Studies in the Diabetic Mutant Mouse: V. Glucose Tolerance in Mice Homozygous and Heterozygous for the Diabetes (*db*) Gene*

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Summary. Mice homozygous and heterozygous for the diabetes (*db*) gene were studied to determine: 1. whether latent carbohydrate intolerance is present in young normoglycemic diabetic mutants (*db/db*); 2. whether normoglycemic food restricted diabetic mutants are carbohydrate intolerant; and 3. whether mice heterozygous for the *db* gene (*db/+*) manifest abnormalities in glucose tolerance, serum IRI levels or body weights. Blood glucose levels were determined 0, 1/2, 1, 2 and 3 h following intraperitoneal administration of 2 mg glucose/g body weight. Normals (+/+) and diabetics (*db/db*) showed similar glucose tolerance curves during the first two weeks of life; however, both were markedly glucose intolerant compared to normal adult mice. At 3 weeks a small number of mutants had higher 3 h levels than any achieved in normal mice. By 4 weeks the average value for diabetics prior to glucose loading (0 time) was significantly ($P < 0.02$) elevated (*db/db* = 144 mg glucose/100 ml, +/+ = 124 mg glucose/100 ml). Although food restriction reduced blood glucose concentration at 0 time, persistence of carbohydrate intolerance was readily demonstrable following glucose loading. — Abnormalities in heterozygotes (*db/+*), 3 to 16 months of age, were primarily restricted to male mice, which showed moderate, but statistically significant elevations in blood glucose both at 0 time and following glucose administration. Forty percent of male heterozygotes had higher serum IRI levels than any observed in normal control males. Body weights of male heterozygotes were significantly greater ($P < 0.01$) than those in age-matched normals.

Etudes de la mutation *dbdb* chez la souris: V. Tolérance au glucose dans la souris homozygote et hétérozygote pour le gène diabétique (*db*)

Résumé. Des souris homozygotes et hétérozygotes porteurs du gène du diabète (*db*) ont été étudiées dans le but de déterminer: 1. si une intolérance latente aux hydrates de carbone existe chez la jeune souris *dbdb* normoglycémique, 2. si la souris *dbdb* normoglycémique ayant une alimentation réduite présente une intolérance aux hydrates de carbone, et 3. si la souris hétérozygote pour le gène *db* (*db/+*) montre des anomalies de la tolérance au glucose, des taux d'IRI sériques et du poids corporel. Les taux de glucose sanguin ont été mesurés 0, 30, 60, 120 et 180 min après l'injection intrapéritonéale de 2 mg glucose/gramme de poids corporel. Les souris normales (+/+) et diabétiques (*db/db*) ont des courbes de tolérance au glucose

semblables au cours des 2 premières semaines de leur vie. Il apparaît pourtant qu'elles manifestent une intolérance marquée au glucose si on les compare avec la souris normale adulte. Un petit nombre de souris *db/db* âgées de 3 semaines présentent des glycémies plus élevées que celles observées chez les souris normales à la 180ème minute du test. A 4 semaines, la glycémie moyenne avant la charge glucosée (0 min) est significativement plus élevée ($P < 0.02$) chez les animaux diabétiques *db/db* (144 mg glucose %) que chez la souris normale +/+ (124 mg glucose %). Bien que la réduction de nourriture diminue la glycémie à 0 min, l'intolérance au glucose est clairement manifeste après administration du glucose. Chez les animaux hétérozygotes (*db/+*) âgés de 3 à 16 mois, les anomalies de la tolérance au glucose sont surtout décétables chez les mâles qui montrent une élévation modérée mais statistiquement significative des taux de glucose sanguins à 0 min et après l'administration du glucose. 40% des mâles hétérozygotes ont des taux d'IRI sériques plus élevés que ceux mesurés chez les animaux mâles normaux. Le poids corporel des mâles hétérozygotes est significativement plus élevé ($P < 0.01$) que celui des mâles normaux du même âge.

Der Diabetes der *dbdb*-Maus: V. Glucosetoleranz homozygoter (*dbdb*) und heterozygoter (*db/+*) Tiere

Zusammenfassung. In den vorliegenden Studien an homozygoten (*db/db*) und heterozygoten (*db/+*) Mäusen des Diabetes-Stammes wurde untersucht: 1. ob bei den jungen, noch normoglykämischen homozygoten Tieren bereits eine Glucosetoleranz nachzuweisen sei; 2. ob Kalorien einschränkung die Glucosetoleranz homozygoter Tiere beeinflusse; und 3. ob bei heterozygoten Anomalien des Zuckerstoffwechsels nachweisbar seien. Bis zum Alter von zwei Wochen war die Glucosetoleranz homozygoter diabetischer Tiere (*db/db*) derjenigen homozygot normaler Tiere (+/+) vergleichbar. In beiden Fällen war sie aber deutlich schlechter als bei normalen erwachsenen Mäusen. Ab der dritten Woche zeigten einige der künftigen diabetischen Tiere gegenüber der Norm erhöhte 3-Stundenwerte. Im Alter von 4 Wochen war bei den diabetischen Tieren der Nullwert signifikant erhöht. Kalorienrestriktion beeinflusste die Glucosetoleranz nicht, obwohl die Blutzuckerkonzentration vor Belastung deutlich niedriger war als bei normal ernährten Tieren. Heterozygote (*db/+*) Weibchen (3–16 Monate alt) waren völlig normal, während bei Männchen vor und nach Glucosebelastung signifikant erhöhte Blutzuckerkonzentrationen und, in 40% der Fälle, auch erhöhte Serum-IRI-Konzentrationen gemessen wurden. Das Körpergewicht männlicher Heterozygote war signifikant höher als dasjenige gleichaltriger Normaltiere.

Key-words: DBM mouse, diabetic mutant mouse, genotype: C57BL/Ks-*db/db*, inherited diabetes, diabetic heterozygote, glucose tolerance, serum insulin.

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The diabetes gene (*db*) in the mutant mouse (C 57 BL/Ks-*db/db*) has been previously characterized as a unit autosomal recessive with complete penetrance [10]. Hyperinsulinemia and obesity represent the earliest documented abnormalities in the syndrome [4]; both appear to precede the development of sustained hyperglycemia, which in most animals first becomes clearly evident at approximately 5 to 6 weeks of age [10, 4, 5, 1]. However, if the food intake of young mutants is restricted, hyperglycemia is either prevented or markedly attenuated [4, 5, 1].

Because of the limited blood volumes in neonatal mice, serial studies of carbohydrate tolerance by means of repeated blood glucose determinations following glucose loading have not previously been feasible. An ultramicro-adaptation of the glucose oxidase method [9] requiring only 5 μ l of whole blood was therefore developed in our laboratory in order to permit examination of glucose tolerance in mice from 1 week of age through full maturity.

The specific objectives of the present studies were to determine: 1. whether latent carbohydrate intolerance is present in young diabetic mutants prior to the onset of sustained hyperglycemia; 2. whether carbohydrate tolerance in normoglycemic food restricted diabetic mutants remains impaired; and, 3. whether mice heterozygous for the *db* gene (*db/+*) manifest abnormalities in glucose tolerance, serum immunoreactive insulin (IRI) levels or body weights.

Materials and Methods

Animals

Mice homozygous and heterozygous for the diabetes (*db*) gene were either from the original strain of diabetics (C 57 BL/Ks-*db*) described by Hummel, Dickie and Coleman [10], or from a balanced stock (DBM) derived by crossing the original strain with mice homozygous for the misty (*m*) coat color gene (C 57 BL/6J-*m/m*), as described in an accompanying publication in this volume [2].

Both DBM diabetic mutants and DBM heterozygotes were produced by matings in our laboratory. The remainder of the animals used were either obtained from The Jackson Laboratory, Bar Harbor, Maine, or were also produced by matings in our laboratory. These included normal control mice (C 57 BL/Ks, young unbred mice, and old retired breeders), diabetic mutants (C 57 BL/Ks-*db/db*) from the original strain, and *db* heterozygotes (C 57 BL/Ks-*db/+*, old retired breeders) from the original strain.

Diet

Animals produced by matings in our laboratory were weaned at 3 weeks of age. All mice were fed with Old Guilford mouse pellets (7.5% fat) and were allowed free access to water. A group was placed on dietary restriction by feeding for only an 8-h period on Monday, Wednesday and Friday of each week, between the seventh and eleventh weeks of life [1].

Serum insulin

Blood samples for serum IRI determinations were obtained at the time of sacrifice by decapitation. IRI was measured in duplicate 50 μ l aliquots of serum by the double antibody method [13, 15], using purified mouse insulin [7] as a standard.

Glucose tolerance tests

Glucose tolerance tests were initiated at approximately the same time each morning. In the case of neonatal animals, the mother was placed in a separate cage during the test. Blood samples were collected directly from the tail using 5 μ l heparinized capillary tubes (Drummond). Immediately following the collection of control (0 time) blood samples, animals were injected intraperitoneally with a pyrogen-free 20% aqueous solution (w/v) of dextrose, 2 mg/g body weight, using a microliter syringe (Hamilton Company) fitted with a 30 or 32-gauge needle. Subsequent blood samples were collected at 1/2, 1, 2 and 3 h following glucose administration.

Ultramicro glucose oxidase method

The 5 μ l samples of heparinized blood were expressed into 6 $\times$ 50 mm glass test tubes containing 70 μ l of 2% perchloric acid, thoroughly mixed, and centrifuged to separate the precipitated protein. Fifty microliters of supernatant fluid was added to 1 ml of 0.2 M phosphate buffer, pH 7.0, containing glucose oxidase, 25 mg/100 ml (Sigma Chemical Co., Type II, specific activity 14 750 units/g), horseradish peroxidase, 2 mg/100 ml (Sigma Chemical Co., Type II, specific activity 200 purpogallin units/mg), and o-dianisidine dihydrochloride, 5 mg/100 ml (Eastman Kodak Co.). The glucose standards analyzed with each assay consisted of identically treated duplicate 5 μ l samples containing 0, 62.5, 125, 250, 500, and 1000 mg glucose/100 ml double distilled water. Samples were incubated for 30 min at 30°C. Optical density was read at 436 m μ , using a Beckman DU spectrophotometer (Gilford modification). The optical densities of the standards were plotted as a function of the glucose concentrations and the slope of this line determined. The glucose concentrations of the unknowns were computed by multiplying the optical densities of the unknowns (minus optical density of the blank) by the slope. Results were expressed as mg glucose/100 ml whole blood.

Results

A. Glucose tolerance in young DBM mutants

Serial glucose tolerance tests were conducted at weekly intervals in DBM mutants, beginning at 1 week of age. Results were compared to serial tests in normal C 57 BL/Ks mice (Fig. 1). Both diabetic and normal mice had similar glucose tolerance curves during the first 2 weeks of life. In fact, values for the diabetic group were occasionally lower than those for the normal mice. Perhaps more surprising was the finding that following glucose administration, values in both groups

were markedly elevated compared to those in normal adult mice. By 3 weeks of age, although the average values for diabetics and normals were similar, a small number of diabetics had higher 3-h levels than any achieved in normal mice. The mean 0 time level for the 3 week old diabetics was in fact lower than normal, probably reflecting the fact that IRI levels rise very early in the syndrome, prior to the onset of sustained hyperglycemia [4]. By 4 weeks of age mild hyperglycemia was apparent in some diabetics even prior to glucose loading (0 time), resulting in a significant elevation ($P < 0.02$) of the average value for the entire group of 12 diabetics ($\text{DBM} = 144 \pm 5 \text{ mg glucose/100 ml}$, control = $124 \pm 5 \text{ mg glucose/100 ml}$ blood). Even in those 4 week old mutants with normal baseline

Monday morning feeding period a marked increase in plasma IRI concentration occurs in food restricted mutants, with a concomitant failure of blood glucose levels to rise [6]. In order to evaluate the effects of acute carbohydrate loading in food restricted mutants in detail, glucose tolerance tests were performed in eleven-week old DBM mutants, and in 3 mutants of the same age from the original (C 57 BL/Ks-*db*) strain. After 4 weeks of food restriction, although 0 time glucose levels had fallen to normal, marked elevations were still apparent following glucose administration. Three of the DBM mutants studied had also been tested at 7 weeks of age, just prior to beginning the 4-week period of food restriction. In all 3 mice glucose tolerance at 11 weeks of age appeared more impaired than

Fig. 1. Comparison of glucose tolerance in young diabetic mutant (DBM) and normal mice. Values are means $\pm$ S.E.M. The small numerals indicate the number of animals for each point. The probability (P) for the difference between the normals and diabetics is shown for each point N.S. = difference not significant at the 0.05 level ($P > 0.05$)

glucose levels, significant impairment of glucose tolerance was demonstrable following glucose loading. As might be predicted, by 5 weeks of age, with the progression of sustained hyperglycemia (rise in 0 time levels above control values), even a more marked impairment in glucose tolerance became apparent.

B. Effects of food restriction on glucose tolerance in diabetic mutants

The fall in blood glucose concentrations in food restricted diabetic mutants from the original strain and the rise following the return to an *ad lib* feeding regimen have already been discussed in an accompanying article in this volume [1]. Interestingly, Coleman and Hummel have found that 2 h after initiation of the

at 7 weeks, despite the fact that 0 time values had decreased to normal (Fig. 2). In addition, in 2 of these mice, and in a third not previously tested at 7 weeks, the shape of the tolerance curve was altered. Following the initial rise in blood glucose concentration at $\frac{1}{2}$ h, these animals showed a leveling off of the glucose concentration at 1 h, followed by a secondary rise at 2 h. This secondary rise was not observed at 7 weeks of age (Fig. 2).

C. Abnormalities in mice heterozygous for the diabetes (*db*) gene

Body weights, glucose tolerance and serum IRI were determined in four groups of animals (Table 1). Heterozygotes from both the original mutant strain

(Group II) and from the DBM colony (Group IV) were studied. Only older retired breeders were available for inclusion in Group II, and therefore the control animals for this group consisted of older normal mice (Group I) which were also retired breeders. A second group of younger normal mice (Group III), never bred,

Body weights

Results (Table 2) indicate that both male *db* heterozygotes and male DBM heterozygotes were significantly heavier than normal control males. There was no significant difference between female heterozygotes and normals, however.

Table 1. Groups of heterozygotes and normal control mice used to study abnormalities in animals heterozygous for the diabetes (*db*) gene

Group	Number of mice male	female	Genotype ^a	Age	Comment
I Normal	7	4	+/+/+	8½ mo.	Retired breeders, control for Group II.
II <i>db</i> heterozygote	17	15	+/+ <i>db</i> +	6 to 15½ mo.	Retired breeders.
III Normal	10	8	+/+/+	11 wk.	Control for Group IV.
IV DBM heterozygote	8	9	+ <i>m</i> / <i>db</i> +	11 wk.	—

^a *db* = diabetes allele; *m* = misty coat color allele; + = wild type alleles for *db* and *m*.

Fig. 2. Effect of food restriction on glucose tolerance in a diabetic mutant (DBM) mouse. At 11 weeks, the peak glucose level is delayed

were used as controls for the younger (11 week old) DBM heterozygotes (also never bred).

Data for male and female animals were analyzed separately, since it was apparent that differences in body weight and glucose tolerance existed within the two groups of heterozygotes which were related to the sex of the mice. Since some heterozygotes included in Group II were older than any of the normal control mice in Group I, data for these heterozygotes were further analyzed according to the individual ages of these mice. No deterioration of glucose tolerance or significant alteration in body weight were discernible with increasing age. We therefore concluded that data for animals of different ages within Group II could be pooled and compared to those for the normals in Group I.

Table 2. Comparison of body weights in heterozygotes (*db*/+) and normal control (+/+) mice

Group	Age	Body weight (g) ^a	
		male	female
I Normal	8½ mo.	30.5 ± 0.3 (7) <i>P</i> < 0.01	29.7 ± 0.7 (4) N.S.
II <i>db</i> heterozygote	6–15½ mo.	35.5 ± 0.9 (17)	29.7 ± 0.7 (15)
III Normal	11 wk.	24.8 ± 0.4 (10) <i>P</i> < 0.01	20.7 ± 0.4 (8) N.S.
IV DBM heterozygote	11 wk.	27.3 ± 0.8 (8)	22.2 ± 0.8 (9)

^a Values are means ± S.E.M. Number of mice in parenthesis.

P is the probability for the difference between the normal and the heterozygote groups, determined from the Student-*t*-test.

N.S. = differences not significant at the 0.05 level (*P* > 0.05).

Glucose tolerance

db heterozygotes. Although baseline (0 time) blood glucose concentrations in both male and female heterozygotes were similar to those in normal animals, significantly greater values in male heterozygotes were evident 1, 2 and 3 h following glucose administration. However, no difference was observed between female heterozygotes and female normals (Fig. 3).

DBM heterozygotes. In contrast to findings in *db* heterozygotes, there was a significant elevation (*P* < 0.01) in baseline blood glucose concentrations of both male and female heterozygotes (Fig. 4). In addition, 2-h levels in male heterozygotes were also significantly elevated compared to those in normal males. Although the remainder of the values were also above normal, the differences did not prove significant at the 0.05 level.

Serum insulin

Serum IRI levels in 18 *db* heterozygotes (12 male, 6 female) from Group II were compared to those in 8 normal control mice (6 male, 2 female) from Group I. With the exception of 5g the male heterozygotes, serum IRI concentrations in all mice studied were less than 36 μ U/ml. These 5 male heterozygotes had values ranging from 61 to 110 μ U/ml. Since the other 7 male heterozygotes had relatively lower levels (range 0 to 36 μ U/ml), the mean value for *all* 12 animals was not significantly elevated (Table 3).

Fig. 3. Comparison of glucose tolerance in *db* heterozygotes and normal mice. Values are means $\pm$ S.E.M. The small numerals indicate the number of animals for each point. The probability (P) for the difference between the normals and heterozygotes is shown for each point. N.S. = difference not significant at the 0.05 level ($P > 0.05$).

Table 3. Comparison of serum IRI levels in *db* heterozygotes and normal mice

Group	Number of Mice		Serum IRI (μ U/ml) ^a	
	male	female	male	female
I Normal	6	2	15 $\pm$ 6 (0-36) N.S.	0 $\pm$ 0 (0-0) N.S.
II <i>db</i> heterozygote	12	6	41 $\pm$ 10 (0-110)	6 $\pm$ 5 (5-32)

^a Mean $\pm$ S.E.M. Range of values in parenthesis. N.S. = difference between the normals and heterozygotes not significant at the 0.05 level ($P > 0.05$).

Discussion

The results indicate that glucose tolerance testing in mice homozygous and heterozygous for the diabetes (*db*) gene can be of value in demonstrating the presence of carbohydrate intolerance under circumstances in which baseline (0 time) glucose levels are still normal.

Examples include both male *db* heterozygotes, and food restricted mutants with apparently normal blood glucose levels which were shown to be carbohydrate intolerant following glucose loading.

The fact that glucose levels in DBM mutants do not exceed those in normal mice until 3 to 4 weeks of age supports the belief that hyperinsulinemia develops prior to the appearance of demonstrable carbohydrate intolerance in these animals. The observation that baseline glucose levels in 3 week old mutants are actually *lower* than normal further supports this hypothesis,

Fig. 4. Comparison of glucose tolerance in DBM heterozygotes and normal mice. Values are means $\pm$ S.E.M. The small numerals indicate the number of animals for each point. The probability (P) for the difference between the normals and heterozygotes is shown for each point. N.S. = difference not significant at the 0.05 level ($P > 0.05$).

and suggests that inappropriately large amounts of insulin are secreted in relation to circulating blood glucose concentrations.

The finding of marked glucose intolerance in *both* normal and diabetic 1 and 2 week old mice is of obvious interest, particularly in view of several reports showing delayed or decreased insulin secretion in newborn humans and in prenatal experimental animals [12, 17, 3, 16]. The possibility that this impairment in insulin secretion is present in neonatal mice is being investigated in our laboratory.

In the case of food restricted mutants, the present study indicates that although the marked increase in serum IRI following 2 h of refeeding noted by Coleman and Hummel [6] was sufficient to maintain normal blood glucose levels, the insulin release following the administration of 2 mg glucose/gbody weight is inadequate to maintain normal glucose tolerance. The fact that in 3 of the 11 food restricted DBM mutants

the peak for glucose concentration occurred at 2 rather than at $\frac{1}{2}$ or 1 h could be explained by an initial high rate of insulin secretion, followed by a large fall in the face of a continued glucose stress.

With regard to studies in mice heterozygous for the *db* gene, the increased body weight, glucose intolerance and in some cases elevated IRI levels indicate that these animals are abnormal. These abnormalities, however, are considerably less severe than those in homozygotes. It is not surprising that we have been unable to document abnormalities in the appearance of the islets of heterozygotes by light microscopy, since the degree of alteration in islet morphology generally correlates with the degree of severity of the diabetic syndrome [2]. The presence of physiological abnormalities in heterozygotes suggests that the normal (+) and the diabetes (*db*) alleles may interact to produce a mild form of diabetes, rather than the normal (+) allele always acting as a complete dominant.

The reasons for these abnormalities being primarily confined to *male* heterozygotes is not entirely clear. In this respect a higher incidence of carbohydrate intolerance has been documented in male mice of both the KK [14] and C 3 Hf $\times$ I F₁ [11] diabetic strains. In addition, a deleterious effect of androgen administration on the evolution of diabetes in subtotally pancreatectomized rats has been recently reported [8].

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