Major Histocompatibility Complex Class II Molecules Can Protect from Diabetes by Positively Selecting T Cells with Additional Specificities

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Summary

Insulin-dependent diabetes is heavily influenced by genes encoded within the major histocompatibility complex (MHC), positively by some class II alleles and negatively by others. We have explored the mechanism of MHC class II–mediated protection from diabetes using a mouse model carrying the rearranged T cell receptor (TCR) transgenes from a diabetogenic T cell clone derived from a nonobese diabetic mouse. BDC2.5 TCR transgenics with C57Bl/6 background genes and two doses of the H-2^{g7} allele exhibited strong insulitis at ~3 wk of age and most developed diabetes a few weeks later. When one of the H-2^{g7} alleles was replaced by H-2^{b}, insulitis was still severe and only slightly delayed, but diabetes was markedly inhibited in both its penetrance and time of onset. The protective effect was mediated by the Aβ^{b} gene, and did not merely reflect haplozygosity of the Aβ^{g7} gene. The only differences we observed in the T cell compartments of g^7/g^7 and g^7/b mice were a decrease in $CD4^+$ cells displaying the transgene-encoded TCR and an increase in cells expressing endogenously encoded TCR α-chains. When the synthesis of endogenously encoded α-chains was prevented, the g^7/b animals were no longer protected from diabetes. g^7/b mice did not have a general defect in the production of A^{g7}-restricted T cells, and antigen-presenting cells from g^7/b animals were as effective as those from g^7/g^7 mice in stimulating A^{g7}-restricted T cell hybridomas. These results argue against mechanisms of protection involving clonal deletion or anergization of diabetogenic T cells, or one depending on capture of potentially pathogenic A^{g7}-restricted epitopes by A^{b} molecules. Rather, they support a mechanism based on MHC class II–mediated positive selection of T cells expressing additional specificities.

The nonobese diabetic (NOD)[1] mouse spontaneously develops a disease with many of the characteristics of insulin-dependent diabetes mellitus (IDDM) in humans (for reviews see references 1 and 2). As such, it has become the most frequently employed small animal model of IDDM, and has permitted some important insights into the complicated pathogenesis of this disorder.

One feature that NOD and human IDDM have in common is that genetic influences on the development and progression of disease are very complex (for reviews see references 3 and 4). Nonetheless, in both cases, the genetics are dominated by one element, the MHC, which accounts for >30% of disease susceptibility (5–9).

The MHC of the NOD mouse is rather peculiar by several criteria. H-2^{g7} is a naturally occurring recombinant locus, such that genes at the K end are of the d haplotype, while at the D end they are of the b haplotype (5). The *A*α gene has the same coding sequence as *A*α^d, but the *A*β gene is a rare allele, otherwise found only in the Biozzi and a few wild strains (10, 11); the resulting AαAβ heterodimer is expressed as usual at the cell surface, but appears to be of unusual conformation (12). The *E*α gene has a promoter-region mutation that precludes its transcription, and thereby expression of an EαEβ heterodimer at the cell surface (5). Certainly, part of the MHC influence on the NOD disease can be attributed to the presence of the A^{g7} complex. Indeed, the Aβ^{g7} chain carries His, Ser residues at positions 56, 57 rather than the Pro, Asp found in most other mouse strains and thought to be diabetes-protective in humans (10, 13). However, another part of the MHC could reflect the absence of other A alleles or of the E complex.

Nishimoto and colleagues were the first to describe the protective effect of introducing non-NOD MHC class II genes onto the NOD genetic background (14–16). When

[1] *Abbreviations used in this paper:* HEL, hen egg lysozyme; IDDM, insulin-dependent diabetes mellitus; KLH, keyhole limpet hemacyanin; NOD, nonobese diabetic; tg, transgenic.

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they introduced an *Eα* transgene, resulting in the surface display of an E complex, they observed a striking protection from insulitis and diabetes. This result has since been reproduced by several other groups using independent *Eα* transgenes (17–19). In addition, there have been several reports of similar findings after the introduction of various A complex alleles or variants, although the extent of disease inhibition was often less striking (8, 18, 20–23).

The mechanism by which non-NOD E and A genes protect from disease in NOD mice remains a mystery. Several possibilities have been raised, most with experimental support both for and against. Possible mechanisms include the following: (*a*) deletion of diabetogenic T cells or inhibition of their selection (supported by references 24 and 25; contested by references 17–19, 22, 26, 27); (*b*) anergization of diabetogenic T cells (contested by references 22, 26, 27); (*c*) diversion of the phenotype of diabetogenic T cells (supported by references 19, 22, 28); (*d*) "capture" of determinants recognized by diabetogenic T cells (supported by references 29, 30; contested by references 19, 26, 27, 31); (*e*) positive selection of regulatory T cells (supported by reference 17; contested by references 26, 32); or (*f*) something else.

Thus far, there is no general agreement on the mechanism responsible for the protection from diabetes afforded by MHC class II molecules. It is not even clear whether the mode of inhibition by different class II molecules is the same. The lack of consensus probably reflects certain problems inherent in the NOD system, i.e., the difficulty of monitoring changes in the behavior of the very rare pathogenic T cells, or the complexities resulting from genetic manipulations.

Our strategy to begin dissecting the mechanism of MHC-mediated protection has been to study the phenomenon in the BDC2.5 TCR transgenic (tg) model of diabetes (27). These mice carry the rearranged TCR genes from a $CD4^+$ T cell clone that is specific for a pancreatic islet β-cell antigen, is restricted by the A^{g7} molecule, and is diabetogenic (33). Although self-reactive, the transgene-encoded specificity is efficiently selected in animals expressing A^{g7} molecules and makes an exaggerated contribution to the peripheral T cell repertoire (27). On the NOD genetic background, there are no signs of disease before 2 wk of age, with a rampant insulitis beginning at three weeks, but no diabetes until many months later (27). On the C57Bl/6 background, the delay between the initiation of insulitis and onset of diabetes is compressed to several weeks, at least in mice carrying two doses of $H\text{-}2^{g7}$ (33a).

We chose to examine MHC-mediated protective effects in BDC2.5/C57 animals because of several advantages afforded by this system. First of all, diabetes occurs quite rapidly (before 8–10 wk of age) and with high incidence (usually 50–75%). In addition, the use of a TCR tg line, with its high numbers of β-cell–reactive T cells, should facilitate elucidation of the mechanisms involved. Finally, using mice with C57 background genes permits one to manipulate the genome relatively easily, which makes use of available congenic and knockout lines without having to perform the extensive serial backcrosses required with animals on the NOD background. Introducing other MHC class II alleles by breeding with MHC congenics rather than tgs has the additional, very important, advantage of avoiding an aberrant phenotype that has been attributed to excess copies of the *Aβ* gene (34, 35)—a problem that has been highlighted in one study (36) but completely ignored by most others.

Here, we describe our initial exploitation of this strategy, the identification of an MHC class II molecule that protects BDC2.5/C57 mice from diabetes, and elucidation of the mechanism of protection at the cellular level.

Materials and Methods

Mice. The generation of BDC2.5 TCR tg mice has been previously described (27). The original founder was bred with a C57Bl/6 mouse and successive offspring were backcrossed onto the C57Bl/6 background for 11 generations. Backcrossed animals were bred with $B6.H\text{-}2^{g7}$ congenic mice (backcrossed >26 generations onto the C57Bl/6 background; a gift from H. Kikutani, Institute for Molecular and Cellular Biology, Osaka, Japan) and the offspring intercrossed to generate $B6.H\text{-}2^{g7}$ (referred to as g^7/g^7), $B6.H\text{-}2^{g7/b}$ (g^7/b) and $B6.H\text{-}2^{b}$ (b/b) littermates, all positive for the BDC2.5 transgenes. All strains used in the other crosses to generate experimental animals have been described before: *Eα16* tg mice, backcrossed to C57Bl/6 for >30 generations (37); mice lacking conventional MHC class II molecules (Aβ°) (38), backcrossed to C57Bl/6 for 10 generations; and mice with a null mutation in the TCR-α locus (39), backcrossed 3 times to C57Bl/6.

Animals were typed for expression of the BDC2.5 transgenes and for MHC status by FACS® analysis of blood lymphocytes (staining for Vβ4 and CD4 for BDC2.5, or with the mAbs Y3P, specific for A^b, and 10.2.16, reactive with A^{g7}, reference 40). Expression of the Eα16 transgene was checked using the mAb 14.4.4, specific for E molecules (41). Mice carrying mutations in the Aβ or TCR-α locus were typed by Southern blot analysis of tail DNA using probes specific for the Aβ° and Cα° mutations, respectively (38, 39).

All animals were maintained in our conventional facility, according to European Economic Community guidelines.

Measurement of Diabetes and Insulitis. Mice were followed for diabetes weekly starting at 3 wk of age by testing urine glucose levels (Uristix; Bayer Diagnostics, Puteaux, France); in addition, during the interval between 3 and 11 wk of age, blood glucose levels were measured every second week (Glucofilm strips read in a Glucometer 3; Bayer Diagnostics). Animals were routinely followed until they were 30 wk old. Mice were considered diabetic if positive in two consecutive readings (urine glucose >10 g/liter, blood glucose >300 mg/dl).

Hematoxylin and eosin staining of thin sections from Bouin's solution-fixed, paraffin-embedded pancreata was performed as previously described (17). For each animal, multiple sections of pancreas, with at least 40 islets, were scored for insulitis. Islets were judged either free of insulitis, or suffering from periinsulitis (when the infiltrate was limited to the surrounding ducts) or from true insulitis (when there was entry of lymphocytes into the islets).

Antibodies and FACS® Analysis. The following mAbs were used for T cell analysis, with immunostainings performed as previously described (38): PE-conjugated anti-CD4 (Caltag, South San Francisco, CA); biotin-conjugated anti-CD8 (Caltag); FITC-conjugated anti-CD69 (PharMingen, San Diego, CA); J11d, specific for heat-stable antigen; FITC-conjugated peanut agglutinin

(Sigma Chemical Co., St. Louis, MO); KT3, specific for CD3; H57-597, specific for TCR-α/β; IM7, specific for CD44; Mel-14, specific for CD62L; FITC-conjugated anti-CD31, clone MEC13.3 (PharMingen); FITC-conjugated anti-CD49d, clone R1-2 (Phar Mingen); 16A, specific for CD45RB; PC61, specific for CD25; PK136, anti-NK1.1 (PharMingen); and B20.1, RR 3-16, KT50, and RR 8-1, specific for Vα2, Vα3.2, Vα8, and Vα11, respectively (for references to mAbs see 27, 42, 43). Biotinylated antibody was revealed with Cy5-labeled streptavidin (Jackson ImmunoResearch Labs., Inc., West Grove, PA), and the other antibodies either with Texas red–labeled anti–rat IgG (Jackson ImmunoResearch Labs., Inc.) or FITC-labeled anti–rat IgG (Caltag).

Production of T Cell Hybridomas and Assays of Reactivity. 6–10-wk-old H-$2^{g7/b}$ non-tg mice were immunized subcutaneously with 50 μg of either hen egg lysozyme (HEL) or keyhole limpet hemacyanin (KLH). 10 d later, the draining lymph nodes were removed and, after in vitro restimulation with the appropriate antigen, activated cells were fused with the myeloma cell line BW5147 following a standard protocol (44). Growing hybridomas were tested in a primary screen for antigen specificity using splenocytes from g^7/b mice as APCs. After 24 h, 50 μl of supernatant was removed and the proliferation of the IL-2–dependent CTLL cell line was used as a readout. Positive hybridomas were subsequently checked for their MHC restriction using either splenocytes from NOD (g^7), B6.H-$2^{g7/b}$ (g^7/b) or B6 (b) mice as APCs, including five serial dilutions of the appropriate antigen. In the case of HEL, most of the hybridomas were restricted to either g^7 or b.

The BDC2.5 hybridoma was stimulated by freshly isolated islet cells from DBA/2 mice (27), and IL-2 production was also measured using the CTLL assay.

Results

The H-2^b Complex, but not E Molecules, Can Protect BDC2.5 TCR tg Mice from Diabetes. To establish a system for studying MHC-mediated protection from diabetes, we evaluated the effect of non-NOD MHC genes on the development of disease in BDC2.5 transgenics carried on the C57 background.

First, we generated cohorts of littermates whose MHC haplotype was either homozygous b/b, homozygous g^7/g^7, or heterozygous g^7/b. As expected from their inability to positively select the BDC2.5 specificity (27), none of the b/b mice showed signs of diabetes or insulitis (data not shown). Both the g^7/g^7 and the g^7/b animals developed diabetes, but there was a marked difference in both the penetrance and time of onset (Fig. 1 *A*). Although g^7/g^7 mice had an average disease incidence of 57% at 12 wk of age, only 21% of their g^7/b littermates were diabetic at this time ($P < 0.0001$). A substantial proportion of g^7/g^7 animals was already diabetic at 3 wk, whereas disease was rarely seen in g^7/b mice before 5 wk of age. In both cases, there were very few additional diabetics after 10–12 wk of age.

To determine whether the significant decrease in diabetes incidence seen for g^7/b mice was accompanied by changes in insulitis, we examined hematoxylin and eosin–stained pancreas sections taken on different days, comparing insulitis scores with reference data on a large number of age-matched g^7/g^7 animals (Fig. 2). As has been reported (33a), g^7/g^7 animals showed almost no infiltration at day 12, but showed an aggressive, overwhelming insulitis at day 18 in the vast majority of individuals. In contrast, in the g^7/b mice only a few islets were infiltrated at day 18, and even at day 24 only a slight to moderate insulitis was observed. However, most of the islets were infiltrated at day 30, eventually showing the same image of aggressiveness as seen for g^7/g^7 animals.

In a second set of experiments, we introduced an *Eα* transgene into the BDC2.5/C57 line, thereby restoring expression of E molecules. We had already shown that the Eα transgene does not influence the slow-onset diabetes in BDC2.5/NOD animals (27), but wondered whether it would affect the more penetrant and more rapid disease in BDC2.5/C57 animals; we also wanted to know whether it would affect the protection seen with H-2^b heterozygosity. As shown in Fig. 1 *B*, we once again observed a marked protection from diabetes in g^7/b versus g^7/g^7 mice, but the expression of E molecules had no detectable influence on diabetes incidence in either g^7/g^7 or g^7/b animals.

Thus, some element encoded in the H-2^b complex affords protection from diabetes in BDC2.5/C57 tg mice; however, the E molecule is unable to do so. Comparing g^7/g^7 and g^7/b BDC2.5/C57 animals provides a system where the only variable is a single dose of the H-2^b complex, offering the advantage that the introduced MHC genes reside in their usual chromosomal context at the normal copy number.

A^b Molecules Are Responsible for the Protective Effect of the H-2^b Complex. Although it seemed likely that the protection afforded by the H-2^b complex was due to class II A^b molecules (18, 20–22), it was possible that other elements might

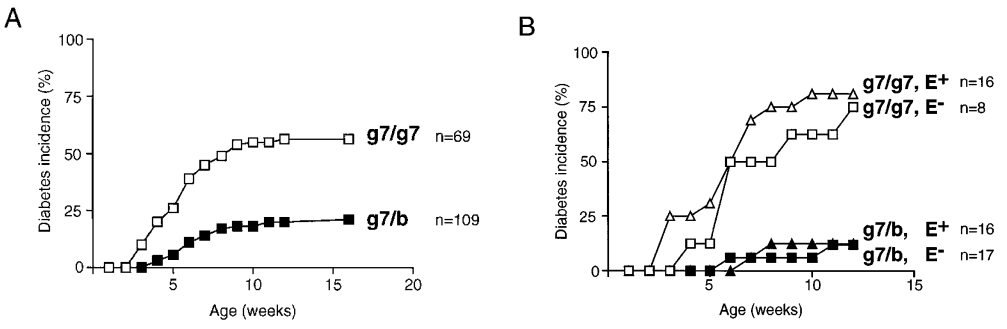

Figure 1. The H-2^b complex, but not the E molecule, protects BDC2.5/C57 tgs from diabetes. (*A*) BDC2.5/C57 g^7/g^7 and g^7/b littermates were followed for diabetes; the percentage of diabetic mice over time is indicated. The data are pooled from several independent cohorts. (*B*) BDC2.5/C57 g^7/g^7 and g^7/b littermates, carrying or not carrying the Eα16 transgene, were monitored for diabetes.

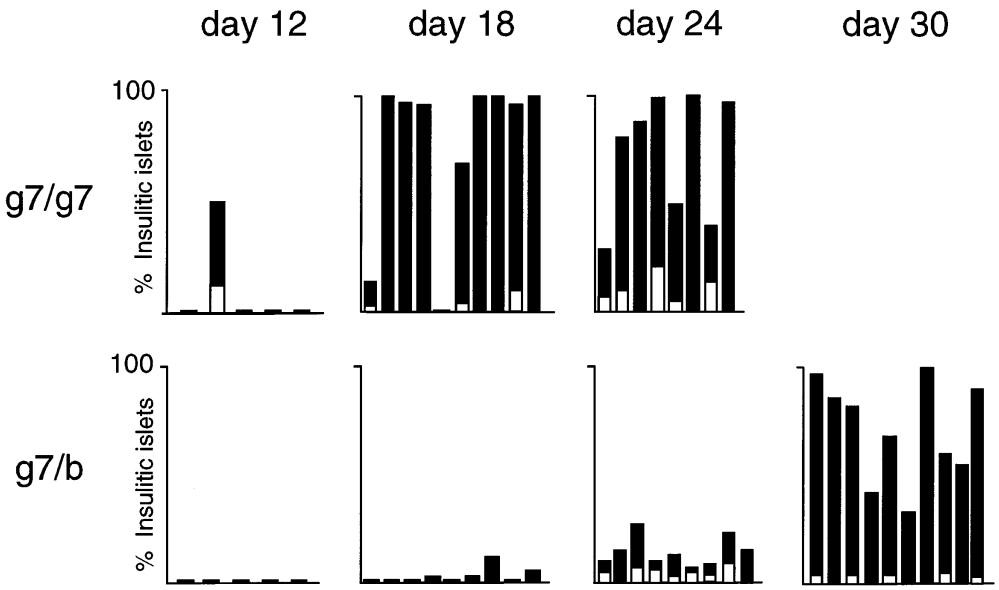

Figure 2. The onset of insulitis is delayed in g^7/b BDC2.5 tgs. BDC2.5/C57 transgenic mice (g^7/g^7 or g^7/b haplotype at the MHC) were killed on days 12, 18, 24, or 30, and the percentage of pancreatic islets showing either periinsulitis or insulitis was quantitated. Each bar represents an individual mouse. The white portion of the bars indicates periinsulitis, the black portion insulitis.

be responsible, in particular, class I molecules (45, 46) or the TNFs (47). To test the role of A^b molecules, we took advantage of the Aβ° line of mice, which carries essentially only C57 background genes (after 10 backcrosses) and all of the genes of the H-2^b complex but has an engineered null mutation at the Aβ locus. By appropriate breedings, we produced BDC2.5/C57 littermates that were either b/b°, g^7/g^7, g^7/b or $g^7/b°$. As can be seen in Fig. 3, the b/b°, g^7/g^7 and g^7/b animals had the expected incidence rates of zero, quite high and low, respectively. The $g^7/b°$ mice, unable to express A^b molecules, showed the same incidence as g^7/g^7 animals.

This result permits us to conclude that the protection from diabetes afforded by the H-2^b complex is due to the presence of A^b molecules. It cannot be attributed to other molecules encoded within H-2^b, nor is it the trivial result of having a single, rather than two, doses of the A^{g7} allele.

g^7/b BDC2.5/C57 tgs have Fewer Mature CD4$^+$ T Cells and More Cells Expressing Endogenously Encoded TCR-α Chains. One of the major advantages of the BDC2.5 TCR tg system is that the exaggerated frequency of T cells displaying the transgene-encoded specificity permits one to study just how MHC-mediated protection impinges on a diabetogenic T cell. Thus, we compared the T cell compartments in the thymus and peripheral organs of 4–6-wk-old nondiabetic g^7/g^7 and g^7/b BDC2.5/C57 tgs, concentrating on cell numbers, expression of the transgene-encoded clonotype versus endogenously encoded TCR chains, and expression of activation markers.

The total number of thymocytes was comparable in g^7/g^7 and g^7/b BDC2.5/C57 mice (Fig. 4 *A*), suggesting that massive clonal deletion did not take place in the latter. The CD4/8 staining profiles of thymocytes from both types of animals revealed a strong bias for CD4$^+$8$^-$ versus CD4$^-$8$^+$ cells (Fig. 4 *B*), as was previously seen for BDC2.5 tgs on the NOD background (27). However, the percentage of mature CD4$^+$ T cells and their total numbers (Fig. 4 *C*) were about twice as high in g^7/g^7 animals as in their g^7/b littermates. This difference was also reflected in the histograms of TCR-α/β expression, where g^7/g^7 animals had more mature cells expressing high receptor levels (Fig. 4 *D*). No differences between the two types of mice were evident in the staining patterns for heat-stable antigen, peanut agglutinin, and CD69, maturation markers correlated with positive selection (data not shown).

The transgene-encoded BDC2.5 TCR carries the Vβ4 and Vα1 variable regions (48). Unfortunately, no anti-Vα1 reagent is presently available, so we could not directly quantitate the BDC2.5 clonotype, but we could estimate the use of endogenously encoded TCR-α chains by staining for Vα2. In both g^7/g^7 and g^7/b mice, >95% of the

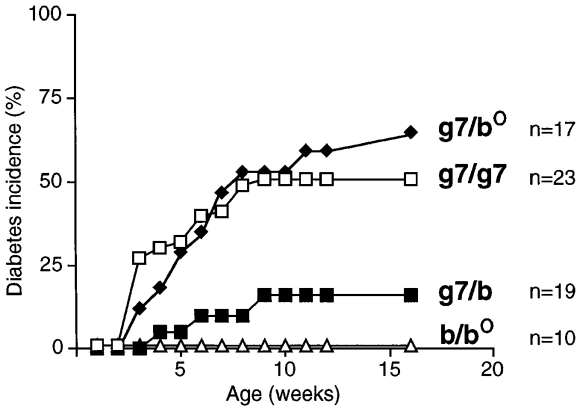

Figure 3. The protective effect of the H-2^b complex can be attributed to A^b molecules. BDC2.5/C57 transgenic mice were bred with g^7, b, or b° haplotypes at the MHC. The latter is a mutant H-2^b complex carrying a null mutation at the Aβ locus (38). Littermates from these crosses were followed for diabetes.

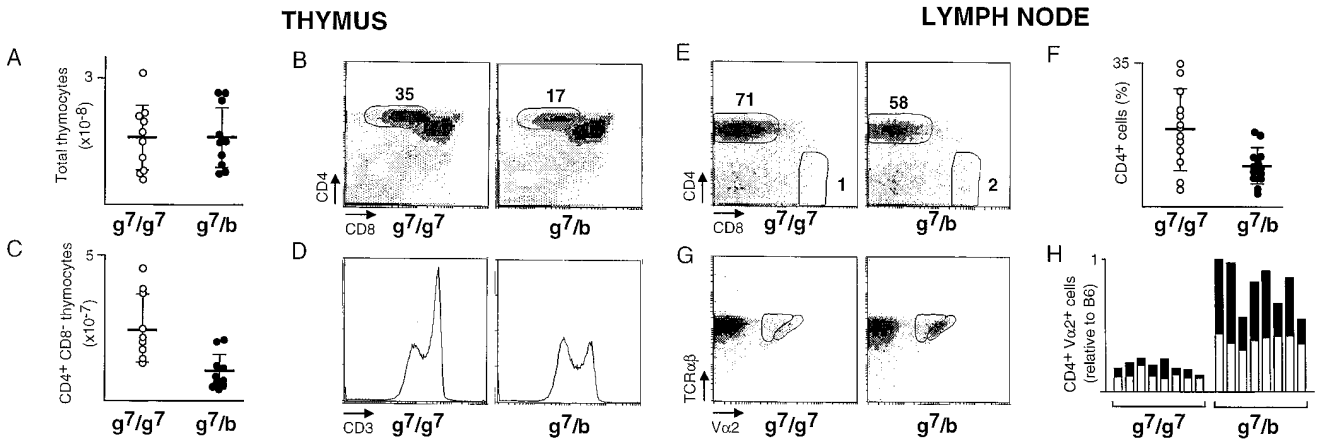

Figure 4. In g^7/b BDC2.5 TCR tg mice, there are fewer $CD4^+$ T cells and more cells that express endogenously encoded TCR-α chains. (*A*) Total numbers of thymocytes in g^7/g^7 and g^7/b littermates from 10 different experiments; each dot represents an individual mouse; the average values are shown (g^7/g^7: 159.5 ± 78.3 millions, g^7/b: 160.4 ± 75.4 millions). (*B*) Representative CD4/CD8 cytofluorimetric profiles; the percentage of $CD4^+8^-$ cells is indicated. (*C*) Numbers of $CD4^+8^-$ cells; the average values are shown (g^7/g^7: 24.8 ± 11.7 millions, g^7/b: 10.3 ± 6.2 millions, $P < 0.003$). (*D*) Representative histogram of CD3 expression on total thymocytes. (*E*) Representative CD4/CD8 profiles of mesenteric lymph node lymphocytes, gated on CD3-positive cells. The percentages of $CD4^+$ and $CD8^+$ cells are indicated. (*F*) Percentage of $CD4^+$ cells for g^7/g^7 and g^7/b littermates; each dot represents an individual mouse; the average values are shown (g^7/g^7: 20 ± 8.9 millions, g^7/b: 11.6 ± 4.1 millions, $P < 0.005$). The total number of lymph node cells did not differ significantly in the different mice. (*G*) TCR-αβ/Vα2 profiles of $CD4^+$ T cells; the two gates delineate the $V\alpha2^{hi}$ and $V\alpha2^{int}$ populations; in normal C57Bl/6 mice taken as a reference, the vast majority (80–90%) of Vα2-positive $CD4^+$ cells fell within this $V\alpha2^{hi}$ gate. (*H*) Black bars represent proportion of cells expressing an endogeneous Vα chain (relative to C57Bl/6); white bars represent proportion of cells expressing low levels of an endogeneous Vα chain (most likely in conjunction with the tg Vα), also relative to C57Bl/6. Each bar represents an individual mouse.

$CD4^+8^-$ thymocytes were positive for Vβ4, demonstrating the almost complete allelic exclusion of the TCR-β gene in both cases (data not shown). In contrast, exclusion at the TCR-α locus differed in the two types of mice: there was repeatably twice the percentage of $CD4^+8^-$ cells expressing Vα2 in g^7/b than in g^7/g^7 animals, signifying a greater usage of endogenously encoded Vα chains in the former (data not shown). Parallel results were obtained using anti-Vα3.2, -Vα8, and -Vα11 reagents, ruling out the possibility that the Vβ4 chain preferentially paired with particular endogenously encoded Vαs (data not shown).

These results were mirrored in the periphery, and sometimes even exaggerated. Total numbers of T cells in the spleen and lymph nodes were comparable in g^7/g^7 and g^7/b littermates (data not shown). The same skewing in favor of $CD4^+$ T lymphocytes was observed (Fig. 4 *E*) and, again, there were more mature $CD4^+$ T cells in g^7/g^7 than g^7/b mice (Fig. 4 *F*). Because there were more $CD8^+$ T cells in the periphery of g^7/b mice, the difference in the CD4/8 ratios was even more striking than in the thymus, averaging about 50 in homozygotes in contrast to only 15 in heterozygotes.

The difference in expression of endogenously encoded TCR-α chains was also more pronounced in the periphery (Fig. 4, *G* and *H*). In the g^7/g^7 BDC2.5/C57 tgs, Vα2 was displayed on only 20% as many T cells as in non-tg C57Bl/6 controls; in g^7/b BDC2.5/C57 mice, 80% as many did. Many more of the T cells in the g^7/b transgenics had intermediate levels of Vα2 expression than is normally found in nontransgenic C57Bl/6 controls. Assuming (*a*) that such cells express two TCRs, the transgene-encoded Vβ4Vα1 receptor and another consisting of Vβ4 paired with an endogenously encoded Vα chain, and (*b*) that there is no preferential pairing, it can be calculated that 80% of the peripheral T cells in g^7/g^7 mice displayed only the BDC2.5 TCR, 12% expressed it together with another receptor, and 8% displayed only another receptor; the contrasting corresponding values for g^7/b mice are 20, 40, and 40%. Thus, a far greater proportion of cells expressing endogenously encoded Vα chains was present in g^7/b animals.

No difference was observed between the two kinds of mice regarding expression of early (CD69, CD25) or late (CD44, CD62L, CD45RB, CD45RC) activation markers, nor regarding the display of adhesion molecules (CD31, CD49d). Both g^7/g^7 and g^7/b transgenics showed a remarkable reduction in NK T cells ($NK1.1^+$, $CD3^+$), probably a reflection of the TCR transgenes, but there was no significant difference between the two (data not shown).

In summary, the only differences we observed in the T cell compartments of g^7/g^7 and g^7/b BDC2.5/C57 littermates were a twofold drop in the number of mature $CD4^+$ cells and a marked rise in the number of cells expressing endogenously encoded TCR-α chains, alterations first observed in the thymus and exaggerated in the periphery.

High Diabetes Incidence Is Restored in g^7/b BDC2.5/C57 tgs Incapable of Expressing Endogenously Encoded TCR-α Chains. The previously described Cα° mutation (39), which eliminates the expression of endogenously encoded TCR-α chains, permitted us to directly test whether there is a causal relationship between the increased expression of additional receptors in g^7/b BDC2.5 transgenics and their reduced incidence of diabetes. The appropriate crosses yielded a panel of littermates that were g^7/g^7 or g^7/b and carried or did not carry the homozygous Cα° mutation. g^7/g^7

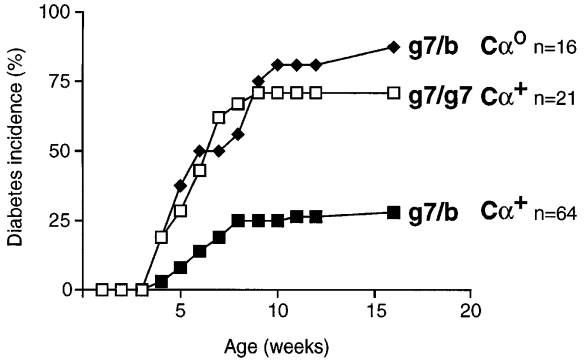

Figure 5. Introduction of the TCR-α° mutation restores high diabetes incidence in $g^{7/b}$ mice. BDC2.5/C57 mice (g^7/g^7 or g^7/b haplotypes at the MHC) were bred with the TCR-α–null mutation. Cα° homozygotes or $C\alpha^+$ littermates were followed for diabetes (since there was no difference between homozygous and heterozygous wild-type animals, the data were pooled, indicated as $C\alpha^+$). The diabetes incidence for Cα° g^7/g^7 mice did not differ from that of $C\alpha^+$ littermates and is therefore not shown here for clarity.

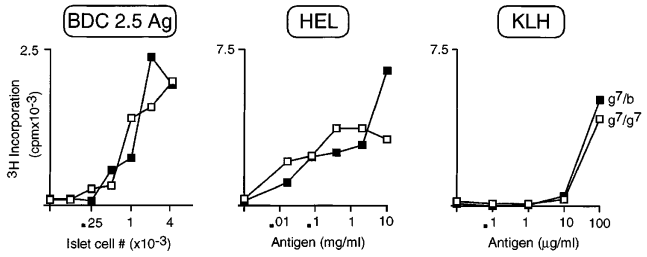

Figure 6. APCs from both g^7/g^7 and g^7/b mice present antigen efficiently to A^{g7}-restricted T cell hybridomas. The BDC2.5 hybridoma or g^7-restricted hybridomas specific for HEL or KLH epitopes [HEL(g^7) 4B12 and KLH(g^7)5D2, respectively] were challenged with APCs from non-tg g^7/g^7 or g^7/b animals. The antigen titration curves were made with islet cells or with HEL or KLH protein, and IL-2 production was read-out as [^{3}H]thymidine incorporation by the IL-2–dependent CTLL cell line.

animals developed diabetes similarly in the presence or absence of the mutation (Fig. 5 and data not shown). Strikingly, the low incidence of disease characteristic of g^7/b mice was reverted in the presence of the Cα° mutation in homozygous form, the time of onset and the penetrance both being equivalent to that of g^7/g^7 animals.

We also assessed the efficiency of selecting $CD4^+$ T cells in the same panel of littermates. g^7/b animals carrying the homozygous Cα° mutation showed the same deficiency in numbers of $CD4^+8^-$ cells in the thymus and $CD4^+$ cells in the peripheral lymphoid organs as did their g^7/b littermates with a wild-type Cα locus (data not shown).

These observations argue that the increase in T cells with additional TCR specificities is responsible for the protection from diabetes in g^7/b BDC2.5/C57 tgs. Were these additional specificities to be causally related to MHC-mediated protection from diabetes, one would expect that g^7/g^7 BDC2.5/C57 tgs carrying an Eα transgene or $g^7/b°$ BDC2.5/C57 animals, neither of which are protected from diabetes, should express low levels of endogenously encoded TCR-α chains, as straight g^7/g^7 TCR tgs do. This expectation proved correct (data not shown).

The A^{g7} Molecules on g^7/b APCs Can Function Effectively in T Cell Selection and Activation. As a first step in defining what underlies the changes in T cell repertoire observed in g^7/b BDC2.5 tgs, we compared the ability of the A^{g7} molecules expressed on g^7/g^7 and g^7/b cells to function in different contexts. It was possible that coexpression of A^b molecules could compromise the ability of A^{g7} molecules to present peptides effectively. Such an effect has been observed with other pairs of class II molecules, concerning both thymic selection of T cells (49) and their peripheral activation (50, 51), and might be expected with A^{g7} molecules, given their unusual conformation at the cell surface probably reflecting poor peptide binding (12).

To test this, we first injected non-tg g^7/b mice with HEL, re-stimulated draining lymph node cells in the presence of HEL, and generated a panel of HEL-specific T cell hybridomas. From 4 experiments, we obtained a total of 31 g^7-restricted and 48 b-restricted hybridomas. This experiment, capable of assessing both thymic selection and peripheral stimulation, but incapable of discriminating between them, ruled out a gross defect in the function of A^{g7} in the presence of A^b molecules.

Secondly, we compared the ability of APCs from non-tg g^7/g^7 and g^7/b animals to present antigen in vitro to different g^7-restricted T cell hybridomas (Fig. 6). APCs from the two types of animals were equally efficient at presenting to HEL- and KLH-specific T cell hybridomas and, most importantly, to the hybridoma expressing the same receptor as the BDC2.5 TCR tg line. This experiment confirmed that A^b molecules do not prevent A^{g7} molecules from effectively presenting antigens to T cells.

Discussion

Exploiting the advantages of the BDC2.5 TCR tg model, we have probed the mechanism of MHC-mediated protection from IDDM. We found that BDC2.5/C57 tgs that carried one H-2^b allele, more specifically, an $A\beta^b$ allele, had a lower penetrance and slower onset of diabetes than littermates homozygous for H-2^{g7}. That this protection was not due to massive A^b-mediated clonal deletion of cells expressing the BDC2.5 specificity is argued by several observations: thymocyte numbers were normal (Fig. 4, *A* and *B*); there seemed to be plenty of clonotype-positive T cells in the peripheral lymphoid organs (Fig. 4, *G* and *H*), and T cells invaded the islets en masse (Fig. 2). T cell homing to and maintenance in the islets of g^7/b mice also suggests that disease inhibition was not a reflection of clonal anergy, at least as conventionally considered. That protection was not due to capture by A^b molecules of the A^{g7}-restricted epitope recognized by BDC2.5 T cells is indicated by their invasion of the islets in g^7/b mice in vivo and the ability of the corresponding hybridoma to be stimulated by hybrid APCs in vitro (Fig. 6).

Rather, our data highlight an effect on positive selection.

A^b-mediated protection from diabetes was accompanied by both a decrease in $CD4^+$ T cells displaying the transgene-encoded clonotype and an increase in cells expressing other clonotypes, specified by endogenously encoded TCR-α–chains. Low diabetes incidence was not always correlated with the former, but always depended on the latter. Thus, in this system, MHC-mediated protection from IDDM reflects the ability of an additional class II molecule to positively select T cells expressing additional TCR specificities.

How might these T cells influence the progression of diabetes? Possible mechanisms depend on whether the disease-modulating cells are those which express only receptors carrying an endogenously encoded TCR-α chain or are those expressing the BDC2.5 clonotype plus such receptors, the distinction being additional cells versus just additional receptors. Additional cells could exert their influence by creating a cytokine/chemokine milieu that alters the phenotype or behavior of the diabetogenic cells, e.g., by skewing their T helper phenotype or influencing their disposition, or that of accessory cells within the islets. They might also act as clonotype-specific suppressor cells, like those described in other systems (52–55). In our system, such suppression could not involve massive deletion of the clonotype-positive targets since they were always present in abundance (Fig. 4 *B*). Additional receptors on cells expressing BDC2.5 TCRs could alter the quality of the response to the β cell antigen. It is now clear that the "strength" with which a T cell is stimulated dictates the subsequent response mode (i.e., how much it proliferates, what cytokines it produces, what other effector functions are mobilized, whether it is activated or anergized [56–58]). As mentioned, anergy induction would seem unlikely in this case, given that the BDC2.5 T cells did invade the islets, but this possibility can be directly tested in ex vivo activation or transfer experiments.

Interestingly, although A^b molecules had a protective effect on diabetes in BDC2.5/C57 tgs, E molecules did not. Two explanations for the difference seem possible. It could be that the mechanism of protection is different with different class II molecules. In fact, this notion has been suggested before to account for the observation that some class II molecules almost completely protect NOD mice from diabetes and insulitis (14–21), whereas others only partially inhibit diabetes and hardly influence insulitis (22, 23). Alternatively, it could be that all of the class II molecules operate by the same mechanism, but that they have different efficiencies, which may be amplified by threshold effects. Specifically in regard to the BDC2.5 TCR tg model, it remains possible that E molecules would exert a negative influence if confronted with less overwhelming numbers of diabetogenic T cells.

Our data contrast with a recent report on similar experiments on MHC-mediated protection using a TCR tg line derived from a different β cell–specific T cell clone (25). In that system, several MHC class II molecules inhibited the development of diabetes, all purportedly because they provoked massive clonal deletion of the diabetogenic T cells expressing the transgene-encoded receptor. As discussed above, substantial clonal deletion did not occur in the BDC2.5 system. The differences between these two sets of results could be an illustration of the notion that different MHC molecules might use different means to protect from disease. However, it seems that clonal deletion is not the major mode of A-mediated protection in the NOD system: $A\beta^b$ (8), $A\beta^k$ (26), $A\beta^d$ (22), and a mutant of $A\beta^{g7}$ (23) inhibit diabetes development, but islet-reactive, potentially diabetogenic T cells remain detectable, as indicated by their ability to infiltrate the pancreatic islets, provoke diabetes upon cyclophosphamide treatment, and/or transfer diabetes to lymphocyte-deficient NOD recipients.

Two features of MHC-mediated protection from diabetes have always been perplexing. First, how can so many different alleles have an inhibitory influence? Second, how can certain molecules inhibit diabetes but exacerbate other autoimmune diseases (for example, see reference 28)? The mechanism we suggest to explain A^b-mediated protection in the BDC2.5/C57 model would provide answers to these questions: the different MHC alleles can all promote the positive selection of T cells with additional specificities; a given MHC molecule could select a repertoire which includes some T cells that are able to downmodulate diabetes, and others that can amplify autoimmune attack on another tissue.

Detailing the mechanism of MHC-mediated protection from diabetes in the BDC2.5/C57 model will hopefully suggest avenues for probing the mechanism in the standard NOD model—and perhaps in humans (for review see reference 29).

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