Genetic Susceptibility and Resistance to Diet-induced Atherosclerosis and Hyperlipoproteinemia

Joel D. Morrisett, Han-Seob Kim, Josef R. Patsch, Surjit K. Datta, and John J. Trentin

To test the hypothesis that genetic susceptibility or resistance to diet-induced atherosclerosis is correlated with serum levels of specific lipids, lipoproteins, or apoproteins, male mice of a genetically susceptible and a genetically resistant strain were fed either a normal or an atherogenic diet. After 20 weeks on a normal diet, neither the resistant nor the susceptible strain mice had atherosclerosis; resistant strain mice had serum cholesterol of 66 ± 11 while the susceptible strain mice had 90 ± 1 mg/dl serum cholesterol, and lipoproteins were dominated by a single α-migrating HDL. After 20 weeks on an atherogenic diet, resistant strain mice had 185 ± 55 mg/dl cholesterol, their lipoproteins remained dominated by α-migrating HDL, and two of eight mice had mild atherosclerotic lesions; susceptible strain mice had 510 ± 94 mg/dl cholesterol, multiple α- and pre-β-migrating lipoprotein species, and all 13 had advanced aortic atherosclerosis. The resistant strain mice had an apolipoprotein E/total lipoprotein protein ratio of 0.42 on the normal diet and 0.53 on the atherogenic diet, while the susceptible strain mice had the significantly lower ratios of 0.07 and 0.31, respectively. These data indicate that genetic resistance to diet-induced aortic atherosclerosis in mice is correlated with capacity to prevent large increases in serum cholesterol, to suppress abnormal α- and pre-β-migrating lipoproteins, and to maintain elevated serum apolipoprotein E/total lipoprotein protein ratios. Our data do not preclude the possibility of additional gene control at the level of arterial end organ response. (Arteriosclerosis 2:312-324, July/August 1982)

It has been known for some time that humans exhibit a wide range of plasma lipid levels, of capacity to catabolize exogenous fat, and of susceptibility to atherosclerosis. A number of animal models have been developed in an attempt to simulate the favorable or unfavorable aspects of these factors. For example, the pig and the monkey have been studied as models of human susceptibility to atherosclerosis, whereas the dog and the rat have been examined as models of resistance to this disease. Recently, it has been demonstrated that a single animal species, the mouse, can exhibit either susceptibility or resistance to atherosclerosis, depending on its genetic makeup. Although the mouse offers the largest number of genetically defined inbred strains and is the most frequently used mammalian animal model in both genetic and nongenetic biomedical research, surprisingly little use has been made of the mouse in atherogenesis research. Early attempts to use the mouse in studies of dietary atherogenesis were disappointing. A variety of high fat "atherogenic" diets were found to cause weight loss or lack of weight gain, severe pathological disturbances, mortality, and variable results regarding atherogenesis. However, Thompson reported that variability could be reduced by use of an inbred (C57BL/6J) strain of mice, and that morbidity and mortality could be greatly reduced by gradually increasing the percentage of a modification of the Hartroft and Thomas thrombogenic rat diet from 70% to 80% to 90% of the mouse diet over a period of 6 days. When phased onto the high fat diet in this manner, one group of C57BL/6J mice fed at the 90% level for 25 weeks showed no mortality and consistently developed atheromatous lesions in the region of the aortic valves. In a test of 13 different inbred strains of mice, Rob-
erts and Thompson\textsuperscript{3} reported that 10 strains tolerat-
ed this dietary regime. Of these strains, the C57BR/cdJ was the most susceptible, and the CBA/J the most resistant to both atheromatous lesions and to elevated serum total cholesterol as a result of the high fat, high cholesterol diet. In a classic genetic study of the F\textsubscript{1} and F\textsubscript{2} hybrids of these two inbred strains of mice, and of the back-cross of the F\textsubscript{1} to the resistant and to the susceptible parent strains, these authors found that susceptibility and resistance to diet-induced elevated serum total cholesterol and atherogenesis were genetically determined by a multifactorial (polygenic) system of inheritance, with the mother and the father exerting an equal influence.\textsuperscript{3}

Our laboratory has had a continuing interest in the effects of diets with various fat content and saturation on the structure and function of the resulting lipids and lipoproteins.\textsuperscript{5-11} It was the purpose of the present study to examine these same two inbred strains of mice not only for their atherogenic and serum cholesterol responses, but also for their serum triglyceride levels and lipoprotein types, to determine if the latter were significantly different in the genetically resistant and susceptible strains fed the

### Methods

The C57BR/cdJ (susceptible) and CBA/J (resistant) strain mice were obtained from the Jackson Memorial Laboratory, and were bred and/or maintained in the animal quarters of the Division of Experimental Biology. Experimental animals were 5- to 6-week-old males which were fed either a normal rodent laboratory chow (Purina 5001) containing 23\% crude protein, 4.5\% crude fat, and 6\% crude fiber, or an atherogenic diet consisting of one part normal chow plus nine parts of an atherogenic concentrated mixture shown in table 1. Our determination of the lipid and fatty acid composition of these diets is shown in tables 2 and 3. The atherogenic diet not only contained a higher weight percentage of cholesterol (table 2) but also a higher level of saturated fat (table 3). The most abundant fatty acyl chain-

<table>
<thead>
<tr>
<th>Table 1. Composition of Atherogenic Concentrated Diet</th>
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<tbody>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>Casein, vitamin free*</td>
</tr>
<tr>
<td>Cocoa butter†</td>
</tr>
<tr>
<td>Cholesterol, U.S.P.‡</td>
</tr>
<tr>
<td>Sodium cholate‡</td>
</tr>
<tr>
<td>D (+) sucrose*</td>
</tr>
<tr>
<td>D (+) dextrose*</td>
</tr>
<tr>
<td>Dextrin, white, technical‡</td>
</tr>
<tr>
<td>Alphacel, non-nutritive*</td>
</tr>
<tr>
<td>Vitamin diet fortification mixture*</td>
</tr>
<tr>
<td>Wesson salt mixture*</td>
</tr>
<tr>
<td>Choline chloride‡</td>
</tr>
</tbody>
</table>

*ICN Nutritional Biochemicals, Cleveland, Ohio.
†Blommer Chocolate Co., Chicago, Illinois.
‡Teklad Test Diets, Madison, Wisconsin.

<table>
<thead>
<tr>
<th>Table 2. Lipid Composition of Normal and Atherogenic Mouse Diets (Weight %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
</tr>
<tr>
<td>Phospholipids</td>
</tr>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
</tr>
<tr>
<td>Triglyceride</td>
</tr>
<tr>
<td>Total lipids</td>
</tr>
</tbody>
</table>

*Approximately 0.5 g portions of each diet were finely divided then extracted three times with CHCl\textsubscript{3}:CH\textsubscript{3}OH (3:1). Extracts were evaporated to dryness, redissolved in isopropanol, and analyzed. Values represent averages of triplicate determinations.

†Contains 10% normal diet plus 90% of the atherogenic concentrated mixture of table 1.
containing lipid types were the triglycerides. These lipids were much richer in the saturated fatty acids C18:0 and C18:2 in the atherogenic diet (30.9% and 33.6%) than in the normal diet (20.4% and 9.4%). Consequently, the P/S ratio for triglyceride in the atherogenic diet was about one-tenth that of the normal diet. The atherogenic concentrated mixture was not fed in pure form, but mixed with 30% normal diet for the first 3 days, with 20% for the next 3 days, and with 10% for the remainder of the study. Food was withdrawn 3 to 5 hours before sacrifice. Animals with 10% for the remainder of the study. Food was withdrawn 3 to 5 hours before sacrifice. Animals were bled from the retroorbital venous sinus and the collected blood was allowed to clot 1 hour at 4°C before centrifugation to obtain individual sera (~ 0.5 ml). The heart of each animal was excised, placed in cold McCoy’s 5A tissue culture medium, and transferred to the Pathology Laboratory where it was processed and embedded using Tissue-Tek II embedding medium (Miles Laboratories, Incorporated, Napaville, Illinois) for frozen tissue specimens. Sections through the aortic sinus were cut at 5 microns on a freezing microtome, stained with Oil Red O (ORO) and counterstained with hematoxylin.

Only sections that contained portions of the valve attachment to the aortic wall were used for evaluation of the atherosclerotic lesions. An ideal section at the right cusp level is illustrated in figure 1 A. The extent of ORO-positive atherosclerotic lesions in the section was arbitrarily divided into one of six groups depending on the sum of the extents of all lesions combined: no lesions (0); combined lesions of the entire circumference equal to less than one-eighth (< 1/8) the distance between the attachment of two adjacent valve cusps; one-eighth to one-quarter (1/8–1/4); one-quarter to one-half (1/4–1/2); one-half to three-quarters (1/2–3/4); and over three-quarters (> 3/4). (The highest possible rating is 3, i.e., lesions of entire circumference.) This differs from the method of Roberts and Thompson3 in which only the largest single lesion, rather than the sum of all lesions, was quantitated.

To minimize proteolysis, individual mouse sera were treated with 5 µl of a 10 mM dioxane solution of phenylmethanesulfonyl fluoride (Pierce Chemical Company, Rockford, Illinois) or 500 K.I. units of Transylol (FBA Pharmaceuticals, New York, New York). Samples were stored at 4°C until used. Typically, 250 µl of serum was extracted with 5 ml of isopropanol for cholesterol and triglyceride analysis performed according to the Lipid Research Clinics protocol.14 For agarose electrophoresis, 5 to 10 µl aliquots were used on the Lipidophor system (Immuno AG, Vienna, Austria).15 After electrophoresis for 90 minutes, gels were treated for 1 hour with heparin/MgCl₂ and overnight with CaCl₂/dextran sulfate before scanning with the Immuno densitometer. Densitometer tracings were digitized with a Hewlett-Packard calculator Model 9100-B equipped with a 9107-A digitizer.

Mouse high density lipoprotein (HDL) was purified by zonal ultracentrifugation of pooled serum (5 ml) obtained from about 10 animals. A 1.0–1.4 g/ml NaBr density gradient and 22-hour spin were used.16 The single HDL species obtained from animals on the normal diet was dialyzed exhaustively against 100 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM Na₃, pH 7.40 (TBS buffer) before further analysis. Molecular weights were estimated by pore gradient gel electrophoresis,17,18 using precast Pharmacia 4% to 30% gels. Bovine albumin,19 human apo E,20 human apo A-I,21 and human apo A-II22 were used as molecular weight standards for apolipoproteins; the Pharmacia mixture of thyroglobulin (MW689,000), apoferritin (MW440,000), catalase (MW232,000), lactate dehydrogenase (MW140,000) and bovine serum albumin (MW67,000) were used as standards for HDL. After electrophoresis, gels were fixed in 10% sulfosalicylic acid, stained in 0.04% Coomassie Blue G-250/7% acetic acid, and destained in 5% acetic acid. Gels were scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Chicago, Illinois). Chemical compositions of lipoproteins were determined by measuring protein by a modification of the Lowry method,23 phospholipid by the Bartlett procedure,24 free and esterified cholesterol using the Boehringer-Mannheim enzymatic method,25 and triglyceride using the Pierce C-37 Rapid Stat kit (Scientific Products). For fatty acid analysis, extracted lipids were separated by thin-layer chromatography, transesterified with BF₃/CH₃OH, and analyzed by gas chromatography.8

**Results**

Whereas both strains of mice on normal chow diet gained progressively in body weight, mice on the high fat diet sometimes showed an initial loss of body weight while adapting to the new diet, followed by a variable period of impaired body weight gain. This was more severe and more prolonged in the sus-

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**Figure 1.** Transverse frozen sections of the aorta at the level of attachment of the aortic valve cusps, stained with Oil-Red-O and counter-stained with hematoxylin. A. Section from a C57BR (genetically susceptible) mouse on the atherogenic diet for 5 weeks, but before development of atherosclerotic lesions. The appearance is essentially that of normal mice of either strain. All three delicate valve cusps and their attachment to the aortic wall are seen. The internal elastic membrane (tortuous due to postmortem contracture of the aorta) is lined by endothelial cells with practically no intervening tissue, x 126. B. Section from a genetically susceptible C57BR mouse on an atherogenic diet for 15 weeks. Thick atheromatous lesions of the aortic sinus wall cover more than three-fourths of the distance between the attachments of adjacent valve cusps. The sparsely cellular atherosclerotic lesions contain much Oil-Red-O positive debris. The intimal lesions encroach upon the media, with an irregular border. x 126.
ceptible C57BR mice, which also had greasy coats and more morbidity and mortality than the resistant CBA mice on the same high fat diet. In the C57BR mice on high fat diet, there was sometimes a "cage effect," some cages of mice showing severe morbidity and mortality, while others did not.

In one experiment in which six mice of each of the four groups were sacrificed at 10 and at 15 weeks, and the remainder at 20 weeks on the diet, there was no additional mortality in any of the 22 CBA mice and 21 C57BR mice started on normal diet; however, one of the 22 CBA mice and three of the 25 C57BR mice started on the high fat diet died between 8 and 15 weeks on diet.

None of the aortic sinuses of 33 C57BR mice and 26 CBA mice examined after 3 to 20 weeks on the normal diet showed any atherosclerotic lesions (table 4). On the high fat, high cholesterol diet, none of 13 CBA mice examined at 3 to 10 weeks showed any atherosclerotic lesions of the aortic sinus walls, but six of 12 mice examined at 15 and 20 weeks showed usually mild atherosclerotic lesions (table 4, figure 1C). In contrast, three of 12 C57BR mice on the same diet showed mild lesions as early as 3 weeks, and by 15 and 20 weeks all 21 C57BR mice examined showed usually severe atherosclerosis (table 4, figures 1B and D). The intimal atherosclerotic lesions consisted of subendothelial accumulations of cells and Oil-Red-O positive lipid globules of various sizes, both intracellular and extracellular. In advanced atherosclerotic lesions the media appeared focally destroyed by the expanding intimal process.

Histopathologic examination of hematoxylin and eosin (H & E) sections of heart, lung, liver, spleen, kidney, and intestine of a genetically susceptible C57BR mouse on the atherogenic diet for 8 weeks showed no significant pathologic lesions in these organs. Microscopic examination of H & E sections of liver from one mouse of each strain on each diet for 29 weeks showed mild fatty change, canalicular bile stasis, and multifocal lymphocytic infiltrate only in the C57BR mouse on the atherogenic diet.

An important step toward understanding the different responses of the genetically resistant and susceptible mouse strains to diet-induced atherosclerosis involved the isolation and characterization of their serum lipoproteins. Whereas this is a relatively straightforward procedure for human and higher primate lipoproteins, this was not true in the present study. Only rather small (~ 0.5 ml) volumes of serum could be obtained from individual mice, and the lipoproteins obtained consisted predominantly of the

Table 4. Summary of Atherosclerotic Involvement of the Aortic Sinus of Susceptible and Resistant Mice on Atherogenic and Normal Diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Weeks on diet</th>
<th>Susceptible C57BR/cdJ strain Fraction of aortic sinus wall involved*</th>
<th>Resistant CBA/J strain Fraction of aortic sinus wall involved*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0  &lt; 1/8 1/8-1/4 1/4-1/2 1/2-3/4 &gt; 3/4</td>
<td>0  &lt; 1/8 1/8-1/4 1/4-1/2 &gt; 1/2</td>
</tr>
<tr>
<td>Atherogenic</td>
<td>3</td>
<td>9  3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6  1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7  3  2  1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1  1  2  9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1  1  2  9</td>
<td>6</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>3</td>
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<tr>
<td></td>
<td>10</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

*Linear sum of all atherosclerotic lesions of the entire circumference of the aorta at the level of the aortic valve cusps, expressed as a fraction of the distance between the points of attachment of two adjacent valve cusps.

Figure 1 continued. Transverse frozen sections of the aorta at the level of the attachment of the aortic valve cusps, stained with Oil-Red-O and counterstained with hematoxylin. C. Section from a genetically resistant CBA mouse on atherogenic diet for 15 weeks. One small and two tiny atherosclerotic lesions (arrows) are seen, but the combined lesions occupy less than one-eighth of the aortic sinus wall between the attachments of two valve cusps. The lesions consist mostly of clusters of cells that are Oil-Red-O positive. × 126. D. From a genetically susceptible C57BR mouse on an atherogenic diet for 20 weeks. The combined atherosclerotic lesions occupy approximately one and one-half times the distance between adjacent valve cusp attachments, extending from approximately 4 to 9 "o'clock". The lesions consist mostly of large collections of lipid-containing cells with little degeneration. Note the variation in the intensity of Oil-Red-O staining of the lesions. Red stained material outside the aorta is periadventitial adipose tissue. × 126.
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high density class. Hence, in the present study the comparison was limited largely to HDL of pooled sera. Zonal ultracentrifugation of pooled sera obtained from a group of susceptible and of resistant 5- to 6-week-old mice on a normal diet suggested the presence of a single density population of HDL particles which banded at a position intermediate between human HDL₂ and HDL₃ (figure 2). These particles were also relatively homogeneous with respect to size as judged by pore gradient gel electrophoresis, with the susceptible strain showing a somewhat broader size distribution (figure 3). The chemical composition of HDL from the resistant and susceptible strains was very similar (table 5). Both populations of particles were rich in protein (46%), phospholipid (~ 27%) and cholesteryl ester (~ 22%), but poor in triglycerides (~ 2.5%) and cholesterol (1.5%). Both HDL populations had particle weights of about 234,000 (figure 3), and calculated hydrated densities of 1.167 g/ml (table 5). SDS-pore gradient gel electrophoresis was used to compare the apoprotein compositions of HDL from the susceptible and resistant strains (figure 4). In each case, the

**Table 5. Chemical Composition of Mouse HDL (Weight %)**

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>Phospholipid</th>
<th>Glycerides</th>
<th>Cholesterol</th>
<th>Cholesteryl ester</th>
<th>Calculated hydrated density (g/ml)</th>
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</thead>
<tbody>
<tr>
<td>Resistant strain</td>
<td>46.2</td>
<td>28.5</td>
<td>3.7</td>
<td>1.3</td>
<td>20.3</td>
<td>1.167</td>
</tr>
<tr>
<td>Susceptible strain</td>
<td>46.2</td>
<td>26.3</td>
<td>1.8</td>
<td>1.7</td>
<td>24.0</td>
<td>1.167</td>
</tr>
</tbody>
</table>

*Pooled sera on ~5 ml from 10 mice on normal diet were subjected to zonal ultracentrifugation (see ref. 16), which yielded a single density population of HDL (figure 4). Fractions from the appropriate zones were pooled and dialyzed exhaustively against Tris-buffered saline before chemical analysis (see refs. 8, 23, 24, 25). Values represent averages of triplicate determinations. Hydrated densities were calculated using constants from Sata et al. (see ref. 34).*
Figure 4. Pore gradient (4%→30%) gel electrophoresis in 0.01% SDS of apoproteins from mouse HDL (normal diet) isolated by rate-zonal ultracentrifugation. The small broad band near the right side of the scan (bottom of the gel) is tracking dye. BSA = bovine serum albumin; E = apoprotein E; A1 = apoprotein A-I; AII = apoprotein A-II.

A major apoprotein was a species migrating virtually the same distance as that of human apo A-I which has a molecular weight of 28,331.21 Hence, the HDL from the genetically susceptible and resistant strains of mice on a normal laboratory chow exhibited a number of very similar physical and chemical properties.

The d = 1.0–1.4 g/ml nonlinear density gradient used to purify HDL yields very low density lipoprotein (VLDL) and low density lipoprotein (LDL) as an unresolved mixture (figures 2 A and 2 B). When these mixtures were recentrifuged on a shallower gradient (d = 1.0–1.3 g/ml) for a shorter time (140 minutes), they were resolved to the extent shown in Panels C and D of figure 2. The amount of material banding in the VLDL range was significantly greater for the resistant strain than for the susceptible strain, but in neither case could we isolate amounts of VLDL or LDL sufficient for careful physical and chemical characterization.

During the course of the study, serum lipids were measured for individual animals of each strain on the two diets. When fed the normal diet, the resistant strain mice maintained an average serum cholesterol of 50%–75 mg% (figure 5 A). On the same diet, the susceptible strain mice maintained a somewhat higher level of about 100 mg% over the entire 20-week period (figure 5 B). When the resistant strain mice were fed the atherogenic diet, serum cholesterol rose to about 185 mg% after 5 weeks and remained at about that level for the rest of the diet period (figure 5 A). In contrast, in only 3 weeks the susceptible strain mice experienced an abrupt increase in cholesterol to about 350 mg% and a further increase at about 15 weeks to about 550 mg%.

The effect of the atherogenic diet on serum triglyceride levels of mice from the two strains is shown in figure 6. The resistant strain mice on the control diet
began the diet period with an average triglyceride level of about 200 mg%, which decreased to about 100 mg% after 20 weeks on either diet. The triglyceride levels of resistant mice on the atherogenic diet were consistently lower than on the normal diet, although not significantly so except at 15 weeks.

In mice from the genetically susceptible strain on the normal diet, the triglyceride levels, except for an initial low value of 50 mg%, declined from 130 mg% at 3 weeks to about 60 mg% at 20 weeks. The triglyceride levels of the susceptible strain, in contrast to the levels in the resistant strain, were elevated by the atherogenic diet, to about 275 mg% at 10 weeks and 20 weeks (figure 6B).

The differing serum lipid responses to the atherogenic diet by mice from the two strains raised the question of what lipoprotein particle(s) in the blood might be responsible for transporting this dietary lip-

**Figure 6.** Serum triglyceride levels (arithmetic mean ± range) of resistant strain mice on the normal diet (n = 4), resistant strain mice on the atherogenic diet (n = 6), susceptible strain mice on the atherogenic diet (n = 8), and susceptible strain mice on the normal diet (n = 4).

**Figure 7.** Agarose gel electrophoretic banding patterns of lipoproteins in sera of mice genetically resistant or susceptible to diet-induced atherosclerosis after having been on diet for 20 weeks. Ten μl aliquots of individual sera were electophoresed in 2% agarose for 90 minutes after which the gels were treated with a solution of 1.8% sodium phosphotungstic acid, 0.18M MgCl₂, 0.07 M NaCl to precipitate the lipoproteins. Gels were scanned with an Immuno lipodipor scanner. Scans of two sera from the resistant strain on the atherogenic diet, both resembling scan C 4, have been omitted. The origin and banding positions of normal mouse HDL, LDL, and VLDL are shown in panel A 4. The numbers 1 to 4 and letters A to E are reference coordinates.
id. The answer to this question was sought using agarose gel electrophoresis (figure 7, 20-week data). The resistant strain mice on the normal diet exhibited low and variable levels of β-migrating and pre-β-migrating lipoproteins, but uniformly high levels of α-migrating HDL. Sera from the susceptible strain mice on the control diet gave electrophoreto grams which, in three of four cases (B1, B2, B3), exhibited increased levels of β- plus pre-β-migrating lipoproteins. Serum from the resistant strain mice on the atherogenic diet contained large amounts of a single, normal α-migrating component, and variable amounts of slow α-, β- and pre-β-migrating species (figure 7, Column C). The electrophoretic patterns for sera from the susceptible strain on the atherogenic diet were remarkably different from that of the three other groups. They were characterized by multiple α-migrating species which banded sharply (figure 7, Columns D and E). These sera were also characterized by the presence of a broad electrophoretic component which migrated more slowly than HDL but faster than LDL, and is, therefore, referred to here as post-α. Lesser and variable amounts of pre-β and β-migrating particles were observed for this group. It would have been desirable to isolate in pure form each of the bands (as many as 7) indicated by agarose electrophoresis for the susceptible strain on the atherogenic diet (figure 7). There are two main problems which prevented us from doing this: 1) obtaining sufficient resolution on a preparative scale to get particle populations which can be regarded as reasonably homogeneous, and 2) obtaining sufficient amounts of each of these different components to permit in-depth chemical and physical studies.

Dietary cholesterol has been shown to affect the plasma levels of apo E in numerous mammalian species. In the present study, possible differences in apo E levels between the two mice strains before and after feeding the atherogenic diet for 20 weeks were investigated using two types of measurements (table 6). In one type, the apo E/apo A-I ratio was determined by SDS-polyacrylamide gel electrophoresis of the d = 1.21 supernatant. The susceptible strain mice on the atherogenic diet had an apo E/apo A-I ratio 11% higher than the same strain mice on a normal diet. For the resistant strain mice on the atherogenic diet, this ratio was slightly lower (5%) than for the same group on a normal diet.

When on the normal diet, the resistant strain mice had a 61% higher apo E/apo A-I ratio than the susceptible strain mice, whereas on the atherogenic diet the resistant strain mice had a 38% higher ratio. In the other type of measurement, electroimmunoassay was used to quantitate apo E in d = 1.21 supernatants (and infranatants) and expressed as a fraction of the total protein. In mice from both the susceptible and the resistant strains, the apo E/total protein ratio in the d = 1.21 supernatant§ was substantially higher when the atherogenic diet was consumed (table 6).

### Table 6. Effect of 20 Weeks of Atherogenic Diet on Mouse Serum Proteins

<table>
<thead>
<tr>
<th>Strain</th>
<th>d = 1.21 fraction</th>
<th>Diet</th>
<th>Apo E (mg/ml)</th>
<th>Apo E* (mg/ml)</th>
<th>Total protein (mg/ml)</th>
<th>Apo E/Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>Top† Bottom§</td>
<td>Normal</td>
<td>0.32 ± 0.010</td>
<td>&lt; 0.010</td>
<td>0.626 ± 0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Susceptible</td>
<td>Top† Bottom§</td>
<td>Atherogenic</td>
<td>0.35 ± 0.02</td>
<td>&lt; 0.010</td>
<td>0.310 ± 0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Resistant</td>
<td>Top† Bottom§</td>
<td>Normal</td>
<td>0.51 ± 0.006</td>
<td>&lt; 0.010</td>
<td>0.197 ± 0.19</td>
<td>0.42</td>
</tr>
<tr>
<td>Resistant</td>
<td>Top† Bottom§</td>
<td>Atherogenic</td>
<td>0.48 ± 0.01</td>
<td>&lt; 0.010</td>
<td>0.245 ± 0.25</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Individual mouse sera of various groups were pooled and ultracentrifuged at d = 1.21 g/ml (SW50.1 rotor, 24 hrs). The supematant containing the total lipoproteins and infranat containing lipid-free serum proteins were analyzed for apo E (* by electroimmunoassay (see ref. 35) using a cross-reacting antibody raised against rat apo E in the rabbit, and for total protein by the method of Lowry (see ref. 23). Values shown for apo E are means ± average deviation for duplicate determinations with undiluted samples. In addition, aliquots of the lipoprotein-containing supernatants were heat-treated in SDS (5 min at 90° and 60 min at 60°), then electrophoresed in SDS on 4% to 30% polyacrylamide pore gradient slab gels, which were stained with Coomassie blue and scanned. Scans were digitized and integrated to obtain ApoE/Apo A-I ratios.

†Because of the technical difficulties in preparatively resolving small amounts (< 1 ml) of serum into its several electrophoretically different lipoprotein components and because of the potential for extensive apo E dissociation from the lipoproteins upon sequential ultracentrifugation at several increasing densities (see ref. 26), it was deemed more appropriate to estimate apo E in the total lipoprotein fraction (d = 1.210 g/ml supernatant).

§Mahley and Holcombe (see ref. 26) have observed that more than 50% of the apo E in rat plasma is dissociated from lipoproteins during repeated ultracentrifugation. In the present study, no mouse apo E was detected in d = 1.21 infranatant after a single 24-hour spin.
Discussion

The present study confirms the genetic susceptibility of the C57BR/cdJ strain and the resistance of the CBA/J strain to diet-induced hypercholesterolemia and atherosclerosis. (In a separate study, we found that this genetic resistance to diet-induced hyperlipidemia and atherosclerosis is unaltered by irradiation; the mice were given four doses of 225 rads at one-week intervals before starting the 20-week atherogenic diet.) It further establishes that the atherosclerotic lesions consist of more than accumulations of Oil Red O positive foam cells. There is significant accumulation of cells in the intima, and focal involvement of the media as well. In addition, significant differences have been observed in the response of these two strains to atherogenic diet in respect to serum triglyceride levels and serum lipoprotein components. Although genetic factors have been standardized by the use of inbred mice of the same strain and sex and responses thereby rendered more uniform, the susceptible strain on a high fat diet still showed a significant range of responses in respect to serum cholesterol level (figure 5), number and distribution of serum lipoprotein components (figures 7 D and E) and atherosclerosis (table 4) from apparently nongenetic variables.

The increased morbidity and mortality observed in the C57BR strain as compared to the CBA strain on the atherogenic diet is consistent with previous reports that nutritionally induced hypercholesterolemia in mice is associated with an impairment of immune ability in vivo, and increased susceptibility to, and mortality from, certain viral and bacterial infections. It is known that certain serum lipoproteins are immunosuppressive both in vitro and in vivo. The “cage effect” in morbidity and mortality of C57BR mice on high fat diet is also consistent with the cage effect due to infection in mice immunosuppressed for other reasons, such as allogenic radiation chimeras suffering from chronic graft versus host reaction.

The C57BR and CBA mouse strains represent wide extremes of genetic susceptibility and resistance to the hyperlipoproteinemic and atherogenic effects of high fat, high cholesterol diet. It is possible that the morbidity and mortality of mice from the highly susceptible C57BR strain on the atherogenic diet can be minimized by further dilution of the atherogenic concentrate with normal chow, and that this will still retain a significant incidence of atherosclerosis. Other investigators have observed that a hypercholesterolemic diet developed for outbred mice was not tolerated by inbred C57BL/6 mice until it had been diluted to 1% cholesterol, 0.5% cholic acid and 18% lard. It should also be noted that other inbred strains of mice have been outcrossed, then genetically selected and inbred to have a high plasma cholesterol level on a normal low fat, low cholesterol diet.

HDL isolated from the genetically susceptible (C57BR/cdJ) and resistant (CBA/J) strains of mice on normal diet were virtually indistinguishable by the criteria of hydrated density, particle size, electrophoretic mobility, lipid composition, and apoprotein composition. However, both these types of mouse HDL differ in one or more of these properties from that of other mouse strains reported to date. Whereas we observed a single electrophoretic component for C57BR/cdJ and CBA/J HDL (figure 7), Ferreri et al. have observed both a diffuse α-migrating band and a sharp pre-α-migrating band in sera of male ICR albino mice. Mice whose HDL contained a larger proportion of the diffuse α-band had significantly higher serum cholesterol levels. HDL isolated from BALB/c mice contained only a single species as determined by analytical ultracentrifugation. The molecular weight was determined to be 300,000, somewhat above the 234,000 measured for the HDL of the susceptible and resistant strains examined in the present study. We obtained a chemical composition for HDL from the male susceptible C57BR/cdJ strain that was significantly different from that reported by Hsu et al. for HDL from pools of male and female BALB/c and C57BL/6 mice. The C57 mice were reported to have an unusually high content of phospholipid (48.7%–51.3%) compared to 26.3% found in the present study. This difference may be due to heterogeneity in sex, strain, and age, or to differences in methods of isolation and computation. Hsu et al. did not indicate what fraction of measured cholesterol is in the esterified form, a factor that can significantly affect the calculated relative abundance of other components.

It is often assumed that the density range appropriate for isolation of human lipoproteins is equally suitable for isolating corresponding lipoproteins from other animals. The zonal ultracentrifugal profiles (figures 2 C and D) illustrate the danger of this assumption. VLDL and LDL from either the susceptible or the resistant strain of mice do not comprise a discrete, well-defined density population, as is the case with the normal human counterparts. Rather, they seem to comprise a continuum of particles extending from d = 1.006 to beyond the conventional LDL density cutoff of 1.063 g/ml. Consequently, the choice of d = 1.006 and d = 1.063 g/ml as the density cutoffs for the isolation of mouse VLDL and LDL must be regarded as somewhat arbitrary and of limited value. The amount of lipoproteins banding in the first 150 ml of the rotor is significantly greater in sera from the resistant strain than from the susceptible strain. Since these lipoproteins are generally rich in triglyceride, this result suggests that the resistant strain has a significantly higher serum triglyceride level. Although mice from both strains exhibited significant changes in triglyceride over the 20-week period, the susceptible strain mice consistently maintained a lower level on normal diet (figure 6). Hsu et al. have reported that the serum triglycerides of the C57BL/6 strain mice decrease (75 mg% → 58 mg%) with increasing age (4–6 months → 24–30 months).

In the present study both strains of mice showed...
generally decreasing levels of triglycerides with age on the normal diet. On the atherogenic diet the resistant strain mice showed comparable and significant decreases of triglycerides, whereas the susceptible strain mice showed significant increases in serum triglycerides. Although the atherogenic diet produced a significant elevation in the serum cholesterol of resistant strain mice, it did not significantly affect serum triglyceride levels in these mice (figure 6). With the exception of the 15-week sampling, both the normal and the atherogenic diets resulted in serum triglyceride ranges which extensively overlapped. This lack of difference cannot be attributed to masking by the nonfasting state of the animals since mice from the susceptible strain did exhibit a significant difference in triglycerides after 5 weeks on these two diets (figure 6); by the end of the 20-week study, susceptible animals on the normal diet had serum triglycerides averaging about 50 mg%, while susceptible animals on the atherogenic diet averaged about 275 mg%.

Feeding the atherogenic diet to the resistant strain mice resulted in an increase of about 100 mg% cholesterol over that of animals maintained on the normal diet (figure 5). This result is entirely consistent with that obtained by Roberts and Thompson with mice of the same strain. However, our results with feeding the atherogenic diet to the susceptible strain mice differ somewhat from theirs (figure 5). Between the start of the experiment and the first sampling after 3 weeks on the atherogenic diet, there was an abrupt increase to about 350 mg% cholesterol, which continued at about that level through the 10-week sampling. By the 15-week sampling, the average value had risen further to about 520 mg% which continued until the experiment was terminated at 20 weeks. In contrast, Roberts and Thompson observed an increase to about 550 mg% by the 5-week sampling, a drop to about 350 mg% by the 10-week sampling, and a rise to about 450 mg% at the terminal 15-week sampling. The reason for these differences in serum cholesterol as a function of time on the atherogenic diet is not readily apparent, but may be related to the amount of food eaten by individual animals and their differing states of health in the two studies.

It is significant that even though the atherogenic diet elevates the average serum cholesterol level of the resistant strain mice to about 185 mg%, this effect is not strongly reflected in the electrophoretic patterns of the individual sera (figure 7C) nor by extensive atherosclerosis in the aortic sinus wall (table 4). However, the much higher elevation of serum cholesterol in the mice from the susceptible strain on the atherogenic diet was accompanied by a striking change in the electrophoretic patterns of all eight sample sera. In turn, these changes could be correlated with a significant atherosclerosis in two animals and more extensive involvement in 11 others at 20 weeks (table 4). The appearance of new α-migrating lipoprotein species suggests the possibility that one or more of these may resemble the cholesterol-rich lipoprotein, HDL₃, produced in the cholesterol-fed rat. HDL₃ migrates with α₁ mobility and contains apo E as its predominant apoprotein. The amount of apo E in plasma of the rat is significantly elevated (29 mg/dl → 47 mg/dl) upon cholesterol feeding. This raised the question of whether the level of apo E might be raised differentially in mice from the resistant and susceptible strains when they were fed the high cholesterol atherogenic diet. When the data are normalized in terms of the apo E/apo A-I or apo E/total protein ratio, then the level of apo E is significantly higher in the resistant strain mice than in the susceptible strain mice on the normal diet (table 7). As determined by electroimmunoassay, the level of apo E rises substantially in mice from the susceptible strain when they are fed the atherogenic diet for 20 weeks; but even after this rise, apo E still is below the level in mice from the resistant strain at the beginning and end of this diet. Hence, it would appear that for animals which already have elevated amounts of apo E necessary for transport and/or cellular catabolism of dietary cholesterol, biosynthesis of only moderate amounts of additional apo E is necessary. For animals which do not have sufficient apo E to satisfy these functions, larger amounts of this apoprotein are synthesized and secreted. Studies of apo E turnover in mice from the resistant and susceptible strains on the normal and atherogenic diets would be of obvious interest for pursuing these possibilities.

**Acknowledgments**

The authors thank A. M. Gotto, Jr. and Jack L. Titus for their advice, support and helpful comments, and Anil Kulkarni and Sulabha Kulkarni for professional assistance in setting up and harvesting the mouse feeding experiment. We also thank Elizabeth Priest, Deborah Wenkert, Susan She, John Gaubatz, Mauro Nava, and Richard Plumlee for technical aid; Yen Chiu Lin Lee and Larry Chan for a gift of rat apo E and anti-apo E; and Sunny Gabriel, Deborah Deiz and Susan McNeeley for typing and graphics assistance.

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Index Terms: lipoproteins • cholesterol • triglyceride • atherogenic diet • genetics
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Arterioscler Thromb Vasc Biol. 1982;2:312-324
doi: 10.1161/01.ATV.2.4.312
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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