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Katalin Kelemen,² Peter A. Gottlieb,² Amy L. Putnam, Howard W. Davidson, Dale R. Wegmann, and John C. Hutton³

Susceptibility to type 1A autoimmune diabetes is linked to expression of particular MHC class II molecules, notably HLA-DQ8 in man and the orthologous I-A\(\beta\)7 in the nonobese diabetic mouse. In the present study, we analyzed two peptide epitopes (peptides 2 and 7) from the diabetes autoantigen phogrin (IA-2β), in the context of their presentation by the I-A\(\beta\)7 and HLA-DQ8 molecules and their role as potential T cell antigenic epitopes in human diabetes. Both of these peptides are targets of diabetogenic CD4⁺ T cell clones in the nonobese diabetic mouse. Transgenic mice expressing HLA-DQ8 as the sole class II molecule generated a robust T cell-proliferative response when primed with peptide 2 or peptide 7 in CFA. Analysis of the IL-2 secretion from peptide 2-reactive T cell hybridomas stimulated with alanine-substituted peptides identified three residues that were crucial to the response. Among 41 islet cell Ag-positive prediabetic human subjects, 36.5% showed PBMC-proliferative responses to peptide 7, 17.1% to peptide 2, and 17.1% to both peptides; no response was seen among 20 matched healthy controls. Stratification of the data based upon HLA haplotype suggested that peptide 7 could be presented by at least one HLA-DR molecule in addition to HLA-DQ8, a finding that was supported by blocking studies with monomorphic mAbs. The results indicate that common phogrin peptides are targeted by autoreactive T cell clones in human and murine type 1A diabetes, and that the responses may in part be associated with the similar peptide-binding specificities of I-A\(\beta\)7 and HLA-DQ8. The Journal of Immunology, 2004, 172: 3955–3962.

Abbreviations used in this paper: NOD, nonobese diabetic; ICA, islet cell Ag; SI, stimulation index; GAD, glutamic acid decarboxylase.

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importance of phogrin as an autoantigen in human type 1A diabetes and the presence of common epitopes that can be presented by the HLA-DQ8 molecule, although are not solely restricted to it.

Materials and Methods

Human subjects

Subjects from the Diabetes Autoimmunity Study in Youth (DAISY) and the Diabetes Prevention Trial-1 (DPT-1) ancillary study no. 9631 were selected for investigation (mean age, 37; range, 5–52; n = 41). Prediabetes in this group of individuals was defined as possession of diabetes susceptibility HLA haplotypes and the presence of islet cell autoantibodies with or without impaired first-phase insulin secretion. HLA and age-matched control subjects were selected from healthy volunteers, the parents, and children in the newborn cohort of the DAISY study and parents of the DAISY sibling/offspring cohort (mean age, 29; range, 3–50; n = 20). All were without recent illness and donated blood for these studies after informed consent was obtained.

Animals

NOD/bdc mice, BALB/c mice, and HLA-DQ8–/– mice, H-2Aβ transgenic mice were bred and maintained in the Barbara Davis Center animal colony, under specific pathogen-free conditions and manipulated in accordance with University of Colorado Health Sciences Center Institutional Animal Care and Use Committee protocols. HLA-DQ8–/–, H-2Aβ mice were generated in the laboratory of Dr. C. David at the Mayo Clinic (Rochester, MN) as described (10). Briefly, the DQA*0301 and DQB*0302 genes were injected into (CBA/J × B10.M)F2 embryos. The transgene-positive founders were identified and subsequently mated to B10.M mice to introduce the HLA-DQ8 transgenes into H-2Aβ mice (20). The animals were backcrossed on to B10.M H-2Aβ mice for six to eight generations before the initiation of the study and during the course of the study were maintained by intercrossing. Flow-cytometric analysis confirmed the segregation of the HLA-DQ8-encoding transgenes and the H-2Aβ mutation, and showed that the HLA-DQ8 mice was present on ∼25% of PBLs (10). The expression of other mouse HLA class II molecules (including H-2Aβ, H-2Kβ) on PBLs was excluded by FACS analyses. Because HLA-DQ8 is the only MHC class II molecule expressed by these animals, any CD4+ T cell response in these mice must reflect the presence of HLA-DQ8 binding sequences within the Ag.

Antigens

Recombinant phogrin C terminus. The cytosolic region of the rat phogrin molecule (aa 629-1004) was cloned into the pGEX-3X expression vector (21) by on-column cleavage with Factor Xa (Boehringer Mannheim, Indianapolis, IN) as previously described (18).

Synthetic peptides. Peptides were generated by custom synthesis (BioWorld, Dublin, OH), purified by HPLC (>95%), and verified by mass spectrometry. The 15-mer phogrin peptides 2 and 7 are targeted by previously generated phogrin-specific NOD T cell clones (19) and represent the dominant I-Aβ-restricted T cell epitopes in the phogrin C terminus. Alanine substitutions in peptide 2 were made in 10 central positions of the 15-mer (Table I). All peptides except 2/10 (which was excluded from the study) were soluble in the assay medium at concentrations of <50 μg/ml.

T cell lines and hybridomas.

Peptide 2- and peptide 7-reactive T cell lines were generated from female HLA-DQ8–/–, H-2Aβ transgenic mice immunized with 50 μg of either peptide 2 or 7 emulsified in CFA (total volume, 50 μl) at the base of the tail. Seven days post-immunization, the draining inguinal and periaortic lymph nodes were harvested, and the resulting cells were washed and cultured in duplicate at 1.0 × 105 cells/well in 96-well microculture plates in Click’s medium (Irvine Scientific, Santa Anna, CA) supplemented with 0.5% mouse serum, 50 μmol/l 2-ME, 10 mmol/l HEPES, 50 μg/ml gentamicin, and 2 mmol/l glutamine, with serial dilutions of the Ag. Phogrin peptides–specific T cell lines were subsequently maintained by in vitro stimulation of 5.0 × 104 T cells with 2.0 × 107 irradiated (3500 rad) syngeneic splenocytes in 20 ml of DMEM/10% FBS containing 50 μg/ml recombinant mouse IL-2 on a 14-day cycle with 10 μg/ml peptide 2 or peptide 7.

Peptide 2-specific T cell hybridomas were produced by fusing T cells derived from HLA-DQ8–/–, H-2Aβ transgenic mice with the BW 5147 TCR αβ T lymphoma using polyethylene glycol (22). The hybridomas were propagated in DMEM/10% FBS, and clones were generated by limiting dilution.

T cell line and hybridoma stimulation assays

T cell lines were assayed by coculture of 2.5 × 105 T cells with 1.0 × 106 irradiated splenocytes, and Ag in DMEM/10% FBS for 72 h with the addition of 0.5 μl of [3H]thymidine 6 h before harvest. T cell hybridomas were assayed by coculture of 1.0 × 106 hybridoma cells with 0.5 × 106 irradiated syngeneic splenocytes or 0.5 × 106 irradiated human PBMCs in DMEM/10% FBS. After 24 h, the secreted IL-2 concentration was measured by ELISA. Briefly, plates were coated with 0.5 μg/ml rat anti-mouse IL-2 (clone JES6-1A12; BD Pharmingen, San Diego, CA) and incubated for 2 h at 37°C with sample, followed by 0.25 μg/ml biotin-conjugated rat anti-mouse IL-2 (clone JES6-5H4; BD Pharmingen). Second Ab binding was determined by time-resolved fluorometry using a streptavidineuroporphyrin conjugate (0.05 μg/ml; PerkinElmer Wallac, Gaithersburg, MD).

Flow cytometry

Flow cytometry was performed on T cell hybridomas (5.0 × 105 cells) using FITC-conjugated rat anti-mouse CD8 (BD Pharmingen) and PE-conjugated rat anti-mouse CD4 (BD Pharmingen) mAbs. Analyses were performed on a BD Biosciences (San Jose, CA) FACS caliber instrument.

Human T cell proliferation assays

PBMCs were isolated from peripheral blood by Histopaque-1077 (density, 1.077 g; Amersham, Piscataway, NJ) gradient centrifugation. A total of 1.5 × 106 PBMCs was cultured in 96-well round-bottom plates (Falcon 3077; Fisher Scientific, Pittsburg, PA) with phogrin peptides 2 or 7 (20 μg/ml) in 200 μl of IMDM (Life Technologies/Invitrogen, Grand Island, NY) containing 5% pooled human serum (Omega Scientific, Tarzana, CA). Tetanus toxoid at 5 μg/ml, and PHA at 2.5 μg/ml were tested on each preparation as controls. Blast population was measured by time-resolved fluorometry using a streptavidin-europorphyrin conjugate (0.05 μg/ml; PerkinElmer Wallac, Gaithersburg, MD).

Table I. Peptides used in this studya

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<th>Peptide</th>
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<td>Peptide 2/position 4</td>
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</tr>
<tr>
<td>Peptide 2/position 15</td>
<td>GADPSATDAEYQEL</td>
</tr>
</tbody>
</table>

a Amino acid sequences of the mouse phogrin epitope peptides 2 and 7 and the alamine-substituted variants of peptide 2 are shown.
munized with 50 g/H9262 transgenic mice. Transgenic animals were immunized with peptide 2 or peptide 7 in CFA, and recall responses to peptide 2 ( ), peptide 7 ( ), and phogrin ( ) were determined. The response in the absence of Ag is indicated by the dashed line. A representative proliferation assay performed in duplicate on cells pooled from four animals is shown. Comparable results were obtained in three separate experiments.

ICA512 autoantibody RIA
Autoantibodies were measured in duplicate as described previously using [35S]methionine-labeled ICA512 produced by in vitro transcription/translation (TNT; Promega, Madison, WI) (23). Results were expressed as an Ab index ((cpm of sample − cpm of negative sera)/cpm reference serum − cpm of negative sera). An Ab index of >0.015 was considered positive.

Results
Proliferative response of lymph node cells from HLA-DQ8+, H-2Aβ0 transgenic mice primed with phogrin epitope peptides 2 or 7

Draining lymph node cells from female HLA-DQ8+, H-2Aβ0 transgenic mice that had been immunized with peptide 2 or peptide 7 in CFA exhibited a robust recall response to the respective peptides with maximal responses observed at concentrations of 33 μg/ml (SI, 10 and 5.5, respectively; Fig. 2). Responses were also observed in each case to recombinant phogrin (maximum response at 33 μg/ml; SI, 6.7), whereas proliferation of T cells to peptide 7 from animals immunized with peptide 2 (Fig. 2A), or of T cells to peptide 2 from animals immunized with peptide 7 (B), did not exceed background levels. The observed responses in the HLA-DQ8β+, H-2Aβ0 transgenic animals were comparable with those observed in NOD mice following immunization with the same peptides (19). This suggests that peptides 2 and 7 are presented efficiently to T cells by HLA-DQ8 molecules, but that no spontaneous responses exist in the inguinal or periaortic lymph nodes of the transgenic animals.

Several phogrin peptide 2-specific T cell lines were established from the draining lymph node cells of five distinct HLA-DQ8β+, H-2Aβ0 transgenic mice. As shown in Fig. 3A, a typical line after four restimulation cycles retained a robust proliferative response to peptide 2 and a weaker response to phogrin.

Characterization of peptide 2-specific T cell hybridomas generated from HLA-DQ8β+, H-2Aβ0 transgenic mice

A panel of 11 peptide 2-specific CD4+ T cell hybridoma clones were generated by limiting dilution (Table II). Clonality was assumed on the basis of reproducible Ag dose-response curve, stability of the clone over long-term culture in vitro, and homogenous population phenotype by FACS analysis. All clones responded to stimulation with peptide 2 (15 μg/ml) by increased IL-2 production in the presence of irradiated spleen cells of HLA-DQ8β+, H-2Aβ0 transgenic mice (Fig. 3B). Irradiated spleen cells from NOD or BALB/c mice were ineffective in the assay (data not shown), suggesting that the response was restricted to HLA-DQ8, and that although the NOD 1-Aγ7 can bind peptide 2, it cannot present to clones that were derived from HLA-DQ8β+, H-2Aβ0 animals.

To further investigate the dependence of the hybridoma clones on presentation by the HLA-DQ8 MHC class II molecule, irradiated PBMCs from three human subjects with different HLA genotypes were evaluated for their ability to present Ag to the hybridomas. Two of these subjects who each expressed HLA-DQ8 in a heterozygous configuration (DQA1*0301-DQB1*0302/DQA1*0301-DQB1*0301 (DQ8/DQ7) and DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 (DQ8/DQ2), respectively) stimulated the hybridoma clones in the presence of Ag (Table III). PBMCs from the third subject with a non-HLA-DQ8 genotype

FIGURE 2. Proliferative response of lymph node cells of HLA-DQ8+, H-2Aβ0 B10.M transgenic mice. Transgenic animals were immunized with 50 μg of peptide 2 (A) or peptide 7 (B) in CFA, and recall responses to peptide 2 ( ), peptide 7 ( ), and phogrin ( ) were determined. The response in the absence of Ag is indicated by the dashed line. A representative proliferation assay performed in duplicate on cells pooled from four animals is shown. Comparable results were obtained in three separate experiments.

FIGURE 3. Responses of peptide 2-specific HLA-DQ8-restricted T cell lines and hybridomas. A, Ag-dependent proliferation of a representative peptide 2-specific T cell line generated from HLA-DQ8β+, H-2Aβ0 B10.M transgenic mice was measured after four rounds of peptide 2/IL-2 stimulation in vitro. The response to phogrin ( ) or peptide 2 ( ) is indicated. B, Eleven clonal peptide 2-specific T cell hybridomas were cultured without ( ) or with peptide 2 ( ) Ag in the presence of HLA-DQ8β+ APCs. Secreted IL-2 was measured as described in Materials and Methods.
(DQA1*0301-DQB1*0301/DQA1*0102-DQB1*0604) were ineffective in the assay. The amount of IL-2 secreted by the hybridomas with human PBMCs as APCs was almost 10-fold lower than that observed with mouse splenocytes. Such differences probably arise from the likely tighter binding of mouse APCs to the murine hybridomas with human PBMCs as APCs was almost 10-fold lower. The reasons for this are unclear, but to allow accurate comparisons, we have excluded the data at the higher peptide concentrations.

Of the 11 clones tested, 9 different patterns of response to alanine substitution were observed, suggesting that the individual TCRs recognized a variety of fine specificities. Substitution at two positions (7 and 14) in the peptide ablated the IL-2 response in every case (Table IV) and corresponded in both instances to a change in an acidic residue. Similarly, loss of Tyr12 caused at least a 50% diminution in response in all cases, whereas Asp5 and Pro5 appeared critical for responses by some, but not all, clones. Interestingly changing Ser5 to alanine significantly enhanced responses from 7/11 clones, but led to a decreased response in two others. Based upon the crystal structure of insulin B9–23 bound to HLA-DQ8 (4) and the sequences of other DQ8-binding peptides, consensus binding motifs for DQ8 have been proposed (24–27). Consideration of these predictions suggests that the most likely MHC contact residues are Ala6 (pocket 1), Thr9 (pocket 4), Ala11 (pocket 6), Tyr12 (pocket 7), and Glu14 (pocket 9). If this alignment is correct, it would explain the effects of alanine substitutions at positions 12 and 14, and suggest that Asp7 is a major TCR contact residue, although this must remain conjectural at this time. The proposed alignment places Asp7, Pro5, and Ser5 outside the core 9-aa binding motif. However, flanking residues likely contribute to both TCR and MHC class II binding (4, 24) and may explain the observed effects of the alanine substitutions (Table IV). Consistent with the proposed conservation between the human and mouse

<table>
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<th>Hybridoma Clone</th>
<th>Phenotype</th>
<th>No Antigen</th>
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\(^a\) Eleven peptide 2-specific T cell hybridoma clones were generated from HLA-DQ8\(^{+}\), H-2\(\text{A}_{\text{B}}\)\(^0\) B10.M transgenic mice. The phenotype (CD4\(^{+}\) or CD8\(^{+}\)) of the clones was determined by flow cytometry, and class II restriction by measuring the release of IL-2 (picograms per milliliter) upon stimulation with Ag and APC from various mouse strains.

### Table III. Characterization of hybridoma clones: release of IL-2\(^a\)

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<th>Hybridoma Clone</th>
<th>Human 0301/0302–0301/0301 PBMC</th>
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\(^a\) Eleven peptide 2-specific T cell hybridoma clones were generated from HLA-DQ8\(^{+}\), H-2\(\text{A}_{\text{B}}\)\(^0\) B10.M transgenic mice. Release of IL-2 (picograms per milliliter) by the hybridoma clones was determined after coincubation with irradiated PBMCs from three human subjects expressing different HLA-DQ genotypes in the presence or absence of 15 µg/ml peptide 2.
responses to peptide 2, substitution of Asp7, Tyr12, and Glu14 with alanine also abolished proliferative responses of two peptide 2-specific I-A\textsuperscript{e}-restricted T cell clones (data not shown).

PBMC-proliferative responses to phogrin peptides 2 and 7 in prediabetic and healthy control subjects

PBMCs from 41 prediabetic and 20 healthy, age- and HLA-matched control subjects were selected to test T cell reactivity to phogrin peptides 2 and 7 (Fig. 4, A and B). DQ8\textsuperscript{+} subjects were highly represented in both groups (80.5 vs 70%) as were DR4\textsuperscript{+} (87.8 vs 70%) individuals. Of the prediabetic subjects, 7 (17%) responded to peptide 2, and 15 (36%) responded to peptide 7. Six (86%) of the peptide 2 responders were HLA-DQ8\textsuperscript{+}, as were 12 (80%) of the peptide 7 responders (Fig. 4, C and D). None of the 20 healthy controls responded to peptide 2, and only one responded to peptide 7 (a DQ8\textsuperscript{+} individual; SI, 3). All of the PBMC preparations showed low proliferation in the absence of Ag (cpm/well, in the range of 12–2496 cpm), and all responded to PHA (2.5 \mu g/ml) used as a positive control (SI; 4–229).

Anti-class II Ab blocking experiments were subsequently performed on a cohort of DPT-1 prediabetic subjects using monomorphic anti-DQ and anti-DR Abs (Fig. 5). The group included three of the peptide 7 responders shown in Fig. 4; however, others were either not available for follow-up (n = 9) or had progressed to clinical diabetes (n = 3). The new group included five positive and two borderline (SI, 2.5–3.0) peptide 7 responders, of whom four also gave SI values >2.0 toward peptide 2. The anti HLA-DQ monoclonal reduced the proliferative response in five of the seven peptide 7 responders by >40%, with >75% inhibition in four cases. A significant exception was an HLA-DR3-homozygous individual (subject 2) who was also homozygous for HLA-DQ2 (Fig. 5B, □). The response to peptide 2 was similarly suppressed by >40% by anti-DQ in three of the four subjects who gave SI values in excess of 2.0, and again unaffected in subject 2 (Fig. 5A, □).

FIGURE 4. Proliferative responses of PBMCs from human subjects to peptides 2 and 7. PBMCs were prepared from peripheral blood of 41 prediabetic and 20 age- and HLA-matched control individuals. Cells were incubated with 20 \mu g/ml phogrin peptide 2 (A and B) or 7 (C and D) as described in Materials and Methods. SI values of >3 were deemed positive. A and C, Shown are the T cell responses for all of the subjects in the prediabetic and control groups. B and D, Shown are the responses only for the 33 prediabetic and 14 control individuals heterozygous or homozygous for HLA-DQ8.

Table IV. Alanine-scanning mutagenesis of peptide 2a

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<th>Clone</th>
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a IL-2 release (picograms per milliliter) from the 11 peptide 2-specific, HLA-DQ8-restricted T cell hybridoma clones was measured upon stimulation with 15 \mu g/ml peptide 2 or peptide 2-derived alanine-substituted variants.
Interestingly anti-HLA-DQ Abs significantly reduced the proliferative response to both peptides by subject 3 who expressed the DR3/5, DQ2/7 haplotype. Given that no effect was observed in the DQ2-homozygous subject 2, this suggests that the antigenic peptides can also be presented by the DQ7 molecules expressed by subject 3, and might therefore contribute to the responses exhibited by subject 1, who similarly expresses the products of the DQA1*0501 and DQB1*0301 alleles (subjects 5 and 6 have the DQA1*0301 allele). In contrast to HLA-DQ8 and I-A^d, the DQ7 molecule has an aspartate at position 57 of the β-chain, suggesting that the peptides are likely to bind in a different register to that adopted when binding to DQ8.

The fact that not all of the peptide 2 and 7 responders in the initial cohort of 41 prediabetics expressed HLA-DQ8 or HLA-DQ7 raised the possibility that these peptides might also be presented by one or more HLA-DR molecules. Studies with the anti-HLA-DR blocking Ab G46-6 suggested that this was indeed the case (Fig. 5, □). In two of four individuals who responded to peptide 2, and seven of seven of those responding to peptide 7, pretreatment with the anti-HLA-DR monoclonal inhibited Ag-dependent T cell proliferation by >50%. Almost complete blockade was observed for subject 2, suggesting that both peptides could be presented by HLA-DR3. Similarly, significant blockade of peptide 7 responses was observed in PBMCs from subjects 5 and 6, indicating that this peptide might also be presented by HLA-DR4 (Fig. 5B, □).

Together, these results suggest that, although HLA-DQ8 is most likely a major determinant in the response to phogrin peptides 2 and 7, these peptides probably contain multiple overlapping epitopes and can be presented on other human class II molecules including HLA-DQ7, -DR3, and -DR4.

### Relationship between T cell reactivity to phogrin and presence of autoantibodies to ICA512 (IA-2)

The group of 41 prediabetic subjects whose T cell responses are described above were originally selected for the DPT-1 trial on the basis of ICA positivity and were subsequently tested for humoral auto-reactivity toward specific molecules (glutamic acid decarboxylase (GAD)65, ICA512, and insulin). Seventeen (41.5%) were positive for ICA512 Abs, and were presumably also anti-phogrin positive, because phogrin and ICA512 autoantiserum cross-react in >90% of cases (17). However, no correlation was detected between the presence or absence of anti-ICA512 Abs, or the magnitude of the autoantibody response, and the primary PBMC-proliferative response to phogrin peptides (Fig. 6). Among the T cell peptide 2 and/or 7 responders, 53.3% were ICA512 autoantibody positive compared with 34.6% in the nonresponder group; the corresponding value for GAD65 autoantibodies were 60 vs 53.8%.

Because the generation of autoantibodies is presumably a T-dependent process, this suggests that, in addition to peptides 2 and 7, other peptides from phogrin and/or IA-2 can also be presented by human MHC class II glycoproteins. Consistent with this conclusion, a recent study identified three additional HLA-DQ8 binding peptides derived from regions of IA-2 that are highly conserved with phogrin (28), but that were not identified in our previous studies of phogrin immunoreactivity in the NOD mouse (19).

### Discussion

The class II region of the MHC is a major diabetes susceptibility locus in both man and the NOD mouse (1). The mechanism by which particular MHC class II molecules, either alone or in combination, influence the development of type 1A diabetes is clearly complex, but presumably involves their distinct binding specificities and affinities for peptides derived from potential diabetic autoantigens. The MHC class II molecule of the NOD mouse model of type 1A diabetes shares structural similarities with HLA-DQ8 (6), and both molecules are associated with disease susceptibility. The question we address here is the extent to which this association is reflected in the specific peptides that can be presented by these molecules. Given the tissue specificity of the autoimmune process, it seems reasonable to propose that disease is initiated by responses directed to one or more islet-specific Ags, but that the resultant destruction may lead to expansion of pathogenic T cells...
FIGURE 6. Correlation between Ab and T cell responses in prediabetic subjects. PBMCs were prepared from peripheral blood of 41 prediabetic and 20 age- and HLA-matched control individuals. All subjects had been screened previously for Abs against ICA512 (IA-2) by RIA, as described in Materials and Methods. Cells were incubated with 20 μg/ml phogrin peptide 2 (A) or 7 (B), proliferation was measured as described previously and is expressed relative to the Ab index of the subject. All of the control subjects had autoantibody indices of <0.015 to this Ag.

targeted at islet proteins that are also expressed in non-β-cells. It is thus likely that multiple Ags, each with multiple epitopes, are involved. Nevertheless, Ag-specific therapy remains an attractive possibility for diabetes prevention, and the identification of the key T cell autoantigenic epitopes recognized by human MHC molecules is an important goal.

One approach is to use T cell epitopes identified from studies using NOD mice to investigate peripheral responses in prediabetic or newly diabetic subjects. Thus, peptides from the humoral autoantigens insulin, GAD65, and heat shock protein 60 (including the immunodominant peptides of these Ags in the NOD mouse) bind to both HLA-DQ8 and I-A\textsuperscript{g7} (29), albeit with differing affinities. Similarly, PBMC responses to (pro)insulin and GAD65 and their corresponding peptide epitopes have been observed (24, 30). Such studies have been facilitated by the availability of HLA-DR and -DQ transgenic mice on multiple genetic backgrounds. These allow investigation of the influence of particular human class II molecules on immune responses and disease development in an in vivo context, thereby providing a stepping stone between the well-established disease models and human subjects.

In previous studies, we identified two peptides from the autoantigen phogrin (IA-2β) that could be processed from the native Ag by NOD APCs, and presented in the context of I-A\textsuperscript{g7} (18, 19). These epitopes did not overlap with the T cell epitopes of the related diabetes autoantigen IA-2 (ICA512) in the NOD mouse (K. Kelemen and J. C. Hutton, unpublished observations), nor those identified in human studies (31) and described in recent analyses using transgenic mice (28). However, the phogrin-derived peptides can lead to expansion of pathogenic T cells capable of destroying pancreatic islets in vivo (18), and might therefore be relevant to the pathogenesis of the disease. In the present study, we show that HLA-DQ8 is also capable of binding the phogrin-derived autoantigenic peptides, and that spontaneous T cell responses to these peptides can be observed in a subset of prediabetic human subjects.

Several lines of evidence support our conclusion that phogrin peptides 2 and 7 can be presented by both I-A\textsuperscript{g7} and HLA-DQ8. Thus, robust Ag-specific T cell responses were obtained following immunization of HLA-DQ8\textsuperscript{−}, H-2A\textsuperscript{a}, and I-A\textsuperscript{g7} transgenic mice (where HLA-DQ8 was the sole functional MHC class II molecule present) with either peptide 2 or 7. The series of peptide 2-specific T cell hybridomas generated from T cells isolated from the transgenic mice were all CD4\textsuperscript{+} and did not respond in assays in which the peptide was presented by either NOD (I-A\textsuperscript{g7}) or BALB/c (I-A\textsuperscript{d}/I-E\textsuperscript{a}) APCs. However human PBMCs from two HLA-DQ8\textsuperscript{+} subjects, but not from a non-HLA-DQ8\textsuperscript{+} individual, were able to present Ag to the hybridomas. These results are consistent with the hypothesis that peptides 2 and 7 are potentially diabetogenic in human subjects that express HLA-DQ8.

Analysis of the HLA-DQ8-restricted hybridomas using alanine-substituted derivatives of peptide 2 identified two positions (aa 7 and 14) that were critical, and a third (aa 12) that was important, for induction of IL-2 release from all clones. At present, the HLA-DQ8 binding motif has not been defined precisely, but the recently described x-ray crystallographic structure of the HLA-DQ8/insulin B9–23 complex (4), together with previous pooled sequencing data (25), suggests that acidic or polar residues are favored at positions P1 and P9, and an uncharged residue at P4. Thus, the diabetogenic B9–23 epitope binds to HLA-DQ8 with the Glu Tyr, and Glu residues at the P1, P4, and P9 sites, respectively (4). For phogrin peptide 2, the alanine-scanning data suggest two possible anchor residues, Asp\textsuperscript{7} and Glu\textsuperscript{14}, respectively. However, if Asp\textsuperscript{7} occupied the P1 pocket, this would place Glu\textsuperscript{10} in P4, which is probably unfavorable, and a hydrophobic residue Leu\textsuperscript{15} in the critical P9 position. In contrast, if Glu\textsuperscript{14} occupied P9, then favorable residues (Thr\textsuperscript{9} and Ala\textsuperscript{11}) would occupy the P4 and P6 positions, respectively, and Tyr\textsuperscript{12} would occupy the P7 position. As P7 is also a class II contact, this might explain the global effect of substituting Tyr\textsuperscript{12} with alanine. Another alternative is that Glu\textsuperscript{13} occupies P9 and Glu\textsuperscript{14} occupies P10, in agreement with the motif proposed by McDevitt and colleagues (24). This would place the potentially favorable Ser\textsuperscript{5} at P1. However, because substitution of this residue resulted in an approximate 3-fold increase in IL-2 production by clone 25/4, but a >50% reduction in secretion by clone 25/5, relative to the wild-type peptide, we believe this residue is unlikely to be a major HLA-DQ8 contact. Proof that our favored assignment (Glu\textsuperscript{14} at P9) is correct must await a crystal structure. However, this alignment would also satisfy the I-A\textsuperscript{g7} binding motif (in which an acidic residue at P1 appears unfavorable (5)), and suggest that the binding of peptide 2 to the human and mouse orthologs is identical, in contrast to the situation for the immunodominant insulin B9–23 peptide which likely uses alternative registers for HLA-DQ8 and I-A\textsuperscript{g7} (4, 5, 32).

Studies of the proliferation of PBMCs isolated from prediabetic subjects to phogrin peptides 2 and 7 showed a significant frequency of spontaneous T cell responses to both of the phogrin peptides. Peptide 7 was targeted more frequently (36% of prediabetics) than peptide 2 (17% of prediabetics), which in part may be related to the greater degree of sequence conservation between human and rodents of the former peptide. Surprisingly, neither the response to peptide 2 nor that to peptide 7 was limited to those individuals that expressed HLA-DQ8. Indeed, preliminary analyses
suggested that a significant proportion of the circulating phogrin-spe-
cific T cells in several subjects was restricted by HLA-DR molecules.
This conclusion was supported by AB-blocking studies in which the
anti-HLA-DR Ab caused a greater degree of inhibition than the
HLA-DQ monoclonal for one of four peptide 2, and four of seven
peptide 7 responses, revealing a considerably more diverse response
to the autoantigenic peptides than anticipated from the results of
the transgenic mouse study. The conclusion that some autoantigenic pe-
tides can be presented by more than one class II molecule has also
been reached from studies of human responses to GAD65 (24). Our
results illustrate both the advantages and limitations of using the NOD
and HLA-DQ8 transgenic mice for the prediction of human autoan-
tigenic T cell epitopes. The orthologous peptides to those identified in
our initial study using NOD mice can clearly be generated and pre-
sented by human APCs and may therefore be involved in the patho-
genesis of the disease, but the use of animals expressing only a single
class II molecule does not permit the diversity of response either to the
parent Ag, or even to 15-residue peptides from that Ag, to be fully
appreciated.

The observation that the same, naturally processed, peptides appear
to be presented by multiple HLA-DQ8 and HLA-DR molecules in
human subjects, may have important ramifications regarding both di-
babetes pathogenesis and potential therapeutic intervention. Given that
the key anchor residues that mediate binding to HLA-DQ8 are distinct
from those that allow interaction with the HLA-DR3 or -DR4 sub-
types expressed by the subjects in the present study (and which also
confer susceptibility) (6), but peptides 2 and 7 bind to all of these class
II molecules, this suggests that the phogrin-derived autoantigenic pe-
tides likely contain multiple overlapping epitopes for both disease-
associated HLA-DR and -DQ molecules. Such peptides, if derived from
proteins that are not expressed at signi-
ficant level in the thymus (allowing cognate T cells to escape negative selection), presumably
exhibit a greater tendency to trigger an autoimmune response, both
within the population as a whole, and in individuals, those that can only bind a single disease-associated class II molecule. Con-
versely, they may also be particularly good candidates as reagents for
therapeutic intervention to induce or restore tolerance, given the likely
higher frequency of individuals possessing T cells capable of respond-
ning to them.

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