

# Hyper- and hypo-responsiveness to dietary fat and cholesterol among inbred mice: searching for level and variability genes

Elizabeth A. Kirk, Gaile L. Moe, Mark T. Caldwell, Johan Å. Lernmark, Deborah L. Wilson, and Renée C. LeBoeuf<sup>1</sup>

Nutritional Sciences and Department of Medicine, University of Washington, Seattle, WA 98195

**Abstract** A concept proposed by Berg (Berg, K. 1989. *Arteriosclerosis*. 9: 1-50-1-58) is that a combination of level and variability genes determine an individual's overall plasma lipid levels and atherosclerotic risk. Our goal was to determine which inbred mouse strains could be used to identify candidate level and variability genes controlling lipid levels and atherosclerosis susceptibility. Nine common inbred mouse strains were examined for responsiveness with respect to plasma lipoprotein and tissue lipid levels upon feeding diets rich in cholesterol and fat. Marked quantitative variations were observed in plasma cholesterol and triglyceride levels among mice fed rodent chow and the high fat test diets. Mice of strains DBA/2 and AKR appeared to be hyporesponsive to diets containing high levels of fat and cholesterol as compared to rodent chow. In contrast, several strains were primarily hyperresponsive to either dietary fat or cholesterol, or both ingredients. Determination of cholesterol absorption for selected strains fed test diets suggested that decreased cholesterol absorption, in part, contributes to hyporesponsiveness as seen in DBA/2 mice. Levels of mRNA for cholesterol 7 $\alpha$ -hydroxylase were estimated and shown to vary markedly among strains. An inverse correlation was seen among strains between cholesterol 7 $\alpha$ -hydroxylase mRNA, and plasma and hepatic cholesterol levels for some diets. ■ Thus, genes controlling cholesterol absorption and bile acid synthesis are candidates for further study as level and variability genes affecting plasma cholesterol levels. Overall, inbred mouse strains will prove useful for identifying genes controlling level and variability traits.—Kirk, E. A., G. L. Moe, M. T. Caldwell, J. Å. Lernmark, D. L. Wilson, and R. C. LeBoeuf. Hyper- and hypo-responsiveness to dietary fat and cholesterol among inbred mice: searching for level and variability genes. *J. Lipid Res.* 1995. 36: 1522–1532.

**Supplementary key words** mouse • nutrition • diet responsiveness • genetics

Differences exist among individuals with respect to responsiveness of plasma lipid levels to changes in diet, occurring within species of laboratory animals (1-3) and among humans (4-6). For instance, high and low responding rhesus monkeys are differentiated based on

large (3.7-fold) and small (1.4-fold) increases in plasma cholesterol levels over basal levels upon feeding a high cholesterol diet (7). Rabbits that are hypo- and hyper-responsive to dietary cholesterol exhibit 6-fold and 19-fold increases in plasma total cholesterol levels, respectively, upon feeding a 0.15% cholesterol-containing diet (8). In humans, individual differences are often smaller than those seen in experimental animals but reproducible, and stable variations in responsiveness have been shown to be a function of changes in dietary cholesterol and dietary fatty acid composition (6).

The metabolic bases of these responses have been examined, and a variety of plausible mechanisms have been proposed to explain the extreme differences among individuals in diet responsiveness. Variations in cholesterol absorption (9, 10), capacity to depress whole-body cholesterol synthesis (5), production and clearance of circulating lipoproteins (11, 12), and plasma lipid hydrolysis and exchange activities (13, 14) contribute to dietary responsiveness. These processes are ultimately controlled by genetic factors, the existence of which is suggested by the observation that extreme differences in responsiveness can be found among inbred strains of the same animal species (1-3). The complexity of the metabolic bases of diet responsiveness suggests the involvement of many genes in determining the response phenotype.

A useful concept introduced by Berg (15) is that of “level genes” and “variability genes” that control risk of atherosclerosis. A level gene is one in which a polymorphism at that locus can be shown to be directly associ-

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; RI, recombinant inbred; C7H, cholesterol 7 $\alpha$ -hydroxylase.

<sup>1</sup>To whom correspondence should be addressed.

ated with heart disease or a known risk factor for heart disease. Such an example is the apoE locus, the alleles of which contribute to determining plasma low density lipoprotein (LDL) cholesterol levels (16). Variability genes are responsible for an individual's responsiveness to environmental stimuli such as diet, and can also contribute to determining atherosclerosis risk. Using within-pair difference analysis among monozygotic twin pairs, Berg (14) showed that cholesteryl ester transfer protein appears to have variability gene effects on LDL and total cholesterol levels. Berg (15) proposed that an individual's total genetic risk of heart disease depends on their combination of level and variability genes. Thus, identification of such genes, and defining an individual's status as a hypo- or hyper-responder to dietary modifications, may be useful in predicting their risk for developing heart disease.

The presence and identity of both categories of genes could be characterized using inbred mice. The hundreds of inbred strains, thousands of chromosomal markers, and ability to rigidly control environmental factors, particularly diet, make the mouse system useful for such genetic studies. The identity and character of level and variability genes can be tested via the now classic candidate gene approach (17), or via positional cloning in which a quantitative trait is mapped to one or more chromosomal sites (18-20). The role of these new genes identified in mice could then be tested for involvement in human diet responsiveness, using homologous gene markers (21).

In this report, we examine nine common inbred mouse strains for responsiveness with respect to tissue lipid and plasma lipoprotein levels upon feeding diets rich in cholesterol and fat. The marked quantitative variations in plasma and hepatic lipid levels observed among strains underscore the potential usefulness of inbred mice for identifying level and variability genes.

## MATERIALS AND METHODS

### Animals

Female mice from 6 to 8 weeks of age were obtained from the Jackson Laboratory, Bar Harbor, ME. Strains used were A/J, AKR/J (AKR), BALB/cByJ (BALB), C57BL/6J (C57BL/6), C3H/HeJ (C3H), DBA/2J (DBA/2), NZB/B1NJ (NZB), SWR/J (SWR), and 129/J. Mice were fed a pelleted rodent chow diet (Wayne Rodent BLOX 8604, Teklad, Madison, WI) for 2 weeks prior to initiation of the diet studies. Mice were maintained in a temperature-controlled (22°C) facility with a strict 12-h light/dark cycle. Mice were given free access to food and water. Food was removed from the mice 16 h prior to the collection of blood from the retroorbital

sinus into tubes containing anticoagulant and antimicrobial agents (1 mM EDTA, 50 µg/ml gentamicin sulfate, 0.05% sodium azide). Plasma was stored for 1 week or less at 4°C prior to analysis. Mice were killed by cervical dislocation and tissues obtained for lipid analyses were placed directly into liquid nitrogen and stored at -70°C. This project was approved by the Animal Care and Use Committee of the University of Washington (Protocol #2140-01).

### Diets and feeding

Five diets were used in this study. The first was pelleted rodent chow (Wayne Rodent BLOX 8604) which served as a reference diet and contained approximately 4% fat, 24% protein, and 4.5% crude fiber. The four remaining diets were high fat, semi-synthetic diets and were prepared in our diet kitchen as described (22). Two high fat diets contained safflower oil and 0.5% sodium cholate with (saff/high) and without (saff/low) added cholesterol (0.5%). The two safflower oil diets were calorically balanced and differed only in cholesterol content. These diets provided approximately 24% kcal from protein, 43% kcal from carbohydrate, and 33% kcal from fat. A detailed description of dietary contents has been given (22). In two other diets (butter/high and butter/low), safflower oil was replaced by butter oil after removal of protein by centrifugation (10,000 *g* for 30 min at 22°C). All diets were stored in air-tight containers at -20°C and fresh diet was provided daily. Animal acceptance of the semi-synthetic diets was improved by gradual introduction of the diet over a 4-day period: 2 days of 50:50 chow to semi-synthetic diet, 2 days of 25:75 chow to semi-synthetic diet, followed by 4 weeks of 100% semi-synthetic diet. All diets were fed concurrently to five groups of mice within each strain.

### Tissue lipid determinations

Lipids were extracted from frozen mouse liver using the method of Folch, Lees, and Sloane Stanley (23), then modified to contain Triton X-100 as described by Carr, Andersen, and Rudel (24). The hepatic cholesterol and plasma total and high density lipoprotein (HDL) cholesterol concentrations were determined using a colorimetric kit (Diagnostic Kit, #236691, Boehringer Mannheim, Indianapolis, IN) with cholesterol standards (Preciset #125512, Boehringer Mannheim). HDL cholesterol values were measured after the selective precipitation of very low density lipoprotein (VLDL)/LDL by phosphotungstate (25). Triglyceride concentrations in liver and plasma were determined after removal of free glycerol (Diagnostic Kit, #450032, Boehringer Mannheim).

## Absorption measurements

Cholesterol absorption was measured as described with modifications (26, 27). After 3 weeks of feeding experimental diets, animals were fasted 16 h and placed into separate metabolic cages. Oral doses of [<sup>3</sup>H]cholesterol and [<sup>14</sup>C]sitosterol (1.5  $\mu$ Ci and 0.2  $\mu$ Ci, respectively, per animal) were administered as part of an experimental diet bolus of 4 g. [<sup>3</sup>H]cholesterol and [<sup>14</sup>C]sitosterol were at least 94% pure, according to the manufacturer (Amersham Corporation, Arlington Heights, IL). Feces were collected for the next 3 days from pans below the metabolic cages and stored at -20°C until ready for analysis, at which time, they were homogenized in PBS. Fifty- $\mu$ l aliquots of a 10% homogenate were dispersed in 5 ml Ecolume (ICN Biomedicals Inc., Costa Mesa, CA), and the radioactivity was measured using a Packard TriCarb 460C liquid scintillation counter. Counting efficiency was determined using the external standard method. No quenching occurred over the range of 0-100  $\mu$ l of the 10% homogenate. Absorption was calculated using the equation:

$$\% \text{ absorbed} = \{1 - [\text{fecal } (^3\text{H}/^{14}\text{C})/\text{diet } (^3\text{H}/^{14}\text{C})]\} \times 100.$$

## mRNA quantification

A 2172 bp cDNA clone of rat cholesterol 7 $\alpha$ -hydroxylase (C7H) in pBluescript SK vector was a generous gift from Dr. David Russell, University of Texas Southwestern Medical Center (28). The probe used was cut from the vector with EcoRI and purified by agarose gel electrophoresis. Total RNA was isolated from mouse livers and quantitated by Northern blotting as previously described (29). In rat liver, the pattern of mRNA for rat C7H consists of four bands of various abundance (28). In mouse liver, three bands (3.6, 2.7, and 1.7 kb) were particularly prominent and were used to quantitate the C7H mRNA. A cDNA probe (Gibco BRL, Gaithersburg, MD) for glycerol-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard. Probes were labeled with [<sup>32</sup>P]dCTP (Amersham) by the random primer method using the Promega Prime-a-Gene sys-

tem. Radioactive signals from Northern blots were quantified using phosphorimaging.

## Statistical analysis

Results are reported as mean  $\pm$  SEM. Statistical differences were determined by ANOVA using SYSTAT for the Macintosh (SYSTAT, Inc., Evanston, IL). Spearman rank order correlation coefficients were used to assess correlations in several cases. Two-way ANOVA was used to determine interactions between strain and diet. Post-hoc analyses of significance were made by Tukey's test for additivity. The Student's *t*-test was used to compare independent means in several cases (absorption). *P* < 0.05 was accepted as statistically significant.

## RESULTS

Results of plasma and hepatic lipid concentrations were examined by two-way ANOVA in terms of the nine strains and five diets. As shown in **Table 1**, main effects and significant 2-way interactions were observed for all parameters. Overall, these results demonstrate that diversity existed among strains with respect to tissue lipid levels, lipid levels were altered by diet, and the extent of change due to diet was strain dependent. The *r*<sup>2</sup> values ranged from 0.726 to 0.966, implying that the variables of strain and diet accounted for the majority of variation within each parameter. Remaining variability is likely to be due to other unidentified environmental and/or experimental factors.

## Plasma cholesterol phenotypes

Inbred strains chosen for this study are progenitors of available recombinant inbred (RI) strains that are useful for gene mapping and genetic analysis of phenotypic traits. Strains were examined with respect to concentrations of plasma total, VLDL/LDL and HDL cholesterol levels.

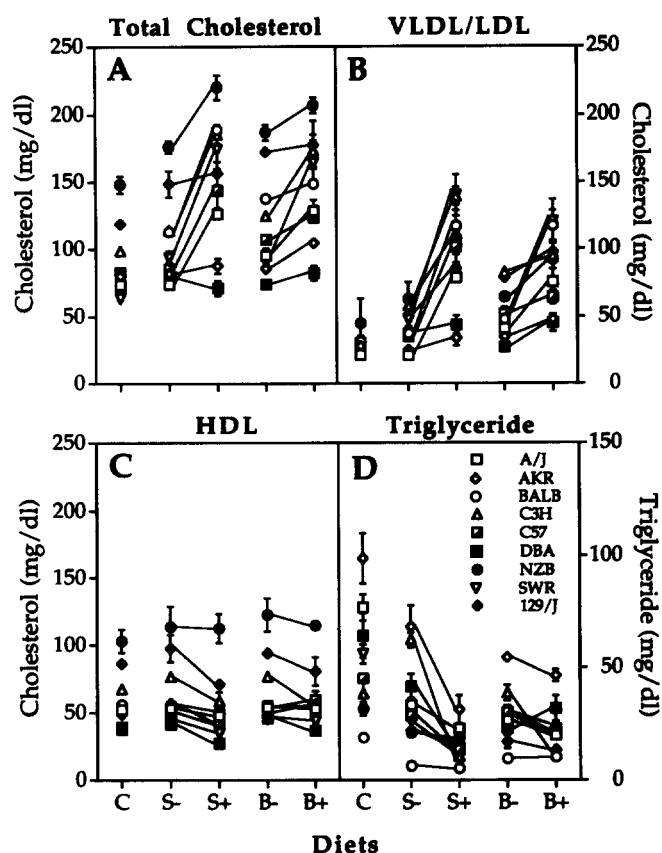
Several phenotypes were observed with respect to plasma total cholesterol levels (**Fig. 1A**). Overall, two strains (NJB and 129/J) exhibited significantly higher

TABLE 1. Statistical parameters describing effects of strain and diet on plasma and liver measurements

ANOVA Factors	Plasma				Liver	
	TC	HDL-C	VLDL/LDL	TG	C	TG
Strain (S)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Diet (S)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
S $\times$ D	< 0.001	0.025	< 0.001	< 0.001	< 0.001	< 0.001
<i>r</i> <sup>2</sup>	0.898	0.896	0.821	0.866	0.966	0.726

Table abbreviations are strain (S), diet (D), total cholesterol (TC), HDL cholesterol (HDL-C), VLDL + LDL cholesterol (VLDL/LDL), cholesterol (C), and triglyceride (TG). ANOVA, analysis of variance for probability > *F*. A 2-factor model was used for initial analysis of plasma and liver parameters.





**Fig. 1.** Plasma cholesterol and triglyceride concentrations among inbred mice fed rodent chow and safflower oil diets. Mice were fed rodent chow (C) or one of four semi-synthetic diets for 4 weeks. The semi-synthetic diets contained either 138 g/kg safflower oil and 5 g/kg sodium cholate without (S-) or with (S+) 5 g/kg cholesterol, or 138 g/kg butter oil and 5 g/kg sodium cholate without (B-) or with (B+) 5 g/kg cholesterol. Animals were fasted 16 h and bled from the orbital sinus between 11 AM and 1 PM. Plasma total (A), VLDL/LDL (B), and HDL (C) cholesterol and triglyceride (D) concentrations were determined as described under Materials and Methods. Each symbol is presented as the mean  $\pm$  SEM obtained for five animals. Significance levels are given in the text.

plasma total cholesterol values than the remaining strains when fed a low-fat rodent chow diet ( $P < 0.001$ ). The levels of plasma total cholesterol of mice fed high-fat diets varied widely (values were from about 70 mg/dl for DBA/2 to 200 mg/dl for NZB).

The "responsiveness" of strains when challenged with high-fat diets without cholesterol also varied widely. Strains BALB and NZB were significantly responsive to safflower oil ( $P < 0.001$  and 0.036, respectively) and strains BALB, NZB, A/J, and 129/J were significantly responsive to butter oil diets ( $P < 0.001$ -0.04) without cholesterol as compared to rodent chow. Increases in plasma total cholesterol of about 30-57 mg/dl were seen. The remaining strains (AKR, C3H, C57BL/6, DBA/2 and SWR) showed no significant differences in total

cholesterol between rodent chow and the high-fat diets without cholesterol.

Upon addition of dietary cholesterol to the safflower oil diet, all strains except AKR, DBA/2, and 129/J showed significant increases in plasma cholesterol levels over mice fed safflower oil alone ( $P < 0.001$ ), with elevations in total cholesterol of 44-81 mg/dl. Feeding butter with cholesterol elicited significant increases in plasma total cholesterol as compared to butter oil alone for A/J, AKR, C3H, and SWR ( $P < 0.001$ -0.04) but not for BALB, C57BL/6, DBA/2, NZB, and 129/J. Thus, in general, BALB and NZB mice were particularly responsive to both fat types, and A/J, C3H, and SWR were particularly responsive to dietary cholesterol when fed with fat. AKR and DBA/2 mice were generally hyporesponsive to both dietary fat and cholesterol.

Although strains differed in responsiveness, significant correlations were seen between chow-fed mice and mice fed high-fat diets with respect to the rank order of strains. Generally, strains with higher plasma total cholesterol values upon rodent chow feeding continued to show the highest plasma cholesterol values regardless of diet changes. For rodent chow versus safflower oil ( $r = 0.935$  at  $P < 0.001$ ), safflower oil with cholesterol ( $r = 0.729$  at  $P < 0.025$ ), butter oil ( $r = 0.905$  at  $P < 0.002$ ), and butter oil with cholesterol ( $r = 0.573$  at  $P = 0.055$ ), correlations were nearly or highly significant.

For mice fed rodent chow, the VLDL/LDL cholesterol values varied over a 2-fold range (from 21 mg/dl for A/J to 45 mg/dl for NZB) (Fig. 1B). This fraction accounted for 30-40% of plasma total cholesterol, except for strain DBA/2 which carried nearly 50% of cholesterol as VLDL/LDL. Changes in the VLDL/LDL cholesterol fraction due to diet mirrored the alterations in plasma total cholesterol.

HDL cholesterol levels also varied over 2-fold among strains fed rodent chow (from 38 mg/dl for DBA/2 to 103 mg/dl for NZB) (Fig. 1C). Overall, HDL cholesterol values were not altered by type of diet. This result is in contrast to previous reports using semi-synthetic high fat diets containing sucrose (30) or commercial diets containing high levels of cocoa butter mixed with rodent breeder chow (31). Thus, for the high fat glucose-based diets used here, HDL cholesterol concentrations were determined by strain alone, while changes in plasma total and VLDL/LDL cholesterol were accounted for by genetic and dietary influences.

### Plasma triglyceride phenotypes

Plasma triglyceride levels among the nine strains fed rodent chow varied over a 5-fold range, from  $19 \pm 1$  mg/dl (BALB) to  $99 \pm 11$  mg/dl (AKR) (Fig. 1D). For most strains fed the high-fat diets without cholesterol, plasma triglyceride levels were markedly reduced as

compared to mice fed the low-fat chow diet (Fig. 1D). Strains showed from 30-50% decreases (10-50 mg/dl) in plasma triglyceride for both high-fat diets without cholesterol ( $P < 0.001-0.05$ ). Exceptions were strains NZB (both oils), C3H (butter oil), and DBA/2 (safflower oil) which showed no significant changes.

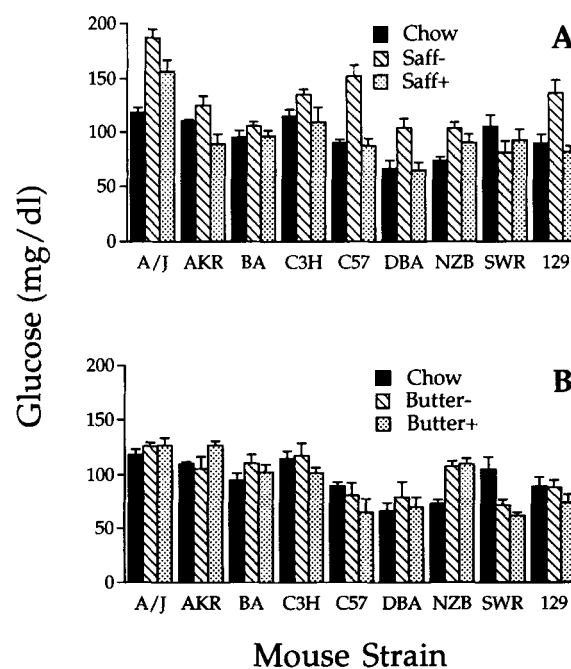
Although further reductions in plasma triglyceride were seen upon addition of dietary cholesterol to the high-fat diets, the largest changes occurred for mice fed safflower oil (Fig. 1D). Five strains (AKR, C3H, C57BL/6, DBA/2, and SWR) showed marked reductions in plasma triglyceride (10-50 mg/dl) upon addition of dietary cholesterol to the safflower oil diet ( $P < 0.001-0.025$ ). In contrast, the addition of cholesterol to butter oil resulted in a reduction in triglyceride (28 mg/dl) only for C3H mice ( $P < 0.001$ ). Thus, in general, most mouse strains were responsive with respect to plasma triglyceride levels upon changing diets from rodent chow to high fat without cholesterol. BALB and 129/J were completely nonresponsive to dietary cholesterol. NZB was nonresponsive to any dietary change.

Significant correlations were seen between chow-fed mice and mice fed high-fat diets with respect to the rank order of strains. Strains with higher triglyceride values upon rodent chow feeding continued to show the highest levels regardless of diet changes. For rodent chow versus safflower oil ( $r = 0.665$  at  $P < 0.025$ ), safflower oil with cholesterol ( $r = 0.832$  at  $P < 0.004$ ), butter oil ( $r = 0.648$  at  $P < 0.03$ ), and butter oil with cholesterol ( $r = 0.871$  at  $P = 0.003$ ), correlations were highly significant.

### Plasma glucose phenotypes

Genetic variation in plasma glucose levels among inbred strains fed rodent chow (32) and a high-fat diet (33) have been observed. To explore phenotypic variations in this study, plasma glucose was quantified among the nine strains fed the five diets. Fasting plasma glucose levels for mice fed rodent chow varied 2-fold among strains (about 66 mg/dl for DBA/2 to 130 mg/dl for A/J) (Fig. 2). A significant positive correlation between plasma glucose and triglyceride levels was observed only for the rodent chow-fed animals (Spearman rank order coefficient was  $r = 0.345$  at  $P < 0.04$ ).

Upon feeding mice the high fat diets, three observations were made. First, dietary fat influenced fasting glucose levels. For diets without cholesterol, safflower oil feeding caused elevations in plasma glucose levels compared to rodent chow for A/J, C57BL/6, NZB, and 129/J mice ( $P < 0.001-0.02$ ). In contrast, butter oil without cholesterol resulted in a higher glucose level for only one strain (NZB;  $P < 0.01$ ). Second, the effect of adding cholesterol to the safflower oil diet was to reduce plasma glucose levels for AKR, C57BL/6, and 129/J compared to safflower oil without cholesterol ( $P < 0.001-$

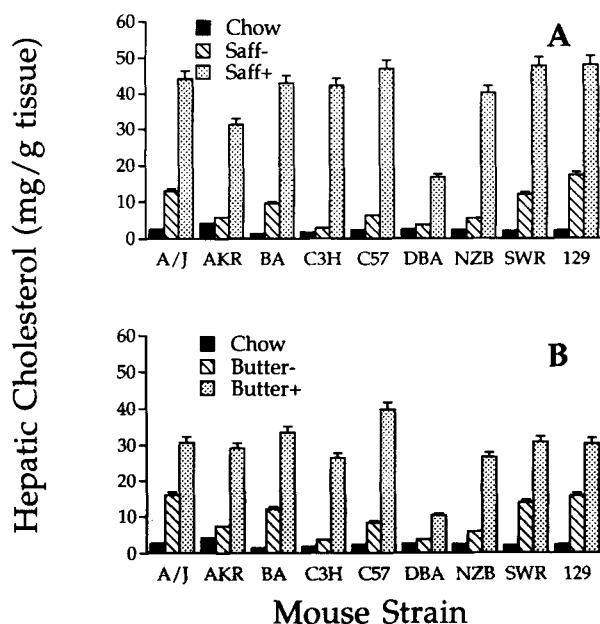


**Fig. 2.** Plasma glucose concentrations among inbred mice fed rodent chow, safflower oil, or butter oil diets. Panel A shows plasma glucose values for mice fed rodent chow (solid bars), safflower oil without cholesterol (Saff-; hatched bars), and safflower oil with cholesterol (Saff+; stippled bars). Panel B shows plasma glucose values for mice fed rodent chow (solid bars), butter oil without cholesterol (Butter-; hatched bars), and butter oil with cholesterol (Butter+; stippled bars). Diets and feeding conditions are as described in Fig. 1 legend. Each bar represents the mean  $\pm$  SEM obtained for five animals. Significance levels are given in the text.

0.02). No significant changes were seen comparing butter to butter with cholesterol. Finally, four strains (BALB, C3H, DBA/2, and SWR) exhibited no significant changes in plasma glucose concentrations regardless of fat type and cholesterol content.

### Hepatic lipid phenotypes

Hepatic cholesterol levels for most strains fed rodent chow were comparable (1.4-2.5 mg/g tissue), except that AKR exhibited a significantly higher value ( $4.0 \pm 0.1$ ) ( $P < 0.001$ ) (Fig. 3). Changing from rodent chow to a high-fat diet without cholesterol resulted in significant increases in hepatic cholesterol levels for A/J, BALB, SWR, and 129/J regardless of fat type ( $P < 0.001-0.05$ ). C3H and C57BL/6 were sensitive to butter oil ( $P < 0.03$ ) but not to safflower oil, and AKR, DBA/2, and NZB were not responsive to either fat. As expected, dietary cholesterol induced marked responses in hepatic cholesterol levels for all strains ( $P < 0.0001-0.02$ ). For all strains but DBA/2, hepatic cholesterol levels ranged from approximately 30 to 50 mg/g tissue when cholesterol was included in the diets. DBA/2 mice were unique



**Fig. 3.** Hepatic cholesterol levels among inbred mice fed rodent chow, safflower oil, or butter oil diets. Panel A shows hepatic cholesterol values for mice fed rodent chow (solid bars), safflower oil without cholesterol (Saff-; hatched bars), and safflower oil with cholesterol (Saff+; stippled bars). Panel B shows hepatic cholesterol values for mice fed rodent chow (solid bars), butter oil without cholesterol (Butter-; hatched bars), and butter oil with cholesterol (Butter+; stippled bars). Diets and feeding conditions are as described in previous legends. Each bar represents the mean value  $\pm$  SEM obtained for five animals. Significance levels are given in the text.

in comparison to other strains showing 11.3 and 16.7 mg/g tissue for butter and safflower oil diets with cholesterol, respectively. Overall, values for safflower oil-fed mice tended to be greater than those of the same strain fed butter oil ( $P < 0.001$ - $0.03$ ) when cholesterol was included in the diets.

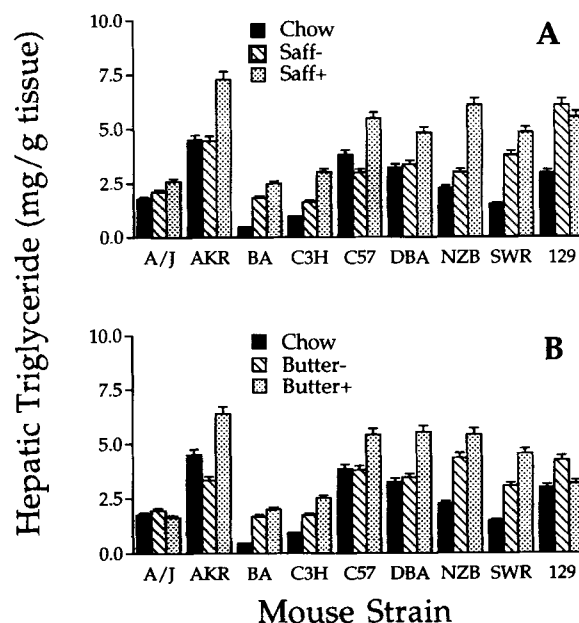
Hepatic triglyceride levels for mice fed rodent chow ranged from about 0.5 mg/g (BALB) to 4.5 mg/g tissue (AKR) (Fig. 4). The responsiveness of hepatic triglyceride levels to changes in diet were modest as compared to responses seen for hepatic cholesterol levels. BALB mice responded significantly to either fat without cholesterol ( $P < 0.002$ ) and AKR mice responded significantly to cholesterol regardless of fat type ( $P < 0.005$ ). Other changes in hepatic triglyceride content due to diet varied. For instance, 129/J mice responded to safflower oil without cholesterol ( $P < 0.05$ ) and SWR mice responded to butter oil without cholesterol ( $P < 0.001$ ). C3H and DBA/2 mice were sensitive to cholesterol in the presence of safflower oil ( $P < 0.005$ ) and butter oil ( $P < 0.04$ ), respectively. A/J, C57BL/6,

and NZB mice were nonresponsive to dietary changes with respect to hepatic triglycerides.

### Plasma total cholesterol levels are influenced by absorption of dietary cholesterol

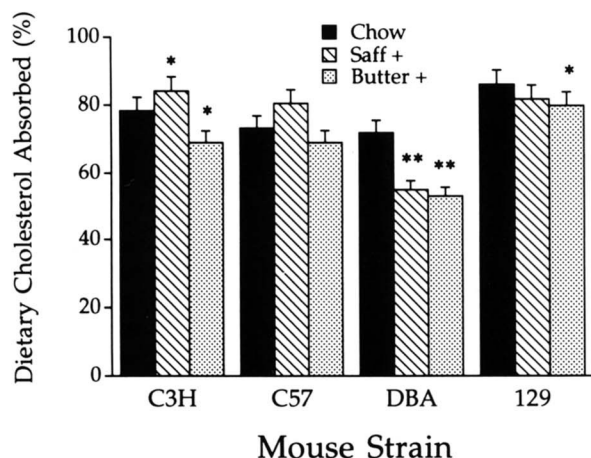
Hypo- and hyperresponsiveness of plasma cholesterol levels to changes in diet has been attributed, in part, to the extent of dietary cholesterol absorbed (9, 10). Thus, the contribution of cholesterol absorption to diet responsiveness was examined among four strains differing in cholesterol responsiveness: DBA/2, C3H, C57BL/6, and 129/J. With respect to plasma cholesterol levels, C3H and C57BL/6 mice were responsive to dietary cholesterol, and 129/J mice were responsive to butter oil (Fig. 1A). Strains C3H, 129/J, and C57BL/6 showed large increases in hepatic cholesterol levels upon feeding fat and cholesterol (Fig. 3). In contrast, DBA/2 mice were hyporesponsive to changes in diet with respect to plasma and hepatic cholesterol levels.

The amount of cholesterol absorbed was approximately 70-80% for all strains fed the rodent chow diet, with significant differences between strains seen only between DBA/2 and 129/J ( $P < 0.005$ ) (Fig. 5). Upon feeding the high-fat diets containing cholesterol, DBA/2 mice showed a marked decrease in cholesterol



**Fig. 4.** Hepatic triglyceride concentrations among inbred mice fed rodent chow, safflower oil, or butter oil diets. Panel A shows hepatic triglyceride values for mice fed rodent chow (solid bars), safflower oil without cholesterol (Saff-; hatched bars), and safflower oil with cholesterol (Saff+; stippled bars). Panel B shows hepatic triglyceride values for mice fed rodent chow (solid bars), butter oil without cholesterol (Butter-; hatched bars), and butter oil with cholesterol (Butter+; stippled bars). Diets and feeding conditions are as described in previous legends. Each bar represents the mean value  $\pm$  SEM obtained for five animals. Significance levels are given in the text.





**Fig. 5.** Percent cholesterol absorbed among inbred strains. Mice were fed diets of rodent chow (solid bars), safflower oil with cholesterol (Saff+; hatched bars), or butter oil with cholesterol (Butter+; stippled bars) for 3 weeks. Animals were fasted 16 h, and placed into separate metabolic cages. Oral doses of [ $^3\text{H}$ ]cholesterol and [ $^{14}\text{C}$ ]sitosterol (1.5  $\mu\text{Ci}$  and 0.2  $\mu\text{Ci}$ , respectively, per animal) were administered within a diet bolus of 4 g. Daily fecal collections were performed for 3 days after administration of the isotopes. Fifty- $\mu\text{l}$  aliquots of a 10% homogenate of the feces were dispersed in 5 ml Ecolume (ICN Biomedicals Inc.), and the radioactivity was measured using a Packard TriCarb 460C liquid scintillation counter. Absorption was calculated using the equation: % absorbed =  $\{1 - [\text{fecal } (^3\text{H}/^{14}\text{C})/\text{diet } (^3\text{H}/^{14}\text{C})]\} \times 100$ . Each bar represents the mean value  $\pm$  SEM obtained for five animals. Significance levels are given with respect to changes from rodent chow values as: \* $P < 0.05$  and \*\* $P < 0.001$ .

absorption to approximately 54% ( $P < 0.004$ -0.05) for both diets. Cholesterol absorption for DBA/2 mice was significantly less for cholesterol-containing diets as compared to the other three strains. Absorption values for C57BL/6 mice showed no significant differences between rodent chow and high-fat diets, and the responses of C3H and 129/J mice varied (Fig. 5).

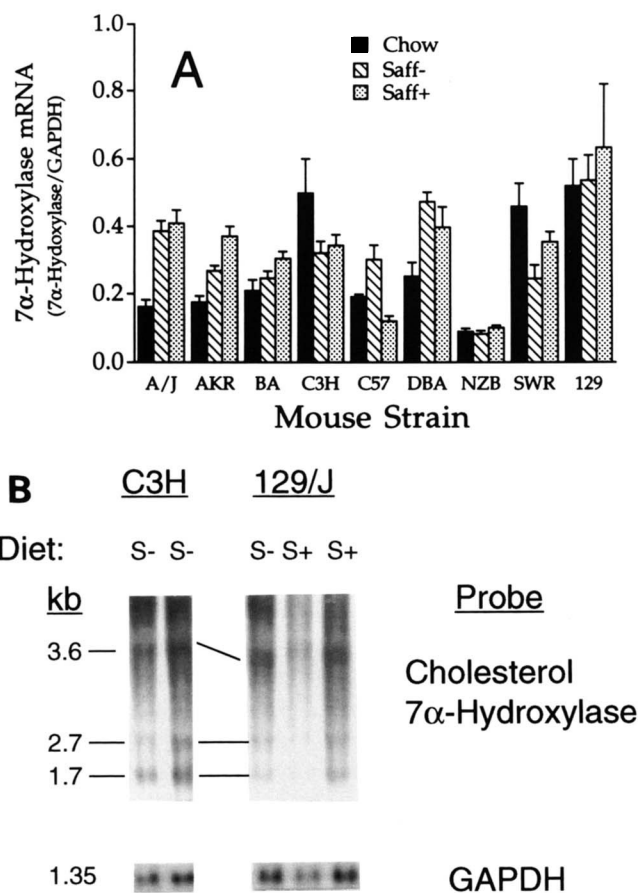
Data from mice fed the two high-fat diets showed that mice absorbing higher amounts of dietary cholesterol exhibited higher plasma total cholesterol levels ( $r = 0.754$  at  $P < 0.02$ ; Spearman rank order correlation coefficient) and higher hepatic cholesterol levels ( $r = 0.802$  at  $P < 0.01$ ). Thus, cholesterol absorption contributes to setting plasma and hepatic cholesterol levels and the dramatic hypo-responsiveness of DBA/2 mice is due in part to lower absorption of dietary cholesterol as compared to other strains.

#### Cholesterol 7 $\alpha$ -hydroxylase does not account for hepatic cholesterol levels

Bile acids are required for cholesterol absorption from the intestine. The effect of diet on the mRNA abundance of cholesterol 7 $\alpha$ -hydroxylase (C7H), the rate-limiting enzyme in conversion of cholesterol into bile acids, was measured to determine whether this

enzyme contributed to diet responsiveness. In this experiment, the focus was on the nine inbred strains fed rodent chow and safflower oil diets.

Mice fed rodent chow exhibited C7H mRNA levels extending over a 5-fold range (Fig. 6A). C7H mRNA appears as multiple bands on Northern blots and values represent the sum of these signals (Fig. 6B). Strains C3H, SWR, and 129/J exhibited significantly higher levels as compared to other strains ( $P < 0.05$ ). Upon feeding safflower oil without cholesterol, C7H mRNA levels increased significantly for A/J, AKR, C57BL/6, and DBA/2 ( $P < 0.001$ -0.04). Interestingly, values tended to decrease for SWR ( $P < 0.02$ ) and C3H (did not reach significance). The addition of cholesterol to the safflower oil diet resulted in a further increase for AKR ( $P$



**Fig. 6.** Hepatic levels of mRNA specific for cholesterol 7 $\alpha$ -hydroxylase (C7H) among inbred strains fed rodent chow and safflower oil-containing diets. Panel A shows mice fed rodent chow (solid bars), or safflower oil diets without (Saff-; hatched bars) or with (Saff+; stippled bars) added cholesterol as described in Figs. 1-4. Total RNA was prepared and probed with a clone for C7H as described in Methods and Materials. Each bar represents the mean value  $\pm$  SEM obtained for five animals. Significance levels are given in the text. Panel B shows Northern blots for selected mice from strains C3H and 129/J fed safflower oil without (S-) or with (S+) cholesterol, as described in Fig. 1. This figure illustrates the multiple banding pattern seen for rodent C7H (28). GAPDH refers to glycerol-3-phosphate dehydrogenase which is used as an internal standard for quantification of C7H mRNA.

$< 0.02$ ) and a decrease for C57BL/6 ( $P < 0.001$ ) compared to safflower oil without cholesterol. Strains BALB, NZB, and 129/J showed no changes in C7H mRNA levels with diet. In summary, initial values and diet responses of C7H mRNA levels varied markedly among strains. AKR and C57BL/6 were hyperresponsive with respect to both fat and cholesterol and BALB, NZB, and 129/J were hyporesponsive. NZB was unique in that C7H mRNA levels remained quite low regardless of diet.

A significant negative correlation was seen between C7H mRNA levels and liver cholesterol ( $r = -0.600$  at  $P < 0.04$ ), but not plasma cholesterol in chow-fed animals. A significant negative correlation was seen between C7H mRNA levels and plasma cholesterol for mice fed safflower oil with cholesterol ( $r = -0.633$  at  $P < 0.04$ ).

## DISCUSSION

Two observations commonly made in human dietary studies are that wide variations in plasma cholesterol levels exist among individual subjects and that responses to dietary change vary among these individuals. In addition, the magnitude of responsiveness to a new test diet is not related to starting values seen on a basal diet. We have recreated these diet study characteristics using nine inbred mouse strains. For instance, plasma total cholesterol levels varied nearly 3-fold among mice fed the rodent chow "basal" diet and the magnitude of responses in plasma cholesterol levels to dietary changes varied among mice from no response (e.g., DBA/2) to marked changes (e.g., BALB). The benefit of using inbred mice for such diet studies is that questions as to possible contributions of particular diet-responsive genes can now be tested easily among crosses made between mice differing in basal or responding cholesterol levels. In addition, the influence of additional unknown genes can be evaluated by positional cloning approaches.

Berg (15) has suggested that a combination of level and variability genes determine an individual's overall plasma lipid levels and atherosclerotic risk. Level genes determine the absolute levels of plasma lipids, while variability genes determine the degree of response to environmental exposures. For level genes, genetic crosses constructed between mice fed rodent chow with the highest (NZB or 129/J) and lowest (A/J, DBA/2, SWR) cholesterol levels, or highest (A/J or AKR) and lowest (BALB, NZB, 129/J) triglyceride levels, could be used. Variability genes determining plasma cholesterol responsiveness to diet could be studied using crosses between responsive strains (e.g., BALB, C3H) and hyporesponsive strains (DBA/2, AKR) fed appropriate diets. To study diet responsiveness of plasma

triglyceride levels, crosses between responsive strains (e.g., AKR, C3H, C57BL/6, DBA/2, or SWR) and those showing more modest changes (BALB, NZB, 129/J) could be used. Interestingly, the fact that plasma cholesterol and triglyceride levels did not correlate among the strains surveyed here and in other studies (1, 3, 34) suggests that level and variability genes controlling plasma cholesterol and triglyceride levels will be distinct.

One approach to characterizing genetic factors contributing to diet responsiveness is to use recombinant inbred strains that are commercially available (35, 36). Level and variability genes have been identified in the mouse in this manner. An example of a level gene is *Apoa2*. Most mouse strains differ 2-fold in apoA-II concentrations, and although apoA-II levels can be altered by diet, the 2-fold difference persists among strains (31). In contrast, the gene *Ath-1* is a variability gene, affecting the levels of plasma HDL cholesterol primarily upon feeding mice diets rich in fat and cholesterol (37).

Our preliminary studies suggest that the use of recombinant inbred (RI) strain analysis for identification of diet-responsive genes will be difficult, as plasma lipid values intermediate between parental levels, indicative of multigenic effects, were seen for several RI sets (C57BL/6 X DBA/2, SWR X C57L, and BALB X C57BL/6; data not shown) fed our test diets. Further, differences in diet responsiveness do not always occur between parental strains of RI strain sets. Thus, the best approach is to use quantitative trait linkage analysis in which diet-responsiveness phenotypes are correlated to genetic markers spanning the mouse genome (18, 21). This approach has been used successfully to simultaneously locate multiple loci involved in determining morphine preference (20), obesity (38), adipose depots, and plasma cholesterol levels (19) in mice.

From this study we are able to identify several metabolic stages in which genetic factors contribute to determining basal and diet responsiveness with respect to tissue lipid levels. From Fig. 1 it is clear that the range of basal (rodent chow) plasma total cholesterol levels among strains was due primarily to the wide differences in HDL cholesterol levels, as VLDL/LDL levels for all strains were quite similar. This suggests that genes controlling basal lipid levels in the mouse are those controlling plasma HDL cholesterol levels. In contrast, diet-responsive genes will be those controlling VLDL/LDL cholesterol levels, as this combined lipoprotein fraction was most altered by altering the diet.

Another metabolic stage contributing to diet responsiveness is cholesterol absorption. The extent of cholesterol absorption among four inbred strains showing a range of plasma cholesterol levels when fed rodent chow was shown to be nearly identical (Fig. 5), arguing against cholesterol absorption as a point of level gene control



for the trait of plasma total cholesterol. However, the percent of cholesterol absorbed by hyporesponsive strain DBA/2 after the feeding of high fat/high cholesterol diets was significantly lower than seen for three diet-responsive strains, suggesting that genetic factors controlling cholesterol absorption participate in diet responsiveness. Cholesterol absorption is a complex process which is influenced by many factors including bile acid content of the gut (39-41), pancreatic cholesterol esterase (40, 41), acyl-CoA:cholesterol acyltransferase (42, 43), sphingomyelin content of brush-border membranes (44), and proteoglycan-bound cholesterol binding enzymes (41). Thus, multiple candidate variability genes should be tested for their role in determining DBA/2 hyporesponsiveness.

However, it is unlikely that cholesterol absorption was the only contributor to hyporesponsiveness in DBA/2 mice. Although DBA/2 exhibited lower cholesterol absorption as compared to other strains, the absolute amount of dietary cholesterol absorbed by DBA/2 mice was not decreased upon changing from rodent chow to 0.5% cholesterol diets. Rodent chow contains approximately 0.03% by weight of cholesterol, and food intake for mice is approximately 5 g/day (22). Thus, approximately 0.11 g/day of cholesterol derived from the diet was absorbed by mice of the four strains examined. The high-fat diets contained 0.5% by weight cholesterol, so DBA/2 mice fed such diets absorbed about 10 times the amount of cholesterol as rodent chow-fed mice (1.35 g/day cholesterol). Overall, the remaining strains (C3H, C57BL/6, 129/J) retained high levels of cholesterol absorption (approximately 78%) and absorbed approximately 2.0 g/day of cholesterol, nearly double that for DBA/2. This analysis suggests that cholesterol absorption combined with other regulatory events contributed to hyporesponsiveness in DBA/2 mice. Further examination of DBA/2 mice with respect to plasma lipoprotein clearance and bile acid synthesis is warranted.

Another metabolic stage contributing to hepatic and plasma lipid levels and to cholesterol absorption is the activity of C7H, the rate-limiting enzyme in bile acid synthesis. Levels of C7H have been reported to be a factor determining plasma cholesterol levels in nonhuman primates (45) and contributing to apoB levels in mice (46). As the regulation of C7H by cholesterol and other agents is largely at the level of transcription (28, 47, 48), we examined mRNA levels for C7H among nine inbred strains fed rodent chow and safflower oil diets to estimate the contribution of C7H to dietary hypo- and hyperresponsiveness among strains. An inverse trend between C7H mRNA levels and either plasma or hepatic cholesterol was seen in some cases. This suggests that the C7H gene can contribute to setting both level and diet variability traits. Of note were the low C7H mRNA

levels observed regardless of diet for NZB, the strain showing the highest plasma cholesterol levels. The combination of low C7H mRNA and high plasma cholesterol levels may result from poor uptake of plasma lipoproteins via the LDL receptor and thus, decreased flux of cholesterol through the liver via C7H. In contrast, hyporesponsive strain DBA/2 exhibited elevations in C7H mRNA levels with diet, which may have contributed to the reduced hepatic cholesterol levels seen in this strain as compared to others (Fig. 3). Whether DBA/2 maintains low plasma cholesterol levels because of reduced relative cholesterol absorption (Fig. 5) or an increased flux through the plasma compartment remains to be established. These phenotypes for C7H suggest that major differences in C7H activities exist among strains and suggest a role for C7H in determining responsiveness to diet.

Although it has been thought that elevations in bile acid availability contribute to increased cholesterol absorption, this was recently refuted in inbred mice (46). Duelland et al. (46) demonstrated that the capacity for cholesterol absorption by BALB and C57BL/6 was identical in the absence and presence of exogenously supplied taurocholate. However, dietary bile acid completely blocked the ability of these strains to induce C7H by dietary cholesterol. Our data obtained for BALB and C57BL/6 mice fed high fat diets containing 0.5% bile acid are supportive of these findings, as marked increases in C7H for these strains were not observed. However, significant increases in C7H mRNA levels were seen for several strains (notably A/J, AKR, and DBA/2), as well as significant decreases (notably C3H and SWR). It would be of interest to examine C7H mRNA levels for these mice fed high-fat diets without added bile acid to determine whether bile acid effects on C7H are strain specific.

In summary, we have identified several strains that could be used to identify level and variability genes controlling plasma and hepatic lipid levels. Further, several of these strains will be useful for examining different features of cholesterol absorption and bile acid synthesis. The best genetic approach may be quantitative trait linkage analysis among mice varying in basal lipid levels and in their responsiveness to dietary challenges. Identifying variability genes involved in responsiveness to diet may provide bases for the identification of syntenic genes in humans. ■

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