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Prevention of type I diabetes in nonobese diabetic mice by allogeneic bone marrow transplantation

(autoimmunity/pancreatic islitis)

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ABSTRACT **An animal model [the nonobese diabetic (NOD) mouse] for type I diabetes features a striking infiltration of T cells into the pancreatic islets. This infiltration selectively destroys beta cells. Most of the T cells are Lyt-1$^+$, but some are Lyt-2$^+$,3$^+$. Transfer experiments using parabiosis revealed that insulitis can be transferred within 2 weeks after parabiosis to immunoincompetent thymectomized mice. When NOD mice (6 mo old) were irradiated and reconstituted with bone marrow cells from young BALB/c *nu/nu* mice (<2 mo old), the NOD mice exhibited neither insulitis nor overt diabetes. Deposits of immunoglobulin in mesangial areas of the glomeruli disappeared within 3 mo after bone marrow transplantation in such irradiated allogeneic bone marrow reconstituted mice. Assays for immunological functions, including mitogen response and mixed lymphocyte reaction, revealed that both T- and B-cell functions were increased in NOD mice with overt diabetes. NOD mice reconstituted with BALB/c *nu/nu* bone marrow cells displayed normal T- and B-cell functions. The newly developed T cells in the allogeneic bone marrow recipients are tolerant to cells with both donor- and host-type major histocompatibility complex determinants. These results suggest that bone marrow transplantation may ultimately be developed as a component of a strategy to be employed for treatment of type I diabetes in humans.**

Diabetes mellitus is a heterogenous disorder, and its pathogenesis remains an enigma. It has been classified into two types (1). Type I is insulin-dependent, often juvenile-onset, nonobese, and ketosis-prone diabetes. Type II is non-insulin-dependent, adult-onset, obese and non-ketosis-prone diabetes. Animal models of spontaneously developing diabetes mellitus are useful for studying the pathogenesis of human diabetes mellitus and its complications. Many animal models for diabetes mellitus have been reported (2). However, most of them are models for type II diabetes. Only strain BB Wistar rats have been presented as a model for type I diabetes (3). A mouse model for type I diabetes has been sought for some time because mice are well suited for genetic and immunologic analyses.

Nonobese diabetic (NOD) mice were found in 1974 by Tochino among Jcl-ICR mice with cataract and have been established recently as an inbred strain by Makino and co-workers (4). Insulitis was observed in more than 90% of both male and female NOD mice at the age of 200 days. The overt diabetic symptoms such as polyuria, polyphagia, hyperglycemia, glycosuria, and ketosis, however, appeared in 90% of the females but only 20% of the males at the age of 250 days (5). In breeding experiments with NOD and C57BL/6J mice, Makino *et al.* (6) found that insulitis in NOD mice is controlled by two recessive genes on independent chromosomes. Fujita *et al.* (7) reported that lymphocyte infiltration into the islets selectively destroys beta cells. They also found retrovirus-like particles in pancreatic beta cells of NOD mice (8).

We have recently demonstrated that allogeneic bone marrow transplantation (ABMT) can treat systemic autoimmune diseases in MRL/1 and BXSB specific-pathogen-free mice without inducing graft-versus-host reaction, provided that bone marrow cells of young *nu/nu* mice or T-cell-depleted bone marrow cells are used (9). These observations prompted us to examine whether or not insulitis as an organ-specific autoimmune phenomenon can also be treated by ABMT. In the present communication, we show that the lymphocytes infiltrating into the islets are T cells, and that ABMT can, indeed, treat insulitis and prevent overt diabetes.

MATERIALS AND METHODS

Mice. Female NOD mice were used. The pedigree of the NOD mice is as follows: In 1966, cataract mice (Jcl-ICR) were found among outbred ICR mice. Since cataract is often observed in diabetic patients, selective breeding was performed to obtain euglycemic and hyperglycemic mice. At 6 generations of sister × brother mating, a strain, CTS, with cataract developed. After more than 20 generations of mating, a diabetogenic subline (NOD strain) was established in 1980. The NOD mice were maintained under conventional conditions for 5 generations. Between the fifth and sixth generations, the pups were delivered by Caesarean section under aseptic conditions and foster nursed on a specific pathogen-free (SPF) ICR mother. Under both conventional and SPF conditions, the NOD mice showed identical manifestations of diabetes. Thereafter, NOD mice were studied under conventional conditions.

Bone Marrow Transplantation. The NOD (H-2^g) mice (>5 mo old) were exposed to 850 rads (8.5 grays) from a ^{60}Co source and reconstituted by intravenous injection of 2×10^7 bone marrow cells from young BALB/c *nu/nu* (H-2^d) mice (<2 mo old). The mice were usually sarificed more than 3 mo after bone marrow transplantation. Testing with anti-H-2 sera and complement established that more than 90% of spleen cells were of donor type.

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Abbreviations: NOD, nonobese diabetic mouse strain; ABMT, allogeneic bone marrow transplant; MHC, major histocompatibility complex; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide.

Histopathological Study. Major organs were obtained at autopsy, and sections were stained with periodic acid/Shiff reagent and hematoxylin/eosin.

Immunofluorescence and Immunohistochemical Studies. Specimens were immediately embedded in optimum cutting temperature (OCT) compound and frozen in dry ice/acetone. Two-micrometer cryostat sections were used for immunofluorescence (10) and immunohistochemical (11) studies, as previously described.

Cell Separation. The spleens were removed aseptically, minced, and gently passed through a fine-mesh stainless-steel sieve into phosphate-buffered saline.

Mitogen Response. The mitogenic reactivity was determined by measuring incorporation of [^{3}H]thymidine into DNA, as previously described (12). In brief, triplicate cultures were set up in wells of flat-bottom microtiter plates, each containing 5×10^5 cells in 0.2 ml of RPMI 1640 medium with 5% human plasma. The cells were cultured in the presence of phytohemagglutinin P (PHA; Difco) at 25 μg/ml, concanavalin A (Con A; Calbiochem) at 5.0 μg/ml, or *Escherichia coli* lipopolysaccharide (LPS; Difco) at 25 μg/ml. The cultures were incubated for 72 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. [^{3}H]Thymidine (0.5 μCi in 20 μl; 1 Ci = 37 GBq) (New England Nuclear) was present during the last 4 hr of the culture period. The ^{3}H incorporated into trichloroacetic acid-insoluble material was measured with a liquid scintillation counter.

Mixed-Lymphocyte Reaction. This reaction was examined by measuring the incorporation of 0.5 μCi of [^{3}H]thymidine into DNA, as previously described (13). In brief, triplicate cultures were set up in 96-well round-bottom microtiter trays (Corning 25850). Each well contained 2×10^5 responder cells and 1×10^5 stimulator cells in a total volume of 0.2 ml of RPMI 1640 medium with 5% heat-inactivated human serum and 20 μM 2-mercaptoethanol (Wako Pure Chemical Industries, Tokyo). Stimulator cells were treated with mitomycin C at 50 μg/ml for 30 min at 37°C. The cultures were incubated for 96 hr in a humidified atmosphere of 5% CO_2 in air. [^{3}H]Thymidine was present during the last 4 hr of the culture period.

Cytotoxicity Test. Cytotoxicity testing using monoclonal antibody plus complement was performed as previously described (14).

Each group (nontreated or ABMT-treated NOD mice) consisted of more than 15 mice. Since the results were uniform, representative data are reported.

RESULTS

Involvement of T Cells in Insulitis. Using immunofluorescence and immunohistochemical techniques, we confirmed that lymphocytes infiltrating into pancreatic islets are Thy-1.2$^+$ T cells; most of them are Lyt-1$^+$ and some are Lyt-2$^+$,3$^+$.

The next step was to determine whether insulitis can be transferred or not. NOD mice were neonatally thymectomized. At the age of 2 mo, biopsies were performed to make sure that insulitis in the mice had not developed. The mice then were prepared as parabionts with NOD mice that had insulitis. Two weeks later, the mice were sacrificed to investigate whether or not the insulitis had been transferred from the insulitis-positive parabiont to the insulitis-negative parabiont. We found that in this model system the neonatally thymectomized mice now also showed insulitis. Thus, insulitis can be transferred after the animals have been in a parabiotic union for only two weeks.

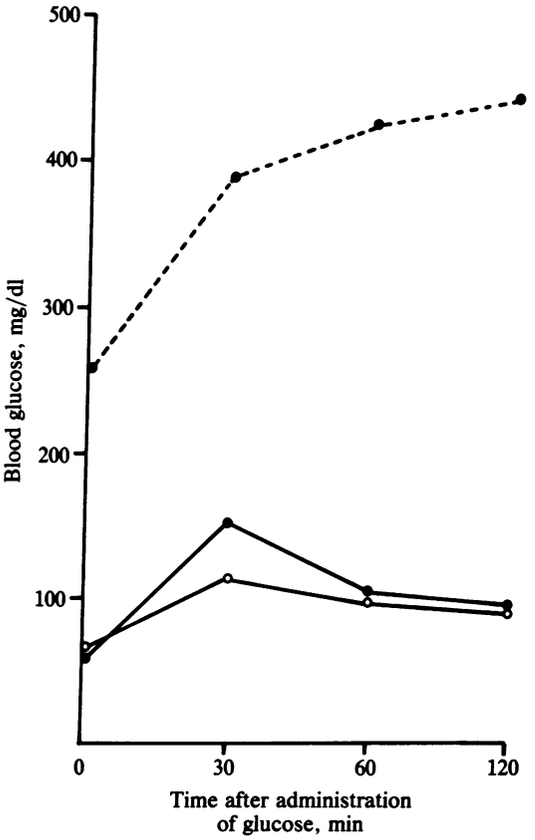

FIG. 1. Glucose tolerance test. Glucose was administered intraperitoneally at 1 g/kg of body weight. Eight-month-old nontreated NOD mice (●--●) display impaired glucose tolerance. NOD mice (●—●) reconstituted with BALB/c *nu/nu* bone marrow show the same pattern as normal ICR mice (○—○).

Glucose Tolerance Test. One day prior to sacrifice, glucose tolerance was tested. As shown in Fig. 1, nontreated NOD mice (8 mo old) display a glucose tolerance similar to that seen in type I human diabetes. By contrast, 9-mo-old NOD mice (3 mo after ABMT) that had been irradiated and reconstituted with BALB/c *nu/nu* bone marrow cells showed the same histologic appearance as normal ICR mice.

Histological Findings in the Islets. As shown in Fig. 2*A*, a marked infiltration of T cells was observed in the pancreatic islets of nontreated NOD mice (6 mo old) in the prediabetic stage. Immunohistochemical study using the Nakane staining procedure (11) revealed that beta (insulin-producing) cells had been selectively destroyed by T cells (Fig. 2*A*), whereas delta (somatostatin-producing) cells (Fig. 2*B*) and alpha (glucagon-producing) cells (Fig. 2*C*) were not destroyed. By contrast, the islets of NOD mice (9 mo old), 3 mo after ABMT, showed no lymphocytic infiltration, and immunohistochemical examinations indicated the presence of intact beta cells as well as alpha and delta cells (Fig. 2*D*).

Immunofluorescence Study of the Kidney. Diabetic nephropathy is a critical complication in patients with diabetes. Since we know that ABMT can decrease deposits of

FIG. 2 (*on opposite page*). Histological findings in the islets. Beta cells are stained bluish gray, alpha cells are brown, and delta cells are pink. (×900.) (*A–C*) Pancreatic islet of a nontreated NOD mouse (6 mo old) in the prediabetic stage. (*A*) Marked infiltration of T cells. Insulin-producing cells (beta cells) were not found. (*B*) Somatostatin-producing cells (delta cells) were not destroyed in the islet of this nontreated NOD mouse. (*C*) Glucagon-producing cells (alpha cells) were detected in this islet of the same mouse. (*D*) NOD mouse with ABMT showed no lymphocytic infiltration in the pancreatic islet. Intact alpha, beta, and delta (arrow) cells were observed.

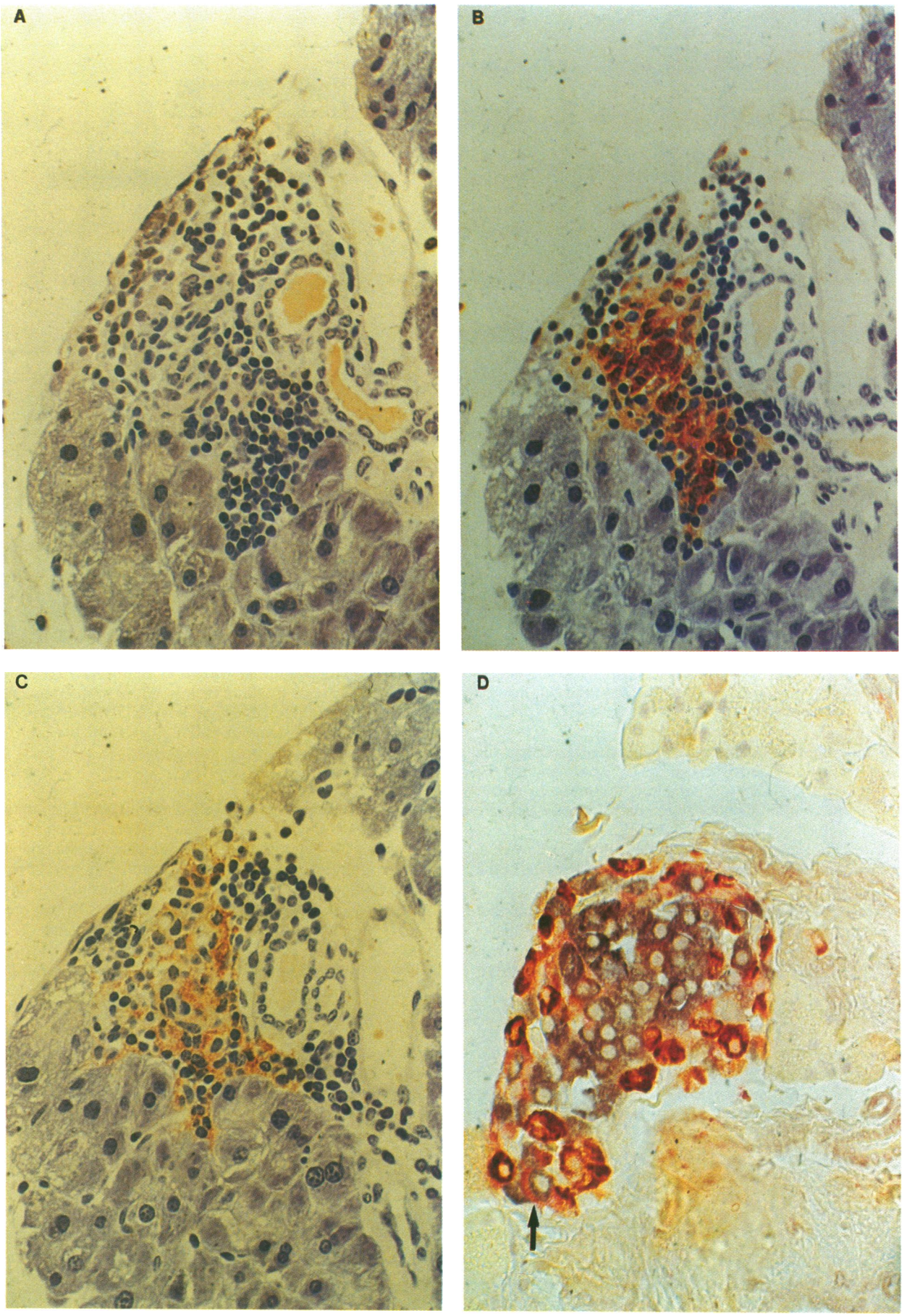

FIG. 2. (*Legend appears at the bottom of the opposite page.*)

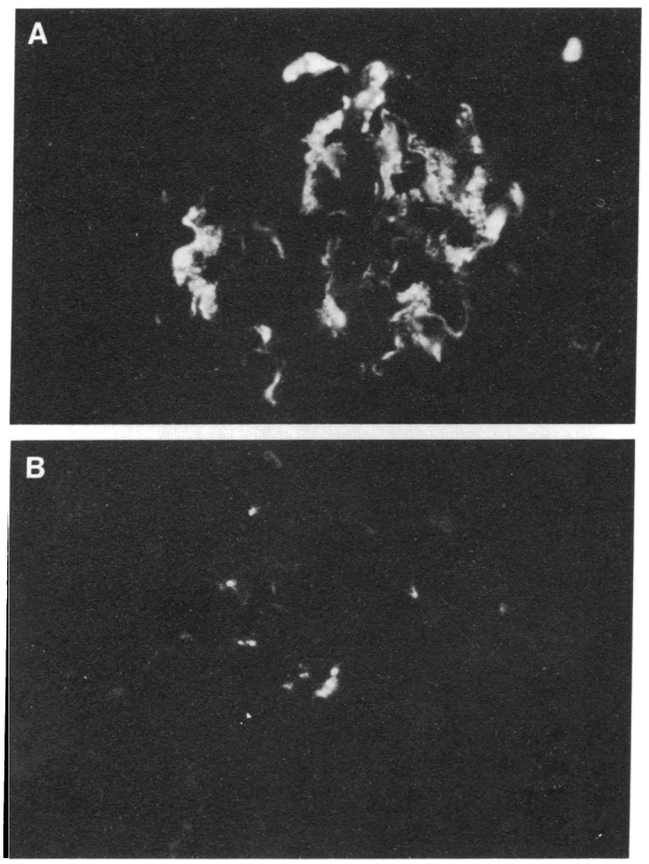

FIG. 3. Immunofluorescence study of the kidney. (×900.) (*A*) Nontreated NOD mouse showed mesangial and capillary deposits of IgG in the glomerulus. (*B*) NOD mice with ABMT had only minimal deposits of IgG in the glomerulus.

immunoglobulin and complement component 3 (C3) in the glomeruli of MRL/1 and BXSB mice (9), we examined the glomeruli of NOD mice by using immunofluorescence techniques. Nontreated NOD mice (8 mo old) showed extensive mesangial deposits of IgG (Fig. 3) and IgA, but not of C3 or IgM. In ABMT-treated NOD mice (9 mo old), however, neither immunoglobulin nor C3 deposits were observed in the glomeruli.

Immunological Functions. Cytotoxic tests revealed that non-treated NOD mice (8 mo old) showed a markedly higher percentage of Thy-1.2$^+$ cells (52.9%) in the spleen than 2-mo-old BALB/c (27.8%) and 9-mo-old ABMT-treated NOD mice (18.4%) (Table 1).

With respect to mitogen stimulation, as shown in Table 1, nontreated NOD mice (8 mo old) with diabetes showed stronger responsiveness to both T-cell mitogens (PHA and Con A) and B-cell mitogen (LPS) than age-matched control ICR mice (8 mo old) and even young BALB/c mice (2 mo old). In contrast, 9-mo-old NOD mice with ABMT exhibited responsiveness to T- and B-cell mitogens similar to that of 8-mo-old ICR mice.

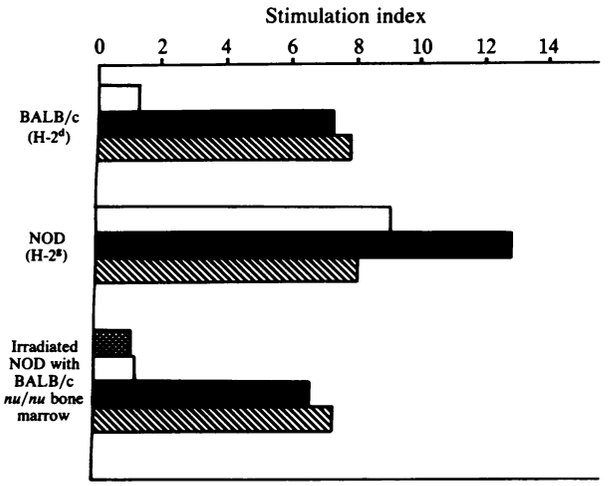

FIG. 4. Mixed lymphocyte reaction. Stimulator cells were as follows: empty bars, BALB/c; filled bars, C57BL/6J; hatched bars, C3H/HeN; stippled bar, NOD. Responder cells are listed on the left. Nontreated 8-mo-old NOD (H-2^g) mice with overt diabetes showed a marked alloreactivity to BALB/c, C57BL/6J, and C3H/HeN. Lymphocytes from NOD mice irradiated and reconstituted with BALB/c *nu/nu* (H-2^d) bone marrow cells were tolerant to MHC determinants of both bone marrow donor (BALB/c) and host (NOD).

In MLR, as shown in Fig. 4, nontreated 8-mo-old NOD (H-2^g) mice with H-2^g overt diabetes showed alloreactivity similar to that observed with young BALB/c mice (2 mo old). NOD mice reconstituted with BALB/c *nu/nu* (H-2^d) bone marrow cells were found to be tolerant to both bone marrow donor (BALB/c) H-2^d-type and host (NOD) H-2^g-type major histocompatibility complex (MHC) determinants.

DISCUSSION

It has been thought that type I diabetes occurs as a consequence of autoimmune phenomena. Gepts (15) reported that patients with juvenile diabetes had peri-insular and intra-insular lymphocytic infiltration. Recently, he reported also that lymphocytes infiltrated into islet regions destroyed only beta cells and not alpha or delta cells (16). In the present study, we demonstrated, using NOD mice, that T cells infiltrate into the islets, where they selectively destroy beta cells as in humans (Fig. 2). In addition, we showed that insulitis can be transferred into young thymectomized NOD mice within 2 weeks after initiating parabiosis with NOD mice that express islitis. It has been reported that Lyt-1$^+$ cells play a crucial role in the pathogenesis of organ-specific autoimmune diseases (17). Furthermore, using immunohistochemical techniques, we demonstrated that most of the T

Table 1. Increase in both T-cell and B-cell functions in NOD mice

Mouse strain	Age, mo	Glycosuria	Thy-1$^+$, %	[^{3}H]Thymidine incorporation, cpm × 10^{-3}				Stimulation index		
				Background	PHA	Con A	LPS	PHA	Con A	LPS
BALB/c	2	−	27.8 ± 4.3	1.3 ± 0.2	38.5 ± 4.3	77.1 ± 5.4	19.9 ± 3.2	29.6	59.3	15.3
ICR	8	−	22.9 ± 3.2	1.2 ± 0.1	7.6 ± 0.5	27.8 ± 3.2	16.3 ± 4.1	6.3	23.2	13.6
NOD	8	+++	52.9 ± 6.3	2.3 ± 0.2	43.0 ± 4.5	122.3 ± 6.4	95.0 ± 5.8	18.7	53.2	41.3
NOD*	6 → 9*	−	18.4 ± 2.8	4.6 ± 0.3	27.8 ± 3.2	77.7 ± 8.2	65.6 ± 2.1	6.0	16.9	14.3

Results are presented as mean ± SD. Mitogen concentrations are given in the text. The stimulation index is [^{3}H]thymidine incorporation relative to background.

*NOD mice (6 mo old) were irradiated and reconstituted with BALB/c *nu/nu* bone marrow cells. The NOD mice were sacrificed at the age of 9 mo.

cells infiltrating into islets are Lyt-1$^+$ cells, and that some are Lyt-2$^+$,3$^+$ cells.

In humans, autoantibodies against islet cell antigens (ICAs) as well as islet cell surface antigens (ICSAs) have been considered to be involved in the development of type I diabetes. Such antibodies were reportedly found also in NOD mice (18). Although we did not demonstrate such antibodies in the present study, we think that antibody-mediated cytotoxicity, cell-mediated cytotoxicity, or both are responsible for the development of type I diabetes because (*i*) B-cell functions as well as T-cell functions increase in NOD mice, as shown in Table 1; and (*ii*) in the late stage of overt diabetes in NOD mice, we could find neither B cells nor lymphocyte infiltration into the pancreas. The lymphocytic infiltration thus correlated temporally with destruction of the host B cells. Since we have recently established that T cells, B cells, and macrophages develop in recipient mice within 3 mo after ABMT, and that ABMT can prevent and treat systemic autoimmune diseases in MRL/1 and BXSB mice (9), we attempted to prevent insulitis and overt diabetes in NOD mice by ABMT. Similarly, Naji *et al.* (19) have reported that MHC-compatible bone marrow transplantation will correct T-cell functions and microenvironmental defects in BB rats. However, these investigators did not describe an influence of bone marrow transplantation on the islets of these rats. In the present study, we clearly demonstrated that MHC-incompatible ABMT can prevent insulitis and overt diabetes, which appears to be an organ-specific autoimmune disease in NOD mice. However, we have thus far not been able to treat diabetes that is already advanced in NOD mice. This may be the case because such mice have often lost their islets. It may be possible to treat such NOD mice by using a combination of bone marrow and islet transplantation.

It has been reported that, in humans, development of type I diabetes is related to viral infections (20). Since Fujita *et al.* (7) reported the presence in NOD mice of retrovirus-like particles in pancreatic beta cells, vertically transmitted viral infection from dam to pup must be considered in the pathogenesis of insulitis and insulitis-associated diabetes in NOD mice. It remains to be determined how the virus infection triggers this autoimmune response and how it is that ABMT can prevent insulitis. Perhaps in crossing major histocompatibility barriers we have introduced resistance genes that, by immunological or nonimmunological mechanisms, can inhibit or suppress the virus infection. Further experiments will be necessary to test this possibility.

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