Loci controlling plasma non-HDL and HDL cholesterol levels in a C57BL/6J × CASA/Rk intercross

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Abstract Plasma non-HDL and HDL cholesterol levels are predictors of cardiovascular diseases. We carried out a genetic cross between two laboratory inbred mouse strains, C57BL/6J and CASA/Rk, to detect loci that control the plasma levels of non-HDL and HDL cholesterol. With regard to non-HDL cholesterol, chow-fed CASA/Rk males and females had 87% and 25% higher levels, respectively, than did C57BL/6Js. The levels of non-HDL cholesterol in F1s were similar to C57BL/6J. There was no strain difference in HDL cholesterol levels. An intercross between F1s was performed, and plasma non-HDL and HDL cholesterol was measured in 185 male and 184 female mice. In both male and female F2 mice, plasma non-HDL and HDL cholesterol levels were unimodally distributed; however, in both cases the values for females were significantly lower than for males. Therefore, linkage analysis was performed with sex as a covariate. Significant linkage for non-HDL cholesterol was found on chromosome 6 at 49 cM (LOD 5.17), chromosome 4 at 55 cM (LOD 4.22), and chromosome 8 at 7 cM (LOD 3.68). Significant linkage for HDL cholesterol was found on chromosome 9 at 14 cM (LOD 7.52) and chromosome 8 at 76 cM (LOD 4.69). A significant epistatic interaction involving loci on chromosomes 2 and 5 was also observed for non-HDL cholesterol.

In summary, linkage analysis was performed with sex as a covariate. Significant linkage for non-HDL cholesterol was found on chromosome 6 at 49 cM (LOD 5.17), chromosome 4 at 55 cM (LOD 4.22), and chromosome 8 at 7 cM (LOD 3.68). Significant linkage for HDL cholesterol was found on chromosome 9 at 14 cM (LOD 7.52) and chromosome 8 at 76 cM (LOD 4.69). A significant epistatic interaction involving loci on chromosomes 2 and 5 was also observed for non-HDL cholesterol.

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Plasma levels of non-HDL and HDL cholesterol have been shown to modulate the risk for cardiovascular diseases. Increased plasma levels of non-HDL cholesterol, especially in the form of LDL cholesterol, and decreased levels of HDL cholesterol are associated with increased risk for these diseases. Multiple studies have shown large individual-to-individual variation in plasma non-HDL and HDL cholesterol levels (1). The causes of this variation and the regulation of non-HDL and HDL cholesterol levels are only partially understood. Current understanding supports a complex interaction between environmental and genetic determinants. Yet, whereas much is known about the nature and effect of environmental factors including cigarette smoking, total dietary fat intake, types of dietary fatty acids, physical activity, and alcohol consumption, relatively little is known about the genetic basis of this variation. Data from twin and family studies have shown that ~50% of the interindividual variability in LDL cholesterol and HDL cholesterol can be ascribed to genetic determinants; however, only some of the genes involved have so far been identified (2–6). Studies in pedigrees that segregate mutations in critical genes largely expand the understanding of the physiological and metabolic aspects of lipoprotein carriers of non-HDL and HDL cholesterol. Yet, although in some populations variants of these genes are sufficiently common to have an impact on non-HDL and HDL cholesterol levels, it is likely that other genes are involved.

Previous mapping studies in the mouse have identified loci in linkage with plasma non-HDL and HDL cholesterol levels. For example, Ko et al. used human apolipoprotein B (apoB) transgenics in a C57BL/6 × 129 cross to identify loci on chromosome 6 and chromosome 4 in linkage with plasma apoB levels, a signature apolipoprotein for plasma non-HDL cholesterol lipoproteins (7). Mehrabian et al. used a C57BL/6J × CAST/Ei cross to map loci on chromosomes 2, 3, 5, 8, 9, 14, 16, 17, and 18 in linkage with plasma HDL cholesterol (8). Here we describe two mouse strains, C57BL/6J and CASA/Rk, that, when fed a chow diet, display different plasma levels of non-HDL cholesterol. To examine the genetic basis of this difference, an intercross was performed.

Supplementary key words chromosome • logarithm of odds • cardiovascular disease

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Abbreviation: LOD, logarithm of odds.
and linkage for non-HDL cholesterol as well as HDL cholesterol assessed in the F2 progeny. We identified three loci, on chromosomes 6, 4, and 8, in linkage with plasma non-HDL cholesterol, and two loci on chromosomes 9 and 8 in linkage with HDL cholesterol. Moreover, analysis of loci interactions identified a significant epistatic interaction that affects plasma non-HDL cholesterol levels.

MATERIALS AND METHODS

Animals, diet, and lipoprotein separation

The inbred mouse strains C57BL/6j and CASA/Rk were purchased from The Jackson Laboratories (Bar Harbor, ME). CASA/Rk males were mated with C57BL/6j females to generate F1 animals. F1 males were intercrossed with F1 females to generate 369 F2 animals (185 males and 184 females). All animals were bred and housed in a single humidity- and temperature-controlled room with a 12 h dark-light cycle (6 AM–6 PM light-dark cycle) at the Laboratory Animal Research Center at Rockefeller University and fed with a single lot of Picolab Rodent Chow 20 (catalog #5053) pellet containing 0.02% w/w cholesterol. At the age of 11 weeks, food was removed from the cages at 10 AM and the animals were allowed access to water. At 3 PM, the mice were anesthetized with ketamine/xylazine, tail dipped for DNA extraction, and blood samples collected by heart puncture into EDTA-containing tubes. Plasma was immediately separated, plasma density was adjusted to d = 1.063 g/ml, and non-HDL and HDL fractions (d ≤ 1.063 g/ml and d > 1.063 g/ml, respectively) were isolated after overnight spinning at 40,000 rpm (Beckman L8-55M ultracentrifuge, rotor type 42.2 Ti) at 4°C. The non-HDL and HDL fractions were separated and kept at −80°C for analysis of cholesterol concentration as described below. All experiments were approved by the Institutional Animal Care and Research Advisory Committee.

Plasma total, non-HDL, and HDL cholesterol measurements

Total plasma cholesterol, non-HDL, and HDL cholesterol levels were determined enzymatically using a Sigma kit. It is of note that plasma total cholesterol, non-HDL, and HDL cholesterol levels in C57BL/6j, CASA/Rk, and F1 males and females were measured in one assay, whereas the measurements in F2 males and F2 females were determined in another assay. The profile of plasma lipoprotein cholesterol was determined by on-line postcolumn analysis of Superose 6 gel-filtration as described previously (9).

Genotyping

Tail tips from parents, F1, and F2 mice were digested with proteinase K, and DNA was precipitated with ethanol. Fluorescently labeled primers corresponding to 350 markers shown to be polymorphic between C57BL/6j and CAST/Ei by Iakoubova et al. (10) were tested to see if they were also polymorphic between C57BL/6j and CASA/Rk. This resulted in the identification of 255 markers that were used for genotyping in the current cross. The average spacing between these markers was 5.9 cM. Markers were subjected to PCR amplification using fluorescently labeled primers, and PCR products were analyzed by capillary electrophoresis using the ABI 3700 DNA sequencer. All PCR reactions and electrophoresis were automated using the Tercan, Genesis RST 100, and Robbins Scientific Hydra 384 robots and carried out by the Starr Center Genotyping Core Facility at the Rockefeller University. Allele scores were analyzed using the ABI Genotype 3.6 NT software. The marker positions in cM correspond to mapping data found in the Mouse Genome Informatics Database at http://www.informatics.jax.org.

Statistical analyses

Differences in plasma non-HDL, HDL, and total cholesterol levels between parentals and F1s and comparisons of plasma non-HDL and HDL cholesterol levels for F2s with the various combinations of genotypes at D6Mit63, D4Mit46, D8Mit171, D9Mit325, and D8Mit91 were analyzed using one-way ANOVA with Tukey’s posttest. Differences in plasma non-HDL and HDL cholesterol between F2 males and females were analyzed using the unpaired Student t-test. Linkage, interval mapping (using the maximum likelihood algorithm), and loci interactions (using the Haley-Knott regression) were analyzed with the R/qtl software package, Version 0.94-17, with sex as a covariate. R/qtl was also used to permute the actual data sets for F2s to determine the significant LODs at the 95% genome-wide threshold level. In addition, R/qtl was used to identify locus-locus interactions by computing a joint LOD score. This joint LOD score represents a composite of the proportion of the trait variance explained by each locus, which together correspond to the additive component of the interaction and the proportion explained through epistasis. Finally, R/qtl was used to permute the actual data sets for F2s to determine the threshold LOD score for the joint and epistasis LODs at which 95% significance is achieved. This software package, developed by Karl Broman and Gary Churchill, is publicly available at http://www.biostat.jhsph.edu/~kbroman/software. The C57BL/6j and CASA/Rk alleles at chromosome 6, 4, and 8 for non-HDL cholesterol and chromosomes 9 and 8 for HDL cholesterol were assessed separately in F2 males and F2 females for additivity and dominance using the Map Manager QTxb10 version 0.19 software.

RESULTS

To determine the plasma levels of non-HDL and HDL cholesterol, we subjected the plasma of chow-fed male and female C57BL/6j, CASA/Rk, and F1s to density ultracentrifugation, and measured the concentrations of non-HDL cholesterol (d ≤ 1.063 g/ml) and HDL cholesterol (d > 1.063 g/ml) in each group. As shown in Table 1, CASA/Rk males and females displayed plasma non-HDL cholesterol levels that were 87% and 25% higher than the corresponding values in C57BL/6j males and females, respectively. Furthermore, the levels of non-HDL cholesterol in F1 males and females were similar to those in C57BL/6j animals, ruling out a codominant effect. As for HDL cholesterol, although females tended to display levels that were lower than the corresponding values in males, no significant differences were found between C57BL/6j, CASA/Rk, and F1. For further characterization of cholesterol distribution among different classes of lipoproteins, we subjected the plasma of C57BL/6j, CASA/Rk, and F1 males and females to superose gel chromatography. As shown in Fig. 1, when compared with C57BL/6j and F1, CASA/Rk males and females displayed increased VLDL cholesterol, with no major differences in either LDL or HDL cholesterol levels. To examine the inheritance of plasma non-HDL and HDL cholesterol levels, F1 animals were intercrossed, and the concentrations of non-HDL and HDL cholesterol were determined in 369 F2 mice (185 males and 184 females).

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To control for possible gender effects, we examined the concentrations of non-HDL and HDL cholesterol in F2 males and females and found that, compared with F2 females, F2 males display significantly higher non-HDL and HDL-cholesterol levels (non-HDL cholesterol of 17.3 ± 6.0 mg/dl vs. 13.9 ± 3.8 mg/dl; *P* < 0.0001, and HDL cholesterol of 44.6 ± 10.9 mg/dl vs. 32.4 ± 7.7 mg/dl; *P* < 0.0001, in males and females, respectively). Figure 2 shows a unimodal distribution of plasma non-HDL and HDL cholesterol in F2 males and females, with lower levels in females. These findings suggest that in F2s, plasma non-HDL and HDL cholesterol levels are probably controlled by more than one gene. Moreover, the data clearly show a gender effect on plasma non-HDL and HDL cholesterol levels, and justify linkage analysis with sex as a covariate.

To identify loci that control plasma levels of non-HDL and HDL cholesterol, a whole genome scan was done on the F2 mice. For non-HDL cholesterol, quantitative trait locus mapping using the R/qtl program with sex as a covariate revealed significant linkage on chromosomes 6, 4, and 8. The interval maps for these chromosomes are shown in Fig. 3. With regard to chromosome 6, the analysis revealed a peak of linkage at 49 cM, with a maximum LOD score of 5.17 that fell between markers D6Mit37 at 46 cM and D6Mit63 at 50 cM. With regard to chromosome 4, the analysis showed a peak of linkage at 55 cM with a maximum

**TABLE 1.** Plasma non-HDL cholesterol, HDL cholesterol, and total plasma cholesterol levels in parental and F1 animals

|                 | Non-HDL Cholesterola | HDL Cholesterolb | Total Cholesterolc |
|-----------------|-----------------------|------------------|--------------------|
|                 | mg/dl                 | mg/dl            | mg/dl              |
| **Males**       |                       |                  |                    |
| C57BL/6J (n = 5)| 13.8 ± 1.8            | 64.2 ± 4.6       | 78.1 ± 5.9         |
| CASA/Rk (n = 4)| 25.8 ± 3.9            | 60.3 ± 7.9       | 89.1 ± 4.7         |
| F1 (n = 5)      | 15.7 ± 1.3            | 60.9 ± 5.8       | 75.5 ± 7.9         |
| **Females**     |                       |                  |                    |
| C57BL/6J (n = 10)| 19.1 ± 2.4            | 53.9 ± 6.1       | 73.0 ± 7.4         |
| CASA/Rk (n = 5)| 23.8 ± 4.0            | 55.2 ± 2.2       | 79.0 ± 3.3         |
| F1 (n = 5)      | 16.2 ± 2.5            | 53.3 ± 2.0       | 69.6 ± 4.4         |

One-way ANOVA:
a Overall males: *P* = 0.0012; C57BL/6J versus CASA/Rk, *P* < 0.01; CASA/Rk versus F1, *P* < 0.01. Overall females: *P* = 0.0022; C57BL/6J versus CASA/Rk, *P* < 0.05; CASA/Rk versus F1, *P* < 0.01.
b Overall males and overall females: NS.
c Overall males: NS; CASA/Rk versus F1, *P* < 0.05. Overall females: NS.

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Fig. 1. Lipoproteins cholesterol profile in C57BL/6J, CASA/Rk, and F1 males and females. Animals fed with chow diet were fasted, plasma samples isolated, the plasma of animals in each group were pooled, and plasma lipoprotein cholesterol profiles were analyzed as described in Material and Methods (n = 10 animals per group).

Fig. 2. Distribution of plasma non-HDL cholesterol and HDL cholesterol in F2 males and females. F2 animals (185 males and 184 females) were fasted, plasma samples collected, and non-HDL cholesterol, and HDL cholesterol measured as described in Materials and Methods.
LOD score of 4.22 that fell between markers D4Mit46 at 51 cM and D4Mit204 at 62 cM. As for the locus on chromosome 8, the analysis found a peak of linkage at 7 cM.

Linkage analysis of HDL cholesterol with sex as covariate revealed significant linkage on chromosomes 9 and 8. The interval maps for these chromosomes are shown in Fig. 4. With regard to chromosome 9, the analysis revealed a peak of linkage at 19 cM with a maximum LOD score of 7.52 that fell between markers D9Mit325 at 14 cM and D9Mit140 at 25 cM. Finally, for chromosome 8, the analysis disclosed a peak of linkage at 69 cM with a maximum LOD score of 4.69 that fell between markers D8Mit91 at 67 cM and D8Mit56 at 73 cM.

At the peaks of linkage for plasma non-HDL cholesterol, the genotypic means in F2s, represented by the closest markers, were calculated for F2 males and F2 females and are shown in Table 2. For the loci on chromosome 6 and chromosome 4, homozygotes for the C57BL/6J allele had higher plasma non-HDL cholesterol levels than did heterozygotes and homozygotes for the CASA/Rk allele. At the chromosome 6 locus, the phenotypic effect of the CASA/Rk allele best fits a dominant mode of inheritance, whereas at the chromosome 4 locus, the CASA/Rk allele best fits a dominant and codominant mode of inheritance in males and females, respectively. At the chromosome 8 locus, homozygotes for the CASA/Rk allele had higher...
plasma non-HDL cholesterol levels than did heterozygotes and homozygotes for the C57BL/6J allele. At this locus, the phenotypic effect of the CASA/Rk allele best fit a codominant mode of inheritance. For the chromosome 8 locus, homozygotes for the C57BL/6J allele had higher plasma HDL cholesterol levels than did heterozygotes and homozygotes for the CASA/Rk allele. At this locus, the phenotypic effect of the CASA/Rk allele best fit a codominant mode of inheritance. For the chromosome 8 locus, homozygotes for the C57BL/6J allele had higher plasma HDL cholesterol levels than did heterozygotes and homozygotes for the CASA/Rk allele. At this locus, the phenotypic effect of the CASA/Rk allele best fit a codominant mode of inheritance. For the chromosome 8 locus, homozygotes for the C57BL/6J allele had higher plasma HDL cholesterol levels than did heterozygotes and homozygotes for the CASA/Rk allele.

At the peaks of linkage for HDL cholesterol, the genotypic means in F2s, represented by the closest markers, were calculated for F2 males and F2 females and shown in Table 3. For the locus on chromosome 9, homozygotes for the CASA/Rk allele had higher plasma HDL cholesterol levels than did heterozygotes and homozygotes for the C57BL/6J allele. At this locus, the phenotypic effect of the CASA/Rk allele best fit a codominant mode of inheritance. For the chromosome 8 locus, homozygotes for the C57BL/6J allele had higher plasma HDL cholesterol levels than did heterozygotes and homozygotes for the CASA/Rk allele.

Locus interactions in determining plasma non-HDL and HDL cholesterol levels were next determined. The R/qtl software program output includes the positions of the interacting loci, the joint LOD score of the interaction, the new LOD score of locus 1 in the presence of locus 2, the new LOD score of locus 2 in the presence of locus 1, and an LOD score of epistasis. For non-HDL cholesterol, significance for a joint LOD score was achieved for the interactions of four pairs of loci. These were primarily of an additive nature (data not shown). In contrast, as shown in Table 4, a fifth interaction was observed between loci on chromosomes 2 and 5, for which the joint LOD was not significant but the epistasis LOD of 7.47 was significant.

For HDL cholesterol, significance for a joint LOD was achieved for the interactions of 19 pairs of loci, which were different plasma levels of non-HDL cholesterol. Utilizing these strains in a genetic cross, we have mapped loci in linkage with plasma non-HDL and HDL cholesterol levels. This analysis revealed three loci on chromosomes 6, 4, and 8 in linkage with plasma levels of non-HDL cholesterol, and two loci on chromosomes 9 and 8 in linkage with plasma levels of HDL cholesterol. In addition, for plasma non-HDL cholesterol levels, a significant epistatic interaction was detected.

Previous studies in the mouse have identified loci in linkage with non-HDL cholesterol levels. Ko et al. performed an intercross between C57BL/6J × 129 mouse strains with the F2s hemizygous for a human apoB transgene (7).
Utilizing human apoB as a surrogate marker for plasma non-HDL lipoproteins, they found linkage on chromosome 6 that peaked at D6Mit55 (49.7 cM) and on chromosome 4 that peaked at D4Mit27 (42.5 cM). Their peak on chromosome 6 coincides with our non-HDL cholesterol peak on chromosome 6 at 49 cM, while their peak on chromosome 4 is about 13 cM proximal to our peak on chromosome 4 at 35 cM. It is of note that in both studies, the 129 and CASA/Rk alleles decrease the plasma levels of apoB and non-HDL cholesterol, respectively. In the Ko study, the genotypic effects of both the chromosome 6 and chromosome 4 129 alleles appear to act in a codominant fashion. In contrast, in our study the chromosome 6 and chromosome 4 CASA/Rk alleles appear to act mainly in a dominant mode (Table 2). Possible interpretations of this difference are: i) the 129 and CASA/Rk segregate different alleles at the loci on chromosome 6 and chromosome 4, ii) the 129 and CASA/Rk segregate the same allele that acts differently under the two genetic backgrounds, and iii) the linkage in the two crosses is to different genes in each interval. The first possibility is conceivable given that 129 and CASA/Rk represent different subspecies of Mus musculus, separated by approximately one million years of evolution (11). The non-HDL cholesterol locus we mapped to chromosome 8 has not been reported in previous studies.

Previous studies in the mouse have identified loci in linkage with HDL cholesterol levels. Mehrabian et al. fed C57BL/6J × CAST/Ei F2s with a chow diet for 13 weeks and measured HDL cholesterol, then switched the animals to an atherogenic diet for an additional 5 weeks and remeasured HDL cholesterol (8). After chow feeding, they found linkage on chromosome 9 that peaked at D9Mit2 (17 cM), and on chromosome 8 that peaked at D8Mit14 (67 cM). Their peaks on chromosome 9 and chromosome 8 coincide with our HDL cholesterol peaks on chromosome 9 at 19 cM and on chromosome 8 at 69 cM. In their cross, the chromosome 9 locus CAST/Ei allele increased and the chromosome 8 locus CAST/Ei allele decreased HDL cholesterol levels. Both loci appeared to act in a codominant fashion. This is similar to what we observed for the CASA/Rk alleles in our cross. This is quite plausible since the inbred strains CAST/Ei and CASA/Rk were both derived from the same pool of castaneus wild-type mice at the Jackson laboratories (http://jaxmice.jax.org/library/notes/456e.html) and may have fixed the same alleles at the chromosome 9 and chromosome 8 loci.

It is noteworthy in our study that parental CASA/Rks have higher non-HDL cholesterol levels than parental C57BL/6J, yet QTL analysis in the F2s revealed dominant CASA/Rk alleles at both chromosome 6 and chromosome 4 that decrease non-HDL cholesterol levels (Tables 1, 2). This type of finding has been observed in other QTL analyses (12), and presumably means that the CASA/Rk alleles at these loci are phenotypically silent in the context of the CASA/Rk genome, but decrease non-HDL cholesterol levels in the presence of one or more C57BL/6J alleles. This implies the presence of significant gene interactions.

The Ensembl mouse genome database was searched for known genes in the intervals of loci for non-HDL cholesterol on chromosome 6 (38–68 cM), chromosome 4 (31–81 cM), and chromosome 8 (1–18 cM). The chromosome 6 interval contains the peroxisome proliferator activated receptor γ (pparγ) gene at 50.5 cM, which encodes an important transcriptional factor that regulates glucose and fatty acid metabolism. This interval also contains the apob mRNA-editing protein (apobec1) gene at 59 cM, which encodes an important constituent of the complex that postsynthetically edits apob-100 mRNA into apob-48 mRNA. The pparγ gene is very close to the chromosome 6 peak at 49 cM, whereas the apobec1 gene is 10 cM distal. The chromosome 4 interval contains the leptin receptor (lepr) gene at 47 cM, which encodes the receptor for leptin, a hormone produced by adipose tissue that regulates satiety and energy metabolism; the carnitine o-palmitoyltransferase II (cd2) gene at 47 cM, which encodes an important protein in insulin signaling pathway. This gene is very close to the chromosome 8 peak at 7 cM. The Ensembl database was also searched for candidate genes in the intervals of loci for HDL cholesterol on chromosome 9 (4–29 cM) and chromosome 8 (52–73 cM). The chromosome 9 interval contains the apoa4, apoc3, apoa1, and apoa5 locus at 27 cM. The genes encoded at this locus are known to play important physiological roles in plasma HDL cholesterol metabolism. Apoa4 is the major structural protein of HDL, and apoa1 and apoa5 have been shown in both mouse and human studies to regulate triglyceride levels, which are often inversely correlated with HDL cholesterol levels. This locus is 8 cM distal to the chromosome 9 peak at 19 cM. The chromosome 8 interval did not contain any genes known to play a prominent role in lipoprotein or lipid metabolism.

In summary, a cross between two strains that differ in plasma non-HDL cholesterol revealed loci on chromosomes 6, 4, and 8 that control the plasma levels of non-HDL cholesterol, and loci on chromosomes 9 and 8 that control the plasma levels of HDL cholesterol. Moreover, we found a significant epistatic interaction that controls the levels of plasma non-HDL cholesterol. It is hoped that the discov-
ery of genes at these loci may explain at least part of the variability in non-HDL and HDL cholesterol levels in humans and lead to the development of novel therapies for dyslipidemic states.

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