Inhibition by 6-Fluoromevalonate Demonstrates That Mevalonate or One of the Mevalonate Phosphates Is Necessary for Lymphocyte Proliferation*

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The sterol synthesis inhibitor 6-fluoromevalonate (Fmev) was used to explore the role of mevalonate products in lymphocyte proliferation. Fmev blocks the synthesis of isopentenyl pyrophosphate and all more distal products in the sterol pathway. When cells were cultured in lipoprotein-deficient medium, Fmev (200 μM) completely inhibited mitogen-stimulated human lymphocyte proliferation, quantified by measuring DNA synthesis. The addition of low density lipoprotein (LDL) restored lymphocyte responses to normal, whereas mevalonate was totally ineffective. Similar results were obtained with concentrations of Fmev up to 1 mM. These results contrast with those observed when sterol biosynthesis was blocked with lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. When lymphocyte proliferation was blocked with lovastatin (5 μM), either high concentrations of mevalonate or LDL together with low concentrations of mevalonate was required to restore responses. In contrast, neither LDL nor low concentrations of mevalonate when alone was able to restore lymphocyte DNA synthesis in cultures blocked with 5 μM lovastatin. The effect of Fmev on the capacity of exogenous mevalonate to restore proliferation of lovastatin-blocked lymphocytes was directly examined. Fmev had no effect on the capacity of LDL plus low concentrations of mevalonate to restore DNA synthesis to lovastatin-blocked lymphocytes, indicating that the synthesis of the necessary factor from mevalonate was unaltered by Fmev. Fmev profoundly blocked lymphocyte endogenous sterol synthesis, decreasing incorporation of radiolabeled acetate into digitonin-precipitable sterols by up to 98%. LDL did not alter the capacity of Fmev to block sterol synthesis. The possibility that Fmev allowed shunting of endogenous mevalonate into essential lipid products was assessed by examining the incorporation of radiolabeled mevalonate. Fmev (200 μM) inhibited the incorporation of mevalonate into all lipids, including ubiquinone, dolichol, and other non-sterol lipids by up to 98%, and this was not altered by LDL. Furthermore, Fmev (200 μM) suppressed the incorporation of radiolabeled mevalonate into protein by up to 97%. These data confirm that a product of mevalonate is essential for cell proliferation. However, the results indicate that the required product is directly synthesized from mevalonate or mevalonate phosphates rather than from a more distal isoprenoid metabolite.

Proliferation of all cells is known to require at least two products of mevalonate metabolism. Although one of these has been clearly shown to be cholesterol (1-4), the other product has only been defined indirectly and has not been identified (5-9). Thus, when mevalonate synthesis is inhibited by blocking the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, cellular proliferation is completely prevented (5-7). The addition of exogenous mevalonate is able to restore proliferation (5-7). However, cholesterol alone is ineffective, thereby demonstrating the need for a second product of mevalonate metabolism other than cholesterol (5-7). The identification of the non-sterol product of mevalonate that is necessary for cellular proliferation has proven to be difficult.

Among the mevalonate-derived products that may be important for cellular proliferation are transfer RNA species, isoprenoids, and proteins that are post-translationally modified by mevalonate-derived moieties (10-12). Studies in baby hamster kidney cell lines initially suggested that adenine, containing an isopentenyl group originating from mevalonate, was the factor necessary for DNA synthesis (13). However, the capacity of isopentenyl adenine to rescue proliferation of cells blocked by inhibitors of HMG-CoA reductase has not been confirmed, suggesting that other metabolites of mevalonate might be required (14). Candidate compounds include the lipids, dolichol and ubiquinone, that are synthesized from isoprene groups originating from mevalonate (15-19). Dolichol plays a role in the glycosylation of proteins and ubiquinones are involved in the mitochondrial respiratory chain (20, 21). More recent studies have shown that certain proteins are post-translationally modified by the covalent attachment of mevalonate-derived isoprene groups (22-26). The identification of some of these proteins as nuclear lamin B (22, 23) and guanine nucleotide-binding proteins (24-26) has led to the speculation that one or more of these proteins may be the mevalonate product necessary for cellular proliferation (9, 23-26). None of these compounds, however, has been clearly identified as the mevalonate-derived factor required for cell growth.

* The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Fmev, 6-fluoromevalonate; LDL, low density lipoprotein; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.
Mitogen-stimulated lymphocytes provide an ideal model system for examining various aspects of sterol metabolism (27-29). The current studies utilized this model system to characterize the non-sterol product of mevalonate necessary for cellular proliferation. The effect of the sterol synthesis inhibitor, 6-fluoro-2-mevalonate (Fmev) (30, 31), that blocks decarboxylation of mevalonate-5-pyrophosphate to isopentenyl pyrophosphate (32, 33), was compared with lovastatin, an inhibitor of HMG-CoA reductase (34). The results are consistent with the conclusion that the mevalonolactone itself or one of the mevalonate phosphates is the direct precursor of the metabolite required for lymphocyte proliferation.

MATERIALS AND METHODS

Cell Preparation and Culture—Peripheral blood mononuclear cells (PBMC) were isolated from anticoagulated venous blood of normal adults and from a patient with low density lipoprotein (LDL) receptor-negative homozygous familial hypercholesterolemia, as described previously (28, 29). Cells were cultured in RPMI 1640 medium (Hazelton Biologies Inc., Kansas City, MO) supplemented with 1% lipopolysome-poor plasma (prepared as described previously (29)). Phytomannoglutinin (PHA, Wellcome Reagents Ltd., Research Triangle Park, NC) or phytohemagglutinin (PHA, Sigma Chemical Co., St. Louis, MO) was used as the mitogenic stimulus for all experiments. Where indicated, cultures were further supplemented with Fmev, a generous gift of Dr. Gary Quistad (Sandoz Crop Protection, Palo Alto, CA), the sodium salt of mevalonate, lovastatin (Merck, Sharp and Dohme), or human LDL (d = 1.020-1.050 g/ml) prepared as described previously (29). Incubations were carried out in sterile microtiter plates for assays of lymphocyte DNA synthesis, in 17×100-mm polystyrene tubes for quantification of endogenous sterol synthesis and in tissue culture flasks for measurement of the incorporation of radiolabeled mevalonolactone. Lymphocyte DNA synthesis and rates of endogenous sterol synthesis were measured as described previously (29).

Incorporation of Mevalonate into Cellular Lipids and Proteins—PBMC were cultured with or without PHA, lovastatin, Fmev, and LDL as indicated in the individual experiments. Incorporation of radiolabeled mevalonolactone into lipid products was determined by a modification of the method of Faust et al. (16). Briefly, cells were incubated with [5-3H]mevalonolactone (specific activity varying between 10 and 1000 Ci/mmol; Du Pont-New England Nuclear) and harvested after 24-h incubation. Following extensive washing, cell pellets were solubilized and lipids extracted after the addition of unlabeled mass quantities of sterols and intermediates together with [14C]cholesterol or [14C]cholesterol ester (Du Pont-New England Nuclear). After evaporation to dryness, the extracted lipids were separated on polyethylene-layer chromatography, with development of chromatograms carried out in 100% chloroform. Lipids were identified by iodine visualization, and the chromatograms were subjected to fluorography after treatment with Enhance (Du Pont-New England Nuclear). The fluorographically identified and iodine-sensitive spots were cut out and counted by liquid scintillation spectrophotometry. The incorporation of radiolabeled mevalonolactone into various lipid products was then calculated and the recovery of added [14C]cholesterol or [14C]cholesterol ester was used to correct for procedural losses. The results are presented as picomoles incorporated per million cells cultures. In some experiments, after lipid extraction the aqueous phase was evaporated to dryness, resuspended in water/methanol (3:1, v/v), spotted onto polyethyleneimine-cellulose, and chromato- graphed using a 0.75 M Tris, 0.45 M NaCl solvent system. Radiolabeled standards that were co-chromatographed were [5-3H]mevalonolactone, [5-14C]mevalonate-5-phosphate (Amersham Corp.), and [5-14C] mevalonate-5-pyrophosphate (Amersham Corp.).

Incorporation of [5-3H]mevalonolactone into protein was determined by a modification of the method of Schmidt et al. (12). PBMC were incubated with [5-3H]mevalonolactone (specific activity 25 mCi/ 

RESULTS

Inhibition of Lymphocyte Proliferation by Lovastatin—PBMC were cultured in lipidprotein-deficient medium, and HMG-CoA reductase activity was inhibited with lovastatin. The addition of low concentrations of lovastatin (0.5 μM) decreased PHA-stimulated lymphocyte DNA synthesis, measured by the incorporation of [3H]thymidine, by 94%. A 10-fold higher concentration of lovastatin (5 μM) caused 96% inhibition of proliferation (Fig. 1). These concentrations of lovastatin inhibited PHA-stimulated lymphocyte sterol synthesis by 78% ± 3% (mean ± S.E., n = 8), and 96% ± 1% (mean ± S.E., n = 6), respectively. The addition of mevalonate (10 mM) restored lymphocyte DNA synthesis, regardless of the concentration of lovastatin used. In contrast, the addition of LDL cholesterol (10 μg/ml) completely prevented the inhibitory effect of low concentrations of lovastatin (0.5 μM) but was unable to restore reponsiveness inhibited by 5 μM lovastatin (Fig. 1). Increasing the LDL concentration to 50 μg/ml also was unable to restore proliferation of lymphocytes blocked by 5 μM lovastatin (data not shown). Inhibition reflected a suppression of cell proliferation and not cell death as there was no decrease in the number of cells originally cultured.

The ability of limiting concentrations of mevalonate to restore lymphocyte proliferation was also examined (Fig. 2). Added alone, mevalonate (1 mM) could not support lymphocyte DNA synthesis when HMG-CoA reductase was inhibited with either concentration of lovastatin. However, the addition of mevalonate (1 mM) together with LDL cholesterol (10 μg/ml), restored lymphocyte DNA synthesis to control levels regardless of the concentration of lovastatin. These results demonstrated that PHA-stimulated lymphocytes required a soluble supernatant, the extracted lipids and the remaining precipitate was counted by liquid scintillation spectroscopy. The results are presented as femtomoles incorporated into the various fractions per million cells cultured. In some experiments, the lipid-extracted precipitate was solubilized by heating in sample buffer containing 2% (w/v) SDS, 50 mM 2-mercaptoethanol and 25 mM NaOH. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (35), using a 4% polyacrylamide stacking gel and a 12.5% polyacrylamide separating gel. Gels were stained with Coomassie Blue, destained, permeated with Enhance, dried, and exposed to Kodak XAR-5 film at -80 °C for 7–14 days with intensifying screens.

Fig. 1. Mevalonate but not LDL restores lymphocyte proliferation blocked by 0.5 μM lovastatin. PBMC were cultured in lipoprotein-deficient medium, with or without PHA, LDL (10 μg cholesterol/ml), and mevalonate (10 mM) as indicated. HMG-CoA reductase activity was inhibited with lovastatin (0.5 or 5 μM) as shown. Lymphocyte DNA synthesis was assayed after 4 days by measuring the incorporation of [3H]thymidine. In the absence of PHA, DNA synthesis was <500 cpm and was unchanged by additions. Results are mean ± S.E. of triplicate determinations.
DNA synthesis (Fig. 3), whereas mevalonate over a broad addition of Fmev inhibited PHA-stimulated lymphocyte DNA synthesis. PBMC were cultured in lipoprotein-deficient medium, the receptors. Thus, LDL could not restore proliferation of Fmev-cholesterol always prevented the inhibition of lymphocyte proliferation by Fmev, whereas mevalonate did not. Regardless of the concentration of Fmev employed, LDL cholesterol alone sufficed to restore lymphocyte DNA synthesis.

Reversal of Fmev mediated inhibition of lymphocyte DNA synthesis by LDL required the presence of functional LDL receptors. Thus, LDL could not restore proliferation of Fmev-blocked PBMC from a patient with LDL receptor-negative familial hypercholesterolemia (Fig. 4). The effect of Fmev itself was comparable with that noted with normal PBMC, inhibiting responses by 89%. However, the capacity of LDL to overcome inhibition was minimal as proliferation remained inhibited by more than 70% despite supplementation of cultures with 50 μg/ml LDL cholesterol. By contrast, as little as 5 μg/ml LDL cholesterol overcame inhibition of the proliferation of normal PBMC by Fmev.

**Fmev Inhibits Lymphocyte DNA Synthesis**—In the next experiments, Fmev, an inhibitor that blocks endogenous sterol synthesis by preventing the decarboxylation of mevalonate-5-pyrophosphate to isopentenyl pyrophosphate (32, 33) and thus acts more distally than lovastatin, was examined. When PBMC were cultured in lipoprotein-deficient medium, the addition of Fmev inhibited PHA-stimulated lymphocyte DNA synthesis in a concentration-dependent manner (Fig. 3). LDL cholesterol totally restored PHA-stimulated lymphocyte DNA synthesis blocked by Fmev, whereas mevalonate did not. Regardless of the concentration of Fmev employed, LDL cholesterol always prevented the inhibition of lymphocyte DNA synthesis (Fig. 3), whereas mevalonate over a broad range of concentrations (data not shown) was ineffective.

Fig. 3. LDL always restores lymphocyte proliferation blocked by fluoromevalonate. PBMC were cultured in lipoprotein-deficient medium with or without PHA, LDL (50 μg cholesterol/ml), mevalonate (10 mM), mevalonate (10 mM), and varying concentrations of Fmev as shown. [3H]Thymidine incorporation was assayed after 4 days. Results are mean ± S.E. of triplicate determinations.

Mevalonate and Lymphocyte Proliferation

**Fmev Inhibits Lymphocyte Sterol Synthesis**—The effect of Fmev on the endogenous synthesis of sterols from labeled acetate was next examined. These experiments were undertaken to estimate the ability of Fmev to block the conversion of endogenously synthesized mevalonate to metabolic products. Measuring sterol synthesis was a convenient means to estimate the rate of incorporation of mevalonate into the major metabolic products. As seen in Fig. 5, Fmev (200 μM) inhibited [1-14C]acetate incorporation into digitonin-precipitable sterols by 93 ± 2% (mean ± S.E., n = 4) in unstimulated PBMC. In PHA-stimulated cells, 200 μM Fmev inhibited [1-14C]acetate incorporation by 94 ± 1% and was thus as effective as high concentrations of lovastatin (see above). Furthermore, 1 mM Fmev inhibited endogenous sterol synthesis by 98 ± 1% (Fig. 5). These results indicate that Fmev prevented synthesis of sterols from acetate precursors nearly completely and was therefore as potent an inhibitor of endogenous sterol biosynthesis as lovastatin. The inhibitory effect of Fmev was similar when LDL was present. Thus, the addition of LDL (50 μg of cholesterol/ml) decreased endogenous sterol synthesis by 80% (6.9 ± 0.1 → 1.4 ± 0.1 pmol/h/10^6 cells) in unstimulated cells and by 70% (161.2 ± 0.5 → 49.1 ± 0.8 pmol/h/10^6 cells) in PHA-stimulated PBMC. Fmev (1 mM) inhibited cholesterol biosynthesis in PHA-stimulated cells cultured in lipoprotein-deficient medium with or without PHA, fluoromevalonate (200 μM), and LDL in varying concentrations as indicated. [3H]Thymidine incorporation was assayed after 4 days. Results are mean ± S.E. of triplicate determinations.

Fig. 4. LDL receptors are necessary for restoration of proliferation by lymphocytes blocked with fluoromevalonate. PBMC obtained from a normal control and from a patient with LDL receptor-negative familial hypercholesterolemia (FH) were cultured in lipoprotein-deficient medium with or without PHA, fluoromevalonate (200 μM), and LDL in varying concentrations as indicated. [3H]Thymidine incorporation was assayed after 4 days. Results are mean ± S.E. of triplicate determinations.

Fig. 5. Fluoromevalonate inhibits endogenous sterol synthesis. PBMC were cultured in lipoprotein-deficient medium with or without PHA and fluoromevalonate (200 μM or 1 mM) as indicated. After 24 h, rates of endogenous sterol synthesis were measured by the incorporation of [1-14C]acetate into digitonin-precipitable sterols. Results are expressed as picomoles ([14C]acetate incorporated into digitonin-precipitable sterols)/h/10^6 cells and are mean ± S.E. of four separate experiments.
deficient medium by 98% (161.2 ± 0.5 → 3.2 ± 0.1 pmol/h/10⁶ cells) and decreased rates of endogenous sterol synthesis by 99% in LDL-containing cultures (49.1 ± 0.8 → 0.6 ± 0.1 pmol/h/10⁶ cells). In unstimulated PBMC, Fmev completely blocked cholesterol synthesis both in lipoprotein-deficient medium (6.9 ± 0.1 → 0.1 ± 0.1 pmol/h/10⁶ cells) and with LDL supplementation (1.4 ± 0.1 → 0.1 ± 0.1 pmol/h/10⁶ cells). The data thus suggest that 6-fluoromevalonate is an extremely potent inhibitor of endogenous mevalonate metabolism and indeed is equivalent to lovastatin in its ability to block the production of sterol lipid metabolites of mevalonate.

**Fmev Does Not Alter the Capacity of Exogenous Mevalonate to Support Proliferation of Lovastatin-blocked Lymphocytes**—The next experiments were carried out to explore the effect of Fmev on the synthesis of the putative mevalonate-derived product necessary for lymphocyte proliferation directly. In these studies, lymphocytes were cultured in lipoprotein-deficient medium and endogenous sterol synthesis was blocked by suppressing HMG-CoA reductase activity with high concentrations of lovastatin (5 µM) or with Fmev (1 mM) or both. The inhibitory effects of this concentration of lovastatin could be overcome by large concentrations of mevalonate (10 mM), but not by LDL cholesterol alone (Fig. 6), whereas the inhibitory effects of Fmev alone could be reversed by LDL cholesterol alone, but not mevalonate.

By adding Fmev to cultures also blocked with lovastatin, the ability of exogenous mevalonate to provide the factor necessary for proliferation of lymphocytes could be assessed. When both inhibitors were present, LDL cholesterol alone could not restore DNA synthesis, demonstrating again the requirement for a non-sterol product of mevalonate when large concentrations of lovastatin are employed (Fig. 6). In addition, 10 mM mevalonate alone was unable to restore lymphocyte responses, confirming the ability of Fmev to inhibit the conversion of mevalonate to sterols. In contrast, the addition of both LDL cholesterol (10 µg/ml) and limiting concentrations of mevalonate (1 mM) could restore lymphocyte responses blocked by lovastatin and Fmev (Fig. 6). These results demonstrate that Fmev does not prevent conversion of mevalonate into the non-sterol product necessary for cellular proliferation.

**Fmev Blocks Mevalonate Incorporation into Cellular Lipids**—The following experiments explored the effect of Fmev on the synthesis of lipid products other than sterols. PBMC were cultured in lipoprotein-deficient medium and incubated with [5-3H]mevalonolactone for 24 h in the presence or absence of PHA, LDL, or inhibitors. The incorporation of radiolabeled mevalonolactone into cellular lipids was assessed.

As seen in Fig. 7, mevalonolactone was incorporated into cholesterol, intermediates in the sterol metabolic pathway, and into ubiquinone and dolichol, in both unstimulated and PHA-stimulated PBMC. Stimulation of PBMC with PHA only modestly increased incorporation (Figs. 7–9). LDL decreased the incorporation of labeled mevalonolactone into total lipids, by 23.5 ± 7.0% (mean ± S.E., n = 5) in unstimulated cells and by 25.2 ± 7.8% in PHA-stimulated PBMC. This down-regulatory effect of LDL could be accounted for by a decrease in incorporation of labeled mevalonate into sterols, with 42.6 ± 6.3% inhibition in unstimulated cells and 37.0 ± 7.3% inhibition in PHA-stimulated PBMC. By contrast, LDL caused a moderate increase in the incorporation of mevalonate into cholesteryl esters and a decrease in the incorporation into ubiquinone and dolichol, with modest increases and decreases observed with PBMC isolated from different individuals (Fig. 7).

Fmev (200 µM) decreased synthesis of all mevalonate-derived cellular lipids (Figs. 7–9). In unstimulated and PHA-stimulated PBMC, Fmev decreased the incorporation of labeled mevalonate into cholesterol, intermediates, and dolichol. In these experiments, Fmev inhibited the conversion of mevalonate to sterols, with modest increases and decreases observed with PBMC isolated from different individuals (Fig. 7).

**FIG. 6.** Fluoromevalonate does not alter the capacity of exogenous mevalonate to support proliferation of lovastatin-blocked lymphocytes. PBMC were cultured in lipoprotein-deficient medium with or without PHA and inhibitors (lovastatin 5 µM or fluoromevalonate 1 mM) as indicated. Cultures were supplemented as shown with mevalonate alone (10 mM) or LDL alone (10 µg cholesterol/ml) and mevalonate (1 mM). [3H]Thymidine incorporation was assayed after 4 days in culture. Results are mean ± S.E. of six separate experiments.

**FIG. 7.** Fluoromevalonate blocks mevalonate incorporation into cellular lipids. PBMC were cultured in lipoprotein-deficient medium containing 1 mM [3H]mevalonolactone, with or without PHA, LDL (50 µg cholesterol/ml), and fluoromevalonate (200 µM) as indicated. After 24 h, cellular lipids were isolated, separated by thin-layer chromatography, and fluorograms prepared. The nonpolar lipid fraction included squalene (at the solvent front) and cholesteryl ester; the intermediate lipid fraction contained ubiquinone and dolichol, with unidentified digitonin-precipitable sterol migrating between them. **Upper panel,** representative experiment using [3H]cholesterol as an internal recovery standard; **Lower panel,** representative experiment using [14C]cholesterol as an internal recovery standard.
Fmev also Blocks Mevalonate Incorporation into Protein—The final experiments examined the possibility that incorporation of exogenous mevalonate was decreased to 10\% of Fmev was least effective at blocking incorporation of labeled mevalonate into 3\'-OH sterols and polar lipids (Fig. 9), with a mean inhibition of 85.5-90.8% in three separate experiments when 1 mM mevalonate was used. In these experiments, incorporation into cholesterol was also measured separately from incorporation into lanosterol and the unidentified 3\'-OH sterol that migrates between lanosterol and cholesterol (see Fig. 7). Fmev inhibited mevalonate incorporation into cholesterol by 82.6 \pm 1.7% and into the other 3\'-OH sterols by 92.9 \pm 0.6%. When the concentration of exogenous mevalonate was decreased to 1 \mu M, Fmev suppressed mevalonate incorporation into cholesterol by 98.4 \pm 0.5% (mean \pm S.E., n = 2) and similarly inhibited incorporation into other 3\'-OH sterols (data not shown). Of note, lovastatin had no effect on the incorporation of mevalonolactone into cellular lipids and did not alter the inhibitory effect of Fmev (data not shown). These experiments demonstrate that mevalonate does not become preferentially incorporated into any lipid product other than cholesterol in the presence of Fmev.

FIG. 8. Inhibition of mevalonate incorporation into lipids by fluoromevalonate. PBMC were cultured in lipoprotein-deficient medium containing 1 mM [3H]mevalonolactone (upper panel) or 10 \mu M [3H]mevalonolactone (lower panel) with or without PHA, LDL (50 \mu g cholesterol/ml), and fluoromevalonate (200 \mu M) as indicated. After 24 h, cellular lipids were isolated and quantified. Results are expressed as picomoles ([3H]mevalonate incorporated into all lipids)/10^6 cells cultured and are mean \pm S.E. of five (upper panel) and two (lower panel) separate experiments. Numbers in parentheses are percentage inhibition.

FIG. 9. Fluoromevalonate blocks incorporation of mevalonate into each lipid fraction. PBMC were cultured in lipoprotein-deficient medium containing 1 mM [3H]mevalonolactone, with or without PHA, LDL (50 \mu g cholesterol/ml), and fluoromevalonate (200 \mu M) as indicated. After 24 h, cellular lipids were isolated, separated, and quantified. Lipids were grouped into nonpolar, intermediate, sterol, and polar fractions as indicated in Fig. 7. Results are expressed as picomoles ([3H]mevalonate incorporated into all lipids)/10^6 cells cultured and are mean \pm S.E. of three separate experiments. Numbers in parenthesis (fluoromevalonate, right panel) are percentage inhibition.

FIG. 10. Fmev blocks the incorporation of mevalonate into all cellular fractions. PBMC were cultured in lipoprotein-deficient medium containing 0.4 \mu M [3H]mevalonolactone, with or without PHA and fluoromevalonate (200 \mu M) as indicated. After 24 h, washed cells were separated into trichloroacetic acid soluble (Aqueous) and insoluble (Lipid and Protein) fractions. Lipids were extracted from the insoluble fraction and each fraction was quantified. Results are expressed as femtomoles ([3H]mevalonate incorporated into each fraction)/10^6 cells cultured and are mean \pm S.E. of three separate experiments.

As shown in Fig. 10, Fmev (200 \mu M) inhibited mevalonate incorporation into lipid by 97.4 \pm 1.0\% (mean \pm S.E., n = 3) in unstimulated PBMC and by 97.5 \pm 1.6% in PHA-induced cells. The effect of Fmev on mevalonate incorporation into protein was similarly suppressed (94.2 \pm 1.8% in unstimulated PBMC and 97.4 \pm 0.5% in PHA-induced cells). When cultures were supplemented with LDL, the results were similar (data not shown). In contrast, mevalonate incorporation into the aqueous fraction was markedly enhanced by Fmev, increasing by 19.4 \pm 8.2-fold in quiescent cells and by 25.5 \pm 7.2-fold in PHA-stimulated PBMC. When analyzed by polyethylene-imine chromatography, the vast majority of the material in the aqueous fraction could be accounted for by cell-associated mevalonate 5-phosphate and mevalonate 5-pyrophosphate as...
well as mevalonate; in the absence of Fmev, only labeled mevalonate was detected (data not shown). These results demonstrate that Fmev causes the accumulation of phosphorylated derivatives of mevalonate and not only blocks mevalonate incorporation into cellular lipids but also incorporation of mevalonate into protein.

The inhibitory effect of Fmev on protein labeling by [3H] mevalonate was additionally examined by gel electrophoresis of lipid-extracted trichloracetic acid precipitates (Fig. 11). Mevalonate labeling of proteins was observed in unstimulated PBMC (lane 1), with the majority of the labeled proteins migrating between 16,000 and 24,000 daltons. PHA stimulation increased mevalonate incorporation into proteins by 2-fold (lane 2), with a particularly striking increase in the labeling of a protein with migration characteristics similar to that of nuclear lamin B (22, 23). Fmev completely inhibited mevalonate incorporation into all proteins (lanes 3–6) and prevented the increase in labeling associated with PHA stimulation (Figs. 10 and 11). Thus, Fmev effectively blocked the incorporation of mevalonate into both cellular lipids and cellular proteins in unstimulated and PHA-stimulated PBMC.

**DISCUSSION**

In the current studies, a new approach was used to characterize the non-sterol product(s) of mevalonate essential for cellular proliferation. When mevalonate metabolism was blocked at the level of conversion of mevalonate 5-pyrophosphate to isopentenyl pyrophosphate by a fluorinated derivative of mevalonate, Fmev, synthesis of lipids and mevalonate-labeled proteins was inhibited almost completely. However, synthesis of the non-sterol product(s) of mevalonate necessary for cell growth was not inhibited, since proliferation was always restored by supplementing the medium with LDL cholesterol alone. In contrast, mevalonate itself was unable to restore growth to Fmev-blocked cells. The unexpected finding that LDL cholesterol completely reversed all the inhibitory effects of Fmev suggested that either mevalonate or one of its phosphorylated products, mevalonate 5-phosphate and mevalonate 5-pyrophosphate, was the factor or the direct precursor of the factor necessary for cellular proliferation. This conclusion was supported by the finding that Fmev had no effect on the ability of exogenous mevalonate to provide the necessary factor to lovastatin-blocked cells. These results indicate that a previously unsuspected product of mevalonate metabolism more proximal than isopentenyl pyrophosphate is required for lymphocyte proliferation.

The existence of two important products of mevalonate necessary for cellular proliferation has been suggested by previous results (5–9). Thus, a number of investigators have demonstrated that inhibition of the endogenous synthesis of mevalonate by inhibiting HMG-CoA reductase activity blocks cell proliferation when no exogenous sources of cholesterol are present (1–4). In our previous studies, we have demonstrated that mitogen-stimulated T lymphocytes are able to utilize lipoproteins as a source of cholesterol when endogenous sterol synthesis is blocked (28, 29). The existence of another product of mevalonate necessary for cellular proliferation became apparent after the introduction of extremely potent inhibitors of the endogenous synthesis of mevalonate. These inhibitors, such as compactin and lovastatin, were able to inhibit endogenous synthesis of mevalonate almost completely (5, 8, 9, 34). Under these conditions, cholesterol alone could not restore proliferation (5–7), indicating that another product of mevalonate metabolism had to be actively synthesized for cellular proliferation to proceed. Investigation of other products of mevalonate metabolism, in addition to sterols, led to the identification of a number of substances potentially necessary for cellular proliferation (10–12). These include transfer RNA modified with an isopentenyl group and the polyisoprenoids, dolichol and ubiquinone. However, none of these is consistently able to restore proliferation when endogenous mevalonate metabolism is suppressed. More recently, cellular proteins modified post-translationally by covalent linkage to mevalonate-derived isoprenyl groups have also been described. Identification of some of these proteins as nuclear lamins (22, 23) and guanine nucleotide-binding proteins such as the cellular homologue of the ras proto-oncogene (24–26) raises the possibility that the non-sterol product of mevalonate necessary for cellular proliferation may be a mevalonate-modified protein.

Mitogen-stimulated PBMC are similar to other cells in that they require both cholesterol and a non-sterol product of mevalonate for continuing proliferation. Thus, when endogenous synthesis of mevalonate was blocked by a high concentration of lovastatin (5 μM) in PBMC, LDL cholesterol did not suffice to restore mitogen-stimulated DNA synthesis. Exogenous mevalonate, when provided at a large enough concentration, was able to restore cellular responses. The addition of LDL cholesterol together with a limiting concentration of mevalonate was also able to support DNA synthesis by PHA-stimulated PBMC, although neither alone was effective. Thus, mitogen-stimulated PBMC are similar to other cultured cells and provide a model system to evaluate the mevalonate products necessary for cellular DNA synthesis and proliferation.

The approach used in our studies was to utilize another sterol synthesis inhibitor. The mevalonate analogue, 6-fluoromevalonate, unlike lovastatin, only inhibited cellular responses by blocking synthesis of cholesterol. Fmev thus differs markedly from lovastatin in its effects on cellular proliferation and mevalonate metabolism, although the two compounds

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**Fig. 11. Fmev blocks mevalonate labeling of proteins.**

PBMC were cultured in lipoprotein-deficient medium containing 0.4 μM [3H]mevalonolactone, with or without PHA and fluoromevalonate (200 μM) as indicated. After 24 h, washed cells were suspended in trichloroacetic acid and trichloroacetic acid precipitates obtained. Lipids were extracted from the precipitates, the proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorograms prepared. Upper panel, Coomassie Blue stain; lower panel, fluorograms.
prevent the inhibitory effects of Fmev, even when present at a small molar excess. This was demonstrated by the use of digitonin-precipitable sterols. Furthermore, at higher concentrations (1 mM), sterol synthesis was almost completely inhibited. Fmev also suppressed the accumulation of mevalonate-derived sterols, although not quite as completely as it inhibited acetate conversion to sterols. Additionally, Fmev inhibited the conversion of mevalonate to identifiable intermediates in the sterol pathway, including squalene, lanosterol, and a 3α-OH sterol transitional compound. These data indicate that Fmev is an extremely effective inhibitor of endogenous sterol synthesis and that the capacity of LDL alone to restore proliferation to Fmev-blocked cells is not likely to result from incomplete inhibition of mevalonate metabolism.

The intriguing finding that cholesterol alone was the only exogenous factor necessary to restore proliferation to lymphocytes blocked with any concentration of Fmev suggested that the non-sterol product of mevalonate necessary for proliferation was in fact derived directly from mevalonate and not from an isoprene compound. An alternative explanation, however, was that measurement of sterol synthesis did not accurately reflect synthesis of non-sterol products. Theoretically, shunting of mevalonate away from cholesterol may have allowed sufficient endogenously derived product to be formed, although insufficient cholesterol was synthesized. In support of this possibility is the evidence that, in fibroblasts, there is a relative enrichment of mevalonate conversion to ubiquinone when cells are cultured in the presence of exogenous LDL cholesterol (16). In a similar manner, the trace amounts of mevalonate escaping the effect of Fmev may have been preferentially incorporated into a putative essential lipid. In the current experiments, incorporation of radiolabeled mevalonolactone was used to examine this possibility. The results demonstrated that in human T lymphocytes, as opposed to fibroblasts, there was only modest regulation of the fate of mevalonate by LDL and that was largely confined to changes in the synthesis of sterols. When HMG-CoA reductase in PBMC was blocked with lovastatin, there was a similar lack of regulation of the fate of labeled mevalonate. Thus, the findings demonstrate that there is no shunting of mevalonate into an essential lipid other than cholesterol. Shunting of mevalonate therefore cannot explain the results.

Mitogenic stimulation did not consistently increase the incorporation of mevalonate into any lipid product, although enhanced labeling of ubiquinone and dolichol was observed in some individuals. The addition of LDL cholesterol only modestly affected the incorporation of mevalonate into sterols, generally increasing the ester fraction and decreasing the free sterol fraction. More importantly, in the presence of LDL there was no preferential shunting of mevalonate into intermediates or branch pathway products, regardless of the presence of lovastatin or PHA. These results suggest that, in PBMC, the pathways involved in the conversion of mevalonate to cholesterol and other lipid intermediates are less regulated because none is required for lymphocyte proliferation. In contrast to the lack of the effects of LDL and lovastatin, Fmev markedly suppressed the incorporation of radiolabeled mevalonate into all lipid products. There was no detectable evidence of shunting of radiolabeled mevalonate into any of the potential alternative pathways. Thus, the ability of LDL cholesterol alone to restore proliferation to cells blocked by Fmev could not be explained by shunting of mevalonate into a necessary lipid product. The absolute incorporation of [3H] mevalonolactone into lipids was highly dependent on the concentration of mevalonate. There was increased production of mevalonate-derived lipids as the concentration of mevalonate was increased until very high exogenous concentrations (5 mM) were reached. This observation is consistent with the conclusion that metabolic events subsequent to HMG-CoA reductase are not rate-limiting steps in endogenous mevalonate metabolism, since the enzymes beyond HMG-CoA reductase were only substrate limited. The relative incorporation of [3H] mevalonate into the various fractions was unaltered by substrate concentration or by the presence of lovastatin. As with the lack of effect of LDL on non-sterol lipid synthesis, these findings are consistent with the conclusion that none of the lipid fractions, apart from sterols, is more important than the others. Taken together, therefore, the data indicate that mevalonate itself or one of its phosphorylation products, mevalonate 5-phosphate or mevalonate 5-pyrophosphate, may be the metabolite required for lymphocyte proliferation. Whether the same product is also required for proliferation of other cell types remains to be determined.

One alternative explanation for the current results is that a product of mevalonate is required for protein modification and that generation of this product is less inhibited by Fmev than lipid intermediates (37). Covalent modification of a number of proteins with a mevalonate-derived lipid has been demonstrated (12, 22-26). The identification of some of these proteins as nuclear lamins and guanine nucleotide-binding proteins has led to the speculation that a mevalonate-modified
protein may be the essential mevalonate product necessary for cellular proliferation. Recent studies have demonstrated that the guanine nucleotide-binding protein p21 ras is covalently modified by the attachment of an isoprenyl-derived farnesyl moiety (24–26). The current experiments establish that PBMC also incorporate mevalonate into proteins. In addition they indicate that mitogenic stimulation increases mevalonate labeling of proteins. Fmev effectively blocked all mevalonate incorporation into proteins. Furthermore, at concentrations comparable with those employed in this report, Fmev inhibits mevalonate incorporation into isoprenylated proteins by 99% (36). Taken together with the results presented above, the findings indicate that the non-sterol product of mevalonate essential for cellular proliferation is neither a lipid nor an isoprenylated protein.

In summary, the data strongly suggest that mevalonate itself or one of its phosphorylated derivatives, mevalonate 5-phosphate or mevalonate 5-pyrophosphate, is the source of a cellular product needed for proliferation. Products of mevalonate distal to its decarboxylation to isopentenyl pyrophosphate are unlikely sources of this product.

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