

Major DQ8-Restricted T-Cell Epitopes for Human GAD65 Mapped Using Human CD4, DQA1*0301, DQB1*0302 Transgenic IA^{null} NOD Mice

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The 65KD isoform of GAD is considered to be a major target autoantigen in many humans with autoimmune prediabetes or diabetes. The major histocompatibility complex class II allele *DQA1*0301*, *DQB1*0302*, which encodes HLA-DQ8, confers susceptibility to type 1 diabetes and occurs in up to 80% of affected individuals. To map T-cell epitopes for GAD65 restricted to the diabetes-associated DQ8 heterodimer, we generated transgenic NOD mice expressing HLA-DQ8 and human CD4 while having the mouse class II gene (*IA_β*) deleted. These mice were immunized with full-length purified recombinant GAD65, and the fine specificity of T-cell responses was mapped by examining recall responses of bulk splenocytes to an overlapping set of 20-mer peptides encompassing the entire GAD65 protein. Four different peptides (P121-140, P201-220, P231-250, and P471-490) gave significant T-cell recall responses. P201-220 and P231-250 have been shown previously to bind DQ8, whereas the other two peptides had been classified as nonbinders. Interestingly, the peptide giving the greatest response (P201-220) encompasses residues 206–220 of GAD65, a region that has been shown to be a dominant T-cell epitope in wild-type IA^{g7} NOD mice. Overlap in this T-cell epitope likely reflects structural similarities between DQ8 and IA^{g7}. The fine specificity of antibody responses in the GAD65-immunized mice was also examined by testing the antisera by enzyme-linked immunosorbent assay (ELISA) against the same overlapping set of peptides. The two dominant B-cell epitopes were P361-380 and P381-400; P121-140 and P471-490 appeared to correspond to both B- and T-cell epitopes. Although the NOD human CD4, DQ8, IA^{null} transgenic mice generated in these studies do not develop autoimmune diabetes either spontaneously or after cyclophosphamide treatment, they can be used to

map DQ8-restricted T-cell epitopes for a variety of human islet autoantigens. They can also be used to test T-cell-specific reagents, such as fluorescently labeled DQ8 tetramers containing GAD65 peptides or other β-cell peptides, which we believe will be useful in analyzing human immune responses in diabetic and prediabetic patients. *Diabetes* 48:469–477, 1999

Type 1 diabetes is a T-cell-mediated autoimmune disease directed against the pancreatic β-cell (1). Several β-cell-specific proteins have been identified as targets of the autoimmune response in humans. One leading candidate autoantigen is the enzyme GAD65; >80% of prediabetic patients and most recent-onset diabetic patients have autoantibodies against GAD65 (2), and up to half of new-onset type 1 diabetic patients show T-cell proliferative responses to GAD65 (3–5). Importantly, human GAD65 (or the closely related mouse GAD67 protein) have been used in tolerogenic regimens to prevent disease in susceptible NOD mice (6–11).

Whereas GAD67 appears to be the predominant GAD isoform expressed in rat and mouse islets, GAD65 predominates in human islets (12,13), and therefore the 65-kDa isoform is of particular interest as an autoantigen in human type 1 diabetes. Unfortunately, widespread studies of anti-GAD65 T-cell responses in human autoimmune diabetes have been hampered by the lack of abundant quantities of purified and soluble recombinant GAD65. In contrast to mouse GAD67 (which is relatively easy to express at high levels in a variety of heterologous systems [8,11]), human GAD65 is expressed at very low levels in *Escherichia coli* and transgenic plants (J.F.E., unpublished observations). In addition to the relatively modest amounts of GAD65 required by investigators for human T-cell studies, much larger quantities of human GAD65 may at some point be required for prevention trials. This GAD65 “supply problem” could potentially be overcome if synthetic peptides could be substituted for the full-length protein, but in designing such peptides, it is critical that the T- and B-cell epitopes be mapped, particularly for the disease-associated major histocompatibility complex (MHC) class II molecules.

Although multifactorial, susceptibility to type 1 diabetes is most significantly influenced by MHC class II genotype; in particular, HLA-DQ haplotypes such as HLA-DQB1*0302, which carry a neutral residue at position 57 of the DQ_β chain (14), are associated with susceptibility to disease. Additional studies

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Received for publication 24 June 1998 and accepted in revised form 13 November 1998.

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ANOVA, analysis of variance; APC, antigen-presenting cell; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HBV, hepatitis B virus; HPLC, high-performance liquid chromatography; MBP, myelin basic protein; MHC, major histocompatibility complex; OD, optical density; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with Tween-20; SI, stimulation index; TFA, trifluoroacetic acid

have demonstrated that susceptibility to type 1 diabetes is strongly associated with the DQ8 (DQA1*0301, DQB1*0302) and DQ2 (DQA1*0501, DQB1*0201) haplotypes, and resistance is associated with the DQ6 (DQA1*0102, DQB1*0602) haplotype (15–17).

A key approach to help delineate the role of MHC molecules in type 1 diabetes, as well as to provide reagents for immunologic investigations and possible prevention trials, is to identify from target autoantigens such as GAD65 the immunodominant epitopes that can be presented by disease-associated HLA molecules. However, it is difficult to do this in human subjects because there is a paucity of autoreactive T-cells in the peripheral circulation, and because the establishment and long-term maintenance of human T-cell clones is difficult. In addition, the presence of multiple HLA molecules in each individual makes the determination of MHC restriction elements problematic. To circumvent many of these problems, we generated human CD4, HLA-DQ8 transgenic, IA knockout NOD mice, immunized them with recombinant human GAD65, and used synthetic peptides to define both T- and B-cell immunodominant epitopes for GAD65.

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. Creation of the original human CD4, HLA-DQ8 NOD transgenic line was a joint effort between R. Flavell and L. Burkley, who prepared the DNA; H. Chen and M. Trumbauer, who completed the microinjections; and L. Wicker and L. Peterson, who established and characterized the transgenic line. In brief, a DNA fragment consisting of the human CD4 cDNA driven by the human CD2 promoter (18) was mixed with genomic fragments encoding DQA1*0301 and DQB1*0302 (isolated as *SalI* fragments from the cos-mids H11A and X10A [19]) and the DNA mixture was microinjected into NOD/MrkTacBR single-cell embryos. Founders were identified using standard methods, and a single founder was used to establish the transgenic NOD line that was homozygous for human CD4 and DQ8. These animals showed a low level of expression of human CD4 on all T-cells. Although levels of expression of IA^{NOD} and HLA-DQ8 were comparable, virtually all of the CD4⁺ T-cell responses were IA restricted, and the diabetes incidence was similar to that of the parental NOD strain (L. Wicker, personal communication).

The NOD class II knockout animals used in these studies have been described previously (20). Briefly, IA_β^{null} animals created in the 129 background (21) were backcrossed onto NOD/ITJ through 11 generations. As expected, the IA^{null} NOD animals had no circulating CD4⁺ T-cells, and they failed to develop diabetes.

To generate the NOD human CD4^{+/+} DQ8^{+/+} IA^{null} animals, the NOD IA_β^{null} animals were crossed with the NOD human CD4, DQ8 line, the F1 animals were intercrossed, and F2 animals were screened by fluorescence-activated cell scanning (FACSscan) for the absence of IA^{NOD} and the presence of two copies of DQ8 (Fig. 1A and B). The simple assumption was made that the human CD4 would be passed along with the DQ8 genes, and this proved to be the case (Fig. 1D). A brother-sister pair from the F2 generation was used to establish the NOD human CD4^{+/+} DQ8^{+/+} IA^{null} line.

Dot blot analysis of transgenic mice. Mouse genomic DNA was prepared using standard methods (22), and 10 μg was denatured, neutralized, and applied to nitrocellulose using a Minifold I Microsample Filtration Manifold (Schleicher & Schuell, Keene, NH). The blot was air-dried, baked at 80°C under vacuum, pre-hybridized, hybridized (50% formamide, 42°C), and washed using standard conditions (23). The probe was a ³²P-labeled cDNA encoding DQA1*0301.

Antibodies and FACS analysis. Hybridomas were grown in bulk in roller bottles, and monoclonal antibodies were purified by affinity chromatography over protein A sepharose. Aliquots of the purified antibodies were conjugated to fluorescein isothiocyanate (FITC) or to phycoerythrin (PE) using standard methods (24). The following antibodies against MHC class II were used in this study: FN81-1 (DQ monomorphic from S. Funderud [25]), SPV-L3 (DQ monomorphic from H. Spits [26]), 4D12 (DQ7,8,9 specific), L243 (DR monomorphic), 10-3-6 (IA specific, including IA^{NOD}), and 14-4-4 (IE specific). The latter four hybridomas were all obtained from ATCC (Rockville, MD), as was H25B10 (isotype control for DQ blocking, specific for hepatitis B virus [HBV] surface antigen). The B220, GK1.5, Ly-2, and F4/80 hybridomas were obtained from D. Denney. For staining, a single cell suspension was prepared and resuspended in phosphate-buffered saline/0.5% bovine serum albumin (PBS/BSA) containing saturating concentrations (25 μg/ml) of labeled monoclonal antibodies. Cells were incubated on ice for 30 min, washed three times in PBS/BSA, and analyzed by FACSscan (Becton Dickinson, Mountain View, CA).

Production of purified recombinant human GAD65. For expression of full-length human GAD65, we created a new T7-based *E. coli* expression vector, pT7j1h. This vector was created from pT7-7His6 (8) by making the following changes: 1) deleting the *XbaI* site between the T7 promoter and initiation codon, 2) adding codons for the epitope tag MRGS at the beginning of the (His)6 segment, 3) expanding the polylinker, and 4) adding a downstream T7 transcription terminator. A full-length cDNA encoding GAD65 was isolated from a human islet cDNA library that was constructed and cloned in pJFE14 using methods described previously (27). The minimal coding region for GAD65 was modified by polymerase chain reaction using Pfu DNA polymerase (Stratagene, La Jolla, CA), and the resulting fragment was cloned into pT7j1h using the *XbaI* and *NotI* sites to generate a fusion with the (His)6 coding segment (NH₂-terminus of the recombinant protein is MRGSHHHHHHSRMASP, etc., where the first methionine of GAD65 is underlined). The COOH-terminus of the recombinant protein was the same as in native GAD65.

The final GAD65/pT7j1h construct was transformed into *E. coli* MC1061/pT7POL23 (28), and grown with shaking at 28°C in 2xYT media containing ampicillin plus kanamycin (23). For large-scale protein expression, a scraping from the glycerol stock corresponding to the isolated colony that gave the highest levels of GAD65 expression on a pilot study was used to generate a 1-liter culture (grown at 28°C in 2xYT media containing ampicillin plus kanamycin), which was used to inoculate a 50-liter fermentor containing the same medium. Once the optical density at 600 nm (OD₆₀₀) of the fermentor culture reached 0.40, the temperature was rapidly shifted from 28 to 42°C by heating the jacket with steam, and the culture was continued at 42°C for 3 h. The biomass was harvested by centrifugation, bacterial cells were opened using a French press (twice at 16,000 psi) in the presence of DNase (4 μg/ml), RNase (2 μg/ml), and phenylmethylsulfonyl fluoride (1 mmol/l), and inclusion bodies were harvested by centrifugation (5,000g for 30 min). The inclusion bodies were dissolved in a solution containing PBS/Tris HCl pH 8.0 (20 mmol/l) and guanidinium hydrochloride (6 mol/l), loaded onto a Ni-NTA column, and eluted using a stepwise pH gradient (pH 8.0, 6.3, 5.8, 5.3, 4.5, 3.5) in 8 mol/l urea essentially as described for GAD67 (8). The eluted protein (typically eluted at pH 5.8 and 5.3) was dialyzed against 1x Laemmli running buffer, against the same buffer with 1/10 the standard amount of SDS, and finally against 4 mmol/l HEPES pH 7.4 (8). Typical yields were ~100 mg of GAD65 per 50-liter fermentor run. The protein was concentrated by lyophilization (but not to complete dryness) before running on high-performance liquid chromatography (HPLC).

The recombinant GAD65 was further purified by reverse-phase HPLC; ~2 mg protein in 1 ml 16 mmol/l HEPES pH 7.4 was boiled for 90 s and then loaded immediately on a C18 column charged with 0.1% trifluoroacetic acid (TFA) in water. The column was run with a linear gradient of acetonitrile/water (0.1% TFA), with GAD65 typically eluting at 75–81% acetonitrile. HPLC column fractions (1 ml) were frozen at –70°C, lyophilized to dryness, and resuspended in 1x Laemmli running buffer (endotoxin free); an aliquot of each fraction was analyzed by SDS-PAGE. Fractions containing GAD65 were pooled and dialyzed against Laemmli buffer with 1/10 the standard amount of SDS, and finally against 4 mmol/l HEPES pH 7.4, with care taken to ensure that all buffers were endotoxin free (8). The final protein was shown to be essentially endotoxin free (< 0.3 EU/ml) as assayed by Limulus lysate assay (Sigma, St. Louis, MO). Yields after HPLC were typically 60–70% of the starting material.

GAD65 peptides. Fifty-eight overlapping peptides (20-mers, each overlapping by 10 residues) spanning the entire human GAD65 sequence were purchased commercially. Peptides were custom synthesized by Chiron Mimotopes (Clayton, Australia) using the PIN synthesis technique. Peptides are indicated by placing a “P” before the residue positions. Although synthesized independently, the peptide set used in these experiments corresponds exactly to the set for which DQ3.2 (i.e., DQ8) binding data are available (29). Two control peptides were also synthesized to verify purity and assess yield. Lyophilized peptides were resuspended in PBS and stored at –70°C.

Measurement of T-cell proliferative responses. Methods to measure T-cell proliferative responses have been described previously (30). Briefly, adult transgenic mice of either sex were immunized subcutaneously in the hind footpad and at the flank of the belly with a total of 50 μg of recombinant GAD65 (1 mg/ml) emulsified in an equal volume of complete Freund's adjuvant (CFA) (DIFCO, Detroit, MI). After 2–8 weeks, spleens were removed, and a single cell suspension was prepared. Proliferation assays were performed in flat-bottom 96-well microtiter plates, using 0.5 · 10⁶ cells per 200-μl well, and culturing at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL, Bethesda, MD) containing 5% FCS, 5 · 10^{–5} mol/l 2-ME, 2 mmol/l L-glutamine, 100 U/ml of penicillin, and 100 μg/ml streptomycin. Cultures were set up in triplicate with either no antigen added, with purified GAD65 at a range of concentrations, or with peptides at 20 μg/ml. ³H-labeled thymidine (0.5 μCi/well) was added after 72 h, and 16 h later, cultures were harvested onto glass fiber mats using a Packard Micromate 196 cell harvester (Packard, Melbourne, Australia). Filter disks were transferred to vials, scintillation liquid was added, and radioactivity was counted using a Beckman

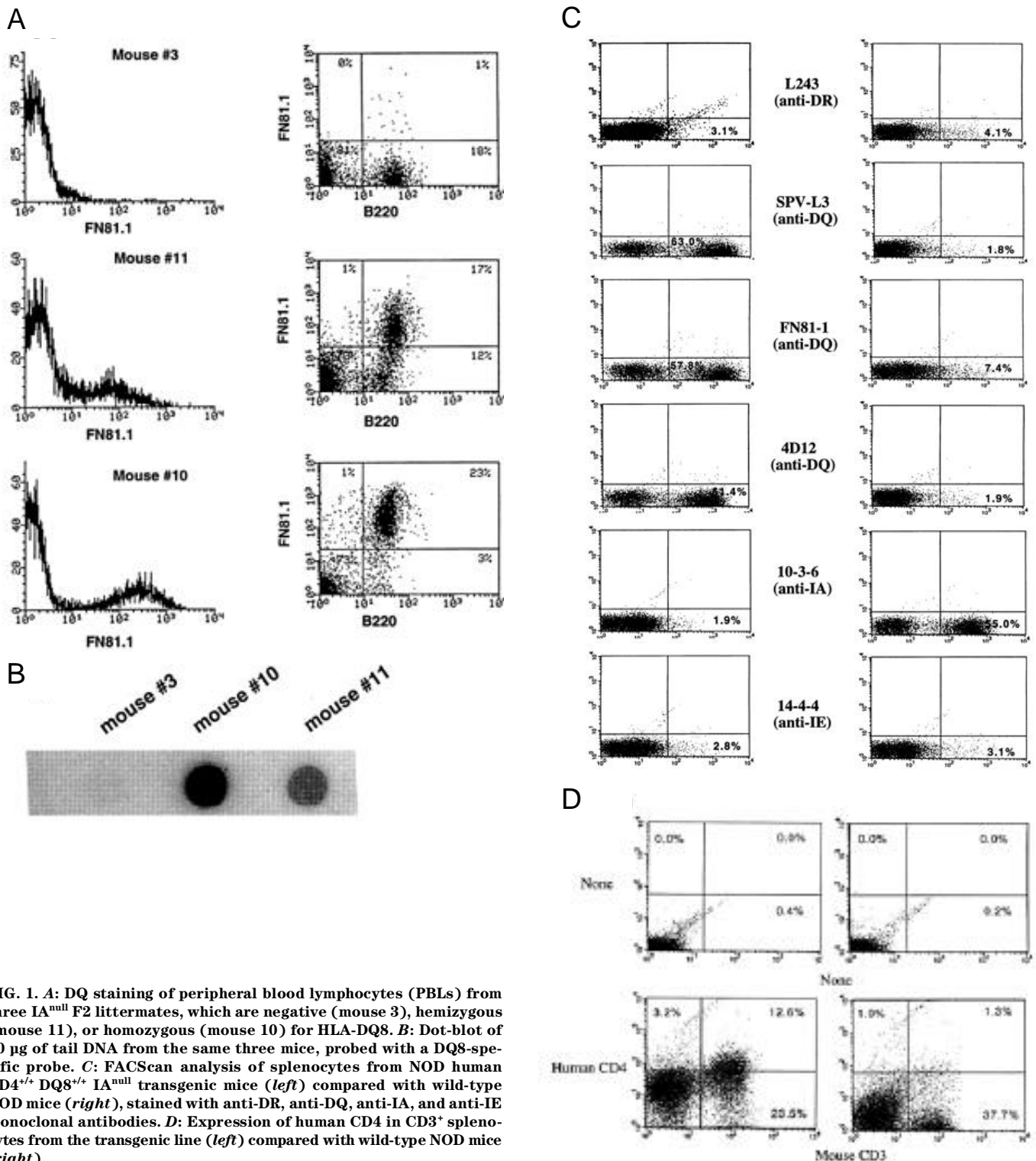


FIG. 1. *A*: DQ staining of peripheral blood lymphocytes (PBLs) from three IA^{null} F2 littermates, which are negative (mouse 3), hemizygous (mouse 11), or homozygous (mouse 10) for HLA-DQ8. *B*: Dot-blot of 10 μ g of tail DNA from the same three mice, probed with a DQ8-specific probe. *C*: FACScan analysis of splenocytes from NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice (*left*) compared with wild-type NOD mice (*right*), stained with anti-DR, anti-DQ, anti-IA, and anti-IE monoclonal antibodies. *D*: Expression of human CD4 in CD3⁺ splenocytes from the transgenic line (*left*) compared with wild-type NOD mice (*right*).

LS6000TA beta counter (Beckman, Palo Alto, CA). The proliferative response was considered proportional to the ³H-thymidine incorporation and was expressed as the mean of the counts per minute of the triplicate cultures. Stimulation index (SI) was calculated to be the proliferative response in the presence of antigen divided by the proliferative response in the absence of antigen.

Generation of GAD65-specific T-cell line. This antigen-specific T-cell line was used in the antibody blocking experiments. Splenocytes from GAD65-immunized mice were resuspended at $2 \cdot 10^6$ cells \cdot ml⁻¹ in complete DMEM containing recombinant GAD65 at a concentration of 5 μ g/ml. After 3 days, viable cells were separated from dead cells by centrifugation over Ficoll-Paque gradients (Phar-

macia, Uppsala, Sweden). Live cells were washed and incubated for 3 days ($1 \cdot 10^6$ cells \cdot ml⁻¹) in complete DMEM containing 15% interleukin-2 supernatant (supernatant from normal mouse splenocytes [$2 \cdot 10^6$ cells \cdot ml⁻¹] incubated 48 h in complete DMEM with 10 μ g/ml Concanavalin A), and dead cells were again removed from live cells by passage over Ficoll-Paque gradients. The resulting T-cell blasts were restimulated with GAD65 (5 μ g/ml) in the presence of fresh irradiated syngeneic (i.e., DQ8 transgenic) splenocytes at $1 \cdot 10^6$ cells \cdot ml⁻¹. This dual cycle of antigen-specific stimulation followed by antigen-nonspecific stimulation was repeated a total of five times, after which the specificity of the resulting cell line was tested using the proliferation assay described above.

ELISA. As described previously (31), flat-bottomed 96-well microtiter plates (Falcon, Becton Dickinson) were pretreated with 0.2% glutaraldehyde in water (50 μ l \cdot well \cdot 30 min⁻¹ at room temperature). Each synthetic peptide was diluted to 1 μ g/ml and recombinant GAD65 to 5 μ g/ml in carbonate buffer pH 9.6 (coating buffer). The glutaraldehyde solution was drained from each well, and the solution of peptide or protein in coating buffer was added (50 μ l/well). After overnight incubation at 4°C, plates were washed three times with PBS containing 0.1% Tween-20 (PBS-T) and were blocked with 2% BSA (Sigma) in PBS (BSA-PBS) at 37°C for 1 h. The drained plates were incubated with mouse serum (1:1,000 dilution in BSA-PBS, 50 μ l/well) for 1 h at 37°C and were washed three times with PBS-T. Appropriately diluted biotinylated goat anti-mouse immunoglobulin G (1:3,000 in BSA-PBS, 50 μ l/well) (Sigma) was added to the drained plates, which were incubated 1 h at 37°C, washed three times with PBS-T, and finally incubated with streptavidin-horseradish peroxidase (1:3,000 in BSA-PBS, 50 μ l/well) (Jackson ImmunoResearch, West Grove, PA) for 30 min at 37°C. After three final washes with PBS-T, specific antibody binding was visualized using 2,2'-azino-bis(3-ethylbenz-thiazoline-6 sulfonic acid) (Sigma) as a substrate, and the OD at 405 nm was measured using a Dynatech microtiter plate reader (Burlington, MA).

RESULTS

Establishment and characterization of the NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic line. Figure 1A shows FACS plots for three representative F2 animals that arose by intercrossing the (NOD IA^{null} \times NOD human CD4, DQ8) F1 animals. All three animals were negative for IA (data not shown), and they displayed zero (e.g., mouse 3), one (e.g., mouse 11), or two (e.g., mouse 10) chromosomal copies of the DQ8 transgene complex, as reflected by the DQ expression levels measured by staining with the anti-DQ monoclonal FN81-1, as well as by the intensity of the hybridization signal obtained from dot-blot analysis of tail DNAs probed with an anti-DQ probe (Fig. 2B). Figure 1C shows a comparison of the final NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic line with wild-type NOD animals to demonstrate that whereas the wild-type NOD animals express only IA, the transgenic line expresses DQ (as assessed by three different anti-DQ antibodies) in the absence of IA. Levels of expression of DQ8 in the transgenic animals are roughly equivalent to levels of IA seen in the wild-type NOD animals. Figure 1D compares expression of human CD4 in wild-type NOD animals and in the transgenic line and demonstrates that, as expected (18), there is low-level expression of human CD4 on nearly all CD3⁺ T-cells in the transgenic animals. As with DQ expression levels (Fig. 1A), hemizygous animals demonstrated an intermediate level of staining for human CD4 (data not shown).

Once a stable colony of NOD human CD4^{+/+} DQ8^{+/+} IA^{null} animals was established, representative animals were killed and various subpopulations of immune cells were characterized by FACS analysis (Fig. 2). These subpopulations were compared with the cell populations found in the original NOD IA^{null} mice to determine whether the immune makeup of the knockout animals had been changed by the addition of the DQ8 molecule. The most striking difference was in the CD4 T-cell population, which was essentially absent in the IA^{null} animals but reappeared both in the periphery and in the spleen of the DQ8^{+/+} IA^{null} animals. The absence of CD4 T-cells in the IA^{null} animals was consistent with our failure to obtain any primed T-cell responses in these animals (data not shown).

In contrast to wild-type NOD mice or to the original human CD4, HLA-DQ8 NOD transgenic line, the NOD human CD4^{+/+} DQ8^{+/+} IA^{null} animals did not develop spontaneous diabetes at any time during their lives (up to 1 year of age), nor did they become diabetic after one injection, or even two injections, of cyclophosphamide (data not shown). However, many of the

older female animals did show cardiac immunopathology, and this finding will be the subject of a separate report.

Fine specificity of T-cell responses to GAD65 in the transgenic animals. To determine whether T-cell recall responses to GAD65 could be obtained in the NOD human CD4^{+/+} DQ8^{+/+} IA^{null} mice, transgenic animals were immunized with GAD65 in CFA, and cells from the draining lymph nodes and spleens were prepared at various times after immunization. Strong proliferative responses to GAD65 were observed at 2–6 weeks after antigen injection in both lymph node cells and splenocytes. For example, Table 1 shows recall responses in splenocytes observed 2 weeks after

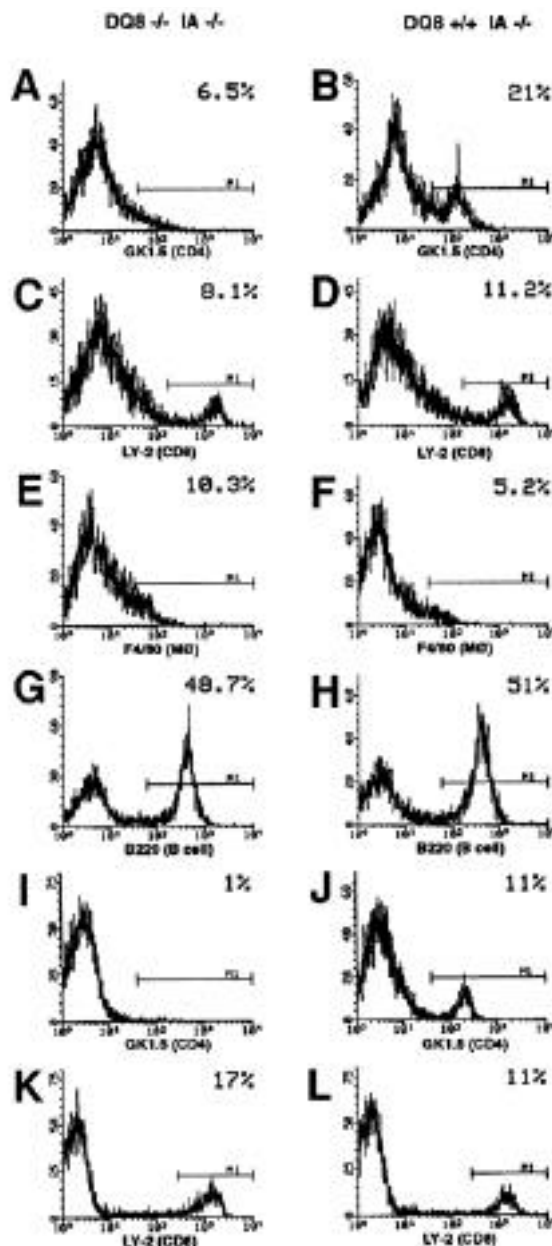


FIG. 2. Comparison of various populations of immune cells found in NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice versus IA^{null} NOD mice. Splenocytes (A–H) and peripheral blood lymphocytes (PBLs) (I–L) were purified and stained with FITC-conjugated antibodies as follows: GK1.5 (A, B, I, J), LY-2 (C, D, K, L), F4/80 (E, F), and B220 (G, H). IA^{null} NOD mice (A, C, E, G, I, K) were compared with CD4^{+/+} DQ8^{+/+} IA^{null} NOD mice (B, D, F, H, J, L). Portions of cells staining within the M1 gate are given as percentages for each FACS plot.

TABLE 1
GAD65-specific dose-dependent T-cell proliferative responses after priming with antigen

Antigen	Counts per minute (SI)
None	1,093 ± 161
GAD65 (µg/ml)	
5	2,225 ± 177 (2.1 ± 0.1)
10	4,683 ± 129 (4.3 ± 0.1)
20	7,985 ± 233 (7.3 ± 0.2)
MBP (µg/ml)	
5	1,533 ± 73 (1.4 ± 0.1)
10	1,481 ± 190 (1.4 ± 0.2)
20	1,458 ± 65 (1.3 ± 0.1)

Data are means ± SE of triplicate wells. Two weeks after immunization with GAD65 (50 µg) in CFA, splenocytes were harvested and tested for proliferative responses to GAD65 or MBP (purified guinea pig) in a 72-h proliferation assay.

immunization and indicates that the magnitude of the proliferative response depends on antigen concentration.

To further delineate the fine specificity of the DQ8-restricted T-cell reactivity to GAD65, the T-cell proliferative response of splenocytes from GAD-immunized animals was measured against each individual peptide from an overlapping set of synthetic peptides encompassing the whole GAD65 sequence. As can be seen from Fig. 3A, a dominant proliferative response was directed to peptide 21 (P201-220), which gave an SI of 5.1 ± 0.23 . Lesser (but still significant) responses were also observed against peptides P121-140 (peptide 13), P231-250 (peptide 24), and P471-490 (peptide 48) with SIs of 3.7 ± 0.35 , 3.3 ± 0.18 , and 3.1 ± 0.23 , respectively (Fig. 3A). Table 2 summarizes the background counts and the average counts per minute obtained for the various positive peptides, analyzed using splenocytes from 30 GAD-immunized mice, each tested individually. In addition, for each positive peptide, the number of mice (out of the total of 30 tested) that gave stimulation indices of ≥ 3.0 is tabulated.

The MHC-restricted nature of the anti-GAD65 and anti-P201-220 responses was further verified by antibody-blocking experiments. For these experiments, a GAD65-spe-

TABLE 2
Splenocyte proliferative responses in GAD65-immunized transgenic mice

Recall antigen	Counts per minute*	Mice with SI ≥ 3.0
None	1,760 ± 93	NA
GAD65 (20 µg/ml)	17,565 ± 1,045	30/30
121-140 (20 µg/ml)	6,547 ± 180	24/30
201-220 (20 µg/ml)	8,993 ± 319	30/30
231-250 (20 µg/ml)	5,741 ± 212	24/30
471-490 (20 µg/ml)	5,491 ± 358	18/30
Negative peptides†	2,061 ± 114	NA

Data are means ± SE or proportion. A total of 30 mice were tested individually using overlapping peptides encompassing the entire human GAD65 molecule. *Counts per minute represent the average value for all 30 mice, regardless of whether SI ≥ 3.0 . †Average of values obtained for all of the other peptides. NA, not applicable.

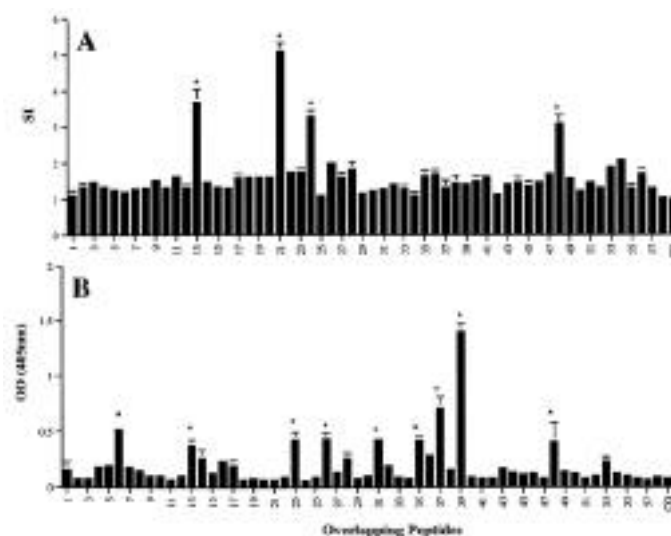


FIG. 3. **A:** Fine specificity of anti-GAD65 T-cell responses in GAD65-immunized NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice. Mice were sacrificed 2-8 weeks after immunization, and splenocytes were prepared and tested against the overlapping peptide set (20 µg/ml) in a 72-h proliferation assay. Peptides are numbered consecutively from the NH₂-terminus to the COOH-terminus (e.g., peptide 3 corresponds to residues 21-40, and peptide 10 corresponds to residues 91-110). Data are derived from five separate experiments involving a total of 30 immunized animals; splenocytes from each individual animal were tested on the entire peptide set. Asterisks indicate responses that differ significantly ($P < 0.05$) using analysis of variance (ANOVA) with post hoc test. Table 2 shows representative values for counts per minute obtained in these experiments. **B:** Fine specificity of antibody responses in GAD65-immunized NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice. Serum was diluted 1:1,000 and tested by ELISA against the overlapping peptide set. Data are expressed as mean OD ± SE. Data were obtained from three separate experiments, involving a total of 12 animals. Asterisks indicate responses that differ significantly ($P < 0.05$) using ANOVA with post hoc test.

cific T-cell line was established from the transgenic mice by harvesting splenocytes from GAD65-immunized animals and repeatedly culturing the cells in the presence of antigen and syngeneic antigen-presenting cells (APCs) (see METHODS). Once established, the cell line was tested against whole antigen and against the P201-220 peptide in the presence or absence of various monoclonal antibodies (Fig. 4). Only the anti-DQ antibody (4D12), but not the anti-IA^{NOD} antibody (10-3-6) nor the isotype-matched control antibody (H25B10), blocked the response of the T-cell line to GAD65 or to the peptide. A similar DQ-specific blocking effect was seen with the other three major stimulatory peptides (numbers 13, 24, or 48; data not shown), although the magnitude of the antipeptide responses from the cell line was less in these cases.

Fine specificity of the anti-GAD65 B-cell responses in the transgenic animals. When immunized with GAD65, the NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice developed strong anti-GAD antibody responses (Fig. 5). In all, 12 high-titer serum samples collected from GAD65 injected animals 3-9 weeks after immunization were tested individually against the entire panel of overlapping GAD65 peptides, and the results of the ELISA assays are summarized in Fig. 3B. The strongest reactivity (OD = 1.40 ± 0.08) was observed against residues 381-400 (peptide 39), with a less reactive epitope (OD = 0.71 ± 0.10) located between amino acids 361-380

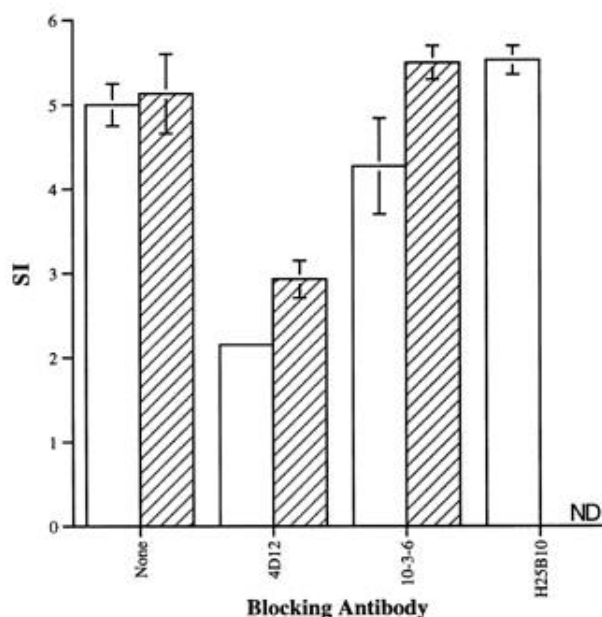


FIG. 4. Anti-DQ antibodies block T-cell responses to GAD65 (□) and to the immunodominant peptide P201-220 (▨). A GAD65-specific T-cell line was established from GAD65-immunized NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice, and T-cells tested in a 72-h proliferation assay in the presence of syngeneic irradiated APCs and GAD65 (5 μg/ml) or peptide (20 μg/ml), with or without purified monoclonal antibodies at 100 μg/ml. Antibodies tested included 4D12 (anti-DQ), 10-3-6 (anti-IA), and H25B10 (anti-HBV surface antigen, which provides isotype control for 4D12). ND, not done.

(peptide 37). Other epitopes with minor reactivities were detected within peptides P51-70 (peptide 6), P121-140, P221-240, P251-270, P301-320, P341-360, and P471-490 (peptide 48). The corresponding OD₄₀₅ values for the ELISAs were, respectively, 0.52 ± 0.03 , 0.37 ± 0.05 , 0.42 ± 0.07 , 0.43 ± 0.05 ,

TABLE 3
Epitopes identified in this study

Position	Sequence
T-cell	
121-140	YVVKSFDRST.KVIDFHYPNE
201-220	NTNMFTYEIA.PVFVLLLEYVT
231-250	PGGSGDGIFS.PGGAISNMYA
471-490	VDKCLELAEY.LYNIKNREG
B-cell	
51-70	DAEKPAESGGSQPPRAAARK
121-140	YVVKSFDRSTKVIDFHYPNE
221-240	LKKMREIIGWPGGSGDGIFS
251-270	MMIARFKMFPEVKEKGMAL
301-320	LIKCDERGGKMPDLERRIL
341-360	YGAFDPLLAVADICKKYKIW
361-380	MHVDAAWGGGLLSRKHKWK
381-400	LSGVERANSVTWNPHKMMGV
471-490	VDKCLELAEYLYNIKNREG

Underlined regions for T-cell epitopes represent best fit with the core consensus motif suggested for DQ8 by Godkin et al. (33). Note that this prediction is theoretical and that core motifs would have to be confirmed by fine mapping. Periods separate the peptides in half, thus indicating the COOH- and NH₂-termini of the immediately adjacent overlapping peptides.

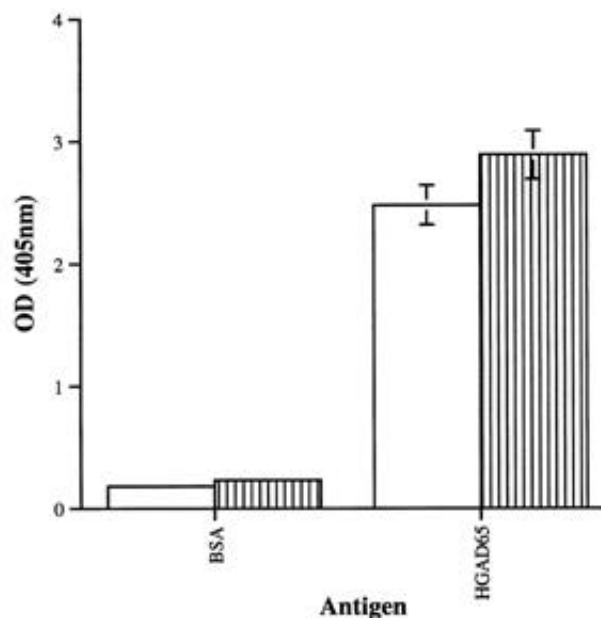


FIG. 5. Antibody responses against GAD65 after immunization of NOD human CD4^{+/+} DQ8^{+/+} IA^{null} transgenic mice with recombinant GAD65. Serum was collected 3 weeks (□) and 9 weeks (▨) after immunization and was tested by ELISA against GAD65- and BSA-coated plates. Bars represent the means of six independent sera, expressed as OD \pm SEM, with each serum tested at a 1:1,000 dilution. Pre-immune serum produced only background reactivity against GAD65 (data not shown).

0.42 ± 0.03 , 0.41 ± 0.04 , and 0.40 ± 0.17 . Interestingly, P121-140 (peptide 13) and P471-490 (peptide 48) were detected as both T- and B-cell epitopes (Fig. 3A). The T- and B-cell epitopes identified for human GAD65 in this study are summarized in Table 3.

DISCUSSION

Type 1 diabetes is a tissue-specific autoimmune disease in which the target tissue has been clearly determined to be the pancreatic β -cell. Because of the well-known association between HLA DR4/DQ8 and type 1 diabetes, considerable attention has focused on mapping DR4- and DQ8-restricted T-cell epitopes in general, and epitopes in islet autoantigens such as GAD65 and proinsulin in particular. For the case of DQ8 (DQA1*0301, DQB1*0302) epitopes, various approaches have been taken, including 1) purification of DQ8 molecules and elution and sequencing of naturally processed peptides (32,33), 2) in vitro binding studies of synthetic peptides on purified class II molecules (29,33,34), 3) establishment of human T-cell clones (35), and 4) characterization of T-cell recall responses in DQ8, IA^{null} transgenic mice. The latter approach was pioneered by David and colleagues (36,37), who first realized that removing the endogenous mouse class II molecules was the key to generating DQ8-restricted T-cell responses in their DQ8 transgenic animals. These investigators have subsequently used their DQ8, IA^{null} transgenic mice to map DQ8-restricted T-cell epitopes from a variety of antigens, including the hypervariable region of various DRB1 molecules (38,39), house dust mite allergen (40), and human preproinsulin (41).

Because we were interested in the possibility of establishing a DQ8 transgenic mouse model for spontaneous autoim-

mune diabetes, and because the necessary transgenic and knockout mice were readily available to us, we established a completely independent new line of DQ8, IA^{null} transgenic animals, this time directly in the diabetes-prone NOD background. Unlike the original line described by David and colleagues (36,37), our animals also expressed the human CD4 molecule, which likely contributes to positive selection of CD4⁺ T-cells restricted to DQ8. Using these animals, we were able to obtain a robust recall response against purified human GAD65, and significant responses against four separate 20-mer peptides derived from GAD65. However, these animals did not show any signs of autoimmune diabetes. In retrospect, this is not surprising, because the original IA_β deletion was done in an ES cell line of 129 origin (21), and in crossing the IA_β deletion onto the NOD background, we have no doubt selected for an extended region of the I29 MHC, notably including the class I genes as well as the TNF α locus, which very likely must be the NOD genotype to see the diabetic phenotype. The possibility also exists that as the sole expressed class II molecule, the DQ8 heterodimer is unable to generate the spontaneous autoimmune/diabetic phenotype, although it is clearly sufficient to mediate collagen-induced arthritis (36), as well as a variety of other antigen-induced 'autoimmune' syndromes (42,43). Resolution of this question will require the generation of NOD ES cells.

Although there have been several reports of GAD65 T-cell epitopes restricted to IA^{g7} (6,9,44,45) and to DR4 (29,46–48), to our knowledge this is the first report of GAD65 T-cell epitopes restricted to the DQ8 heterodimer. The four epitopes identified complement information generated by binding studies using GAD65 peptides and purified DQ8 molecules (29,34), as well as more general attempts to identify a DQ8 "binding motif" (32–34). To ensure that the epitopes identified represent those that are naturally processed, we primed mice with the whole GAD65 protein, rather than with individual peptides (or a mixture) from an overlapping peptide set. Interestingly, two of the peptide epitopes we discovered in the present study (P201-220 and P231-250) were found in a previous study to bind DQ8 with relatively high affinity, whereas the other two peptide epitopes we describe (P121-140 and P471-490) showed no significant binding to DQ8 (29). This finding is consistent with the idea that class II binding is only one criterion by which potential T-cell epitopes can be evaluated; clearly, there are examples of immunodominant peptide epitopes that have a low affinity for class II (e.g., the immunodominant myelin basic protein [MBP] Acl-11 has a low affinity for IA^u, whereas other higher-affinity MBP peptides do not represent T-cell epitopes in IA^u mice [49]). Although it is possible that additional minor DQ8-restricted epitopes may exist in GAD65, which might be better identified using T-cell hybridomas or T-cell clones, the four epitopes identified in the present study (using splenocyte recall responses) are likely to predominate no matter which approach is used.

Another interesting outcome of this study is that two of the peptides (P121-140 and P471-490) contained both T- and B-cell epitopes. Whether the T- and B-cell epitopes are completely overlapping or simply in close proximity within the 20-mers will need to be clarified by finer mapping of each region. It is of note, however, that both of these peptides are classified as DQ8 nonbinders; perhaps their relatively poor class II binding is compensated for by highly efficient capture and presentation by B-cells. In this regard, we assume that because

our recombinant GAD65 antigen is not folded, following vaccination we would expect significant extracellular degradation, and capture of proteolytic fragments (rather than the whole GAD65 antigen) by B-cells is a real possibility. Overlapping or contiguous T- and B-cell epitopes (i.e., occurring within a single 20-mer peptide) have been reported for other autoantigens, including myelin oligodendrocyte glycoprotein (31,50) and the acetylcholine receptor (51).

In general, the DQ8-restricted GAD65 T-cell epitopes discovered in the present study bear little relationship to the GAD65 epitopes described for other class II molecules, with one important exception. The major DQ8 epitope identified in this study (P201-220) overlaps with the major IA^{g7}-restricted T-cell epitope for GAD65, described by Chao and McDevitt (44) as residues 206–220 and by Singh and colleagues (45) as residues 207–221. Determination of whether the DQ8 and IA^{g7} epitopes are identical in this region will require further fine mapping using GAD-specific T-cell hybridomas generated from the DQ8 transgenic animals. However, their being identical is clearly a possibility, because the DQ8 and IA^{g7} molecules share many structural and biochemical features (14,52), and our best guess at the core DQ8 motif for P201-220 (underlined in Table 3) overlaps with one suggested by Singh and colleagues (45) for IA^{g7}. On the other hand, the second predominant IA^{g7}-restricted GAD65 T-cell epitope described by both McDevitt and Singh (residues 221–235 or 217-236) does not appear in our studies to be a major DQ8 epitope, suggesting that there are also subtle differences between these diabetes-associated mouse and human class II molecules.

Whether the DQ8 T cell epitopes identified in this study will ultimately be useful depends on whether the same epitopes are also recognized specifically by DQ8 diabetic patients who show high anti-GAD65 T-cell responses. In this regard, we are encouraged by the fact that a DRB1*0401-restricted GAD65 epitope (P271-285) mapped using DR4 transgenic mice (48) is essentially identical to the peptide epitope (P270-283) recognized by DR4-restricted anti-GAD65 T lymphocyte lines established from recent-onset type 1 diabetic patients (46). Because DQ-restricted responses have historically been more difficult to elicit than DR-restricted responses (particularly from peripheral blood mononuclear cells [53,54]), other potentially more sensitive and more specific approaches may be required to detect DQ8-restricted T-cells. One promising new approach involves staining peripheral T-cells using fluorescently labeled DQ8 tetramers containing GAD65 peptides (55). Creation of such tetramers requires information on the DQ8-restricted peptide epitopes, such as those described in the present study. Indeed, such DQ8 tetramers have not yet been made, and creating them may present a technical challenge—thus, the DQ8 transgenic mice described in this paper should also be useful for testing such tetramers to prove that they can reliably stain reactive T-cells. With this staining reagent will come the opportunity for repeated analyses over time and for specific selection of GAD65-reactive, DQ8-restricted T-cells in diabetic and prediabetic patients. Such studies may well lead to new insights into disease pathogenesis and progression.

ACKNOWLEDGMENTS

This work was supported by operating grants from the Canadian Diabetes Association and the Juvenile Diabetes Foun-

dation International to J.F.E., and by a Juvenile Diabetes Foundation/Medical Research Council of Canada Research Network Program grant to A.M.J. In addition, J.F.E. is a Senior Research Scholar of the Alberta Heritage Foundation for Medical Research (AHFMR), J.L. is supported by a Multiple Sclerosis Society of Canada Fellowship, and L.E.P. was supported by an AHFMR Studentship.

We thank K. Marlin for preparation of the antibodies used in these studies, D. Denney for the hybridomas, and L. Wicker for the human CD4, DQ8 transgenic NOD line. We acknowledge the contributions of R. Flavell, L. Burkley, H. Chen, M. Trumbauer, L. Wicker, and L. Peterson to the generation of these transgenic animals. We thank L. Wicker, B. Singh, and G. Nepom for supplying peptides during our preliminary studies and A. Godkin for suggestions on the DQ8 motifs.

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