Genetic Analysis of the Difference in Diet-Induced Atherosclerosis Between the Inbred Mouse Strains SM/J and NZB/BlNJ

Wendy A. Pitman, Mark H. Hunt, Cynthia McFarland, Beverly Paigen

Abstract—To identify the genetic factors affecting susceptibility to atherosclerosis, we studied the inheritance of plasma total cholesterol (TC) and HDL cholesterol (HDL-C) concentrations and susceptibility to atherosclerotic lesion formation in an (SM/J[SM]$\times$NZB/BlNJ[NZB]) outcross, an (SM$\times$NZB)F1[F1]$\times$SM backcross, and the NXSM recombinant inbred (RI) strain set. After 18 or 26 weeks on the atherogenic diet, lesion sizes in female mice were $160\pm110$ (SE) $\mu$m$^2$ for NZB, $100\pm60$ for F1, and $3800\pm920$ for SM. After 0, 4, or 26 weeks on the atherogenic diet, NZB had higher TC and HDL-C levels than either SM or F1. The F1 progeny had TC and HDL-C levels slightly higher than or similar to the SM/J parent, while lesion size in the F1 progeny was more similar to the NZB parent. Among the 15 RI strains, 8 resembled NZB and F1, 3 resembled SM, and 4 were intermediate between NZB and SM for lesion size. For the (SM$\times$NZB)F1$\times$SM backcross offspring, 26 resembled NZB and F1, 7 resembled SM, and 6 were intermediate between NZB and SM for lesion size. There was poor correlation between lesion size and plasma TC or HDL-C in the parental strains and the backcross. These data suggest that resistance to atherosclerosis is determined by at least one major dominant gene contributed by the NZB strain, which we have named Ath8. Ath8 segregates independently of genes controlling TC and HDL-C levels. (Arterioscler Thromb Vasc Biol. 1998;18:615-620.)

Key Words: genetics ■ quantitative trait loci analysis ■ atherosclerosis ■ recombinant inbred strains ■ lipids

Atherosclerosis is an important disease affecting millions of Americans. Many factors, including genetics, behavior, and environment, contribute to the risk of developing atherosclerosis, and great progress has been made in identifying many of these risk factors. The mouse has become a particularly useful tool for identifying genetic factors contributing to many diseases, including atherosclerosis,1-4 because of the existence of inbred, RI, and mutant strains5 and the development of molecular techniques allowing for the creation of transgenic6 and knockout mice.7 Also, recent developments in mapping technologies have resulted in the development of high-resolution genetic8 and physical maps of the mouse genome.5-10

Inbred mouse strains differ in plasma lipoprotein concentrations, responsiveness to high-fat, high-cholesterol diets, and susceptibility to atherosclerotic lesion development,11,12 thus providing useful tools for identifying genetic factors responsible for these differences.11,13 Two of these inbred strains, SM and NZB, differ in plasma TC and HDL-C concentrations11,14 and in susceptibility to atherosclerotic lesions.11 When female mice are fed either a chow or a high-fat, high-cholesterol diet, NZB have higher plasma TC and HDL-C concentrations than SM. When consuming a chow diet, neither strain develops atherosclerosis, but when fed the high-fat, high-cholesterol diet, NZB females are resistant to lesion formation, whereas SM females develop large lesions. Because high HDL-C levels are thought to be protective against atherosclerosis, the high HDL-C concentrations in the NZB mice may be responsible for their resistance to the disease.

A recent QTL analysis in 184 F2 progeny of an NZB$\times$SM intercross identified several genetic loci linked to levels of plasma TC, HDL-C, triglycerides, free fatty acids, and apo A-II.15 However, susceptibility to lesions was not measured in this study, leaving unanswered questions about the differences in susceptibility to lesions and the relationship between lesion formation and plasma lipids. Therefore, to determine the genetic factors responsible for the difference in susceptibility to atherosclerotic lesions between the NZB and SM inbred mouse strains and to ascertain whether the differences in plasma lipids correlate with the difference in susceptibility to lesions, we have conducted an analysis of inheritance patterns for plasma TC and HDL-C concentrations and lesion size in (1) an SM$\times$NZB outcross, (2) the NXSM RI strain set, and (3) a small (SM$\times$NZB)F1$\times$SM backcross.

Methods

Mice and Diets
SM, NZB, and NXSM RI mice were obtained from The Jackson Laboratory, Bar Harbor, Me. SM females were mated to NZB males

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to produce the F1 progeny, and F1 females were mated to SM males to produce the backcross progeny. Mice were housed in a climate-controlled facility with a 14-hour light and 10-hour dark cycle. After weaning at 21 days, mice were maintained on a chow diet (Old Guildford 234A, Guilford, Conn) and offered free access to food and water throughout the experiment. All experiments were approved by the Institution’s Animal Care and Use Committee.

The atherogenic diet contained (wt/wt) 15% dairy fat, 50% sucrose, 20% casein, 0.5% cholic acid, 1.0% cholesterol, and cellulose, vitamins, and minerals. The source of chemicals and the diet have been described previously. After reaching 6 to 8 weeks of age, female mice were fed the atherogenic diet for periods of 18 or 26 weeks. There were two sets of mice each for NZB, SM, and F1; one set was fed the atherogenic diet for 18 weeks and the other for 26 weeks. All lipid values cited in this study for the parentals and F1 come from the 26-week experiment. For the NZB and F1, lesion sizes for the 18- and 26-week experiments were not significantly different; therefore, lesion data are from both experiments. For the SM strain, the 26-week lesions were smaller than we normally observe in this strain and were significantly smaller than the 18-week lesions; therefore, SM lesion data cited in this study are from only the 18-week experiment. For the small backcross, all data are from one 18-week experiment; for the RI strain set, all data are from one 26-week experiment.

Lipid Measurements
Mice were fasted for 12 hours before blood was collected for lipid determinations. All bleeds were performed in the morning. Blood was collected by retro-orbital bleed into EDTA-coated tubes and plasma was separated by centrifugation at 1500 rpm, 5 minutes at 4°C. Plasma total cholesterol and HDL cholesterol concentrations were measured by commercial colorimetric enzymatic assay as described in “Methods.”

Measurement of Atherosclerotic Lesions
Susceptibility to atherosclerotic lesions was determined by measurement of lesion size as previously described in detail. Briefly, under Avertin (2,2,2-tribromoethanol) anesthesia (0.4 mg/g), the aorta was rounded and valves appeared distinctly and through to the end point where the valves disappeared, a distance of approximately 350 μm. Lesion size is expressed as mean±SEM in μm² based on five sections per mouse.

Figure 1. Plasma total and HDL cholesterol concentrations and HDL/total cholesterol ratios for SM, NZB, and F1 progeny after 0, 4, or 18 to 26 weeks on the atherogenic diet. a, Plasma total cholesterol; b, plasma HDL cholesterol; c, ratio of HDL cholesterol to total cholesterol. Female mice were fed the atherogenic diet for 0, 4, or 26 weeks. Total and HDL cholesterol concentrations, expressed in mmol/L, were determined by commercial colorimetric enzymatic assay as described in “Methods.” d, Lesion size, expressed as μm². Female mice were fed the atherogenic diet for 18 or 26 weeks as explained in “Methods.” Lesion size was measured as described in “Methods.” Solid bars indicate NZB; shaded bars, F1; and open bars, SM. Significantly different are indicated as follows: a, significantly different from SM, F1 (P<.001); b, significantly different from SM (P<.03 or less); c, significantly different from NZB (P<.01); and d, significantly different from NZB, F1 (P<.02 or less). For lipid data, n=6 for NZB and SM and n=5 for F1. For lesion data, n=10 for NZB, n=9 for F1, and n=13 for SM.

QTL Analysis
QTL analysis in the NXSM RI strain set was performed using Map Manager QTb8 for Macintosh. Strain distribution patterns for the NXSM RI set were retrieved from the Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Me; World Wide Web URL: http://www.informatics.jax.org (February, 1997). There were a total of 160 markers on the RI strain set at the time of this analysis. The number of markers, and therefore the resolution, varies by chromosome. Most chromosomes contain a minimum of 5 markers, with the exception of chromosomes 3, 14, 18, 19 and X, which have 4, 1, 2, 0, and 3 markers, respectively. Strain distribution patterns and data for lesion size, total cholesterol, and HDL cholesterol were entered into the Map Manager QTb8 program and tested for linkage with the QT Links Report function using free regression with no control for other QTLs.

Statistical Analysis
Statistical analyses were done using Statview II (Abacus Concepts Inc, Berkeley, Calif) for Macintosh. Between-group comparisons were analyzed by one-way ANOVA using Fisher’s least significant difference test to determine statistical significance. All values are expressed as mean±SE. The number of mice used for each experiment is specified in individual figures and tables. Correlations between lesions and lipid measurements were tested using simple linear regression analysis.

Results
The inbred mouse strains SM and NZB and their F1 progeny differed in plasma TC and HDL-C concentration, HDL-C/TC
ratio, and susceptibility to atherosclerosis as shown in Fig 1a through 1d. Regardless of whether mice were fed a chow diet (week 0) or an atherogenic diet (weeks 4 and 26), the NZB strain had significantly higher concentrations of TC (Fig 1a) and HDL-C (Fig 1b) than the SM strain or the F1 progeny. The F1 progeny generally had TC and HDL-C levels that were slightly higher than or similar to the SM parent, although F1 TC levels were significantly higher than SM at week 0 and F1 HDL-C levels were significantly higher than SM at weeks 0 and 26.

The strain differences in TC and HDL-C were reflected in the HDL-C/TC ratios for both parental strains and F1 progeny. When consuming a chow diet, SM, NZB, and F1 mice had similar HDL-C/TC ratios (Fig 1c). When consuming the atherogenic diet, this ratio was significantly lower for SM than NZB at 4 weeks and significantly lower than both NZB and F1 at 26 weeks.

As depicted in Fig 1d, SM mice were susceptible to atherosclerosis, with mean lesion size of $3800 \pm 920 \, \mu m^2$; NZB and F1 mice were resistant, with lesion sizes of $160 \pm 110$ and $100 \pm 60 \, \mu m^2$, respectively. These data suggest that the resistant phenotype is dominant and contributed by the NZB parent.

As shown in Fig 2, the distribution of lesion size among individual animals was nearly identical in NZB and F1 progeny and highly variable among SM. Since only 9 of 13 SM mice had lesion sizes greater than 1200 $\mu m^2$, this phenotype could be considered poorly penetrant.

Linear regression analysis of 26-week lesion size versus 0-, 4-, and 26-week TC and HDL-C for the SM and NZB parentals and their F1 progeny revealed only one significant correlation: between F1 lesion size and 4-week HDL-C levels ($r = .90, P < .05$). The lack of association between plasma TC and HDL-C and lesions strongly suggests that $Ath8$ affects lesion size independently of plasma TC or HDL-C levels.

In the NXSM RI set, susceptibility to lesions segregated as shown in Fig 3. Eight of 15 strains had mean lesion sizes similar to NZB and F1, ranging from 0 to 123 $\mu m^2$. Four strains had mean lesion sizes that were intermediate between NZB and SM, ranging from 397 to 686 $\mu m^2$. The remaining 3 strains had mean lesion sizes more similar to SM, ranging from 1534 to 3324 $\mu m^2$. A linkage analysis in the RI strain set using Map Manager QTb8 failed to identify any significant linkages to lesion size. The failure to find linkage may have been due to the limited number of RI strains in this set and the small number of previously typed loci.

The NXSM RI strains differed in TC and HDL-C as shown in Table 1. Linear regression analysis revealed significant correlations between lesion size and 0-week TC ($r = .33, P < .005$) and HDL-C ($r = .30, P < .01$) and 26-week TC ($r = .38, P < .002$). Linkage analysis for TC and HDL-C at 0, 4, or 26 weeks on diet resulted in the identification of several suggestive ($2 < LOD < 3$) but no significant (LOD $> 3$) loci; LOD indicates likelihood of odds. Suggestive loci included $Mmv14$ (chromosome 1, 91.6 centimorgan) for TC at 0 weeks on high-fat diet and for HDL-C at 0 and 4 weeks on high-fat diet; $Apoa2$, $Mtv27$, $Pmv44$ (chromosome 1, 92.6 to 96.8 centimorgan) for TC at 0 weeks on high-fat diet and for HDL-C at 0 and 26 weeks on high-fat diet.
cM), and the carbonic anhydrase genes, Car1 and Car2 (chromosome 3, 10.5 cM) for HDL-C at 4 weeks on high-fat diet. Although the Ath1 (chromosome 3, 10.5 cM) for HDL-C at 4 weeks on high-fat diet. Although the Ath1 gene has not been mapped on the NXSM RI set, Ath1 does map in the vicinity of the suggestive loci identified on chromosome 1.

In a small backcross of (SM×NZB)F1×SM, susceptibility to lesions segregated as shown in Table 2. Of 39 female progeny collected, 26 had no lesions at all or mean lesion sizes similar to NZB and F1 (range 0 to 120 μm²), 6 had mean lesion sizes intermediate to NZB and SM (200 to 1200 μm²), and 7 had mean lesion sizes similar to SM (range 1410 to 12 110 μm²). Regression analysis showed poor correlation between lesion size and TC, HDL-C, or HDL-C/TC at 0, 4, and 18 weeks on the atherogenic diet (r<.3 for all comparisons), suggesting that the gene(s) determining susceptibility and plasma TC and HDL-C levels were segregating independently.

**TABLE 1. Lesion Size and Total and HDL Cholesterol Levels for RI Strain Sets**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lesion Size</th>
<th>Total Cholesterol</th>
<th>HDL Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 0</td>
<td>Week 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZB</td>
<td>160±110</td>
<td>4.24±0.34</td>
<td>7.24±0.26</td>
</tr>
<tr>
<td>SM</td>
<td>3800±920</td>
<td>1.50±0.10</td>
<td>4.60±0.31</td>
</tr>
<tr>
<td>F1</td>
<td>100±60</td>
<td>2.28±0.10</td>
<td>4.70±0.31</td>
</tr>
<tr>
<td>C</td>
<td>2286±1311</td>
<td>2.30±0.13</td>
<td>4.01±0.21</td>
</tr>
<tr>
<td>D</td>
<td>1535±439</td>
<td>3.57±0.13</td>
<td>6.96±0.41</td>
</tr>
<tr>
<td>E</td>
<td>59±40</td>
<td>2.92±0.10</td>
<td>5.92±0.16</td>
</tr>
<tr>
<td>F</td>
<td>3324±857</td>
<td>4.29±0.10</td>
<td>6.85±0.16</td>
</tr>
<tr>
<td>I</td>
<td>612±265</td>
<td>1.24±0.05</td>
<td>5.22±0.31</td>
</tr>
<tr>
<td>L</td>
<td>123±75</td>
<td>3.33±0.18</td>
<td>6.38±0.26</td>
</tr>
<tr>
<td>N</td>
<td>12±12</td>
<td>2.28±0.10</td>
<td>5.35±0.65</td>
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<tr>
<td>P</td>
<td>575±353</td>
<td>1.66±0.05</td>
<td>4.24±0.34</td>
</tr>
<tr>
<td>Q</td>
<td>6±6</td>
<td>2.15±0.08</td>
<td>4.50±0.16</td>
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<tr>
<td>T1</td>
<td>686±403</td>
<td>2.77±0.16</td>
<td>4.68±0.16</td>
</tr>
<tr>
<td>T2</td>
<td>0±0</td>
<td>3.13±0.10</td>
<td>6.70±0.21</td>
</tr>
<tr>
<td>U</td>
<td>88±59</td>
<td>2.22±0.05</td>
<td>4.97±0.21</td>
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<tr>
<td>W</td>
<td>0±0</td>
<td>1.76±0.16</td>
<td>4.76±0.39</td>
</tr>
<tr>
<td>T1</td>
<td>21±16</td>
<td>3.00±0.21</td>
<td>5.46±0.52</td>
</tr>
<tr>
<td>Z</td>
<td>397±231</td>
<td>2.59±0.10</td>
<td>5.15±0.34</td>
</tr>
</tbody>
</table>

**TABLE 2. Distribution of Lesion Size, and Total and HDL Cholesterol Levels in (SM×NZB)F1×SM Backcross Progeny**

<table>
<thead>
<tr>
<th>Lesion Size μm²</th>
<th>No. of Progeny</th>
<th>Total Cholesterol (mmol/L)</th>
<th>HDL Cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 0</td>
<td>Week 4</td>
</tr>
<tr>
<td>0–200</td>
<td>26</td>
<td>1.7</td>
<td>5.1</td>
</tr>
<tr>
<td>201–800</td>
<td>2</td>
<td>1.5</td>
<td>5.3</td>
</tr>
<tr>
<td>801–1200</td>
<td>4</td>
<td>1.9</td>
<td>6.0</td>
</tr>
<tr>
<td>1201–2000</td>
<td>2</td>
<td>1.7</td>
<td>4.2</td>
</tr>
<tr>
<td>&gt;2000</td>
<td>5</td>
<td>2.1</td>
<td>5.7</td>
</tr>
</tbody>
</table>

**Discussion**

This analysis was undertaken to identify the genetic factor(s) responsible for the difference in susceptibility to diet-induced atherosclerosis between the inbred mouse strains SM and NZB and to determine whether differences in plasma TC and HDL-C between these two strains are correlated with susceptibility to lesion formation. The data presented here allow for several significant conclusions to be drawn regarding the nature of this genetic difference. First, there is at least one major gene responsible for the difference in susceptibility to diet-induced lesions between the SM and NZB inbred strains. We have tentatively named this major gene atherosclerosis 8 (Ath8). However, we cannot rule out the possibility that there may be other genes that contribute to this phenotype. Second, the Ath8 allele, which confers resistance to diet-induced atherosclerosis, is contributed by the NZB strain and is
inherited in a dominant fashion. Finally, \( \text{Ath}8 \) segregates independently of plasma TC and HDL-C levels, indicating that differences in susceptibility to lesion formation are not related to the differences in plasma TC or HDL-C between these two strains.

The lesion data presented in Fig 2 are consistent with previous observations that the SM and NZB strains differ in susceptibility to lesion formation.\(^{11}\) Although neither strain develops atherosclerotic lesions when fed a low-fat, low-cholesterol diet, when fed an atherogenic diet, SM is susceptible to lesion formation, whereas NZB is almost completely resistant. Fig 2 also demonstrates another important characteristic of the atherosclerosis phenotype in the SM strain; ie, incomplete penetrance. It is common to find no lesions in an SM mouse despite its having been fed an atherogenic diet. The fact that there is incomplete penetrance reduces the power of linkage analyses by weakening the correlation between genotype and phenotype. However, the similarity in the mean lesion size and the distribution of lesion size between the F1 and NZB demonstrate that the alleles conferring resistance to lesion formation are inherited in a dominant fashion from the NZB parent.

In an attempt to clarify the number of genes contributing to resistance to diet-induced lesions, we characterized the distribution of lesion size in the NXSRI strain set and in a small (SM×NZB)×SM backcross. The phenotypic distributions obtained do not resemble the one-to-one distributions that are expected for a trait determined by a single gene. These unexpected patterns could be due to the presence of another major gene or to the effects of the incomplete penetrance masking some of the effect of \( \text{Ath}8 \). The failure to find any linkage for \( \text{Ath}8 \) in the RI strain set using Map Manager QTb8 could be due to the incomplete penetrance, the small number of RI strains, the limited coverage of previously typed loci in this strain set, or a combination of these factors.

Plasma TC, HDL-C, and HDL-C/TC for the parental strains and F1 progeny are shown in Fig 1a through 1c. In this study, as in those previously reported from this laboratory\(^{11}\) and by Purcell-Huynh et al,\(^{15}\) the SM and NZB inbred strains differ markedly in plasma TC and HDL-C levels when consuming either chow or an atherogenic diet. While our lipid values for SM and NZB fed the chow diet and for NZB mice fed the high-fat diet are similar to those reported by Purcell-Huynh et al,\(^{15}\) lipid values for our SM mice fed the high-fat diet tended to be lower. However, this small difference is not surprising considering the lower level of cholesterol in the diet (1.00% versus 1.25%) and the difference in carbohydrate and fat sources.\(^{15}\) Plasma TC and HDL-C for the F1 progeny of the SM×NZB outcross are intermediate to the two parental strains but more closely resemble the SM than the NZB parent, suggesting that genes controlling plasma lipid levels are inherited in different fashion than those determining resistance to lesion development. To test this hypothesis, we performed linear regression analysis to examine the correlation between plasma lipid levels and resistance to lesion development in the NZB, the SM, the F1 progeny, the NXSRI RI set, and the small backcross. Plasma lipid values for the RI set are shown in Table 1 and for the backcross offspring (grouped according to lesion size) in Table 2. Although we did obtain significant correlations between F1 lesion size and 4-week HDL-C and between NXSRI lesion size and 0-week TC and HDL-C and 26-week TC, there were no other significant correlations between lesion size and plasma TC or HDL-C in either the parental strains, the NXSRI RI set, or the backcross progeny. These limited and somewhat arbitrary correlations suggest to us that TC and HDL-C levels are not related to the increased susceptibility to lesion development. Rather, we conclude that \( \text{Ath}8 \) is segregating independently of genes determining plasma TC and HDL-C levels and that differences in plasma TC and HDL-C levels between these two strains are not responsible for the difference in susceptibility to lesion development. This does not, however, rule out the possibility that susceptibility to lesions is determined by a factor related to other aspects of lipid transport or metabolism.

A linkage analysis for the NXSRI RI set identified a region on distal chromosome 1 near the \( \text{Apoa2} \) locus that was linked (2<LOD<3) to TC levels for chow-fed animals and to HDL-C levels for both chow- and high-fat-fed animals. This analysis confirms linkages for TC and HDL-C on distal chromosome 1 (D1Mit36, 92.3 cM) near the \( \text{Ath}1 \) and \( \text{Apoa2} \) loci as reported earlier for an F2 cross using the NZB and SM strains.\(^{13}\) This analysis also identified an additional locus near the carboxic anhydrase genes, \( \text{Car}1 \) and \( \text{Car}2 \), on chromosome 3 (10.5 cM) that was linked to HDL-C levels at 4 weeks on high-fat diet (2<LOD<3).

In summary, this genetic analysis of a cross between the SM and NZB inbred strains of mice has resulted in the identification of at least one major dominant gene that determines resistance to atherosclerosis and segregates independently of genes determining plasma TC and HDL-C levels. Given the lack of association between \( \text{Ath}8 \) and plasma TC and HDL-C levels, we find this gene particularly interesting in terms of identifying nonlipid factors that are involved in determining lesion development. A mapping strategy using a more extensive backcross has been initiated to identify the map position of \( \text{Ath}8 \). Preliminary analysis of approximately 200 mice of a large backcross strongly supports our previous conclusion that the difference in susceptibility to lesions between these two strains is due to a single major gene.

Acknowledgment

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References

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