

SYNGENEIC TRANSFER OF AUTOIMMUNE DIABETES FROM DIABETIC NOD MICE TO HEALTHY NEONATES

Requirement for Both L3T4⁺ and Lyt-2⁺ T Cells

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The nonobese diabetic (NOD) mouse was established as an inbred strain in 1980 and proposed as a model of type I diabetes mellitus (1). By 6–8 wk of age, mononuclear cells start infiltrating the periphery of pancreatic Langerhans' islets of both males and females. Progressive invasion inside the islets occurs later and is correlated with selective destruction of insulin-producing β cells and with the onset of clinically overt diabetes. Diabetes is first observed at 12 wk of age and strongly predominates in females. By 30 wk of age, ~70% of females have become diabetic, while <20% of males develop overt disease (2). Several lines of evidence suggest that diabetes in the NOD mouse is an autoimmune disease mediated by T cells: First, Thy-1,2⁺ cells predominate in the cellular islet infiltration (3); second, neonatal thymectomy prevents the disease (4); third, NOD *nu/nu* mice do not develop diabetes (5).

Further identification of immune cells involved in the destruction of insulin-producing cells has been hindered by lack of suitable *in vivo* transfer models. The NOD mouse has a particular MHC haplotype due to unique I-A specificity (6) that prevents the inoculation of NOD lymphoid cells into MHC-compatible strains. Attempts to derive lines of nondiabetic mice from the original NOD nucleus have also been unsuccessful. Recently, it has been shown (7) that the transfer of spleen cells from diabetic mice into diabetes-prone NOD adults greatly promoted the onset of overt diabetes, provided that the recipients had been sublethally irradiated. In addition, adoptive transfer required recipients older than 6–8 wk who, presumably, had already begun to self-damage their pancreatic islets as inferred from histological studies (2). This latter condition limits the validity of the model to account for the whole history of β cell destruction, particularly in its initial stages.

In this study we show that diabetes can be adoptively transferred to NOD neonates by spleen cells from diabetic NOD donors. Overt diabetes, which is correlated with >90% of β cell destruction (8, 9) occurred as early as 21 d of age, at a time when pancreases of noninjected mice were still free of histological changes. The susceptibility of the recipients to the transfer was limited in time and declined after 3 wk of age. We also show that the neonatal model of transfer

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provides a sensitive *in vivo* assay to estimate the autoreactive potential of lymphocytes from prediabetic NOD mice. Finally, we demonstrate that diabetes can be transferred by purified T cells and that both L3T4⁺ and Lyt-2⁺ T cells are necessary to mediate the destruction of insulin-producing cells.

Materials and Methods

Mice. NOD mice were bred in our own facilities under specific pathogen-free conditions. The spontaneous incidence of diabetes in our colony reaches 40% by 25 wk in females, whereas overt diabetes remains rare in males. Histological examination shows that up to 92% of males and 100% of nondiabetic females present destructive insulinitis by 25 wk of age, although histological alterations are usually less severe in males.

Cells. Single cell suspensions were aseptically prepared in HBSS. 4–15 spleens were pooled for each experiment. Diabetic donors were female mice used 1–3 wk after the onset of glycosuria.

Cell Fractionation. T cells were purified by filtration through nylon wool columns and subsequent panning for 45 min at 4°C over Petri dishes coated with rabbit anti-mouse Igs (Miles Scientific Div., Naperville, IL) to remove residual contaminating B cells. Depletion of Thy-1,2⁺ cells, L3T4⁺ cells, or Lyt-2⁺ cells was accomplished by incubating cells at a density of 2×10^7 /ml with relevant mAbs (see below) diluted in HBSS for 45 min at 4°C. The cells were then pelleted, resuspended at a density of 2×10^7 /ml in rabbit complement diluted 1:30, and incubated for 30 min at 37°C. Treated cells were washed three times before injection into experimental animals. Rat IgM mAbs used for cell depletion were culture supernatants diluted 1:2 from clone 4.221 for Thy-1,2⁺ cells (10), clone 3.155 for Lyt-2⁺ cells (11), and clone LICR-LAU-RL172.4 for L3T4⁺ cells (12) (with kind permission from Dr. MacDonald, Ludwig Institute for Cancer Research, Lausanne, Switzerland). For Lyt-2⁺ cell depletion, YTS 169.4, a rat IgG2b antibody with specificity against Lyt-2⁺ cells (Biosys, Compiègne, France), was added as an ascites fluid diluted 1:100 together with the supernatant of clone 3.155.

Monitoring of Purified Subpopulations. Contamination of purified subpopulations was monitored by membrane fluorescence analysis, using fluorescein-conjugated Fab fragments of sheep anti-mouse Igs (Biosys) for membrane Ig⁺ cells, and fluorescein-conjugated purified ascites from clone 30-H12 for Thy-1,2⁺ cells (13), 53-6.7 for Lyt-2⁺ cells (13), and GK 1.5 for L3T4⁺ cells (14). Since 30-H12 and 53-6.7 compete, respectively, with 4.221 and 3.155 for immunofluorescence staining, an additional control was included. A fluorescein-conjugated mouse anti- κ mAb specific for rat light chain (Biosys) was used to detect cells that might have escaped lysis by complement despite rat IgM- κ 4.221 or 3.155 mAbs fixation. In all depletion experiments, <1% residual cells (among $3\text{--}4 \times 10^2$ counted cells) were stained with either the corresponding antibody or the anti-rat κ light chain antibody. T cells purified after filtration through nylon wool columns and panning over Petri dishes coated with rabbit anti-mouse Igs were composed of >90% Thy-1,2⁺ and <1% membrane Ig-bearing cells.

Neonatal Injection Protocol. Within 24 h after birth, neonates subjected to hypothermic anesthesia (4 min at -20°C) were injected into the periorbital superficial vein with 0.05 ml cell suspension at the appropriate concentration under microscopic control. For experiments designed to compare the effects of selective cell depletions or of various cell numbers, representative treatments and controls were equally distributed within each litter. Control litters were injected with spleen cells from nondiabetic NOD female mice (8–15 wk old). Moreover, to monitor any potential consequences of the neonatal manipulation on the subsequent onset of diabetes, additional controls included littermates exposed to hypothermic anesthesia and injected with HBSS.

Monitoring of Neonatally Injected Mice. Mice were tested for glycosuria three times weekly (Glukotest; Boehringer-Mannheim, Mannheim, Federal Republic of Germany) and glycosuric mice were controlled for hyperglycemia using teststrips and a quantitative colorimetric assay (Haemoglukotest and Reflolux F, Boehringer-Mannheim). Diabetic mice showed permanent fasting hyperglycemia above 3 g/liter (normal 0.88 ± 0.08), and

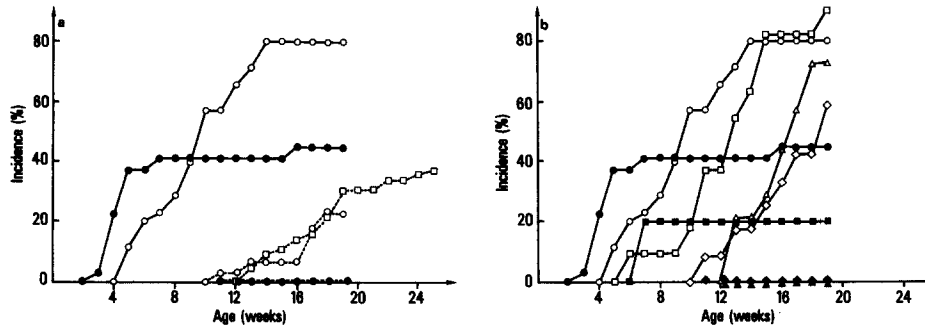


FIGURE 1. Incidence of diabetes in NOD females (open symbols) and males (closed symbols). (a) Broken lines, spontaneous incidence of diabetes in control groups: Neonatally HBSS-injected mice (○, n = 31; ●, n = 38), noninjected females (□, n = 64). Continuous lines, incidence of diabetes in mice neonatally injected with 20 × 10⁶ cells from pooled adult diabetic female spleens (○, n = 35; ●, n = 27). (b) Comparison of the autoimmune potential of prediabetic NOD mice spleen cells versus that of various doses of diabetic spleen cells: Mice neonatally injected with splenocytes from diabetics, 20 × 10⁶ cells (○, n = 31; ●, n = 38), 5 × 10⁶ cells (□, n = 11; ■, n = 10), 1.25 × 10⁶ cells (◇, n = 12; ◆, n = 10), or 20 × 10⁶ spleen cells from prediabetic 8–15-wk-old females (△, n = 14; ▲, n = 16).

TABLE I
Age-related Susceptibility of Young NOD Recipients to Diabetes Transfer

Age	Successful transfers of total mice injected			
	Males		Females	
	n	%	n	%
1d	11/27	42	20/35	57
2d	5/10	50	6/8	75
3d	3/7	43	2/9	22
4d	2/4	50	2/4	50
3 wk	1/19	5	13/31	42
5 wk	0/29	0	1/11	9

20 × 10⁶ spleen cells from a pool of diabetic NOD mice, injected intravenously. Successful transfers were scored up to 10 wk of life for mice injected at 1 and 3 wk and up to 15 wk of life for mice injected at 5 wk.

usually died within 2–8 wk with overt diabetic symptoms, including polyuria, polydipsia, and severe weight loss.

Histopathology. Paraffin sections (2 μM) of Bouin-fixed pancreases were stained with hematoxylin and eosin. At least 20 Langerhans islets were examined for each specimen.

Results

Adoptive Transfer of Diabetes. Diabetes can be adoptively transferred to NOD neonates by injection of adult female diabetic NOD spleen cells. As shown in Fig. 1 and Table I, up to 50% of mice injected neonatally with 20 × 10⁶ cells became diabetic by 10 wk of age. At this age spontaneous diabetes is still not observed in the control littermates or in the other reference groups of the colony. This age was thus chosen as the upper limit for scoring successful transfers. On the other hand, >90% of the control mice of both sexes present already histological signs of insulinitis (data not shown) and therefore this parameter cannot

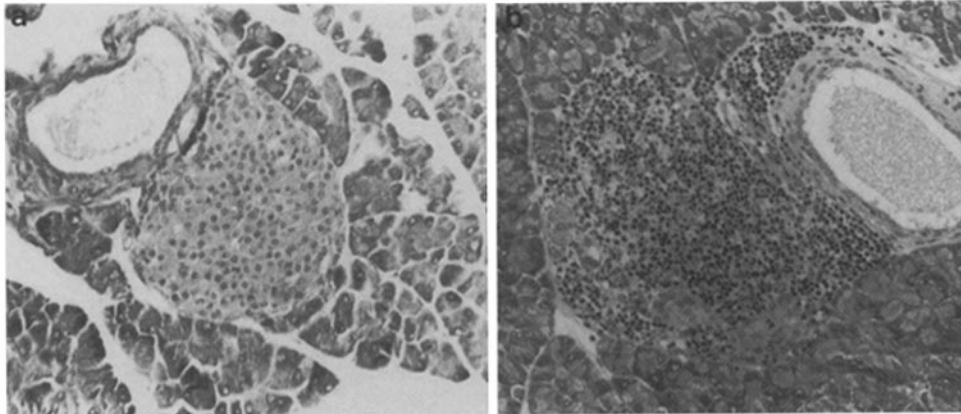


FIGURE 2. Photomicrographs of NOD mice pancreases. (a) Normal features in 4-wk-old NOD mice; islets of Langerhans are intact and free of mononuclear cell infiltration. (b) Lesions observed in a 4-wk-old male recipient neonatally injected with 20×10^6 spleen cells from diabetic adult females; severe mononuclear cell infiltration and destruction of the islet.

be used as a criterion of transfer among such animals. Some of the injected animals showed the first symptoms of diabetes as early as the third or fourth week of age. Destructive lymphocytic insulinitis was observed in these mice whereas a control group of 10 males and 10 females did not present any detectable histological changes in their pancreas at 4 wk of age (Fig. 2). Importantly, diabetes could be transferred in both diabetes-prone females and diabetes-resistant males. At 10 wk, 42% of injected males and 57% of females had become diabetic. However, a marked difference between males and females occurred later. The incidence of diabetes among females increased continuously until it reached 80%, whereas it remained unchanged in males (Fig. 1).

Diabetes Transfer by Spleen Cells from Diabetics is a Dose-dependent Phenomenon. Only 19% of mice injected with 5×10^6 cells had become diabetic by 10 wk. Males and females showed similar proportions of successful transfers, but again the rate of diabetes continued to increase after 10 wk in females, whereas it remained constant in males (Fig. 1). Diabetes was not adoptively transferred with 1.25×10^6 cells. However this dose produced a significant increase in the subsequent rate of diabetes in females but not in males (Fig. 1).

Neonatal Injection of Spleen Cells from Nondiabetic NOD Mice. Groups of newborn mice were also injected with spleen cells from young nondiabetic females of 8, 10, 12, or 15 wk of age. The results obtained in these experiments were pooled to provide a substantial control group (Fig. 1). As expected, no adoptive transfer, as defined above, was observed. However a striking acceleration of the onset of diabetes was noted in these animals after 10 wk, as compared with the noninjected control groups. The incidence rate was strikingly superimposable to that observed after injection with the low dose of 1.25×10^6 spleen cells from diabetics. Interestingly, spleen cells from older nondiabetic females of 18–19 wk of age could successfully transfer the disease into newborn mice. 6 of 12 recipients became diabetic within 10 wk (data not shown).

Diabetes Transfer Depends upon the Age of the Recipients. To determine whether

TABLE II
Effect of Selective Thy-1,2⁺ Spleen Cell Depletion
on Diabetes Transfer

Injected cells	Successful transfers of total mice injected
Anti-Thy-1,2 + C'	0/6*
C'	7/11

20 × 10⁶ pretreated viable spleen cells from a pool of six diabetic NOD mice, injected intravenously into 1-d-old recipients (two litters born on the same day).

* *p* < 0.04 (Fisher exact test).

TABLE III
Effects of Selective L3T4⁺ and Lyt-2⁺ T Cell Depletion on Diabetes Transfer

Exp.	Anti-L3T4 + C'	Anti-Lyt-2 + C'	C'
1	—	0/6*	6/11
2	0/6	—	1/6
3	0/3	0/4	4/4 [‡]
4	0/6	0/6	2/7
Total			
L3T4 ⁺ T cell depletion	0/15	—	7/17 [§]
Lyt2 ⁺ T cell depletion	—	0/16	12/22 [§]

Each experiment required simultaneous intravenous injection at birth of two litters with pretreated viable spleen cells from a pool of 5–15 diabetic NOD mice. Exps. 1 and 3, 1 × 10⁷ nylon wool-purified T cells, intact or after subset depletion; Exps. 2 and 4, 2 × 10⁷ spleen cells, intact or after subset depletion.

* Successful transfers of total mice injected

[‡] Cells injected were a mixture of 5 × 10⁶ purified T cells depleted of L3T4⁺ cells plus 5 × 10⁶ purified T cells depleted of Lyt-2⁺ cells.

[§] *p* < 0.02 and *p* < 0.001, respectively, for L3T4⁺ and Lyt-2⁺ T cell depletion (Fisher exact test).

the transfer of diabetes was due to a particular susceptibility of newborn mice, older recipients were also injected with 20 × 10⁶ spleen cells from diabetic mice. The results of these experiments are shown in Table I. Animals injected at the age of 1, 2, 3, and 4 d showed a similar incidence of successful transfer at 10 wk of age. Older animals were also tested as recipients. Females were still sensitive to diabetes transfer at 3 wk of age, but became refractory around 5 wk. Males, on the other hand, were already found resistant to adoptive transfer at 3 wk of age.

Lymphocyte Subsets Depletion Experiments. Table II shows that splenocytes from diabetic mice, depleted of Thy-1,2⁺ cells no longer transfer the disease. Conversely, positively selected nylon T cells are effective in transferring diabetes (Table III, Exps. 1 and 3). Table III compares, in addition, the capacity of T cell subsets to transfer the disease. Both L3T4⁺ cell-depleted and Lyt-2⁺ cell-depleted splenocytes failed to transfer the disease, whereas complement-treated control spleen cells transferred diabetes. When the two T lymphocyte subsets, obtained after nylon wool passage and either anti-L3T4 or anti-Lyt-2 plus complement treatment, were mixed together, total cell number being constant

among the injected groups, successful transfer of the disease was restored (Table III, Exp. 3).

Discussion

Increasing evidence for the role of T cells in organ-specific autoimmunity has accumulated over the past few years (reviewed in reference 15). Cell-transfer experiments have provided the first direct evidence for T cell involvement in various experimental autoimmune diseases. Similar evidence has been obtained in the spontaneous autoimmune disease of the BB rat, another model of type I diabetes mellitus. The disease could be transferred from diabetic to nondiabetic BB rats provided that the injected spleen cells had been previously activated *in vitro* with Con A (16). However, the cells mediating the adoptive transfer have not been further characterized.

The rationale for our experiments with NOD mice was to take advantage of the delayed histological and clinical expression of the disease to develop a neonatal model of adoptive transfer that could account for the whole history of β cell autoimmune destruction. We report here several findings concerning the successful adoptive transfer of NOD diabetes to neonates, the sensitivity of the neonatal model as an *in vivo* assay to test the autoimmune potential of nondiabetic NOD mice, the age-related susceptibility of recipients to the transfer of the disease, and the lymphocyte subsets involved in the cellular events leading to β cell destruction.

First, type I diabetes mellitus was transferred with spleen cells from diabetic NOD mice. However, a considerable number of spleen cells were necessary to produce a significant effect. Up to 20×10^6 cells were required to destroy, in 50% of the cases, the β cells of a neonate. Several hypotheses may explain this finding: (a) spleen may not be the elective organ for activated autoimmune cells; attempts to transfer diabetes with lymphocytes isolated from the pancreas itself are in progress and should provide a more accurate estimation of the autoimmune potential of diabetic NOD mice lymphocytes; (b) β cell destruction in NOD mice, as well as in BB rats and in diabetic humans, follows a chronic course so that relatively few autoimmune cells may be needed at a given moment of the disease; and (c) pancreases from neonates present two distinctive features, as compared with adults, namely an increased ratio of endocrine cells and substantial regeneration potencies, as suggested by the presence of mitoses among islet cells (our histological observations) and by functional regeneration experiments (17).

Of interest was the finding that successful transfer could be achieved into both males and females, whereas only females spontaneously develop overt diabetes in our breeding colony. However, the occurrence of diabetes in the male group stopped abruptly at 7 wk after transfer, whereas the incidence in females continuously increased. Moreover, older male recipients became resistant to the transfer earlier than females, at 3 wk of age. These features are probably related to the natural resistance of males to overt diabetes. Sexual hormones, which influence the incidence of diabetes in NOD mice (18), might account for these differences. In addition, suppressor mechanisms are probably involved in the resistance of males, as suggested by the promotion of overt diabetes after cyclophosphamide treatment (19). Our results suggest that these putative mech-

anisms may influence the long-term fate of the injected cells, but do not operate in the first weeks of life.

Second, the neonatal model of transfer provides a sensitive assay to evaluate the pathogenic effect of NOD mouse lymphocytes. Although as few as 1.25×10^6 spleen cells from diabetic mice do not transfer the disease within the first 10 wk, they nevertheless increase the rate of diabetes in female recipients. This effect probably reflects the interference between adoptive transfer and spontaneous occurrence of the disease. Moreover, the finding that transfer of 20×10^6 spleen cells from 8–15-wk-old nondiabetic females can induce changes similar to those provoked by 1.25×10^6 cells from diabetics strongly suggests that the autoimmune process has already begun in prediabetic animals. Cells from older prediabetic females of 18–19 wk were able to transfer diabetes to neonate recipients with a roughly equal rate of success as spleen cells from overtly diabetic mice, indicating that the capacity of transferring the disease is not necessarily linked with the presence of overt diabetes but that it is more probably related to the duration and the severity of the insulinitis as observed among 18–19-wk-old prediabetic donors (data not shown). Altogether, these data indicate that the neonatal model of transfer provides a suitable *in vivo* assay to explore the autoimmune potential of NOD mice at various stages of their natural history.

Third, transfer experiments with recipients of various ages suggest that the susceptibility to diabetes transfer is limited to the first weeks of life. 5-wk-old animals of both sexes appeared to be refractory to the transfer, a result which is in keeping with the work of Wicker et al. (7). However, it seems paradoxical to observe that animals of both sexes become resistant to diabetes transfer at the very age when they notoriously begin to self-damage their pancreatic islets. At variance with the adult models of transfer in NOD mice (7) and in BB rats (16), the transfer in NOD newborns does not require prior irradiation of the recipients or prior *in vitro* activation of the injected cells. These important features probably reflect the unique immune status of the neonate. In addition, the susceptibility of the neonate to the disease transfer provides direct evidence for the expression early in life of the pancreatic self antigen(s) involved in the autoimmune process. Therefore, the recent observations obtained in a transgenic model of antiislet autoimmunity, suggesting that the delayed expression of a (transgenic) self antigen could be responsible for the occurrence of autoimmune lesions (20), cannot be extended to the NOD mouse model.

Fourth, transfer experiments with fractionated cell subsets yield clear-cut conclusions. Both L3T4⁺ and Lyt-2⁺ T cells are necessary to successfully transfer diabetes to neonates, and the mixture of the two separated subsets transfers the disease. These results show that the neonatal transfer is T cell mediated and probably involves cooperation between L3T4⁺ and Lyt-2⁺ T cells. This dual requirement has not been observed in other models of T cell-mediated organ-specific autoimmunity in which the effector cells have been characterized, such as experimental autoimmune encephalomyelitis, adjuvant arthritis, and experimental autoimmune thyroiditis. L3T4⁺ cells from spleen, lymph node, or cell lines and clones, presumably acting as inducer T cells, have been shown to mediate these diseases, whereas Lyt-2⁺ cells did not seem to represent effectors in these models (15).

Identification of the phenotype of the T cells involved in the transfer does not allow, however, assigning a function to these T cell subsets *in vivo*. It is now admitted that the expression of L3T4 or of Lyt-2 molecules is correlated respectively with MHC class II or class I restriction of the T cell receptor rather than with the helper versus cytotoxic or suppressor functions (21). Therefore, it may only be inferred from these results that β cell destruction in the pancreas implies the presentation of autoantigen(s) in the context of class I and class II molecules at some stages of the autoimmune process. It is likely that L3T4⁺ cells act as helper cells cooperating with activated Lyt-2⁺ cytotoxic cells, for example by providing expansion signals such as IL-2. The absence of transfer with L3T4⁺ cells alone would therefore indicate that the young recipients lack recruitable Lyt-2⁺ cells. On the other hand, autoreactive Lyt-2⁺ effector cells are probably in too limited a number in the inocula to produce the disease by themselves. Alternatively, other roles may be putatively assigned to L3T4⁺ and Lyt-2⁺ cells in the destruction of β cells. L3T4⁺ cells could act as class II-restricted autoreactive cytolytic cells, as has been shown in other systems (22), and Lyt-2⁺ cells could inhibit the secretion of insulin as indicated by *in vitro* experiments (23).

The identification of two cell subsets responsible for diabetes transfer does not preclude the recruitment of host lymphocytic or nonlymphocytic cells. The helper T cells might cooperate with host B lymphocytes and in turn mediate complement-dependent or antibody-dependent cell-mediated cytotoxicity. Although antiislet cell autoantibodies are detected in NOD mice (24), several lines of evidence argue against humoral effector mechanisms: (a) diabetes is not transferred by mothers to their offspring (our personal observation of five litters from insulin-treated diabetic mothers); (b) spleen cells depleted of B lymphocytes are as efficient as whole spleen cells in transferring the disease; and (c) B cells are underrepresented among locally infiltrating cells in the pancreas (3). Host macrophages might also be involved in the process leading to the destruction of β cells. T helper cells may secrete factors, such as macrophage-activating factor or interferon, that promote macrophage cytotoxicity. In addition, IL-1 has been shown to be selectively cytotoxic for β cells (25).

Therefore, the diabetes of the NOD mouse may result from a complex autoimmune process mediated by distinct T cell subsets. The neonatal model of transfer should provide a basis for useful and more definitive studies of the cellular events involved in the onset and regulation of autoreactivity against insulin-producing cells in type I diabetes mellitus.

Summary

We have developed a model of syngeneic adoptive transfer for type I diabetes mellitus of NOD mice. This model consists in injecting spleen cells from diabetic adult mice into newborn NOD recipients. 50% of recipients inoculated with 20×10^6 cells develop diabetes within the first 10 wk of life, at a time when none of the control littermates have yet become diabetic. The earliest successful transfers are observed at 3 wk of age, at a time when controls do not even exhibit histological changes in their pancreas. In addition we have shown that: (a) both males and females can be adoptively transferred, despite the fact that males rarely develop spontaneous diabetes in our colony; (b) diabetes transfer is a dose-

dependent phenomenon that provides an *in vivo* assay for comparing the autoimmune potential of spleen cells from mice at various stages of their natural history; (c) the susceptibility of the recipients to the transfer is limited in time and declines after 3 wk; and (d) both L3T4⁺ and Lyt-2⁺ T cell subsets are necessary for the successful transfer. The neonatal syngeneic transfer provides an effective model for studies of the cellular events involved at regulatory and effector stages of autoimmune type I diabetes.

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