

EXPRESSION OF GENETICALLY DETERMINED DIABETES
AND INSULITIS IN THE NONOBESSE DIABETIC
(NOD) MOUSE AT THE LEVEL OF
BONE MARROW-DERIVED CELLS

Transfer of Diabetes and Insulitis to Nondiabetic (NOD × B10) F₁
Mice with Bone Marrow Cells from NOD Mice

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The nonobese diabetic (NOD)¹ mouse is a model of human type I diabetes mellitus (1-4). The diabetic processes that occur in both species appear to reflect an autoimmune response to the insulin-producing β cells within the islets of the pancreas (5-7). Helper and cytotoxic T lymphocytes are found within the affected islets in the NOD mouse (6, 8). We (9), and Bendelac et al. (10), have recently demonstrated that both the CD4 and CD8 T cell subsets are necessary to transfer diabetes in the NOD mouse. As in man, diabetes in the NOD mouse is controlled by multiple genetic loci, including one that is linked to, or is located within, the MHC (11-13). Although the stimulus for the induction of an autoimmune response in the NOD mouse is still unknown, periductal and perivascular infiltrates and insulitis are observed within the pancreas beginning at 3-5 wk of age (2, 3).

To determine whether the primary genetic defect that causes insulitis and diabetes in the NOD mouse resides in the immune system or, alternatively, in the tissue that is the target of the autoimmune response, we have initiated a series of studies using irradiated (NOD × B10)F₁ mice that have been reconstituted with NOD bone marrow cells. (NOD × B10)F₁ mice do not develop diabetes or display insulitis (12). In addition, since (NOD × B10)F₁ mice are semisyngeneic at the MHC with the transferred NOD bone marrow cells, an efficient immune response can be mounted in the chimeras. In this study, we demonstrate that a majority of irradiated (NOD × B10)F₁ mice that receive NOD bone marrow cells develop insulitis and some become diabetic. These NOD \rightarrow F₁ chimeras not only destroy their own β cells but also reject the β cells present in transplanted pancreata obtained from newborn NOD

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¹ Abbreviation used in this paper: NOD, nonobese diabetic.

and B10 mice. These data indicate that the genetically determined insulinitis and diabetes present in NOD mice are expressed at the level of the hematopoietic stem cells.

Materials and Methods

Animals. An NOD (K^d , $I-A^{NOD}$, D^b) breeding nucleus was kindly provided by Dr. Yoshihiro Tochino (Aburabi Laboratories, Shionogi and Co., Osaka, Japan) and C57B1/10SnJ (B10) (K^b , $I-A^b$, D^b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). (NOD \times B10) F_1 mice were bred in our animal facility in Rahway, NJ. Mice used in this study were housed under specific pathogen-free conditions.

Preparation and Analysis of Chimeras. Bone marrow cells were harvested from 5–7-wk-old female donors. To remove mature T cells, bone marrow cells were incubated at 4°C for 30 min with a mixture of T cell-specific mAbs: anti-Thy-1.2 (HO-13-4) (14) (TIB 99, American Type Culture Collection, Rockville, MD), anti-Lyt-1.2 (C3PO.13) (15) (kindly provided by Dr. Eli Sercarz, University of California at Los Angeles), and anti-Lyt-2 (3.155) (16) (TIB 211, ATCC). The cells were then incubated with absorbed guinea pig complement at 37°C for 40 min. 5–7-wk-old females were irradiated (1,000 rad from a 137 Cesium source, Gammacell 40, Atomic Energy of Canada, Ltd., Ottawa, Ontario) and injected intravenously with $10\text{--}20 \times 10^6$ bone marrow cells. Chimeras are designated as bone marrow donor \rightarrow irradiated recipient. For example, an irradiated F_1 recipient that has received NOD bone marrow is represented as NOD \rightarrow F_1 .

After a period of at least 2 mo, representative chimeras from each group were tested for their extent of chimerism. Spleen cells from chimeras were typed with class I- and class II-specific antibodies: anti- $K^{b,q}$, D^d , $H\text{-}2^r$ (34-4-20S) (17) (Litton Bionetics, Charleston, SC), anti- K^d (2.7.2) (18) (kindly provided by Dr. Terry Potter, Albert Einstein College of Medicine, Bronx, NY), anti- $I\text{-}A^{b,d}$, $H\text{-}2^{p,q}$ (34-5-3S) (17) (Litton Bionetics, Charleston, SC), which is not reactive with the NOD I-A product, and anti- $I\text{-}A^{k,r,f,s,u}$ (10-2.16) (19) (TIB 93, American Type Culture Collection), which is reactive with the NOD I-A product but not the B10 I-A product. The cells were incubated with 34-5-3S, 10-2.16, or 34-4-20S for 30 min, washed, and then incubated with FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) that had been absorbed with a sepharose-conjugated mouse IgM protein, MOPC 104E (Litton Bionetics). Cells incubated with 2.7.2 were washed and then incubated with FITC-conjugated monoclonal anti-rat κ chain (MAR 18.5, Becton Dickinson & Co., Mountain View, CA). Cells were analyzed by flow cytometry on the FACS IV (Becton Dickinson & Co.).

Normal and chimeric mice were monitored for the development of diabetes by testing for urinary glucose with Tes-Tape (Eli Lilly, Indianapolis, IN). Animals were classified as diabetic after producing consistent Tes-Tape values of 1+ or higher. To allow sufficient time for the development of insulinitis and/or diabetes, normal animals were killed at 7 mo old or older, and chimeras were killed no sooner than 7 mo after construction. Some chimeras were allowed to reach the age of 17 mo post-construction.

Neonatal Pancreas Transplantation. Selected NOD \rightarrow F_1 chimeras received transplants of pancreata (20) obtained from NOD and B10 mice that were <24 h old. Each host received three pancreata from newborn NOD mice under one renal capsule and three pancreata from newborn B10 mice under the other renal capsule.

Histology. Pancreata were excised and fixed in buffered 10% formalin and processed for paraffin sectioning. Tissue sections (5 μm) were stained with hematoxylin and eosin and microscopically evaluated for the presence of mononuclear cell infiltration. Histological specimens were interpreted without knowledge of the treatment. Three noncontiguous sections of each pancreas (45–50 μm between sections) were examined. Animals received a positive score for insulinitis if infiltrating mononuclear cells were observed within islets. Frozen sections of pancreata were prepared for immunohistochemical staining of the L3T4 and Lyt-2 T cell subsets. Ascites fluid containing anti-L3T4 antibodies (GK1.5) (21) was prepared as previously described (9) and anti-Lyt-2 antibodies were obtained from Becton Dickinson & Co. Sections were developed using the Vectastain ABC kit specific for rat IgG (Vector Laboratories, Inc., Burlingame, CA).

4–5 wk after transplantation, chimeras that received pancreas grafts were killed, and the grafts were fixed in buffered 10% formalin and embedded in paraffin. Specimens were sectioned (5- μ m thickness) and stained with hematoxylin and eosin to assess lymphocytic infiltration. Pancreatic beta cells within grafted tissue were identified with anti-insulin antibodies in an indirect peroxidase anti-peroxidase method (DAKO PAP Kit, Accurate Chemical, Westbury, NY).

Results

Experimental Protocol. The NOD mouse develops insulinitis by 3 mo of age and nearly 70% of female NOD mice become diabetic between 3 and 7 mo of age (1). In contrast, B10 mice do not display insulinitis or develop diabetes. The expression of insulinitis and diabetes is recessive since (NOD \times B10) F_1 mice do not become diabetic or develop insulinitis (12). To ask whether the failure of (NOD \times B10) F_1 mice to express insulinitis and develop diabetes can be attributed to the immune system or whether the F_1 fails to express appropriate target tissues for the autoimmune response, lethally irradiated F_1 mice were reconstituted with NOD bone marrow. To demonstrate that the experimental manipulations alone did not induce diabetes or insulinitis, F_1 mice were irradiated and reconstituted with bone marrow obtained from B10 or F_1 mice. To confirm that the manipulations also did not prevent the development of diabetes, NOD \rightarrow NOD bone marrow chimeras were constructed.

Extent of Chimerism. 2 mo after construction, two mice from each group of non-syngeneic chimeras, NOD \rightarrow F_1 (three groups constructed) and B10 \rightarrow F_1 (one group constructed), were analyzed for their extent of chimerism. In addition, a majority of the NOD \rightarrow F_1 chimeras were also analyzed when they were killed (>7 mo post-construction). Fluorescence profiles from an MHC typing experiment of a representative NOD \rightarrow F_1 chimera are shown in Fig. 1. Greater than 95% of the spleen

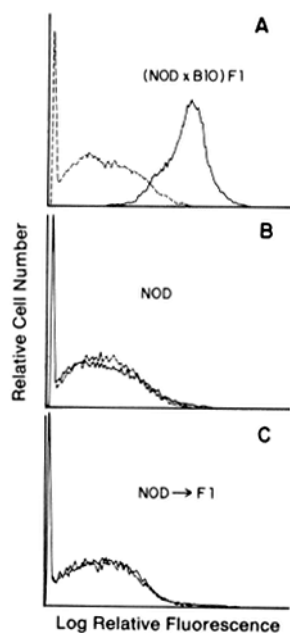


FIGURE 1. Spleen cells from (A) (NOD \times B10) F_1 , (B) NOD, and (C) NOD \rightarrow F_1 mice were tested for the presence of the K^b class I antigen using mAb 34-4-20S and anti-mouse IgG (FITC conjugate) as indicated by the solid lines. The dashed lines represent cells incubated with counterstain alone.

TABLE I
Prevalence of Diabetes and Insulinitis in Normal and Chimeric Mice

Strain	Number with insulinitis/ total observed		Number diabetic/ total observed	
		%		%
NOD	27/27	(100)	72/108	(67)
(NOD × B10)F ₁	0/54	(0)	0/100	(0)
B10	0/11	(0)	0/100	(0)
NOD→NOD	8/8	(100)	8/8	(100)
NOD→F ₁	16/24	(67)	5/24	(21)
B10→F ₁	0/8	(0)	0/15	(0)
F ₁ →F ₁	0/6	(0)	0/6	(0)

All mice represented in this table were females. Mice were evaluated at the onset of diabetes or at >7 mo old or 7 mo after construction. Chimeras became diabetic between 2 and 10 mo after construction. The average age of NOD mice at the onset of diabetes was 4 mo.

cells from all NOD→F₁ and B10→F₁ chimeras tested expressed the class I and class II MHC types of the bone marrow donor.

Expression of Insulinitis and Diabetes in Radiation Bone Marrow Chimeras. Results from chimeras constructed to examine the influence of the experimental manipulations on the development of insulinitis and diabetes validated the use of this methodology to study disease development. All NOD→NOD chimeras ($n=8$) became diabetic 3–7 mo after bone marrow transfer (Table I), and F₁→F₁ and B10→F₁ chimeras did not become diabetic and did not display insulinitis (Table I).

Insulinitis was observed in 67% ($n=24$) of NOD→F₁ chimeras and five of these mice (21%) developed diabetes within 9 mo after construction. From histologic observation, the insulinitis seen in NOD→F₁ chimeras did not appear to be different from the spontaneous insulinitis of NOD mice (Fig. 2 A). In addition, immunohistochemical analysis of pancreatic inflammation demonstrated that islet infiltrates were composed primarily of L3T4⁺ and Lyt-2⁺ lymphocytes (Fig. 2, B and C). These same cell populations predominate in infiltrated NOD mouse islets (6, 8). These data demonstrate that NOD bone marrow cells transfer the ability to mount an autoimmune response to the pancreatic β cells present in normally nondiabetic (NOD × B10)F₁ mice.

Rejection of Normal Islets by NOD→F₁ Chimeras. To demonstrate that normal pancreatic β cells can be the targets of the autoimmune process present in the NOD mouse, pancreata from B10 and NOD newborn donors were transplanted under the renal capsules of three diabetic and four nondiabetic NOD→F₁ chimeras (Table II). 4–5 wk after transplantation, no insulin-containing β cells were observed in three diabetic recipients (numbers 1–3) that had received B10 and NOD pancreas grafts. Lymphocytic infiltration in the grafts ranged from mild to extensive. Both the NOD and B10 β cells were rejected in two nondiabetic recipients (numbers 4 and 5) as evidenced by the lack of insulin-positive cells within the islets found in the grafted pancreata. In a third nondiabetic recipient (number 6), the NOD β cells were rejected whereas insulin-positive cells within islets were observed in the B10 graft. Although β cells were observed in the B10 graft in recipient number 6, a high level

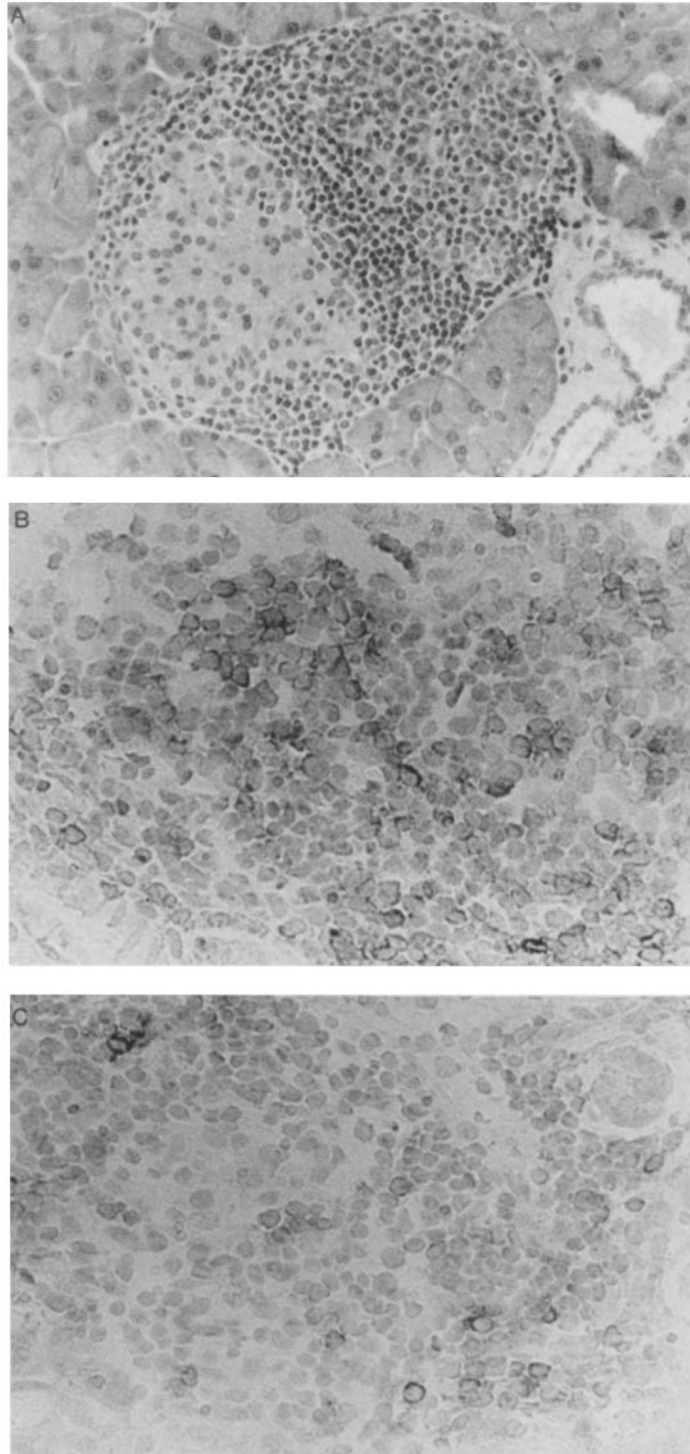


FIGURE 2. Histology of infiltrated islets of NOD \rightarrow F₁ chimeras. (A) Hematoxylin and eosin staining showing insulitis ($\times 100$). Immunoperoxidase staining demonstrating (B) L3T4⁺ and (C) Lyt-2⁺ cells within islet infiltrates ($\times 200$).

TABLE II
*Transplantation of Pancreata from Newborn B10 and NOD Mice to Diabetic
 and Nondiabetic NOD→F₁ Chimeras.*

Recipient status	Recipient pancreas	Histology			
		NOD graft		B10 graft	
		β cells	Lymphocytic infiltrates	β cells	Lymphocytic infiltrates
1. Diabetic	++++	-	+	-	+++
2. Diabetic	++++	-	++	-	++
3. Diabetic	++++	-	+++	-	+
4. Nondiabetic	+++	-	+	-	+++
5. Nondiabetic	+++	-	++	-	+
6. Nondiabetic	+++	-	+++	+	+++
7. Nondiabetic	+++	+	++	+	+

Pancreata from newborn B10 and NOD mice were grafted under the kidney capsules of NOD→F₁ chimeras. Recipients were killed 4-5 wk after transplantation. The host pancreas was examined histologically with (++++) massive insulinitis and/or lack of intact islets consistent with overt diabetes and (+++) the presence of moderate to severe insulinitis. Grafts were examined for the presence (+) or absence (-) of insulin-containing β cells within defined islets. The level of lymphocytic infiltration within the grafts was scored as follows: (+) few infiltrating lymphocytes, (++) moderate infiltration, and (+++) intense infiltration.

of lymphocytic infiltration within the graft (score of +++) suggests that this graft would have been rejected at a later point in time. A fourth nondiabetic recipient (number 7) failed to reject β cells in either graft since insulin-positive islets were observed under both renal capsules, and only mild to moderate lymphocytic infiltration was observed. As technical controls, syngeneic grafts of BALB/c pancreata to BALB/c recipients and NOD and B10 pancreata to normal (NOD × B10)F₁ recipients showed very good islet structure, insulin-positive β cells within the islets, and very few infiltrating lymphocytes (data not shown). In addition, we have previously shown that young NOD mice (5 wk old), which were free of insulinitis at the time of analysis, failed to reject the β cells within pancreas transplants from newborn donors (20). Therefore, these data, showing that NOD→F₁ chimeras can reject pancreatic beta cells from newborn B10 mice, indicate that the autoimmune response in the NOD mouse is not specific for a β cell-associated antigen unique to the NOD since β cells in the pancreata of normal B10 mice are also targets of the autoimmune response.

Discussion

In the current report, we demonstrate that reconstitution of irradiated (NOD × B10)F₁ mice with NOD bone marrow cells causes insulinitis in a majority of the recipients and diabetes in some. The insulinitis in NOD→F₁ chimeras is similar to that observed in the NOD mouse since CD4 and CD8 T cells are observed within the mononuclear cell infiltrates in both cases. In contrast, (NOD × B10)F₁ mice reconstituted with bone marrow cells from F₁ or B10 mice did not display either insulinitis or diabetes. These data strongly suggest that the hematopoietic stem cells of the NOD mouse contribute to the development of insulinitis and diabetes and that this genetically determined disease phenotype is not expressed by the hematopoietic

stem cells of (NOD \times B10) F_1 mice. The F_1 mouse, however, does express appropriate target tissues for the initiation of the autoimmune process, since transfer of hematopoietic stem cells from NOD mice results in insulinitis and diabetes. In addition, we have demonstrated that the effector cells of the autoimmune destruction in the NOD mouse can also recognize the pancreatic β cells from normal B10 mice. Therefore, it is unlikely that an abnormal β cell is a primary cause of diabetes in the NOD mouse. However, it is still possible that although normal islet tissue can be the target of the autoimmune response, tissue present in normal mice may not be able to initiate the autoimmune process. We are currently preparing a congenic mouse strain, B10. H -2^{NOD}, to address this issue. This strain will be histocompatible with the NOD mouse and will express "normal" tissue (in contrast to the (NOD \times B10) F_1 , which could express a tissue-related diabetogenic phenotype inherited from the NOD). If normal tissue is able to initiate the diabetogenic process in the presence of NOD bone marrow cells, then NOD \rightarrow B10. H -2^{NOD} chimeras should develop insulinitis and diabetes.

The rate of onset of diabetes (2–10 mo after bone marrow reconstitution) observed in both NOD \rightarrow NOD and NOD \rightarrow F_1 chimeras is consistent with the observation that normal NOD mice do not become diabetic before 3 mo of age. Both in normal mice and in radiation bone marrow chimeras, the maturation of lymphocytes and the development of an autoimmune response must occur before the onset of diabetes. This relatively slow onset of disease contrasts with our previously reported model for the adoptive transfer of diabetes (9). In this model, diabetes was induced in 4–5-wk-old irradiated NOD mice 2–3 wk after receiving splenic T cells from diabetic donors. We demonstrated that both CD4 and CD8 cells were required to transfer diabetes and that both subsets had to be from "primed" (diabetic) donors.

Our observation that NOD \rightarrow (NOD \times B10) F_1 chimeras can reject newborn B10 pancreas grafts is consistent with the findings of Terada et al. (20) that suggest that the effector cells of the autoimmune process in the NOD mouse are restricted by class I gene products and that the target antigens of the autoimmune response are found on the beta cells of normal mice. In the Terada et al. study, diabetic NOD mice, which were treated with cyclosporin to prevent allograft rejection, received transplants of pancreata from newborn mice with various MHC haplotypes. C57BL/6 (which shares D^b with NOD) and BALB/c (which shares K^d with NOD) grafts were both rejected, whereas CBA (which has a complete mismatch at the MHC with NOD) grafts were accepted. Thus, the rejection of newborn B10 grafts by NOD \rightarrow F_1 chimeras observed in the current study could be mediated by effector cells restricted by the class I product of the D^b locus. The existence of class I-restricted effector cells in the NOD mouse is also supported by the observation that Lyt-2⁺ T cells (usually class I restricted), in addition to L3T4⁺ T cells (usually class II restricted), are required to adoptively transfer diabetes in the NOD mouse (9, 10).

The observation that only 21% of NOD \rightarrow F_1 chimeras became diabetic as opposed to the high incidence (100%) in NOD \rightarrow NOD control chimeras suggests that the full potential of the NOD bone marrow cells is not reached in the F_1 environment. We (12) and others (11, 13) have shown that the genetic control of diabetes in the NOD mouse involves at least three functionally recessive genes, including one linked to the MHC. Thus, the most obvious distinction between F_1 - and NOD-irradiated recipients is that both H -2^{NOD} and H -2^b class I and class II restriction elements are

expressed on all appropriate nonhematopoietic tissues in NOD \rightarrow F₁ chimeras, whereas in NOD \rightarrow NOD chimeras only *H-2^{NOD}* restriction elements are expressed. The presence of non-*H-2^{NOD}* restriction elements on various cell types may contribute to the less severe autoimmune response observed in NOD \rightarrow F₁ chimeras. For example, a lower density of *H-2^{NOD}* class I antigens on β cells may make them less susceptible to destruction by class I-restricted effector cells. Alternatively, in NOD \rightarrow F₁ chimeras, radiation-resistant F₁ APC bearing both *H-2^b* and *H-2^{NOD}* MHC antigens, which are present for varying periods of time after the construction of chimeras (22, 23), may not be as effective in inducing an autoimmune response as APC that express only *H-2^{NOD}* MHC antigens. There is also expression of MHC antigens from both haplotypes on thymic epithelial cells of NOD \rightarrow F₁ chimeras. Thus, thymic learning would be altered and may cause the autoimmune response to be less pronounced (less help, more suppression) in NOD \rightarrow F₁ chimeras as compared with NOD \rightarrow NOD chimeras. Studies with appropriate MHC congenic strains will be needed to address these various alternatives.

In this study, we have demonstrated that the hematopoietic stem cells of the NOD mouse contribute to the development of diabetes. The studies of Ikehara et al. (24) also support this hypothesis since they demonstrated that NOD mice which were lethally irradiated and reconstituted with allogeneic bone marrow cells did not develop insulinitis or diabetes. Further experiments to define how the diabetic genotype of the NOD mouse is expressed via the immune system are clearly required. The development of congenic strains separating the unlinked diabetogenic loci in the NOD mouse will facilitate the discovery of the underlying mechanisms responsible for the development of diabetes.

Summary

The development of autoimmune diabetes in the nonobese diabetic (NOD) mouse is controlled by at least three recessive loci, including one linked to the MHC. To determine whether any of these genetic loci exert their effects via the immune system, radiation bone marrow chimeras were constructed in which (NOD \times B10)F₁-irradiated recipients were reconstituted with NOD bone marrow cells. Unmanipulated (NOD \times B10)F₁ mice, or irradiated F₁ mice reconstituted with F₁ or B10 bone marrow, did not display insulinitis or diabetes. In contrast, insulinitis was observed in a majority of the NOD \rightarrow F₁ chimeras and diabetes developed in 21% of the mice. These data demonstrate that expression of the diabetic phenotype in the NOD mouse is dependent on NOD-derived hematopoietic stem cells. Diabetogenic genes in the NOD mouse do not appear to function at the level of the insulin-producing β cells since NOD \rightarrow F₁ chimeras not only developed insulinitis and diabetes but also rejected β cells within pancreas transplants from newborn B10 mice. These data suggest that the β cells of the NOD mouse do not express a unique antigenic determinant that is the target of the autoimmune response.

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Note in added proof: Similar findings have been recently reported by Serreze et al. (25). They demonstrated the transfer of diabetes to irradiated (NOD × NON)F₁ hybrids with bone marrow cells from NOD mice.

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