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Spontaneous T-cell proliferation in the non-obese diabetic mouse to a peptide from the unique class II MHC molecule, I-A^{g7}, which is also protective against the development of autoimmune diabetes

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Abstract

Aims/hypothesis. Major histocompatibility complex class II molecules present antigenic peptides to T-cells and have an important role in T-cell thymic education. The mechanism by which major histocompatibility complex alleles confer a high genetic risk for autoimmune diabetes is not known. One hypothesis is that during positive thymic selection, the peripheral T-cell repertoire is modelled by major histocompatibility complex-restricted presentation of self major histocompatibility complex molecule-derived peptides, some of which mimic tissue autoantigens. The sequence similarity between a known T-cell epitope of glutamic acid decarboxylase-65, 509:VPPSLRTLED and the non-obese diabetic mouse class II major histocompatibility complex molecule I-A^{g7} 86:VPTSLRRLEQ is consistent with this.
Methods. We measured spontaneous proliferation of peripheral T-cells from non-obese diabetic mice and other, non-diabetes-prone strains, to the I-A$^{g7\ 86–101}$ and glutamic acid decarboxylase-65$^{509–524}$ peptides, binding of these peptides to intact I-A^{g7} and assessed the effect of tolerance induction on diabetes development, by injecting young non-obese diabetic mice with high doses of peptide.
Results. T-cells from non-obese diabetic, but not other mice strains, spontaneously proliferate to the I-A$^{g7\ 86–101}$ and glutamic acid decarboxylase-65$^{509–524}$ peptides, but not control peptides. Both test peptides bind I-A^{g7}. Tolerance induction prolongs diabetes-free survival in non-obese diabetic mice when either the I-A$^{g7\ 86–101}$ or glutamic acid decarboxylase-65$^{509–524}$ peptide, but not control peptide, is used.
Conclusion/interpretation. A peptide from the unique class II major histocompatibility complex, diabetes-susceptibility molecule, I-A^{g7}, presented by I-A^{g7} is a target of T-cell responses in diabetes-prone non-obese diabetic mice and tolerance induction against the peptide offers appreciable protection against the development of diabetes. [Diabetologia (1999) 42: 560–565]

Keywords NOD mice, class II MHC, I-A^{g7}, T cells, peptide therapy, tolerance, GAD-65.

In autoimmune diseases such as Type I (insulin-dependent) diabetes mellitus in man and spontaneous diabetes in the non-obese diabetic (NOD) mouse, the major component of genetic susceptibility maps to the MHC class II region [1]. Specifically, the higher risk of Type I diabetes is conferred by the possession of genes encoding non-Asp57 DQβ/Arg52 DQα chains in man [2] and by the close murine homologue I-A^{g7} in NOD mice [3]. The mechanism by which these molecules confer a risk of disease is not clear. The classical view of the relation between MHC-determined vulnerability and autoimmune disease is that susceptibility molecules offer optimum binding characteristics for autoantigenic peptides to be presented to autoreactive T-cells in the target organ [4]. An alternative,

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Abbreviations: NOD, Non-obese diabetic; TCR, T-cell receptors; GAD65, glutamic acid decarboxylase-65.

but not mutually exclusive view, is that the MHC susceptibility is programmed in the thymus [5].

Positive selection of CD4 + T-cells in the thymus depends upon T-cell receptors (TCR) having a moderate to weak affinity for MHC-peptide complexes displayed by thymic antigen presenting cells (APCs) [6]. Since the majority of self-peptides thus presented derive from MHC molecules themselves [7–10], it is probable that MHC/MHC-peptide complexes are a dominant template for selection of the peripheral T-cell repertoires. It follows that T-cell epitopes on antigens encountered post-thymically will be biased towards those that most closely mimic self-MHC peptides. Mimicry of MHC could apply equally to T-cell epitopes of autoantigens and foreign antigens and we have argued that this provides an explanation for MHC-associations with autoimmune disease [11]. In this scenario, MHC associations would be dependent upon the availability of susceptibility allele-specific peptide mimics, as well as the control of their presentation by the susceptibility MHC molecule.

To date, numerous examples of potential mimicry between MHC molecules and known T-cell and B-cell epitopes within antigens have been noted [11], but experimental evidence of their disease relevance has been lacking. One of the most remarkable examples to arise is that in the NOD mouse, in which a region of the I-A^{g7} beta-chain (shared only with one other, minor, allele, I-A^{u}) 86:VPTSLRRLEQ is similar to a sequence in glutamic acid decarboxylase-65 (GAD65), 509:VPPSLRTLED [12]. GAD65 is a key autoantigen in the development of autoimmune diabetes in the NOD mouse and the peptide commencing at amino acid 509 is a known early T-cell epitope in the onset of the disease [13,14]. In this study, we have focused on the questions of whether the 86–101 peptide from the I-A^{g7} beta-chain is a T-cell epitope in NOD mice and whether abrogation of T-cell responses to this peptide affects the development of spontaneous diabetes in this model.

Materials and methods

Animals. The NOD mouse colony at King's College School of Medicine and Dentistry (London, UK) has an incidence of diabetes of approximately 90–95 % at 25 weeks of age in both females and males [15]. Diabetes is diagnosed using urinary glucose testing (values > 8.3 mmol/l on two occasions 72 h apart) and confirmed by blood glucose measurement over 16.7 mmol/l. The mice were obtained originally as a breeding nucleus from Dr. M Hattori (Joslin Diabetes Center, Boston, Mass., USA) and are maintained in a clean but not specifically pathogen free environment and fed ad libitum. Protective conditions include limited access, use of protective clothing, separate food stores, autoclaved drinking water and regular monitoring for infectious pathogens. We purchased BALB/c (H-2^{d}) mice from Charles River (Kent, UK) and DBA/2 ($H2^{b}$), C57BL/6 ($H2^{b}$) and SJL ($H2^{s}$) mice from Harlan (Oxford, UK). All experiments were licensed and carried out according to the guidelines of the Animals (Scientific Procedures) Act 1986.

Peptides. All peptides (GAD65, I-A^{g7}, control peptides) were synthesised by standard Fmoc chemistry using an Applied Biosystems 432A peptide synthesiser (Perkin Elmer, Warrington, UK). After de-protection, peptide purity was checked by HPLC and was always more than 95 %. Peptide mass was confirmed by matrix assisted laser desorption time-of-flight mass spectrometry (Perseptive Voyager Elite, Perkin Elmer). The following test peptides were used: murine $GAD65^{509–524}$ VPPSLRTLEDNEERMS; NOD mouse class II MHC β chain I-$A^{g7\ 86–101}$ VPTSLRRLEQPNVAIS. In addition, two control peptides were used; QYLERTRAELDTVCRY corresponding to I-$A^{U\ 64–79}$ (selected as an irrelevant sequence from an I-A β chain that is similar to I-A^{g7}, differing only in positions 76 (A) and 79 (H)) and VNQSLRPTPLEISVRA, corresponding to a random scramble of the test I-A^{g7} peptide.

T-cell proliferation assay. In preliminary experiments, limiting dilution analysis was used to assess the responder frequency of splenic T-cells. The frequency of spontaneous responses to candidate peptides was found to be in the range 1/300,000 to 1/1,000,000 (data not shown). For this reason, to assess spontaneous proliferative responses to peptides, a modified proliferation assay carried out under limiting dilution conditions was used, similar to that used to measure spontaneous peptide specific T-cell responses in human peripheral blood. Spleen mononuclear cells were isolated from the NOD mice and four strains of non-NOD mice (BALB/c, DBA/2, C57BL/6 and SJL) by mechanical disruption and Lymphoprep (Nycomed, Birmingham, UK) density gradient separation. After washing, cells were resuspended in RPMI 1640 supplemented with 10 % fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 U/ml), 25 mmol/l HEPES and 2 mmol/l l-glutamine and 3×10^5 cells dispensed into wells of a 96-well flat-bottomed tissue culture plate and incubated with the relevant peptide (100 μmol/l final concentration) or medium alone at 37 °C in 5 % CO_2 for 5 days. All peptide assays were carried out as 15-well replicates. Concanavalin A (Sigma Chemical, Poole, UK) was used (2.5 ng/ml) as a positive control. At 18 h before harvesting, 18.5 Kbq (0.5 μCi) [^{3}H]-thymidine (Amersham International, Little Chalfont, Buckinghamshire, UK) was added to each well, and isotope incorporation measured. For each mouse, the mean and standard deviation (SD) background counts per minute (cpm) for 15 wells of cells incubated in the presence of media alone were calculated. Typically, background counts ranged between 65–652 cpm, mean 406, SD 134 (results from a representative experiment). Results for peptide assays were calculated as the number of wells in which cpm exceeded the mean + 2 SD of the background.

Tolerization regime. To induce tolerance, 4-week-old NOD mice were injected subcutaneously with 100:1 of 0.28 mmol/l peptide (GAD65, I-A^{g7} or control peptide) in incomplete Freund's adjuvant (IFA; Sigma), as described previously [16].

Peptide binding assay. The relative affinity of test and control peptides for I-A^{g7} was measured by a europium-streptavidin dissociation enhanced lanthanide fluoroimmunoassay (DELFIA), as described [17]. NOD spleen cells were fixed in 0.5 % paraformaldehyde in phosphate buffered saline (PBS) for 30 min on ice, followed by one wash with RPMI 1640/10 % FCS and two washes with PBS. Cells were resuspended at 1×10^7 cells/ml in 0.15 mol/l NaCl, 0.01 % sodium azide and 1 mol/l citrate/phosphate buffer pH 5.5. We added 100 μl of the cell suspension to each well of a round bottom 96-well

plate. Biotinylated positive control peptide was added to the cells at a concentration of 10 μmol/l, followed by the addition of the non-biotinylated test peptides in serial dilution from 100 μmol/l. The positive control peptide was synthesised as above, and comprised the GAD65$^{524–543}$ peptide, sequence SRLSKVAPVIKARMMEYGTT, a strong I-A^{g7} binder (E.P. Reich, personal communication). The I-Eα$^{52–68}$ peptide, sequence FAKFASFEAQGALANIA which does not bind (E.P. Reich, personal communication), was used as a negative control. Triplicate samples were tested in each case. The cell/peptide mixture was incubated at 37 °C for 18 h. After incubation, the plates were centrifuged for 10 min at 400 *g*. The supernatant was removed and the cells were lysed using 60 μl/well of lysis buffer (0.5 % NP-40 in 0.1 % bovine serum albumin, 5 % non fat dried milk powder, 0.1 % sodium azide). The cells were incubated on ice for 30 min with mixing every 15 min followed by centrifugation for 10 min at 400 *g* to obtain a clear lysate. We added 50 μl of the clear lysate to 96-well microtitre plates pre-coated with 100 μl/well of a 50 mg/ml solution of anti-murine MHC class II (I-A) antibody (ATCC clone 10.2.16) in PBS. After 2 h at 4 °C, plates were washed several times in 50 mmol/l Tris HCl, pH 7.5, 0.15 mol/l NaCl containing 0.1 % Tween-20. We added 100 μl of enhancement solution (2 mmol/l acetate at pH 3.1, 0.05 % Triton X-100, 60 mmol/l benzoyl trifluoroacetone, 8.5 mmol/l yttrium oxide in ddH_2O) to each well and the plate was rocked at room temperature, then read in a time delay fluorometer.

Statistical analysis. The mean frequency of positive wells detected in groups of mice by peptide proliferation assays was compared using the Student's *t* test, with *p* less than 0.05 considered significant. Individual mice were considered to have shown a significant proliferative response to a peptide if more than 6/15 (> 40 %) of wells had cpm above the mean + 2 SD of the background ($p < 0.05$ by χ^2 test), as reported previously [18]. The effect of peptide tolerization on time to development of diabetes in the three groups was examined using survival curves and the logrank (Mantel-Cox) test, calculated with the StatView (Version 4.5) program (Cherwell Scientific, Oxford, UK).

Results

Spontaneous T-cell response to GAD65$^{509–524}$ and I-A$^{g7\ 86–101}$ peptides. Spleen cells from non-diabetic NOD mice (aged 8–10 weeks $n = 12$) were stimulated in vitro with GAD65$^{509–524}$, I-A$^{g7\ 86–101}$ and control peptides and T-cell responses measured as the number of wells positive (positive = cpm greater than the mean + 2 SD of the background cpm measured in the presence of medium alone).

Comparing the mean percentage of wells positive, proliferation responses to GAD65$^{509–524}$ and I-A$^{g7\ 86–101}$ peptides were higher than to the control peptide (means ± SEM; 33.0 ± 5.7 % for GAD65$^{509–524}$, 36.5 ± 6.4 % for I-A$^{g7\ 86–101}$ and 15.0 ± 5.2 % for the I-A^{U} control peptide, $p < 0.05$ in both cases, Fig. 1). Similar results were obtained with the scramble control peptide (data not shown).

We observed spontaneous proliferative responses (number of wells positive > 40 % in an individual mouse) to GAD65$^{509–524}$ and I-A$^{g7\ 86–101}$ peptides in 5/12 and 7/12 NOD mice, respectively, compared with 1/12 for the control peptide ($p = 0.06$ and $p < 0.01$, respectively).

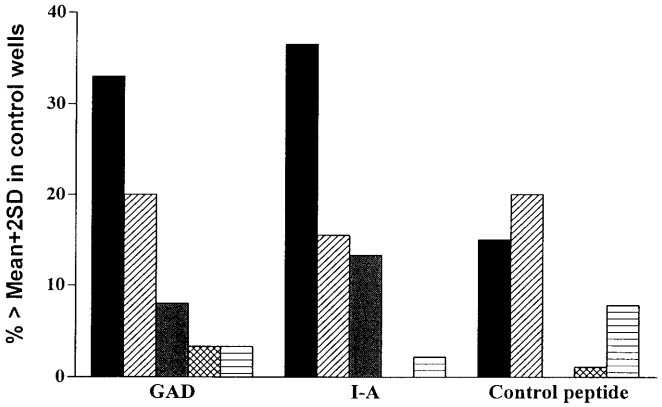

Fig. 1. Spontaneous T-cell proliferation to GAD65$^{509–524}$, I-A$^{g7\ 86–101}$ and control peptide (I-A^{u}) in NOD and non-NOD mice. Bars represent mean results from 12 NOD (■) mice and 6 each of BALB/c (▨), DBA/2 (▩),C57BL/6 (▩) and SJL (▤) mice. The height of the bar is the group mean percentage of wells in which cpm were greater than the means + 2 SD of control wells (background). Proliferative responses to the GAD65 and I-A^{g7} peptides in NOD mice were higher than those to the control peptide ($p < 0.05$). In non-NOD mice, proliferative responses were similar for each peptide

Much lower responses (mean < 20.0 % wells positive in all cases) to these peptides were observed in the four strains of non-NOD mice (BALB/c, DBA/2, C57BL/6 and SJL; $n = 6$ for each group, Fig. 1). None of the individual non-NOD mice showed spontaneous proliferative responses (number of wells positive > 40 % in an individual mouse) to any of the peptides.

Levels of I-A$^{g786–101}$ and GAD$^{509–524}$ peptide-induced T-cell stimulation were higher in NOD mice compared with control peptide and medium alone (Fig. 2). In non-diabetic strains, levels of stimulation were similar for medium alone, test and control peptides (Fig. 2).

Binding of peptides to I-A^{g7}. The GAD65$^{509–524}$ and I-A$^{g7\ 86–101}$ test peptides bound to I-A^{g7}, displacing the positive control peptide used in the assay with equal affinity but the control peptide was no more effective at displacing the positive control peptide than was the I-Eα$^{52–68}$ negative control peptide (Fig. 3). Binding assays were not done for the I-A^{U} control peptide.

Effect of tolerization with peptides on diabetes onset. NOD mice aged 4 weeks were subjected to a standard tolerizing regime with test and control peptides and monitored for diabetes development to 25 weeks of age. At 25 weeks, 21 of 34 (61.7 %) NOD mice remained non-diabetic in the group receiving GAD65$^{509–524}$ peptide. From 25 NOD mice, 15 (60.0 %) remained non-diabetic in the group receiv-

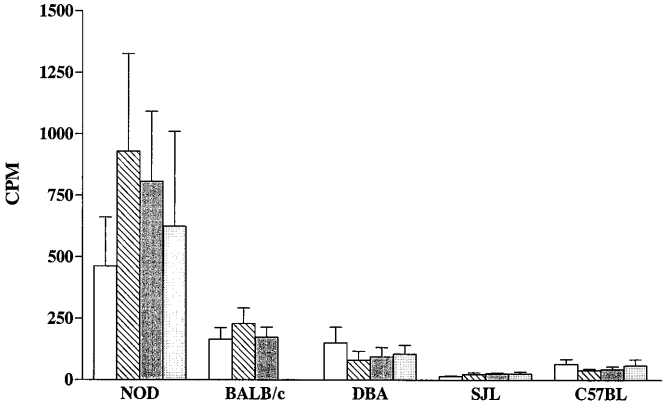

Fig. 2. Spontaneous T-cell proliferation to test and control peptides in NOD and non-NOD mice, shown as the means ± SEM level of T-cell stimulation (cpm) in the presence of medium alone (□), I-A$^{g7\ 86-101}$ (▨) GAD65$^{509-524}$ (■) and I-A^{u} (▩) control peptide. Note that BALB/c mice were not tested against control peptide

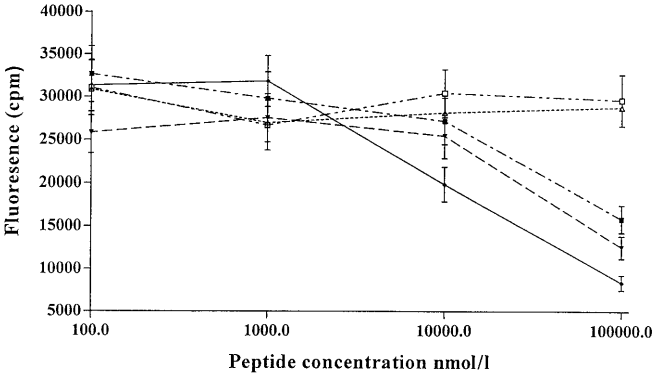

Fig. 3. Competitive I-A^{g7} peptide binding assay for test GAD65$^{509-524}$ and I-A$^{g7\ 86-101}$ peptides. Error bars represent means ± SEM. Graph shows binding of positive biotinylated control peptide (GAD65$^{524-543}$) to I-A^{g7} in the presence of different concentrations of test peptides (GAD65$^{509-524}$ —◆—; I-A$^{g7\ 860-101}$ –▼–; control scramble peptide _.□._; and the negative control peptide I-Eα^{52-68} --△--. Competitive binding is seen for GAD65$^{509-524}$ and I-A$^{g7\ 86-101}$ peptides, similar to that for the non-biotinylated GAD65$^{524-543}$ positive control peptide (_.■._) whereas the control scramble peptide is similar to the I-Eα^{52-68} negative control peptide

ing I-A$^{g7\ 86-101}$ peptide. Diabetes-free survival in both of these groups was prolonged compared with NOD mice receiving I-A^{U} control peptide (7/33, 21.2%; $p < 0.0005$ vs GAD65$^{509-524}$ and $p < 0.005$ vs the I-A$^{g7\ 86-101}$ peptides) (Fig. 4). A similar lack of protection was observed with the scramble control peptide, although this failed to reach statistical significance compared with the test peptides due to the smaller number of mice in this control group (3/10, 30% surviving at 25 weeks; $p = 0.05$ vs GAD65$^{509-524}$ peptide, $p = 0.1$ vs I-A$^{g7\ 86-101}$ peptide).

Discussion

The key genetic factor in the development of autoimmune diabetes in the NOD mouse is the presence of the unique class II MHC molecule, I-A^{g7} [3]. In this study we show that a peptide from I-A^{g7} which shares considerable sequence identity with the immunodominant region of GAD65 (a key autoantigen in the pathogenesis of autoimmune diabetes) is itself presented by I-A^{g7}, recognised by NOD T-cells and capable of modulating diabetes development when delivered as a tolerogen.

The beta-chain of I-A^{g7} shares in common with the human Type I diabetes-susceptibility MHC molecule (DQ8), a non-aspartate at position 57. Murine transgenic studies in which this residue has been mutated to the more common aspartate reduces the incidence of diabetes, indicating a key role for this residue [19]. The mechanism by which it exerts this effect is, however, not known. It is unlikely to relate to restricted presentation of exogenous peptides, since diabetes incidence in this model depends upon the animals being maintained in pathogen-free environments.

We have suggested a general role for class II MHC in susceptibility to autoimmune disease arising from what is known about T-cell selection in thymic ontogeny [11]. This notes that peripheral T-cells are selected in the thymus for moderate affinity recognition of class II MHC presented self-peptides [6]. The majority of self-peptides presented in the thymus are probably derived from MHC molecules themselves (class I and class II), since such peptides are those most commonly eluted from class II MHC [7–10]. This is probably because MHC molecules constitute major components of membrane proteins recycled into the endocytic compartments during antigen processing. It would follow from this that dominant T-cell epitopes of foreign antigens or autoantigens or both mimic sequences within MHC molecules, and that where such sequences were allele-specific, disease susceptibility potentially resides in the primary sequence of MHC alleles (which give rise to presented peptides) as well as in the restriction of their presentation.

In the case of I-A^{g7}, we have noted that the 10-mer VPTSLRRLEQ in I-A^{g7} is remarkably similar to a sequence in (GAD65) VPPSLRTLED [12], known to be a dominant T-cell epitope in the onset of autoimmune diabetes in the NOD mouse [13, 14]. This similarity forms the basis for our current study, in which we test the hypothesis that T-cells responding to the I-A^{g7} presented I-A^{g7} peptide 86–101 are present and have a role in the development of diabetes by virtue of this mimicry with a key islet autoantigen. We show that this peptide is indeed capable of binding to I-A^{g7}, there is spontaneous T-cell proliferation to this peptide in the NOD mouse but not in non-diabetic strains and induction of tolerance to the peptide

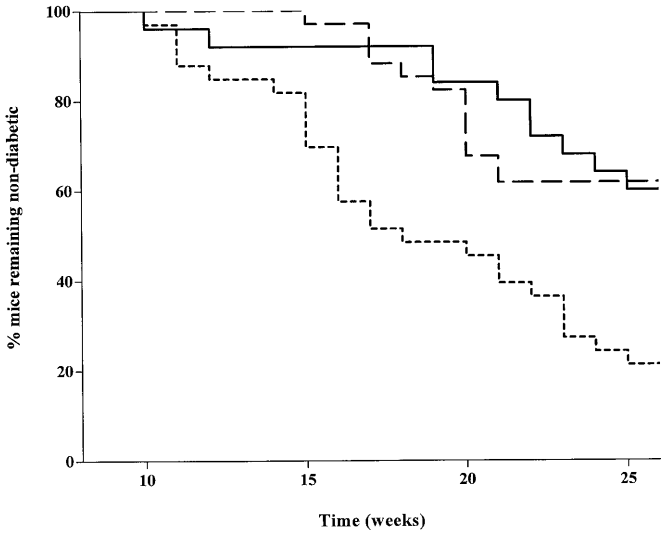

Fig. 4. Survival curves for NOD mice treated at 4 weeks of age with subcutaneous injections of GAD65$^{509–524}$ (——), I-A$^{g7\ 86–101}$ (– – –), or control I-A^{u} peptide (-----). Diabetes-free survival was prolonged in GAD65$^{509–524}$ and I-A$^{g7\ 86–101}$-treated mice, compared with controls ($p < 0.0005$ and $p < 0.005$, respectively)

has a profound protective effect on the development of autoimmune diabetes. In all of these experiments, the I-A^{g7} peptide was at least as biologically active as the GAD65 peptide it resembles; this GAD65 sequence derives from the major immunodominant region within this autoantigen. The frequency of T-cells reactive to either peptide appears low (less than 1/300,000). This may reflect that reactivity to single peptides was analysed and by necessity we have studied splenic T-cells. Islets of NOD mice at the ages studied are likely to be more heavily infiltrated by autoreactive T-cells.

The protection against the onset of diabetes using a tolerizing regime (high doses of peptide during early life) is likely to reflect true anergy, since, as we have previously reported, animals remaining non-diabetic beyond 25 weeks after I-A^{g7} or GAD65 peptide therapy become diabetic within days after treatment with high dose IL-2 [12]. Such treatment is known to overcome peripheral T-cell tolerance and in man can lead to the emergence of clinical autoimmune disease.

Notwithstanding that spontaneous T-cell proliferation is seen to each of the mimicking peptides, and not to the control peptides, this does not prove that there is a single population of autoreactive T-cells capable of responding to both peptides. It is well established [20] that a single selecting MHC-peptide complex in the thymus can select peripheral TCRs of apparently diverse peptide specificities. Thus cross-reactive peptides are likely to be partial rather than true mimics, as would be the case for I-A$^{g7\ 86–101}$ and GAD65$^{509–524}$ which differ at several residues. In this case, T-cell reactivity to the selecting and mimicking peptides are possibly subtly different in degree, as we observed. The trigger for the development of diabetes in the NOD mouse is not known, although the fact that mice reared germ-free have a high incidence of the disease suggests that it is endogenous. Whatever the initial pathological process in the islets, we can speculate that the ready availability of islet autoreactive T-cells, selected in the thymus as a result of cross-reactivity with MHC peptides, would be an important factor in amplifying and perpetuating the disease process. If the hypothesis tested in this study were to be correct, then it raises the question of how to account for early reactivity directed against more than one islet autoantigen during the development of autoimmune diabetes. The targeting of GAD67 as an autoantigen is not surprising, since it has a sequence homology to GAD65 in the region of similarity to I-A^{g7} (GAD65$^{509–518}$ VPPSLRTLED; GAD67$^{517–526}$ IPQSLRGVPD). Of greater interest are similarities between the same I-A^{g7} peptide and regions in two other islet autoantigens, proinsulin and peripherin (proinsulin^{52}-PKSRREVEDPQ, compared with I-A$^{g7\ 87}$ PTSLRRLEQPN; peripherin^{50}-VRFLEQQNAAL, compared with I-A$^{g7\ 90}$ LRRLEQPNVAI). The concepts developed here in relation to autoimmune diabetes are now being explored by other groups in relation to other autoimmune diseases. For example, in the case of rheumatoid arthritis, synovial T-cells of patients but not controls, proliferate to a peptide of the Dna J heat shock protein of *E coli*, that bears a sequence corresponding to the "common epitope" found in alleles of HLA-DR4 associated with susceptibility to the disease [21]. Also, and of particular interest, T-cells from patients with autoimmune uveitis, who are HLA-B27 positive, proliferate to a uveitogenic peptide of the retinal S-antigen and to a peptide of HLA-B27 that resembles it [22]. Furthermore, in a preliminary clinical study, induction of mucosal tolerance to the HLA-B27 peptide resulted in disease remission in all of a group of HLA-B27 patients with autoimmune uveitis [23]. This raises the possibility that human Type I diabetes might be prevented by tolerance induced to peptides of HLA-DR and -DQ susceptibility molecules.

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