Transfer of Autoimmune Diabetes Mellitus With Splenocytes From Nonobese Diabetic (NOD) Mice

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SUMMARY

The nonobese diabetic (NOD) mouse, a model of human type I diabetes, develops insulitis beginning at 4–6 wk of age. By 30 wk of age, 72% of females and 39% of males develop spontaneous diabetes, apparently because of an overwhelming autoimmune response to the insulin-producing β-cells within the islets. To identify the immune mechanism responsible for destruction of β-cells in the NOD mouse, we developed an adoptive transfer protocol that induces diabetes in NOD mice at an age when spontaneous diabetes is rarely observed.

Splenocytes from overtly diabetic NOD mice were unable to transfer diabetes to very young (≤6 wk) irradiated NOD mice but effectively transferred diabetes to irradiated NOD mice >6 wk of age. In such transfers, overt diabetes was induced within 12–22 days in >95% (79/82) of the recipients. Thus, transfer of splenocytes to young mice induces them to become diabetic at a higher frequency and at a younger age than their untreated littermates. Equally successful transfers with as few as 5 × 10^6 spleen cells have been performed in male and female NOD mice, even though males display a lower spontaneous incidence of diabetes than females. Splenocytes obtained from diabetic mice maintained on insulin for up to 2 mo also transferred diabetes.

Because NOD mice display increasing levels of insulitis with age, spleen cells obtained from nondiabetic NOD mice of different ages were tested for their ability to transfer diabetes. Spleen cells obtained from 7-wk-old nondiabetic donors were unable to transfer disease, suggesting that high numbers of effector cells are not yet present in the spleens of young NOD mice. In contrast, spleen cells obtained from most nondiabetic NOD mice >15 wk of age were able to transfer diabetes, indicating that the donor need not be overtly diabetic at the time of transfer.

Our study provides direct demonstration of the immunological nature of the diabetic disease process in the NOD mouse and provides a method to determine which cells are the effectors of β-cell destruction.

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The nonobese diabetic (NOD) strain of mouse was developed in Japan from a female mouse displaying severe glycosuria. After brother-sister mating for over 20 generations, an inbred strain was established in 1980.[1,2] The incidence of spontaneous diabetes is influenced by sex; by 30 wk of age, ~80% of females and <20% of males become overtly diabetic, characterized by polydipsia, polyuria, hyperglycemia, glycosuria, and in some mice, ketonuria.[2] If not treated with insulin, all diabetic NOD mice display severe weight loss and die within several weeks of displaying initial symptoms of overt diabetes. A regulatory role for sex hormones has been suggested by the observations that castration of male NOD mice increases the incidence of diabetes, whereas castration of females lowers their incidence of disease.[3]

Despite the failure of all NOD mice to become overtly diabetic, >95% of male and female mice display a mononuclear cellular infiltration within their pancreatic islets by 30 wk of age.[2,3] At 3 wk of age, no insulitis is observed; however, by 6 wk, initial insulitis is seen in >50% of NOD mice of both sexes.[2] This initial insulitis is periductal and perivascular and invasion of the islets by lymphocytes is minimal.[4] At ~8 wk of age, lymphocytes begin to invade the islets. The amount of intraislet insulitis increases with age until the islets are massively infiltrated. The observed immune response is specific for the insulin-producing β-cells within the islet and results in the destruction of these cells.[4] Immunohistochemical studies of the pancreas at 22 wk of age reveal that most of the infiltrating cells are IgM^+ B cells; however, Ly-1^+ T cells and Lyt-2^+ T cells are also present in the intraislet infiltrates.[5]

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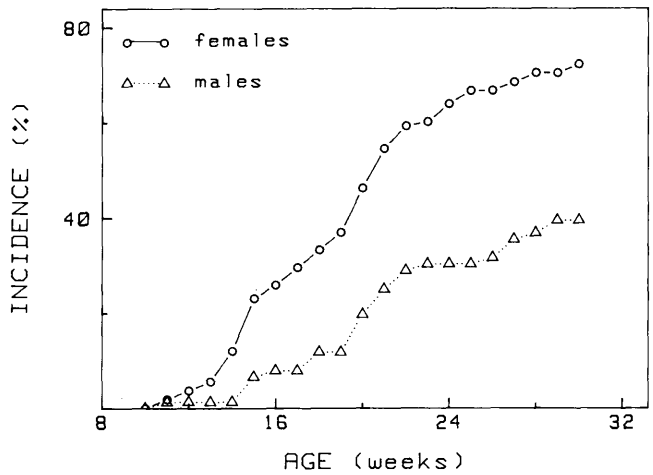

FIG. 1. Frequency of spontaneous diabetes in NOD mice. Female (*N* = 108) and male (*N* = 76) NOD mice were monitored for development of spontaneous diabetes until they were 30 wk of age. Age refers to age of mice at day of glycosuria onset.

The mechanism of islet cell destruction in the NOD mouse has not been elucidated; however, a cellular effector is suggested by the studies of Maruyama et al.,[6] who reported the existence of islet-specific cytotoxic cells in prediabetic NOD mice. The occurrence of antilymphocyte antibodies and anti-islet cell surface antibodies (ICSA) in some NOD mice supports the hypothesis that antibodies may be involved in β-cell destruction.[5] Recently, a naturally occurring monoclonal ICSA was derived from an NOD mouse.[7] Finally, a role for suppressor T cells in the regulation of overt diabetes in the NOD mouse has been suggested by studies in which treatment with cyclophosphamide induced overt diabetes in both male and female mice.[8]

Thus, the NOD mouse appears to be a model for human type I diabetes.[9] In humans, anti-islet cell antibodies are found in many recently diagnosed diabetics, and their presence can help predict future insulin dependency in healthy relatives of type I diabetics.[10,11] However, T cells rather than islet cell–specific antibodies may mediate islet cell destruction in human type I diabetes. The finding that cyclosporine may stop or retard the islet cell destruction leading to diabetes supports this hypothesis.[12] In the Wistar BB rat, the only other animal model of spontaneous type I diabetes, concanavalin A (ConA)–activated T-cell blasts derived from diabetic rats were shown to transfer disease to young, nondiabetic BB rats.[13] Disease in the BB rat is also dramatically reduced by treatment with cyclosporine.[14]

Therefore, if the insulitis and overt diabetes observed in the NOD mouse are immune based, the disease should be transferrable with lymphocytes or serum derived from diabetic mice. In the current study, we found that splenocytes from diabetic NOD mice can induce diabetes in irradiated NOD recipients >6 wk of age.

MATERIALS AND METHODS

Animals. An NOD breeding nucleus was kindly provided by Dr. Yoshihiro Tochino (Aburabi Laboratories, Shionogi, Osaka, Japan). Mice used in this study were bred under specific pathogen-free conditions and did not display antibody titres to Sendai virus or mouse hepatitis virus. NOD mice were tested weekly for urinary glucose with Tes-Tape (Lilly, Indianapolis, IN). Animals showing Tes-Tape values of 1+ or higher were classified as overtly diabetic. In diabetic mice treated with insulin, 2 U (Lente 40, Lilly) were injected subcutaneously each afternoon. Diabetic animals have been maintained >3 mo with this protocol.

TABLE 1
Spleen cells from diabetic female and male NOD mice transfer diabetes to irradiated NOD mice

Experiment no.	Sex*	Age of recipients (wk)	Cells transferred	Diabetic recipients	
				+ /total	Day of onset
1	F	9–15	50×10^6	3/3	16 ± 2.3
2	F	8–10	0	0/5	
	F	8–10	43×10^6	4/4	17 ± 0
3	M	9–10	0	0/5	
	M	10	43×10^6	4/4	12 ± 3.7
4	F	9	40×10^6	4/4	13 ± 0.5
	F	9	20×10^6	4/4	12 ± 0
5	F	8	20×10^6	4/4	16 ± 1.5
6	F	7–9	20×10^6	4/4	15 ± 1.0
	F	7–9	10×10^6	3/3	19 ± 2.0
7	F	11	10×10^6	4/5	17 ± 0.8
	F	7–10	5×10^6	4/4	18 ± 1.4
	F	7–10	2×10^6	1/4	20
8	M	8–10	10×10^6	5/5	16 ± 0.5
	M	8–10	5×10^6	4/4	17 ± 0.8
	M	8–10	2×10^6	2/4	21 ± 0

In each experiment (except no. 7), spleen cells pooled from 2 or more recently diagnosed (within 1 wk) diabetic male or female mice were transferred i.v. to irradiated (775 R) sex-matched NOD mice. In experiment 7, spleen cells from insulin-treated diabetic mice were transferred to irradiated sex-matched recipients. Recipients were considered to be overtly diabetic when urinary glucose was 1+ with Tes-Tape. Day of onset of overt diabetes is represented by the mean ± SE.
*Same sex in both donor and recipient.

TABLE 2
Transfer of diabetes is dependent on dose of irradiation and age of recipient

Dose of irradiation (R)	Age of recipients (wk)	Cells transferred	Diabetic recipients	
			+/total	Day of onset
Experiment 1				
0	7–11	15×10^6	0/4	
350	7–11	0	0/3	
350	7–11	15×10^6	0/7	
600	7–11	0	0/8	
600	7–11	15×10^6	7/8	18 ± 1.2
600	4	15×10^6	0/4	
Experiment 2				
775	9	16×10^6	3/3	19 ± 0
775	7	16×10^6	4/4	17 ± 1.0
775	6	16×10^6	1/4	15

In experiment 1, spleen cells were obtained from spontaneously diabetic male NOD mice and male recipients were used. In experiment 2, female NOD donor and recipient mice were used.

Spleen cell transfer and assessment of diabetes. Recipients were irradiated (775 R from a ^{137}cesium source, Gammacell 40, Atomic Energy of Canada, Ottawa, Ontario), injected intravenously within 2 h of irradiation with donor splenocytes suspended in 0.2 ml of phosphate-buffered saline (PBS), and tested daily with Tes-Tape for the development of glycosuria.

If recipients had not become diabetic within 28 days after transfer, they were scored negative but were still tested weekly for the development of glycosuria. However, no consistent pattern of late-onset glycosuria was apparent after cell transfer.

RESULTS

Splenocytes from diabetic NOD mice transfer diabetes to nondiabetic, irradiated NOD recipients. To characterize the effectors of β-cell destruction in the NOD mouse strain, young, nondiabetic NOD mice were used as recipients of cells from diabetic donors. Figure 1 summarizes the age of onset and the spontaneous incidence of diabetes for male and female mice in our NOD colony. At 30 wk of age, the incidence of overt diabetes is 72 and 39% for female and male NOD mice, respectively. Because a 10% incidence of spontaneous diabetes is reached at 14 wk for female NOD mice and 18 wk for male NOD mice, most adoptive transfer experiments used recipient mice that were <11 wk of age.

Initial attempts to transfer diabetes to young nondiabetic NOD mice with splenocytes derived from diabetic NOD mice failed. However, if recipient mice were irradiated with 775 R, diabetes developed 12–22 days after the transfer (Table 1). Irradiation alone did not induce diabetes in young NOD mice (Table 1, experiments 2 and 3). As seen in spontaneously diabetic NOD mice, adoptive transfer recipients that became overtly diabetic displayed glycosuria, hyperglycemia, polydipsia, polyuria, and severe weight loss. In preliminary experiments, assessment of diabetes by glycosuria was compared with assessment by blood glucose levels in nonfasting adoptive transfer recipients. A total of 20 glycosuria-positive and 40 glycosuria-negative recipients were analyzed. All glycosuria-negative recipients had morning nonfasting blood glucose levels <134 mg/dl with a mean of 98 ± 3 (SE). All glycosuria-positive mice had blood glucose levels >320 mg/dl with a mean of 436 ± 14. Histological examination of 14 mice that became diabetic after transfer of cells from diabetic mice displayed massive intraislet insulitis (70–100% infiltration in all islets) and little intact islet material remained. Examination of the pancreata of 7 age-matched control mice receiving irradiation alone showed that a majority of the islets were 30–70% infiltrated. These histological findings are consistent with those obtained from spontaneously diabetic mice and unmanipulated age-matched control mice, respectively. Because it is difficult to delineate between the levels of intraislet-insulitis seen in overtly diabetic and nondiabetic mice, we did not use this relatively subjective criterion.

Transfer of diabetes was not limited to female donors and recipients because splenocytes from diabetic male mice consistently induced diabetes in irradiated NOD males (see Tables 1, 2, and 4). This is noteworthy because male NOD mice do not develop spontaneous diabetes as frequently as females. In a series of cell titration experiments, we found that as few as 5×10^6 cells obtained from male or female diabetic mice could transfer diabetes to irradiated recipients (Table 1, experiments 4, 6, 7, and 8).

In a titration of the irradiation dose, it was found that at least 600 R was required to prepare the recipient for successful transfer of diabetes. Mice receiving either no irradiation or 350 R did not become diabetic within 28 days of splenocyte transfer (Table 2, experiment 1). In these experiments, the age of the irradiated recipient also appeared to be critical because diabetes could not be transferred to mice that were 4 wk of age (Table 2, experiment 1 and unpublished observations). To identify the age at which NOD mice first become competent recipients, spleen cells pooled from three diabetic mice were transferred to 6-, 7-, and 9-wk-old irradiated NOD recipients (Table 2, experiment 2). All 7- and 9-wk-old recipients developed overt diabetes within 19 days; however, only 1 of 4 6-wk-old recipients became diabetic.

After destruction of the insulin-producing cells in the islet, NOD mice can be maintained on daily injections of insulin. NOD mice that have been insulin dependent for 2 mo should lack β-cells within their islets and presumably fail to express the antigenic stimulus for the autoimmune response. Spleen cells taken from mice maintained on daily insulin injections for 2 mo were found to transfer diabetes to irradiated recipients, suggesting that long-lived memory cells exist in insulin-dependent NOD mice (Table 3). The ability to transfer diabetes with spleen cells from long-term diabetic mice is also confirmed in the experiments represented in Tables 1 (experiment 7) and 4.

Transfer of spleen cells from young and old nondiabetic NOD mice. Older NOD mice not exhibiting glycosuria display massive infiltration of their islets, indicating that a vigorous inflammatory response is in progress.[4] The processes that prevent the final destruction of such infiltrated islets in NOD mice are unknown, but a recent report demonstrating induction of diabetes after treatment with cyclophosphamide suggests that suppressor cells may mediate the prevention of diabetes in some NOD mice.[8] In contrast, the pancreata of NOD mice <8 wk of age display low to moderate levels of insulitis, most of which is perivascular or periductal, not intraislet.[4] These previous histological observations on the pancreas of the NOD mouse have been confirmed in our own laboratories (unpublished observations).

TABLE 3
Spleen cells from long-term insulin-treated diabetic NOD mice transfer diabetes

Spleen cell source	Cells transferred	Diabetic recipients	
		+ /total	Day of onset
Recent-onset NOD*	20×10^6	4/4	18 ± 2.3
Insulin-treated NOD†	20×10^6	5/5	14 ± 0

*Recent-onset NOD mice (pool of 2) were not treated with insulin. Spleen cells were transferred to irradiated (775 R) 8-wk-old female recipients.
†Insulin-treated NOD represents a pool of 2 diabetic female NOD mice that had been treated with 2 U insulin daily for 2 mo.

Spleen cells obtained from nondiabetic mice of various ages were tested for their ability to induce diabetes in irradiated young NOD recipients (Table 4). Spleen cells obtained from 7-wk-old nondiabetic mice were unable to transfer diabetes to irradiated recipients, suggesting that an insufficient number of effector cells are present in the spleen at this age (experiments 2–4). Variable results were obtained when spleen cells from nondiabetic NOD mice ≥16 wk of age were tested for their ability to transfer diabetes. In experiment 1, spleen cells pooled from two 22-wk-old nondiabetic NOD mice transferred diabetes as efficiently as their diabetic littermates. However, spleen cells from a 25-wk-old donor failed to transfer diabetes (experiment 4). In experiment 5, spleen cells from six mice ranging from 16 to 28 wk of age were individually tested for their ability to transfer diabetes. All donors had normal blood sugar levels and displayed a high degree of intraislet insulitis at the time of transfer. However, only four of six donors transferred diabetes. Thus, spleen cells from some nondiabetic NOD mice can transfer the disease; however, massive insulitis alone is not sufficient to predict a successful transfer.

DISCUSSION

In our study we demonstrated that spleen cells obtained from diabetic NOD mice can transfer diabetes to irradiated nondiabetic NOD mice that are at least 7 wk of age. Greater than 95% (79/82) of the recipients became diabetic within 3 wk of transfer. This high rate of successful transfer occurred consistently in both male and female donor-recipient combinations with as few as 5×10^6 transferred spleen cells. Thus, young NOD mice were induced to become diabetic at a higher frequency and at a younger age as a result of the transfer of spleen cells from diabetic donors.

Transfer of spleen cells from 7-wk-old nondiabetic mice failed to induce diabetes in irradiated recipients. At this age, donors display only low levels of insulitis and little intraislet infiltration is observed.[4] Thus, it would not be expected that 7-wk-old NOD mice possess large numbers of effector cells capable of transferring diabetes. Nondiabetic NOD mice >15 wk of age possess high levels of intraislet insulitis, but the ability of their spleen cells to transfer diabetes is variable. In one experiment (Table 4, experiment 5), when six older nondiabetic mice were tested individually for their ability to transfer diabetes, spleen cells obtained from only four of the six donors induced diabetes, even though all the donor mice displayed massive intraislet insulitis. Thus, spleen cells from mice that do not present signs of overt diabetes (hyperglycemia, glycosuria) but display high levels of insulitis can transfer diabetes in some cases. However, the presence of severe insulitis alone does not guarantee a successful trans-

TABLE 4
Transfer of diabetes with spleen cells from nondiabetic NOD mice

Donor spleen cell source	Age of donors (wk)	Cells transferred	Diabetic recipients	
			+ /total	Day of onset
Experiment 1				
Nondiabetic	22	20×10^6	3/3	21 ± 1.4
Diabetic	22	20×10^6	3/3	20 ± 1.2
Experiment 2				
Nondiabetic	7	11×10^6	0/4	
Diabetic	>20	11×10^6	4/4	15 ± 1.2
Experiment 3				
Nondiabetic	7	10×10^6	0/4	
Diabetic	>20	10×10^6	4/5	17 ± 0.8
Experiment 4				
Nondiabetic	7	10×10^6	0/4	
Nondiabetic	25	10×10^6	0/4	
Diabetic	>20	10×10^6	5/5	16 ± 0.5
Experiment 5				
Nondiabetic	28	20×10^6	2/3	18 ± 0.5
Nondiabetic	28	20×10^6	0/3	
Nondiabetic	28	20×10^6	3/3	20 ± 3.0
Nondiabetic	28	20×10^6	0/3	
Nondiabetic	18	20×10^6	2/3	17 ± 0
Nondiabetic	16	20×10^6	2/3	18 ± 0
Diabetic	>20	20×10^6	2/3	17 ± 1.0

In experiment 1, spleen cells from 2 male diabetic NOD mice and spleen cells obtained from 2 male nondiabetic littermates were prepared and transferred into irradiated (775 R) male recipients (9–11 wk old). In experiment 2, spleen cells pooled from 2 male diabetic mice treated with insulin and spleen cells from a male nondiabetic mouse were transferred to irradiated male recipients (7–10 wk old). In experiment 3, spleen cells from insulin-treated female diabetic mice (pool of 2) and a female nondiabetic mouse were transferred to irradiated female recipients (7–11 wk old). In experiments 4 and 5, spleen cells from individual male mice that were either nondiabetic or diabetic were transferred into irradiated male recipients (8–10 wk old).

fer. This finding suggests that in at least some older nondiabetic NOD mice, either an insufficient number of effectors are present in the spleen or suppressor cells can interrupt effector cell function.

Although this transfer protocol only accelerates the disease process in the NOD mouse, it will be useful in elucidating the effector cell responsible for β-cell destruction. Purified cell populations isolated from the spleen are currently being tested for their ability to transfer diabetes. We are also breeding a nondiabetic strain that possesses the NOD major histocompatibility complex, because it is important to determine whether the autoimmune disease can be transferred to a mouse strain that normally does not display overt diabetes. Preliminary attempts to transfer spleen cells from diabetic NOD mice to irradiated (C57BL/10 × NOD)F1 mice failed due to complications arising from graft-versus-host (GVH) disease (unpublished observations). It is possible that in vitro stimulation of NOD splenocytes with ConA, a procedure developed in the BB rat model,[13] may eliminate the GVH reaction.

The necessity to irradiate the adoptive transfer host with at least 600 R may simply provide "room" for the transferred splenocytes in the host or, alternatively, might be explained by the presence of irradiation-sensitive suppressor T cells in the recipient. Several adoptive transfer protocols require that the recipient be immunosuppressed to successfully transfer immune reactivity.[15-17] The existence of suppressor T cells that modulate the diabetic disease process in NOD mice has been suggested by studies with cyclophosphamide.[8] This agent, which is thought to act primarily by eliminating suppressor T cells, induces an increased number of male and female NOD mice to become diabetic. Thus, suppressor cells may protect the islets against destructive effector cells. The failure of suppression would result in complete β-cell destruction and overt diabetes. The observation that a majority of male NOD mice, as well as a small number of females, never become diabetic is consistent with a role for suppressor cells. Evidence for suppressor cell activity has also been demonstrated in the diabetes-prone BB rat, in which T cells transferred from diabetes-resistant W-line rats were able to block the development of overt diabetes in BB rats.[18]

Two hypotheses could explain the finding that NOD mice ≤6 wk of age do not consistently become diabetic after transfer of splenocytes from diabetic mice. It is possible that the NOD β-cell may not express critical antigenic determinants until 4–6 wk after birth. This corresponds to the time at which initial insulitis is observed.[2,4] Thus in very young adoptive transfer hosts, the β-cells either lack the target structure for which the transferred splenocytes are specific or fail to shed the target antigen in sufficient quantity to attract transferred effector cells. Alternatively, the influence of age on the transfer of diabetes could be explained by a maturational step in the recipient's immune or hormonal system that is necessary before the successful transfer of diabetes can be accomplished. Such a proposed step would be completed by 7 wk of age in NOD mice.

The ability of splenocytes from long-term insulin-treated NOD mice to transfer diabetes indicates that memory effector cells are present at least several months after the destruction of β-cells. A similar phenomenon has been observed in human pancreas transplantation. A long-term insulin-dependent patient receiving a pancreas transplant from an identical twin will still reject the islet tissue even though the original antigenic stimulus has been absent for years.[19]

In conclusion, the described protocol for the transfer of autoimmune diabetes offers the opportunity to study the immunological basis for the destruction of islets in the NOD mouse model of diabetes. In addition, this relatively simple and reproducible transfer of the autoimmune diabetic disease process offers an excellent model to test immunoregulatory compounds that might be useful in modifying the disease process.

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