Islet-Infiltrating Lymphocytes from Prediabetic NOD Mice Rapidly Transfer Diabetes to NOD-*scid/scid* Mice

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In an effort to study the development of diabetes in NOD mice, our laboratory developed a novel adoptive transfer model using NOD-*scid/scid* (NOD-*scid*) mice as recipients of islet-infiltrating lymphocytes from donor prediabetic female NOD mice. We first confirmed previous results that demonstrated that splenocytes of diabetic and prediabetic female NOD mice could transfer diabetes to NOD-*scid* mice. We demonstrated that the kinetics of disease transfer were dependent on the age of transferred lymphocytes and reiterated the kinetics of diabetes in conventional female NOD mice. We then demonstrated that islet-infiltrating lymphocytes from prediabetic female NOD mice could transfer diabetes. In contrast with the age-dependent transfer of diabetes seen using splenocytes, islet-infiltrating lymphocytes obtained from prediabetic female NOD mice aged ≥40 days rapidly transferred diabetes to NOD-*scid* recipients. The time required to transfer insulin-dependent diabetes mellitus (IDDM) using islet-infiltrating lymphocytes from young prediabetic mice (25 ± 9 days) was not statistically different from the time required to transfer IDDM using splenocytes from overtly diabetic mice (32 ± 5 days). Cotransfer of splenocyte cells or $CD4^+$, but not $CD8^+$ spleen cells, from 60- to 80-day-old prediabetic female NOD mice together with either splenocytes from diabetic mice or islet-infiltrating lymphocytes from prediabetic NOD mice delayed the rapid transfer of IDDM, suggesting that $CD4^+$ cells mediated immunoregulation. Use of the NOD-*scid* islet-infiltrating lymphocyte-adoptive transfer model should help elucidate the pathophysiology of the early inflammatory events leading to insulitis and subsequent β-cell destruction. Use of the NOD-*scid* adoptive model in cotransfer experiments should help identify the immunoregulatory cells that delay development of IDDM in conventional NOD mice. *Diabetes* 44:550–554, 1995

Human insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease targeted at the insulin-secreting cells in the pancreatic islets of Langerhans (1). The NOD mouse provides a model to study human IDDM (2). In both NOD mice and humans, lymphocytes begin to infiltrate the islets of Langerhans (insulitis) before expression of overt diabetes. Histological analyses of pancreatic islets from NOD mice show periductal and perivascular accumulations of mononuclear cells by 4 weeks of age. Insulitis begins at 5 weeks and is well established by 6–8 weeks. β-cell destruction and hyperglycemia, however, occur at ~15 weeks of age (3). T-cells are responsible for both insulitis and β-cell destruction in NOD mice; humoral immunity does not appear to play a major role in the effector phase of diabetes (4–7).

Direct evidence for both autoimmunity and T-cell involvement in insulitis and diabetes in NOD mice has been provided by adoptive transfer experiments using both splenocytes and highly purified T-cell populations (8–10,12). These studies have demonstrated that adoptive transfer of diabetes requires both $CD4^+$ and $CD8^+$ T-cell subsets and that lymphocytes from diabetic NOD mice were more efficient than lymphocytes from younger nondiabetic NOD mice (8–10).

Recently, an NOD strain congenic for the severe combined immunodeficiency (*scid*) mutation has been developed to study diabetogenesis in NOD mice (11). The *scid* mutation has rendered these mice immunodeficient by preventing normal rearrangement of functional T-cell receptor and immunoglobulin genes; thus, NOD-*scid/scid* (NOD-*scid*) mice lack functional lymphocytes. Neither insulitis nor diabetes is observed in NOD-*scid* mice. Transfer studies using peripheral T-cells from diabetic NOD.NON-*Thy*-1^a mice, congenic mice expressing the Thy-1.1 allotype (N11F3), established that diabetes could be transferred into NOD-*scid* mice and that donor Thy-1.1^+ T-cells were responsible for insulitis and diabetes in recipient NOD-*scid* mice (12).

In an effort to further develop a transfer model useful to examine the early autoimmune events that initiate diabetes in NOD mice and to identify the immunoregulatory cells that control progression to diabetes, we studied the transfer of islet-infiltrating lymphocytes, as well as splenic lymphocytes, from NOD mice of various ages into NOD-*scid* recipients. Data presented in this study suggest the existence in prediabetic mice of immunoregulatory $CD4^+$ lymphocytes that regulate the severity of insulitis and, ultimately, the development of IDDM.

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FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; IDDM, insulin-dependent diabetes mellitus; PBS, phosphate-buffered saline; *scid*, severe combined immunodeficiency.

RESEARCH DESIGN AND METHODS

NOD mice and NOD-*scid* mice (10th backcross generation) were maintained in a breeding colony in the conventional animal facility in the Department of Laboratory Animal Medicine, Stanford University. In this NOD colony, ~70% of the female mice were diabetic by 6 months of age and <20% of the male mice were diabetic by 6 months of age. Mice from our NOD-*scid* colony have never developed diabetes spontaneously. For certain experiments, female NOD mice were purchased from Taconic Farms (Germantown, NY).

NOD and NOD-*scid* mice were bled weekly to detect hyperglycemia. Plasma glucose levels were determined using the One Touch II meter (Johnson & Johnson, Milpitas, CA). Mice with two consecutive plasma glucose readings (48 h or more apart) of >400 mg/dl were considered diabetic.

Splenocyte preparation. Spleens from four groups of five or six female NOD mice (20, 40, 60, and 80 days of age) from a single litter (or, in two instances, from two litters) were removed and teased apart into single cell suspensions. Lymphocytes were isolated by density-gradient centrifugation (Lympholyte M, Cedarlane, Hornby, Ontario, Canada) as previously described (13). The cells were washed three times with RPMI-1640 (BioWhittaker, Walkersville, MD)/2% fetal calf serum (FCS) and kept on ice until time of transfer.

In those experiments using 100-day-old mice or diabetic mice, spleen cells from two to six mice were pooled before lymphocyte isolation by density-gradient centrifugation.

Preparation of splenocyte subsets. $CD4^+$ or $CD8^+$ subsets were prepared by magnetic separation using the MiniMACS System (Miltenyi Biotec, Auburn, CA). Briefly, splenocytes were incubated with anti-CD4 or anti-CD8 magnetic microbeads (Miltenyi Biotec) for 15 min at 4°C, washed, and collected on a magnetic flow-through column. Purified cells (>95% purity) were then resuspended in medium.

Islet isolation. Each donor NOD pancreas was perfused in situ with 0.625 mg/ml collagenase P (Boehringer Mannheim, Indianapolis, IN) dissolved in Hanks' balanced salt solution (HBSS) medium (HBSS, 1% penicillin-streptomycin [BioWhittaker], 4 mmol/l sodium bicarbonate [J.T. Baker, Phillipsburg, NJ], and 0.22 g/l bovine albumin [Sigma, St. Louis, MO]). Excised NOD donor tissue was then incubated for 25 min at 37°C, washed, passed through a strainer, and placed in a discontinuous gradient of Ficoll (Sigma) at 27, 25, 23, and 11% to isolate the islets from the exocrine pancreatic digest. After centrifugation, the islets were removed from the 23–11% interface and washed in HBSS. Free islets were handpicked under a dissecting microscope to a purity of >97%.

Single islet cell suspensions. Handpicked pancreatic islets (~800–900 islets, isolated from 8–10 similarly aged female NOD donor mice) were washed in HBSS medium, resuspended in $Ca^{2+}Mg^{2+}$-free 0.53 mmol/l EDTA/0.05% trypsin solution (Sigma), and incubated at 37°C for 10 min. After incubation, cold CMRL-1066 (Gibco, Grand Island, NY)/10% FCS was immediately added, the islets were disrupted by successive aspirations using 19- and 21-gauge needles, and the single islet cells were washed three times with HBSS medium. Viability was determined by trypan blue exclusion and on average was >90%.

Transfers. Immediately before transfer, the single islet cells were washed and resuspended in 0.5 ml of 0.9% sterile endotoxin-free saline (Abbott, North Chicago, IL). Cells (1×10^6) were transferred to NOD-*scid* recipients by intraperitoneal injection.

In experiments using splenocytes, the cells were similarly washed, resuspended in 0.5 ml of 0.9% sterile endotoxin-free saline, and immediately transferred by intravenous or intraperitoneal injection.

Cell staining. The relative T-cell frequency in the islet cells was determined by fluorescence-activated cell sorter (FACS) analysis. Cells were stained with fluorescein isothiocyanate–conjugated anti-Thy-1.2 and were analyzed on a modified dual laser FACSCAN (Becton Dickinson, Mountain View, CA).

Statistical analysis. Student's *t* test for small samples was used for comparisons of the means.

RESULTS

Splenocytes from diabetic and prediabetic NOD mice transfer diabetes to NOD-*scid* recipients. To confirm the findings of previous studies that splenocytes from prediabetic female NOD mice could transfer disease to NOD-*scid* mice (12), donor splenocytes were harvested from groups of five or six 20- to 80-day-old female NOD mice and transferred one-to-one to female NOD-*scid* recipients aged 30–60 days.

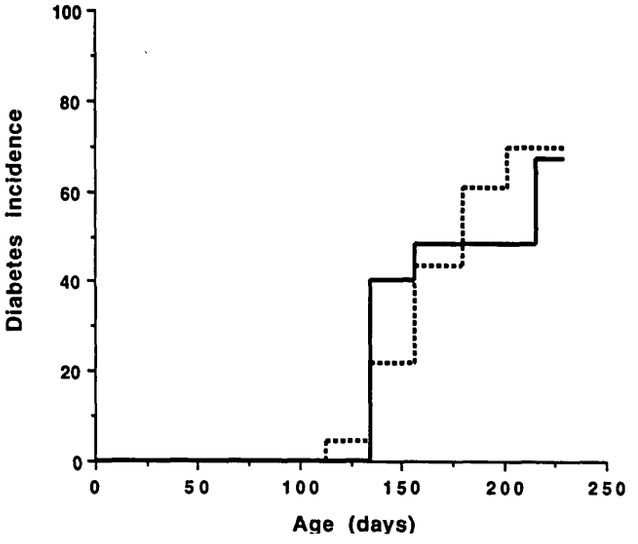

FIG. 1. Incidence of diabetes in female NOD-*scid* splenocyte transfer recipients and female NOD mice. The control group (———) consisted of 15 female NOD mice monitored for hyperglycemia weekly for >200 days. The experimental group (- - - -) consisted of 23 NOD-*scid* recipients (30–60 days old) monitored for hyperglycemia >200 days after transfer of 40–60 × 10^6 lymphocytes from prediabetic female NOD mice (four groups of 20-, 40-, 60-, or 80-day-old mice, generally littermates). The incidence of diabetes (as a percentage) is plotted as a function of time to onset of diabetes. Time to onset of diabetes in the NOD-*scid* recipients (donor age [days] + number of days after adoptive transfer until onset of hyperglycemia in NOD-*scid* recipients) and time to onset of diabetes in the NOD control group (donor age [days]) is indicated along the *x*-axis.

Plasma glucose levels of recipient mice were analyzed weekly for ~200 days, at which time the remaining normoglycemic recipients were killed. Diabetes, as indicated by glucosuria, hyperglycemia, polydipsia, polyuria, and severe weight loss, was successfully transferred using splenocytes from each age-group. Overall, 16 of 23 (70%) NOD-*scid* recipients of prediabetic NOD splenocytes became diabetic. The time to onset of diabetes in the NOD-*scid* recipients (donor age [days] + number of days after adoptive transfer until onset of hyperglycemia) and time to onset of diabetes in a control group of 15 female NOD mice (donor age [days]) paralleled one another (Fig. 1).

In a separate set of experiments, pooled splenocytes from 100-day-old prediabetic female NOD mice, rather than splenocytes from individual mice, were transferred to NOD-*scid* recipients and 6 of 6 became diabetic an average of 47 days later (data not presented).

Transfer experiments using splenocytes ($4.0–6.0 \times 10^6$ per female NOD-*scid* recipient [n = 8]) from overtly diabetic female NOD mice resulted in rapid adoptive transfer of disease to all NOD-*scid* recipients in 32 ± 5 days, which confirmed previous studies (12).

Islet-infiltrating lymphocytes from prediabetic NOD mice ≥40 days old rapidly transfer IDDM to recipient NOD-*scid* mice. After achieving these results, we asked whether islet cells containing infiltrating lymphocytes from NOD mice of various ages would transfer disease.

To obtain single islet cells containing infiltrating lymphocytes, we isolated islet tissue from the pancreases of age-grouped female NOD mice. We initially chose to study donor mice at ages at which lymphocytic infiltration was demonstrable histopathologically (≥40 days old) to ensure transfer of infiltrating lymphocytes (3). Age-grouped islets were isolated from 8–10 40-, 60-, and 80-day-old female NOD donor

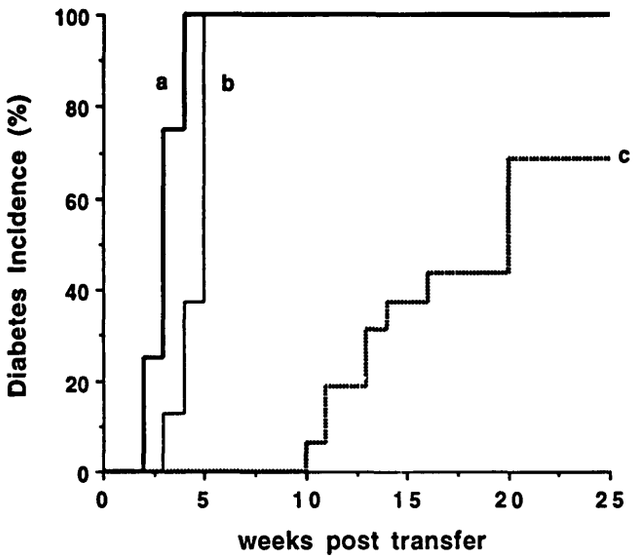

FIG. 2. Prediabetic islet-infiltrating lymphocytes rapidly transfer diabetes into NOD-*scid* recipients. NOD-*scid* female recipients were injected with 10^6 prediabetic NOD (age 40–80 days) single islet cells containing infiltrating lymphocytes (n = 8) (a), 4–6 × 10^6 splenocytes from diabetic NOD mice (n = 8) (b), or 40 × 10^6 splenocytes from prediabetic (age 40–80 days) NOD mice (n = 16) (c). The incidence of diabetes in the recipients is shown plotted against time. The time to onset of diabetes was significantly different in recipients of islet-infiltrating lymphocytes compared with recipients of splenocytes from age-matched donors. Islet-infiltrating lymphocytes from prediabetic mice transferred diabetes as effectively as did splenocytes from diabetic NOD mice.

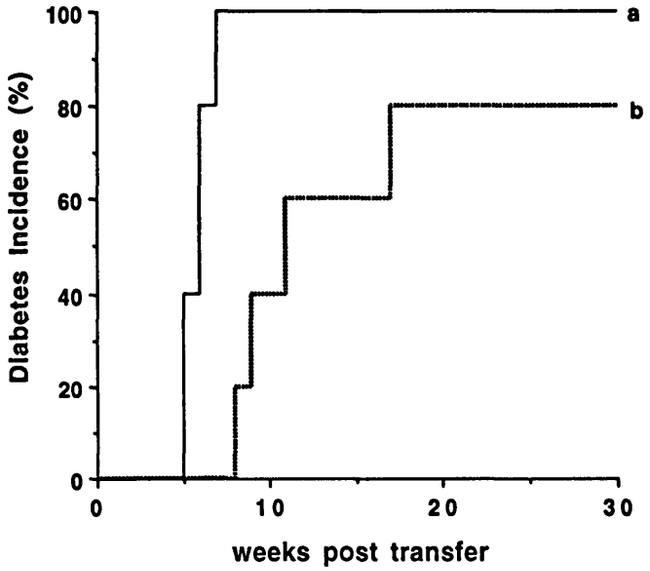

FIG. 3. Splenocytes delay adoptive transfer of IDDM to NOD-*scid* recipients. Two groups of five male NOD-*scid* recipients received either an aliquot of ~6 × 10^6 splenocytes pooled from four diabetic female NOD mice (140–160 days old) (———) (a) or a similar inoculum of donor splenocytes plus ~6 × 10^6 splenocytes pooled from three prediabetic female NOD mice (aged 60–80 days) (- - - -) (b). The incidence of diabetes is shown as a function of time (weeks).

mice, dispersed into a single islet cell suspension, and transferred (~1 × 10^6 pooled cells) to female NOD-*scid* recipients by intraperitoneal injection. By FACS analysis, we determined that islet cells from 60- to 80-day-old female NOD mice contained ≤4% Thy-1.2$^+$ T-cells. The NOD-*scid* recipients of islet cells containing infiltrating lymphocytes from 40- to 80-day-old female NOD mice became diabetic within 25 ± 9 days (Fig. 2). Transfer of islet cells from 20- or 30-day-old NOD mice did not transfer diabetes or destructive insulitis to NOD-*scid* recipients.

Splenocytes from diabetic NOD-*scid* islet cell recipients transfer IDDM. Additional confirmation that infiltrating lymphocytes within the islet cells transferred IDDM into the NOD-*scid* recipients was generated by secondary transfer experiments. Transfer of as few as 2 × 10^6 splenocytes from diabetic, NOD-*scid*, islet cell recipients resulted in IDDM in the secondary naive NOD-*scid* recipients between 24 and 30 days post-transfer (n = 5).

CD4$^+$ spleen cells delay disease transfer. We next asked whether splenocytes from prediabetic NOD mice could delay disease transfer using lymphocytes from overtly diabetic NOD mice in the NOD-*scid* lymphocyte adoptive transfer model. A total of 6 × 10^6 splenocytes from diabetic NOD female mice were transferred into recipient NOD-*scid* female mice with or without cotransfer of 6 × 10^6 spleen cells from 60- to 80-day-old prediabetic female mice. Cotransfer of splenocytes from 60- to 80-day-old prediabetic female NOD mice with lymphocytes from diabetic NOD mice prolonged the time to transfer and decreased the incidence of IDDM when compared with the NOD-*scid* recipients of lymphocytes from diabetic NOD mice in the absence of cotransferred spleen cells from prediabetic NOD mice (Fig. 3). In addition, cotransfer of lymphocytes from 60- to 80-day-old prediabetic NOD mice delayed the onset of diabetes in NOD-*scid* recipients of islet-infiltrating lymphocytes from 60- to 80-day-old prediabetic NOD mice when compared with control recipients of islet-infiltrating lymphocytes alone (data not presented).

To analyze which subset of splenocytes was involved in the delay in onset of diabetes in NOD-*scid* recipients, splenocyte populations were fractionated into CD4$^+$ and CD8$^+$ subsets. As demonstrated by data in Fig. 4, by 7 weeks post-transfer, all of the mice that received splenocytes from recently diabetic NOD females plus phosphate-buffered saline (PBS) or splenocytes from recently diabetic NOD female plus CD8$^+$ cells in cotransfer became diabetic. However,

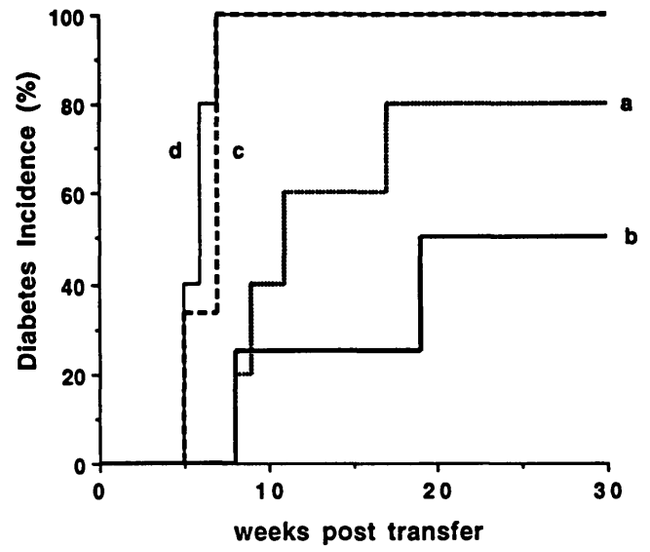

FIG. 4. CD4$^+$, but not CD8$^+$, splenocytes delay adoptive transfer of IDDM to NOD-*scid* recipients. Splenocytes (6 × 10^6) from diabetic NOD mice were transferred into NOD-*scid* recipients along with isolated subsets (CD4$^+$ or CD8$^+$) of splenocytes from young (60- to 80-day-old) prediabetic NOD mice. Cotransferred cell populations were (a) splenocytes (6 × 10^6; n = 5), (b) CD4$^+$ splenocytes (4 × 10^6; n = 4), (c) CD8$^+$ splenocytes (4 × 10^6; n = 3), and (d) PBS only (n = 5).

cotransfer of $CD4^+$ splenocytes or unfractionated splenocytes from prediabetic mice significantly delayed the onset of diabetes and reduced the incidence (Fig. 4).

DISCUSSION

We have developed a cellular adoptive transfer model to examine the pathophysiological events that initiate anti–β-cell autoimmunity in NOD mice. In these studies, we confirmed previous results that splenocytes from prediabetic female NOD mice could transfer IDDM to NOD-*scid* mice (12). The 70% incidence of diabetes in the recipient NOD-*scid* mice was similar to that found in the control group (67%, Fig. 1) as well as that in historical control female mice in our NOD colony (70%). Interestingly, the younger the donor NOD mouse, the longer the time to transfer of IDDM in the NOD-*scid* recipient. The cumulative time to onset of disease (age of the splenocytes at time of transfer plus the length of time required to transfer disease; ~160 days) paralleled the age of onset of disease in the group of control female NOD mice (Fig. 1). These data suggest that the pathophysiological mechanisms responsible for diabetes in NOD mice may be recreated in the NOD-*scid* splenocyte adoptive transfer model.

This age dependence of time to transfer of IDDM was not seen in transfers of islet-infiltrating lymphocytes from similarly aged donor NOD female mice. The times required to transfer disease using islet-infiltrating lymphocytes resident in islet cell suspensions from NOD mice 40–80 days old or splenocytes from overtly diabetic female NOD mice were almost identical (25 ± 9 vs. 32 ± 5 days, respectively). These results suggest that the islets are infiltrated with the necessary effector lymphocytes to mediate β-cell destruction at an early age; however, the development of diabetes is blocked in prediabetic NOD mice. This observation is consistent with previous observations that regulatory cells within the NOD animal might prevent the rapid destruction of β-cells after inflammatory insulitis (14–17). To study this possibility, we performed cotransfer experiments. In these studies, lymphocytes from prediabetic NOD mice delayed the onset of IDDM when cotransferred with lymphocytes from overtly diabetic mice (Fig. 3).

The characteristics of regulatory T-cells that may have an impact on the development of autoimmune diabetes in NOD mice are not known. Experimental data, based on studies that demonstrate that cyclophosphamide can induce early diabetes in young male and female NOD mice (14) and the requisite irradiation before transfer of disease to male NOD mice (8,9), have indirectly suggested that suppressor cells play a role in protection from diabetes. Furthermore, Strom and colleagues (18) demonstrated that transfer of a noncytolytic $CD8^+V\beta11^+$ T-cell clone into prediabetic NOD mice could block diabetogenic autoimmunity in the recipient. In other studies, transfer of $CD4^+$ T-cells from 2-month-old NOD mice prevented the accelerated onset of diabetes in irradiated transfer recipients of splenocytes from diabetic donors (15). Monoclonal anti–major histocompatibility complex class II antibody treatment has been suggested to prevent IDDM through an active immunosuppressive mechanism that depends on the presence of $CD4^+$ T-cells (19). Data presented above on cotransfer of subsets of lymphocytes from prediabetic NOD mice into NOD-*scid* recipients suggested that $CD4^+$, but not $CD8^+$, lymphocytes were able to mediate immunoregulation (Fig. 4). These results suggest that the islets are infiltrated with effector lymphocytes capable of destroying β-cells during the early stages of NOD insulitis, but the capacity of these cells in prediabetic NOD mice to destroy β-cells is blocked by $CD4^+$ immunoregulatory cells. We suggest that the development of IDDM in NOD mice depends on an imbalance between diabetogenic and regulatory lymphocytes. Use of the NOD-*scid*, islet-infiltrating, lymphocyte-adoptive transfer model should help elucidate the pathophysiology of the early inflammatory events leading to insulitis and subsequent β-cell destruction. Use of the NOD-*scid* adoptive model in cotransfer experiments should help identify the immunoregulatory cells that delay development of IDDM in conventional NOD mice.

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