Atherosclerosis Susceptibility Differences among Progenitors of Recombinant Inbred Strains of Mice

Beverly Paigen, Brian Y. Ishida, Judy Verstuyft, Robert B. Winters, and Deborah Albee

Female mice of 16 inbred mouse strains were fed an atherogenic diet for 14 weeks and were then evaluated for atherosclerotic lesions in the aorta. Strains C57BL/6, C57BR/cd, C57L, and SM were very susceptible to atherosclerosis, with lesion area/aortic cross-sections in the range of 4500 to 8000 μm². Strain C58 and SWR were intermediate in susceptibility, with lesion area/sections in the range of 1670 to 1690 μm². Strains 129, AKR, DBA/2, and BALB/c had only small lesions in the range of 20 to 350 μm²/section; strains C3H, NZB, CBA, HRS, SJL, and A had no lesions after 14 weeks. Lesion formation in five strains was compared at several time points. Strain C57BL/6 mice developed lesions by 7 weeks, and these continued to grow until all mice had large atheromatous plaques in the aorta and coronary arteries. Strains AKR and DBA/2 also had fatty streak lesions as early as 7 or 8 weeks, but these lesions had not progressed in size by 14 weeks. Strains BALB/c and C3H, which were both resistant to lesion formation at 14 weeks, diverged from each other as time progressed. By 1 year, BALB/c mice had large lesions, but C3H mice had none. Most of the inbred strains chosen for evaluation are the progenitors of recombinant inbred sets of strains, a genetic tool that greatly facilitates the analysis of strain differences. This survey indicates seven additional recombinant inbred sets of strains whose progenitors differ in atherosclerosis susceptibility: BXD, AKXL, SWXJ, NXS, 129XB, NXSM, and B6N2AKRN. An analysis of these recombinant inbred strains may reveal additional mouse genes affecting atherosclerosis susceptibility.

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BALB/c mice at 14 weeks. Resistance to atherosclerosis in both strains is associated with the quantity of high density lipoprotein (HDL) cholesterol, and BALB/c consistently had HDL cholesterol levels that were 30% lower than those of C3H. These observations led us to follow the time course of lesion formation in strains C57BL/6, BALB/c, and C3H over the course of 12 months.

The data obtained from the time course studies and from a comparison of 16 strains at 14 weeks suggest RI sets with progenitors that differ in atherosclerosis susceptibility.

**Methods**

**Animals**

Female mice of strains C57BL/6J, C57BR/cdJ, C57L/J, C58/J, C3H/HeJ, BALB/c, AKR/J, SWR/J, SJL/J, SM/J, DBA/2J, 129/J, A/J, NZB/BINJ, HRS/J, and CBA/J were obtained from the Jackson Laboratory, Bar Harbor, ME, and were provided with an atherogenic diet at 6 to 12 weeks of age. The animals were housed in a temperature-controlled facility with 12-hour dark and light cycles. The mice were fed ad libitum and fasted overnight before sacrifice by carbon dioxide asphyxiation. The experimental protocol received approval from the Institute’s Animal Care and Use Committee.

**Chemicals and Diet**

The source of chemicals has been described previously. The atherogenic diet based on the Thomas-Hartroft diet was obtained from Teklad, Madison, WI. This was mixed with Purina breeder chow (#5015) containing 10% fat in the ratio of three parts breeder chow to one part Thomas-Hartroft diet to obtain a final concentration of 15% fat, 1.25% cholesterol, and 0.5% sodium cholate. The polyunsaturated/saturated fat ratio was 0.7, and the fatty acid composition has been described earlier.

**Experimental Design**

The reproducibility of lesion formation was as follows: 10 C57BL/6 female mice were provided with an atherogenic diet and were sacrificed for evaluation of atherosclerotic lesions at 14 weeks. The lesion data from this set of animals provided the information for Tables 1 and 2. To demonstrate the reproducibility of lesion formation, data from all other experiments during the same calendar year which had a group of female C57BL/6 mice fed the same diet for 14 weeks are also given in Table 3.

The time course of lesion formation in strains C57BL/6, AKR, and DBA/2 was as follows: 20 female mice of each strain were provided an atherogenic diet, and five mice from each strain were sacrificed at intervals of 7, 10, 12, and 14 weeks with two exceptions. AKR was sacrificed at 8 weeks rather than 7, due to an error, and the cage of C57BL/6 mice for 10 weeks was lost due to a water bottle leak. The time course of lesion formation in strains C57BL/6, BALB/c, and C3H was as follows: 14 mice of strains C3H and BALB/c were provided an atherogenic diet, and five mice from each strain were sacrificed at 14 weeks and three mice at 6, 7, or 12 months. All C3H mice survived the entire year; two deaths occurred in BALB/c mice, leaving only one mouse at 12 months. Two aortas were torn, one each for BALB/c and C3H at 6 months, so that these lesions could not be evaluated. For C57BL/6 mice, the 7- and 14-week time points are the data for the time course experiment described above and came from five mice per group. Because we knew that C57BL/6 mice would not survive the atherogenic diet for a whole year, 30 additional mice were fed an atherogenic diet for subsequent time points; three were sacrificed at 6 months, three at 7.5 months, and two at 9.75 months. Mortality was high: 50% of the mice not selected for sacrifice had died by 30 weeks, 75% by 24 weeks, and 100% by 40 weeks.

The comparison of 16 strains of mice was as follows: female mice from each strain were fed the atherogenic diet for 14 weeks and were sacrificed; their aortas were evaluated for lesion formation. The goal was to test 10 mice per strain, but these mice were not always available in the numbers required, so several strains fell short of that goal, and the size of the group tested varied from 5 to 15. No mice were lost due to death in this short experiment.

**Evaluation of Atherosclerosis**

The heart and attached section of aorta were placed in 0.9% saline for 1 hour while still beating to eliminate red blood cells. This permits the heart muscle to relax, which facilitated subsequent sectioning of the aorta. The hearts were fixed in 4% formaldehyde and embedded in gelatin as described previously. The embedded tissue was stored in 10% formaldehyde at 4°C until processed.

For a quantitative evaluation of the lesions, each heart was frozen on a cryostat and sectioned at a thickness of 10 μm. The preparation for sectioning and the plane at which the first cut was made have been described in detail. The region of the aorta that was sectioned began at the juncture of the aorta to the heart and continued toward the aortic arch for a distance of approximately 350 μm. Every other 10 μm section was collected, was fixed on gelatin-coated microscope slides, was stained with oil red O and hematoxylin and eosin. For a quantitative evaluation of the lesions, the hearts were embedded in paraffin, sectioned at 5 μm, and stained with oil red O and hematoxylin and eosin. The mice used for morphologic evaluation were sampled at various intervals from a large group of mice fed the atherogenic diet.
The reproducibility of lesion formation in C57BL/6 mice from experiment to experiment is shown in Table 3. The only difference between experiments was the batch of diet, time of year, and, to a limited extent, age of mice, which varied between 2 and 4 months of age at the start of the diet. Although some variation was observed, C57BL/6 mice always had lesions of substantial size, and no significant differences between experiments occur.

### Results

#### Reproducibility of Lesion Formation In C57BL/6 Mice

The same person measured the lesions in all slides. To determine the reproducibility of measurements by that one observer, the same set of slides from C57BL/6 mice were read and then reread 12 months later. The two measurements were very similar (Table 1). The same set of slides were evaluated again for lesions, but measurements began at the aortic cross section 20 μm further toward the aortic arch, so that each subsequent aortic cross-section was also displaced 20 μm; thus lesion area was measured in five different sections than those measured in evaluation 1. Such a procedure is possible because every 10 μm section is preserved and stained, but sections used for measurement are at 80 μm intervals. The total lesion area was indistinguishable from that found in the first two evaluations (Table 1). Thus, the actual measurement of lesion size is quite reproducible.

Lesions were more likely to occur in the aortic sections closer to the heart as compared to those closer to the aortic arch. The differences in lesion area per section were compared for the five sections from the same 10 mice measured for Table 1. These data demonstrate that lesions are larger in the first section than in the last (Table 2). More variation occurred from section to serial section in the same mouse than between the sections located in a comparable position in different mice. We have begun to question whether the fifth section should be measured at all, since only two of the 10 mice had any lesion in the fifth section. The presence of so many fifth sections with no lesions means that the standard deviation of measurements is high.

The reproducibility of lesion formation in C57BL/6 mice from experiment to experiment is shown in Table 3. The

Table 1. Lesion Formation in Set of Mice Evaluated In Various Ways

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Lesion area/section in μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluation 1</td>
<td>2670±510</td>
</tr>
<tr>
<td>Evaluation 2 (1 year later)</td>
<td>2950±600</td>
</tr>
<tr>
<td>Evaluation 3 (sections offset 20 μm)</td>
<td>2530±550</td>
</tr>
</tbody>
</table>

The values are means±SEM based on 50 aortic cross-sections, five from each of 10 C57BL/6 mice fed the atherogenic diet for 14 weeks.

Table 2. Lesion Area as Function of Location In Aorta

<table>
<thead>
<tr>
<th>Cumulative μm from start</th>
<th>Lesion area/section in μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>6290±1610</td>
</tr>
<tr>
<td>80</td>
<td>4030±1070</td>
</tr>
<tr>
<td>160</td>
<td>2200±650</td>
</tr>
<tr>
<td>240</td>
<td>710±260</td>
</tr>
<tr>
<td>320</td>
<td>120±90</td>
</tr>
</tbody>
</table>

The values are the means±SEM.

*This is the first aortic cross-section after the aortic sinus. It is recognizable by the fact that the perimeter of the aorta is round rather than irregular and bulging like the perimeter of the aortic sinus and by the layers of elastin fibers. These data are from the first evaluation shown in Table 1.

Table 3. Reproducibility of Lesion Formation In C57BL/6 Mice

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Lesions/section in μm²</th>
<th>Number of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2670±510</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>4200±860</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>3360±640</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>3260±730</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>3810±610</td>
<td>12</td>
</tr>
</tbody>
</table>

The values are the means±SEM.

*Experiment 1 is from Table 1, experiment 2 is from Table 4, and experiments 3 to 5 are other experiments conducted in our laboratory during the same calendar year. The data from experiments 3 to 5 will be published in other reports and are included here to demonstrate the spread in values.

#### Time Course of Lesion Formation In Strains C57BL/6, AKR, and DBA/2

Strain C57BL/6 was significantly different from AKR and DBA/2 in atherosclerosis susceptibility (Figure 1). Lesions in C57BL/6 mice had developed by 7 weeks and continued to grow throughout the 14 weeks. Lesions in strains AKR and DBA/2 were also apparent by 7 to 8 weeks, but these lesions had not progressed in size by 14 weeks. Thus, the set of RI strains derived from C57BL/6 and DBA/2 were useful in examining genes affecting atherosclerosis susceptibility. In contrast, both AKR and DBA/2 were relatively resistant to atherosclerosis and were indistinguishable from each other with regard to lesion formation, indicating that the RI set derived from AKR and DBA/2 was not useful in the analysis of genes affecting atherosclerosis susceptibility. The lesion areas at 7 weeks for DBA/2 and 10 weeks for AKR were significantly greater than the lesion areas for both strains at 14 weeks (p<0.05), suggesting that fatty streaks were formed during the initial adjustment to the atherogenic diet but later regressed. We do not know whether this apparent regression would be confirmed in a more detailed time course with larger numbers of mice, but these data do demonstrate that C57BL/6 is more susceptible to atherosclerosis than are AKR and DBA/2.

#### Time Course of Lesion Formation In Strains C57BL/6, C3H, and BALB/c

In this experiment strains C57BL/6, C3H, and BALB/c were examined for lesion formation at intervals over 1 year (Figure 2). As described previously, C57BL/6 mice did not survive but died between 28 and 40 weeks. Mice sacrificed during this period of high mortality had large atheromatous plaques in the aortas and occlusion of the coronary artery. Most BALB/c mice survived the year, but did not develop lesions, although to a lesser extent than C57BL/6 mice. C3H mice survived the entire year and did not develop any lesions at all.
**Figure 1.** Development of atherosclerotic lesions in strains C57BL/6, DBA/2, and AKR while consuming the atherogenic diet for 14 weeks. The means and error bars represent the lesion area/cross-section for groups of five mice from each strain at each time point.

During this experiment, the progression of lesion pathology from a thickened wall to fatty streak to atherosclerotic plaque was observed in C57BL/6 mice. Compared to normal arterial wall (Figure 3A), the thickened wall (Figure 3B) had proliferating cells as demonstrated by the rows of 3 to 5 nuclei between successive elastin fibers in the space usually occupied by one nucleus. These dividing nuclei in a thickened arterial wall often were observed before any lipid deposition, but Figure 3B shows a thin layer of fat-filled cells at the surface. In the fatty streak depicted in Figure 3C, a thick layer of lipid-filled foam cells with cuboidal nuclei overlies the elastin fibers. Figure 4 shows the lesion from an animal fed the atherogenic diet for 9 months. A fibrous cap consisting of layers of smooth muscle cells, which are recognizable by the flat pancake-shaped nuclei, covers the deeper layer of the lesion, which has relatively fewer cells, almost no intact elastin fibers, and areas of free cholesterol deposits, which appear as clear or light gray areas. Beneath the lipid layer is the remainder of the arterial wall.

The contrast between lesions in the different strains is depicted in Figure 5. The lesion in BALB/c after 1 year of the atherogenic diet (Figure 5A) is a substantial layer of lipid-filled cells at the surface of the arterial wall. This layer covers the entire inner surface of the aortic wall (not shown) so that the total lesion area is substantial. However, the lesion has not invaded the arterial wall, has caused little observable destruction of elastin fibers, and has no atheromatous layer with free cholesterol deposits. In contrast, the aorta from a C57BL/6 mouse fed the diet for 8.5 months (Figure 5B) has two lesions. The fatty streak lesion on the left resembles the BALB/c lesion except that some invasion of the arterial wall has already occurred. The lesion on the right has a layer of lipid-filled cells overlying an atheromatous layer with few cells and deposits of free cholesterol. The elastin fibers have been digested so that only a thinned arterial wall remains. C3H mice, even after 1 year of atherogenic diet consumption, had a normal arterial wall (Figure 5C). Figures 5A and 5B are stained with oil red O to emphasize the lipid, but the aorta from a C3H mouse (Figure 5C) has been stained with hematoxylin and eosin to emphasize structure and to demonstrate that there is not even any sign of dividing nuclei.

**Comparison of Atherosclerosis in Inbred Strains at 14 Weeks**

Female mice from 16 inbred strains were fed the atherogenic diet and were evaluated for lesion formation after 14 weeks. A wide range in atherosclerosis susceptibility was observed (Table 4). To have some reference for a comparison of lesion size to other features in the aortic anatomy, the width of the aortic wall is approximately 20 μm, and the internal lumen of the aorta is approximately 600 000 μm² in C57BL/6 mice of 20 to 24 g.
Figure 3. Early changes in aortic wall that resulted from feeding the atherogenic diet to C57BL/6 mice. A. A normal arterial wall from a chow-fed mouse. B. An arterial wall from a mouse fed the atherogenic diet for 7 weeks. C. An arterial wall from a mouse fed the atherogenic diet for 14 weeks. In B, the nuclei are more frequent, the cellular volume between successive elastin fibers is expanded, and the arterial wall is thicker when compared to the normal wall. C depicts a fatty streak and shows a thick layer of lipid-filled cells, most of which have cuboidal nuclei. The fatty streak has caused some erosion of the elastin fibers since this layer is thinner than that shown in A or B. The lumen is at the top of all photos. Stain: hematoxylin and eosin. Bar=25 μm.

Figure 4. An atherosclerotic lesion in a C57BL/6 mouse fed the diet for 9 months. The fibrous cap of smooth muscle cells covering the deeper layers of the lesion is 3 to 4 cells in thickness. The next layer down contains degenerating foam cells and cellular debris. Below that layer are deposits of lipid, much of it in the form of cholesterol crystals. The lowest layer contains the elastin fibers and smooth muscle cells. Stain: hematoxylin and eosin. Bar=25 μm.

Discussion

This report demonstrates that susceptibility of a mouse strain to atherosclerosis is a relative term and that a gradation of response was observed. The relative susceptibility of inbred strains to atherosclerosis as determined by lesion size in this report agrees remarkably well with our previous data, but the use of a better method of quantitating atherosclerosis and following lesions over time permitted the discrimination of certain strains which previously appeared to be similar. For example, the previous method of counting the number of lesions without regard to size showed C57BL/6, DBA/2, AKR, and 129 to be similar with mean lesion numbers of 1.8±0.3, 1.0±0.3, 0.7±0.3, and 0.9±0.4, respectively; this report showed clear differences with lesion size/cross-sections of 4200±860, 200±80, 44±24, and 350±120, respectively. Previously we had suggested that strain SWR was resistant to atherosclerosis, but that conclusion was based on lesion data at a single time point (10 weeks), which is earlier than our usual time for evaluation (14 weeks). The data in this report show that SWR was susceptible to atherosclerosis although much less so than C57BL/6. BALB/c, and C3H, which were both resistant at 14 weeks, showed quite a different picture at 1 year. C3H was still resistant, but BALB/c had a substantial area of the aorta involved in lesions. The conclusion that these two strains differ from C57BL/6 by a single gene Ath-1 is still true for mice fed an atherogenic diet for 14 weeks. Two possible explanations may account for the difference in susceptibility at 1 year. The first is that other genetic factors may play a role in lesion formation as time progresses and that at least one of these factors differs between BALB/c and C3H. The second explanation is that the alleles of Ath-1 in BALB/c and C3H may be different; this alternative could be tested by constructing congenic strains of C57BL/6 carrying the Ath-1' allele from BALB/c or the Ath-1' allele from C3H and comparing these congenic strains to each other.

The difference in susceptibility, at least for the six susceptible strains C57BL/6, C57BR, C57L, C58, SM, and SWR, appears to be a difference in the rate of lesion formation rather than a difference in lesion structure. However, this conclusion must necessarily be tentative since it is based on lesion characteristics at a time when all lesions were fatty streaks; it might not be true if atherosclerosis-susceptible strains were compared after 6 months or more of the atherogenic diet. Furthermore, these comparisons were made using the oil red O stain,
which emphasizes the presence of lipid rather than with stains which would emphasize lesion morphology.

Total cholesterol, triglycerides, and levels of the apolipoproteins, B48, B110, and E, have been published for most of these stains. No correlation between total cholesterol or triglycerides with susceptibility to lesion formation has been found. There is a tendency for strains that are resistant to atherosclerosis, such as A, BALB/c, C3H, and SJL, to have relatively high levels of HDL cholesterol, but this is not true for all resistant strains.

The strains C58, C57BR, C57L, and C57BL/6 are related since they came from common ancestors. It is likely that all these strains carry the susceptible allele of the Ath-1 gene, although only C57BL/6 has been tested directly by an analysis of backcrosses or RI strains. Likewise C3H, BALB/c, A, and CBA share common ancestors. The first three strains all carry the resistant allele at Ath-1 and so CBA may also carry Ath-1, although this had not been tested directly. The remaining strains listed in Table 4 are of independent origin, and are the most likely strains to carry uncharacterized genes affecting atherosclerosis susceptibility.

With the exception of strains C57BR and CBA, which were tested because so many laboratories have used these strains, mouse strains selected for evaluation of atherosclerosis susceptibility are the progenitors of RI strains. RI strains are a relatively recent tool in mouse genetics that greatly facilitates characterizing and mapping new genetic variants. RI strains are constructed by crossing two progenitor strains and establishing a series of new homozygous inbred strains from their progeny. Each new RI strain consists of a unique mixture of genes in a homozygous state derived from the two parental strains.

The major reason for this study was to determine the susceptibility or resistance of progenitors of RI sets to determine which sets might have undiscovered genes affecting atherosclerosis. To answer this question, the existing sets of RI strains with at least six strains in the set were examined to determine those which had progenitors that differed significantly in atherosclerosis...
susceptibility (Table 5). Of the 12 RI sets listed in Table 5, three sets have already been examined. The BXH set and the CXB set differ in Ath-1, a gene that affects the levels of HDL cholesterol. The set of AXB and BXA strains differs in both Ath-1 and Ath-2. Ath-2 is also a gene that affects the levels of HDL cholesterol. In our judgment, the SWXL progenitors are so close in atherosclerosis susceptibility that segregation analysis would be difficult even though the difference between the parents is statistically significant. The C57BL/6NXC3N set is essentially the same as the BXH set except that different sub-lines of C57BL/6 and C3H were used for the progenitors: NIH sub-lines for the former and Jackson sub-lines for the latter. Although these sub-lines may differ somewhat, they are so closely related that they probably have similar alleles at the Ath-1 locus. Thus, seven remaining RI sets hold interest for further studies: BXD, AKXL, SWXJ, NX8, 129XB, NXSM, and C57BL/6NXAKRN. An analysis of these sets of RI strains may reveal additional mouse genes affecting atherosclerosis susceptibility.

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Table 5. Recombinant Inbred Sets with Differences in Atherosclerosis Susceptibility

| Designation | No. of strains | Susceptible | Resistant | Lesions in µm²† |
|-------------|----------------|-------------|-----------|----------------|---|
| BXD         | 26             | C57BL/6J    | DBA/2J    | 4200±          | 200|
| AVXIL       | 17             | C57L/J      | AKR/J     | 6130±          | 44 |
| BXH         | 12             | C57BL/6J    | C3H/HeJ   | 4200±          | 0  |
| CXB         | 7              | C57BL/6By   | BALB/cBy  | 4200†          | 201|
| SWXL        | 6              | C57L/J      | SWR/J     | 6130±          | 1690|
| SWXJ        | 14             | SWR/BmJ     | SJL/BmJ   | 1690†          | 0  |
| NX8         | 6              | C58/J       | NZB/Idc   | 1670±          | 0  |
| NXSM        | 15             | SM/J        | NZB/B1N   | 4490±          | 0  |
| AXB,BXA     | 45             | C57BL/6J    | A/J       | 4200±          | 0  |
| 129XB       | 13             | C57BL/6JPas | 129/SvPas | 4200†          | 350†|
| B6NXAKN     | 15             | C57BL/6N    | AKR/N     | 4200†          | 441|
| B6NXC3N     | 20             | C57BL/6N    | C3H/HeN   | 4200†          | 0  |

*The standard errors for these measurements are given in Table 4.
†These values are for the Jackson sub-line, not for the sub-line that is the progenitor for this RI set.
ATHEROSCLEROSIS SUSCEPTIBILITY IN MICE


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