

## Hyperinsulinemia in Pre-Weaning Diabetes (*db*) Mice\*

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**Summary.** Two new strains of diabetes mice (C57BL/KsJ-*m db* and C57BL/6J-*m db*) have been developed with diabetes (*db*) and the closely linked misty (*m*) gene coupled on the same chromosome. This permits identification of diabetes mice (*m db/m db*) by their gray coat color and white feet as early as age 4 days, well before obesity or signs of diabetes are visible. Hyperinsulinemia was evident in diabetes mice of both strains at 10 days of age and the severity increased progressively with age. These elevated concentrations of plasma insulin were associated with mild hypoglycemia. No significant differences were observed with respect to the degrees of hyperinsulinemia or hypoglycemia between diabetes mice of the two

strains. The mild hypoglycemia observed in diabetes mice in the presence of a pronounced hyperinsulinemia suggests that insulin resistance occurs as early as age 10 days and may itself be a factor in causing hyperinsulinemia. The sequence of early events occurring in diabetes mice appears to be hyperinsulinemia leading to mild hypoglycemia, followed by compensatory hyperphagia which in turn results in enhanced insulin secretion and obesity.

**Key words:** Diabetes mice, pre-clinical diabetes, hyperinsulinemia, insulin resistance

The mutant gene, diabetes (*db*), causes an obesity-diabetes syndrome characterized by hyperphagia, obesity, mild to severe hyperglycemia, hyperinsulinemia, and characteristic morphological changes in the islets of Langerhans [2]. The severity of the diabetic syndrome depends on the inbred strain in which the mutation is maintained [6]. The disease in the strain of origin, C57BL/KsJ (BL/Ks) is characterized by rapid weight increase, marked hyperglycemia, and transient hyperinsulinemia coupled with atrophy of the islets of Langerhans [2, 6]. Death usually occurs between 6 and 8 months in these severely diabetic mice. On the other hand, diabetes mice on the C57BL/6J (BL/6) background become grossly obese, have mild hyperglycemia, but sustained hyperinsulinemia associated with marked hyperplasia of  $\beta$ -cells and hypertrophy of the islets of Langerhans [6]. The difference between the diabetes mice of the two strains appears to be in the ability continuously to control excessive rises in blood sugar concentrations.

Diabetes mice of each strain can be identified at 3–4 weeks of age by marked depositions of adipose tissue in the axial and inguinal regions. Most studies, to date, have dealt with the development of the diabetes syndrome in the post-weaning period because identification of diabetes homozygotes prior to the onset of obesity was not possible. In a previous study [3] we presented statistical evidence that hyperinsulinemia occurs as early as 2 weeks of age. Plasma immunoreactive insulin (IRI) determinations were

made on 38 individual offspring from heterozygous matings. Of these, 25% would be expected to be homozygous for the diabetes gene and therefore should exhibit any abnormalities. When the 38 mice were classified with respect to plasma IRI, they could be divided into two groups, of which one group of 10 (28%) had plasma insulin concentrations significantly higher than the other group of 28. The 10 hyperinsulinemic mice were presumed to be the diabetes homozygotes.

The gene, diabetes, is located in Chromosome 4,  $1.4 \pm 0.74$  recombination units away from the coat color gene, misty (*m*) [6]. We have taken advantage of this close linkage in developing two new congenic strains BL/Ks-*m db* and BL/6 *m db* in which misty (*m*) in coupling with diabetes (*db*) is used for a marker. In these strains 1/4 of the mice are gray and homozygous for both genes (*m db/m db*) and the other 3/4 are black and either heterozygous for both genes or carry neither gene. The *m db/m db* young can be distinguished as soon as their lighter coat color and characteristic white feet are visible, usually at 4–5 days.

This paper reports studies of these *m db/m db* mice beginning at age 8 days in an attempt to detect changes that occur prior to the onset of obesity.

### Materials and Methods

The misty gene (*m*) was introduced into our diabetes (*db*) strains by crossing BL/Ks-*db/+* and BL/6-*m/m* mice. Brother-sister matings of progeny established a balanced stock in which only very few of the misty phenotypes would be of the crossover genotype heterozygous for *db* (*m db/m +*). One such misty was found among many tested. This mouse

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was the progenitor of a stock in which essentially all the misty mice ( $98 \pm$  percent) exhibited the diabetic syndrome. Black mice from this stock, known to be *m db/+ +* by progeny testing, were mated to mice of the BL/Ks and BL/6 inbred strains and after 5 cycles of cross intercross breeding the two congenic strains BL/Ks—*m db* and BL/6—*m db* used in this study were considered to be inbred. Experimental mice were gray (*m db/m db*) and controls were black littermates, a mix of genotypes *m db/+ +* and *+ +/+ +* in the expected proportions, 2/3 and 1/3 respectively. All mice were housed in stainless steel pens on pine shavings with pelleted food (19% protein, 6% fat, Emory Morse Company, Guilford, Conn.), and tap water available at all times.

One of our most immediate concerns was whether the introduction of the gene for misty had any effect on the expression of diabetes in either strain. 7 to 10 misty diabetes mice of each sex and strain were therefore set aside at weaning for routine body weight, blood sugar, and plasma insulin determinations.

Breeding pens of *m db/+ +* mice were checked daily, except Saturday and Sunday, to ascertain the dates of birth of litters required for the pre-weaning studies. In most cases, birth dates were accurate to within one-half a day. Fed and fasted blood sugar determinations were made on blood obtained from the orbital sinus of individual *m db/m db* and like-sexed littermates ranging from 12 to 18 days of age. Fasting was accomplished by taking equal numbers of misty and like-sexed littermates from their mothers and placing them in a clean cage for 7 h. Plasma IRI was determined using pooled blood, obtained by decapitation, from misty diabetes and like-sexed littermates ranging in age from 8 to 28 days. Blood from a minimum of 6 mice of the youngest age (8 to 10 days) was pooled to obtain sufficient plasma (0.3 ml) for triplicate IRI determinations, whereas blood from individual mice sufficed at 21 days of age. Mice of both phenotypes were weighed at time of blood sugar determination or at time of sacrifice if insulin determinations were made. All analytical and histological procedures were as previously described [2].

## Results

### Post-Weaning Studies

BL/Ks—*m db/m db* mice developed hyperglycemia (average greater than 400 mg per 100 ml) between 10 and 15 weeks similar to that reported for BL/Ks—*db/db* mice [2, 6]. Obesity and hyperinsulinemia were evident at 3 to 4 weeks and the degree of each increased until the average blood sugar concentrations exceeded 400 mg/100 ml after which time plasma insulin concentrations dropped to near normal. The average maximum weight occurred at 13 weeks for males (39.4 g) and at 17 weeks for females (48.5 g), after which average weights decreased. The mice were

killed at 6 months for histological examination of the pancreas, and as in BL/Ks—*db/db* mice, islet atrophy and marked  $\beta$ -cell degranulation were the characteristic morphological findings. BL/6—*m db/m db* mice showed the developmental pattern typical of BL/6—*db/db* mice [6]. Hyperglycemia was mild and transient, the average maximal blood sugar concentration for males (293 mg/100 ml) and for females (242 mg/100 ml) occurred at 6 weeks of age after which average values gradually decreased to normal at 16 weeks. Hyperinsulinemia was marked and sustained, and at the time of sacrifice (6 months) usually exceeded 1500 microunits per ml. Histological examination of the pancreases of representative mice revealed the expected hypertrophy and hyperplasia of the islets of Langerhans. The average weights for both sexes were over 50 g at sacrifice, at which time individual weights were still increasing. These are the typical and expected patterns for BL/Ks and BL/6 diabetes mice and no effect of misty (*m*) on the development of diabetes could be discerned in either strain.

### Pre-Weaning Studies

Mice of the BL/Ks—*m db* strain proved to be more productive than mice of the BL/6—*m db* strain in which neonatal mortality was common. Therefore, we have more information on BL/Ks—*m db* mice and only comparative data for the most important critical period of development (10–18 days) in the BL/6—*m db* mice. Body weights of both phenotypes at any given age were highly variable depending on the litter size. Also when compared with like-sexed littermates, *m db/m db* mice tended to be smaller. This slight decrease in body weight may be associated with the misty gene but this possibility was not pursued further.

The changes in plasma IRI with age in both phenotypes of both strains are shown in Table 1. At 8 days of age, diabetes and control BL/Ks—*m db* mice had nearly identical average plasma insulin concentrations. By 10 days of age, the average plasma IRI value of the diabetes mice was significantly elevated over that observed in their controls. Plasma insulin in control BL/Ks—*m db* mice remained reasonably constant throughout the period studied and the average value obtained (44.7  $\mu$ U/ml) was within the range observed in adult BL/Ks mice (30 to 70  $\mu$ U/ml) in this laboratory. On the other hand, plasma insulin concentrations in the *m db/m db* mice increased with age until at age 21 days the average value obtained was three times that of controls (154.2 vs. 51.7  $\mu$ U/ml).

BL/6—*m db/m db* mice also had a statistically significant elevation of average plasma IRI value age at 10 days and the degree of this elevation became more pronounced with age although it appeared that the degree was not as great as that seen in like-aged BL/Ks—*m db/m db* mice. Again, the average preweaning IRI concentration of the controls (39.8

$\mu\text{U/ml}$ ) was within the range observed for adult BL/6 mice (30 to 80  $\mu\text{U/ml}$ ).

Average fed blood sugar concentrations for diabetes and control mice of the two strains are shown in Table 2. Diabetes mice, aged 12 to 18 days, of both strains had small but significantly decreased blood sugar concentrations when compared with littermate controls. This slight hypoglycemia in diabetes mice is probably a response to the developing hyperinsulinemia. No strain differences in blood sugar concentrations were observed. The mild hypoglycemia observed in diabetes mice in the presence of a pronounced hyperinsulinemia suggested that the infant diabetes mice might be responding to the hyper-

and their average blood sugar concentrations at 28 days typically exceeded that of controls (Table 2). However, since the diabetic syndrome did not develop uniformly in all diabetes mice, individual normoglycemic diabetes mice were not uncommon at 28 days.

## Discussion

The data presented here establish hyperinsulinemia as the first detectable abnormality in diabetes (*db/db*) mice of both inbred strains. Hyperinsulinemia is evident as early as age 10 days and the degree of it increases progressively with age. Diabetes mice of

Table 1. Average plasma immunoreactive insulin concentrations in diabetes and control mice

Age	BL/Ks- <i>m db</i>			BL/6- <i>m db</i>		
	<i>m db</i> / <i>m db</i>	+ + / ?	n <sup>a</sup>	<i>m db</i> / <i>m db</i>	+ + / ?	n <sup>a</sup>
days	$\mu\text{U/ml}$			$\mu\text{U/ml}$		
8	47.0 $\pm$ 7.2	45.7 $\pm$ 4.5	6	—	—	—
10	61.4 <sup>b</sup> $\pm$ 8.3	43.7 $\pm$ 3.5	7	49.0 <sup>b</sup> $\pm$ 9.9	31.7 $\pm$ 3.7	7
12	57.3 <sup>c</sup> $\pm$ 6.9	38.7 $\pm$ 4.8	6	65.4 <sup>c</sup> $\pm$ 10.5	34.9 $\pm$ 6.0	7
14	82.2 <sup>c</sup> $\pm$ 7.9	40.8 $\pm$ 5.7	10	67.0 <sup>c</sup> $\pm$ 9.9	37.7 $\pm$ 4.5	7
16	90.2 <sup>c</sup> $\pm$ 21.2	43.9 $\pm$ 4.7	7	75.0 <sup>c</sup> $\pm$ 5.2	55.0 $\pm$ 2.1	8
18	83.6 <sup>c</sup> $\pm$ 8.7	48.2 $\pm$ 7.9	5	72.7 <sup>c</sup> $\pm$ 7.1	39.0 $\pm$ 5.9	6
21	154.2 <sup>c</sup> $\pm$ 22.9	51.7 $\pm$ 7.3	6	143.2 <sup>c</sup> $\pm$ 19.8	29.0 $\pm$ 2.8	6
28	363.2 <sup>c</sup> $\pm$ 43.0	52.2 $\pm$ 9.9	7	359.0 $\pm$ 126	48.3 $\pm$ 5.9	10

<sup>a</sup> Number of separate pools of plasma from each genotype used for the insulin determinations.

<sup>b</sup> Values different from control at  $P < 0.05$  level of significance.

<sup>c</sup> Values different from control at  $P < 0.01$  level of significance.

Table 2. Average blood sugar concentrations in fed diabetes and control mice

Age	BL/Ks- <i>m db</i>			BL/6- <i>m db</i>		
	<i>m db</i> / <i>m db</i>	+ + / ?	n	<i>m db</i> / <i>m db</i>	+ + / ?	n
days	$\text{mg}/100 \text{ ml}$			$\text{mg}/100 \text{ ml}$		
12	115.1 <sup>a</sup> $\pm$ 3.7	128.7 $\pm$ 4.5	13	107.6 <sup>a</sup> $\pm$ 2.3	111.1 $\pm$ 1.6	11
14	109.0 <sup>a</sup> $\pm$ 3.0	125.6 $\pm$ 3.2	15	122.8 <sup>a</sup> $\pm$ 3.9	137.8 $\pm$ 2.9	12
16	121.4 <sup>a</sup> $\pm$ 3.0	133.9 $\pm$ 3.0	9	112.8 <sup>a</sup> $\pm$ 3.8	132.5 $\pm$ 4.1	10
18	130.3 <sup>a</sup> $\pm$ 7.6	160.4 $\pm$ 6.5	20	—	—	—
28	187.0 $\pm$ 18.1	153.1 $\pm$ 4.9	20	204.1 <sup>a</sup> $\pm$ 7.7	148.0 $\pm$ 5.4	20

<sup>a</sup> Values significantly different from control ( $P < 0.05$ ).

insulinemia by consuming more food. If so, fasting for a few hours might cause a precipitous drop in blood sugar concentration. This was not found to be the case, since a 7 h fast in 12, 14 and 16-day old mice resulted in average decreases in blood sugar concentrations that did not differ significantly in the two phenotypes. The combined average decreases for both strains for days 12, 14, and 16 were 28.1  $\text{mg}/100 \text{ ml}$  for *m db/m db* mice, and 24.9  $\text{mg}/100 \text{ ml}$  for controls, suggesting that compensatory hyperphagia does not occur in diabetes mice prior to 16 days of age. Apparently, insulin resistance is already present in diabetes mice as early as age 12 days, even though excess fat deposition is not yet apparent. At weaning, all diabetes mice were recognizable by increased deposition of fat

both strains become hyperinsulinemic at about the same age but the degree of hyperinsulinemia appears to be less pronounced in the BL/6-*m db* strain. This is of interest since BL/6-*m db/m db* mice are able to control hyperglycemia by increasing insulin secretion throughout life. It may be that if the  $\beta$ -cells are over-stressed during some critical developmental period, they lose their capacity to proliferate and secrete insulin in sufficient quantities to control hyperglycemia in later life as occurs in BL/Ks-*m db/m db* mice. Variations in weight, however, of individual mice in litters and variations of litter size make it impossible to rule out variable nutritional status of the pre-weaning mice as a cause of any strain difference in average plasma insulin values.

Insulin resistance is a pronounced feature of diabetes mice which is recognizable as soon as obesity becomes apparent and which increases with age, becoming almost total in the later stages of the syndrome [2, 3]. Restoration of body weight to near normal by restricting food intake has been shown to result in a marked increase in insulin sensitivity suggesting that the insulin resistance is related to adiposity [3]. The higher insulinogetic index observed as early as age 10 days in diabetes mice compared to that of controls suggests that insulin resistance occurs prior to obesity and may itself be a factor in causing hyperinsulinemia and the resultant diabetic syndrome.

Preclinical studies of the phenotypically similar obese (*ob/ob*) mouse failed to demonstrate either hyperinsulinemia or insulin resistance until about 4 weeks of age [5, 7]. However, increased fat cell size was evident as early as 13 days of age [7]. Comparative measurements of fat cell size were not undertaken in our study but if the fat cells are enlarged in diabetes mice, the enlargement must have taken place simultaneously with, or as a result of, the development of hyperinsulinemia. Studies from our laboratory [4, 6] have shown that obese (*ob*) and diabetes (*db*) mice in the post weaning period are phenotypically identical with respect to the expression of the disease syndrome if on the same inbred background. One difference between obese and diabetes mice was detected after parabiosis of the two mutants. Obese mice, like normal mice, when coupled to a diabetes partner stop eating and die of starvation within 2 to 4 weeks [1]. Our interpretation of these results is that diabetes mice produce satiety factor but lacking a functional satiety center cannot respond to the presence of satiety factor whereas obese mice, like normal mice, have fully responsive satiety centers and starve to death when in parabiosis with a diabetes partner. The results of our study with infant diabetes (*db/db*) mice and those of a study [7] with infant obese (*ob/ob*) mice demonstrate another difference between these two mutants. In the diabetes mouse hyperinsulinemia precedes any other abnormality yet detected, whereas in the obese mouse hyperinsulinemia does not occur until age 4 weeks, well after adipocyte enlargement is

apparent [7]. The cause of the hyperinsulinemia in diabetes mice has not been established but it could result either from a defective pancreas with over-responsive  $\beta$ -cells, or from neural over-stimulation of the pancreas by the hypothalamus.

Our studies establish hyperinsulinemia, accompanied by mild hypoglycemia, as the earliest abnormality occurring in the diabetes mouse. The sequence of events might be hyperinsulinemia leading to mild hypoglycemia and compensatory hyperphagia resulting in enhanced insulin secretion and obesity, with or without a severe diabetic syndrome depending on the inbred background.

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