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Direct evidence for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice

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Abstract

The non-obese diabetic (NOD) mouse is an excellent animal model of autoimmune diabetes associated with insulitis. The progression of insulitis causes the destruction of pancreatic β cells, resulting in the development of hyperglycemia. Although it has been well documented that T cells are required for the development of insulitis and diabetes in NOD mice, the importance of B cells remains unclear. To clarify the role of B cells in the pathogenesis of NOD mice, we therefore generated B cell-deficient NOD (B^-NOD) mice. Surprisingly, none (of 13) of the B^-NOD mice developed diabetes by 40 weeks of age, while the control littermates with B cells (B^+NOD) suffered from a high proportion (43 of 49) of diabetes. The insulin reactivity of B^+NOD mice was significantly impaired, while the B^-NOD mice showed a good insulin response, thus suggesting the pancreatic β cell function to be well preserved in B^-NOD mice. Although B^-NOD mice did develop insulitis, the extent of insulitis was significantly suppressed. These data thus provide the direct evidence that B cells are essential for the progression of insulitis and the development of diabetes in NOD mice.

Introduction

The non-obese diabetic (NOD) mouse develops diabetes concomitant with the progression of insulitis, thus providing an excellent animal model of human autoimmune diabetes mellitus (1–3). It has been proven that T cells are required for the development of insulitis and diabetes in NOD mice (3–15). Insulitis and diabetes did not develop in congenitally athymic nude mice and neonatally thymectomized NOD mice (4,5). Immunohistological studies revealed the predominant accumulation of T cells in the insulitic lesion (6,7). Treatment of mice with anti-T cell antibody prevented the development of the disease (8–10). In addition, adoptively transferred T cells could successfully induce insulitis and diabetes in irradiated young and newborn NOD mice (11–14).

Although these previous studies revealed the significant role of T cells, the role of B cells in the development of insulitis and diabetes remains obscure. B cells were not required for the transfer of diabetes (15), while immunohistochemical studies of insulitis revealed the accumulation of B cells in the later stage of insulitis (6,7). In addition, production of autoantibodies to pancreatic β cells is predictive of the later development of diabetes (16,17). These data raise the possibility that B cells may also play some role in the pathogenesis of insulitis and diabetes in NOD mice.

In the present study, in order to clarify the involvement of B cells in the development of insulitis and diabetes in NOD mice, we have generated B cell-deficient NOD mice by

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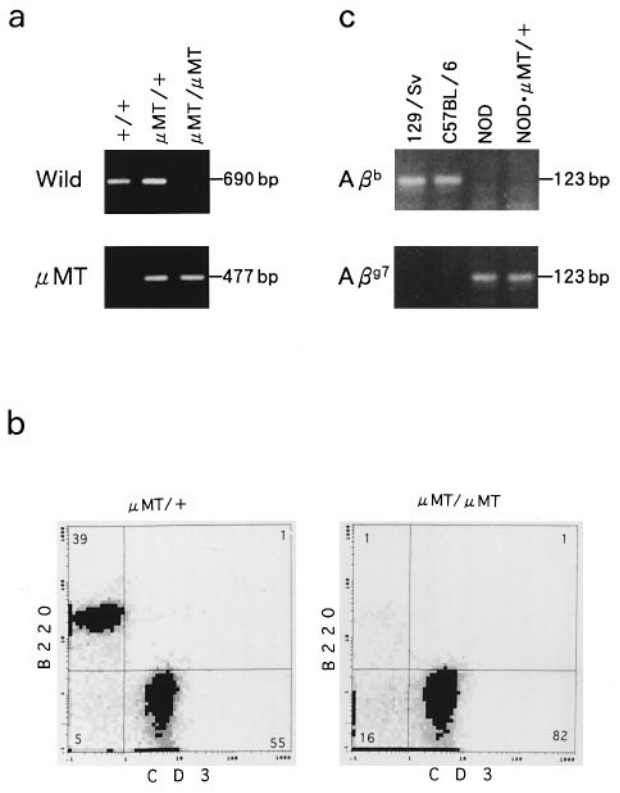

Fig. 1. Establishment of B cell-deficient NOD mice. μMT/+ B6 mice were backcrossed to +/+ NOD mice 8 times and μMT/+ NOD mice were produced. μMT/μMT NOD mice were obtained by crossing μMT/+ mice. (a) Homozygocity of the μMT allele was determined in a μMT/μMT NOD mouse. (b) Flow cytometric analysis revealed the absence of $B220^+$ B cells in peripheral blood mononuclear cells from a μMT/μMT NOD mouse. (c) The I-A_β gene of the class II molecules in the parental μMT/+ NOD mouse was I-A_β^7 as assessed by PCR.

backcrossing mice with the null μMT mutation to NOD mice. In the B cell-deficient NOD mice, diabetes did not develop over a 40-week observation period and the progression of insulitis was significantly suppressed, indicating the importance of B cells in the pathogenesis of insulitis and diabetes in NOD mice.

Methods

PCR to detect μMT and I-A_β^7 genes

The μMT allele was detected by PCR using genomic DNAs from tails. We used primer sets P-1988 (5′-TACAGCTCAGCTGTCTGTGG-3′) and P-neo^r (5′-TCTATCGCCTTCTTGACGAG-3′) to amplify the 477 bp products from the μMT allele where the neo^r gene was inserted in the membrane exon of the μ chain. Primer sets P-3542 (5′-CTGTCTTGCTTGCTCTGCTC-3′) and P-1988 were used to amplify 690 bp products from the wild allele. The PCR was performed at 95°C for 60 s, 65°C for 60 s and 72°C for 90 s, for 40 cycles on a DNA thermal cycler. PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. I-A_β genes of the class II molecules were determined as described previously (18). Genomic DNA (1 μg) was used for PCR by primers with additional *Eco*RI sites specific for I-A_β^b (5′-A_β^b: 5′-TCTAGAATTCACAGCGACATGGGCGAGC-3′ and 3′-A_β^b: 5′-TCTAGAATTCCGTAGTTGTGTCTGCACA-3′) and I-A_β^{g7} (5′-I-A_β^{g7}:5′-TCTAGAATTCACAGCGACGTGGGCGAGT-3′ and 3′-A_β^{g7}: 5′-TCTAGAATTCCGTAGTTGTGTCTGCACG-3′). I-A_β genes were amplified as follows: denaturing at 94°C for 1.5 min, annealing at 55°C for 1.0 min and extension at 72°C for 1.0 min, for 25 cycles on a DNA thermal cycler.

Flow cytometric analysis

The peripheral blood mononuclear cells of NOD mice were stained with phycoerythrin-labeled anti-B220 (Dainippon, Osaka, Japan), FITC-labeled anti-B220 (PharMingen, San Diego, CA) and biotinylated anti-CD3ε (PharMingen) mAb. The cells were washed with PBS containing 2% fetal bovine serum and 0.1% NaN_3, followed by streptavidin–Red670 (Gibco, Grand Island, NY) staining. The cells were then fixed with 1% paraformaldehyde and analyzed by a flow cytometer.

Assessment of serum Ig levels

Serum Ig levels were determined by ELISA. Briefly, sera for the Ig assay were collected from mice at 30 weeks of age. Plastic plates coated with anti-Ig (Rougier Biotech, Canada) were used after blocking with 5% non-fat milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0). After washing the plate with TBST, diluted serum samples were added to the plates and incubated at 37°C for 2 h. The bound IgM or IgG was detected with horseradish peroxidase-labeled goat anti-mouse IgM antibody (Tago, Burlingame, CA) or goat anti-mouse IgG antibody (Zymed, San Francisco, CA), and developed with TMB with the TMB microwell peroxidase substrate system (Kirkegaad & Perry, Gaithersburg, MD).

Glucose tolerance test and assay for serum insulin levels

Mice at 40 weeks of age were i.p. injected with 1 g/kg body weight of glucose and blood samples were then obtained at 0, 60 and 120 min after injection. The concentrations of insulin in the samples were determined by ELISA (Morinaga Biochem., Yokohama, Japan). All data are the means ± SEM, $n = 3$ per group.

Histological analysis

Blocks of pancreas were obtained at 12 and 30 weeks of age. For light microscopy, the pancreases were fixed with formalin and embedded in paraffin. Then, 4 μm thick sections made at 15 μm intervals for each pancreas were stained with hematoxylin & eosin. All islets were evaluated and the insulitis scores were determined as follows: 0: no mononuclear cell infiltration; 1: mild peri-insular mononuclear cell infiltration; 2: moderate mononuclear cell infiltration into the islets (granulation of $<$50%); 3: severe massive cell infiltration (granulation of $>$50%).

Results

Generation of B cell-deficient NOD mice

B cell-deficient (μMT/μMT) [129/Sv×C57BL/6 (B6)] mice were originally produced by disrupting the membrane exon of the IgM gene by gene targeting (19). μMT/+ B6 mice were

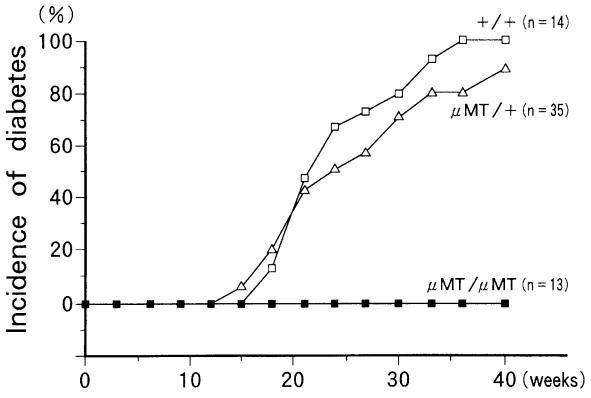

Fig. 2. Cumulative incidence of diabetes in the B⁻NOD and control B⁺NOD mice. Thirteen B⁻NOD (μMT/μMT) mice and control B⁺NOD littermates (15 +/+ and 34 μMT/+) were monitored for blood glucose levels for 40 weeks and the cumulative incidence of diabetes was determined. Open squares, +/+ B⁺NOD mice; open triangles, μMT/+ B⁺NOD mice; closed squares, μMT/μMT B⁻NOD mice.

previously generated by six backcrosses to B6 mice. μMT/+ B6 mice were backcrossed to +/+ NOD mice 8 times and μMT/+ mice with 99.6% NOD background were obtained. μMT/+ B⁺NOD males and females were crossed and assessed for their genetic background and also for the presence of B cell deficiency.

Thirteen μMT/μMT B⁻NOD mice and control B⁺NOD littermates (15 +/+ and 34 μMT/+) were obtained. The B⁻NOD and B⁺NOD mice were bred under specific pathogen-free conditions at the Kyushu University Animal Center. The mice were genotyped for μMT and wild-type alleles by PCR (Fig. 1a). Flow cytometric analysis of peripheral blood mononuclear cells of B⁻NOD mice revealed the absence of $B220^+$ B cells (Fig. 1b). The levels of serum IgG and IgM in B⁺NOD mice were 12.50 ± 3.73 and 0.80 ± 0.05 g/l respectively, while those of B⁻NOD mice were <0.4 g/l for IgG and <0.02 g/l for IgM, thus confirming the lack of Ig production in B⁻NOD mice. The I-A_β gene of the class II molecules in the parental B⁻NOD mouse was determined to be I-A_β^7, which is a unique class II gene of original NOD mice (20), according to the PCR findings (Fig. 1c).

Development of diabetes in B⁺NOD and in B⁻NOD mice

The development of diabetes in wild-type (+/+), μMT/+ B⁺NOD and μMT/μMT B⁻NOD mice was followed for 40 weeks. Every 3 weeks blood samples were monitored for blood glucose levels and diabetes was diagnosed when the blood glucose levels were >200 mg/dl. As shown in Fig. 2, diabetes developed in wild-type (+/+) and μMT/+ B⁺NOD mice beginning at 15 weeks of age and 87.8% (43 of 49) of B⁺NOD mice developed diabetes up to 40 weeks of age. In contrast, none (of 13) of the B⁻NOD mice developed diabetes by 40 weeks of age. Thus, the development of diabetes was prevented in B⁻NOD mice ($P < 0.0001$ as assessed by the Fisher's exact test), indicating that B cells are critically important for the development of diabetes in NOD mice.

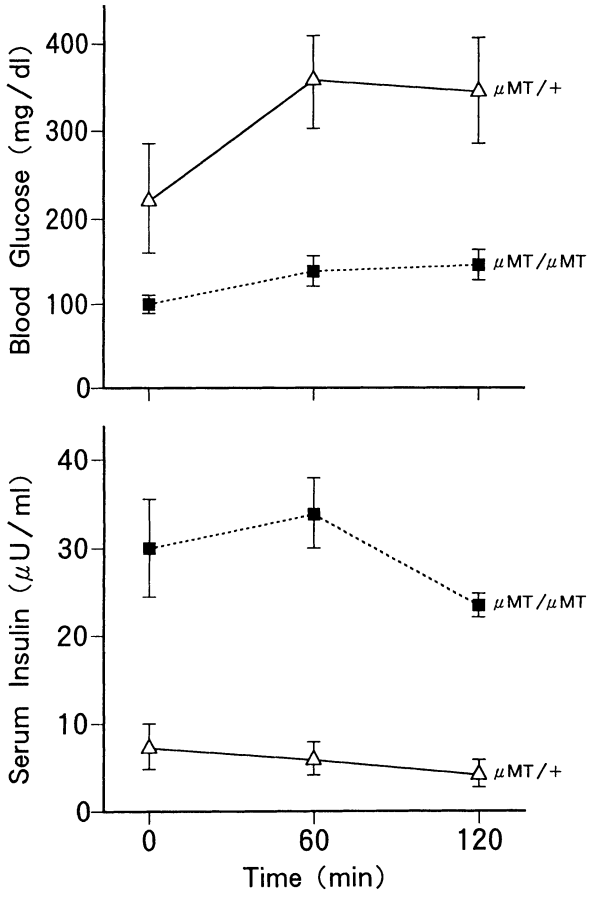

Fig. 3. Glucose and insulin reactivity in the B⁻NOD mice (μMT/μMT) and control B⁺NOD (μMT/+) mice after glucose loading. All data are the means ± SEM, $n = 3$ per group. Open triangles, μMT/+ B⁺NOD mice; closed squares, μMT/μMT B⁻NOD mice.

Glucose response and insulin reactivity after glucose loading in B⁺NOD and in B⁻NOD mice

B⁺NOD mice as well as the B⁻NOD mice were i.p. challenged with 1 g/kg body wt of glucose at 40 weeks of age. The blood glucose levels of the B⁺NOD mice showed a diabetic pattern >200 mg/dl at 120 min, while the B⁻NOD mice maintained normal glucose levels (Fig. 3). Although the insulin reactivity of B⁺NOD mice was significantly impaired, the B⁻NOD mice showed a good insulin response (Fig. 3), thus suggesting pancreatic β cell function to be well preserved in B⁻NOD mice.

Development of mild insulitis and suppression of the progression of insulitis in B⁻NOD mice

Although the development of mild insulitis was observed in B⁻NOD mice at 12 weeks of age, the extent of insulitis was lower in B⁻NOD mice than in B⁺NOD mice (Table 1). B⁻NOD mice showed mild insulitis at 30 weeks of age, at which time mild peri-insular mononuclear cell infiltration often associated with mild lymphocytic infiltration into the islets was seen (Fig. 4, left). In contrast, B⁺NOD mice developed moderate to severe insulitis at the same age, in which moderate mono-

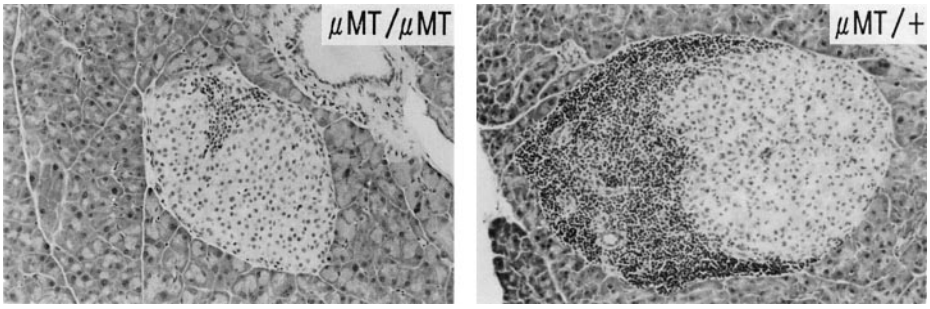

Fig. 4. Representative histopathology of insulitis in a B⁻NOD (μMT/μMT) mouse and a B⁺NOD (μMT/+) mouse at 30 weeks of age. Left: mild insulitis (insulitis score: 1) of a B⁻NOD (μMT/μMT) mouse: hematoxylin & eosin, ×200. Right: moderate insulitis (insulitis score: 2) of a B⁺NOD (μMT/+) mouse: hematoxylin & eosin, ×200.

Table 1. Incidence and grading of insulitis in B⁻NOD mice and B⁺NOD control littermates

Mice	Age (weeks)	No. of islets analyzed	Total no. of islets graded (percent of total): insulitis score			
			0	1	2	3
B⁺NOD (μMT/+)	12	300	99 (33.0)	77 (25.7)	67 (22.3)	57 (19.0)
B⁻NOD (μMT/μMT)	12	300	230 (76.7)	42 (14.0)	24 (8.0)	4 (1.3)
B⁺NOD (μMT/+)	30	300	80 (26.7)	68 (22.7)	79 (26.3)	73 (24.3)
B⁻NOD (μMT/μMT)	30	300	246 (82.0)	44 (14.7)	9 (3.0)	1 (0.3)

nuclear cell infiltration into the islets producing a lymphfollicle-like appearance (Fig. 4, right). Consistently, the insulitis scores of the B⁻NOD mice were significantly lower ($P < 0.0001$ as assessed by the Mann–Whitney test) than those of the B⁺NOD mice at either 12 or 30 weeks of age (Table 1), thereby indicating that the progression of insulitis was significantly suppressed in B⁻NOD mice.

Discussion

The present study demonstrates that diabetes did not develop during the 40 weeks observation period in B cell-deficient NOD mice, while as high as 90% of control NOD mice with B cells developed diabetes. In addition, although the B cell-deficient NOD mice did develop insulitis, the extent of insulitis was significantly suppressed, thereby indicating that B cells are crucial for the progression of insulitis as well as the development of diabetes in NOD mice.

Recently, Serreze *et al*. that reported neither insulitis nor diabetes developed in B cell-deficient (μMT/μMT) NOD mice, drawing a conclusion that B cells were required for the initiation of T cell-mediated autoimmunity in NOD mice (21). They interpreted the data that B cells might play an important role, at least as antigen-presenting cells to T cells in the initiation of insulitis (21). In this study, we demonstrated that mild insulitis was present in the B⁻NOD mice as early as 12 weeks of age, although the extent of insulitis was limited. Accordingly, it is suggested that initial activation of T cells against islets might occur, but further expansion of T cells might be impaired in B⁻NOD mice. Since antigen-presenting cells such as macrophages and dendritic cells are intact in B cell-deficient mice, the initial activation of T cells would be operative in B cell-deficient mice. Consistently, previous studies have reported that T cells were normally primed with peptide or parasite antigens in B cell-deficient mice and that peripheral tolerance was also successfully achieved (22–24). The reason for the discrepancy regarding the development of insulitis in our study and the previous study is not clear. This may be because they evaluated the insulitis only at 20 weeks of age, when insulitis may have not become evident or disappeared in the B⁻NOD mice they used. Alternatively, this may be due to differences in some unidentified genetic background between mice they used and ours.

Although the development of mild insulitis in B⁻NOD mice was observed in our study, progression of insulitis was significantly suppressed. Since it was reported that T cells predominantly infiltrated into the insulitic lesion of NOD mice (6,7), the expansion of T cells might be suppressed in B⁻NOD mice. We found the predominant accumulation of $CD4^+$ T cells in the insulitic lesion of islets in B⁺NOD mice (K. Anzai *et al*., manuscript submitted), while the proliferation of the $CD4^+$ T cells in the insulitic lesion was prominently suppressed in B⁻NOD mice. Recently, several reports have indicated the critical role of B cells in the expansion of $CD4^+$ T cells (23–25). In a parallel study, we created B cell-deficient B6 *lpr* mice, and found both lymphadenopathy and lymphoproliferation to be significantly suppressed in such animals, concomitant with the suppression of proliferation of $CD4^+$ T cells (I. Akashi *et al*., manuscript submitted). Based on these data, it is reasonable to postulate that the initial activation of T cells might be preserved in B⁻NOD mice, while the expansion of autoreactive $CD4^+$ T cells might be impaired, preventing a further progression of insulitis.

It was reported that anti-B7-2 (a CD28 ligand)-mediated co-stimulatory signal through CD28 expressed on T cells plays an important role in the proliferation of autoreactive T

cells in NOD mice (26). Indeed, recent advances suggested that B7-1- and B7-2-mediated co-stimulatory signals play a role in the proliferation and differentiation of T cells (27). In addition, it was also recently reported that CD40 engagement by the CD40 ligand (CD40L) expressed on antigen-activated T cells is critical for the up-regulation of B7 molecules on antigen-presenting B cells for both T cell proliferation and differentiation (28). These observations taken together suggest the presence of a T cell–B cell augmentation circuit mediated by T cell–B cell interaction, which is operative by CD40L–CD40 ligation, followed by the up-regulation of B7 molecules on B cells, with the subsequent stimulation of T cells via CD28 expressed on T cells. Accordingly, the prevention of progression of severe insulitis in B^-NOD mice in our study might be due to the lack of B cell-mediated co-stimulatory signals which might be essential for the late activation and/or proliferation of autoreactive T cells against pancreatic β cells.

The question as to whether or not antigen-specific clonal T cell expansion was regulated by B cells should be clarified in further studies, since clonal T cells specific for islet cell antigen could successfully transfer diabetes in NOD mice (12–14). Another possible contribution of B cells in NOD diabetes is that B cells may play an important role as antigen-presenting cells, especially in the shift of the T cell repertoires which are responsible for the development of diabetes from the stage of mild insulitis to severe insulitis (29,30). It is also possible that autoantibodies produced by B cells against certain islet β cell-associated antigens may contribute to the progression of diabetes (16).

Although further detailed analysis is required to clarify the precise role of B cells in the pathogenesis of NOD mice, the present study demonstrates that B cells are essential for the progression of autoimmunity in NOD mice, thus providing new insight into the development of autoimmune diseases with respect to the role of B cells in addition to autoantibody production. A better understanding of the role of B cells in autoimmunity will therefore help to promote the development of new treatment strategies for autoimmune diseases using B cell-directed therapy.

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Abbreviations

CD40L	CD40 ligand
NOD	non-obese diabetic mice

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