Induction of Insulitis by Glutamic Acid Decarboxylase Peptide–specific and HLA-DQ8-restricted CD4$^+$ T Cells from Human DQ Transgenic Mice

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Abstract

Insulin-dependent diabetes mellitus in humans is linked with specific HLA class II genes, e.g., HLA-DQA1*0301/DQB1*0302 (DQ8). To investigate the roles of HLA-DQ8 molecules and glutamic acid decarboxylase (GAD) in disease development, we generated DQ8$^+$/I-Ab^o transgenic mice expressing functional HLA-DQ8 molecules and devoid of endogenous mouse class II. DQ8$^+$/I-Ab^o mice produced antigen-specific antibodies and formed germinal centers after immunization with GAD65 peptides. Two GAD peptide–specific (247–266 and 509–528), DQ8 restricted Th1 CD4$^+$ T cell lines, were generated from immunized DQ8$^+$/I-Ab^o mice. They induced severe insulitis after adoptive transfer into transgene positive (but not negative) mice who were treated with a very low dose of streptozotocin that alone caused no apparent islet pathology. In addition to CD4, islet mRNA from these mice also showed expression of CD8, IFNγ, TNFα, Fas, and Fas ligand. Our data suggest that a mild islet insult in the presence of HLA-DQ8 bearing antigen-presenting cells promotes infiltration of GAD peptide reactive T cells into the islet. (*J. Clin. Invest.* 1998. 102:947–957.) Key words: transgenic mice • HLA-DQ8 • glutamic acid decarboxylase • T cells

Introduction

Type 1 or insulin-dependent diabetes mellitus (IDDM)[1] results from the interplay of genetic, environmental, and autoimmune factors that lead to the selective destruction of pancreatic β cells by the immune system, especially by T cells. In both humans and the nonobese diabetic (NOD) mouse, an animal model of IDDM, glutamic acid decarboxylase (GAD), an enzyme mainly restricted to brain and β cells, has been implicated in the pathogenesis of the disease (1, 2). Antibody responses to GAD are amongst the earliest markers for the subsequent development of disease in first degree relatives of patients with IDDM (3–5). Moreover, T cell reactivity to GAD (6, 7), as well as to GAD-65 peptides (8) can be seen in prediabetic subjects (defined by the presence of serum autoantibodies) and newly diagnosed patients. The significance of these observations remains unclear, however, since there is no direct evidence, in humans, that GAD reactive T cells have a primary role in the β cell-directed autoimmune process.

The dominant locus defining genetic susceptibility to human IDDM is encoded within the MHC region on chromosome 6 (9). Although controversy still exists over which locus or loci within the MHC are linked to IDDM susceptibility, most data indicate that alleles encoding MHC class II molecules, whose primary function is to present antigenic peptides to CD4-positive T cells, have the greatest importance. Although the DR locus may be involved, current data suggest that the DQA1 and DQB1 regions are more closely linked to IDDM and thus are likely to encode key susceptibility/resistance determinants (10–12). In NOD mice, the predisposing MHC allele is I-A^{g7}, which like the DQ allele, is characterized by the unusual substitution of serine for the charged residue aspartic acid at position 57 of the β chain which is thought to have a protective effect (13). However, the picture is more complex. A single residue does not appear to determine susceptibility and the combination of both DQA1 and DQB1 determinants may be important (10, 12, 14–16).

It is noteworthy that the most common haplotype seen in Caucasian patients with IDDM is DQA1*0301-DQB1*0302 or DQ8 haplotype. Kwok et al. (17) and Wicker et al. (18) have reported that DQA1*0301-DQB1*0302 molecules preferentially bind, in vitro, several diabetes-associated peptides derived from GAD. This raises the possibility that the binding of GAD peptides to DQA1*0301-DQB1*0302 molecules in vivo may be involved in immunopathogenesis of IDDM. This study was therefore undertaken to characterize the recognition of GAD65 peptides by T cells in the context of HLA-DQA1*0301-DQB1*0302 (DQ8) molecules using an in vivo system and the role of these T cells in the development of islet autoimmunity.

Methods

Generation of HLA-DQ8 transgenic mice. To generate mice transgenic for human DQA1*0301-DQB1*0302 (DQ8), intact DQA1*0301-DQB1*0302 genes were inserted into cosmids PDCβ2 (kindly provided by Dr. Jack Strominger) and excised by cleavage of a 30-kb fragment using PvuI and ClaI. The fragments were purified and injected into fertilized eggs of (CBA/Ca × C57BL/6)F2 mice. This transgene contained 649 bp of pBR322 sequence and the transgene-positive founders were genotyped by Southern blot analysis of tail DNA using a 626-bp PvuI/EcoRI fragment derived from the cosmid. The transgenic line was then backcrossed to C57BL/6 (N6, five generation-backcross), to remove mouse MHC class II I-E^k (equivalent to

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1. *Abbreviations used in this paper:* GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; STZ, streptozotocin.

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human MHC class II DR) that was derived from CBA/Ca, before the initiation of this study. All the mice used in this study were littermates derived from the same breeders that were the progeny of N6.

The transgenic line expresses HLA-DQ in both the cortical epithelial and the medullary bone marrow derived cells of the thymus with a profile similar to control I-A^b. The DQA1*0301-DQB1*0302 molecules are also expressed on the cell surface of B220+ peripheral lymphocytes analyzed by flow cytometry using HLA-DQ-specific mAb (IVD12; Becton Dickinson Co., CA), indicating expression on mouse antigen-presenting cells. The homozygosity for the transgene was determined by the level of DQA1*0301-DQB1*0302 expression on B220+ cells and by crossing the homozygous transgenic mouse to a wild-type C57BL/6 mouse. All the pups from this mating expressed the transgene at a moderate level.

Homozygous HLA-DQA1*0301-DQB1*0302 transgenic mice were mated with MHC class II mutant mice (I-A$\beta^{-/-}$, C57BL/6, kindly provided by Diane Mathis) (19) to generate DQ transgenic mice devoid of murine MHC class II expression. F1 offspring of this mating were screened for DQA1*0301-DQB1*0302 expression by flow cytometric analysis of PBL. DQA1*0301-DQB1*0302-positive/I-A$\beta^{+/-}$ mice were selected and backcrossed to I-A$\beta^{-/-}$ mice (C57BL/6). The resulting mice included both DQA1*0301-DQB1*0302-positive/I-A$b^{-/-}$ mice (DQ^+/class II^o) and DQA1*0301-DQB1*0302-negative/I-A$b^{-/-}$ (DQ^-/class II^o). The DQ^+/class II^o mice were then intercrossed to generate mice homozygous for the transgene. Homozygous DQ^+/class II^o and DQ^-/class II^o mice (both sexes) 6–12 wk old were used.

Immune responses upon immunization. DQ^+/class II^o and DQ^-/class II^o mice were immunized subcutaneously with murine $GAD65_{247–266}$ and $GAD65_{509–528}$ peptides (50 μg per foot pad) in the presence of CFA. After 3 wk they were boosted with the same amount of the peptide in IFA and then were killed 5–7 d later. The sera were tested before and after immunization for GAD peptide–specific antibody by ELISA (20, 21). Half of the spleen from each mouse was snap frozen in optimal compound temperature for germinal center staining (22) and the remainder was used for proliferation assays.

Generation and propagation of T cell lines. Lymphocytes were harvested from popliteal lymph nodes and spleens of DQ^+/class II^o mice that had been immunized with GAD peptides and were cultured in Click's modified Eagle's medium (EHAA) containing IL-2 (EL-4 supernatant; 5 U/ml). After 7 d, the cultured cells were stimulated with the peptide (10 μg/ml) in the presence of irradiated DQ^+/class II^o splenocytes as APCs. The cell cultures were maintained on the medium described above and restimulated with cycles of antigen at 2-wk intervals. Two stable T cell lines (S.511 and L.17) were established from mice immunized with GAD65 peptide 247–266 and 509–528, respectively. TCR usage was examined by flow cytometry using a panel of FITC-conjugated mAbs to Vβ2 to 14, and 17 (PharMingen, San Diego, CA).

Proliferation and inhibition assays. T cells (10^4/well for T cell lines or 2×10^5/well for cells from spleen or lymph node) were incubated 72 h with antigen(s) in the presence of irradiated DQ^+/class II^o or DQ^-/class II^o splenocytes (10^5/well) as APCs. The cultures were pulsed with 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA) and harvested 16 h later. Blocking assays were performed with the addition of mAbs to CD4 (GK1.5), HLA-DQ (IVD12), TCRβ (H57), CD8 (TIB 105), mouse MHC class I (28-8-6S) and I-A^b (212-A1). Monoclonal abs (used at concentration of 10 μg/ml) were kindly provided by Dr. Charles Janeway, Jr. except for the mAb to HLA-DQ (Becton Dickinson).

Cytokine detection. Cytokine mRNA was detected by RT-PCR (see below). Secreted cytokine proteins were measured by ELISA using mAbs and the recommended protocols (PharMingen). Recombinant IFNγ and IL4 (Gibco Laboratories, Gaithersburg, MD) were used as standards.

Kinetic study of streptozotocin (STZ) effect. Genetic background and gender (male > female) may influence the susceptibility of mice to STZ-induced diabetes (23–26). Although the susceptibility to low-dose STZ treatment of the C57BL/6 (H-2^b) strain, to which our transgenic mice were backcrossed, is not high (relative to some other strains) and comparable to CBA/J (H-2^k) (27), preliminary STZ dose response studies were conducted to determine the susceptibility of the DQ^+/class II^o mice to STZ induced β cell destruction. The goal was to develop an experimental model system with STZ in which there was very mild subclinical islet injury before injection of GAD peptide–specific T cells. For this purpose, six groups of sex-matched mice ($n = 4$ for each group [two males and two females]) except one group ($n = 6$ [three males and three females]) were injected with STZ (Boehringer Mannheim, Mannheim, Germany; dissolved in citrate buffer) daily for 1–6 d (40 mg/kg per d, i.p.). They were killed 8 wk after the first injection unless diabetes occurred. Mice given over four injections developed either insulitis or diabetes (blood glucose > 250 mg/dl), whereas none of the mice treated with STZ on three occasions or less developed insulitis and their pancreatic islets were free of monocytic infiltration (H+E staining, data not shown). To further confirm this result, two doses (50 mg/kg per d) of STZ were given to a separate sex-matched group ($n = 10$ for each sex) and the period of observation was extended to 16 wk; none developed either insulitis or diabetes. This indicates that the presence of any CBA-derived genetic elements or gender influence must be minimal. Therefore, two injections at 50 mg/kg per d (i.p.) were used for the adoptive transfer experiments (see below).

Adoptive transfer experiments. Irradiated (600 rads) DQ^+/class II^o and DQ^-/class II^o mice (both sexes, 8–14 wk old) were used as recipients in the adoptive transfer experiments. In each experiment, some of the mice were treated on two successive days with low-dose STZ 24 hours before irradiation. 1 d later, 10×10^6 GAD peptide–reactive T cells were injected intravenously. As negative controls, mice were injected with either purified $CD4^+$ cells (naive) (using CD4 T cell isolation columns; Pierce Chemical Co., Rockford, IL) from DQ^+/class II^o splenocytes or with Con A (Gibco; at 2.5 μg/ml) stimulated (48 h at 37°C; 5% CO_2) purified $CD4^+$ cells (activated). One group of mice was also injected with normal saline as another control. All the mice were monitored for glycosuria and the experiments were terminated 8 weeks after adoptive transfer. Pancreas, kidney, liver and salivary gland from each recipient were fixed in formalin and examined with standard hematoxylin-eosin staining. An average of 10 islets/mouse was evaluated under light microscope and insulitis was scored according to Huang et al. (28).

Effect of STZ on lymphocytes and tissue organs. Both DQ^+/class II^o ($n = 2$, male) and DQ^-/class II^o ($n = 2$, male) mice were treated twice with STZ, as described above, and killed 24 h later. Pancreas, kidney, liver were fixed and snap-frozen in OCT compound (Miles, West Haven, CT), as described previously (29). (7 μm) frozen sections were analyzed by immunohistochemical staining (22). Splenocytes were stained with a panel of mAbs against MHC molecules (AF6-88.5 for K^b from PharMingen and IVD12 for DQ from Becton Dickinson). T cell markers (CD3, CD4, and CD8, PharMingen), costimulatory and adhesion molecules (B7.1, B7.2, LFA-1, ICAM-1 and LPAM, PharMingen) and analyzed by flow cytometry.

RT-PCR for islet cytokine, Fas, and Fas-ligand (FasL) mRNA. Pancreatic islets were isolated as previously described (29) at the termination of experiments 8 wk after adoptive transfer (see above). Pancreata from most of the recipients in the adoptive transfer experiments were used for the histopathological study (see above). However, pancreatic islets were also obtained from six groups of mice ($DQ8^+$ and $DQ8^-$; three groups each) that received (*i*) saline; (*ii*) twice STZ; (*iii*) twice STZ and S.511 cell transfer (see above adoptive transfer experiments). Total RNA was prepared from these handpicked islets by Trizol (Gibco Laboratories) and ~ 3 μg of RNA was reverse transcribed into single-strand cDNA using an oligo(dT) primer (Pharmacia Fine Chemicals, Piscataway, NJ) and MMLV reverse transcriptase (Gibco Laboratories). 2 μl of the cDNA (100 μl) was amplified with primers specific for IFNγ, TNFα, Fas, FasL, CD4 and CD8, together with hypoxanthine phospho ribosyl transferase as a "housekeeping" control in the presence of 100 ng of the 5′ and 3′

primers, 1 μl dNTPs (10 mM), 1.5 mM $MgCl_2$, and 1 unit of Taq polymerase (Boehringer Mannheim). The PCR reactions were denatured at 94°C for 5 min followed by 35 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 1 min and a final extension at 72°C for 7 min. The annealing temperature of PCR reactions for CD4 and CD8 was 61°C and $MgCl_2$ concentration was 2.5 mM. PCR products were analyzed on 1.5% agarose gels. Primers were synthesized in the Keck Facility of Yale University. The primer sequences were previously published (30, 31).

Results

Generation and expression of the human DQ transgene in mice lacking endogenous mouse MHC class II molecules. Mice homozygous for both DQ transgene and the I-Aβ mutation (DQ^+/class II^o), and control transgene negative mice, homozygous for the I-Aβ mutation (DQ^-/class II^o), were studied (see Methods). The possibility that transgenic mice expressed hybrid I-Aα-DQB1*0302 molecules was excluded by negative staining of PBL with mAb specific for I-$A\alpha^b$ (AF6-120.1; PharMingen), indicating the only MHC class II molecule expressed is the human transgene DQ, as C57BL/6 mice do not express I-E due to a mutation in the promoter of the Eα gene (32).

The homozygous expression of the human DQA1*0301-DQB1*0302 transgenes (Fig. 1 *A*) in mice deficient in murine MHC class II molecules partially restored $CD4^+$ T cells (10–15% of total lymphocytes (B and T cells) compared with ~ 1% in DQ^-/class II^o mice, Fig. 1 *B*). Analysis of the TCR Vβ repertoire of the DQ^+/class II^o mice showed no significant differences between transgenic and wild type (MHC class II-sufficient) mice (C57BL/6), except that the level of $CD4^+$ T cells was lower in transgenic (15±5% of $CD3^+$ T cells; $n = 10$) than in wild type mice (56±8% of $CD3^+$ T cells; $n = 4$). On the other hand, as seen in MHC class II-deficient mice (19), the percentage of $CD8^+$ T cells was higher in transgenic mice (76±8% of $CD3^+$ T cells; $n = 10$) than in wild type (24±4% of $CD3^+$ T cells; $n = 4$). Furthermore, T cells from the transgene-positive mice did not express the DQ8 molecules. This is noted because human T cells can express MHC class II molecules when activated. In addition, it also shows that the transgene is expressed appropriately only on professional antigen-presenting cells.

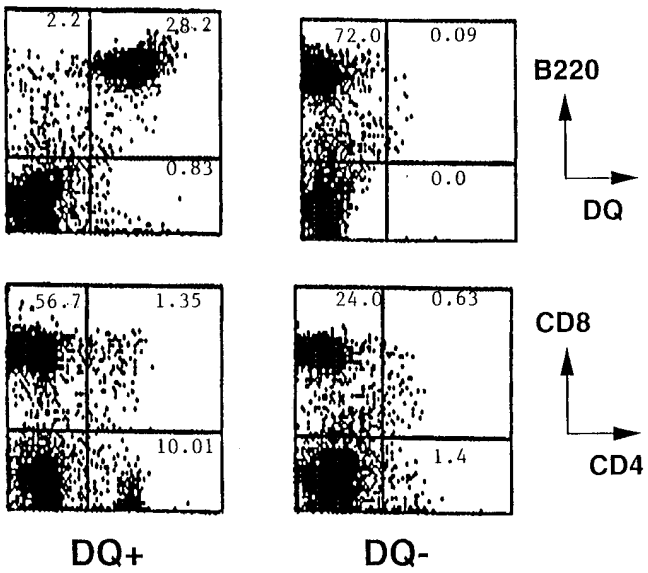

Figure 1. Comparison of HLA-DQ expression and CD4 restoration in transgenic-positive and -negative mice. Splenocytes were harvested from 6-wk-old mice and stained with mAbs as described in the text and analyzed by flow cytometry.

Function of the human DQ transgene: production of GAD specific antibody and development of germinal centers. While B cell development in MHC class II-deficient mice appears normal (33), they can neither mount specific antibody responses against thymic-dependent antigens nor generate germinal centers (GCs) (19), the hallmark of both T–B cell interactions and B cell affinity maturation. To investigate whether the HLA-DQA1*0301-DQB1*0302 could restore this functional B cell defect, we immunized the DQ transgene positive or negative MHC class II-deficient mice with GAD peptide 247–266 and 509–528, respectively. As shown in Fig. 2 *A*, the immunized DQ^+/class II^o mice (three mice/group) produced GAD65 peptide–specific antibodies, whereas DQ^-/class II^o mice (three mice/group) did not. Moreover, GC formation was also seen in spleens from all immunized DQ^+/class II^o mice (6/6), but not DQ^-/class II^o mice (0/6; Fig. 2 *B*). Thus, B cells lacking MHC class II failed to be activated by T cells in a cognate interaction and this defect is abrogated by human DQA1*0301-DQB1*0302 molecules. Moreover, the $CD4^+$ T cells restored by the expression of human MHC molecules appear to function normally and this may also contribute to the rescue of B cell responses.

T cell response to GAD peptide. To study the T cell immune responses of immunized DQ^+/class II^o transgenic mice against $GAD65_{247–266}$ and $GAD65_{509–528}$ peptides, in vitro proliferation assays were performed. Cells derived from either draining lymph nodes (popliteal LN) or spleen from immunized DQ^+/class II^o transgenic mice (three mice/group) mounted a specific proliferative response against the GAD peptides, while cells from draining popliteal LN or spleen of immunized DQ^-/class II^o mice (three mice/group) did not (data not shown), implying that they are $CD4^+$ and DQA1*0301-DQB1*0302 restricted.

The immunized DQ^+/class II^o mice were used to generate two DQA1*0301-DQB1*0302 restricted, GAD peptide specific $CD4^+$ T cell lines, one from popliteal lymph nodes (L.17, specific for peptide 247–266) and one from spleen (S.511, specific for peptide 509–528). FACS analysis of TCR Vβ usage of these two T cell lines using a panel of mAbs against murine TCR Vβs showed that S.511 expressed Vβ7 and Vβ12 (Fig. 3 *A*), whereas L.17 did not show dominant staining by any of the TCR Vβ mAbs used (data not shown). However, L.17 and S.511 T cell lines proliferated specifically to $GAD65_{247–266}$ and $GAD65_{509–528}$ peptide, respectively (Fig. 3 *B*, solid symbols), and not to $GAD65_{524–543}$ peptide or control peptides of the reverse sequence to $GAD65_{247–266}$ or $GAD65_{509–528}$ (data not shown). Neither showed reactivity to whole islets isolated from DQ^+/class II^o or DQ^-/class II^o mice (data not shown), perhaps explained by the fact that mouse islets express extremely low levels of both GAD isoforms (34). However, unexpectedly, the two T cell lines also did not respond to purified GAD from rat brain on several testings using three different batches of GAD antigen preparation at a concentration of 0.3–30 mg/ml (data not shown). There is complete sequence homology in the region of $GAD65_{247–266}$ peptide between mouse and rat, whereas

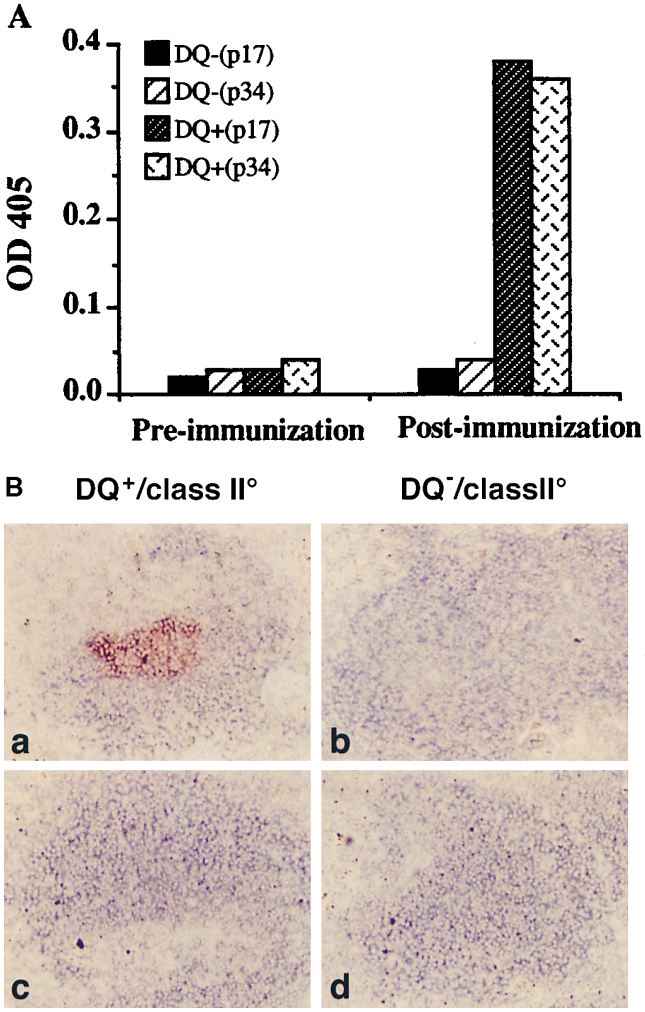

Figure 2. Comparison of B cell immune responses to GAD peptide immunization in transgene-positive and -negative mice. (*A*) GAD peptide–specific antibody production. $GAD65_{247–266}$- and $GAD65_{509–528}$-specific IgG production was detected by ELISA (see Methods) in the sera (diluted 1:100; shown as mean of three) of immunized mice ($n = 3$ for each group). (*B*) Germinal center (GC) formation. GC, detected with PNA, which stained red-brown, was found in $GAD65_{509–528}$-immunized $DQ8^+$ mice (*a* represents one of the three mice), but not in immunized $DQ8^-$ mice (*b* represents one of the three mice) or unimmunized mice, $DQ8^+$ (*c* represents one of the two mice) or $DQ8^-$ (*d* represents one of the two mice). Primary follicular B cell areas were detected in *a–d* with anti-B220 (blue). GCs were also found in $GAD65_{247–266}$-immunized $DQ8^+$ mice (data not shown). ×40.

there is one amino acid sequence difference in the region of $GAD65_{509–528}$ peptide between mouse and rat (at position 515: Threonine for mouse and Valine for rat; [35]), which may have contributed to the unresponsiveness in the latter experiments. Alternatively, the two GAD peptide–reactive T cell lines may reflect T cells with a "type B response," that are peptide specific but not whole antigen specific (36). The specific responses of L.17 and S.511 to $GAD65_{247–266}$ and $GAD65_{509–528}$ peptides, respectively, were HLA-DQA1*0301-DQB1*0302 restricted, as there was (*i*) no proliferation when DQ^-/class II^o (Fig. 3 *B*, *open symbols*) or when wild type (C57BL/6, data not shown) splenocytes were used as APCs and (*ii*) complete inhibition of the response (Fig. 3 *C*) by the addition of anti-CD4 (GK1.5), anti-HLA-DQ (IVD12) and anti-TCRβ (H57) mAbs but not control antibody rat IgG (Fig. 3 *C*). In contrast, anti-CD8 (TIB 105), anti-class I (28-8-6S) and anti-$I\text{-}A^b$ (212-A1) had no inhibitory effect (data not shown). In vitro cytokine production following stimulation with $GAD65_{247–266}$ and $GAD65_{509–528}$ peptides at both the mRNA and secreted protein level (Fig. 3 *D*), demonstrated typical Th1 cytokine profiles, namely high levels of IFNγ and negligible levels of IL-4.

Induction of insulitis by DQ restricted GAD peptide specific CD4 T cells. To test whether the specific interaction of DQA1*0301-DQB1*0302 molecules with GAD peptides alone might be sufficient to promote islet autoimmunity, without confounding interference from background insulitis from other genetic causes (C57BL mice develop neither insulitis nor diabetes), the $GAD65_{247–266}$ and $GAD65_{509–528}$ peptide–specific Th1 cell lines, L.17 and S.511, were adoptively transferred into recipient DQ^+/class II^o and DQ^-/class II^o mice (both male and female), respectively. Considering that (*i*) GAD is an intracellular enzyme; (*ii*) GAD is expressed at very low levels in mouse islets as compared with that seen in humans and the rat (34); and (*iii*) mouse β cells do not express MHC class II molecules (37, 38), we anticipated that our GAD peptide–reactive CD4+ T cells might not be able to recognize their antigen under normal conditions in mice that do not develop spontaneous islet pathology. For the above reasons, we treated one group of mice ($n = 34$, 16 males and 18 females) with two low doses of STZ. This, we hypothesized, might allow GAD-reactive T cells to gain better access to antigen presenting cells expressing the DQ transgene, which could have bound GAD peptides released by mild β cell injury (39, 40). Irradiated DQ^+ and DQ^- recipients (±STZ) were given an intravenous injection of $GAD65_{247–266}$ peptide reactive T cells or $GAD65_{509–528}$ peptide–reactive T cells, or normal saline (as a control). Although diabetes was not observed in any of the mice during the 8-wk study period, only the STZ treated DQ^+/class II^o mice given GAD peptide–reactive T cells, regardless of whether peptide 247–266 specific or 509–528 specific, developed insulitis (Fig.

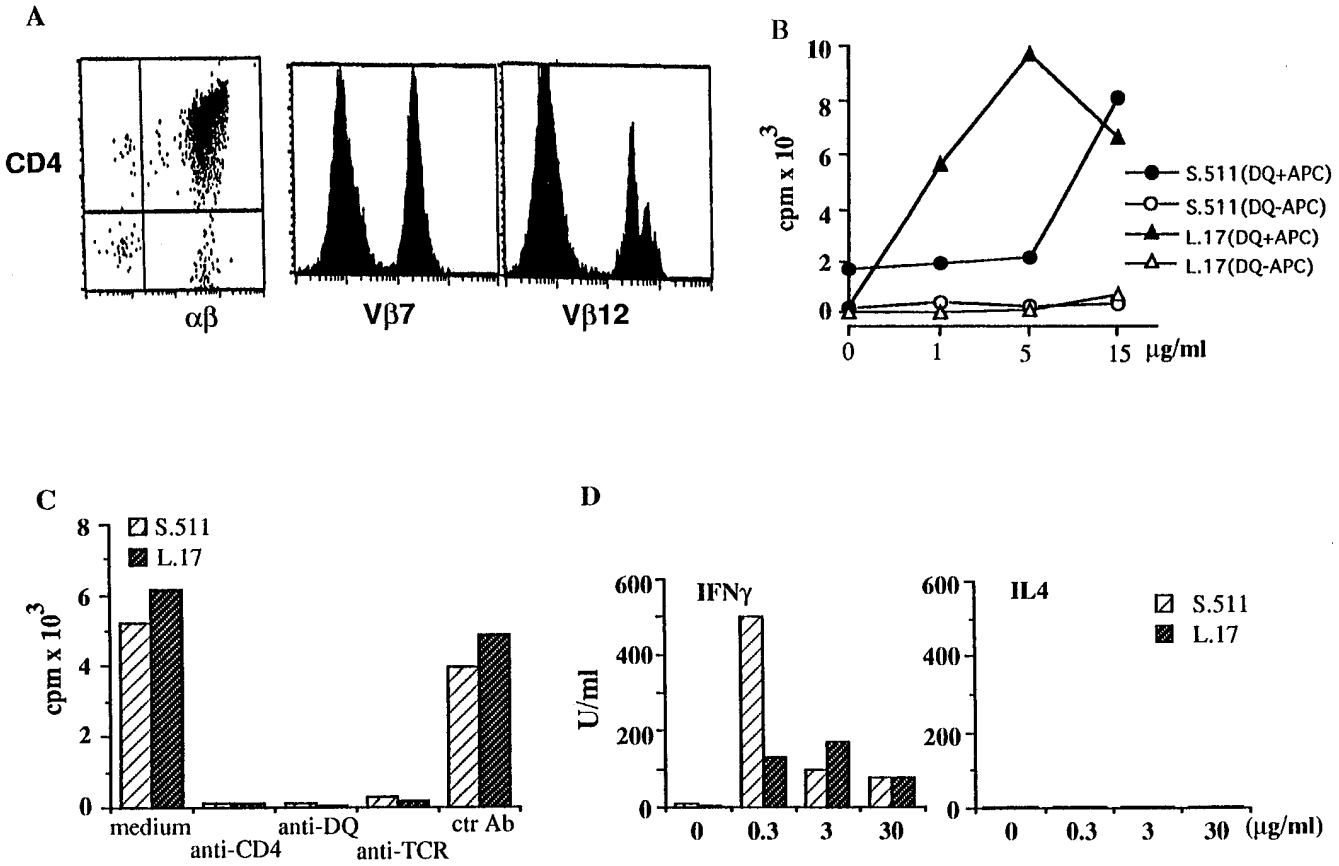

Figure 3. Characterization of GAD peptide–reactive T cell lines. (*A*) Phenotype analysis. Cells from S.511 were stained with mAbs to PE-conjugated CD4, FITC-conjugated TCRαβ, TCR Vβ2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 17 and examined on FACScan. (*B*) Proliferative response of the T cell lines (*circle*: S.511; *triangle*: L.17; 10^4/well, in triplicates) to $GAD65_{247–266}$ and $GAD65_{509–528}$, respectively, in the presence of APCs from DQ+ (*solid symbols*) or DQ− (*open symbols*) mice. (*C*) Antibody-blocking assay. The GAD peptide specificity of the T cell lines S.511 and L.17 (10^4/well, in triplicates) was tested in the presence of mAbs (10 μg/ml) to HLA-DQ, CD4, TCRαβ. (*D*) Cytokine profiles. Supernatants were harvested from the proliferation assay (as described in *B*) and IFNγ and IL-4 production was measured by ELISA (see Methods). *Filled* and *hatched bars* represent the cytokine production by line S.511 and L.17, respectively.

4). The insulitis was HLA-DQA1*0301-DQB1*0302 restricted as no obvious insulitis occurred in either STZ treated or untreated DQ^-/class II^o mice (Fig. 4; Table I) although occasionally very few scattered mononuclear cells could be found in rare islets. Insulitis was also not detected in STZ-treated DQ^+/class II^o mice given saline, indicating that these small doses of STZ per se are insufficient to provoke islet-directed destructive immune responses. However, STZ was required for the insulitis caused by the GAD peptide–reactive T cells, since no perceptible insulitis was observed in DQ^+/class II^o mice given GAD peptide reactive T cells without prior STZ treatment (Fig. 4; Table I). Thus, the insulitis appears to require both a mild islet insult and the expression of HLA-DQA1*0301-DQB1*0302 molecules.

To examine whether insulitis could be attributed to the nonspecific entry of T cells into the islet following mild local injury by STZ, adoptive transfer experiments were also performed using purified splenic $CD4^+$ T cells, showing no reactivity to GAD or its peptides, that were obtained from unmanipulated DQ^+/class II^o mice. All the recipients (DQ^+/class II^o, [n = 3, two males, one female] and DQ^-/class II^o [n = 3, one male, two females] mice) were treated with STZ before irradiation and adoptive transfer of $CD4^+$ T cells ($\sim 8 \times 10^6$/mouse). Insulitis was not observed in any of the mice, despite STZ treatment (data not shown). Considering that purified splenic $CD4^+$ T cells used in the above transfer experiments are likely to consist mainly of quiescent cells, unlike the GAD peptide specific T cell lines that were periodically in vitro activated with the peptides, we performed additional adoptive transfer experiments using Con A–stimulated purified splenic $CD4^+$ T cells obtained from unmanipulated DQ^+/class II^o mice. These cells showed no reactivity to GAD or its peptides either before or after Con A stimulation. The recipients (DQ^+/class II^o, [n = 3, one male, two females] and DQ^-/class II^o [n = 2, 1 male, 1 female] mice) were treated with two doses of STZ before irradiation and adoptive transfer of activated $CD4^+$ T cells ($\sim 8 \times 10^6$/mouse). Insulitis was not observed in these mice 5 wk post-adoptive transfer, despite Con A stimulation of the donor $CD4^+$ T cells (data not shown).

Direct evidence that STZ treatment is required to induce GAD peptide–reactive T cells to home to pancreatic islets was derived from cell tracing studies in which 10^7 S.511 T cells were labeled with the fluorescent dye, DiI (29) and transferred into DQ^+/class II^o and DQ^-/class II^o mice with or without STZ treatment. In all mice, the labeled T cells were found in the spleen (data not shown). This is expected, as they are lym-

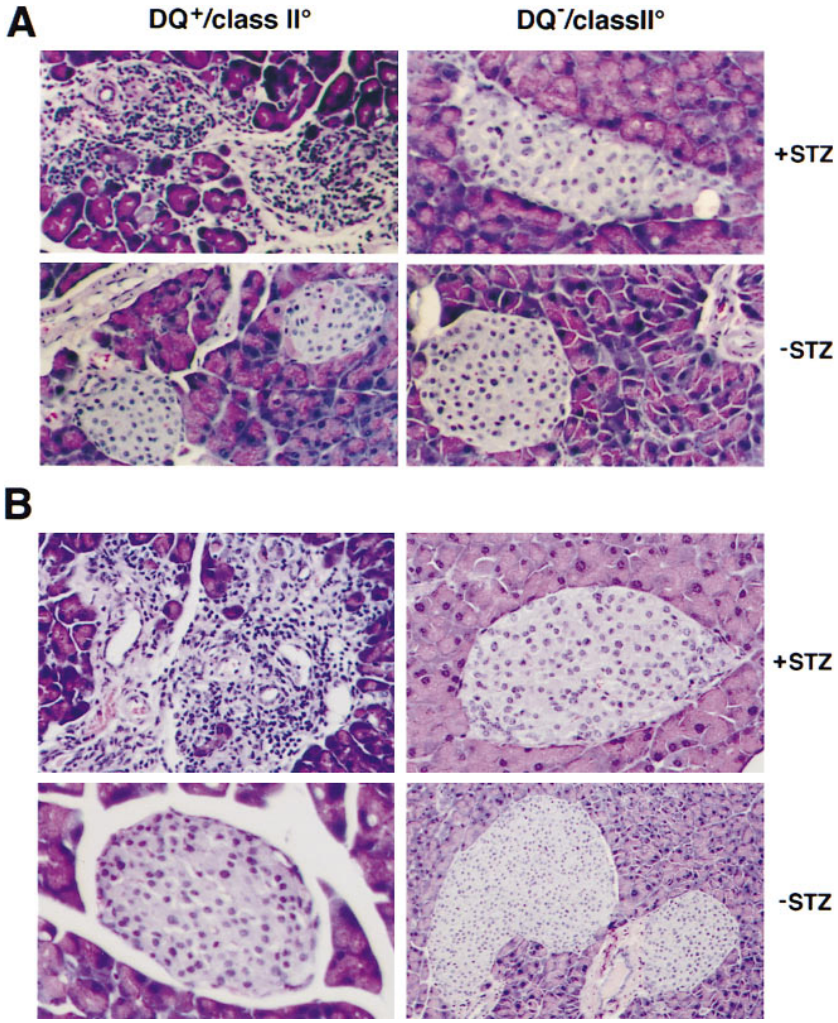

Figure 4. Histopathology of insulitis postadoptive transfer. Hematoxylin–eosin staining of islets in pancreata of DQ8+ and DQ8− mice with or without STZ treatment. (*A*) Mice which received S.511 cells; (*B*) mice which received L.17 cells. Pictures shown in this figure were taken from a representative mouse in each group. ×100, apart from the bottom right (×40) as the size of the islets on that section were much larger than the rest of the sections.

phoid cells and their trafficking to lymphoid organs is not dependent on the expression of HLA-DQA1*0301-DQB1*0302 or STZ treatment. However, the appearance of labeled transferred GAD peptide–reactive T cells within the pancreatic islets occurred only in the STZ-treated DQ^+/class II^o mice (Fig. 5), providing direct evidence that the insulitis in these mice is mediated by the GAD peptide–reactive T cells and requires STZ. This phenomenon was DQ restricted as labeled T cells did not migrate to the pancreatic islets of DQ^-/class II^o mice, even when they were pre-treated with STZ (data not shown).

Table I. Induction of Insulitis by DQ-restricted GAD Peptide–Specific $CD4^+$ T Cells

	DQ+/class II°				DQ−/class II°			
	+++	++	+	−	+++	++	+	−
2× STZ + S.511 (n = 8)	36/80	19/80	11/80	16/80	0/80	0/80	6/80	74/80
2× STZ + L.17 (n = 4)	16/40	10/40	3/40	11/40	0/40	0/40	2/40	38/40
S.511 (n = 6)	0/60	0/60	0/60	60/60	0/60	0/60	0/60	60/60
L.17 (n = 3)	0/30	0/30	0/30	30/30	0/30	0/30	0/30	30/30
2× STZ (n = 4)	0/40	0/40	2*/40	38/40	0/40	0/40	3*/40	37/40
None (n = 4)	0/40	0/40	0/40	40/40	0/40	0/40	0/40	40/40

Four groups of sex- and age-matched mice from each genotype (DQ^+ or DQ^-) were used in the adoptive transfer experiments. All the mice were irradiated 1 d before the adoptive transfer and some of the mice were treated twice with STZ (50 mg/kg per d) before irradiation (see Methods). Glycosuria was monitored three times per week using Diastix (Bayer, Elkhart, IN). Pancreata were removed and fixed in 10% formalin and embedded in paraffin when the experiments were terminated (8 wk postadoptive transfer). Histopathology of pancreatic islets were examined on H+E stained tissue sections. An average of 10 islets/mouse was evaluated and insulitis was scored according to Huang et al. (28). *Occasionally, very few scattered mononuclear cells could be found in rare islets of 2× STZ-treated transgene-positive and transgene-negative recipients. We graded as "+," although it is much milder than the "+" score by Huang et al. (28).

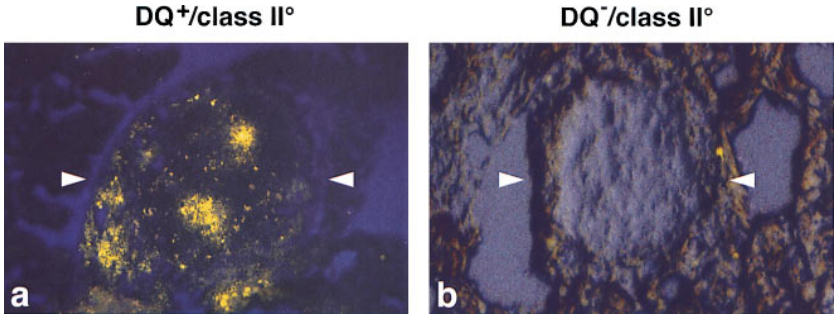

Figure 5. GAD peptide–specific T cells home to islets. Pancreatic sections taken at 10 d after adoptive transfer of S.511 cells labeled with DiI, show labeling of the cells within the islet in the $DQ8^+$ mouse (*a*), but not in the $DQ8^-$ mouse (*b*).

Upregulation of MHC molecules, T cell markers and adhesion molecules by STZ. To investigate how STZ treatment might promote the pathogenicity of the DQ8-restricted, GAD peptide specific T cells, we examined the expression of a variety of immune markers on splenocytes, pancreas and other organs after STZ treatment in both DQ^+/class II^o and DQ^-/class II^o mice (see Methods). Interestingly, the expression of mouse MHC class I (K^b) and human MHC class II (DQ) on splenocytes was upregulated by STZ treatment, particularly MHC class I (Fig. 6 *A*). The expression of CD3 T cell receptor and CD8, but not CD4, coreceptor was also upregulated by STZ treatment (Fig. 6 *A*). The expression of both costimulatory molecules (B7.1 and B7.2) and adhesion molecules (LFA-1, ICAM-1 and LPAM) on spenocytes was not significantly affected by STZ treatment (data not shown). By contrast, the expression of the adhesion molecules ICAM-1 (Fig. 6 *B*) and VCAM-1 (data not shown) on the endothelial cells of the pancreas and epithelial cells of the kidney tubules was increased by STZ treatment. The enhanced expression of the adhesion molecules was more distinct on the epithelial cells of the kidney tu-

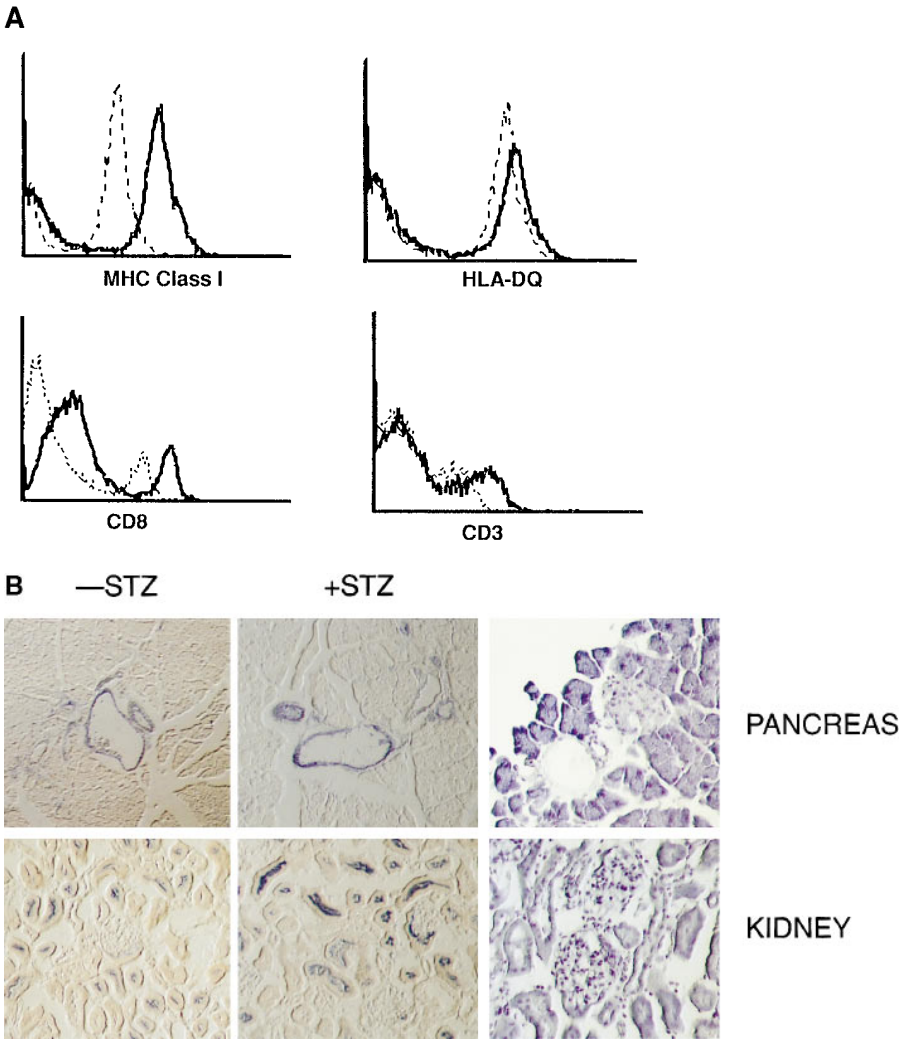

Figure 6. Effect of STZ treatment. (*A*) FACS profiles of splenocytes before and after STZ treatment. Splenocytes from transgene-positive mice were stained with mAbs to FITC- or PE-conjugated MHC class I (K^b), HLA-DQ, CD3, and CD8 and examined on FACScan. *Dotted lines*, splenocytes of a mouse that had not received STZ; *solid lines*, splenocytes from a mouse after STZ treatment. Similar upregulated expression of K^b, CD3, and CD8 was also seen in transgene negative splenocytes post STZ treatment. (*B*) Immunohistochemical staining of ICAM-1 before and after STZ treatment. Frozen section (7 μm) of pancreas and kidney were stained with biotin conjugated mAb to ICAM-1 (PharMingen) at dilution 1:100, followed by AP-conjugated streptavidin (Southern Biotechnology, Birmingham, AL). Fast BB blue was used as substrate as described previously (22). The panel on the right shows the hematoxylin staining of pancreas and kidney from the same tissue shown in the middle panel. ×50.

bules (Fig. 6 *B*). The effect of STZ was not different between transgene-positive and transgene-negative mice (data not shown). The STZ treatment used here did not induce insulitis as shown by several experiments described earlier. However, the upregulation of MHC molecules, T cell markers on lymphocytes and adhesion molecules on endothelial or epithelial cells indicates that STZ could facilitate the homing of GAD peptide–reactive T cells to pancreatic islets.

Gene expression of cytokines and other molecules after adoptive transfer of GAD peptide–reactive T cells. To evaluate the molecular mechanism(s), in situ, for the development of insulitis, we analyzed the mRNA expression of cytokines (IFNγ and TNFα), Fas and FasL as well as CD4 and CD8 coreceptors in hand picked islets of the recipients at the termination of the adoptive transfer experiments after 8 wk using S.511 T cells ($GAD65_{509–528}$ peptide reactive) as the growth of S.511 T cells in vitro was better than L.17 cells and therefore more S.511 T cells were available for the transfer experiment. Three groups of DQ^+/class II^o mice (n = 2/group; STZ+S.511; STZ+saline; saline) were used for this experiment. Although IFNγ, TNFα, and FasL gene products were not detected in the cDNA samples derived from islets of the saline-treated mice, regardless of the expression of DQ transgene, the expression of Fas gene product was readily detected in the same cDNA samples (Fig. 7 *a*). Samples from DQ^+/class II^o mice treated with STZ, but without receiving adoptive cell transfer, showed increased expression of Fas in conjunction with the expression of IFNγ and TNFα gene products (Fig. 7 *b*). Adoptive transfer of GAD peptide reactive T cells in vivo not only increased the expression of IFNγ and TNFα gene products, but also caused the expression of FasL gene product in the cDNA sample from DQ^+/class II^o STZ-treated recipients with insulitis (Fig. 7 *c*). As expected, the CD4 gene product was detected in these DQ^+/class II^o STZ-treated mice (data not shown). However, CD8 mRNA was also present in the islet, implying that the insulitis lesion provoked the entry of CD8 T cells as well into the islet (data not shown). It is interesting that low levels of cytokine and CD8, but not CD4, mRNA were also revealed from the cDNA samples of DQ^-/class II^o islets (data not shown), suggesting the recruitment of small numbers of CD8 T cells after STZ treatment in DQ^-/class II^o mice. These data further support the findings described above, namely that STZ upregulates the expression of the CD8 coreceptor and other molecules, thereby allowing GAD-reactive (S.511 and L.17) and nonreactive (of as yet unknown antigen specificity) CD8 T cells to home to pancreatic islets. Consistent with the cytokine production data, mRNA of S.511 T cells showed abundant gene expression of the cytokines IFNγ and TNFα. Fas, FasL, and CD4 (but not CD8) (data not shown). The specificity of Fas and FasL mRNA amplification was corroborated using the cDNA samples prepared from splenocytes of *lpr* (Fas-deficient) and *gld* (FasL-deficient) mice (C57BL/6 background), respectively (data not shown).

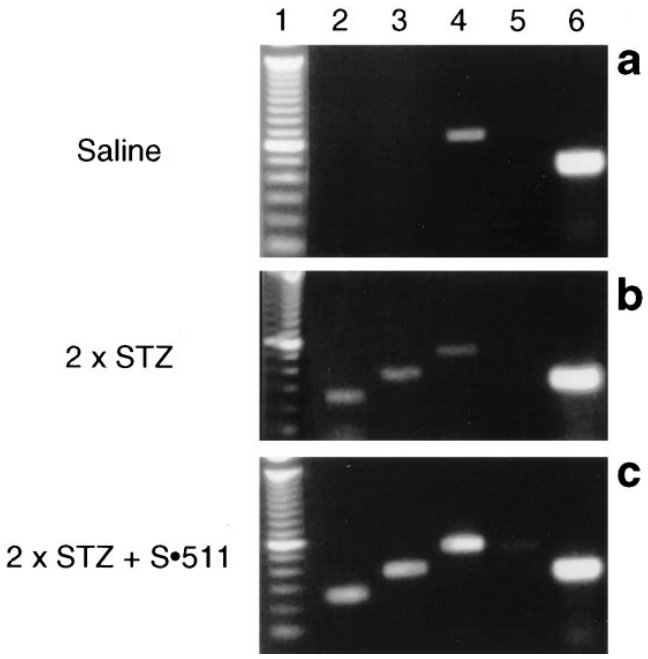

Figure 7. The profiles of cytokine (IFNγ and TNFα), Fas and FasL in islet β cells and S.511 cells obtained from DQ8+ mice. Expression of cytokines, Fas and FasL mRNA was assessed by RT-PCR, as shown in the ethidium-bromide stained gel (see Methods). Lane *1*, 100-bp ladder; lane *2*, IFNγ; lane *3*, TNFα; lane *4*, Fas; lane *5*, FasL; lane *6*, HPRT.

Discussion

This study makes several significant observations. First, we demonstrate that IDDM-linked human DQA1*0301-DQB1*0302 molecules are able to present GAD peptides to mouse $CD4^+$ T cells, both in vivo and in vitro, in the absence of mouse MHC class II molecules. Second, we show that GAD peptide specific, DQA1*0301-DQB1*0302 restricted Th1 T cells have the capacity to induce insulitis in vivo. Finally, we provide a model supporting the hypothesis that an external or environmental insult to islet β cells could play a key role in the initiation of disease in genetically susceptible (such as the DQA1*0301-DQB1*0302 bearing) individuals.

The primary function of MHC class II molecules is to bind and present peptides to and thereby activate $CD4^+$ T cells, which then proliferate and potentiate the immune response. It has been suggested that differences in peptide binding could be one of the major factors responsible for MHC-linked susceptibility to autoimmune diseases, such as IDDM (41, 42). It is, therefore, possible that DQA1*0301-DQB1*0302 molecules may be more effective either in binding a pancreatic autoantigen, such as GAD, or in interacting with the receptor of diabetogenic T cells, thereby increasing disease susceptibility. Nabozny et al. (43) have recently reported, using a similar transgenic mouse model, that DQA1*0301-DQB1*0302 molecules are critical in the development of collagen-induced experimental arthritis. In studies related to diabetes, Kwok et al. (17) have shown that these same MHC class II molecules have a high degree of allelic specificity for binding the $GAD65_{247–266}$ peptide, in vitro, using EBV-transformed human B cells of known HLA type and a lesser degree of binding to other GAD peptides. The present study extends this work by showing that DQA1*0301-DQB1*0302 molecules can present this peptide and one of the other GAD peptides in the in vivo setting, and more importantly by showing that their interaction with $CD4^+$ T cells induces a potent anti-GAD Th1 response with significant biological consequences.

Among the putative autoantigens implicated in IDDM, GAD is thought to be particularly important (1–8). GAD au-

toantibodies are detected in most newly diagnosed IDDM patients and in a majority of prediabetic subjects (44). T cell reactivity to GAD65 and several GAD peptides has also been detected in peripheral blood samples from IDDM patients (45, 46) although the disease specificity study of GAD-reactive T cells obtained from human blood samples and analyzed in a 'blind' manner, is inconclusive (Fifteenth Immunology of Diabetes Workshop, Canberra, Australia, 1996). Furthermore, tolerization of GAD-reactive cells in NOD mice results in the prevention of insulitis and diabetes (1). In spite of this growing body of indirect evidence supporting a role of GAD autoimmunity in the pathogenesis of human IDDM, the biological activity of GAD or GAD peptide–reactive T cells in inducing disease is uncertain. The current data, however, provide evidence for induction of insulitis by GAD peptide–reactive, DQA1*0301-DQB1*0302-restricted Th1 $CD4^+$ T cells in DQ8 transgenic mice. Interestingly, we have recently shown, in a separate study using the NOD mouse model, that another COOH-terminal GAD65 peptide, 524–543, specific T cell line could induce the development of diabetes in NOD/SCID recipients by adoptive transfer (47).

It should be noted that there are two amino acids that are different in $GAD_{247–266}$ peptide between the murine and the human sequence (at position 252: M for human and L for murine; at position 256: F for human and Y for murine) and one amino acid different in $GAD_{509–528}$ peptide between the murine and the human sequence (at position 509: I for human and V for murine). The murine sequence was chosen for our studies as this is a murine model system. Moreover, it might be possible that the human sequence may be seen by murine T cells as foreign, thereby triggering a nonspecific response. However, the differences between the sequences are unlikely to contribute to our findings in this study as their structures and properties of side chains are very similar. In fact, the $GAD_{247–266}$ peptide specific T cell line (L.17) was also reactive equally well to the human sequence of this peptide (data not shown). The difference in $GAD_{509–528}$ peptide is located at the first amino acid of the peptide, which is outside the proposed binding motif of DQ8 (17).

Like human and rat GAD65, mouse $GAD_{247–266}$ peptide also contains a region of sequence similarity with Coxsackie virus (34). Analysis of peptides bound to DQ8 molecules has identified a binding motif which may be selectively bound by these molecules and presented to T cells (17). Although our experiments were not designed for the study of "molecular mimicry" and the mice were kept under an extremely restricted SPF condition, the induction of insulitis by the $GAD_{247–266}$ peptide specific T cell line L.17, after the mild islet "insult," supports the potential role of environmental factors in the initiation of disease development (see below).

The Th1 phenotype of our GAD peptide–reactive T cell lines is consistent with previously reported data indicating that the Th1 immune response to GAD develops in NOD mice at the same time as the onset of insulitis (1). Our data also support the hypothesis that GAD-reactive T cells release proinflammatory cytokines that might serve to recruit and/or activate additional immune cells which in turn exacerbate the inflammatory lesion. The proinflammatory cytokines produced by GAD peptide reactive T cells could also upregulate the expression of Fas on islet β cells, which in turn, renders islet β cells more susceptible to apoptosis via Fas-FasL interaction (48, 49). In recent studies using transgenic NOD mice that express functional FasL specifically in islet β cells, Chervonsky et al. (50) have provided strong support for the concept that the Fas–FasL interaction is critical for the development of diabetes. The current data are consistent with this view as Fas expression by islet cells increases and FasL gene expression by invading T cells in the islets is seen in our mice after development of insulitis. Our data, however, do not exclude the participation of macrophage or T cell–derived cytokines, such as TNFα and IFNγ.

In this report, we used a non–diabetes-prone mouse strain, C57BL/6, that expresses functional human diabetes susceptible MHC class II molecules, DQA1*0301-DQB1*0302. In contrast to NOD mice, C57BL mice develop neither insulitis nor diabetes, as they lack a number of disease susceptibility genes (51). There are two principal reasons that we elected to maintain our DQ^+/class II^o mice on a nondiabetogenic background (C57BL/6). Firstly, we aimed at dissecting the specific role of DQA1*0301-DQB1*0302 molecules in disease development in the absence of any potential influence by other IDDM-susceptible genes, such as numerous Idd loci found in the NOD mouse which are not identical to those linked to human IDDM. Secondly, our goal was to study human MHC class II DQ molecules in the absence of the potential influence by either mouse MHC class II molecules, I-A (homologue of human DQ) or I-E (homologue of human DR). As mentioned earlier, C57BL/6 mice do not express I-E molecules due to a gene mutation, and targeting the I-Aβ gene on C57BL background, therefore, provides a mouse deficient in both MHC class II molecules. Although NOD mice also do not express I-E molecules, $I\text{-}A^{NOD}$ ($I\text{-}A^{g7}$), the homologue of DQA1*0301-DQB1*0302 molecules, is unique, and the I-Aβ knockout mutation does not give rise to selective deficiency of MHC class II expression in the NOD strain as there is also a change in one of the MHC class I molecules (K^d vs. K^b), which might be expected to impact on disease development (29, 52–54). On the other hand, if our transgene for DQA1*0301-DQB1*0302 molecules was created on a NOD background without crossing to the I-Aβ knockout mutation, it would be expected to result in coexpression of $I\text{-}A^{g7}$ and human DQA1*0301-DQB1*0302 molecules, thereby confounding the interpretation of results.

Epidemiological and experimental evidence implicate environmental factors, such as viral infection or chemical toxins in precipitating IDDM (55, 56). Previous studies using a multiple low-dose streptozotocin injection protocol have demonstrated that toxin-induced islet damage can trigger an autoimmune, T cell–mediated model of diabetes that is genetically restricted (38, 39). Our data in this study are consistent with the possible role of environmental factors in development of the disease, as adoptive transfer of GAD-specific, DQ-restricted, Th1 T cells produces insulitis only in STZ treated recipients. The double low-dose injection of STZ used in this study did not produce insulitis, nor promote insulitis after adoptive transfer of nonspecifically activated $CD4^+$ T cells from DQ8 transgenic mice. However, it upregulated MHC molecules, T cell markers on lymphocytes, and adhesion molecules on pancreatic endothelial cells. Thus, the STZ treatment used in this study was probably sufficient to cause a subtle "insult" to islet β cells, which may have attracted a small number of host CD8 T cells and allowed GAD to be presented by local APCs, thereby inducing GAD-specific T cells to home to the pancreatic islets and mediate a local inflammatory immune response. In line with this, Herold et al. (57) and Huang et al. (28) have reported that

STZ treatment could induce the production of inflammatory cytokines by the islet infiltrate. Our data presented here also demonstrate that STZ could not only induce TNFα production, but also upregulate the expression of Fas; and the interactions of both TNFα-TNFR and Fas-FasL are powerful agents causing the apoptotic death of target cells, such as islet β cells.

Our data suggest that the insulitis induced in the DQ transgenic mice is T cell dependent, GAD autoantigen specific, susceptible to MHC gene product DQA1*0301-DQB1*0302, and dependent on environmental factors. Although animal models have provided an important means for studying the pathogenic process of autoimmune diseases, a common criticism is that they may not be representative of the human disease. The transgenic mice used here to study IDDM and other strains used to study arthritis in previous reports (43, 58, 59) suggest that this model system may provide a useful tool to study human autoimmune processes. Moreover, the data provided by the current human HLA transgenic model support the hypothesis that GAD plays an important role in the pathogenesis of human IDDM.

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