Spontaneous mutation in the db gene results in obesity and diabetes in CD-1 outbred mice

JULIE A. BROWN, STREAMSON C. CHUA, J R., SHUN MEI LIU, MATTHEW T. ANDREWS, AND JOHN G. VANDENBERGH

Spontaneous mutation in the db gene results in obesity and diabetes in CD-1 outbred mice. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R320–R330, 2000.—Five allelic mutants of the diabetes (db) gene have been previously described in mice and rats causing obesity, infertility, and varying degrees of diabetes. We have identified a new, spontaneous mutation resulting in obesity and diabetes in a colony of CD-1 outbred mice, Mus musculus domesticus. Genetic complementation studies indicated that the new mutation was an allele of the diabetes locus. Sequence analysis of cDNA fragments showed a deletion of one G residue located in exon 12 of the leptin receptor gene. The mutation, Lepr<sup>db-NCSU</sup>, results in a frameshift and reduces Lepr transcript levels 10-fold. Mutant mice drank up to four times more water and were up to two times heavier than wild-type mice. Blood glucose and plasma insulin and leptin concentrations were sexually dimorphic among affected mice, suggesting an effect of sex steroids. Mortality of affected males was 100% by 5 mo, whereas affected females survived up to 10 mo of age.

METHODS

Animals. The mice used in this study were derived from Charles River Laboratories outbred CD-1 strain. All animals were individually housed, except when paired, in 28 × 18 cm clear cages at ~23°C and kept on a 12:12-h light-dark cycle.
Water and food (LabDiet 5,015, PMI Feeds, St. Louis, MO) were provided ad libitum, except during fasts.

Breeding experiments. To determine whether the spontaneous obese/diabetic phenotype was the result of a mutation, sibling matings were conducted within a family showing such traits. All animals were paired for 2 wk, and females were examined daily for the appearance of vaginal plugs. All obese animals from the original litter were paired with unafflicted siblings. The lean male was mated sequentially to his unaffected sisters.

Mice heterozygous for the db and ob mutations were obtained from the Jackson Laboratory. C57BLKs/j db heterozygotes and C57BL/6j ob heterozygotes were paired with possible lean heterozygotes from our original litter.

Genomic DNA preparation. Genomic DNA was prepared from the brain and liver of mutant and WT mice according to the protocol described in unit 2.2 of Ausubel et al. (3). Total RNA from normal and affected mice was prepared by a modification of the method developed by Chomczynski and Sacchi (8) where brain tissue was homogenized in 4 M guanidinium isothiocyanate followed by addition of sodium acetate to a final concentration of 0.2 M. Samples were extracted by water-saturated phenol and chloroform-isoamyl alcohol (50:49:1) followed by two extractions with phenol-chloroform-isooamy alcohol (50:49:1). All extractions were centrifuged through Phase Lock Gel (5 Prime). The WT allele (Leprdb-NCSU) produces a 195-bp fragment that is cut by MspI and size fractionated on agarose gels. The mutant allele (Leprdb-NCSU) allele produces a 195-bp fragment that is cut by Taq I into two fragments of 165 and 30 bp.

Alternatively, the db-NCSU mutation can also be detected by amplification with NCSU-Msp (AGA AAA ATG GAT GGG GAC GTT ACT CCG) and Ex12R (GTC AGC TCT GAC AAC CAC AT). Amplification is performed with a hot-start modification of a standard 20 µl PCR (Superscript II, GIBCO, Gaithersburg, MD) in a 20-µl reaction according to the manufacturer's instructions. The samples were diluted with 80 µl of water. Serial twofold dilutions of the cDNA, starting at the equivalent of 100 ng of RNA, was used for hot-start amplification with Taq DNA polymerase using the specified primers in 25-µl reactions. Both primer pairs span at least two introns. Lepr cDNA was amplified for 35 cycles while actin cDNA was amplified for 20 cycles. Control amplification reactions of RNA without reverse transcription showed no products. The products were size fractionated by agarose gel electrophoresis (2% agarose gel) and visualized by ethidium bromide fluorescence on a Bio-Rad Gel Doc 1,000. The fluorescence intensity of the bands was quantified with the QuantOne software (Bio-Rad, Hercules, CA). Lepr cDNA and actin cDNA are presented as fluorescence units. Input RNA (log scale) is plotted against the logarithm of amplified Lepr or actin cDNA to accommodate the disparity of Lepr mRNA copy number.

Estimation of Lepr mRNA by semiquantitative RT-PCR. The quantity of amplified products after a number of PCR cycles can be expressed by the following equation

$$ T_j = T_0 \ast (1 + E_1)^j $$

where T_0 is the initial amount of template, j is the number of PCR cycles, E_1 is the mean efficiency of amplification per cycle, and T_j is the amount of product after j cycles (28). Taking the logarithm of equation 1, we obtain

$$ \log T_j = \log T_0 + j \log (1 + E_1) $$

Given that equation 2 holds within the linear range of amplification, a plot of \( \log T_0 \) to \( \log T_j \) should yield a straight line. Samples with equivalent amounts of the template mRNA will yield superimposable lines with similar slopes and intercepts. Samples with varying amounts of the template mRNA will yield parallel lines with identical slopes (identical amplification efficiencies) but different intercepts (varying \( T_0 \)). Although this procedure does not provide absolute quantification, relative differences in the fractional contents of a given mRNA species between various samples can be inferred. The procedure of using a standard housekeeping gene provides data equivalent to high throughput real-time RT-PCR methods (16, 19, 26, 34).

Ten micrograms of total brain RNA was used for cDNA synthesis with an RNaseH negative reverse transcriptase (Superscript II, GIBCO, Gaithersburg, MD) in a 20-µl reaction according to the manufacturer's instructions. The samples were diluted with 80 µl of water. Serial twofold dilutions of the cDNA, starting at the equivalent of 100 ng of RNA, was used for hot-start amplification with Taq DNA polymerase using the specified primers in 25-µl reactions. Both primer pairs span at least two introns. Lepr cDNA was amplified for 35 cycles while actin cDNA was amplified for 20 cycles. Control amplification reactions of RNA without reverse transcription showed no products. The products were size fractionated by agarose gel electrophoresis (2% agarose gel) and visualized by ethidium bromide fluorescence on a Bio-Rad Gel Doc 1,000. The fluorescence intensity of the bands was quantified with the QuantOne software (Bio-Rad, Hercules, CA). Lepr cDNA and actin cDNA are presented as fluorescence units. Input RNA (log scale) is plotted against the logarithm of amplified Lepr or actin cDNA to accommodate the disparity of Lepr mRNA copy number.

Primers sequences. The primer sequences were as follows: mLepr-58, ATT TTG GAG AAA AAT GGA TGG G; mLepr-59, AAG GTA AGG TTA AAA TTC ACA AG; actin-1, CTG GAG AAC AGG CAT TAT GCT GTG; actin-3, CTC CTG CTG CCT GAT CCA CAT CAT C.

Physiological experiments. Because Lepr<sup>db-NCSU</sup> mutants are infertile, a colony of heterozygotes was maintained and bred to produce affected animals. Mice were mated until vaginal plugs were found or pregnancy was palpable.

On postnatal day 7, all pups were toe clipped for identification and weighed. Mice were weaned and weighed on postnatal day 21, and pups were tail clipped at this time for DNA analysis. Urinary glucose was measured beginning on day 28 and at 7-day intervals thereafter until age 49 days or glucose was detected. Urinary glucose was measured using urinary reagent strips (Multistix 7, Bayer, Elkhart, IN).

Animals heterozygous for the mutation (+/Lepr<sup>db-NCSU</sup>) were bred throughout the experimental period to ensure the continued production of affected animals. Therefore, most of the phenotypic experiments described below included only WT (+/+) and mutant (Lepr<sup>db-NCSU</sup>) mice. Heterozygous females, aged 6 mo old, were included in measurement of leptin concentrations.

Affected and WT pups were randomly placed into three “treatment” groups. Groups underwent similar tests, but at different ages. Because the db mutation shows autosomal recessive inheritance, approximately one-quarter of the animals per litter were expected to express the diabetic phenotype. Animals were treated as follows.
Group 1 consisted of seven affected males, six affected females, five WT males, and five WT females. Mice were weighed weekly from day 7 through 56 days of age. Response to insulin was measured at 1 and 2 mo of age (~35 and 54 days). Animals were fasted for 10 h, and blood glucose concentrations were determined using tail cuts to obtain blood samples, followed immediately by injection of porcine insulin at a concentration of 1 IU/kg. Blood glucose was measured using a glucometer (One Touch Basic by Lifescan, Johnson and Johnson, Milpitas, CA). Blood glucose was measured at 15 and 30 min after injection (14). Glucose concentrations at time 0 were considered basal fasting concentrations for these animals.

Food and water consumption were measured daily over a 3-day period beginning at ~37 days of age. All mice received 100 g of mouse chow pellets, and the amount of decrease in the weight of the food was measured daily to determine the amount consumed (g). Food spillage was not controlled. Graduated water bottles provided continuous measurement of each animal’s daily water consumption.

At 2 mo (~56 days), mice were killed by cervical dislocation after a 12-h fast. With the use of cardiac puncture, blood was collected for insulin measurement. Insulin concentrations were measured using a rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO).

Group 2 consisted of eight affected males, six affected females, five WT males, and five WT females. Mice were weighed at 84 days (~3 mo) and 112 days (~4 mo). Animals were subjected to tests similar to those described for animals in group 1, using techniques previously described. The ages, however, at which animals underwent each test varied as follows.

Insulin resistance was measured at ~4 mo of age (~110 days). Food and water consumption were measured from days 87 to 90. Mice were killed by cervical dislocation at 4 mo of age (day 112). Blood was collected for insulin and leptin measurement. Plasma leptin concentrations were measured using a mouse leptin radioimmunoassay kit (Linco Research).

Group 3 consisted of five affected females and four WT females. Basal blood glucose and insulin resistance were measured at 6 mo of age (~165 days). Mice were killed by cervical dislocation at 6 mo (168 days). Blood was again collected for insulin and leptin measurement. Due to high morbidity of affected males, all males were killed at 2 or 4 mo of age.

In addition, plasma leptin concentrations of heterozygotes were measured using a mouse leptin radioimmunoassay kit (Linco Research). Four heterozygous females were killed at ~6 mo of age for comparison with homozygous mutant and WT females from group 3. Due to their value in the production of affected mutants, no heterozygotes younger than 6 mo were killed.

For some tests, the sample sizes vary from the numbers indicated above because of experimental error or inadequate amount of blood or plasma obtained for radioimmunoassay. Sample sizes for each test are indicated in the legends of Figs. 4, 6, 7, and 8.

Statistical analysis. All statistical analysis was done with Statistical Application Software programming. Reported means are least square means ± SE.

Body weights were analyzed by age as a two-way ANOVA. Group main effect and interactions were included but removed from the model due to lack of significance. Litters were included as a random effect. Sex and category (affected vs. WT) main effects and interaction were tested against a between-litter error.

Glucose, insulin, and leptin concentrations were also analyzed by ANOVA. Litters were included as a random effect. The litter-by-sex interaction term and category (affected vs. WT or heterozygote) interactions were pooled and treated as random to represent a between-litter error term. Sex and category main effects and interactions were tested against a between-litter error. Data were analyzed similarly to weight, except that age was included as a within-subject factor. Age main effect and interactions were included in the model.

Insulin response data were analyzed similarly to glucose, except that the means tested were differences in glucose concentration after insulin injection. Differences at both 15 and 30 min after injection were analyzed. Age of onset of hyperglycemia was analyzed for correlation with weight at weaning using linear regression.

RESULTS

New mutation is allelic to Lepr^db^ An apparent spontaneous mutation in our breeding colony of CD-1 mice resulted in a litter containing two obese females, one obese male, six lean females, and one lean male. At 60 days of age, the affected mice were almost double the weight of their unaffected siblings and age-matched colony members. Obese animals drank approximately four times more water than normal siblings and showed urinary glucose concentrations >2,000 mg/dl.

All obese animals proved to be infertile based on 2-wk matings with unaffected siblings, regardless of sex combinations. Mating the unaffected male sequentially to his unaffected sisters showed that the male was heterozygous for the trait because two of the sisters produced affected offspring at a ratio of three normal to one obese (12 of 49 pups, not different from the expected ratio). This indicated that the male and two females were heterozygous for the trait. A single autosomal recessive gene mutation was the most parsimonious explanation for these results. Complementation mating of our heterozygous mice with mice known to be heterozygous for the db mutation produced 8 of 23 affected pups. When mated with ob heterozygous mice, no affected pups out of 18 born were produced. These results indicated that the spontaneous mutation was allelic to the db mutant because ~34% of the offspring were obese (not significantly different from the expected 25%). In comparison, none of the ob offspring were affected (significantly different from the expected 3:1 ratio; Fisher’s exact test P < 0.005).

Identification of the mutation in the Lepr gene. The coding sequence of the leptin receptor gene was analyzed from genomic DNA and whole brain cDNA of affected animals. Sequence analysis of cDNA fragments obtained by amplification of brain cDNA showed a deletion of one G residue located in exon 12 of the affected animals. Sequence analysis of cDNA fragments obtained by amplification of brain cDNA showed a deletion of one G residue located in exon 12 of the Lepr gene (11). Because the normal sequence is AAGGAG, whereas the mutant sequence is AAGAG, it is impossible to determine whether nucleotide 2022 or 2023 was deleted (Fig. 1A). This deletion results in a shift of the reading frame, causing a substitution of 11 amino acids followed by premature termination of translation before the membrane-spanning domain. No other differences were found in the Lepr coding sequences between affected and lean (+/+) mice.
Two PCR-based assays were developed to detect the missing base pair in genomic DNA without resorting to sequence analysis. One primer, NCSU-Msp (Fig. 1A), was designed to introduce an Msp I site uniquely into the WT sequence, whereas a similar primer, NCSU-Taq (Fig. 1A), was made to introduce a Taq I site solely into the mutant allele. These primers, in conjunction with a primer based on intronic sequence 3' of coding exon 12 (Ex12R), were used to amplify a fragment from the genomes of lean (+/+), affected mice. Digestion of the amplification products with the appropriate enzymes (Msp I or Taq I) yielded the expected restriction fragments so that use of this assay allowed identification of mice heterozygous for the mutation (Fig. 1B). The mutation, designated Lepr-db-NCSU, terminates translation before the transmembrane region (11). Thus any protein product would not be membrane bound nor would it contain the critical STAT3 box. The mutation should be considered a null allele, similar to the mouse db-Pas and the rat allele fa-f (35). The new allele, expressed on a CD-1 outbred genetic background, produces a novel sexually dimorphic obesity/diabetes syndrome described below.

Effects of the db-NCSU mutation on Lepr expression. We examined the effect of the db-NCSU mutation on Lepr gene expression by RT-PCR analysis of total brain RNA. Initial analysis using NCSU-Taq and mLepr-59 as primers for amplification indicated that the mutation significantly decreased Lepr mRNA levels (data not shown). However, because the NCSU-Taq primer has two mismatches compared with the authentic sequence, it was possible that the mismatches caused preferential amplification of the WT Lepr cDNA. Therefore, we performed a second series of RT-PCR analyses with two primers that flank the db-NCSU mutation, mLepr-58 and mLepr-59. In this case, we obtained amplification products for both the WT and mutant Lepr mRNAs (Fig. 2A). There was significantly less Lepr cDNA product from the mutant brain RNA, whereas amplification of actin cDNA indicated equivalent amounts of total RNA in the WT and mutant samples (Fig. 2B). Densitometric analysis of the Lepr cDNA amplification products showed there was a significant reduction (15-fold) in Lepr mRNA due to the db-NCSU mutation (Fig. 2, C and D).

Onset of obesity and hyperglycemia. Mice containing the Lepr-db-NCSU mutation were significantly heavier than WT animals at an early age. Mutant females were heavier than WT females when first weighed at 7 days of age (P < 0.02; data not shown) and remained heavier in body mass through 6 mo of age (Fig. 3). The body mass of mutant males did not differ significantly from WT males until 35 days (P < 0.002). Unlike female mutants, however, male mutant animals showed a steep decrease in body mass from 56 to 70 days of age, which continued to decline throughout the 4-mo data
collection period (Fig. 3). By 84 days of age, body weights of affected and WT males were no longer significantly different. The loss of weight in males was associated with a dramatic increase in glycemia and decrease in circulating insulin (see below). There was no difference between weights of affected males and females through 2 mo of age. However, female mutants were heavier than male mutants at 3 and 4 mo of age \((P < 0.0002)\).

The mean age of diabetes onset, as determined by urinary glucose, was 32.3 days for affected mice (Fig. 4). The mean weight at age of onset was 34.7 g, with no sexual dimorphism in either variable. There was also a correlation between age of onset and body weight at weaning \((P < 0.02;\) Fig. 5). Mice that were heavier at weaning showed earlier onset of urinary hyperglycemia and higher blood glucose concentrations at 35 and 56 days of age than lighter db weanlings. There were no significant effects of sex on age of onset of glycosuria.

Morbidity and mortality rates of diabetic males were significantly greater than diabetic females at 3–4 mo of age. Mortality of males was 100% by 5 mo of age. Therefore, data obtained at 6 mo were based on female diabetic mice only.

Food and water consumption. Water consumption differed significantly between diabetic mutants and WT mice when tested at both 1 and 3 mo. There was no effect of sex on water consumption. Mean water consumption for affected animals at 1 mo \((n = 12)\) was 38.7 ± 2.4 ml, whereas WT mice \((n = 10)\) consumed a mean of 8.03 ± 2.7 ml at this age \((P < 0.0001)\). At 3 mo of age, mean water consumptions for affected \((n = 13)\) and WT mice \((n = 10)\) were 36.8 ± 2.4 and 7.15 ± 2.7 ml, respectively \((P < 0.0001)\). Mean food consumption for affected animals at 1 mo was ~0.290 ± 0.01 g/g body wt, whereas mean food consumption was 0.221 ± 0.01 g/g body wt for 1-mo-old WT animals \((P < 0.0002)\). At 3 mo, mean food consumptions for affected and WT
animals were 0.198 ± 0.01 and 0.129 ± 0.01 g/g body wt, respectively (P < 0.0002). Where standard error bars are absent, errors were too small to appear on graph. Data at 6 mo were based on females only. Sample sizes are as follows: affected females: 1 mo (6), 2 mo (6), 3 mo (6), 4 mo (6), 6 mo (5); affected males: 1 mo (7), 2 mo (7), 3 mo (8), 4 mo (8); WT females: 1 mo (5), 2 mo (5), 3 mo (5), 4 mo (5), 6 mo (4); WT males: 1 mo (5), 2 mo (5), 3 mo (5), 4 mo (5).

Blood glucose and insulin measurement. At 1 mo of age, there were no significant effects of sex on blood glucose concentrations. The mean blood glucose concentrations of mutant males and females were greater than those of sex-matched WT animals at 1 mo (P < 0.02) and remained significantly higher throughout the test period (Fig. 6A).

At 2 mo of age, mean glucose concentrations of affected males were higher than those of affected females (P < 0.04; Fig. 6A). From 1 to 2 mo of age, blood glucose concentrations increased twofold for male mutants, and 1.5-fold for female mutants (P < 0.0001 and P < 0.008, respectively). Again, both affected male and female glucose concentrations were higher than those of WT mice (P < 0.0001 and P < 0.0001, respectively).

Insulin concentrations of affected females did not significantly exceed those of WT females at 2 mo of age (Fig. 6B).

At 4 mo of age, affected mice again exhibited sex differences in blood glucose, with male mutants displaying higher concentrations than their female counterparts (P < 0.002). Both male and female mutants remained hyperglycemic compared with WT mice (P < 0.0001 and P < 0.0001, respectively; Fig. 6A). However, although insulin concentrations of affected males remained low at 4 mo, the hyperglycemia of mutant females persisted despite high plasma insulin concentrations (Fig. 6B). Plasma insulin concentrations of affected females were higher than those of WT females.
Because of increased morbidity at earlier ages, no males were available for blood glucose measurement at 6 mo of age. Mean blood glucose concentrations of female mutants remained higher than WT females (P < 0.0001; Fig. 6A). Plasma insulin concentrations of affected females also remained higher than WT females at 6 mo (P < 0.0001; Fig. 6B).

Insulin response. There were no effects of sex on insulin response at 1, 2, or 4 mo of age. Affected and WT animals responded similarly to insulin injection at 1 mo of age. Both groups were insulin sensitive, with corresponding decreases in blood glucose concentrations after insulin injection at 15 and 30 min postinjection (Fig. 7).

Insulin sensitivity changed by 2 mo of age. WT mice showed steady declines in blood glucose concentrations, whereas mutant animals showed increases in glucose concentration at 15 and 30 min postinjection (Fig. 7). The increase was likely due to both increased insulin resistance and increased production of glucose due to the stress of injection. Thus at 15 and 30 min after insulin injection, mutant animals were significantly more resistant than WT mice (P < 0.0002 and P < 0.0002, respectively).

At 4 mo of age, diabetic mice again exhibited significantly greater insulin resistance than WT animals (Fig. 7). WT mice showed steady declines in blood glucose concentration, whereas mutant animals continued to show increases in glucose concentrations at 15 and 30 min postinjection (Fig. 7).
glucose at 15 and 30 min postinjection. Conversely, 4-mo-old affected mice again exhibited increases in glucose concentration. Differences in insulin response between affected and WT animals at each time interval were significant (P < 0.05 and P < 0.0005 at 15 and 30 min, respectively). There was no sexual dimorphism in response to insulin injection among affected animals at 1, 2, or 4 mo of age.

At 6 mo of age, mean blood glucose concentrations of WT females again decreased after insulin injection, whereas those of affected females increased steadily, indicating greatly reduced insulin sensitivity. These responses to insulin at 15 and 30 min after injection were significantly different for diabetic versus WT females at 6 mo of age (P < 0.007 and P < 0.0005; Fig. 7).

Plasma leptin concentrations. Plasma leptin concentrations of affected male mice, aged 4 mo, ranged from 5.4 to 31.4 ng/ml, with a mean of 13.5 ng/ml. This value was higher than age-matched WT mice (P < 0.04; Fig. 8). Plasma leptin concentrations of all affected females, aged 4 and 6 mo (n = 11), were higher than the maximum detectable value (20 ng/ml) of the radioimmunoassay kit used. When diluted two times, values of affected females were still higher than the maximum, indicating that affected females had plasma leptin concentrations >40 ng/ml (Fig. 8). Because of insufficient plasma samples, no further dilutions could be made. The available data suggest that leptin concentration is sexually dimorphic among affected animals at 4 mo of age. The mean leptin concentration of 6-mo-old female heterozygotes (n = 4) was 10.5 ± 2.5 ng/ml. Age-matched WT females (n = 4) exhibited a mean leptin concentration of 5.8 ± 2.9 ng/ml. This difference was not statistically significant.

**DISCUSSION**

History of the Swiss albino mouse. The CD-1 mice, an outbred stock of albino mice, are based on the Swiss mouse colony maintained at The Rockefeller Institute for Medical Research, starting from two males and seven females in 1926 (24). This stock was derived from a colony of 200 mice obtained by investigators at the Pasteur Institute from a local dealer. Genetic analysis supports the claims of commercial breeders that colonies of outbred Swiss mice have not been supplemented by non-Swiss mice, as Swiss mice are genetically distinct from other mouse stocks (29). The Swiss mouse stocks may be considered an island population, isolated from migration. The C57BL/6 inbred mouse strain was established in 1921 from a breeder pair (female 57 and male 52) from Abbie Lathrop’s mouse colonies. The C57BLKS/J inbred strains are a derivative strain of C57BL/6 that was genetically contaminated, probably by DBA/2 (25). Information about other strains of mice relevant to this discussion can be obtained from a listing by Dr. Michael Festing maintained by the Mouse Genome Informatics Group at the Jackson Laboratories.

**Lepr<sub>db-NCSU</sub> phenotype.** The purpose of this study was to provide a phenotypic description of outbred mice with a novel, spontaneous mutation in the Lepr gene. The Lepr<sub>db-NCSU</sub> mutation exhibits sexual dimorphism in the CD-1 outbred strain, produces a phenotype in CD-1 females unlike previously described db mutations, and may serve to provide a more complete understanding of leptin’s physiological effects.

The earlier appearance of diabetic symptoms in pups that were heavier at weaning indicates that obesity plays a significant role in the disease process of Lepr<sub>db-NCSU</sub> mutant animals. It is also clear that the early onset of urinary hyperglycemia affects disease progress later in life, because mice with the earliest onset of diabetes symptoms had the highest blood glucose at 35 and 56 days of age. However, it is not clear from these data that obesity actually precedes diabetes. Although female mutants displayed greater body mass than control animals by postnatal day 7, male mutants were not significantly heavier than WT males until day 35, at which time affected animals were already hyperglycemic compared with WT animals. Urinary hyperglycemia as an indication of diabetes onset may be problematic as well. Coleman and Hummel (14) found hyperinsulinemia in db mice as early as 10 days of age, long before evidence of hyperglycemia became apparent.

Sex differences became apparent during the study and indicated variability in the progress of diabetes in the mutant mice. Coincident with decreasing weight in diabetic males after 2 mo of age were decreasing plasma insulin and increasing hyperglycemia. Mutant males were insulin resistant at 2 and 4 mo of age, although their plasma insulin concentrations at these ages were low. Insulin resistance increased during the lifetimes of the mutants. This is consistent with the
observation that mice with severe insulin resistance due to combined haploinsufficiency for the insulin receptor and insulin receptor substrate-1 do not exhibit abnormal insulin tolerance tests until 4–6 mo of age (4). The severe weight loss and early mortality of affected males seem to be due to their decreased insulin production. The mean decrease in plasma insulin concentration of affected males at 4 mo is the probable result of islet degeneration, as affected males were not hyperinsulinemic compared with controls at 2 or 4 mo of age. Data on insulin production of db-NCSU mice aged 1 mo and younger will be necessary to determine if males are hyperinsulinemic before 2 mo of age. In contrast, affected CD-1 females showed oversecretion of insulin without apparent loss of insulin secretory capacity. Although females maintained high glucose concentrations, their continued weight gain and longer life spans seem to be due to maintained insulin secretion.

Sexual dimorphism among Lepr<sup>db-NCSU</sup> mutants may be due to differing sex steroid concentrations. Prochazka et al. (27) reported that homozygous db mutants fed estrone in their diets did not develop hyperinsulinemia, hyperglycemia, or islet atrophy. Leiter (21) also noted a marked sexual dimorphism in the diabetes syndrome of db/db animals of the subline CBA/Lt. Female db/db animals of this strain were obese and hyperinsulinemic, but only slightly hyperglycemic, in contrast to the sustained hyperglycemia in Lepr<sup>db-NCSU</sup> mutant females. Males of the CBA/Lt strain exhibited only moderate obesity but severe hyperglycemia and 100% mortality by 6 mo. CBA/Lt mutant males were hyperinsulinemic at 2 mo, but exhibited normal plasma insulin levels by 4 mo, a progression similar to our mutant males. Leiter (21) found that injections of 17β-estradiol and progesterone resulted in remission of diabetes symptoms in males.

The increased plasma leptin concentrations exhibited by male and female Lepr<sup>db-NCSU</sup> mutants were not unexpected, because the db mutation blocks leptin signal transduction. Differences in leptin concentrations between affected males and females were most likely due to the decreased body weights, possibly corresponding to fat mass, of mutant males. The Lepr<sup>db-NCSU</sup> mutation is unique in its effects on CD-1 female mice. Obese CD-1 males exhibit the hyperglycemia and early mortality due to loss of insulin secretory capacity similar to db/db males of the CBA/Lt and C57BLKS/J strains. However, obese CD-1 females behave differently than db/db females of the CBA/Lt and C57BLKS/J strains. Obese (db/db) CBA/Lt females are hyperinsulinemic and normoglycemic, similar to obese (db/db) C57BL/6j females. Obese (db/db) females of the C57BLKS/J strain are hyperglycemic and progress to decreased insulin production and body weight. In contrast to these strains, mutant CD-1 females are hyperglycemic but maintain high body weight and insulin secretory capacity. A further analysis of well-characterized Lepr<sup>db</sup> strains may be useful for comparison of mutation effects on different genetic backgrounds.

Phenotypic comparisons of 10 congenic Lepr<sup>db</sup> strains. The summary data in Table 1 provide an overview of information about the effects of Lepr deficiency in 10 mouse strains at 4–5 mo of age (this paper, 2, 22, 23; insulin values were converted using 25 µU = 1 ng). It is clear that the db mutation causes obesity independent of genetic background, although severe insulinopenia with hyperglycemia is associated with lower body weights. The average body weights for lean mice of all strains is 33 g for males and 30 g for females. Taking into account that the following comparisons are based on historical data rather than data obtained from concurrent experiments, it appears that the diabetes subphenotypes of obese CD-1 mice are similar to the subphenotypes of obese mice of the DBA/2J and CBA/Lt strains. The hyperglycemia and insulinopenia of obese males of these three strains result in high early mortality, whereas the insulin levels in obese females of the three strains minimize mortality. However, the DBA-db congenic strains were discontinued, whereas the CBA-db strain is only available as frozen embryo stocks. This sexual dimorphism is not present in the C57BLKS/J strain, with obese animals of both sexes being hyperglycemic with insulinopenia and having similar mortality rates. Mortality appears to be minimized if insulin levels are maintained near 20 ng/ml (~500 µU/ml), as in the case for obese mice of the C57BL/6J, C3H, MA/MyJ, SWR/J, and 129J strains. One should note that high insulin levels (>100 ng/ml) are not strictly associated with euglycemia, as exemplified by a comparison of SWR/J mice and 129J mice. This information regarding the influence of strain background is a useful base for efforts to identify

### Table 1. Characteristics of 4- to 5-mo-old obese mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Weight, g at 4–5 mo</th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
<th>Mortality, % at 4–5 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-1 db-NCSU</td>
<td>M</td>
<td>61</td>
<td>352</td>
<td>11.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>51</td>
<td>219</td>
<td>1.8</td>
<td>80</td>
</tr>
<tr>
<td>CBA/Lt</td>
<td>M</td>
<td>43</td>
<td>219</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>51</td>
<td>211</td>
<td>1.4</td>
<td>80</td>
</tr>
<tr>
<td>C3H/HeB/Fj</td>
<td>M</td>
<td>43</td>
<td>252</td>
<td>1.2</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>51</td>
<td>211</td>
<td>1.2</td>
<td>35</td>
</tr>
<tr>
<td>C57BL/6j</td>
<td>M</td>
<td>44</td>
<td>174</td>
<td>1.8</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>51</td>
<td>211</td>
<td>1.8</td>
<td>35</td>
</tr>
<tr>
<td>C57BLKS/J</td>
<td>M</td>
<td>44</td>
<td>174</td>
<td>1.8</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>51</td>
<td>211</td>
<td>1.8</td>
<td>35</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>M</td>
<td>49</td>
<td>463</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>55</td>
<td>365</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td>DW/Pas db-Pas</td>
<td>M</td>
<td>67</td>
<td>365</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>76</td>
<td>126</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td>MA/MyJ</td>
<td>M</td>
<td>64</td>
<td>149</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>76</td>
<td>126</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td>SWR</td>
<td>M</td>
<td>51</td>
<td>137</td>
<td>1.8</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>71</td>
<td>102</td>
<td>1.8</td>
<td>73</td>
</tr>
</tbody>
</table>

Data presented are for obese mice between 4 and 5 mo of age and derived from cited literature (see text). Insulin values were converted to ng/ml using 25 µU = 1 ng. All values are based on 4- to 5-mo-old animals. Data are provided for coisogenic strains (db-NCSU, db-Pas, and db-3j) or congenic db strains. Strains in which mutation is not specified are db congenic strains. NA, not available; M, male; F, female.
genetic loci and their allelic variants that modulate glycemia and insulin sensitivity.

Diabetes susceptibility is not associated with a specific Lepr isoform. Mutations (db-NCSU, db-Pas, db-3) that result in the loss of all membrane-bound Lepr isoforms (Lepra, Leprb, Leprc) produce obesity equivalent to the loss of the Leprb isoform only (db mutation). There is no correlation of the presence/absence of non-Leprb isoforms with susceptibility to develop hyperglycemia and/or insulinopenia. The db-NCSU, db-Pas, and db-3 mutations abrogate the synthesis of all membrane-bound Lepr isoforms, whereas obese CD-1-db-NCSU mice are severely hyperglycemic but obese DW-db-Pas and obese 129-db-3) mice are nearly euglycemic. In support of this observation, the sole lack of Leprb causes obese C57BL/6J-db mice to remain euglycemic while obese C57BLKS/J-db mice suffer the most severe hyperglycemia of all Leprb congenic mice.

The Leprdb-NCSU mutation provides the opportunity to further clarify the effects of the diabetes mutation on the diabetic phenotype of the mouse and to investigate the various associated physiological mechanisms that produce the sexual dimorphisms apparent in this strain. The severity of diabetes produced by the db mutation is clearly affected by genetic background, and the placement of the db-NCSU mutation into an inbred mouse strain may further distinguish these background effects. Phenotypic characterizations of this and other db alleles play an integral role in our understanding of the structure and function of the leptin receptor.

We thank Vernon Bauer, Brenda Hill, and Martha Poe for excellent technical assistance; Trudy Mackay for advice on breeding protocols; and CAVL Browne for statistical analysis and assistance. This work was supported in part by North Carolina Agricultural Research Service Project NC06263 and the W. M. Keck Foundation to M. T. Andrews, and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-47473 to S. C. Chua and the New York Obesity Research Center.

The GenBank accession number for the mouse Leprdb-NCSU mutation reported in this paper is AF152957.


Received 15 March 1999; accepted in final form 30 August 1999.

REFERENCES


