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28. All $V_\gamma 9/V_\delta 2$ T cells react strongly to these antigens, whereas the majority of $V_\delta 1^+$ (including $V_\gamma 9/V_\delta 1$) T cells do not. Preliminary results suggest that very rare $V_\gamma 9^+/V_\delta 1^+$ T cell clones [as determined by MAbs (*11*)] may react with lower affinity.
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30. Proliferative assays were performed with 10^5 to 1.5×10^5 irradiated (120 Gy) stimulator cells and 5×10^4 to 10^5 responder T cells per flat bottom well of a 96 well microtiter plate. After 48 hours, the cells were pulsed with [^{3}H]thymidine (1 μCi per well) for 24 hours, the plates were harvested and the samples counted by liquid scintillation. The [^{3}H]thymidine uptake into the irradiated stimulator cells alone (wells without the responding T cells) was subtracted. For some clones, the proliferative response to Daudi and mycobacterial antigens is only detected in the presence of low concentrations of IL-2 (5 units/ml). When tested early after the initial clonal expansion, all $V_\gamma 9/V_\delta 2$ clones responded to Daudi and some mycobacterial antigens.
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Diet-Induced Hypercholesterolemia in Mice: Prevention by Overexpression of LDL Receptors

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The current studies were designed to determine whether chronic overexpression of low density lipoprotein (LDL) receptors in the liver would protect mice from the increase in plasma LDL-cholesterol that is induced by high-fat diets. A line of transgenic mice was studied that express the human LDL receptor gene in the liver under control of the transferrin promoter. When fed a diet containing cholesterol, saturated fat, and bile acids for 3 weeks, the transgenic mice, in contrast to normal mice, did not develop a detectable increase in plasma LDL. The current data indicate that unregulated overexpression of LDL receptors can protect against diet-induced hypercholesterolemia in mice.

DIETS HIGH IN CHOLESTEROL AND saturated fats increase the plasma level of low density lipoprotein (LDL), owing to a combination of increased cholesterol content of precursor particles, increased synthesis of particles, and decreased hepatic degradation (*1*). Degradation is mediated by LDL receptors, most of which are located on hepatocytes (*2*). When high-cholesterol diets are ingested, hepatic LDL receptors are suppressed, and this contributes to the subsequent elevation in plasma LDL levels (*3–5*).

The question arises as to whether forced overexpression of LDL receptors in liver would ameliorate or prevent diet-induced hypercholesterolemia. Although a stimulation of LDL receptor production is one means by which drugs lower plasma LDL concentrations (*2*), it is not known whether such an approach would succeed during feeding of a high-fat diet. Now we attempt to answer this question by using transgenic mice that abnormally overexpress human LDL receptors.

We have previously achieved overexpression of LDL receptors by injecting mouse eggs with a cDNA encoding the human LDL receptor under control of the mouse metallothionein-I promoter (designated transgene-1) (*6*). Mice expressing this transgene had high levels of LDL receptor activity in liver when the metallothionein promoter was induced through the administration of $CdSO_4$. As a consequence, the hepatic uptake of LDL increased. When the animals ate a low-fat diet, the plasma concentration of the apoproteins (apo) B and E fell to virtually undetectable levels (*6*). We could not use transgene-1 for long-term studies, since high-level expression of this transgene requires induction with $CdSO_4$ (*6*), and chronic treatment with such heavy metals is toxic to the animals. To circumvent this problem, we have established a new strain of transgenic mice that express a human LDL receptor minigene at high levels without the need for metal induction. The human LDL receptor minigene, designated transgene-3 (Fig. 1), is contained on a 15.5-kb Not I fragment that includes 3 kb of the mouse transferrin promoter and 27 bp of 5′ untranslated region from the human LDL receptor gene followed by the first four exons and introns of this gene. This sequence is followed by contiguous exons 5 to 18 contained in a single fragment derived from the LDL receptor cDNA. As with other transgene constructions (*7*), the minigene gave much higher levels of expression than did the earlier transgene that contained only a cDNA sequence with no intron sequences (*6*). Previous studies in transgenic mice showed that the 3-kb transferrin promoter segment used in the current study gave expression of human growth hormone that was high in liver and low in brain and kidney (*8*). We confirmed high-level expression of the human LDL receptor in the transgenic mouse livers by blot hybridization of mRNA, immunoblot analysis of receptor protein, and immunofluorescence (*9*). Expression of the human receptor protein in the liver of the transgenic mice was four to five times as high as that of the endogenous receptor protein in the normal mouse liver.

When maintained on a normal laboratory diet (*10*), mice expressing LDL receptor

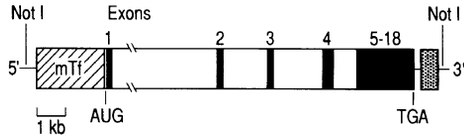

Fig. 1. Human LDL receptor transgene-3. The mouse transferrin (mTf) promoter and transcription initiation site were contained in a 3-kb Bam HI fragment (striped box) derived from the plasmid that includes mTf and human growth hormone (pmTf-hGH) (*8*). This fragment was fused to a 12.5-kb fragment containing a human LDL receptor minigene (*20*) and the transcription termination signal from the human growth hormone gene (stippled box) (*6*). Exons 1 to 18 and introns 1 to 4 are indicated by filled-in and open boxes, respectively. The construct contained 27 bp of 5′ untranslated region from the human LDL receptor gene (*21*). The thin lines represent polylinker sequences. A total of 436 eggs from C57BL/6 × SJL F_2 hybrid mice were microinjected with transgene-3 contained in a 15.5-kb Not I fragment and transferred into pseudopregnant females (*22*). Among 60 offspring, 23 (38%) contained the transgene as determined by dot-blot hybridization of DNA from tail tissues. Of these 23 mice, 6 showed a chronic absence (<1 mg/dl) of apo B-100 in plasma as measured by rocket immunoelectrophoresis (*6*). A strain of heterozygous mice derived from founders (line 212-5) was used in this study. Sibling mice that lacked the transgene (as determined by dot-blot hybridization) were used as controls.

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transgene-3 had total plasma cholesterol levels that were less than 50% of those in their normal siblings (Table 1). The results were similar in three experiments (A to C). Since the bulk of cholesterol in the plasma of normal mice is contained in HDL (*11*), the measurement of total cholesterol underestimates the differences in atherogenic lipoproteins such as LDL and IDL (*11*). So we used fast protein liquid chromatography (FPLC) to fractionate by size the lipoproteins from the normal and transgenic mice from two experiments (Fig. 2, A and C, respectively). The cholesterol content in each fraction was measured. In normal mice fed a normal diet, HDL was the major lipoprotein (Fig. 2A). Only small amounts of cholesterol were found in lipoproteins of a size corresponding to VLDL, IDL, or LDL. In the transgenic mice fed a normal diet, the amount of HDL was reduced, and there was no significant amount of cholesterol in any of the larger lipoprotein fractions (Fig. 2C). The low level of HDL in the transgenic mice has been postulated to be due to increased receptor-mediated clearance of HDL particles that contain apo E (*6*) (Fig. 3).

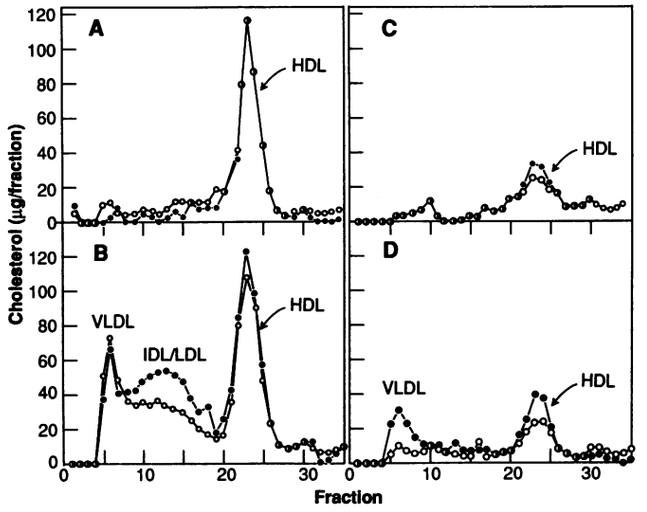

Fig. 2. Fractionation by FPLC of plasma lipoproteins from normal and transgenic mice fed different diets. A sample (1.25 ml) of the pooled plasma from experiments A (●) and B (○) in Table 1 was subjected to ultracentrifugation at $d = 1.215$ g/ml (*23*). The resulting lipoprotein fractions ($d < 1.215$ g/ml) were adjusted to a final volume of 3 ml with buffer A [0.15 M sodium chloride, 0.01% (w/v) sodium EDTA, and 0.02% (w/v) sodium azide at pH 7.2], and a sample (2 ml) was subjected to gel filtration with an FPLC apparatus that had a Superose 6B column at room temperature. The column was eluted with buffer A at a constant flow rate of 1 ml/min. Fractions of 2 ml were collected, and a sample (200 μl) of each fraction was used for measurement of total cholesterol content (*17*). The positions of elution of the major lipoprotein classes are indicated.

To put stress on the cholesterol transport system, we fed the mice a diet that has been used by others to raise the plasma cholesterol level (*12, 13*). This diet contains 0.6% (w/w) cholesterol, 3.6% (w/w) saturated fat in the form of cocoa butter, and 0.24% (w/w) cholic acid. We refer to this diet as the high-fat diet. When the normal mice from three experiments were fed the high-fat diet for 3 weeks, their plasma cholesterol levels rose by an average of 70 mg/dl (Table 1). In the transgenic mice, the rise in plasma cholesterol was much less, averaging 26 mg/dl (Table 1).

In the normal mice, the increase in plasma cholesterol was explained by a major increase in cholesterol content of lipoproteins corresponding to particles in the VLDL and IDL to LDL size range (Fig. 2B). In transgenic mice fed the high-fat diet, small amounts of cholesterol appeared in the VLDL fraction, but no significant amount was present in the IDL or LDL fractions (Fig. 2D). Thus, in the transgenic mice fed the high-fat diet, none of the increase in cholesterol could be attributed to an increase in LDL or IDL.

The profile from SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the apoproteins contained in the various lipoprotein fractions separated by FPLC is shown (Fig. 3). In the normal mice fed a normal diet, apo B-100 and apo B-48 were both found in lipoprotein particles that corresponded to the size range of LDL (Fig. 3A). Most of the apo E was present in HDL. Very few apoproteins were found in the fractions corresponding to the size range of VLDL or IDL (Fig. 3A). When the normal mice were fed the high-fat diet, the total amount of apo B-48 appeared to increase, and it was shifted to larger lipoproteins in the VLDL to IDL

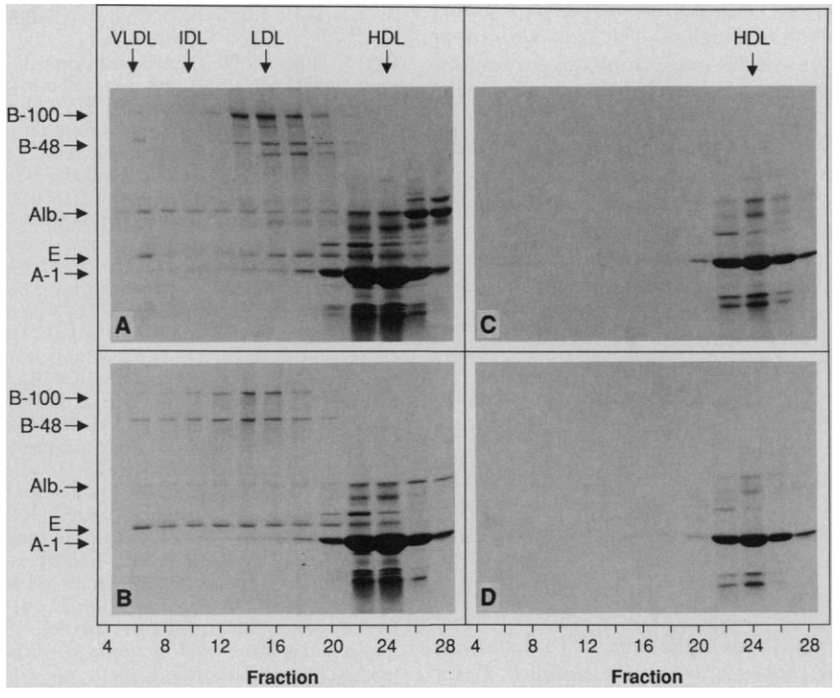

Fig. 3. Profile from SDS-PAGE of plasma lipoproteins from normal and transgenic mice fed different diets. A sample (850 μl) from each of the FPLC fractions from experiment B of Table 1 was mixed with 150 μl of 100% (w/v) trichloroacetic acid and placed on ice for 30 min. The precipitated apoproteins were collected by centrifugation and washed twice with 1 ml of ice-cold acetone to extract the lipids. The pellets were solubilized in 100 μl of buffer (*24*) containing 0.1 M dithiothreitol. After boiling for 3 min, each sample was loaded onto a 3 to 15% gradient polyacrylamide gel and subjected to electrophoresis (30 mA, 3 hours, and 20°C). The gel was stained with Coomassie blue. Each gel was calibrated with molecular size standards that were run in an adjacent lane. The positions of migration of apo B-100 (~512 kD), apo B-48 (~250 kD), albumin (66 kD), apo E (35 kD), and apo A-I (19 kD) are denoted at the left. The peak fraction of elution of VLDL, IDL, LDL, and HDL (see Fig. 2) is shown.

Table 1. Cholesterol content of plasma and livers of normal and transgenic mice fed different diets. Male transgenic and normal sibling mice 8 to 10 weeks of age, weighing 22 to 25 g, were fed a normal diet or a high-fat diet (*10*) ad libitum for 20 days. On day 21 after 5 hours without food, the mice were anesthetized with pentobarbital and killed by exsanguination. The blood from each mouse (250 to 750 μl) was mixed with 1.5 mg of potassium EDTA, and the plasma was obtained by centrifugation. A sample (20 μl) of the plasma from each mouse was used for measurement of its total cholesterol content by the cholesterol oxidase method (*17*). The cholesterol content of liver was measured as follows: 0.2 g of liver tissue from each mouse was homogenized with a Polytron homogenizer in 4 ml of chloroform/methanol (2/1, v/v) (*18*), after which 0.8 ml of 50 mM NaCl was added. A sample (50 μl) of the organic phase was mixed with 7.5 mg of Triton X-100 (*19*). After evaporation of the organic solvents, the lipid in the detergent phase was used for measurement of total cholesterol content by the cholesterol oxidase method (*17*). Each value is the mean ± SEM of data obtained from five to nine animals. ND, not done.

Diet	Total cholesterol content			
	Control mice		Transgenic mice	
	Plasma (mg/dl)	Liver (mg/g of tissue)	Plasma (mg/dl)	Liver (mg/g of tissue)
Experiment A				
Normal	76 ± 2.6	2.8 ± 0.6	29 ± 1.5	3.1 ± 0.09
High fat	159 ± 15.9	16 ± 1.9	85 ± 20	12 ± 1.9
Experiment B				
Normal	103 ± 8.1	2.7 ± 0.1	43 ± 7.6	2.7 ± 0.3
High fat	183 ± 18.5	15 ± 3.6	52 ± 8.7	12 ± 2.3
Experiment C				
Normal	101 ± 3.4	ND	39 ± 2.5	ND
High fat	147 ± 8.1	ND	51 ± 6.6	ND

size range (Fig. 3B). There was an apparent increase in the total amount of apo E, which was also shifted from the HDL and LDL fractions in mice fed the control diet (Fig. 3A) to the VLDL to IDL range in the mice fed the high-fat diet (Fig. 3B). In the transgenic mice, no apoproteins could be detected in the VLDL to IDL size range when the animals were fed either the normal diet (Fig. 3C) or the high-fat diet (Fig. 3D). In particular, we did not detect apo B-100, apo B-48, or apo E in any of the lipoprotein fractions by this technique.

In two additional experiments not shown (*9*), groups of six transgenic mice were fed high-fat diets containing 1.2 and 2.4% cholesterol with proportionate increases in cocoa butter and cholic acid. Although the VLDL peak from FPLC was somewhat higher than that observed in the transgenic mice fed 0.6% cholesterol, there were still no detectable IDL and LDL peaks despite the higher fat diets. Moreover, SDS-PAGE of the FPLC fractions in both experiments showed results that were similar to those in Fig. 3.

The transgenic mice appeared to absorb the cholesterol in the high-fat diet as indicated by a rise in the cholesterol content of the liver (Table 1). In the normal mice, hepatic cholesterol was five times as high when the high-fat diet was administered. In the transgenic mice, a similar increase was seen (Table 1).

The current data indicate that unregulated overexpression of human LDL receptors in the liver of the mouse can prevent the increase in IDL and LDL that results from the ingestion of a diet rich in cholesterol, saturated fats, and bile acids. Since the transgenic mice absorbed the dietary cholesterol, it is likely that they synthesized cholesterol-rich lipoproteins. However, the LDL receptors cleared them so rapidly from the circulation that they were not detectable in plasma. The receptor clearance may have been so efficient that the lipoproteins never even emerged from the liver, or it may have occurred after the lipoproteins circulated briefly.

Our data with the C57BL/6 × SJL hybrid mice are consistent with previous findings of Lusis *et al.* (*14*), who showed that the apo B-100 content of plasma did not rise when these strains of mice were fed a high-fat diet, whereas the apo B-48 content was several times higher. In this regard the mouse resembles the rat (*15*) and differs from other species such as the rabbit (*1, 3*), the dog (*1, 3*), and primates (*5*).

In contrast to other species, the liver of the rat produces apo B-48 as well as apo B-100 (*16*). If the same is true in the mouse, this would explain the elevation of apo B-48 in response to cholesterol feeding (*14*) (Fig. 3). In addition, it is likely that some of the apo B-48 was synthesized in the intestine. No matter what the source of the apo B-48, the LDL receptor seems to be rate-limiting for its catabolism. Because of this rate-limiting effect, increasing LDL receptor expression above its normal level in the mouse can prevent hypercholesterolemia even in the face of a diet that contains large amounts of cholesterol, saturated fat, and bile acids.

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10. The normal mouse diet, obtained from Teklad Premier Laboratory Diets (Madison, WI), contained 1% (w/w) animal fat, <0.03% (w/w) cholesterol, no sodium cholate, and no casein. The high-fat diet, a modification (*13*) of a diet originally described by Thomas and Hartroft (*12*), contained 0.6% (w/w) cholesterol, 3.6% (w/w) cocoa butter, 0.24% (w/w) sodium cholate, and 3.6% (w/w) casein. It was prepared by mixing 7.3 parts of normal mouse diet with 1 part of the Thomas-Hartroft diet. The latter was obtained from Teklad (diet TD 78399).
11. The four major classes of lipoprotein in animal plasma are VLDL, very low density lipoprotein [density (*d*) < 1.006 g/ml]; IDL, intermediate density lipoprotein (*d*, 1.006 to 1.019 g/ml); LDL, low density lipoprotein (*d*, 1.019 to 1.063 g/ml); and HDL, high density lipoprotein (*d*, 1.063 to 1.215 g/ml).
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