Maternal Factors in a Model of Type 1 Diabetes Differentially Affect the Development of Insulitis and Overt Diabetes in Offspring

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Type 1 diabetes, a multifactorial disease involving genetic and environmental factors, results from the destruction of pancreatic β-cells. The maternal environment has been suggested to be important in the development of diabetes. To assess the role of maternal factors in the development of insulitis and overt diabetes, we transplanted pre–implantation stage embryos of nonobese diabetic (NOD) mice, a model of type 1 diabetes, into the uterus of each recipient. Recipients were ICR and DBA/2J mice without diabetic genetic predisposition and NOD mice not exhibiting overt diabetes during the experiment; offspring were designated as NOD/ICR, NOD/DBA, and NOD/NOD, respectively; unmanipulated NOD offspring were also examined. NOD/ICR and NOD/DBA offspring developed insulitis significantly earlier than NOD/NOD offspring. However, overt diabetes was significantly suppressed in NOD/ICR and NOD/DBA offspring in comparison with NOD/NOD offspring. Insulin autoantibodies (IAAs) were undetectable in ICR and DBA/2J surrogate mothers and in NOD/ICR and NOD/DBA offspring at the onset of insulitis, suggesting that maternal factors other than transmitted IAAs induced the earlier onset. The present study indicates that altered maternal factors modify the immune response to islets, which in turn might affect the pathogenic course from insulitis to overt diabetes. Diabetes 54:2026–2031, 2005

Human type 1 diabetes is characterized by the establishment of autoimmune insulitis, which precedes β-cell destruction, culminating in hypoinsulinemia and hyperglycemia. Human type 1 diabetic patients spontaneously develop a diabetic syndrome that is controlled by both genetic and environmental factors (1,2). The environmental triggers of islet autoimmunity and type 1 diabetes are essentially unknown but are considered likely to operate early in life, possibly even in utero (3). Previous studies have linked the risk of type 1 diabetes to congenital rubella infection, but non-congenital infection does not appear to convey this risk, indicating the importance of pre- or perinatal events as triggers of islet autoimmunity (4–6). It has been suggested that maternal environment and maternally transmitted insulin autoantibodies (IAAs) in particular modify the risk of the development of autoimmune diabetes in offspring (7), however, it was recently reported (8) that fetal exposure to IAAs did not increase the risk of diabetes development in nonobese diabetic (NOD) mice. The exact role of maternal environmental factors and their relationship in the initiation and modification of the pathogenesis of diabetes in each diabetic individual remain largely unknown. In our previous study (9), by transplanting NOD embryos into the uterus of a recipient ICR mouse, we analyzed genetic predisposition versus diabetic environment and the interaction between these two factors in NOD embryos as causes of congenital malformation.

In the present study, using the same experimental strategy, the onset and incidence of insulitis and overt diabetes were examined in offspring produced from NOD embryos transplanted into the uteri of recipient ICR and DBA/2J mice. Our results indicate that maternal factors control the onset of both insulitis and overt diabetes, two major checkpoints in the pathogenesis of type 1 diabetes (10). Surprisingly, maternal factors were found to differentially affect the development of insulitis and overt diabetes in NOD offspring.

RESEARCH DESIGN AND METHODS

NOD mice from 2 to 40 weeks of age were used in this study and were originally obtained from the Shionogi Research Laboratory (Tokyo, Japan). The Jcl:ICR and DBA/2J mice were purchased from CLEA Japan (Tokyo, Japan) and were used as surrogate mothers for embryo transfer in this study when they were between 8 and 14 weeks old. The animals were maintained at the Institute of Experimental Animals at Shimane University and were handled in accordance with the institutional animal care guidelines. The animals were kept in a room maintained at 24 ± 2°C with 60–70% relative humidity. The room was illuminated by artificial light from 0900 to 2000. We transplanted the pre–implantation stage NOD embryos into the uteri of recipient pseudo-pregnant NOD, ICR, and DBA/2J mice (see below) and designated the offspring NOD/ICR, NOD/ICR, and NOD/DBA, respectively. We only investigated the female offspring. The mice were deeply anesthetized by an intraperitoneal injection of 70 mg/kg pentobarbital sodium (Abbott, North Chicago, IL) and then killed by cervical dislocation to obtain the embryos and/or tissues for the analyses described below. The experimental design and numbers of dams used for transplantation and offspring obtained are shown in Fig. 1. Six of 20 surrogate NOD mothers developed overt diabetes during pregnancy and thus delivered no offspring. Fewer numbers of blastocysts were transplanted to NOD and DBA/2J surrogate mothers than to...
ICR surrogate mothers, since this is empirically known to work better in raising the ratio of live offspring obtained to transplanted embryos. Thus, fewer numbers of offspring were obtained from NOD and DBA/2J surrogate mothers than those from similar numbers of ICR surrogate mothers.

**Embryo transfer.** The optimal timing and yield of fertilized embryos from the superovulated females were induced by intraperitoneal injection of a donor female NOD mouse with 5 units pregnant mare’s serum (Teikokuzokki, Tokyo, Japan). Two days later, 5 units human chorionic gonadotropin (Sankyo, Tokyo, Japan) was injected intraperitoneally and the female was placed with a potent NOD male. When a vaginal plug was confirmed the following morning, this day was defined as day 0 of gestation (E0). Embryo transfer was performed utilizing well-established techniques (9,11). In one method (9), eight cell-stage embryos were collected in modified Whitten's medium by flushing the ampullae of the oviducts with modified Whitten's medium under a dissecting microscope. The embryos were cultured in modified Whitten's medium in an atmosphere of 5% O2/5% CO2/90% N2 at 37°C. The cultured embryos, which developed to the blastocyst stage after 24 h, were transferred to the uteri of pseudopregnant NOD, ICR, and DBA/2J female mice. Recipient mice that were plugged by vasectomized stud males were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium, and the embryos were introduced into the uterine horns with a glass pipette. In another method (11), the eggs were collected from the ampullae of the oviducts with M2 medium, and the fertilized embryos were selected under a microscope. The recipient mice were prepared and anesthetized as above. A glass pipette containing fertilized eggs was inserted under a stereomicroscope into a small opening torn in the bursa over the infundibulum, and the eggs were blown in. Upon successful completion of gestation, the pups were born and were nursed normally by the surrogate mother. There were no significant differences between the offspring obtained by these two techniques, and, therefore, the results are described collectively.

**Histological observation of insulitis.** The pancreata from the unmanipulated NOD, NOD/NOD, NOD/ICR, and NOD/DBA offspring at 2–40 weeks after birth were used for histological analysis. The pancreata were fixed in 10% formalin neutral buffer solution and embedded in paraffin. Serial sections were cut at 5 μm, stained with hematoxylin and eosin, and observed by a light microscope using sections every 200 μm. We observed ~50 islets per pancreas and investigated the incidence and degree of insulitis with lymphocyte infiltration using a grading system in which there was 0 or <25, 25 or <50, 50 to <75, and 75 to ≤100% infiltration of each islet. An islet that showed lymphocyte infiltration was defined as having insulitis, regardless of the infiltrating cells. In Fig. 2, each mouse is shown as a mark, and its percentage indicates the ratio of islet with insulitis in the 50 islets observed in the mouse. Insulitis levels, i.e., degrees of insulitis, were semiquantitatively analyzed by Ridit analysis (12) at 3–5 weeks after birth. We examined the islets from 5 to 10 offspring in each group each week. Sections from mice at 40 weeks were stained immunohistochemically with an insulin antibody using the standard avidin-biotin-peroxidase complex method.

**Overt diabetes by checking urinary glucose levels.** Urinary glucose levels were checked with PRETEST (Wako, Osaka, Japan) once weekly until the onset of glucosuria, when mice were diagnosed with overt diabetes.

**IAA assay.** For the IAA assay, blood was collected from surrogate mother NOD, ICR, and DBA/2J mice aged 14–20 weeks and within 3 weeks of giving birth and from NOD/ICR, NOD/DBA, and NOD/NOD offspring at 3–5 weeks after birth. IAA levels were measured with a 96-well filtration plate micro-IAA assay (13). 125I-insulin (Amersham Pharmacia Biotech, Piscataway, NJ) of 20,000 cpm was incubated with 5 μl serum with and without cold human insulin, respectively, for 3 days at 4°C in buffer A (20 mmol/l Tris-HCl buffer, pH 7.4, containing 150 mmol/l NaCl, 1% BSA, 0.15% Tween-20, and 0.1% sodium azide). Fifty microliters of 50% Protein A Sepharose 4FF/8% Protein G-Sepharose 4F (Amersham Pharmacia Biotech, Upsala, Sweden) were added to the incubation in a MultiScreen-DV filtration plate (Millipore, Billerica, MA) that was precoated with buffer A. The plate was shaken for 45 min at 4°C, followed by two cycles of four washes each cycle with cold buffer B (the same

**FIG. 2.** Incidence of insulitis. Each offspring is shown as a symbol, and its percentage indicates the ratio of islets with insulitis among the 50 islets observed. At 3 weeks after birth, insulitis was already present in the NOD/ICR (C) and NOD/DBA (D) offspring, whereas it did not appear until 4 weeks after birth in the NOD/NOD offspring (E) and NOD control offspring (F). At 4 and 5 weeks after birth, the ratio of mice with insulitis was significantly higher among the NOD/ICR and NOD/DBA offspring than among the NOD/NOD and NOD offspring. Between 5 and 10 offspring were examined in each group each week. *P < 0.01, **P < 0.05.
buffer as buffer A except for 0.1% BSA). After washing, 40 μL scintillation liquid (Microscint-20; Packard Instrument, Meriden, CT) was added to each well, and the radioactivity was determined directly in the 96-well plate with a TopCount (96-well plate β-counter; Packard) scintillation counter. The results were calculated based on the difference in counts per minute (Δcpm) between the wells with and without cold insulin and are expressed as an index: \[ \text{index} = \frac{\text{sample} \times \Delta \text{cpm} - \text{human negative control} \times \Delta \text{cpm}}{\text{human positive control} \times \Delta \text{cpm} - \text{human negative control} \times \Delta \text{cpm}} \]. The limit of normal (0.010) was chosen by the analysis of IAAs in nondiabetic strain mice (13).

Leptin radioimmunoassay. For the leptin assay, blood was collected from pregnant (E18) surrogate NOD (n = 7), ICR (n = 7), and DBA/2J (n = 5) mice from 11–17 weeks of age. The blood was immediately centrifuged, and the serum was frozen at −80°C until the assay. We performed a radioimmunoassay to examine the serum leptin levels using the Mouse Leptin Kit (Linco Research, St. Charles, MO) that utilizes 125I-labeled mouse leptin and a mouse leptin antiserum.

Statistics. The Mann-Whitney U test was used in the statistical analyses, except for those regarding the degree of insulitis, and \( P < 0.05 \) was regarded as significant. Insulitis levels were analyzed by Ridit analysis (12), and a level of \( T > 1.96 \) was regarded as significant.

RESULTS

Onset of insulitis in NOD/ICR, NOD/DBA, and NOD/NOD offspring. At 3 weeks after birth, insulitis had already appeared in the offspring of ICR and DBA/2J mice (NOD/ICR and NOD/DBA offspring, respectively), while it appeared starting at 4 weeks after birth in the offspring of NOD mice not exhibiting overt diabetes (NOD/NOD offspring) (Fig. 2). At 4 and 5 weeks, the ratio of mice having insulitis was significantly higher in the NOD/ICR and NOD/DBA offspring than in the NOD/NOD offspring (\( P < 0.01 \) and \( P < 0.05 \), respectively) (Fig. 2), and the degree of insulitis was significantly more advanced in the NOD/ICR and NOD/DBA offspring than in the NOD/NOD offspring at 5 weeks after birth (Fig. 3A). Additionally, insulitis occurred significantly earlier in the NOD/ICR and NOD/DBA offspring than in the NOD/NOD offspring. By histological observation, most of the insulitis appeared to be peri-insulitis in offspring of all groups at 5 weeks after birth.

Onset and incidence of overt diabetes. The onset of overt diabetes was significantly later and the incidence of overt diabetes significantly lower in NOD/ICR (n = 28) and NOD/DBA (n = 12) offspring than in NOD/NOD (n = 10) or unmanipulated NOD offspring (n = 58) (Fig. 4).

Serum IAA levels in surrogate mothers and offspring. IAAs were not detected in the ICR and DBA/2J surrogate mothers, while one of seven NOD surrogate mothers showed IAAs (Fig. 5). The IAA levels in offspring were examined during the early stages of insulitis. At 3–5 weeks after birth, no IAAs were detected in the NOD/NOD or NOD/ICR offspring, while minimal levels were detected in two of nine NOD/DBA offspring.

Serum leptin levels in pregnant surrogate mothers. The endocrine environment may affect the immune reaction (rev. in 14) and thus the pathogenesis of insulitis. We therefore investigated serum leptin levels in pregnant (E18) surrogate mothers to elucidate possible differences in the maternal hormonal environment, as leptin may be transmitted to offspring perinatally and enhance the Th1...
response (see DISCUSSION). Serum leptin levels (Fig. 6) were found to be significantly higher in ICR and DBA/2J surrogate mothers than in NOD surrogate mothers at E18 ($P < 0.01$).

**Insulitis at 40 weeks after birth.** Insulitis was still present at 40 weeks of age in nondiabetic NOD/ICR and NOD/DBA offspring, and their $\beta$-cell mass was larger than in both nondiabetic NOD/NOD (Fig. 7) and unmanipulated NOD offspring (data not shown).

**DISCUSSION**

Both genetic and environmental factors have been reported to be involved in the pathogenesis of type 1 diabetes (1,2), and maternal factors represent some of the most salient environmental influences during the earlier stages of life (rev. in 14). Using transplantation techniques similar to ours in addition to other complementary strategies, Greeley et al. (7) report that NOD progeny implanted in mothers of a nonautoimmune strain were protected from spontaneous diabetes and suggest that the transmission of NOD maternal IAAs is critical for the progression of diabetes in NOD offspring. However, Koczwarz et al. (8) subsequently reported that no significant differences in diabetes development were observed between female NOD progeny of IAA-positive NOD dams, negative NOD dams, and NOD dams that had antibodies against exogenous insulin, indicating that fetal exposure to IAAs does not increase the risk of diabetes development and that some maternal factors other than IAAs may protect NOD mice from the development of overt diabetes. Several studies have demonstrated that B-cell–deficient NOD mice have little insulin and a markedly reduced incidence of diabetes, indicating that B-cells and perhaps antibodies play a role in the initiation of diabetes (15–18). In the present study, using the same embryo transfer strategy as that in our previous study (9), we analyzed the effect of alterations in the maternal environment on the initiation of diabetes, particularly on the onset of insulitis.

Our results demonstrate that changing the maternal environment from NOD to ICR or DBA/2J by embryo transplantation results in protection from overt diabetes, a fact that is consistent with the results reported by Greeley et al. (7). One of the most remarkable findings in the

**FIG. 4.** Onset and incidence of overt diabetes. Urinary glucose levels were checked once weekly until the onset of glucosuria. The onset of overt diabetes was significantly later and the incidence of overt diabetes significantly lower in NOD/ICR (○) and NOD/DBA (□) offspring than in NOD/NOD (■) or NOD (●) offspring.

**FIG. 5.** IAA levels. For the IAA assay, blood was collected from surrogate mother NOD, ICR, and DBA/2J mice aged 14–20 weeks and within 3 weeks of giving birth, and from NOD/ICR, NOD/DBA, and NOD/NOD offspring at 3–5 weeks after birth. IAA levels were measured with a 96-well filtration plate micro IAA assay. IAAs were detected in only one of seven NOD mothers. At 3–5 weeks after birth, no IAAs were detected in NOD/ICR offspring, though minimal levels were detected in two of nine NOD/DBA offspring.

**FIG. 6.** Serum leptin levels determined by radioimmunoassay in pregnant surrogate mothers (E18). Serum leptin levels were found to be higher in ICR (■) and DBA/2J (□) mice than in NOD (●) mice (means ± SD). *$P < 0.01$.
present study is the acceleration of the onset of insulitis in nondiabetic NOD/ICR (e and g) and NOD/DBA (d and h) offspring, and their β-cell mass was larger than that in nondiabetic NOD/NOD (b and f) offspring. NOD/NOD-DM (a and e) indicates NOD/NOD with overt diabetes. Scale bars, 400 μm (a–d) and 50 μm (e–h).

FIG. 7. Photomicrographs of insulitis at 40 weeks after birth. Insulitis was still present at 40 weeks of age in nondiabetic NOD/ICR (e and g) and NOD/DBA (d and h) offspring, and their β-cell mass was larger than that in nondiabetic NOD/NOD (b and f) offspring. NOD/NOD-DM (a and e) indicates NOD/NOD with overt diabetes. Scale bars, 400 μm (a–d) and 50 μm (e–h).

It is possible for the acceleration of the development of insulitis and protection from the development of overt diabetes to coexist. Immunization of young NOD mice with an islet autoantigen such as insulin B-chain peptide prevents autoimmune diabetes (24) but accelerates the development of insulitis in NOD mice (N.A., K. Fukushima, M.K., unpublished observations), and the administration of IL-4 prevents autoimmune diabetes but enhances pancreatic insulitis in NOD mice (25). In the present study, it was observed that insulitis was still present at 40 weeks of age in nondiabetic NOD/ICR and NOD/DBA offspring and that their β-cell mass was larger than in nondiabetic NOD/NOD offspring. The insulitis at 40 weeks after birth that is tolerated might be established by a change in maternal environment caused by transplantation, such that checkpoint 2 is halted without the development of overt diabetes. Yu et al. (13) report that the early expression of IAA in NOD mice correlates with the early development of type 1 diabetes, suggesting that the program for developing diabetes in NOD mice is relatively fixed early in life. The maternal environment may play a critical role in this fixed program even in utero.

In the present study, IAA was not detected in NOD/NOD or NOD/ICR offspring showing early signs of insulitis. Therefore, transmission of NOD maternal IAA to offspring during late gestation is unlikely to directly influence the onset of insulitis.

We examined whether the maternal endocrine environment may affect the immune reaction, as previously suggested (rev. in 14), and thus the pathogenesis of insulitis. We investigated serum leptin levels in surrogate mothers since leptin has been reported to be transmitted via mother's milk (26) and the administration of leptin to young (up to 6 weeks of age) but not adult female NOD mice has been found to accelerate the development of insulitis with an increased Th1 response and γ interferon–mRNA expression (27). In a previous study (26), maternal plasma leptin levels were reported to correlate significantly with those in their respective pups during lactation. In the present study, the serum leptin level was higher in pregnant ICR and DBA/2J surrogate mothers than in the corresponding NOD mice. Therefore, differences in the maternal environment, including the concentrations of maternal hormones such as leptin, between pregnant ICR and DBA/2J mice and NOD surrogate mothers might be related to the earlier induction of T-cell–mediated immunity and the development of insulitis in offspring.

In conclusion, the present study has revealed that the maternal environment modulates the immune response leading to the early onset of insulitis and suggests that changes in immune response brought about by maternal factors during the perinatal period might further affect progression to overt diabetes.

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