The Influence of the Major Histocompatibility Complex on Development of Autoimmune Diabetes in RIP-B7.1 Mice

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The most important genetic susceptibility factor for type 1 diabetes is encoded in the major histocompatibility complex (MHC). The nonobese diabetic (NOD) mouse, which develops spontaneous diabetes, expresses H-2^{g7} comprising the MHC class I molecules K^d and D^b and the MHC class II molecule I-A^{g7}. However, neither B6.H-2^{g7} mice, in which H-2^{g7} is expressed on the C57BL/6 genetic background, nor the nonobese resistant (NOR) mouse, in which H-2^{g7} is expressed on a genetic background that is 88% similar to NOD mice, develop diabetes. Immune tolerance can be broken in these diabetes-resistant mice expressing H-2^{g7} if the costimulatory molecule B7.1 is present on the islet β cells. This does not occur if only single MHC class I components of the H-2^{g7} haplotype are present, such as K^d in BALB/c mice or D^b in C57BL/6 mice, both of which develop only a low level of diabetes when B7.1 is expressed. The presence of I-A^{g7} leads to the development of an autoimmune T-cell repertoire, and local costimulation of CD8 T-cells precipitates aggressive diabetes. This implies that a major role of the MHC class II molecules in diabetes is the development of an autoreactive T-cell repertoire. *Diabetes* 54:2032–2040, 2005

T-cell activation is dependent on at least two signals: the first is the specific stimulation through the T-cell receptor, and the second is a costimulatory signal expressed on antigen-presenting cells (1). In vitro studies have suggested that CD8 T-cells require costimulation through CD28 to become activated, but once effector cells are generated, there is less requirement for costimulation for full effector function (2). Tissue cells are normally protected from attack by self-reactive CD8 T-cells by a low expression of major histocompatibility complex (MHC) class I and the absence of costimulatory molecules, which prevents activation of naive T-cells (3). CD8 T-cells will become activated and proliferate with CD4 T-cell help, which can provide interleukin-2 (4) or induce CD40 activation on antigen-presenting cells, which can then "license" the CD8 T-cells (5–7).

Previous studies have shown that the expression of the costimulatory molecule B7.1 alone on islet β-cells, using the rat insulin promoter (RIP) on an autoimmune diabetes–resistant genetic background, does not break tolerance to the tissue (8–10). In these models where RIP-B7.1 is expressed on a B6 or bm1 background, a second transgene that predisposed to inflammation, such as TNF-α (tumor necrosis factor-α) (9) or the local expression of interleukin-2 (10), was required to break islet immune tolerance.

However, the expression of the B7.1 molecule on the pancreas will allow development of spontaneous diabetes in B6 mice if the MHC class II molecule (in this case, I-A^b) is replaced by human MHC class II molecules that predispose to autoimmune diabetes (11,12). Similarly, on the highly susceptible NOD background, the expression of RIP-B7.1 will significantly accelerate diabetes (10,13).

Susceptibility to autoimmune diabetes is clearly controlled by a number of factors. Under normal circumstances both genetic factors and environmental modifiers are required for development of disease. However, although the MHC is a major genetic factor, the expression of predisposing MHC molecules on an otherwise nonsusceptible genetic background is not sufficient for diabetes to occur. Diabetes does not develop in congenic mice that express MHC H-2^{g7} on a C57BL/6 background. Similarly, the nonobese resistant (NOR/Lt) mouse, though sharing the MHC with NOD mice (14,15), is a diabetes-free strain that differs from the NOD mouse over a number of chromosomes approximating 11.6% of the genome (15). Previous studies had shown that NOR/Lt mice were distinguished from NOD mice by expressing peri-insulitis that does not progress further to diabetes (16) and by exhibiting more robust suppressor T-cell function (14). Thus, in these mice, the presence of the predisposing MHC is not sufficient to overcome the resistance factors derived from the C57BL/KsJ mouse.

It is clear that both CD4 and CD8 T-cells are required for spontaneous diabetes in the NOD mouse. The activation of CD8 T-cells possibly occurs outside the islets because there is no evidence that islet cells express costimulatory molecules, even under inflammatory conditions (17). It is likely that one of the major functions of the predisposing MHC class II molecule in the NOD mouse is to select for and activate autoreactive CD4 T-cells, which then facili-

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MHC, major histocompatibility complex; RIP, rat insulin promoter.

tate the traffic of the CD8 T-cells to the islets. In the RIP-B7.1 transgenic model, the requirement for CD4 T-cells is not absolute (18,19), but a combination of factors that will allow CD8 cytotoxic T-cells to traffic to the islets and become activated considerably increases the efficiency and speed of disease development. A high incidence of rapid-onset spontaneous diabetes could be induced in C57BL/6 mice in the presence of both B7.1 and TNF-α transgenes expressed on the islets. In this case, accelerated diabetes was found as early as 3 weeks of age, implying that expression of inflammatory cytokines was necessary to attract the autoreactive T-cells to the islets (9).

In this study, we have examined the effect of expression of a number of MHC haplotypes on different background strains in facilitating the development of islet autoreactivity and diabetes in the presence of the RIP-B7.1 transgene, which alone is insufficient to precipitate diabetes. We provide evidence that the expression of predisposing H-2^{g7} alone is sufficient to cause ultimate islet β-cell destruction in the presence of a costimulatory molecule that can activate autoreactive CD8 T-cells. We suggest that the MHC class II molecules play a definitive role in facilitating the development of autoimmune diabetes by selecting an autoimmune repertoire that can facilitate diabetes, given the appropriate stimulus to the CD8 T-cells.

RESEARCH DESIGN AND METHODS

C57BL/6 mice (B6) expressing B7.1 costimulatory molecules on pancreatic β-cells under the RIP were generated as previously described (20). These mice are designated B6/RIP-B7.1 mice. BALB/c mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and bred with our B6/RIP-B7.1 mice. To obtain BALB/c/RIP-B7.1 mice, we backcrossed BALB/c × B6/RIP-B7.1 F1 mice to BALB/c mice for 10 generations. B6 congenic mice expressing NOD MHC genes (B6.H-2^{g7}) were kindly provided by Diane Mathis and bred with our B6/RIP-B7.1 mice. Because both strains were on the same B6 genetic background, we only needed to intercross the F1 generations to obtain B6.H-2^{g7}/RIP-B7.1 mice. NOD congenic mice expressing the MHC haplotype $H2^{i7}$ ($K^bD^dI\text{-}A^b$) were kindly provided by Linda Wicker. To generate NOR/Lt mice expressing RIP-B7.1, we bred NOR/Lt mice (Jackson Laboratories) to B6/RIP-B7.1 mice. The F1 progeny were further backcrossed to the NOR/Lt genetic background for six generations.

The RIP-B7.1 transgene was also introduced into NOD.scid mice (previously obtained from Jackson Laboratories). NOD.scid/RIP-B7.1 mice were obtained by repeated backcross of B6/RIP-B7.1 mice onto NOD.scid mice, a process that was commenced in the Jackson Laboratories and continued at Yale University. The RIP-B7.1 transgene expression in each strain of mouse used in the study was detected by PCR amplification of the B7.1 gene, using genomic DNA isolated from tail biopsies. We screened for the expression of H-2^{g7} in the B6.H-2^{g7}/RIP-B7.1, NOR/RIP-B7.1, and NOD.scid/RIP-B7.1 mice, using flow cytometric analysis of peripheral blood lymphocytes with monoclonal antibodies to K^d (SF-1.1.1; BD Bioscience, San Diego, CA), D^b (KH95; BD Bioscience), and I-A^k (10.3.6 cross-reactive to I-A^{g7}; BD Bioscience). We screened for the scid mutation by the absence of both T-cells (CD3, 2C11; BD Bioscience) and B-cells (B220, RA3–6B2; BD Bioscience) in peripheral blood lymphocytes, using flow cytometry. Diabetes development was assessed by weekly screening for glycosuria, and the disease was confirmed by measurement of blood glucose >13.9 mmol/l (>250 mg/dl). The mice were maintained in specific pathogen–free facilities at Yale or Bristol University, and the experiments were undertaken in accordance with approved Yale animal care and use committee protocols and U.K. Home Office–approved protocols.

Reagents. Human (soluble) and bovine/porcine (Lente) insulin were purchased from Eli Lilly (Indianapolis, IN). All of the fluorochrome-conjugated or biotinylated monoclonal antibodies used in the study were purchased from BD Bioscience. Bruff's medium and FCS were purchased from Invitrogen (Carlsbad, CA) and Gemini (Woodland, CA), respectively.

Proliferation assays. Splenocytes (10^5 per well) were assayed for antigenic response against islets by culturing them in the presence of irradiated (4,000 rads) handpicked pancreatic islets (21) in 96-well round-bottomed plates in triplicate. After 72 h, the wells were pulsed with 0.5 μCi [^{3}H]thymidine and cultured for a further 14–16 h, after which the plates were harvested and counted in a β-plate counter. Measurements in counts per minute (cpm) were converted to Δcpm, calculated as: cpm with islets − (cpm without islets + cpm of islet cells alone). To investigate the subset of T-cells that were responsive to islet autoantigens, we also performed inhibition assays, in which the B6.H-2^{g7} or NOR Splenocytes (10^5 per well) were cultured with irradiated handpicked pancreatic islets in the presence or absence of anti-CD4 (GK1.5), anti-CD8 (TIB 105), or control rat IgG.

CD4 and CD8 T-cell depletion. Splenocytes were incubated with either anti-CD8 antibody (TIB105) or anti-CD4 antibody (GK1.5) for 30 min on ice. Cells were washed and then incubated with BioMag goat anti-rat Ig–conjugated beads from Qiagen (Valencia, CA) for 45 min while shaking on ice. CD8- or CD4-depleted splenocytes were then washed and used for adoptive transfer experiments.

Adoptive transfer of diabetes. Diabetic splenocytes (10^7 per recipient) from different donors were injected into B6.scid, CB17.scid, NOD.scid, and NOD.scid/RIP-B7.1 mice (5–7 weeks of age) as the recipients for the adoptive transfer of diabetes. Irradiated (650 rads) B6.H-2^{g7}/RIP-B7.1 and NOR/RIP-B7.1 mice were also used as adoptive transfer recipients. All of the mice were monitored for glycosuria, and the experiments were terminated 15 weeks after adoptive transfer unless the mice developed diabetes, which was confirmed by blood glucose (>13.9 mmol/l), and they were killed immediately.

Further adoptive transfer experiments using splenocytes (10^7 per recipient) from 6- to 10-week-old B6, BALB/c, NOR, B6.H-2^{g7}, and NOD mice were transferred into NOD.scid/RIP-B7.1 recipients (5–7 weeks of age). All of the mice were monitored for glycosuria, and the experiments were terminated 12 weeks after adoptive transfer unless the mice developed diabetes, which was confirmed by blood glucose (>13.9 mmol/l), and they were killed immediately. To confirm the role for both CD4 and CD8 T-cells in the adoptive transfer of diabetes, CD4-depleted NOR and B6.H-2^{g7} spleen cells and CD8-depleted B6.H-2^{g7} spleen cells were also individually adoptively transferred into NOD.scid/RIP-B7.1 recipients, and the mice were observed for diabetes. Splenocytes from NOR, B6.H-2^{g7}, and NOD mice were also transferred into NOD.scid and CB17.scid recipients (10^7 per recipient). The recipients were also monitored for diabetes development as described above.

Histology. Paraffin sections of pancreas were prepared and stained with hematoxylin and eosin and scored for insulitis. Some pancreata were fixed in paraformaldehyde lysine periodate–based fixing buffer, sucrose infused, and frozen in optimal cutting temperature compound (21). Cryosections were stained for anti-CD4, anti-CD8, or anti-B220, using biotinylated antibodies from BD Bioscience.

RESULTS

B6.H2^{g7}/RIP-B7.1 mice develop a high incidence of spontaneous diabetes. It is known that in addition to the MHC, other factors also contribute to the development of autoimmune diabetes. To evaluate the effect of islet microenvironmental change in promoting autoimmune diabetes, we used B6 congenic mice (B6.H-2^{g7}) that express NOD MHC (H-2^{g7}) but are resistant to autoimmune diabetes, and we compared them with B6.H-2^{g7}/RIP-B7.1 and B6/RIP-B7.1 transgenic mice. We generated cohorts of mice that were either homozygous (g7/g7) or heterozygous (b/g7) for H-2^{g7} and were expressing the B7.1 transgene on their pancreatic islets. The natural history of spontaneous diabetes development was studied in these mice. Despite the presence of the diabetes-resistant B6 genetic background, all of the groups of B6.H-2^{g7}/RIP-B7.1 mice, homozygous (g7/g7) or heterozygous (b/g7) for H-2^{g7}, developed spontaneous diabetes, as shown in Fig. 1*A*. It is interesting that irrespective of whether the mice were homozygous or heterozygous for H-2^{g7}, diabetes developed with a similar incidence in the female mice, but there was a delay in the onset of diabetes in the male mice heterozygous for H-2^{g7}. This difference was statistically significant ($P = 0.0004$). In contrast, none of the B6.H-2^{g7} mice, either homozygous or heterozygous for H-2^{g7}, developed autoimmune diabetes in the absence of the RIP-B7.1 transgene (Fig. 1*B*). Pancreatic sections stained with hematoxylin and eosin were examined at the termination of

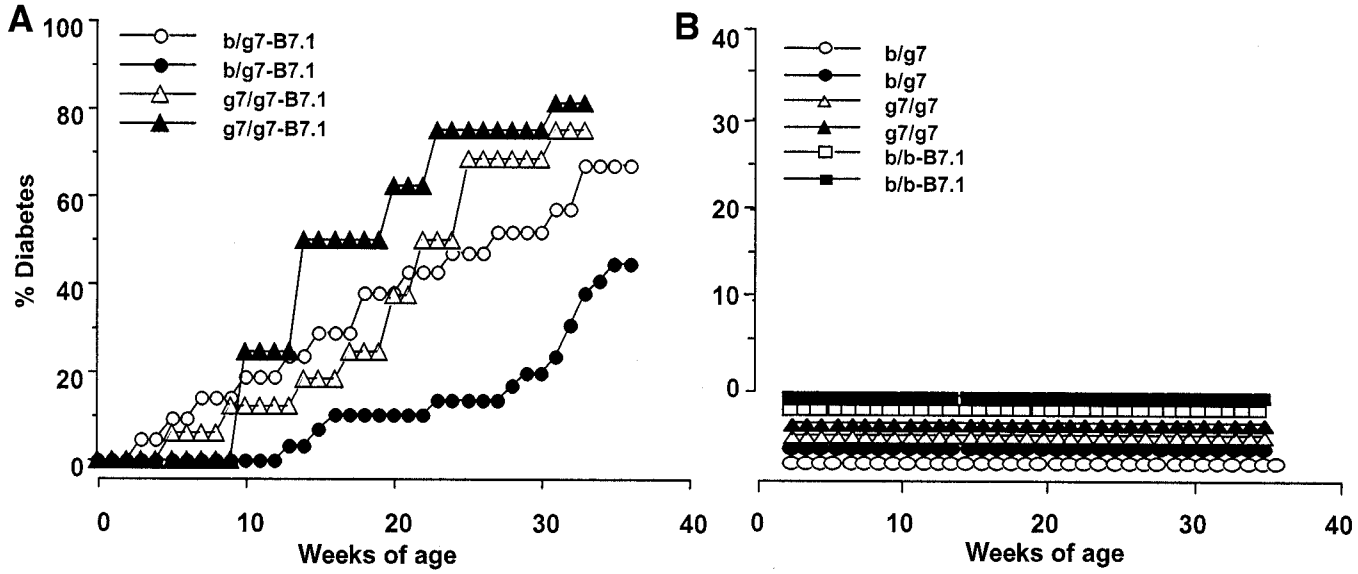

FIG. 1. Diabetes development in B6.H-2^{g7}/RIP-B7.1 and control mice. *A*: Groups of mice were observed for diabetes over 36 weeks. B6.H-2^{g7} mice were crossed with B6/RIP-B7.1 mice, and the B6.H-2$^{b/g7}$/RIP-B7.1 (F1) and B6.H-2$^{g7/g7}$/RIP-B7.1 (F2) mice observed. Female mice are shown in open symbols and males in closed symbols. Mice were positive for the RIP-B7.1 transgene and homozygous H-2$^{g7/g7}$ (shown in triangles; females n = 16, males n = 16) or heterozygous H-2$^{g7/b}$ (shown in circles; females n = 21, males n = 29). The only statistically significant difference in the survival (log rank test) was between the male homozygous H-2$^{g7/g7}$ and male heterozygous H-2$^{g7/b}$ groups (P = 0.0004). Diabetes was diagnosed by the presence of glycosuria and confirmed by a blood glucose measurement >13.9 mmol/l. *B*: Control groups consisted of mice that did not express the RIP-B7.1 transgene and B6 mice that express the RIP-B7.1 transgene. Female mice are shown in open symbols and males in closed symbols. Mice homozygous for H-2$^{g7/g7}$ are shown in triangles (females n = 20, males n = 20). Mice heterozygous for H-2$^{g7/b}$ are shown in circles (females n = 28, males n = 32). The B6/RIP-B7.1 mice (H-2$^{b/b}$) are shown in squares (females n = 20, males n = 20).

the experiments, and none of the control B6.H-2^{g7} or B6.H-2$^{g7/b}$ mice examined developed insulitis (data not shown).

NOR/RIP-B7.1 mice also develop a high incidence of spontaneous diabetes. To investigate whether the expression of the B7.1 molecule on pancreatic β-cells could break organ-specific peripheral tolerance in diabetes-resistant NOR mice, we generated NOR/RIP-B7.1 mice by repeated backcross (six generations) of B6/RIP-B7.1 mice to the NOR/Lt strain. It is interesting that a higher incidence and slightly earlier onset of spontaneous diabetes was found in NOR/RIP-B7.1 mice, even in the first generation (F1) (Fig. 2). It appears that further backcross to the NOR strain did not obviously increase the incidence of diabetes or accelerate the time of disease onset (Fig. 2). Instead, there was a tendency for reduced diabetes incidence in later backcross generations. As seen in other RIP transgenic systems, there was no obvious sex preference in the accelerated disease development.

Interestingly, we observed that the coat color of all the F1 progeny was black, not agouti, and even at the N4 generation of backcross to NOR, 2 of 11 mice had a black coat color. This phenomenon indicates the presence of the C57BL/KsJ genes in the NOR strain, as reported previously (14). However, from the N5 generation onwards, all of the progeny were albino as in the parental NOR/Lt strain.

BALB/c/RIP-B7.1 and B6/RIP-B7.1 mice are resistant to the development of autoimmune diabetes. To test whether the expression of the B7.1 costimulatory molecule on pancreatic β-cells could also break peripheral immune tolerance in other diabetes-resistant strains, we introduced the RIP-B7.1 transgene into BALB/c mice by backcross of B6/RIP-B7.1 mice to the BALB/c genetic background for 10 generation. We then observed the natural history of diabetes development in 62 BALB/c/RIP-B7.1 mice (35 females and 27 males) for 30 weeks (Fig. 3). As seen previously in B6/RIP-B7.1 mice, a relatively low number of mice developed spontaneous diabetes; 4 of 35 (11.4%) female BALB/c/RIP-B7.1 mice became diabetic (Fig. 3). However, none of the 40 B6/RIP-B7.1 mice (20 of each sex) developed diabetes in this study (data not shown). Thus, when only K^d or D^b is expressed (MHC class I components of the H-2^{g7} haplotype) diabetes does not occur, or it occurs at a low incidence (in the case of K^d). BALB/c mice express the MHC class II molecules I-A^d and I-E^d, and B6 mice express I-A^b but not the MHC class II molecule of the H-2^{g7} haplotype, I-A^{g7}.

Diabetes can be transferred to nondiabetic B6.H-2^{g7}/RIP-B7.1, nondiabetic NOR/RIP-B7.1, or NOD.scid/RIP-B7.1 recipients. To test whether the disease could be transferred by diabetogenic lymphocytes with enhanced potency in the presence of the RIP-B7.1 transgene, we performed adoptive transfer experiments using splenocytes from diabetic B6.H-2^{g7}/RIP-B7.1 or diabetic NOR/RIP-B7.1 mice. Four types of scid mice were used as recipients: B6.scid, CB17.scid, NOD.scid, and NOD.scid/RIP-B7.1. In addition, irradiated B6.H-2^{g7}, irradiated B6.H-2^{g7}/RIP-B7.1, and irradiated NOR/RIP-B7.1 mice were also used as recipients. As shown in Fig. 4*A* diabetic B6.H-2^{g7}/RIP-B7.1 splenocytes could transfer diabetes to NOD.scid/RIP-B7.1 and irradiated B6.H2^{g7}/RIP-B7.1 mice. Similarly, diabetic NOR/RIP-B7.1 splenocytes also transferred diabetes only to NOD.scid/RIP-B7.1 mice and irradiated NOR/RIP-B7.1 mice (Fig. 4*B*). Both diabetic B6.H-2^{g7}/RIP-B7.1 splenocytes and diabetic NOR/RIP-B7.1 splenocytes failed to transfer the disease to either NOD.scid (K^d,D^b), B6.scid (D^b), or CB17.scid (K^d) recipients. Furthermore, diabetes could not be transferred to irradiated B6.H-2^{g7} mice that

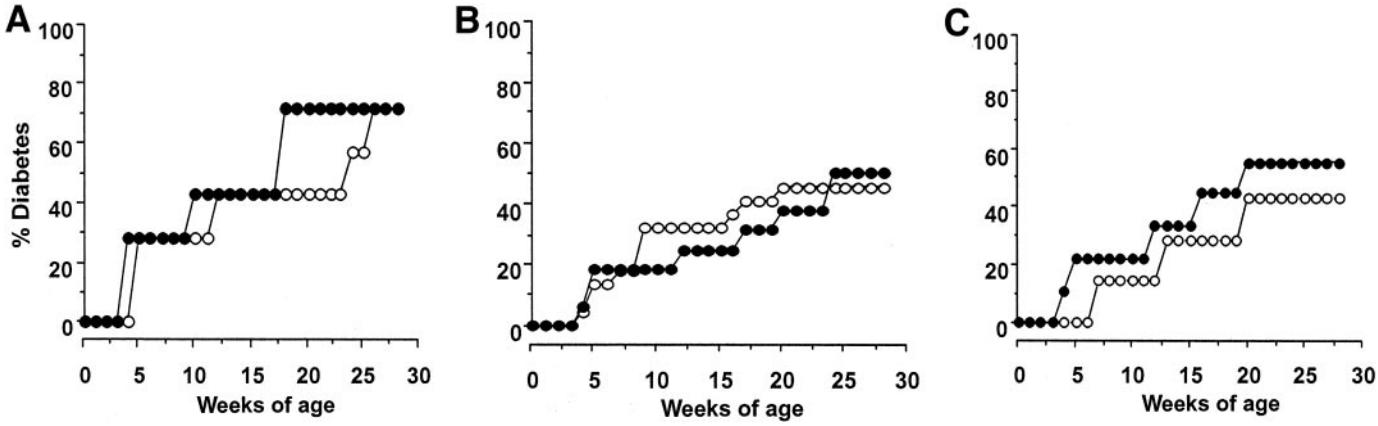

FIG. 2. Diabetes development in NOR/RIP-B7.1 mice. NOR mice were crossed with B6.RIP-B7.1 mice and then backcrossed to NOR mice. Results are from the first cross (F1) (*A*) and then after two backcrosses (N3) (*B*) and after four backcrosses (N5) (*C*) to NOR mice. In the F1 generation, 7 females and 7 males were observed; in the N3 generation, 16 females and 22 males were observed; and in the N5 generation, 9 females and 7 males were observed. Diabetes was diagnosed by the presence of glycosuria and confirmed by a blood glucose measurement >13.9 mmol/l. ●, Female NOR-B7.1 mice; ○, male NOR-B7.1 mice.

did not express the RIP-B7.1 transgene. Because expression of the RIP-B7.1 transgene is required for adoptive transfer in the RIP-B7.1 transgenic systems (10,18), we then used irradiated, but MHC partially mismatched, B6/RIP-B7.1 mice (K^bD^b, 6 weeks, $n = 6$, 650 rads) as recipients for the transfer of disease. Despite the expression of the RIP-B7.1 transgene, irradiated B6/RIP-B7.1 recipients did not develop diabetes after adoptive transfer of B6.H-2g7/RIP-B7.1 splenocytes. The MHC was only matched at the D^b locus in this case, and therefore it is not surprising that diabetes could not be transferred. The reconstitution after adoptive transfer in different recipients was comparable in the period of experiments because the cell count from the spleen of different recipients ranged from 7×10^6 to 10×10^6 cells. However, diabetes could easily be transferred into NOD.scid recipients expressing the RIP-B7.1 transgene (NOD.scid/RIP-B7.1), even though apart from MHC genes, the rest of the genetic backgrounds were very different between the donor (B6) and the recipients (NOD). The results from the adoptive transfer experiments suggest that the expression of matched MHC and RIP-B7.1 transgene are the only requirements for disease transfer in this model system.

Spontaneous islet autoreactivity in mice expressing MHC H-2^{g7} on diabetes-resistant genetic backgrounds. Even though NOR and B6.H-2^{g7} mice are normally resistant to the development of diabetes, in the presence of RIP-B7.1, these mice develop a high incidence of spontaneous diabetes. However, B6 and BALB/c mice are also resistant to diabetes development, and the presence of RIP-B7.1 does not cause them to develop a high incidence of spontaneous diabetes. It is possible that this could be related to the pre-existence of an autoreactive T-cell repertoire that can be stimulated to become diabetogenic effector cells. To investigate whether there is a difference in the islet autoreactivity in the four parental mouse strains that could explain the different incidence of diabetes in the presence of RIP-B7.1, we performed in vitro proliferation assays using splenocytes from the different strains of mice used in this study. It is interesting that islet autoreactivity was clearly associated with the expression of MHC H-2^{g7} ($n = 2$–4 mice for each strain) (Fig. 5) because spontaneous islet autoreactivity could be readily detected in diabetes-resistant B6.H-2^{g7} and NOR mice. However, B6 or BALB/c mice did not show obvious spontaneous autoreactivity to islets. To further confirm the association of MHC H-2^{g7} with islet autoreactivity, we tested an NOD congenic strain, NOD^{i7}, that has the NOD genetic background but has mismatched MHC and does not express I-A^{g7}. As shown in Fig. 5, islet autoreactivity in NOD^{i7} mice was very similar to that seen in B6 or BALB/c mice. The NOD genetic background, without the NOD MHC haplotype, did not predispose the mice to development of spontaneous islet autoreactivity.

To investigate the T-cell subset(s) responsible for the islet autoreactivity in B6.H-2^{g7} and NOR mice, we performed antibody blocking assays, in which splenocytes from B6.H-2^{g7} and NOR mice ($n = 2$ for each strain) were cultured with irradiated islets in the presence or absence of anti-CD4, anti-CD8, and control rat IgG (all at a concen-

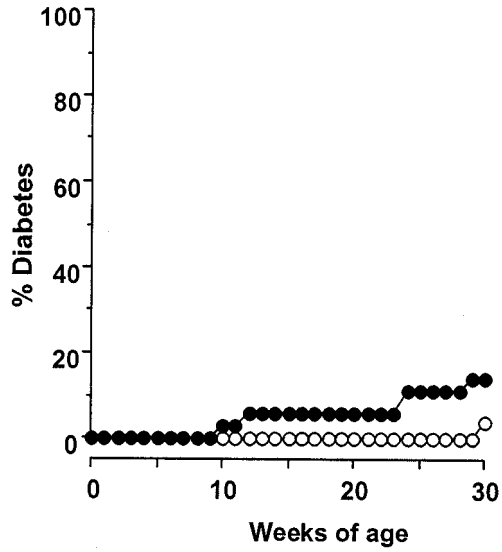

FIG. 3. Diabetes development in BALB/c/RIP-B7.1 mice. BALB/c mice were crossed with B6.RIP-B7.1 mice and then backcrossed to BALB/c mice for 10 generations. The mice were observed for diabetes over 30 weeks. Diabetes was diagnosed by the presence of glycosuria and confirmed by a blood glucose measurement >13.9 mmol/l. ●, Female BALB/c-B7.1 mice ($n = 35$); ○, male BALB/c-B7.1 mice ($n = 27$).

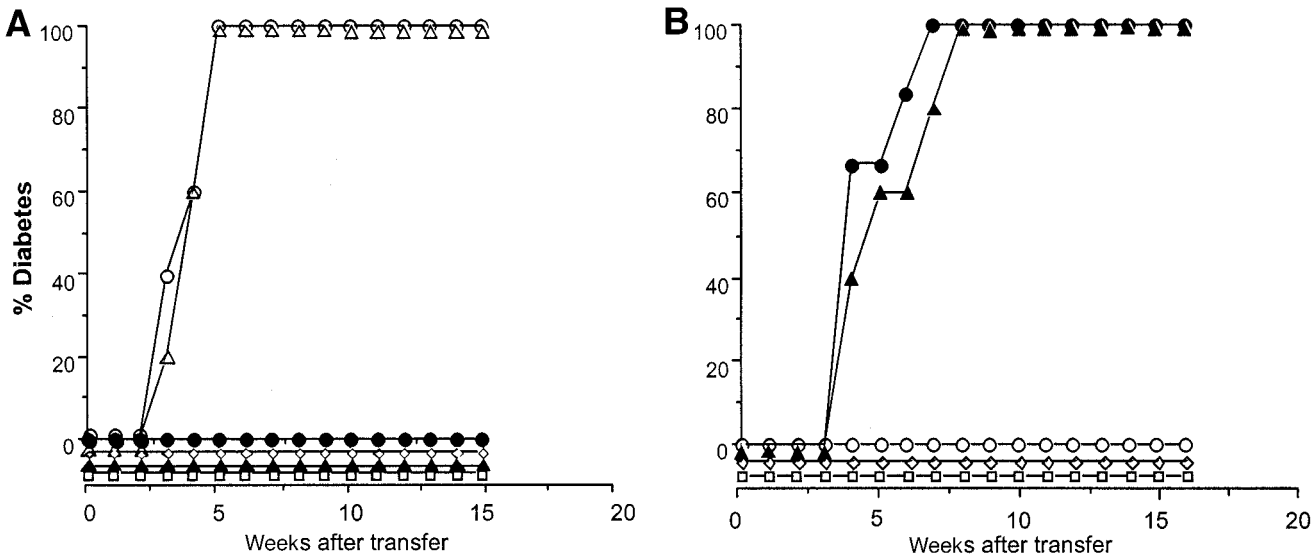

FIG. 4. Adoptive transfer of diabetes. *A*: Diabetic spleen cells from B6.H-2^{g7}/RIP-B7.1 mice were adoptively transferred into six types of mice, irradiated B6.H-2^{g7} mice (●, n = 5), irradiated nondiabetic B6.H-2^{g7}/RIP-B7.1 mice (○, n = 5), B6.scid (□, n = 6), CB17.scid (◇, n = 4), NOD.scid (▲, n = 8), and NOD.scid/RIP-B7.1 mice (△, n = 10). *B*: Diabetic spleen cells from NOR/RIP-B7.1 mice were transferred into five types of recipients, irradiated nondiabetic NOR/RIP-B7.1 mice (▲, n = 5), B6.scid (□, n = 6), CB17.scid (◇, n = 4), NOD.scid (○, n = 15), and NOD.scid/RIP-B7.1 mice (●, n = 15). Diabetes was monitored by the presence of glycosuria and confirmed by blood glucose measurements >13.9 mmol/l.

tration of 15 μg/ml). The blocking experiments suggested that both CD4 and CD8 T-cells were involved in the spontaneous islet autoreactivity seen in these two strains of mice (Fig. 6).

Spontaneous diabetes development in NOD.scid/RIP-B7.1 mice after receiving splenocytes expressing MHC H-2^{g7}. Diabetes-resistant B6.H-2^{g7} and NOR mice express obvious islet autoreactivity but remain insulitis and diabetes free, unless the B7.1 costimulatory molecule is expressed on islet β-cells, when both strains develop a high incidence of diabetes with early onset. In contrast, B6 and BALB/c mice, each expressing one of the MHC class I alleles present in H-2^{g7}, do not have detectable islet autoreactivity, and diabetes rarely occurs, even when the B7.1 costimulatory molecule is expressed on islet β-cells. We therefore hypothesized that any islet-reactive CD8 T-cells in B6.H-2^{g7} and NOR mice are quiescent, and their full differentiation into efficient effector diabetogenic cells would require both a secondary signal and CD4 T-cell help. To test this hypothesis, we conducted a series of adoptive transfer experiments in which NOD.scid/RIP-B7.1 mice were used as recipients and they received splenocytes from five strains of mice (B6.H-2^{g7}, NOR, NOD, B6, and BALB/c). We found that 100% of the NOD.scid/RIP-B7.1 mice developed diabetes if B6.H-2^{g7}, NOR, or nondiabetic young NOD splenocytes were adoptively transferred. However, no diabetes was found in the NOD.scid/RIP-B7.1 recipients if they received B6 or BALB/c spleen cells (Fig. 7*A*). Again, the reconstitution after adoptive transfer was comparable among the recipients in the period of experimentation. These results not only support our hypothesis but also mirror the natural history of diabetes development in the four strains of mice that carry the RIP-B7.1 transgene. To further confirm the requirement for CD4 T-cells in disease development, NOD.scid/RIP-B7.1 recipients were infused with CD4-depleted spleen cells from B6.H-2^{g7} or NOR mice (depletion >95% data not shown). As illustrated in Fig. 7*B*, without CD4 T-cells, neither B6.H-2^{g7} nor NOR splenocytes were able to induce diabetes development in NOD.scid/RIP-B7.1 mice. However, insulitis was still present in these mice when analyzed by histopathology (data not shown).

Although CD4 T-cells are important for diabetes development in this model system, it is known that the expression of RIP-B7.1 recruits and/or augments the diabetogenic CD8 T-cells as well. Our previous studies had shown that in the absence of CD8 T-cells in the NOD/RIP-B7.1 model, where mice were generated by crossing the NOD/RIP-B7.1 mice with NOD.β-2microglobulin$^{-/-}$ mice,

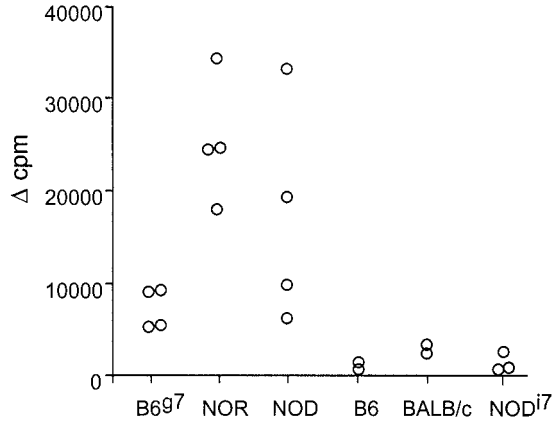

FIG. 5. Proliferative responses of splenocytes to islets. Splenocytes (10^5) were incubated with dispersed islet β-cells (25,000 per well) in triplicate. [^{3}H]thymidine was added after 72 h. Cells were harvested and counted after a further 14 h. These results represent 2–4 mice of each strain. The results are expressed in Δcpm, defined as the cpm with islets − (cpm without islets + cpm of islet cells alone), to facilitate comparison between experiments. Baseline counts without the addition of antigen ranged from 703 to 4,155 cpm for B6.H-2^{g7} (B6^{g7}) cells, from 2,032 to 5,500 cpm for NOR cells, from 4,210 to 10,504 cpm for NOD cells, from 712 to 1,174 cpm for B6 cells, from 1,225 to 3,774 cpm for BALB/c cells, and from 1,950 to 6,200 cpm for NOD.H-2^{i7} (NOD^{i7}) cells.

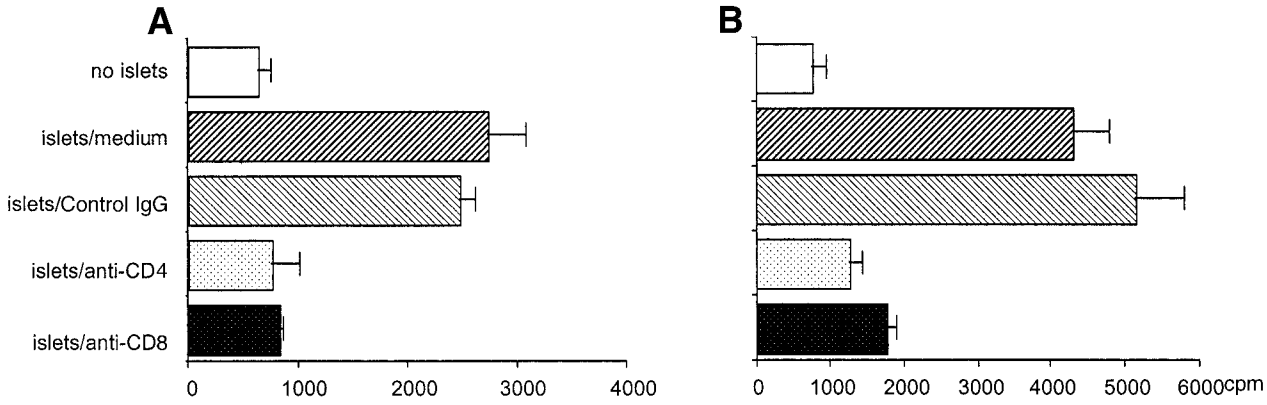

FIG. 6. Blocking assay proliferation of splenocytes to islets. B6.H-2^{g7} (*A*) or NOR (*B*) splenocytes (2×10^5) were incubated with irradiated islets (12 islets per well) in triplicate in the presence or absence of monoclonal antibody to CD4 (GK1.5), CD8 (TIB 105), and control rat IgG (all at the concentration of 15 mg/ml). [^{3}H]thymidine was added after 72 h, and cells were harvested and counted after a further 14 h. These results represent two separate experiments with two mice of each strain in each group.

a very low incidence of diabetes occurred (18). To confirm the importance of CD8 T-cells in the current RIP-B7.1 models, we adoptively transferred CD8-depleted B6.H-2^{g7} splenocytes into NOD.scid/RIP-B7.1 recipients. The depletion was 100%, as confirmed by fluorescence-activated cell sorter analysis (data not shown). As expected, after removing CD8 T-cells, B6.H-2^{g7} splenocytes lost the ability to induce diabetes in the NOD.scid/RIP-B7.1 recipients (Fig. 7*B*). Our results suggest that even in the RIP-B7.1 model system, both CD4 and CD8 T-cells are required for the disease to occur. This is further demonstrated by immunohistochemical analysis. In the adoptive transfer experiments using nondiabetic young NOD splenocytes transferred to either NOD.scid or NOD.scid/RIP-B7.1 recipients, frozen pancreatic sections were taken and stained for infiltrating CD4 and CD8 T-cells as well as B cells. As shown in Fig. 7*C*, in addition to many CD4 T-cells in the islets of both diabetic recipients, there were considerably more infiltrating CD8 T-cells in the diabetic NOD.scid/RIP-B7.1 recipients. This suggests that CD8 T-cells are critical for diabetes development in this model because the NOD.scid/RIP-B7.1 recipients developed 100% diabetes by 29 days after adoptive transfer (Fig. 7*A*) compared with 75% of NOD.scid recipients, which developed diabetes within 54–78 days ($n = 4$) (data not shown).

To strengthen the hypothesis that expression of RIP-B7.1 transgene augments and/or activates CD8 T-cells locally, we examined the number of CD8 T-cells and their activation markers in spleen and various lymph nodes from NOD.scid and NOD.scid/RIP-B7.1 recipients after transferring B6.H-2^{g7} or NOR splenocytes. It is interesting that the expression of RIP-B7.1 promoted the expansion of CD8 T-cells only in pancreatic lymph nodes but not in spleen or other peripheral lymph nodes, such as axillary lymph nodes (Fig. 8). However, there was no obvious difference between CD4 and CD8 T-cells in terms of the expression levels of activation markers in either spleen or lymph nodes (data not shown).

DISCUSSION

In this study we have shown that in the presence of H-2^{g7} in B6.H-2^{g7} mice, spontaneous autoreactivity to islet β-cell antigens can be generated. However, this alone is not sufficient to cause diabetes in the absence of other predisposing factors. In B6.H-2^{g7} mice, the background genes are identical to the B6 mouse, which is insulitis and diabetes resistant. Similarly, our studies showed that B6.H-2^{g7} mice do not have insulitis, nor do they develop diabetes. However, expression of the costimulatory molecule B7.1 on the islets promoted diabetes development in these congenic B6.H-2^{g7} mice. Furthermore, when autoreactive T-cells from young B6.H-2^{g7} mice were transferred into MHC-matched NOD.scid/RIP-B7.1 mice, diabetes developed rapidly. This showed that the autoreactive T-cells present in the B6.H-2^{g7} mice are fully capable of being stimulated to become diabetogenic. In addition, spleen cells from diabetic B6.H-2^{g7}/RIP-B7.1 mice can transfer the disease into both irradiated nondiabetic syngeneic recipients (B6.H-2^{g7}/RIP-B7.1) and MHC-matched NOD.scid/RIP-B7.1 mice. These results agree with previous studies showing that H-2^{g7} on the NOD background together with RIP-B7.1 leads to much-accelerated diabetes (10,13). However, unlike the previous study, the other genetic factors for diabetes in the NOD strain were not present in the mice in the current study, and this suggested that H-2^{g7} together with the local expression of the B7.1 molecule is sufficient to cause diabetes. The factors that normally protect the B6.H-2^{g7} mice can clearly be overcome within the mice.

A similar effect was also found when the RIP-B7.1 transgene was introduced to the NOR genetic background. NOR mice share ~88% of the genome with NOD mice, differing at *Idd 4*, *5*, *9*, *11*, and *13* (14,15). An early study suggested that NOR mice were insulitis free (15). However, later investigation showed that NOR mice do develop peri-insulitis (16), although this appears to be different in composition to that of NOD insulitis, consisting of primarily antigen-presenting cells (16). It is intriguing that cells from these NOR mice were fully capable of transferring diabetes to NOD.scid/RIP-B7.1 mice and with an accelerated disease rate, similar to that seen when the RIP-B7.1 transgene is expressed on the NOD genetic background, and with no sex preference. Thus, again, the presence of the RIP-B7.1 molecule overrides the protective effect of the genes that appear to prevent T-cell migration into the islets in the NOR mice, and it facilitates the development of disease. The common factor in both B6.H-2^{g7}/RIP-B7.1 and NOR/RIP-B7.1 mouse strains is that they express

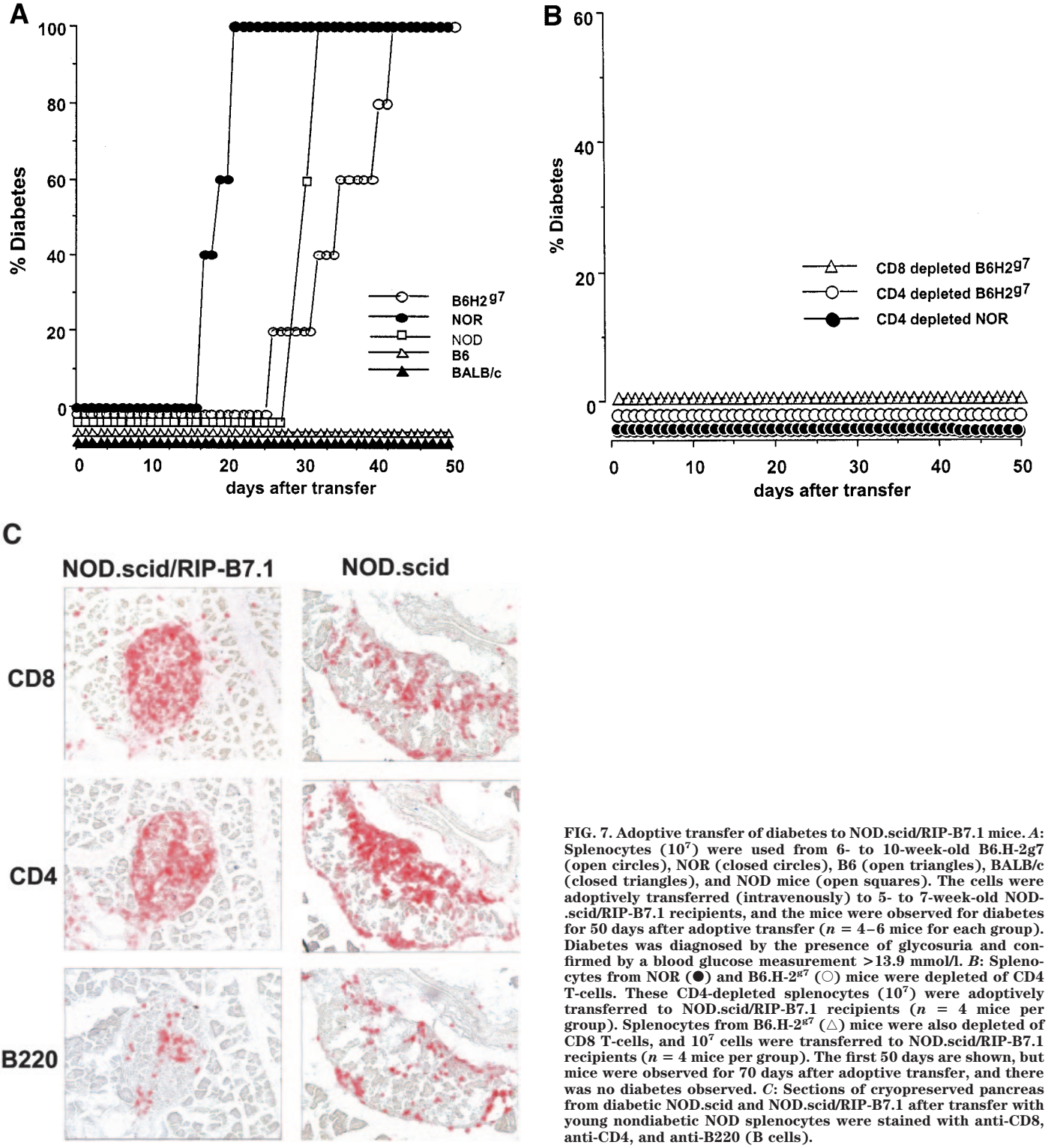

FIG. 7. Adoptive transfer of diabetes to NOD.scid/RIP-B7.1 mice. *A*: Splenocytes (10^7) were used from 6- to 10-week-old B6.H-2g7 (open circles), NOR (closed circles), B6 (open triangles), BALB/c (closed triangles), and NOD mice (open squares). The cells were adoptively transferred (intravenously) to 5- to 7-week-old NOD-.scid/RIP-B7.1 recipients, and the mice were observed for diabetes for 50 days after adoptive transfer (n = 4–6 mice for each group). Diabetes was diagnosed by the presence of glycosuria and confirmed by a blood glucose measurement >13.9 mmol/l. *B*: Splenocytes from NOR (●) and B6.H-2^{g7} (○) mice were depleted of CD4 T-cells. These CD4-depleted splenocytes (10^7) were adoptively transferred to NOD.scid/RIP-B7.1 recipients (n = 4 mice per group). Splenocytes from B6.H-2^{g7} (△) mice were also depleted of CD8 T-cells, and 10^7 cells were transferred to NOD.scid/RIP-B7.1 recipients (n = 4 mice per group). The first 50 days are shown, but mice were observed for 70 days after adoptive transfer, and there was no diabetes observed. *C*: Sections of cryopreserved pancreas from diabetic NOD.scid and NOD.scid/RIP-B7.1 after transfer with young nondiabetic NOD splenocytes were stained with anti-CD8, anti-CD4, and anti-B220 (B cells).

H-2^{g7}, and we have shown that T-cells from both strains have spontaneous islet autoreactivity.

The RIP-B7.1 model is used to study a different type of diabetes compared with the NOD mouse. The presence of RIP-B7.1 provides a means to activate CD8 T-cells locally in the islet. This is not to attempt to mimic spontaneous diabetes, as found in the NOD mouse, because the B7.1 costimulatory molecule is not normally expressed on islet β-cells. Rather, it provides a tool to study mechanisms of loss of tolerance in potentially diabetogenic CD8 T-cells. In the original B6.RIP-B7.1 studies, it was shown that simply conferring antigen-presenting capability to islets was not sufficient to stimulate loss of tolerance (9). However, there are clearly other factors required to break immune tolerance to insulin-producing β-cells, and the current model facilitates the dissection of these other parameters. The effect of local expression of B7.1 in this context has been shown in the past to act mainly on CD8

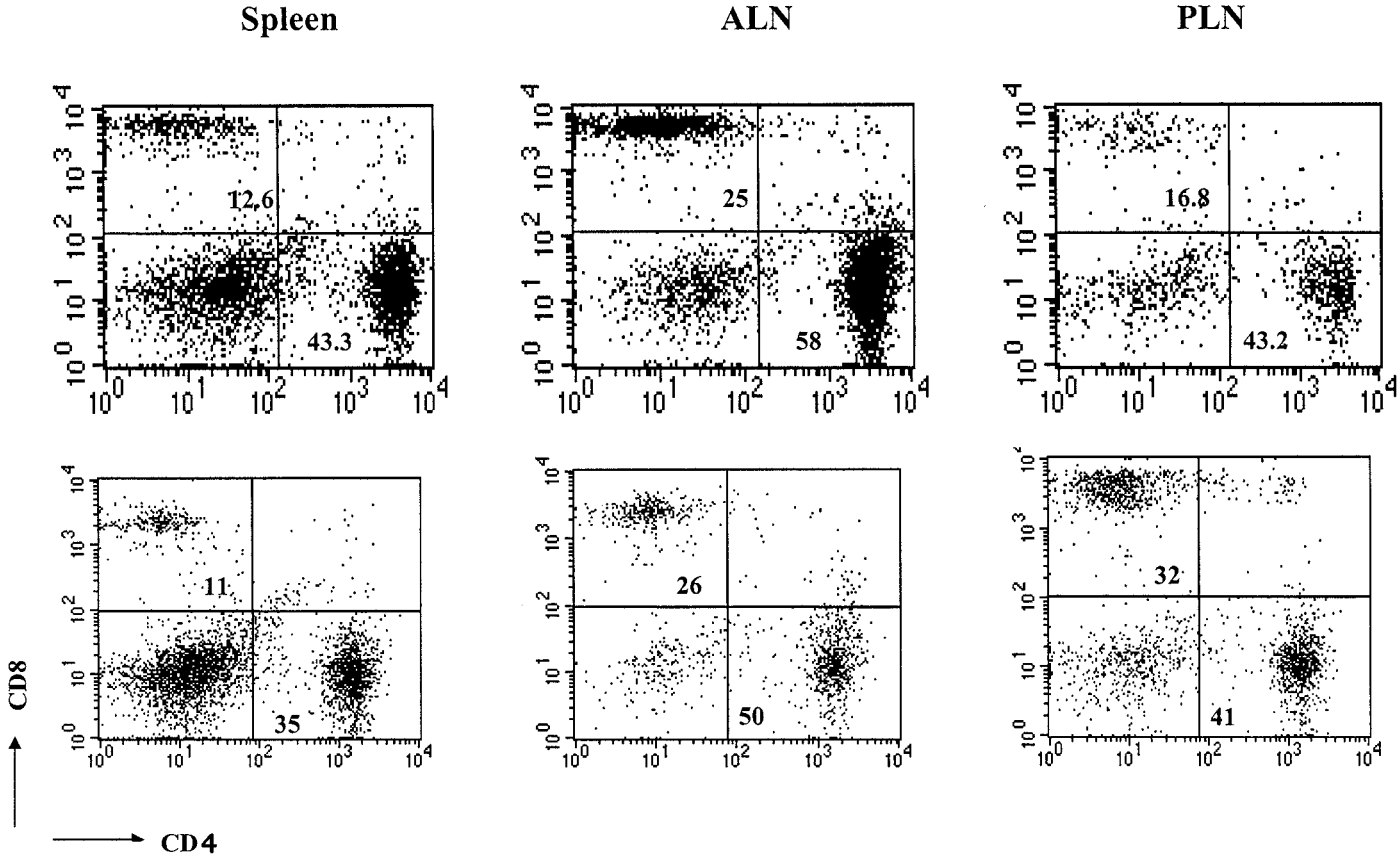

FIG. 8. Fluorescence-activated cell sorter analysis of T-cells from spleen and lymph nodes. Splenocytes (10^7) from B6.H-2^{g7} mice were adoptively transferred (intravenously) to 5- to 7-week-old NOD.scid (*upper panels*) or NOD.scid/RIP-B7.1 (*lower panels*) recipients. At 2 weeks post–adoptive transfer, spleens and lymph nodes (axillary lymph nodes and pancreatic lymph nodes) were harvested from the recipients. Single lymphocyte suspensions were stained with different fluorescence-conjugated antibodies to CD4 and CD8 and then analyzed by flow cytometry. Similar results were obtained after transfer of NOR splenocytes (not shown). ALN, axillary lymph nodes; PLN, pancreatic lymph nodes.

T-cells because there is only a low incidence of diabetes in the absence of CD8 T-cells (18). However, diabetes does develop, although it occurs much later in the absence of CD4 T-cells or MHC class II molecules (18,19). We demonstrated that although BALB/c/RIP-B7.1 mice express K^d and B6/RIP-B7.1 mice express D^b (MHC class I components of H-2^{g7}), the mice develop no diabetes, or they have a lower incidence of diabetes with a later onset. In addition, T-cells from diabetic B6.H-2^{g7}/RIP-B7.1 mice could not transfer diabetes to B6/RIP-B7.1 mice matched only at the D^b locus. This suggested that even in this system designed to break tolerance in CD8 T-cells, predisposing MHC class II molecules (and possibly the absence of protective MHC class II molecules, such as I-E in the BALB/c strain) are important for efficient autoimmune diabetes to develop.

The etiology of autoimmune diabetes is multifactorial. Despite the identification of numerous diabetes susceptibility regions, the *idd1* locus found in NOD mice and *IDDM1* in humans—mapping to the MHC gene locus in both the mouse model and human disease—is the most dominant disease susceptibility gene (22,23). The MHC class II molecule expressed in the NOD mouse, I-A^{g7}, is the homologue of human HLA-DQA1*0301/DQB1*0302 (DQ8), and both molecules are highly associated with autoimmune diabetes (rev. in 24). However, in both mice and humans, the expression of MHC molecules that predispose to diabetes alone is not sufficient to promote autoimmune diabetes. In humans, only 1 in 15 people with the high-risk genotype HLA-DR3-DQ2/DR4-DQ8 develop autoimmune diabetes in most Caucasian populations (24), and in mice no diabetes occurs in a number of I-A^{g7}–expressing congenic strains, including the NOR (15,16) and B6.H-2^{g7} mice shown in our study. However, in the presence of predisposing MHC class II molecules, islet autoreactivity does occur, although this alone is insufficient for the development of diabetes.

Our previous studies and those by other investigators using a similar transgenic system suggested that the expression of the B7.1 costimulatory molecule on pancreatic β-cells certainly facilitates the effect of diabetogenic CD8 T-cells in disease development (10,13,18,25). Our current study indicates that although diabetes may be induced in the absence of detectable autoreactive cells, as in the small percentage of BALB/c/RIP-B7.1 mice that developed diabetes, it is clear that B7.1 facilitates ultimate islet destruction most efficiently when preexisting autoreactive cells are present. The role of the MHC class II molecules in the etiology of autoimmune diabetes has long been a subject for study (26,27), and CD4 T-cells restricted by I-A^{g7} are pivotal in the development of type 1 diabetes in NOD mice. We have shown here that despite different factors giving rise to diabetes resistance, the MHC haplotype predisposes to the development of autoreactive T-

cells that can cause diabetes. This supports a recent study by Stratmann et al. (28) demonstrating that cells that recognize the mimotope peptide stimulating a highly pathogenic NOD CD4 T-cell clone, BDC2.5, can be found in both NOR and B6.H-2^{g7} mice. In the presence of a permissive islet factor that stimulates potentially autoreactive CD8 T-cells to become increasingly pathogenic, diabetes can occur, and this overrides the protective factors derived from both NOR resistance genes and the nonpermissive B6 genetic background. We suggest that the role of CD4 T-cells in this instance is to provide a means to "license" the CD8 T-cells (5–7), and when these CD8 T-cells encounter a much stronger stimulus in the islet, they become potent cytolytic effector cells to damage the β-cells, overriding the protection conferred by their natural environment. We postulate that under normal circumstances, simply having an autoreactive repertoire of T-cells is not sufficient for disease to occur, and that the reason why some animals (or humans predisposed to disease) develop diabetes is because of the presence of a stimulus/stimuli that is sufficient to override the normal protective mechanisms and capable of precipitating disease. The nature of these stimuli has yet to be resolved.

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