Further characterization of diabetes mellitus and body weight loss in males of the congenic mouse strain DDD.Cg-\(\Delta^f\)

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ABSTRACT. The \(\Delta^f\) allele at the agouti locus causes obesity and promotes linear growth in mice. However, body weight gain stops between 16 and 17 weeks after birth, and then, body weight decreases gradually in DDD.Cg-\(\Delta^f\) male mice. Body weight loss is a consequence of diabetes mellitus, which is genetically controlled mainly by a quantitative trait locus (QTL) on chromosome 4. This study aimed to further characterize diabetes mellitus and body weight loss in DDD.Cg-\(\Delta^f\) males. The number of \(\beta\)-cells was markedly reduced, and plasma insulin levels were very low in the DDD.Cg-\(\Delta^f\) males. Using a backcross progeny of DDD \(\times (B6 \times DDD.Cg-\Delta^f) F_1\), we identified one significant QTL for plasma insulin levels on distal chromosome 4, which was coincidental with QTL for hyperglycemia and lower body weight. The DDD allele was associated with decreased plasma insulin levels. When the DDD.Cg-\(\Delta^f\) males were housed under three different housing conditions [group housing (4 or 5 DDD.Cg-\(\Delta^f\) and DDD males), individual housing (single DDD.Cg-\(\Delta^f\) male and single male housing with females (single DDD.Cg-\(\Delta^f\) male with DDD.Cg-\(\Delta^f\) or DDD females)], diabetes mellitus and body weight loss were most severely expressed in individually housed mice. Thus, the severity of diabetes and body weight loss in the DDD.Cg-\(\Delta^f\) males was strongly influenced by the housing conditions. These results demonstrate that both genetic and nongenetic environmental factors are involved in the development of diabetes mellitus and body weight loss in the DDD.Cg-\(\Delta^f\) males.

KEY WORDS: \(\Delta^f\) allele, \(\beta\)-cell loss, DDD.Cg-\(\Delta^f\) mouse, quantitative trait locus (QTL), reduced insulin level


Cpe\(\Delta^f\), Tub\(\Delta^f\), Lep\(\Delta^f\), Lepr\(\Delta^f\) and \(\Delta^f\) have been known as representative single gene obesity mutations in mice [12]. Among these, obesity in mice with \(\Delta^f\) allele is moderate and occurs later compared with that in mice with the other four mutations. The \(\Delta^f\) mice also show metabolic abnormalities, such as hyperglycemia and hyperinsulinemia, thus making them a suitable model system for the study of obesity-associated diabetes mellitus in humans.

In normal mice, the agouti gene is expressed only in the skin [3, 16], and it regulates pigmentation by serving as an inverse agonist of the melanocortin 1 receptor (MC1R) [13, 18]. However, in the \(\Delta^f\) mice, the \(\Delta^f\) allele is associated with a large deletion causes agouti gene expression to be aberrantly controlled by the unrelated Raly gene promoter, leading to its ectopic overexpression [7, 14–16]. As a result, the \(\Delta^f\) mice have a yellow coat color and develop maturity-onset obesity. Obesity in \(\Delta^f\) mice is considered a consequence of the agouti protein serving as a constitutive antagonist of MC3R and MC4R by mimicking the action of the agouti-related protein [4, 10, 17].

In addition to the two commercially available congenic mouse strains for the \(\Delta^f\) allele, B6.Cg-\(\Delta^f\) [C57BL/6J (B6) background] and KK.Cg-\(\Delta^f\) [KK/Ta (KK) background], we have developed an additional congenic strain, DDD.Cg-\(\Delta^f\) [DDD/Sgn (DDD) background] [19]. DDD.Cg-\(\Delta^f\) females are characterized by their massive obesity compared with KK.Cg-\(\Delta^f\) and B6.Cg-\(\Delta^f\) females [20]. Mice carrying the \(\Delta^f\) allele at the agouti locus become obese and are heavier than their non-\(\Delta^f\) littermates with B6 and KK genetic backgrounds. However, in males with the DDD genetic background, e.g., DDD.Cg-\(\Delta^f\) male mice, body weight gain stops between 16 and 17 weeks after birth, and then, body weight decreases gradually [21]. We showed that the body weight loss is a consequence of diabetes mellitus, which is controlled by a major quantitative trait locus (QTL) on chromosome 4. Thus, the DDD strain background has been suggested to be sensitive to obesity-associated diabetes mellitus. This was in contrast to the B6 strain background, which has been considered resistant to obesity-associated diabetes mellitus [5, 6, 9]. We have already shown that DDD.Cg-\(\Delta^f\) males are hyperglycemic [21], but we do not know anything about the other aspects of diabetes mellitus. One of the aims of this study was to examine whether blood insulin levels and/or pancreatic islet histology are normal in DDD.Cg-\(\Delta^f\) males.

During the course of the experiments of our previous study in which 4 or 5 DDD.Cg-\(\Delta^f\) and DDD males were housed together [21], we noticed that the body weight of the DDD.Cg-\(\Delta^f\) males never exceeded 50 g. However, we found obese DDD.Cg-\(\Delta^f\) males weighing more than 50 g in breeding cages in which males and females were housed together. Therefore, we suspected that the growth of the DDD.Cg-\(\Delta^f\) males was strongly influenced by housing conditions. Thus, another aim of this study was to determine whether housing conditions influenced diabetes mellitus and body weight.
loss. For this purpose, we compared and analyzed growth curves and blood phenotypes among DDD.Cg-A' males housed in one of the following conditions: (1) group housing (4 or 5 DDD.Cg-A' males housed together), (2) individual housing (single DDD.Cg-A' male) and (3) single male housing with females (single DDD.Cg-A' male with DDD.Cg-A' or DDD females).

MATERIALS AND METHODS

Mice: The inbred mouse DDD.Cg-A', DDD, B6.Cg-A' and B6 strains were maintained at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). The DDD.Cg-A' mouse strain was established by introgression of the A' allele from the B6-A' strain into the DDD strain by backcrossing for 12 generations [19]. Because the original DDD strain had an albino coat, congenic DDD.Cg-A' mice were further intercrossed between yellow (A') and agouti (A) littersmates to eliminate the Tyr' allele.

All mice were maintained in a specific pathogen-free facility with a regular light cycle and controlled temperature and humidity. Food (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and water were freely available throughout the experimental period. Unless specifically mentioned, 4–5 mice were group-housed during the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of NIAS.

Blood phenotype analyses: At 25 weeks of age, mice were euthanized with an overdose of ether after they were fasted for 4 hr. Whole blood was drawn from the heart into a plastic tube using heparin as an anticoagulant. Blood glucose levels were determined using a clinical colorimetric kit (Glu-Test; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan), according to the procedure described previously [21]. In brief, mice were weaned at 4 weeks after birth. Body weights according to the procedure described previously [21]. In brief, mice were weaned at 4 weeks after birth. Body weights were measured weekly from 5 to 25 weeks of age and determined to the nearest 0.01 g using an electronic balance. The average body weights of mice from 5 to 25 weeks were modeled as the study outcomes using the following equation:

\[
\text{Log (Body weight)} = a + b \times \text{Log (Week)} + c \times [\text{Log (Week)}]^2 + \epsilon
\]

The DDD.Cg-A' males were housed under one of the following three different conditions: (1) group housing: 4 or 5 DDD.Cg-A' males were housed together during the experimental period (DDD.Cg-A' males housed under this condition are hereafter designated as GH); (2) individual housing: a single DDD.Cg-A' male was individually housed during the experimental period (DDD.Cg-A' males housed under this condition are hereafter designated as IH); and (3) single male housing with females: single DDD.Cg-A' male was housed with 1–4 females (DDD.Cg-A' or DDD) of the same litter during the experimental period (DDD.Cg-A' males housed under this condition are hereafter designated as SF).

Growth curves of the DDD.Cg-A' males were analyzed using the model defined by the above equation. Optimum estimates and 95% CI for the parameters were determined by the least-squares method weighted by the number of mice. Statistical significance was set at the α=0.05 level. Data analysis was performed using JMP ver. 9.0.1 statistical software (SAS Institute Inc., Cary, NC, U.S.A.).
**RESULTS**

**Plasma insulin levels were reduced in DDD.Cg-A' males:** Table 1 shows the mean ± SE for plasma insulin levels in several inbred strains and their F1 mice at 25 weeks of age.

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Mean ± SE (ng/ml)</th>
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<tbody>
<tr>
<td>B6.Cg-4' male</td>
<td>12</td>
<td>12.57 ± 3.18</td>
</tr>
<tr>
<td>DDD.Cg-4' male</td>
<td>14</td>
<td>1.68 ± 0.28 a, b</td>
</tr>
<tr>
<td>DDD male</td>
<td>12</td>
<td>7.29 ± 1.14</td>
</tr>
<tr>
<td>(B6 × DDD.Cg-A') F1-A' male</td>
<td>12</td>
<td>23.22 ± 5.22</td>
</tr>
<tr>
<td>(DDD × B6.Cg-A') F1-A' male</td>
<td>9</td>
<td>13.11 ± 1.88</td>
</tr>
<tr>
<td>B6.Cg-4' female</td>
<td>10</td>
<td>9.41 ± 1.34</td>
</tr>
<tr>
<td>DDD.Cg-4' female</td>
<td>10</td>
<td>9.38 ± 2.13</td>
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</tbody>
</table>

Table 1. Plasma insulin levels in several inbred strains and their F1 mice

We compared and analyzed the growth curves and blood phenotypes in the GH, IH and SF mice. Figure 6 shows a plot of the average body weight of the GH, IH (IH_1) and SF mice from 5 to 25 weeks of age. The average body weight of the IH_2 mice at 25 weeks is also presented. The maximum body weight of the GH mice was 43.5 g at 16.7 weeks (Table 3). The maximum body weight of the IH_1 mice was 42.9 g at 14.2 weeks, and that of the SF mice was 49.5 g at 19.3 weeks. Thus, the peak of the growth curve occurred in the order of the GH, IH and SF mice.

**Individual housing significantly decreased and the presence of females significantly increased body weight at 25 weeks:** In the IH_2 mice, body weight was determined only at 25 weeks. The average body weight of the IH_1 mice (40.06 g) was not significantly different from that of the IH_2 mice (37.94 g). Therefore, the data for the IH_1 and IH_2 mice were merged and analyzed (Fig. 7A). The IH mice were significantly lighter than the GH and SF mice. The SF mice were significantly heavier than the GH mice.

**Individual housing substantially increased blood/plasma glucose levels and decreased plasma insulin levels:** The average blood glucose level at 25 weeks in the IH_1 mice (537 mg/dl) was not significantly different from that in the IH_2 mice (592 mg/dl); therefore, the data for the IH_1 and IH_2 mice were merged and analyzed. Because 2 of the 9 IH_1 mice and 14 of the 16 IH_2 mice had blood glucose levels more than the upper limit of the measuring apparatus (600 mg/dl), their blood glucose levels were assumed to be 600 mg/dl. The IH mice had significantly higher blood and plasma glucose levels than the GH and SF mice, and no significant difference was observed between the GH and SF mice (Fig. 7B and 7C). Conversely, the IH mice had significantly lower plasma insulin levels than the GH and SF mice, and no significant difference was observed between the GH and SF mice (Fig. 7D).

**DISCUSSION**

Contrary to the expectation that A' mice are invariably hyperinsulinemic, DDD.Cg-A' males were hypoinsulinemic. In particular, plasma insulin levels were higher in the non-A' DDD males than in the DDD.Cg-A' males. We conclude that diabetes mellitus in the DDD.Cg-A' males is a consequence of decreased insulin levels. Decreased insulin directly sug-
Fig. 1. Comparison of the histology of pancreatic islets (HE staining). (A) DDD male, (B)–(D) DDD Cg-Ay males. No notable abnormalities were found in the islets of the DDD male (A). In contrast, histological changes (surrounded by dotted lines), such as fibrosis (B), vacuolar degeneration (C) and lymphocytic infiltration around the ducts (D), were observed in the islets of the DDD Cg-Ay males. Scale bars=100 μm.

Fig. 2. Comparison of the histology of pancreatic islets (immunostaining). (A) DDD male, (B)–(D) DDD Cg-Ay males. The number of β-cells stained by anti-insulin antibody was markedly reduced in the islets of the DDD Cg-Ay males (B–D) compared with the DDD male (A). Scale bars=100 μm.
gests pathological changes in pancreatic islets. Although the size of the islets varied among the DDD.Cg-A' males, the number of the islets was reduced in DDD.Cg-A' males. All DDD.Cg-A' mice showed islet fibrosis, which is observed in humans and various spontaneous rodent models of Type 2 diabetes mellitus [8]. Furthermore, all DDD.Cg-A' males showed a loss of β-cells.

Because severe diabetes mellitus was not observed in the B6.Cg-A' males, its development is specifically related to the DDD strain background. Thus, the DDD strain can be defined as having a diabetes-sensitive background, while the B6 strain has a diabetes-resistant background [21]. Such genetic background effect suggests the presence of modifier genes that functionally differ between the two strain backgrounds. QTL on chromosome 4, identified in the DDD.Cg-A'

Fig. 3. Quantification of the islets and β-cells. Compared to DDD male, the number of the islets in a section and the average number of β-cells per islet were reduced in DDD.Cg-A' males and correlated positively (r=0.6893).

Fig. 4. A histogram showing the distribution of plasma insulin levels in BC A' mice. The approximate mean insulin values of the parental strains are indicated by arrows.

Fig. 5. QTL mapping of plasma insulin levels. (A) The genome-wide LOD score plot for plasma insulin levels in BC A' mice. A horizontal dotted line indicates the genome-wide threshold LOD score for significant linkage. (B) Identification of a significant QTL on distal chromosome 4. Horizontal dotted lines indicate the threshold LOD scores for significant linkage (P<0.05, upper line) and suggestive linkage (P<0.63, lower line). A horizontal short line indicates 95% CI for the QTL.

Table 2. Phenotypic measurements conducted in terms of the housing conditions of DDD.Cg-A' males

<table>
<thead>
<tr>
<th>Housing condition</th>
<th>n</th>
<th>Analyses</th>
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<tbody>
<tr>
<td>Group housing (GH)</td>
<td>14</td>
<td>Growth curve</td>
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<tr>
<td></td>
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<td>Body weight at 25 weeks</td>
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<td></td>
<td></td>
<td>Blood glucose</td>
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<td>Plasma glucose</td>
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<td></td>
<td>Plasma insulin</td>
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<tr>
<td>Individual housing (first, IH_1)</td>
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<td>○</td>
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<td></td>
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<td>nd</td>
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<tr>
<td>Individual housing (second, IH_2)</td>
<td>16</td>
<td>nd</td>
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<tr>
<td>Single male housing with females (SF)</td>
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<td>○</td>
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○ indicates that the analysis was performed in the DDD.Cg-A' males of the group, nd, not determined.
Ay mice, was exactly the modifier locus that induced severe diabetes mellitus in the presence of the Ay allele. Similar results have been found for the obese gene mutations, Lepob and Leprdb [5, 6, 9]. Lepob or Leprdb mutations produce mild diabetes mellitus with hyperinsulinemia in the B6 background, whereas they produce severe diabetes mellitus with degenerative changes in the islets (including β-cell loss) in the C57BL/KsJ (KSJ) background. The B6 strain has been defined as the diabetes-resistant background, and the KSJ strain has been defined as the diabetes-sensitive background. These results suggest the presence of modifier genes that interact with Lepob or Leprdb mutations; however, such modifier genes or loci have not yet been identified in the KSJ background. Although DDD.Cg-Ay and KSJ-Leprdb/Leprdb mice are similar in the point that both develop diabetes mellitus with β-cell loss, QTL on chromosome 4 is unlikely to serve as a modifier gene in the KSJ background because there are no conspicuous differences in the diabetes-related phenotypes induced by the Ay allele between the B6 and KSJ backgrounds [5].

Many BC Ay mice had higher plasma insulin levels than the parental strains (Fig. 3). We considered that such extreme values might be due to epistatic interactions between the loci. Although the plasma insulin levels did not follow a normal distribution, the results of QTL mapping performed using a parametric procedure were similar to those of QTL mapping performed using a non-parametric one (although the peak LOD score for QTL on chromosome 4 was changed); therefore, we performed pairwise scans. As a result, we did not find any potential interactions (data not shown).

Most importantly, QTL on chromosome 4 was coincident with that for body weight loss and blood/plasma glucose levels. The DDD allele was associated with decreased body weight, increased glucose levels and decreased insulin levels. For glucose levels, QTL on chromosome 4 was suggestive, and there were other suggestive QTLs on other chromosomes. In contrast, for insulin levels, the LOD score of QTL on chromosome 4 was very high, and there were no other QTLs. Taken together, the plasma insulin levels, therefore β-cell loss, were suggested to be controlled solely by the gene on chromosome 4. Inferring from the diabetes-related phenotypes in the KSJ-Leprdb/Leprdb mice, QTL on chromosome 4 will surely be related to the failure of β cell expansion and islet atrophy [5, 6, 9].

In addition to the genetic aspect of diabetes mellitus, we need to pay attention to the non-genetic aspects of diabetes mellitus, because we obtained experimental results that the housing conditions affected the severity of diabetes mellitus.
and resulting body weight loss. The maximum body weight and the weeks at the maximum body weight clearly differed among the housing conditions.

As far as the blood/plasma glucose and plasma insulin levels were concerned, only IH mice were significantly different from the GH and SF mice, and no significant differences were observed between the GH and SF mice. This indicated that SF can be considered as a form of group housing. In other words, the significant difference between the IH and SF mice should not be attributed to the presence of females, but to the number of cohabiting mice. However, it was true that body weight loss was apparently ameliorated by the presence of the females.

Although the factors that link housing conditions and diabetes-related phenotypes were currently unknown, it was evident that the severity of diabetes and body weight loss in the DDD.Cg-Ay males was strongly influenced by the housing conditions. Similar to most spontaneous rodent models of Type 2 diabetes mellitus, diabetes mellitus, and therefore body weight loss, in the DDD.Cg-Ay males were also controlled by both genetic and non-genetic environmental factors.

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REFERENCES


