The Effect of Neonatal Sex Hormone Manipulation on the Incidence of Diabetes in Nonobese Diabetic Mice (43527)

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Abstract. **The nonobese diabetic (NOD) mouse is a model of Type I (insulin-dependent) diabetes. It develops autoimmune pancreatic β-cell lesions characterized by lymphocytic infiltration and β-cell destruction. The incidences of diabetes for male and female NOD mice in our colony were 24% and 73%, respectively. In this study, we investigated the effect of neonatal manipulation of the sex hormone profile on the incidence of diabetes in male and female NOD mice. One day after birth, male mice were castrated and female mice were either ovariectomized, given testosterone, or ovariectomized and given testosterone. The mice were maintained for 140 days and blood samples were collected biweekly starting at 42 days old. Diabetes was determined by three consecutive blood glucose levels >200 mg/dl. Neonatal gonadectomy increased the incidence of diabetes in males but decreased it in females. Females treated with testosterone also had a decreased incidence of diabetes, whereas ovariectomy plus testosterone increased the incidence to 100%. Castration decreased the body weight in males and increased body weight in females. Testosterone treatment with or without ovariectomy also increased body weight. From these studies, we concluded that neonatal hormonal imprinting has a significant influence on the incidence of diabetes in the NOD mouse.**

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The nonobese diabetic (NOD) mouse strain described originally by Makino *et al.* (1) develops diabetes spontaneously and is considered a good model for autoimmune insulin-dependent diabetes mellitus (2, 3). The incidence of diabetes in this model has been shown to be influenced by gender and sex hormones. Female NOD mice have a higher incidence of diabetes than males and castration has been demonstrated to increase the incidence in males and decrease it in females (4–6). Androgen treatment has also been shown to decrease the incidence in females.

Androgens and estrogens have important roles in development and they also influence the developing immune system. Steroid hormones are known to modulate immune responses in a number of animal models (7–10). It is well established that the immune response is more active in females than in males (8), and in the NOD mouse, it has been demonstrated that the administering of testosterone decreased the appearance of diabetes while estrogen injections increased the appearance of diabetes in mature or juvenile mice (4). Since testosterone is a potent determinant of development, it may establish a physiologic imprint that leads to the sexual dimorphism in the development of diabetes in the NOD mice (10).

The gestational period of mice is 21 days, and studies have confirmed that the perinatal period is a critical time in which the neonate is very sensitive to imprinting by sex steroids (11). Neonatal castration on the day of birth deprives the male mouse of testicular androgens and effects not only sexual development, but also establishes female metabolic, neurologic, and behavioral patterns. Treating a normal female mouse with a single dose of androgen at birth or by the fourth day of age has been shown to permanently ablate ovulation and produce constant vaginal estrus. Conversely, castration of the male on the day of birth results in an adult which, if transplanted with ovaries, can exhibit cyclic ovulation and behavioral estrus (11).

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Since the development of diabetes in the mouse is an autoimmune process and the immune system can be modulated by sex steroids, our aim was to study the effect of neonatal castration and ovariectomy with or without testosterone treatment on the incidence of diabetes in male and female NOD mice.

Materials and Methods

Experimental Animals and Surgical Procedures. Our breeding stock of NOD mice was produced from a pair of animals provided as a gift from Clea Japan, Inc. Male and female NOD mice were housed and maintained at 23–25°C, with a 12:12-hr light:dark cycle and free access to food and water. At specified intervals, the mice were mated at 6 weeks of age and pups were sorted into experimental groups. All injections, treatments, and surgical procedures were performed within 24 hr after birth. Male and female pups designated as the control groups each received a single subcutaneous injection of corn oil (25 μl) at the base of the neck. A third group consisting of males was castrated 24 hr after birth. A fourth group of female pups received a single subcutaneous injection of 400 μg of testosterone propionate (TP) in 25 μl of corn oil. A fifth group of females was ovariectomized 24 hr after birth. A sixth group of females was ovariectomized and given an injection of TP (400 μg in 25 μl). Castration and ovariectomy were performed by cooling the neonates shortly before and during surgery. This produced a plane of anesthesia adequate for surgery. A small tub was filled with ice and a stainless steel surgical pan was inverted and placed in the ice. Neonates were initially cooled by placing the pups in a beaker in the refrigerator (4°C). After 5–8 min, when neonates were motionless but a faint heartbeat could be seen, their legs were fastened with tape to the stainless steel pan. Males were positioned on their dorsal surface and gonads were removed from a single ventral incision. Females were positioned on their ventral surface and ovaries were removed from two incisions just below the rib cage. The incision was closed using a single 6-0 silk ligature. The neonates were then placed under a 75 W lamp until they regained consciousness. All pups were returned to mothers when fully recovered from surgery. They were then weaned at 21 days of age and allocated to the appropriate experimental group.

Monitoring and Measuring Blood Glucose and Growth Patterns. Animals were studied for 140 days; during this time, blood samples and body weights were obtained every 2 weeks. Blood samples were collected by orbital sinus bleeding. Blood glucose levels were determined using a glucose oxidase kit (Sigma diagnostics glucose kit, St. Louis, MO). Vaginal smears were performed on TP-injected females between 60 and 68 days of age. All TP-treated animals were found to be in constant vaginal estrus, thus confirming the lack of cyclicity and effectiveness of neonatal testosterone administration. The criteria for establishing diabetes were three consecutive blood glucose determinations greater than 200 mg/dl.

Statistical Analyses. The data were analyzed using either Pearson's chi-square or one-way analysis of variance where appropriate. All statistical analyses were performed using the Statistix computer program (Analytical Software, St. Paul, MN). *P*-values greater than 0.05 were considered statistically significant.

Results

The initial expression of diabetes was observed at approximately 56 days of age, with progressive increases in the incidence to 140 days. The incidences of diabetes at 140 days of age in control male and female groups were 24% and 73%, respectively (Fig. 1). A significant difference between the male and female in the incidence of diabetes was first noted at 84 days (Day 84, $P < 0.01$; Day 140, $P < 0.0005$). Neonatal castration doubled the incidence of diabetes (52%) when compared with controls (24%) at 112 days of age (Fig. 2). This significant difference ($P < 0.04$) was maintained until the end of the experiment (140 days). Neonatal ovariectomy and neonatal testosterone injection of the female significantly decreased the incidence of diabetes in these groups when compared with female controls (Fig. 3). The incidences and significance were 26.3% ($P < 0.002$ vs controls) for the testosterone-injected group and 13% ($P < 0.0001$ vs controls) for the ovariectomized group. Mice that were both ovariectomized and treated neonatally with testosterone showed a significant increase ($P < 0.002$) in the incidence of diabetes when compared

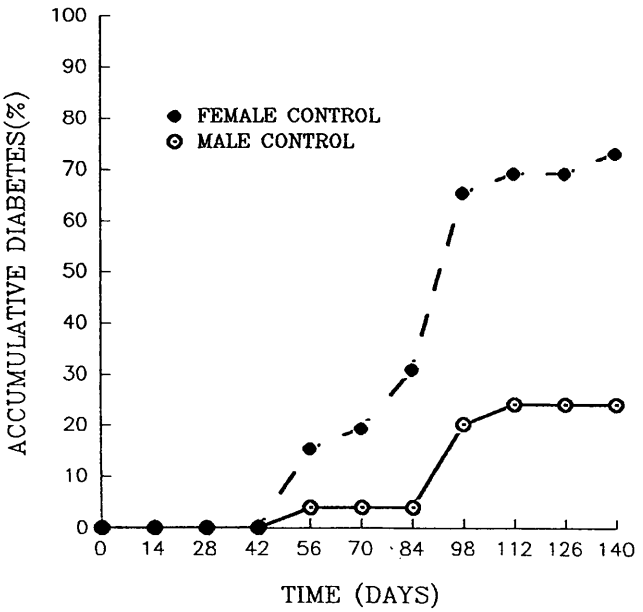

Figure 1. The accumulative incidence of diabetes in male (n = 25) and female (n = 26) NOD mice. Significance, $P < 0.0005$ by Pearson's chi-square.

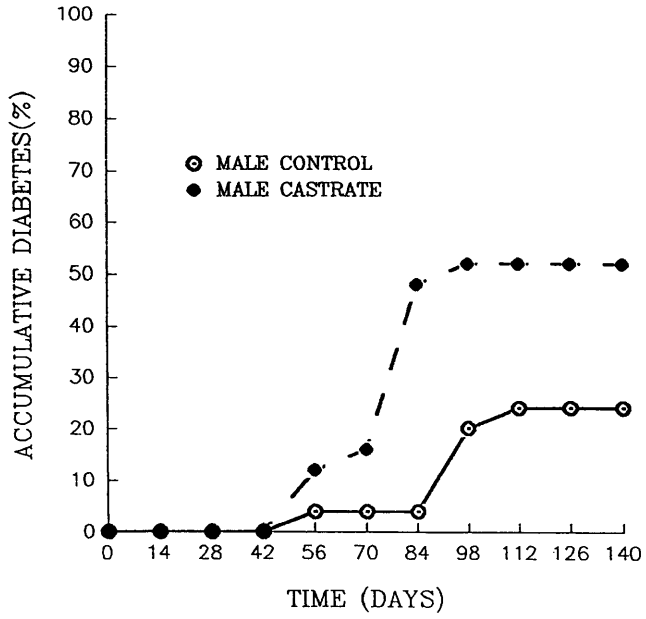

Figure 2. The effect of neonatal castration (n = 25) (24–48 hr after birth) compared with male controls (n = 25) on the subsequent incidence of diabetes in NOD mice. Significance, $P < 0.04$ by Pearson's chi-square.

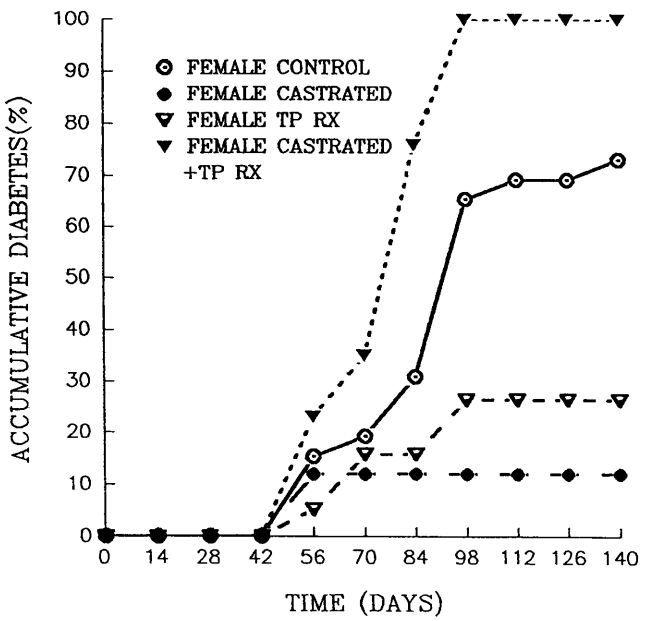

Figure 3. The effect of neonatal (24–48 hr after birth) ovariectomy (n = 25), testosterone treatment (n = 19), and ovariectomy and testosterone treatment (n = 17) compared with female controls (n = 25) on the subsequent incidence of diabetes in female NOD mice. Significance, $P < 0.002$ control versus testosterone; $P < 0.0001$ control versus ovariectomy; and $P < 0.02$ control versus ovariectomy plus testosterone.

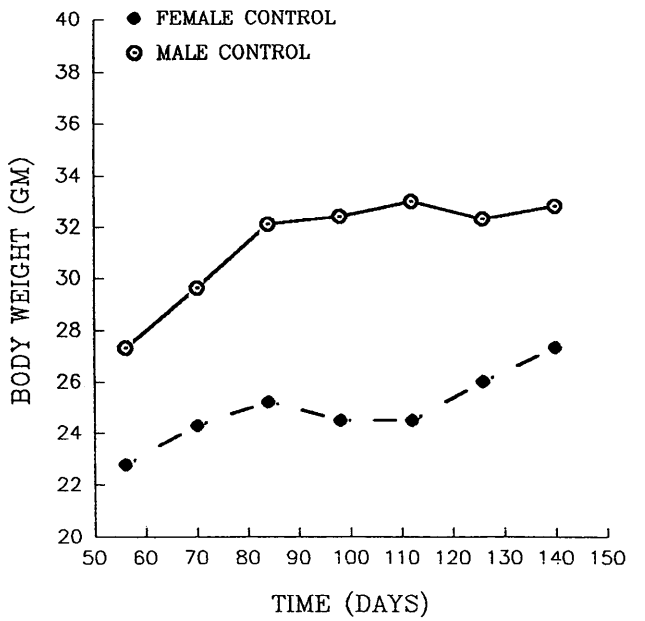

Figure 4. The body weight profile of control male (n = 25) and female (n = 25) NOD mice. $P < 0.001$ analysis of variance.

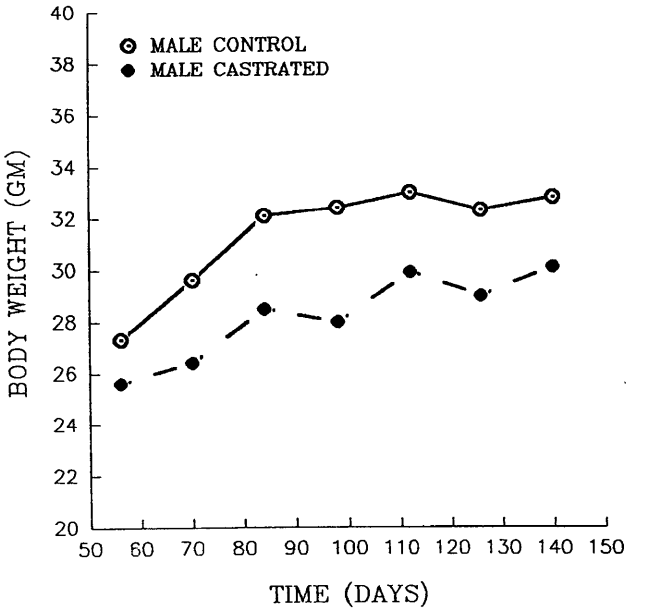

Figure 5. The effect of neonatal castration (n = 25) (24–48 hr after birth) compared with male control (n = 25) NOD mice. $P < 0.01$ analysis of variance.

with controls, with an accumulative incidence of 100% at the end of the 140-day experimental period (Fig. 3).

Male mice were significantly larger ($P < 0.001$) compared with the female mice (Fig. 4). Neonatal castration decreased the body weight of male mice (Fig. 5), whereas ovariectomy and TP treatment of females increased the body weights of female mice (Fig. 6) when compared with their respective controls. Mice that were ovariectomized and testosterone treated had body weights comparable to that of the male controls and significantly higher than that of control females. Neonatal TP-treated intact females had body weight gains comparable to controls. All groups demonstrated an increase in body weight during the course of the experiment.

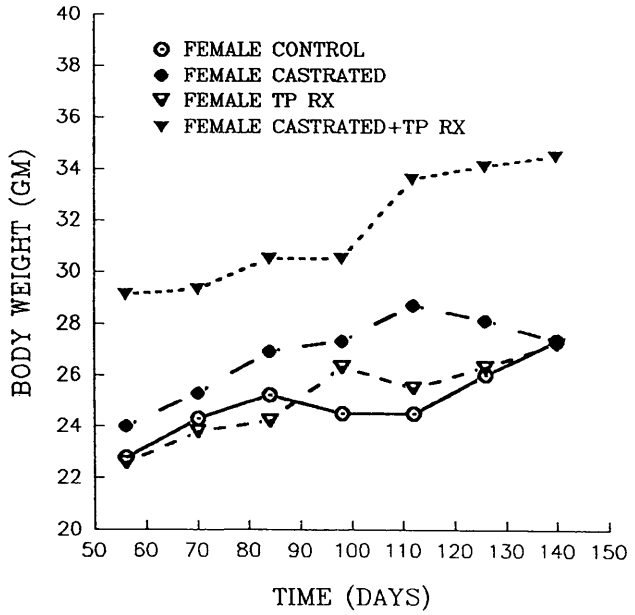

Figure 6. The effect of neonatal (24–48 hr after birth) ovariectomy (n = 25), testosterone treatment (n = 19), and ovariectomy and testosterone treatment (n = 17) compared with female controls on the body weight profile in NOD mice. $P < 0.05$ and 0.0001 for ovariectomy and ovariectomy plus testosterone, respectively.

Discussion

The results from our colony of NOD mice confirm previous observations that female mice had a higher incidence of diabetes when compared with male mice (4). Neonatal castration in males, like castration after weaning (5, 6, 12), increased the incidence of diabetes, and in our study, the incidence was twice that of control animals. Neonatal ovariectomy resulted in a marked decrease in the incidence of diabetes in females. This confirms and extends the observations of others in which ovariectomy was performed after weaning the animals (5, 6, 12). In general neonatal gonadectomy resulted in a more dramatic response in the incidence of diabetes when compared with postweaning gonadectomy.

Alterations in body weight were noted as a result of experimental manipulations. As reported previously for postweaning castration (13), neonatally castrated mice had significantly lower and neonatally ovariectomized animals had significantly higher body weights. When female mice received a single neonatal injection of testosterone, the body weights were similar to those of controls. However, if the animals were also ovariectomized at the time of neonatal testosterone administration, the body weights were significantly greater than those of intact female controls and similar to those of intact males. These findings are similar to previous reports (14). The lack of an effect on body weights for intact female animals receiving neonatal testosterone may have been due to the continuous production of ovarian estrogen because of the animals' inability to ovulate. Estrogen is well known to suppress body weight gain in rodents.

This study partially supports the suggestion of others (6) that testosterone has a protective action on the development of diabetes in the NOD mouse. Our results demonstrate that a single neonatal dose of testosterone lowered the incidence of diabetes in the female while castration increased the incidence in the male. However, since the exposure to testosterone was limited to a single high dose 24 hr after birth, results could not be due to testosterone per se, but may be due to the well-known effects of masculinizing a number of systems of the body. Although evidence is strong for masculinization of the brain and liver by neonatal TP administration (11), it is possible that the immune system and thus the expression of diabetes in the NOD mouse may also be masculinized. The decreased incidence of diabetes in neonatally ovariectomized animals suggests, however, that some form of estrogen production may be needed to fully induce the expression of diabetes in animals predisposed to the disease.

The 100% incidence of diabetes in the ovariectomy plus TP group is not easy to explain. It is possible that the combined treatment removes an inhibitory influence, thus allowing a stimulatory influence to be expressed. In the intact female, neonatal TP administration decreased the incidence of diabetes when compared with the female control. In this group, the animals were under continuous estrogen stimulation, as evidenced by constant vaginal cornification. The administration of estrogen has been reported to increase the incidence of diabetes in the NOD mouse (4, 6). When present in the normally cycling female in an intermittent manner, it has a stimulatory action on the incidence of diabetes. However, when present as in the above study, in a continuous manner, or as after daily injection, or in the constant estrous animal, it may have an inhibitory effect. An understanding of the mechanism of estrogens actions on the expression of diabetes would give us some insight into the sex difference in the expression of diabetes in the NOD mouse. This understanding, however, will require additional investigation.

Another possible clue in explaining the high incidence of diabetes in the neonatal ovariectomized animals given testosterone may come from the observed increase in the body weights of this group. It is known that neonatal testosterone given to female rats results in a growth hormone (GH) secretion pattern similar to that of the male (14–16). This male GH secretion pattern also results in a greater body weight growth similar to that observed by us in the NOD mouse. Growth hormone is known to induce insulin resistance, resulting in an elevation of blood glucose and an increased secretion of insulin (17). In an animal in which the β-cells of the pancreas are already compromised, this additional insult may induce animals that are bor-

derline diabetic to be frankly diabetic, using the criterion we have selected of blood glucose values >200 mg/dl. Since estrogen is known to block the actions of GH on a number of its responses (18), this may explain why intact females given neonatal TP have a lower incidence of diabetes. Future investigations will be directed to evaluating the involvement of GH and the sex steroids in the expression of diabetes in the NOD mouse.

Other investigators have concluded that neonatal imprinting can permanently alter metabolic profiles that regulate growth rates, liver metabolism, and hormone responses (19–21). This study suggests that neonatal exposure to testosterone or the neonatal absence of estrogen or testosterone can permanently alter the sensitivity of the immune response that leads to β-cell destruction and overt diabetes.

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