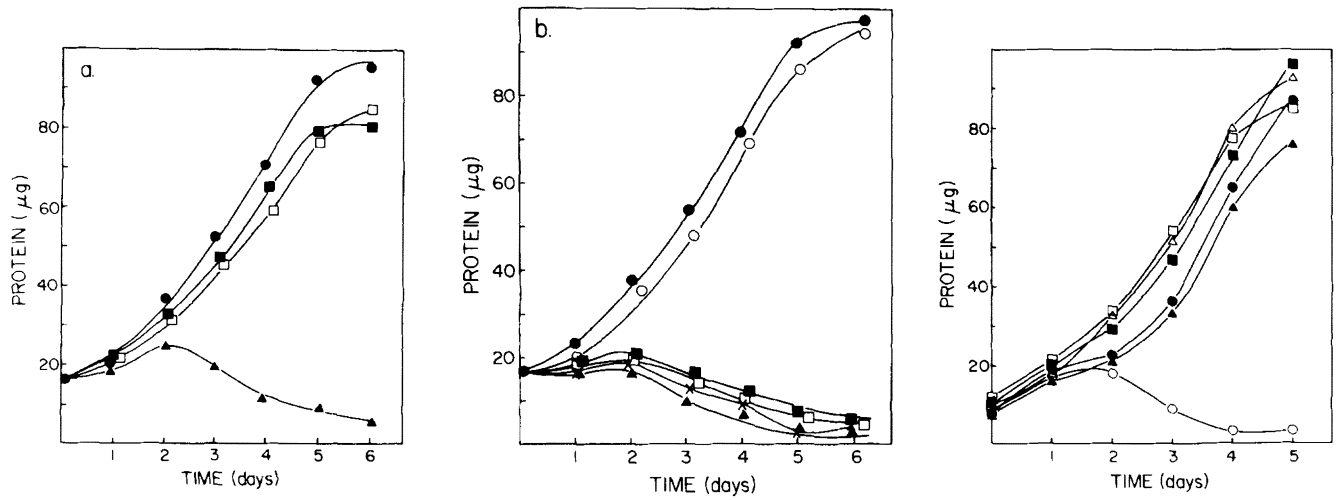

FIG. 1 (*left and center*). **Prevention of ML236B-induced growth inhibition of CHO-K1 cells by products of mevalonate metabolism.** Cells were inoculated at a density of 2.5×10^4 cells/ml into multiwell plates (2 cm^2/well) and were preincubated for 12 h. The medium was then changed to one containing either 0, 8 (*a*), or 50 (*b*) μg/ml of ML236B, plus the indicated supplements. Supplements to the growth medium were made from ethanolic stock solutions, except mevalonic acid lactone which was prepared in 0.05 M potassium phosphate buffer, pH 4.3. Final concentrations of supplements in the medium were: mevalonic acid lactone, 7.7 mM; cholesterol, 10 μg/ml; dolichol, 5 μg/ml; coenzyme Q_{10}, 0.166 μg/ml and isopentenyladenine, 0.5 μg/ml. The ethanol concentration never exceeded 0.2% (v/v) a level which was shown to be noninhibitory to cell growth. Cells were incubated for the indicated time periods, with one refeeding on the 3rd day. Protein values were determined and described under "Experimental Procedures," and are the average of triplicate determinations. Supplements to the growth medium are as follows: *a*: ●——●, no supplement; ▲——▲, 8 μg/ml of ML236B; ■——■, 8 μg/ml of ML236B + cholesterol; □——□, 8 μg/ml of ML236B + cholesterol + dolichol + ubiquinone (coenzyme Q_{10}). *b*: ●——●, no supplement; ▲——▲, 50 μg/ml of ML236B; ■——■, 50 μg/ml of ML236B + cholesterol; □——□, 50 μg/ml of ML236B + cholesterol + dolichol + ubiquinone (coenzyme Q_{10}); ○——○, 50 μg/ml of ML236B + mevalonolactone; ×——×, 50 μg/ml of ML236B + cholesterol + dolichol + ubiquinone + isopentenyladenine.

FIG. 2 (*right*). **Growth properties of CHO-K1 and variant cell lines in the presence of ML236B.** The indicated cell lines were inoculated into the growth medium at a density of 3×10^4 cells/ml/2 cm^2 well plates (1 ml each). After an initial 12-h preincubation of the cells in the absence of ML236B, the medium was removed by aspiration and replaced with fresh medium containing ML236B at 0 or 8 μg/ml. Control cells were removed for analysis at zero time. Cell cultures were incubated for the times indicated with one refeeding after the 2nd day. Cell growth was terminated by aspirating the growth medium from the well plates and washing the cell monolayer with cold phosphate-buffered saline. Protein values were determined and represent the average of triplicate determinations. ●——●, CHO-K1; ■——■, variant-ML; ▲——▲, variant-NS grown in the absence of ML236B; ○——○, CHO-K1; □——□, variant-ML; △——△, variant-NS grown in the presence of 8 μg/ml of ML236B.

insufficient to prevent cell death and suggest that other mevalonate-derived products are required. Our results indicate that addition of ubiquinone, dolichol, and isopentenyladenine with cholesterol does not prevent the cell death induced by these high levels of ML236B. The important point is that at levels of ML236B as high as 50 μg/ml, the inhibitory site remains HMG-CoA reductase.

Selection of CHO-K1 Cell Lines Resistant to ML236B—Having determined a concentration range over which ML236B inhibited CHO-K1 cell growth, selection of resistant cells was initiated. CHO-K1 cells were initially selected for resistance to 1 μg/ml of ML236B. Individual colonies which arose from the selection were isolated and grown to high cell density in the presence of ML236B at 1 μg/ml. These cells were then exposed sequentially to 2, 4, and 8 μg/ml of ML236B and selected as a population of cells surviving after 90% or greater cell death at each increment of increased concentration. The cell line derived from this selection has been designated variant-ML. Another cell line, variant-NS, was obtained independently after selection at 4 and 8 μg/ml of ML236B. Both variant cell lines have been maintained for over 2 years with ML236B present in the culture medium at 8 μg/ml. Variant-NS has been cloned by limiting dilution in medium containing 8 μg/ml of ML236B. Six clones have been examined and appear to be similar to each other with respect to ML236B-resistant growth phenotype and HMG-CoA reductase activity (see below).

Growth Properties of CHO-K1, Variant-NS, and Variant-ML Cell Lines—The growth properties of CHO-K1, variant-ML, and variant-NS cell lines are shown in Fig. 2. Preliminary experiments had shown that CHO-K1 cells were sensitive to 0.1 μg/ml of ML236B. Growth inhibition by ML236B was observed for CHO-K1 cells by 48 h, followed by a subsequent decline in cell protein as cells detached from the plate over the next 3 days. Both variant-NS and variant-ML cell lines exhibited continued cell growth in the presence of 8 μg/ml of ML236B in the culture medium. Essentially identical results were obtained using either the lactone or the salt form of ML236B (data not shown). Thus, the variant cell lines are able to grow in concentrations of ML236B that are nearly 100 times higher than that sufficient to cause growth inhibition of the parental CHO-K1 cell line.

Levels of HMG-CoA Reductase Activity in CHO-K1, Variant-NS, and Variant-ML Cell Lines—The results presented in Fig. 1 demonstrate that growth inhibition of CHO-K1 cells by ML236B is consistent with its known action as a specific competitive inhibitor of HMG-CoA reductase, and consequently mevalonic acid synthesis. One possible mechanism by which the cell could relieve this type of inhibition would be to synthesize additional enzyme and thus overcome the competitive inhibition and the metabolic block (32–35). It was of interest, therefore, to determine the relative levels of HMG-CoA reductase activity in the CHO-K1 and variant cell lines. Representative values for HMG-CoA reductase activities are given in Table I for the three cell lines grown in the presence or absence of 8 μg/ml of ML236B. When assayed after growth in medium supplemented with 8 μg/ml of ML236B, the variant cell lines showed HMG-CoA reductase activities 2- to 6-fold higher than CHO-K1 cells that were also grown in the presence of ML236B. The values of HMG-CoA reductase deter-

mined for cells grown in the presence of ML236B represent the enzyme activities present in the cells after removal of bound ML236B by extensive dilution in preparing the extracts. In addition, the assays have been performed with increasing levels of substrate and no further increase in enzyme activity was observed, suggesting that no residual inhibitor remained bound to the enzyme. When variant cell lines were grown for 5 days in the absence of ML236B and then reassayed for HMG-CoA reductase activity, enzyme levels had declined to values nearly equal to those of the CHO-K1 cell line. This finding suggests that elevated HMG-CoA reductase levels in the variant cell lines require the presence of ML236B in the culture medium.

Also shown in Table I are the HMG-CoA reductase activities for variant and CHO-K1 cell lines grown in the presence of an exogenous sterol source. It has been shown for a number of systems that cholesterol added exogenously to the medium of cultured cells causes a decrease in HMG-CoA reductase activity (1, 15, 16). As shown in Table I, addition of whole serum, containing sterol, to the culture medium reduces HMG-CoA reductase levels regardless of whether or not ML236B is present. These results are consistent with those previously reported by Brown *et al.* (28).

These results demonstrate that variant-NS and variant-ML when grown in the presence of ML236B have HMG-CoA reductase levels that are 2- to 6-fold higher than that of the parental cells grown under the same conditions and 20- to 60-fold higher than that of the parental cell line grown in the absence of ML236B.

HMG-CoA Reductase Levels in CHO-K1, Variant-NS, and Variant-ML Cell Lines after Removal of ML236B from the Culture Medium—The data in Table I demonstrate that variant cells grown in the absence of ML236B for 5 days had decreased levels of HMG-CoA reductase activity. The rate of decline in enzyme levels was determined for the parental CHO-K1 and variant cell lines after removal from medium containing ML236B (Fig. 3). For comparison, CHO-K1 cells grown in the presence of 8 μg/ml of ML236B for 5 days, were also removed from the ML236B containing culture medium and HMG-CoA reductase activities determined. After 5 days in the absence of ML236B, HMG-CoA reductase levels for CHO-K1 cells had dropped from 1.5 nmol/mg/min to 0.15 nmol/mg/min. HMG-CoA reductase levels in the variant cell lines also dropped rapidly from their elevated levels.

TABLE I

HMG-CoA reductase activity in CHO-K1 and variant cells in the presence and absence of ML236B

Cells were grown in flasks (75 cm²) in growth medium containing either 0 or 8 μg/ml of ML236B as indicated.

Cell type	Growth media	HMG-CoA reductase activity	
		0 μg/ml ML236B[a]	8 μg/ml ML236B[a]
		nmol MVA formed/min/mg	
CHO-K1	MEM-DFCS[b]	0.15–0.40	1.2–1.5[c]
	MEM-FCS[d]	0.05–0.06	ND
Variant-ML	MEM-DFCS	0.15–0.19	3.0–7.5
	MEM-FCS	0.02–0.05[e]	0.44–0.88[f]
Variant-NS	MEM-DFCS	0.15–0.40	3.5–9.5
	MEM-FCS	0.10–0.40[e]	0.78–1.2[f]

[a] Concentration of ML236B present in the growth medium. Variant cells had been grown in the absence of ML236B for 3 months prior to assaying.

[b] Minimal Essential Medium supplemented with 5% delipidated fetal calf serum (DFCS) and nonessential amino acids.

[c] CHO-K1 cells were grown in the presence of ML236B for 5 days.

[d] Minimal Essential Medium supplemented with 5% fetal calf serum (FCS) and nonessential amino acids.

[e] Variant cells had been grown in the absence of ML236B for 3 months and in the presence of fetal calf serum for 3 days.

[f] Variant cells had been grown in the presence of fetal calf serum for 3 days.

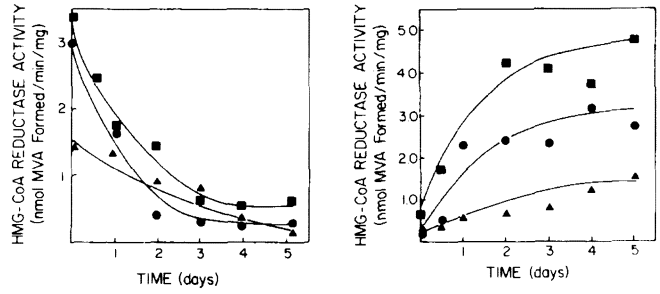

FIG. 3 (*left*). **HMG-CoA reductase activity in cells as a function of time following removal of ML236B from the culture media.** Twelve flasks (75 cm²) were inoculated at a cell density of 6 × 10⁵ cells/flask with either variant-NS or variant-ML cells that had been grown continuously in the presence of 8 μg/ml of ML236B. Each day of the time course indicated, one flask of each cell type was changed from media containing 8 μg/ml of ML236B to one containing no ML236B. All remaining flasks also received fresh media. CHO-K1 cells were treated in a similar manner, with the exception that these cells had been pregrown in the presence of 8 μg/ml of ML236B for 5 days. Five days was the maximum time that CHO-K1 cells could be maintained in the presence of ML236B and still have sufficient cells remaining in the culture for assay. Using this protocol, all flasks were assayed at the same time, having been refed 24 h prior to harvesting. Cell extracts were prepared as described under "Experimental Procedures." The HMG-CoA reductase values presented are the mean of duplicate assays from a single growth flask. ▲——▲, CHO-K1; ●——●, variant-ML; ■——■, variant-NS.

FIG. 4 (*right*). **Increase in HMG-CoA reductase activity as a function of time after addition of ML236B to the culture medium.** Variant-ML and NS cells which had been grown in the absence of ML236B for 4 days were trypsinized and inoculated into flasks (75 cm²) at 6 × 10⁵ cells/flask for variant-ML and 7 × 10⁵ cells/flask for variant-NS. CHO-K1 cells were inoculated at either 1 × 10⁶ or 6 × 10⁵ cells/flask (75 cm²). These inoculum sizes were set so that at the time of assay all cells would be subconfluent. All cells are incubated for 12 h. Medium was then exchanged to include 8 μg/ml of ML236B for one flask of each cell type per day. Remaining flasks also received fresh medium. All cells were fed 24 h prior to harvesting. The 0.5-day determination was initiated by the addition of 1.0 ml of fresh media containing ML236B so the final concentration in the growth media was 8 μg/ml. Cells were harvested and assayed as described under "Experimental Procedures." Values for HMG-CoA reductase are the average of triplicate determinations of varying protein concentrations taken from a single growth flask. ▲——▲, CHO-K1; ●——●, variant-ML; ■——■, variant-NS.

Induction of HMG-CoA Reductase Levels in CHO-K1, Variant-ML, and Variant-NS Cell Lines after Addition of ML236B to the Culture Medium—In order to determine the rate of increase in HMG-CoA reductase activity induced by the addition of ML236B to the culture medium, variant cells were grown in the absence of ML236B for 5 days, sufficient time to allow the decrease in HMG-CoA reductase activities noted in Fig. 3. Variant and CHO-K1 cells were then transferred back to medium containing 8 μg/ml of ML236B. As shown in Fig. 4, the HMG-CoA reductase activities of variant-NS and variant-ML cells increased over 10-fold to a constant level in 3 to 4 days. In contrast, the increase in HMG-CoA reductase activity in CHO-K1 cells is more gradual and reaches a maximal level well below the induced levels of the variant cell lines. HMG-CoA reductase activity of the CHO-K1 cell line could not be measured at longer time periods because of detachment of the cells from the culture flask. These results clearly demonstrate that variant cell lines are able to achieve higher levels of HMG-CoA reductase activity than the parental cell line.

We have examined the long term stability of the variant cell phenotype by maintaining both variant cell lines on

medium in the absence of ML236B for up to 9 months. These cells retained their resistance to ML236B growth inhibition and displayed the same high levels of HMG-CoA reductase activity when ML236B was added to the culture medium.

Levels of HMG-CoA Reductase in CHO-K1, Variant-NS, and Variant-ML Cell Lines Induced by Varying Concentrations of ML236B in the Culture Medium—The HMG-CoA reductase activities determined for the variant cell lines grown in the presence of various concentrations of ML236B are shown in Fig. 5. HMG-CoA reductase activities for the variant cells were essentially equivalent over the 4-fold range of ML236B concentrations tested. Parental CHO-K1 cells exposed to the same range of ML236B concentrations showed a qualitatively similar response, although the magnitude of the elevation in HMG-CoA reductase activity was considerably less than for the variant cell lines. The finding that both 4 and 8 μg/ml of ML236B in the culture medium elicited the same activity of HMG-CoA reductase in CHO-K1 cells, suggests that this level (1.5 nmol/mg/min) may be a maximum level for the parental cell line. No value could be obtained for CHO-K1 cells at 16 μg/ml of ML236B as too few cells survived the induction period. These observations suggest that the high levels of HMG-CoA reductase activity in these cells are not due to differences in the intracellular accumulation of ML236B.

Kinetic Properties of HMG-CoA Reductase from CHO-K1, Variant-NS, and Variant-ML Cell Lines—One possible mechanism for cellular resistance to an enzyme inhibitor and for apparent increases in enzyme activity would be for the cell to produce an enzyme that has altered kinetic properties with respect to substrate and inhibitors. While this seems somewhat unlikely in the case of a competitive inhibitor, we examined this possibility by determining the kinetic properties of the enzyme prepared from CHO-K1 and the variant cell lines. The calculated K_m values for HMG-CoA are 30 μM (average of three determinations) for enzyme from CHO-K1 cells and 55 μM (average of three determinations) for the enzyme from variant-NS. We do not consider these values to differ significantly. The K_I values for ML236B for enzyme from CHO-K1 and variant-NS are 1.9×10^{-8} M and 2.3×10^{-8} M, respectively. It is clear that the resistance of variant-NS, and presumably variant-ML as well, to ML236B is not attributable to lowered sensitivity of the enzyme to the inhibitor.

Synthesis of Cholesterol by CHO-K1 and Variant Cells—The effect of ML236B on the rates of cholesterol and fatty acid synthesis was examined and the results are presented in Table II. CHO-K1 and variant cell lines were assayed both in the presence and absence of ML236B. Cells grown in the presence of ML236B were changed to culture medium containing no inhibitor 1 h prior to incubation with the radioactive precursor to allow full expression of HMG-CoA reductase. Parental CHO-K1 cells never exposed to ML236B received the inhibitor 1 h prior to the incubation period to ensure full inhibition of the enzyme.

Initially, [^{14}C]acetate was used to measure the rates of cholesterol and fatty acid synthesis. When CHO-K1 cells were exposed to 8 μg/ml of ML236B, cholesterol synthesis was inhibited by 95% while the rate of fatty acid synthesis was unaffected. However, under the same assay conditions the variant cell lines displayed elevated rates of both cholesterol and fatty acid synthesis. Specifically, the rates of acetate incorporation into cholesterol and fatty acids for the variant cell lines were 5 times those of CHO-K1 cells incubated in the absence of inhibitor. When the variant cell lines were incubated in the absence of ML236B, the rate of cholesterol synthesis increased to 35 to 40 times that of parental CHO-K1 cells.

The results obtained with [^{14}C]acetate as the metabolic precursor are unclear. The findings suggest that variant cells maintained on ML236B should contain approximately 5 times the levels of cholesterol found in parental cells. However, this "extra" cholesterol was not detected by gas chromatographic analysis of total cellular cholesterol (Table III). There was virtually no cholesterol found in the media after the incubation period (data not shown). It is possible that there are acetate pool fluctuations that account for these results even

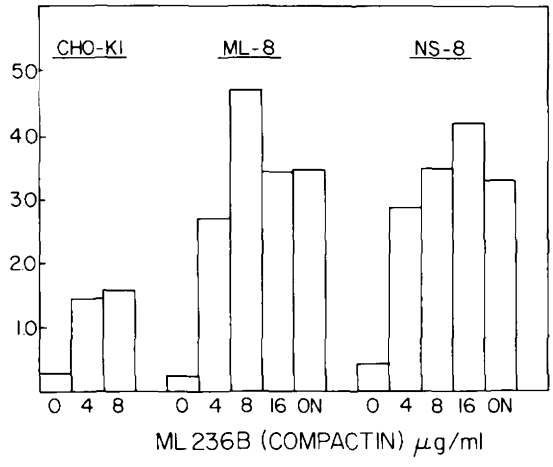

FIG. 5. **HMG-CoA reductase activity of CHO-K1 and variant cells as a function of varying concentrations of ML236B in the culture medium.** Variant cells were grown for 3 days in the absence of ML236B. Variant and CHO-K1 cells were trypsinized and inoculated at 1×10^6 cells/flask into three flasks (75 cm^2) in growth medium containing no ML236B. Cells were incubated for an additional 24 h. Two flasks (one of each variant) were maintained throughout the course of the experiment in media containing 8 μg/ml of ML236B. These control flasks are designated "ON." Experiment flasks were fed with fresh media containing either 0, 4, 8, or 16 μg/ml of ML236B. Cells were fed twice during a 4-day incubation period with the last feeding 24 h before assay. Cell extracts were prepared and assayed for HMG-CoA reductase as described under "Experimental Procedures."

TABLE II

[^{14}C]Acetate versus ^3H_2O incorporated into total cellular cholesterol and fatty acids by CHO-K1 and variant cells

One h prior to the incubation with radioactive precursor, the subconfluent cell monolayers received the appropriate medium, plus or minus 8 μg/ml of ML236B. At the onset of the incubation period, each monolayer received the appropriate media plus 1 mM [^{14}C]-acetate (0.5 μCi/ml) or 10 mCi of ^3H_2O. The incubation period lasted for 1 h in the case of [^{14}C]acetate incorporation and 6 h in the case of ^3H_2O incorporation. The cells were harvested for analysis of their respective ^{14}C- or ^{3}H-labeled total cellular cholesterol and fatty acid content. Each value represents the average of duplicate incubations. For additional details, see "Experimental Procedures."

Cell type	ML236B (8 μg/ml)	[^{14}C]Acetate incorporation		^3H_2O incorporation	
		Cholesterol	Fatty acids	Cholesterol	Fatty acids
		cpm/min/mg		*cpm/h/mg*	
CHO-K1[a]	–	190 ± 19	540 ± 10	650 ± 3	1500 ± 50
CHO-K1[b]	+	10 ± 1	600 ± 10	28 ± 3	1400 ± 2
Variant-NS[c]	–	6700 ± 100	2100 ± 40	2500 ± 80	1500 ± 60
Variant-NS[d]	+	1000 ± 60	2900 ± 70	660 ± 10	1300 ± 10
Variant-ML[c]	–	8000 ± 80	3000 ± 70	ND	ND
Variant-ML[d]	+	1000 + 40	2900 ± 30	650 ± 7	1300 ± 5

[a] ML236B was absent from both the growth medium and the incubation medium.

[b] ML236B was absent from the growth medium, but present in the medium 1 h prior to and during the incubation period.

[c] ML236B was present in the growth medium, but absent from the medium 1 h prior to and during the incubation period.

[d] ML236B was present in both the growth medium and in the incubation medium.

TABLE III

Effect of ML236B on cellular cholesterol levels

Cells were innoculated at a cell density of 1.5 to 2.0 × 10^6 cell/flask (150 cm^2) into growth medium containing the indicated concentrations of ML236B. Cells were incubated for 3 days to approximately 80% confluency. All flasks had been fed 24 h prior to harvesting. Cell cholesterol determinations are described under "Experimental Procedures."

Cell type	Concentration of ML236B in the growth media	Cholesterol/protein[a]
	μg/ml	*μg/mg*
CHO-K1	0	7.3 (1.6)
CHO-K1	8[b]	3.2
Variant-ML	0[c]	11.2 (1.1)
Variant-ML	8	10.0 (2.6)
Variant-NS	0[c]	13.0 (5.6)
Variant-NS	8	8.4 (1.8)

[a] The values presented are the average of three determinations with the maximum range indicated in parentheses. The value for the CHO-K1 + 8 μg/ml of ML236B is the result of a single determination.

[b] Grown in the presence of 8 μg/ml of ML236B for 3 days.

[c] Grown in the absence of ML236B for 7 days.

though we used [^{14}C]acetate concentrations that were saturating. It is also possible that there has been an alteration in some other enzyme(s) of acetate metabolism.

This apparent anomaly prompted us to use another metabolic assay, ^3H_2O uptake, to either corroborate or negate these findings. The data from the ^3H_2O incorporation studies (Table II) are consistent with the results from total cellular lipid analysis. Clearly, both variant cell lines have the same rates of total cellular cholesterol and fatty acid synthesis in the presence of 8 μg/ml of ML236B as do CHO-K1 cells in the absence of inhibitor. The rate of fatty acid synthesis remained unchanged in the presence of ML236B. Variant-NS incubated in the absence of inhibitor displayed a rate of cholesterol synthesis 4 times that of parental CHO-K1. This increased rate presumably reflected the unmasking of the inhibitor-bound HMG-CoA reductase and quantitatively agreed with the elevated specific activity of HMG-CoA reductase in the variant cells.

Cholesterol Levels of the CHO-K1, Variant-NS, and Variant-ML Cell Lines—From the data presented, we conclude that the elevated levels of HMG-CoA reductase in variant cell lines permit the synthesis of sufficient mevalonic acid and other mevalonic acid-derived products in the presence of ML236B. This conclusion would predict that the variant cell lines have sufficient cholesterol for cell growth. Analysis of total cellular lipid was performed and the data are presented in Table III. After 3 days growth in the presence of 8 μg/ml of ML236B the cholesterol level in CHO-K1 cells dropped to less than 50% of the level for cells grown in the absence of ML236B. Variant cells grown in the presence of ML236B had cholesterol levels essentially the same or slightly higher than CHO-K1 cells grown in the absence of ML236B. Thus, there is qualitative agreement among the increases in HMG-CoA reductase activity, cholesterol biosynthesis, and cellular cholesterol levels. The results further demonstrate that the variant cell lines are not sterol-deficient.

DISCUSSION

We demonstrate that the cytotoxicity of ML236B to CHO-K1 cells at a concentration of 8 μg/ml in the culture medium can be prevented by the addition of either mevalonate or cholesterol (Fig. 1). At a concentration of 50 μg/ml of ML236B in the culture medium, cholesterol alone is ineffective in preventing cell death; however, mevalonate remains effective. Addition of other mevalonate-derived metabolites to the culture medium along with cholesterol including ubiquinone, dolichol, and isopentenyladenine, did not prevent the toxic effect of ML236B. It is not clear, however, that the addition of these hydrophobic compounds to the culture medium leads to their proper entry or targeting. There is also the interesting possibility that a certain level of endogenous cholesterol synthesis is required for cell growth. The important conclusion from the present results is that even at the high concentrations of ML236B used for the selection of resistant cells, the site of growth inhibition remains HMG-CoA reductase.

We have selected two cell lines which are resistant to the cytotoxic effects of ML236B and characterized them with regard to HMG-CoA reductase levels, cholesterol biosynthetic rates, and cellular cholesterol levels. The results suggest that the variant cell lines are resistant to the cytotoxic effects of ML236B because of their ability to overaccumulate amounts of HMG-CoA reductase which are sufficient to overcome the inhibition of mevalonic acid production. We base this conclusion on the following lines of evidence. 1) The variant cell lines have 2- to 6-fold higher levels of HMG-CoA reductase activity than CHO-K1 cells have when all cells are grown in the presence of ML236B (Table I, Fig. 4). 2) At the concentration of ML236B that inhibits cholesterol synthesis by 95% in CHO-K1 cells, the variant cells exhibit rates of cholesterol synthesis comparable to the uninhibited rates of CHO-K1 cells (Table II). 3) The cholesterol levels of the variant cells grown in the presence of ML236B are equivalent to or slightly greater than the levels for the CHO-K1 cells grown in the absence of ML236B (Table III).

While the increase in HMG-CoA reductase activity is the probable explanation for the cells resistance to ML236B, the mechanism of this increase is not clear. Several possible explanations exist including changes in the amount of enzyme, changes in the activity of pre-existing enzyme or alteration of enzyme structure and catalytic properties. It seems unlikely that the results can be explained by an alteration of the ratio of inactive enzyme to active enzyme. The method used to prepare the extracts does not include fluoride which is required to maintain the enzyme in the inactive phosphorylated state. Furthermore, the addition of *E. coli* alkaline phosphatase to the cell extract caused no increase in enzyme activity. It is also unlikely that the kinetic properties of the enzyme have been altered, since the K_I and K_m values of the enzyme from resistant cells for ML236B and HMG-CoA, respectively, appear unaltered. Thus, the most likely explanation for the observed increase in enzyme activity would be an increase in the amount of enzyme. This increase in amount of HMG-CoA reductase could result from increased synthesis and/or decreased degradation. One possible mechanism for increased enzyme synthesis is a gene amplification. This seems possible if the decrease in activity seen after removal of ML236B is really a regulatory response to overproduction of mevalonate metabolites rather than a decrease in an amplified gene number. We are currently attempting to distinguish these possibilities.

While the observed increase in HMG-CoA reductase seems the most likely explanation for the resistant phenotype, there are other possibilities which invoke more indirect effects such as changes in cellular uptake of ML236B or the presence of a ML236B detoxification mechanism. Both of these possibilities seem unlikely since addition of ML236B to the culture medium of both resistant and sensitive cells induces an the increase in HMG-CoA reductase activity. Thus, ML236B must enter the resistant cells and remain effective.

Even though the mechanism of the ML236B resistance in the variant cell lines remains unclear, an important practical outcome of this work is that we now have available cell lines

which produce 20- to 60-fold more HMG-CoA reductase than other available cell lines. This will simplify the task of purifying HMG-CoA reductase from these cultured cells, and most importantly, these cell lines or subsequent isolates should provide us with the tools necessary for studying the molecular basis for regulation of HMG-CoA reductase.

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