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Type 1 Diabetes-Predisposing MHC Alleles Influence the Selection of Glutamic Acid Decarboxylase (GAD) 65-Specific T Cells in a Transgenic Model

Roshini S. Abraham, Li Wen, Eric V. Marietta, and Chella S. David

The genetic factors that contribute to the etiology of type 1 diabetes are still largely uncharacterized. However, the genes of the MHC (HLA in humans) have been consistently associated with susceptibility to disease. We have used several transgenic mice generated in our laboratory, bearing susceptible or resistant HLA alleles, in the absence of endogenous MHC class II (A\beta), to study immune responses to the autoantigen glutamic acid decarboxylase (GAD) 65 and its relevance in determining the association between autoreactivity and disease pathogenesis. Mice bearing diabetes-susceptible haplotypes, HLA DR3 (DRB1*0301) or DQ8 (DQB1*0302), singly or in combination showed spontaneous T cell reactivity to rat GAD 65, which is highly homologous to the self Ag, mouse GAD 65. The presence of diabetes-resistant or neutral alleles, such as HLA DQ6 (DQB1*0602) and DR2 (DRB1*1502) prevented the generation of any self-reactive responses to rat GAD. In addition, unmanipulated A\betao/DR3, A\betao/DQ8, and A\betao/DR3/DQ8 mice recognized specific peptides, mainly from the N-terminal region of the GAD 65 molecule. Most of these regions are conserved among patients, mouse, and rat GAD 65. Further analysis revealed that the reactivity was mediated primarily by CD4+ T cells. Stimulation of these T cells by rat GAD 65 resulted in the generation of a mixed Th1/Th2 cytokine profile in the A\betao/DR3/DQ8, A\betao/DR3, and A\betao/DQ8 mice. Thus, the presence of diabetes-associated genes determines whether immune tolerance is maintained to islet autoantigens, but autoreactivity in itself is not sufficient to induce diabetes. The Journal of Immunology, 2001, 166: 1370–1379.

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D4+ T cells play a dominant role in the development of type 1 diabetes and this is not unexpected, due to the strong primary association of susceptibility with the MHC class II molecules, DQ and DR (1). Because CD4 T cells recognize peptide epitopes presented in the context of MHC class II, the presence of disease-associated MHC alleles may select for the presentation of islet-specific self-peptides that are capable of activating autoreactive T cells. There is recent evidence to suggest that diabetes-associated MHC alleles can present peptides from other endogenous MHC molecules (2), which mimic autoantigen-derived peptides (3) and thus may present potential targets for self-reactive T cells. The most significant association of MHC class II molecules with susceptibility to type 1 diabetes are the HLA DQ8 (DQB1*0302) and DQ2 (DQB1*0201) alleles (1). HLA DR3 (DRB1*0301) and DR4 (DRB1*0401) (4) are also increased in frequency in patients with type 1 diabetes as the genes encoding these proteins are in linkage disequilibrium with those encoding the predisposing DQ2 and DQ8 molecules, respectively (5). Epidemiological evidence supports the concept of specific combinations of MHC alleles interacting synergistically, if other genetic and environmental factors are present, to induce disease (6, 7). X-ray crystallographic analysis of peptide-MHC complexes along with the identification of allele-specific peptide-binding motifs have revealed that disease-associated MHC polymorphisms map to the peptide binding site of MHC molecules (8, 9). The association of different MHC alleles with susceptibility to type 1 diabetes implies that the pathogenic T cell response, which is Ag specific, is triggered either by a number of B cell-derived peptides concomitantly or by a single peptide with subsequent inter- and intramolecular spreading of the autoimmune response and expansion of the T cell repertoire (10).

A number of autoantigens have been implicated in the etiopathogenesis of type 1 diabetes. These include islet-specific Ags, like insulin, and other nonislet-specific Ags, like glutamic acid decarboxylase (GAD) (11, 12). Of all the known autoantigens implicated in the disease process, treatment with only insulin, GAD 65, and the heat shock protein (hsp) 60 peptide p277 can protect nonobese diabetic (NOD) mice from disease (13–16). Insulin and GAD 65 are also the most prominent islet autoantigens shown to be recognized by peripheral T cells from type 1 diabetes patients (17, 18).

Therefore, although it is recognized that the genes of the HLA region are a major risk factor for the development of type 1 diabetes, and considerable information is currently available on MHC-peptide binding, there are significant lacunae in our understanding of autoantigen processing and presentation and its role in the pathogenesis of disease. The classification of type 1 diabetes as a T cell-mediated autoimmune disease, although valid, still requires considerable characterization in terms of understanding B cell autoantigen-specific T cell autoreactivity. We have attempted to evaluate the interactions between MHC alleles and an islet autoantigen, GAD 65, in a HLA transgenic model system, in an effort

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2 Abbreviations used in this paper: GAD, glutamic acid decarboxylase; hsp, heat shock protein; GTT, glucose tolerance test; NOD, nonobese diabetic.
to delineate the nature of autoreactive T cell responses that drive autoimmunity in type 1 diabetes.

Materials and Methods

Generation of transgenic mice

The generation of Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice has been previously described (19). Briefly, the DQ8 (DQA1*0301/DQB1*0302) and DR3 (DRA1*0101/DRB1*0301) transgenes were inserted into B10.M (H-2b, I-Ab/I-Eb) embryos, and the resulting progeny were crossed with Aβo (C57BL/6 x 129; H-2b, I-Ab/I-Eb) mice to generate the appropriate transgenic animals deficient in endogenous MHC class II molecules. Similarly, the HLA DR2 (DRB1*1502)-transgenic mice were crossed with Aβo/DQ6 (DQA1*0101/DQB1*0601, a diabetes-neutral allele) to generate Aβo/DR2/DQ6 mice (19). HLA DQ and DR expression was analyzed by flow cytometry and PCR.

Preparation of rat GAD 65

GAD was purified from homogenized rat brains by affinity chromatography using a mAb against GAD 65 (clone GAD6) as previously described (20). Briefly, 25 g of rat brain was homogenized in a potassium phosphate buffer (pH 7.5) followed by centrifugation at 130,000 × g for 1 h at 4 °C for size exclusion affinity chromatography column. The supernatant was loaded onto an affinity column that had been conjugated with the GAD6 mAb to Affi-gel (Bio-Rad, Richmond, CA). After passing the material through at a slow rate, the column was washed and GAD was eluted with elution buffer (0.5 M potassium phosphate buffer containing 0.01 M diethylamine and 0.02 M glutamate, pH 11). The eluted GAD was dialyzed against cold water for 3 days and concentrated by lyophilization. Following resuspension in a small volume of PBS, the protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL). The purity of the preparation was analyzed by both electrophoresis on a 8% denaturing acrylamide gel, followed by silver staining and Western blot using rabbit anti-GAD serum (7673), which recognizes both GAD 65 and GAD 67. Although the mAb, GAD 6, recognizes only GAD 65, a 120-kDa band due to the heterodimeric association of the two native isoforms of GAD 65 and 67 may result in a small amount of GAD 67 in the preparation (21). The rat GAD preparations were negative when tested for endotoxin contamination.

Peptides

GAD peptides were synthesized as described earlier (22). In addition to the 19 peptides previously described, which included both mouse and human sequences, 42 20-mer overlapping peptides of human GAD 65, starting at the N-terminal sequence, 42 20-mer overlapping peptides of human GAD 65, starting at the N-terminal sequence, were used along with Ag for inhibition of proliferation.

Flow cytometry and PCR analysis of transgenic mice

Analysis of HLA expression and absence of endogenous MHC class II by flow cytometry and PCR has been previously described (19).

T cell proliferation assays

Lymphoproliferation assays were performed according to a previously described protocol (19). The age of the mice used for the various T cell experiments ranged from 8 to 12 wk. For Ab blocking experiments, 10 μg/ml of either purified anti-CD4 (GK1.5), anti-CD8 (53.7.62; affinity purified from supernatants of hybridomas, American Type Culture Collection), anti-DQ (clone SPV-L3; Flanders, NJ), and anti-DR (clone TAL8.1) were used along with Ag for inhibition of proliferation.

T cell enrichment by nylon wool

T cells were purified from spleens of transgenic mice (3–4 per group) by using nylon wool columns prepared by a standard protocol (19). The enriched T cells were used with irradiated syngeneic spleen cells as APCs (pooled from two mice per group) for culture in vitro with rat GAD 65.

Cytokine analysis

Culture supernatants were collected from in vitro stimulations of spleen cells or enriched T cells along with irradiated APCs from unmanipulated transgenic mice with rat GAD 65 after 60 h of culture. The supernatants were tested in a cytokine detection ELISA for IL-2, IL-4, IFN-γ, IL-5, IL-6, IL-10, IL-12, and IL-13 (23). Standard curves with the appropriate recombinant cytokines were used in each experiment for quantification as well as internal controls.

Glucose tolerance test (GTT)

Transgenic (Aβo/DR3/DQ8, Aβo/DQ8, and Aβo/DR3) and control mice (Aβo and C57BL/10; The Jackson Laboratory, Bar Harbor, ME) were fasted overnight for 14 h; this was followed by an i.p. glucose injection (2 g/kg body weight) (20% dextrose; Baxter Health Care, Mundelein, IL) (24). Whole venous blood was obtained from the tail vein at 0, 15, 30, 60, and 120 min after injection. Blood glucose was measured by an automatic glucometer (Dex; Bayer, St. Paul, MN). Each group had 8–10 mice, equally divided by sex and between 8 and 10 wk of age.

Results

Spontaneous lymphocytic proliferative response to rat GAD 65

Previously, we reported that mice bearing two diabetes-associated MHC class II alleles, DQ8 and DR3, develop spontaneous T cell responses to human GAD 65 (19). However, single transgenic mice bearing either one of the diabetes susceptibility genes did not show this autoreactive response (19). Because the homology between human and mouse GAD 65 is ~96%, we surmised that by using an Ag-like rat GAD 65, which is more homologous (98%) to the self molecule, mouse GAD 65 (Fig. 1), we might be able to determine whether a single predisposing allele, either DQ8 or DR3, is capable of selecting autoreactive T cells specific for islet autoantigens or whether autoreactivity requires epistatic interactions between DR and DQ susceptibility genes. Spleen cells from unmanipulated Aβo/DR3/DQ8, Aβo/DQ8, and Aβo/DR3 all responded strongly to rat GAD 65 when the Ag was used for stimulation in vitro (Fig. 2). To ensure that the response was truly specific and not incidental, several nonislet-specific Ags were used as controls, such as OVA (Sigma, St. Louis, MO), BSA (Sigma), and hsp 60 (Sigma). The latter two Ags are also considered to be putative type 1 diabetes autoantigens (25, 26). However, there was no detectable response to these Ags (Fig. 2) in the transgenic mice.

To ascertain that the GAD 65-specific response was associated with type 1 diabetes-predisposing haplotypes, we used spleen cells from transgenic mice having either a diabetes-resistant haplotype (Aβo/DQ6, DQB1*0602) or double transgenic mice having both susceptible and resistant or neutral alleles (Aβo/DR2/DQ8 and Aβo/DR2/DQ6 (DQB1*0601)). MHC class II knockout animals (Aβo) were used as an additional control. These mice did not show any T cell reactivity to rat GAD or the other protein Ags tested but had normal responses to the polyclonal T cell mitogen, Con A (data not shown). Therefore, evidence of the autoreactive T cell response was associated with HLA-DQ and resistant or neutral alleles.

T cells are the responder cell population in this autoreactive response

To determine whether the proliferative response was due to autoreactive T cells recognizing appropriate MHC self-peptide complexes, we prepared enriched T cell populations from spleens of unmanipulated Aβo/DR3/ DQ8, Aβo/DR3, and Aβo/DQ8 mice using nylon wool columns. These T cells were used for culture in vitro with irradiated splenocytes as APCs and titrating amounts of rat GAD 65 (Fig. 3A). The enriched T cells from the transgenic mice mounted a response to the rat GAD clearly establishing that autoreactive T cells specific for GAD 65 were being expressed in these animals. Purified T cells from the Aβo/DR3/DQ8 showed a dose-dependent titration of the response (Fig. 3A), whereas the Aβo/DR3 mice had maximal stimulation at a lower concentration (1 μg/ml) of Ag, suggesting that either the frequency of GAD-reactive T cells in these mice is higher or that their affinity for the MHC peptide complex is stronger (than the Aβo/ DR3/DQ8) and higher concentrations of Ag may be inhibitory. Of greater interest was the fact that T cell stimulation in the Aβo/DQ8 mice was considerably higher than the other two transgenic strains and was almost 10-fold greater than the background control at the lowest concentration of Ag tested (0.3 μg/ml) (Fig. 3A).

To identify the phenotype of the responding T cells as well as the MHC restriction, Abs against CD4, CD8, DR, and DQ were used, along with whole spleen cells and rat GAD 65. In addition, an Ab against B220 (CD45R) was used to confirm that the response to rat GAD was not due to B cell proliferation (Fig. 3B, panel 1). In the three responding strains of transgenic mice, Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8, the anti-CD4 Ab blocked the response, whereas the anti-CD8 Ab did not inhibit the response (Fig. 3B, panel 1). Both DR and DQ were capable of presenting rat GAD as they blocked responses in the double transgenic, Aβo/DR3/DQ8 as well as the specific single transgenic, Aβo/DQ8 and Aβo/DR3 mice. The anti-B220 Ab did not block the rat GAD 65-specific response (Fig. 3B, panel 1). Therefore, the autoreactive lymphocytic response to rat GAD 65
GAD 65 appeared to be mediated by CD4+ T cells and was both DR- and DQ-restricted in the Aβ0/DR3/DQ8, and either DR- or DQ-restricted in the appropriate single transgenic mice. Ab blocking was also performed in the enriched T cell stimulations and, in a similar manner, anti-CD4 Ab blocked proliferation of rat GAD-specific T cells, although anti-CD8 did not (Fig. 3B, panel 2). The anti-DQ Ab seemed to block reactivity in the purified T cell fraction in Aβ0/DR3/DQ8 to a slightly greater extent than the anti-DR Ab (Fig. 3B, panel 2).

Identification of regions on the GAD 65 molecule recognized by autoreactive T cells

From the data thus far, it is apparent that HLA DQ8 and DR3 single and double transgenic mice have in their repertoire T cells that are capable of recognizing rat GAD 65, which has high sequence homology with the corresponding self protein. To identify immunogenic peptides that were responsible for this self-reactive response, 20-mer overlapping peptides were synthesized and used for stimulation in vitro at 100 μg/ml with spleen cells from Aβ0/DR3/DQ8, Aβ0/DQ8, and Aβ0/DR3 mice (Fig. 4). Aβ0/ DR3/DQ8 mice stimulated a strong response to peptides covering amino acid residues 21–40 and 281–300. Responsiveness was also seen to peptides 331–350, 361–380, and 250–270 (19). The Aβ0/DR3 mice similarly mounted a response to peptides 21–40, 61–80, and 311–330. In contrast, Aβ0/DQ8 mice appeared to recognize more peptides than either Aβ0/DR3 or Aβ0/DR3/DQ8. Although a very strong response was obtained to peptide 61–80 (Fig. 4), several other peptides also induced proliferation, including 1–20, 21–40, 261–280, 281–300, 311–330, and 381–400. Peptides 21–40, 281–300, and 361–380 have identical amino acid sequences for human, rat, and mouse GAD 65 (Table I), whereas peptides 250–270, 261–280, and 311–330 are the same for rat and mouse protein. Only peptides 1–20, 61–80, and 381–400 differ by a single amino acid residue between rat and mouse GAD 65 molecules (Table I).

Interestingly, the peptides that stimulated lymphocytic responses in the transgenic mice were selected from peptide libraries that are HLA typed and are expected to be in the HLA repertoire of the mouse strains. The peptides that were selected were then tested in vitro in mixed lymphocyte cultures to determine their specificity for mouse T cells. The results of these experiments are shown in Fig. 3B, panel 2. The anti-CD4 Ab blocked proliferation of rat GAD-specific T cells, although anti-CD8 did not (Fig. 3B, panel 2). The anti-DQ Ab seemed to block reactivity in the purified T cell fraction in Aβ0/DR3/DQ8 to a slightly greater extent than the anti-DR Ab (Fig. 3B, panel 2).

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FIGURE 2. Lymphocytic responses to rat GAD 65, BSA, OVA, and Hsp 60 in unmanipulated Aβ/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 transgenic mice. Lymphocytes from spleens of three mice per group were cultured at 3 × 10⁶ cells per well with half-log titrating amounts of rat GAD 65, BSA, OVA, and Hsp 60 for 96 h. Thymidine incorporation shown for rat GAD 65 at an antigenic concentration of 3 μg/ml. Background counts ranged from 1000 to 7000 cpm and were subtracted from the cpm obtained in the presence of Ag. One representative experiment of seven is shown.

mainly in the NH2 terminus and mid-region of the molecule. The interactions of class II molecules with each other seem to dramatically influence the pattern of immune responsiveness to GAD peptides. Peptides 1–20, 261–280, and 381–400 stimulate responses in the Aβo/DQ8 mice, whereas there is no reactivity seen in the Aβo/DR3/DQ8 mice, suggesting that the presence of HLA-DR3 may be in some way suppressing proliferative responses to these peptides. In a similar manner, p61–80 and p311–330 between the medium control and GAD 65-stimulated cells in both Aβo/DR3 and Aβo/DQ8 transgenic mice. Unmanipulated transgenic mice in all groups had background IFN-γ production when compared with the control groups (Fig. 6), indicating that autoreactivity to self-Ags by itself is not sufficient to trigger organ-specific autoimmune dysfunction, although it may be a necessary prerequisite.

Discussion

The studies described in this paper were undertaken to dissect the role of susceptible MHC genes, in the development of autoreactivity to islet Ags and their contribution to β cell function. One of the most reliable ways to ascertain early β cell dysfunction is to perform a Gtt. Mice are injected with glucose, in amounts appropriate to their body weight and metabolism, and then evaluated at defined intervals of time for changes in blood glucose levels. Aβo/DR3/DQ8, Aβo/DQ8, Aβo/DQ6 (control), Aβo (control), and C57BL/10 (control) were evaluated for changes in β cell function (Fig. 6). The three test groups did not show any significant differences in glucose metabolism when compared with the control groups (Fig. 6), indicating that autoreactivity to self-Ags by itself is not sufficient to trigger organ-specific autoimmune dysfunction, although it may be a necessary prerequisite.

Evaluation of β cell function

From our previous studies, it was clear that neither the double (Aβo/DR3/ DQ8) or single (Aβo/DR3 and Aβo/DQ8) transgenic mice develop diabetes (19) even though there was insulin or peri-insulitis to varying extents in all of them. There was no lymphocytic infiltration in control mice lacking both endogenous MHC class II molecules and the human transgenes (Aβo).

Nonetheless, the presence of autoreactive T cells recognizing an islet autoantigen warrants further investigation into subtle loss or alteration of β cell function. One of the most reliable ways to ascertain early β cell dysfunction is to perform a Gtt. Mice are injected with glucose, in amounts appropriate to their body weight and metabolism, and then evaluated at defined intervals of time for changes in blood glucose levels. Aβo/DR3/DQ8, Aβo/DQ8, Aβo/DQ6 (control), Aβo (control), and C57BL/10 (control) were evaluated for changes in β cell function (Fig. 6). The three test groups did not show any significant differences in glucose metabolism when compared with the control groups (Fig. 6), indicating that autoreactivity to self-Ags by itself is not sufficient to trigger organ-specific autoimmune dysfunction, although it may be a necessary prerequisite.

Discussion

The studies described in this paper were undertaken to dissect the role of susceptible MHC genes, in the development of autoreactivity to islet Ags and their contribution to β cell-specific autoimmunity, which causes chronic inflammation and the loss of insulin production. Type 1 diabetes is a fairly common autoimmune disease in children and young adults (28). Over the last several years, significant progress has been made in identifying genetic susceptibility and environmental factors that predispose to islet autoimmunity (29). It is thought that numerous etiological factors feed into a single, common pathogenic pathway that triggers unbridled immune destruction of β cells in the pancreas.

Genome-wide scans of type 1 diabetes sibling pairs have revealed a plethora of genetic intervals that confer risk for disease either alone or in combination (30, 31). Early, as well as more recent, genomic studies have clearly established the primacy of the MHC locus in disease predisposition (32, 33). However, what is still unclear is how MHC genes affect predisposition to type 1 diabetes. From our previous studies with HLA class II transgenic mice, it was evident that the mere presence of at-risk MHC genes cannot induce diabetes in this model, although they have comparable levels of expression of MHC class II molecules and normal numbers of CD4 and CD8 T cells, B cells, and macrophages (19). However, mice bearing either HLA DQA8 or DR3 had islets that showed mild peri-insulitis and/or insulinitis (19). More interestingly,
double transgenic mice having both susceptibility alleles, DR3 and DQ8, developed comparatively more extensive infiltration in the islets and, in addition, had spontaneous T cell reactivity to human GAD 65 (19). It has been reported that T cell responses to GAD 65 in newly diagnosed type 1 diabetic patients are significantly higher than in nondiabetic healthy subjects (34). Therefore, it appears that there is a requirement for either two susceptible MHC genes or epistatic interactions between them to select for an autoreactive
repertoire. Though this in itself does not induce disease, it is able to trigger the initiation of islet pathology, albeit benign. More recently, it was demonstrated that mice transgenic for HLA DQ8, in the absence of endogenous MHC class II and expressing the co-stimulatory molecule B7 only in the islets through the rat insulin promoter, developed spontaneous immune-mediated diabetes, whereas similar rat insulin promoter-B7 mice that had the diabetes-neutral allele $DQ^6 (DQB1*0601)$ did not show disease (35).

So, it is clear that particular MHC alleles are permissive for the development of diabetes if other predisposing genes are present.

The significant homology between rat GAD 65 and its murine counterpart enabled us to use it as a source of self-Ag to further evaluate the role of diabetes-predisposing and diabetes-neutral/resistant MHC class II alleles in the selection and survival of autoreactive T cells. The spontaneous lymphocytic reactivity to rat GAD 65, but not to other nonself-Ags, in the A$b^o/Dr3, A$b^o/Dr3/DQ8, and A$b^o/Dr3/DQ8$ mice clearly established the presence of autoreactive lymphocytes. This selection of self-reactive cells appears to require the presence of diabetes-susceptible MHC alleles as control transgenic mice having either resistant ($A$b$^o/DQ6, $DQ^6$), or combinations of resistant and susceptible alleles ($A$b$^o/Dr2/DQ8; $A$b$^o/Dr3/DQ6$) were unable to mount effective responses to GAD 65. It has been reported that a pancreatic β cell-reactive, I-A$^b$-restricted transgenic TCR that is strongly diabetogenic in NOD mice is negatively selected in the thymus of mice expressing various diabetes-resistant MHC alleles transgenically, along with a single I-A$^b$ allele on the NOD background (36).

FIGURE 4. Peptide responses to rat GAD 65 in unmanipulated A$b^o/Dr3/DQ8, A$b^o/Dr3, and A$b^o/DDQ8$ mice. Spleen cells (3 × 10$^5$ per well) from two to three mice per group were stimulated in vitro with GAD 65 peptides at 100 μg/ml for 96 h. Background counts ranged from 1000 to 7000 cpm and were subtracted from the cpm obtained after Ag stimulation. One representative of three experiments is shown here.

Mice that delete the transgenic TCR develop insulitis to variable degrees that correlate with the extent of thymocyte deletion, but are quite resistant to the development of diabetes (36). Extrapolation of this data to the current findings suggests that disease-resistant MHC class II alleles mediate their protection by not selecting for an autoreactive T cell repertoire.

Also, in the NOD mouse (37), insulitis has been shown to appear de novo at ~3–4 wk of age, after weaning, with progression to diabetes occurring in ~80% of female animals between 10 and 30 wk of age (38, 39). Coincidently, T cell reactivity is seen in NOD mice at about the same time that insulitis develops, and the initial response seems to be against a single autoantigen, which is followed by intra- and intermolecular spreading to other islet Ags (11, 12). In A$b^o/Dr3/DQ8$ mice, where insulitis is comparatively more extensive than in the single transgenic mice, there also appears to be, at least superficially, a temporal correlation between autoreactivity to islet Ag and onset of insulitic changes in the pancreas, which occurs at ~8 wk of age (our unpublished observation). We have not been able to determine any intermolecular spreading of T cell reactivity, as T cells do not mount a response against insulin (R.S.A. and C.S.D., unpublished observation). Stimulation of lymphocytes in vitro from unmanipulated A$b^o/Dr3/DQ8$ mice with human insulin did not induce any detectable T cell responses. This was not very surprising, because it has been documented that although over half of the patients with type 1 diabetes have autoantibodies to insulin (40), rarely have Ag-specific T cell responses been elicited in these patients. In fact, it has been shown (41) that the most immunogenic epitope on the insulin autoantigen, in the context of HLA DR4 (DRB1*0401), is on the preproinsulin and proinsulin molecules at the junction of the C
Table I. GAD 65 peptide sequence comparison for human, mouse, and rat

<table>
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<tr>
<th>GAD65 Sequence</th>
<th>Species</th>
<th>Human Sequence</th>
<th>Rat Sequence</th>
<th>Mouse Sequence</th>
<th>Sequence Variations</th>
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<td>MASPGSGFWSGEDGSQG</td>
<td>MASPGSGFWSGEDGSDP</td>
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<tr>
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</tbody>
</table>


**FIGURE 5.** A, Cytokine analysis of spleen cell responses to rat GAD 65. Supernatants harvested at 60 h from spleen cells of unmanipulated H-2d/D2R3/DQ8, H-2d/D2R3, and H-2d/D2R3 cultured with or without rat GAD 65 (medium control). Supernatants were analyzed in an ELISA for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and IL-12. Sensitivity of the ELISA ranged from 1 to 30 pg/ml. One representative of two experiments is shown here. B, Cytokine analysis of enriched T cell populations stimulated with rat GAD 65. T cells (1 x 10⁶) were cultured with irradiated APCs (2 x 10⁵) from unmanipulated H-2d/D2R3/DQ8, H-2d/D2R3, and H-2d/D2R3 cultured with or without rat GAD 65, Aβ84/DBO3/Q8, Aβ84/DBO3, and Aβ84/DBO3, and cultured with irradiated APCs (2 x 10⁵) for 60 h and analyzed for IL-2, IL-4, IL-10, and IFN-γ. The sensitivity of the ELISA was 30 pg/ml.
female mice and were between 8 and 10 wk of age. before the test was performed. Each group had equal numbers of male and female mice and were between 8 and 10 wk of age.

There have been reports that lend credence to the possibility that selection of the islet Ag-specific T cell repertoire occurs before the influx of lymphocytes into the islet (42). Because lymphocytic infiltration of islets appears to be a highly regulated phenomenon that is initiated at weaning, there is evidence to suggest that it may be due to major immunological changes that occur as a result of changes in diet, which induce broad-spectrum T cell activation and concomitant alteration in the homing potential of T cells, permitting migration into pancreatic tissue (42). Although the formation of an anti-islet Ag repertoire may very likely predate islet invasion, at least in the NOD, these two events appear to coalesce into a single entity with the presence of both autoreactivity and insulitis at the same age. In the DR3 and DQ8 single and double transgenics, it is not clear as yet whether there are two independent events or a single event with two interrelated components, because we have tested lymphocytic responses only in 8- to 12-wk-old animals. As mentioned earlier, on evaluating the kinetics of insulitis in Aβo/DR3/DQ8, we found no histological changes in the islets before 8 wk of age (our unpublished observation), and the extent of insulitis remained static even in older mice. There is sufficient evidence to show in a number of different animal models, including NOD (43), that it is possible for lymphocytic invasion of the islets of Langerhans to be tolerated for long periods of time (44–47) without provoking disease. It has been demonstrated that murine MHC class II I-E molecules, which are homologous to HLA DR, can protect NOD mice from developing diabetes in some situations, although this is not a universal phenomenon. It has been reported that I-E transgenic mice can protect from clinical diabetes but not insulitis. The increase in the extent of insulitis in the Aβo/DR3/DQ8 mice in comparison to the single transgenic Aβo/DR3 or Aβo/DQ8 suggests that DR3 and DQ8 are capable of interacting epistatically, and the absence of overt disease may be due to the presence of other non-MHC protective genes.

A number of studies have reported characterization of GAD 65 T cell epitopes both in NOD mice (11, 48) and in patients with type 1 diabetes (49–51). In a study done on Japanese patients with diabetes, CD4+ T cell clones that were GAD 65 reactive were isolated from PBMCs (52). These T cell clones recognized a number of GAD peptides, including p111–131, p413–433, p200–217, and p368–388, and these epitopes showed a tendency to be restricted by susceptible HLA-DR but not -DQ molecules (52). This finding has also been supported by other studies that demonstrated that the vast majority of human T cell responses are HLA-DR restricted; this may be due to the higher expression of HLA-DR in the periphery in comparison to DQ and vice versa in the thymus (53). Other analyses have similarly identified a variety of T cell epitopes on GAD 65, such as p473–555 (54), p247–279, p146–165 (55), p174–185, p206–225, p270–283, and p555–575 (56). T cell clones from diabetic patients, recognizing peptides 505–519 and 521–535, have also been reported (50). The GAD peptides that stimulate T cells from unmanipulated HLA DR3, DQ8, or DR3/DQ8 transgenic mice recognize partial sequences of peptides: p250–270, p261–280, p281–300, p361–380, and p381–300 identified in patients with diabetes. Epitope analysis with HLA DQ8 transgenic mice using GAD 65-specific T cell hybridomas revealed at least nine immunogenic epitopes on the molecule (57). The vast majority of the T cell clones responded to one of three epitopes in the regions 51–120, 111–180, or 521–585. The other peptides that were stimulatory included 101–115, 126–140, 206–220, 341–345, 461–475, and 536–550 (57). In contrast to the studies described in this paper, the DQ8 transgenic mice used above were made on the NOD background, which could account for the differences in interpretation of GAD epitopes. We have previously shown that non-MHC background genes from the NOD can influence Ag processing and presentation of GAD 65 peptides (22).

Binding studies done with HLA DR3 and GAD 65 peptides demonstrate weak affinity in comparison to other T cell epitopes, but all peptide epitopes recognized by HLA DR-restricted T cells from diabetic patients or GAD 65-immunized DR-transgenic mice bind with high affinity to the appropriate DR restriction molecule (56). However, binding studies are fraught with difficulties in interpretation, because peptides that stimulate CD4+ T cells have dramatically varying affinities for the restricting MHC class II molecules (58, 59). Also, the relationship between T cell responsiveness and peptide binding affinity to MHC is extremely complex, with certain weakly binding self-peptides capable of potent T cell stimulation and, sometimes, even induction of autoimmunity (60). Studies on the peptide binding motif of HLA DQ8 have revealed that pockets 4 and 9 on the MHC molecule, which interact with complementary residues on the peptide, are key sites of disease-associated polymorphisms (61, 62). It has been shown that peptides binding DQ8 with high affinity have large aliphatic side chains in pocket 4 and negatively charged side chains in pocket 9, forming a stable peptide–MHC complex. The presence of an alanine residue (A) at pocket 9 has been shown to bind DQ8 with only low to moderate affinity, quickly dissociating from the MHC class II complex (63). The affinity studies clearly indicate that any self-peptide with a negative charge at residue 9 is likely to be a good binder and would negatively select self-reactive T cells; in contrast, self-peptides with similar motifs but different charge properties would bind with only low or moderate affinity and positively select autoreactive T cells. All of the immunodominant peptides in the HLA-transgenic mice have neutral (tryptophan, phenylalanine, alanine, isoleucine, glycine, and serine) or basic (arginine) residues at position 9 that would allow for low to intermediate affinity interactions and positive selection of T cells.

CD4+ T cells play a key role in the regulation of immune function and responsiveness. There is considerable evidence that suggests the existence of functionally polarized CD4+ T cell responses based on their pattern of cytokine production, both in mice and humans (64). Th1 cells produce IFN-γ, IL-2, and IL-12, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, among others.
65-specific T cells. Unlike the NOD, the A
macrophages produced significant amounts of IL-6 after culture levels of IL-6 (69). In contrast, adherent spleen cells and peritoneal normal, murine freshly isolated T cells revealed either no or ex-
cytokine with biological activity on B cell differentiation and T cell
known to be produced by a variety of hemopoietic cell types in-
were higher in the double transgenics. Although IL-6 was secreted
Th subset cytokines secreted in response to antigenic stimulation.
12). Qualitative analysis of the CD4
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bodies contribute only partially to the overall phenotype of dis-
expression of disease may be independent but not mutually exclusive
bias. This balance between Th1 and Th2 cytokines certainly ap-
and recognize a distinct set of epitopes on short and giant ragweed group 5
in HLA DRD and DQ8 single or double transgenic mice. The presence of both subsets of cytokines may be due to the fact that the rat GAD 65 response is a composite of individual peptide-specific responses that stimulate and terminally differen-
tiate Th1- or Th2-producing T cells.
Therefore, this data suggests that diabetes-susceptible HLA class II molecules are capable of binding and presenting peptides from the autoantigen, GAD 65, and that T cells with the ability to bind self-peptide-MHC complexes exist in the periphery of mice bearing these molecules. So far the story is similar to NOD mice that also have a diabetes-susceptible MHC, I- A<sup>07</sup> (70), and GAD 65-specific T cells. Unlike the NOD, the Aβo/DQ8, Aβo/DR3, or Aβo/DR3/DQ8 mice do not show any overt manifestations of hyperglycemia, which indicates that autoimmunity and progression of disease may be independent but not mutually exclusive phenomena. There is evidence to suggest that MHC class II genes contribute only partially to the overall phenotype of di-
ease susceptibility and that non-MHC genes and other, presum-
ably environmental, factors also participate significantly in de-
termining the ultimate outcome of disease.

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