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Induction of Glutamic Acid Decarboxylase 65-Specific Th2 Cells and Suppression of Autoimmune Diabetes at Late Stages of Disease Is Epitope Dependent

Roland Tisch, Bo Wang, and David V. Serreze

Peptide-based immunotherapy is one strategy by which to selectively suppress the T cell-mediated destruction of β cells and treat insulin-dependent diabetes mellitus (IDDM). Here, we investigated whether a panel of T cell epitopes derived from the β cell autoantigen glutamic acid decarboxylase 65 (GAD65) differ in their capacity to induce Th2 cell function in nonobese diabetic (NOD) mice and in turn prevent overt IDDM at different preclinical stages of disease development. The panel consists of GAD65-specific peptides spanning aa 217–236 (p217), 247–265 (p247), 290–309 (p290), and 524–543 (p524). Our studies revealed that all of the peptides effectively prevented insulitis and diabetes when administered to NOD mice before the onset of insulitis. In contrast, only a mixture of p217 and p290 prevented progression of insulitis and overt IDDM in NOD mice exhibiting extensive insulitis. The highly specific nature of β cell destruction suggests that Ag-specific immunotherapy may prove to be an effective approach to prevent IDDM development. Ag-specific immunotherapy is appealing because it provides a strategy to selectively inactivate, eliminate, or functionally deviate autoreactive T cells while maintaining the function of the remainder of the immune system. Parenteral immunization with autoAg-specific peptides is a further means of enhancing the specificity of T cell tolerization protocols. The strong association found between specific HLA class II haplotypes and susceptibility for chronic inflammatory autoimmune diseases such as IDDM, multiple sclerosis, and rheumatoid arthritis has generated a great deal of interest in peptide-based immunotherapies for clinical application (18). The use of MHC class II-restricted peptides to tolerize autoreactive CD4+ T cells reduces the possibility of eliciting pathogenic autoantibodies and/or cross-reactivity with other self proteins. In addition, the immunogenicity of a peptide can be readily altered, more so than intact Ag. However, the successful application of peptide-based treatment for ongoing autoimmunity is contingent on suppressing reactivity to multiple autoantigens and T cell epitopes. For example, the current criteria for entry into IDDM prevention trials identify individuals who have already developed significant levels of β cell autoimmunity and therefore are in the preclinical stages of IDDM. Deleting and/or energizing specific T cell clonotypes through administration of high doses of peptide may have only limited efficacy in these cases. Recently, studies have reported that IDDM is inhibited in NOD mice immunized with GAD65-derived peptides at an age when insulitis had not yet developed (9, 19). Furthermore, we and others have shown that immunization with intact GAD65 can prevent progression to overt IDDM in older NOD mice that had already developed significant levels of insulitis (20, 21). In both instances, protection was associated with the induction of GAD65-specific Th2 cells which are believed to suppress the activity of GAD65- and other β cell-specific Th1 cells in a general bystander.

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2 This work was supported by National Institutes of Health Grants 5P01 AI41580 and 5R01 DK52365.

3 Insulin-dependent diabetes mellitus (IDDM) is characterized by the CD4+ and CD8+ T cell-mediated destruction of the insulin-secreting β cells found in the islets of Langerhans (1–3). Studies in the nonobese diabetic (NOD) mouse, a spontaneous murine model for IDDM, suggest that at the onset of inflammation (insulitis) only few β cell autoantigens are initially targeted by T cells (4, 5). Among these is glutamic acid decarboxylase 65 (GAD65), which is believed to serve a key role in mediating islet inflammation in humans and NOD mice (6–8). As IDDM progresses, intra- and intermolecular determinant spreading occurs in which infiltrating CD4+ and possibly CD8+ T cells recognize additional epitopes within an autoantigen as well as multiple β cell-specific proteins, respectively (4, 5, 9). This process is thought to amplify β cell destruction and eventually induce overt IDDM. The CD4+ T cells mediating β cell destruction predominantly exhibit a Th1 cell phenotype, characterized by the secretion of large amounts of IFN-γ and IL-2. Conversely, CD4+Th2 cells, characterized by the secretion of IL-4, IL-5, and IL-10, are thought to have a regulatory role in IDDM (10, 11). This is consistent with several studies demonstrating that Th2 cells via IL-4 secretion suppress the development and effector function of Th1 cells in various model systems (12, 13). Indeed, administering IL-4 can prevent diabetes in NOD mice by potentiating Th2 cell function in vivo.
manner. In these studies, the relevant GAD65-specific determinant(s) that elicited Th2 cell function was not determined. Nevertheless, these results suggest that peptide-based immunotherapy may in fact be effective in preventing progression to overt IDDM at the late preclinical stages of disease through immune deviation.

A key issue regarding the applicability of peptide immunotherapy, however, is whether distinct peptide determinants differ in their capacity to elicit Th2 cell function and if so how this may impact on the inhibition of IDDM development at various preclinical stages of disease. For example, the frequency of T cell clonotypes specific for a given peptide and in turn the magnitude of the Th2 cell response may be sufficient to prevent the development of pathogenic effectors early in disease, but not in later stages when a wider array of activated Th1 cells are present. It is also possible that the intrinsic binding properties of a peptide for MHC class II may preferentially promote Th1 rather than Th2 cell development (22, 23). In the present study, our aim has been 2-fold: 1) we have set out to identify, from a selected panel, GAD65-specific peptides involved in the induction of regulatory Th2 effector cell function and subsequent suppression of IDDM in NOD mice; 2) we have explored the possibility that differences exist within this panel of GAD65-specific peptides to induce Th2 cells at various preclinical stages of IDDM development and to prevent progression to overt hyperglycemia. Our results indicate that even after significant levels of insulitis have developed, progression to overt IDDM can be inhibited via peptide immunotherapy, but protection associated with Th2 cell activation required administration of specific combinations of GAD65-derived peptides. A similar stringency was not required to prevent IDDM in NOD mice that had not yet developed significant levels of insulitis.

Materials and Methods

Mice
NOD/Lt mice were housed and bred under specific pathogen-free conditions and allowed access to NIH Diet 31A (Purina). Currently, in our colony maintained at the University of North Carolina, IDDM develops in >85% of female NOD/Lt mice by 1 year of age. NOD mice deficient in IL-4 expression were established by introducing an IL4 allele functionally disrupted by homologous recombination (24) onto the NOD background using the previously described “speed congenic” approach (25). At the 6th backcross generation (N7), mice heterozygous for the IL4mut allele and fixed to homozygosity for microsatellite markers linked to all previously identified Idd loci of NOD origin were intercrossed. The frequency of IDDM development in the resulting intercross progeny that were homozygous for the IL4mut allele was compared with heterozygous segregants and those homozygous for the wild-type IL4 allele. NOD/IL4mut homozygotes were subsequently maintained by brother-sister matings. During the course of establishing this speed congenic stock, segregants homozygous or heterozygous for the IL4mut allele were identified using a three-primer-based PCR assay. The three primers utilized were as follows: primer 77, 5'-GGCGAGACATTTATGGGTC-3'; primer 78, 5'-GGCTGTGAGGACGTGGTGGC-3'; and primer 79, 5'-TCAGGACATTTATGGGTC-3'. Primer pairs 77 and 78 amplify a 444-bp product from wild-type IL4 allele, whereas primer pairs 78 and 79 amplify a 576-bp product from the IL4mut allele.

Assessment of diabetes and insulitis
Mice were monitored weekly for the development of glycosuria with Ames Diastix. Glycosuric values of >3 for two successive measurements was considered diagnostic of diabetes onset. Insulitis was assessed by histology. Pancreases were prepared for histology by fixing in neutral buffered formalin and then embedding in paraffin. The fixed blocks were sectioned and stained with hematoxylin and eosin. A minimum of five sections each differing by 90 μm, were cut for each block, and slides viewed by light microscopy. A minimum of 30 islets was scored for each animal. The severity of insulitis was scored as either perinodal (islets surrounded by a few lymphocytes) or intrinsinalitis (lymphocytic infiltration into the interior of the islets).

Immunizations
Female NOD mice 4 wk of age received two i.p. injections of 50 μg of intact GAD65, carboxypeptidase H (CPH) or peptide emulsified in 0.05 ml IFA 14 days apart. Female NOD mice, 12 wk old, were immunized with three i.p. injections of 200 μg of peptide emulsified in 0.1 ml IFA every 7–10 days. In experiments in which a mixture of peptides was used for injection, 200 μg of each peptide was emulsified in 0.1 ml IFA.

Antigens
The cloning and preparation of murine β cell autoantigens GAD65, CPH, and heat shock protein 60 (HSP60) have been previously described (4). Briefly, the cDNAs were engineered to encode six histidine residues at the COOH terminus of each protein. Recombinant GAD65 and CPH were expressed in a baculovirus expression system and purified using an Ni²⁺-conjugated resin (Qiagen, Chatsworth, CA). HSP60 was produced in an Escherichia coli expression system and similarly purified. Each recombinant protein was further purified by preparative SDS-PAGE, electroeluted and dialyzed extensively against PBS. Peptides were synthesized by using standard fluorenylmethoxycarbonyl chemistry on a Ranin Symphony at the Peptide Synthesis facility at University of North Carolina. The purity of the peptides was verified by reversed phase HPLC and mass spectrometric analysis.

Lymphocyte proliferation assay
Lymphocyte proliferation in response to the panel of β cell autoantigens was determined as previously described (4). Briefly, a spleen cell suspension was prepared from individual mice in ice cold PBS. The spleen cell suspension was immediately centrifuged at 400 × g for 5 min at 4°C and resuspended at 5 × 10⁶ cells/ml in culture medium consisting of RPMI 1640 supplemented with 2% Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN), 5 × 10⁻⁵ M 2-ME (Sigma, St. Louis, MO), 1× nonessential amino acids, 1 mM sodium pyruvate, and 100 U/ml penicillin. Spleen cells (0.1 ml/well) were incubated in a 96-well flat-bottom microtiter plate in triplicate for 72 h in the presence of 20 μg/ml of β cell autoantigen or peptide. Lymphocyte proliferation was assessed by measuring the amount of [³H]thymidine incorporation after an 18-h pulse (1 μCi/well) and expressed as a stimulation index (mean cpm of response to Ag divided by mean cpm with medium only).

Determination of T cell cytokine secretion
CD4⁺ T cells were purified from splenocytes from nondiabetic female NOD mice by magnetic separation using anti-CD4 Ab conjugated to magnetic beads (Miltenyi Biotec, Auburn, CA). T cells were eluted by flushing the magnetic column with PBS containing 0.5% FCS as recommended by the manufacturer. The CD4⁺ T cells (10⁶) were cultured in duplicate with 2 μg/ml of each peptide (5 × 10⁶) in 1.5 ml culture medium supplemented with Nutridoma-SP and 20 μg/ml intact GAD65 or peptide. Culture supernatants were harvested after 48 h. A capture ELISA was used to measure IFN-γ, IL-4, and IL-5 in 0.1 ml of culture supernatant in triplicate. Abs were obtained from PharMingen (San Diego, CA), and the ELISA was conducted as recommended by the manufacturer. Standard curves were established to quantitate the amount of the respective cytokines in the culture supernatants.

Adaptive transfer of diabetes to neonatal NOD mice
Neonatal NOD mice 24–48 h of age received a single i.p. injection of 5 × 10⁶ spleen cells from diabetic donors (26) mixed with or without 10⁶ CD4⁺ T cells purified as above from nondiabetic NOD mice treated with peptide or unimmunized. The number of donor mice immunized at 4 wk of age and used as a source of CD4⁺ T cells were: OVA, n = 3; p217, n = 4; p290, n = 4; and p247/p524, n = 4. The number of donor mice immunized at 12 wk of age and used as a source of CD4⁺ T cells were: OVA, n = 4; and p217/p290, n = 4. Four immununized, nondiabetic female NOD mice 25 wk of age were also used as donor mice to isolate CD4⁺ T cells. Between 15 and 20 diabetic NOD mice were used as donors for diabetogenic spleen cells for each experiment. Recipient mice were monitored for diabetes up to 8 wk of age. Typically, the purity of the CD4⁺ T cells was >95%.

Results
A panel of GAD65-specific peptides can all prevent insulitis and diabetes in NOD mice when administered at 4 wk of age
Our work and that of others have demonstrated that administration of intact GAD65 to female NOD mice either before the onset of insulitis (3–4 wk old) (Fig. 1A) (4, 5) or after significant β cell
autoimmunity has developed (12 wk old) (20, 21) can block progression to overt IDDM. Furthermore, this protection is in part mediated by regulatory GAD65-specific Th2 cells. Importantly, protection is GAD65 dependent in that the candidate β cell autoantigen CPH or IFA alone fail to elicit detectable Th2 effector cell function and prevent overt IDDM (Fig. 1A, Table I) (4, 20). To elucidate the mechanistic basis for disease prevention mediated by GAD65 treatment, we set out to determine whether: 1) GAD65-specific peptide immunotherapy can, through the induction of sufficient Th2 cell activity, prevent progression to overt IDDM at the late preclinical phases of disease development; and 2) protection is dependent on the specific GAD65 peptide(s) administered. A panel of GAD65-specific peptides consisting of aa 217–236 (p217), 290–309 (p290), 247–266 (p247), and 524–543 (p524) were used for this study. Peptides p217 and p290 were selected based on our observation that GAD65-specific T cell clones established from 4-wk-old unimmunized female NOD mice recognize only these two epitopes (our manuscript in preparation). Because spontaneous GAD65-specific T cell proliferation is first detected in spleen cell cultures prepared from 4-wk-old NOD mice, the p217 and p290 determinants may in fact be the initial epitopes targeted on the molecule. p247 and p524 were first reported to elicit T cell-proliferative responses in spleen cell cultures prepared from unimmunized NOD mice (5). Subsequently, it was demonstrated that immunizing 4-wk-old NOD mice with a mixture of these two peptides prevented diabetes (19). In addition, a T cell line established from GAD65-immunized NOD mice and specific for p524 was recently shown to transfer diabetes to NOD.Prkdcscid recipients (27). The OVA-specific peptide 323–339, which is immunogenic in NOD mice, was used as a control for these experiments. Initially, we compared the efficacy of immunizations with p217 or p290 –309 (p290), 247–266 (p247), and 524–543 (p524) were used for this study. Peptides p217 and p290 were selected based on our observation that GAD65-specific T cell clones established from 4-wk-old unimmunized female NOD mice recognize only these two epitopes (our manuscript in preparation). Because spontaneous GAD65-specific T cell proliferation is first detected in spleen cell cultures prepared from 4-wk-old NOD mice, the p217 and p290 determinants may in fact be the initial epitopes targeted on the molecule. p247 and p524 were first reported to elicit T cell-proliferative responses in spleen cell cultures prepared from unimmunized NOD mice (5). Subsequently, it was demonstrated that immunizing 4-wk-old NOD mice with a mixture of these two peptides prevented diabetes (19). In addition, a T cell line established from GAD65-immunized NOD mice and specific for p524 was recently shown to transfer diabetes to NOD.Prkdcscid recipients (27). The OVA-specific peptide 323–339, which is immunogenic in NOD mice, was used as a control for these experiments. Initially, we compared the efficacy of immunizations with p217 or p290 relative to a mixture of p247/p524 to prevent diabetes in young NOD mice. The treatment protocol consisted of two i.p. injections of 50 μg of GAD65-specific peptide(s) emulsified in IFA at 4 and 6 wk of age. IFA alone has no protective effect (Fig. 1A). Fig. 1B demonstrates that 9 of 12 of OVA-treated mice developed overt diabetes. In contrast, a significant reduction in the frequency of diabetes was observed in mice receiving p217 (1 of 12, p = 0.004, χ2), p290 (2 of 12, p = 0.014), or the mixture of p247 and p524 (3 of 12, p = 0.041) (Fig. 1B). Histological analysis of pancreases from nondiabetic mice >1 yr old indicated that the frequency of intrainsulitis in the GAD65-specific peptide-treated animals was also significantly reduced relative to mice immunized with OVA or unmanipulated, nondiabetic female NOD mice 36 wk of age (Table II). Therefore, when administered to young NOD mice the different GAD65-specific peptides exhibited similar efficacy to prevent the initiation of insulitis and the subsequent development of overt diabetes.
Inhibiting progression to overt IDDM at late stages of disease development is dependent on the specific combination of GAD65 peptides administered

We next determined whether GAD65-specific peptide immunizations could suppress progression to overt IDDM in 12-wk-old female NOD mice. At 12 wk of age, female NOD mice in our colony at the University of North Carolina are typically euglycemic yet male NOD mice. At 12 wk of age, female NOD mice in our colony immunizations could suppress progression to overt IDDM in 12-wk-old female NOD mice. This observation suggests that progression of idiopathic diabetes in female NOD mice is dependent on the specific combination of GAD65 peptides administered.

GAD65-specific peptide immunization blocks progression to overt IDDM in NOD mice at a late preclinical stage of disease by inducing regulatory Th2 cells

Previous studies have demonstrated that IDDM can be prevented in NOD mice by immunization with intact GAD65 and that this protection correlates with induction of regulatory Th2 cells (20, 21). In addition, the use of IFA has been reported to skew toward Th2 subset development (28). Therefore, to determine whether the protection seen after GAD65-specific peptide immunization was indeed mediated by Th2-regulatory cells, CD4+ T cells were purified from 56- and 60-wk-old non-diabetic mice that had been immunized at 4 and 12 wk of age, respectively, and cytokine secretion was measured in response to intact GAD65 or GAD65-specific peptides. GAD65-stimulated cultures prepared from non-diabetic NOD mice treated with OVA at either 4 or 12 wk of age exhibited similar cytokine profiles in which significant amounts of IFN-γ, but not IL-4 or IL-5, were produced (Fig. 2). Diabetic NOD mice immunized with OVA responded similarly (data not shown). In addition, IFN-γ secretion, albeit reduced, was detected in cultures stimulated with p217, p290, and p247/p524 (Fig. 2. A and D). These lower levels of IFN-γ may reflect differences in the frequency of clonotypes responding to the specific peptides. As expected, the magnitude of the response to intact protein, which can be recognized by all possible GAD65-specific clonotypes, was greater than that seen for the respective peptides. Regardless, these results suggest that a dominant Th1-like phenotype was maintained in the OVA-treated control NOD mice. This in vitro cytokine profile in response to GAD65 is typical of that observed in spleen cell cultures prepared from unimmunized female NOD mice (Fig. 4.A), and animals treated with IFA alone or a nonprotective β cell autoantigen such as CPH (Table I). In contrast, cultures established from non-diabetic mice treated at 4 wk of age with the GAD65-specific peptides had a cytokine profile characteristic of a Th2-like phenotype (Fig. 2. A–C). Reduced IFN-γ and enhanced levels of IL-4 and IL-5 were detected when cultures were stimulated with the corresponding peptide(s) used for immunization. In addition, intracellular determinant spreading of Th2 cell reactivity was detected in mice immunized with either p217 and p290; i.e., enhanced levels of IL-4 and IL-5 in response to both p217 and p290 in addition to intact GAD65 were detected in an-

### Table II. Frequency of insulitis in nondiabetic NOD mice immunized at 4 weeks of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Infiltration</th>
<th>Periinsulitis</th>
<th>&lt;50%</th>
<th>&gt;50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA (n = 5)</td>
<td>0 (0%)</td>
<td>11 (8.6%)</td>
<td>28 (21.9%)</td>
<td>89 (69.5%)</td>
</tr>
<tr>
<td>p247/p524 (n = 9)</td>
<td>350 (89.7%)</td>
<td>31 (8.0%)</td>
<td>5 (1.3%)</td>
<td>4 (1.0%)</td>
</tr>
<tr>
<td>p217 (n = 11)</td>
<td>415 (87.0%)</td>
<td>43 (9.0%)</td>
<td>12 (2.5%)</td>
<td>7 (1.5%)</td>
</tr>
<tr>
<td>p290 (n = 9)</td>
<td>310 (87.6%)</td>
<td>19 (5.4%)</td>
<td>22 (6.2%)</td>
<td>3 (0.8%)</td>
</tr>
<tr>
<td>Unimmunized (n = 5)</td>
<td>1 (0.5%)</td>
<td>15 (7.3%)</td>
<td>59 (28.8%)</td>
<td>130 (63.4%)</td>
</tr>
</tbody>
</table>

a Insulitis was determined in nondiabetic mice 55 wk of age.

b p < 10−3 vs unimmunized female NOD mice, as determined by χ2 analysis.

c Pancreases were obtained from unmanipulated 36-wk-old non-diabetic female NOD mice.

### Table III. Frequency of insulitis in nondiabetic NOD mice immunized at 12 wk of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Infiltration</th>
<th>Periinsulitis</th>
<th>&lt;50%</th>
<th>&gt;50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA (n = 4)</td>
<td>3 (1.7%)</td>
<td>14 (7.7%)</td>
<td>59 (32.6%)</td>
<td>105 (58.0%)</td>
</tr>
<tr>
<td>p247/p524 (n = 2)</td>
<td>4 (5.3%)</td>
<td>10 (13.3%)</td>
<td>22 (29.3%)</td>
<td>39 (52.0%)</td>
</tr>
<tr>
<td>p217 (n = 4)</td>
<td>11 (7.2%)</td>
<td>15 (9.7%)</td>
<td>53 (34.4%)</td>
<td>75 (48.7%)</td>
</tr>
<tr>
<td>p290 (n = 2)</td>
<td>3 (3.6%)</td>
<td>5 (6.0%)</td>
<td>14 (16.9%)</td>
<td>61 (73.5%)</td>
</tr>
<tr>
<td>p217/p290 (n = 9)</td>
<td>117 (30.6%)</td>
<td>46 (12.0%)</td>
<td>124 (32.5%)</td>
<td>96 (24.9%)</td>
</tr>
<tr>
<td>Unimmunized (n = 5)</td>
<td>90 (35.2%)</td>
<td>25 (9.8%)</td>
<td>75 (29.3%)</td>
<td>66 (25.7%)</td>
</tr>
</tbody>
</table>

a Insulitis was determined in nondiabetic mice 60 wk of age.

b p < 10−3 vs OVA-treated mice as determined by χ2 analysis.

c Pancreases were obtained from unmanipulated 12-wk-old female NOD mice.
animals immunized with either p217 or p290. The cytokine profile of cultures prepared from p247/p524-treated mice, however, differed in that only marginal enhancement of IL-4 and IL-5 could be detected in response to intact GAD65, p217 or p290 (Fig. 2, B and C). Importantly, IFN-γ (3890 ± 429 pg/ml) but not IL-4 (<25 pg/ml) or IL-5 (<25 pg/ml) was detected in OVA-stimulated (10 μg/ml) spleen cell cultures prepared from nondiabetic NOD mice previously immunized with OVA. This finding suggests that the observed immune deviation and associated protection from IDDM is GAD65 peptide dependent.
Cultures established from nondiabetic mice immunized with p217/p290 at 12 wk of age also displayed a Th2-like cytokine profile relative to cultures prepared from mice receiving the OVA (Fig. 2, D–F). In the former, reduced IFN-γ secretion and elevated IL-4 and IL-5 levels were detected in response to intact GAD65, p217, and p290. In addition, intermolecular determinant spreading of the Th2 cell response was observed. Reduced IFN-γ and concomitant increases in IL-4 and IL-5 secretion in response to the two candidate β cell autoantigens HSP60 and CPH were observed in cultures established from NOD mice receiving p217/p290 relative to OVA-treated controls (Fig. 2, D–F).

To determine whether protection from IDDM was directly attributable to Th2 cell reactivity, two different approaches were taken. First, CD4+ T cells were purified from the spleens of nondiabetic mice that had been immunized with peptide and were assessed in an adoptive cotransfer experiment for ability to suppress disease in neonatal NOD mice also receiving diabetogenic spleen cells. The majority of recipient mice developed overt diabetes after receiving diabetogenic spleen cells plus CD4+ T cells purified from animals treated with OVA at 4 (6 of 6) or 12 (9 of 11) weeks of age (Table IV). In contrast, adoptive transfer of IDDM was significantly reduced in recipients receiving a mixture of cells containing CD4+ T cells prepared from mice immunized at 4 wk of age with p217 (0 of 8), p290 (0 of 7), and p247/p524 (2 of 7), or at 12 wk of age with p217/290 (2 of 11) (Table IV). Second, NOD mice homozygous for the IL4null allele were immunized with p217 or p217/p290 at 4 and 12 wk of age, respectively, and then assessed for IDDM development relative to similarly treated wild-type control NOD mice. Unmanipulated NOD.LI4null mice, which are deficient for IL-4 expression and consequently lack typical Th2 effector cell function (24), develop IDDM at a rate and frequency not significantly different from that of wild-type control NOD mice (Fig. 3A) (29). However, in contrast to wild-type control NOD mice, IDDM development was not inhibited in female NOD.LI4null mice immunized with GAD65-specific peptides at 4 (2 of 10 vs 8 of 10, p = 0.025) or 12 (2 of 12 vs 11 of 12, p = 0.011) weeks of age (Fig. 3). These results demonstrate that IL-4-secreting Th2 cells induced by GAD65-specific peptide administration have a key role in preventing and suppressing IDDM.

The efficacy of GAD65-specific peptides to block progression to overt IDDM at a late preclinical stage of disease correlates with the degree of induced hyporesponsiveness to intact GAD65

Peptide immunization may elicit Th2 cell differentiation and effector function by at least two possible mechanisms that are not mutually exclusive. Multiple immunizations with high doses of peptide emulsified in IFA may either: 1) directly induce differentiation of Th2 cells that down-regulate Th1 cell development and function; and/or 2) trigger clonal deletion and/or anergy of Th1 cells which in turn facilitates expansion of Th2 cells. To distinguish between these two alternatives, 12-wk-old female NOD.LI4null mice were immunized as above and IFN-γ secretion in response to intact GAD65 was determined within 10 days of the last peptide injection. The inability of NOD.LI4null mice to produce IL-4 allows one to exclude the possibility that reduced GAD65-specific Th1 reactivity after peptide immunization is directly attributable to Th2-mediated bystander suppression. Importantly, there is no significant difference in the levels of IFN-γ secreted in cultures established from unmanipulated 12-wk-old female NOD and NOD.LI4null mice in response to intact GAD65 or the panel of peptides (Fig. 4A). Fig. 4B demonstrates that immunization with the p217/p290 mixture elicited the greatest decrease in in vitro IFN-γ secretion in response to intact GAD65 (p < 10−5) relative to cultures established from OVA-immunized NOD.LI4null mice. Furthermore, immunization with p247/p524 had minimal effect, whereas treatment with p217 (p < 10−5) or p290 (p < 10−3) reduced GAD65-specific T cell production of IFN-γ, but not as effectively as the combination of the two peptides. A similar profile of GAD65-specific hyporesponsiveness

### Table IV. CD4+ T cells from GAD65-specific peptide-immunized mice block the adoptive transfer of diabetes

<table>
<thead>
<tr>
<th>CD4+ T Cells</th>
<th>Diabetes at 8 Weeks of Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD mice immunized at 4 wk of age</td>
<td></td>
</tr>
<tr>
<td>Unimmunizedb</td>
<td>5/5</td>
</tr>
<tr>
<td>OVA</td>
<td>6/6</td>
</tr>
<tr>
<td>p217</td>
<td>0/8 (p = 0.001)</td>
</tr>
<tr>
<td>p290</td>
<td>0/7 (p = 0.002)</td>
</tr>
<tr>
<td>p247/p524</td>
<td>2/7 (p = 0.039)</td>
</tr>
<tr>
<td>NOD mice immunized at 12 wk of age</td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>9/11</td>
</tr>
<tr>
<td>p217/p290</td>
<td>2/11 (p = 0.011)</td>
</tr>
</tbody>
</table>

* Neonatal recipient mice (24–48 h old) received diabetogenic spleen cells (5 × 106) plus CD4+ T cells (107) prepared from NOD mice immunized with the corresponding peptides at either 4 or 12 wk of age.

* CD4+ T cells were purified from unmanipulated, nondiabetic female NOD mice 25 wk of age. χ² was used for statistical analysis.
FIGURE 4. Protection correlates with GAD65-specific hyporesponsiveness after peptide immunization. A, CD4+ T cells were purified from three unmanipulated wild-type or NOD.IL4null 12-wk-old female mice, and IFN-γ secretion in response to APC plus 20 μg/ml intact GAD65 or 40 μg/ml peptide was measured in duplicate via ELISA. Groups of five 12-wk-old female NOD.IL4 null mice received three i.p. injections of 200 μg of peptide in IFA. CD4+ T cells were purified from individual mice 10 days after the final injection, stimulated with APC plus 20 μg/ml intact GAD65 in duplicate, and IFN-γ (B) and IL-5 (D) measured in culture supernatants via ELISA. C, Individual spleen cell cultures from the same mice were established, and T cell proliferation in response to 20 μg/ml of intact GAD65 in triplicate was assessed via [3H]thymidine uptake. In medium-only cultures 2999 cpm were detected. E, In a separate experiment, groups of three NOD.IL4null 12-wk-old female mice received three i.p. injections of OVA or p217/p290 as above. IFN-γ secretion in response to APC plus intact GAD65 ± 20 U/ml IL-2 was measured in individual cultures of purified CD4+ T cells from the respective treatment groups. F, groups of three female NOD.IL4null mice 12 wk of age were immunized with OVA or p217/p290 as above, and 10 wk after the final injection CD4+ T cells were purified and stimulated with APC plus GAD65, and IFN-γ secretion was determined via ELISA. The results represent the average of individual mice within a given group. The Student t test was used for statistical analysis. *p < 0.001 vs OVA-treated mice.
was observed measuring T cell proliferation (Fig. 4C). Interestingly, a concomitant increase in IL-5 secretion in response to GAD65 was also observed in cultures established from p217-, p290-, and p217/p290-immunized NOD.IL4null mice (Fig. 4D). These results suggest that under the immunization conditions used, a Th2-like population was induced in NOD.IL4null mice independent of IL-4. Similar results have been reported for IL-4-deficient mice when immunized with OVA emulsified in alum, a potent stimulator of Th2 cell activity (30). Furthermore, the IL-5-secreting T cell population found in NOD.IL4null mice exhibited minimal proliferation in response to Ag under the culture conditions used. This in vitro response profile is analogous to what we and others have observed for “typical” CD4+ Th2 cells (4, 5, 19, 20). Supplementing the cultures with 20 U/ml murine IL-2 had no significant effect on the GAD65-specific hyporesponsiveness (Fig. 4E), suggesting that clonal deletion and not anergy of Th1 effector cells may account for the reduced anti-GAD65 reactivity in peptide-immunized NOD.IL4null mice. One and two injections of p217/p290 led to progressively reduced responses, however, induction of efficient GAD65-specific hyporesponsiveness required a minimum of three immunizations. Furthermore, the observed hyporesponsiveness was transient; significant GAD65-specific Th1 cell reactivity was detected in cultures established from NOD.IL4null mice 10 wk after the third immunization with p217/ p290 (Fig. 4F). Therefore, these results indicate that the efficacy of a given peptide treatment to suppress progression to overt IDDM at the late preclinical stages of disease correlates with the degree of in vitro GAD65-specific Th1 cell hyporesponsiveness that is induced. However, expansion and long-term maintenance of Th2 effector cells requires IL-4.

Discussion

In the present study, we have determined that: 1) GAD65-specific peptide immunotherapy is effective in inducing regulatory Th2 cells that can prevent progression to overt IDDM at a late preclinical stage of disease; 2) GAD65-specific peptides differ in their efficacy to induce sufficient Th2 cell reactivity required to suppress established Th1 effector cell function; and 3) there is a correlation between the capacity of GAD65-specific peptides to induce adequate Th2 effector cell function and hyporesponsiveness of GAD65-specific Th1 cells.

Our results demonstrate that under the conditions used, peptide-specific Th2 cells that secrete IL-4 have a critical role mediating protection. First, spleen cell cultures established from animals remaining diabetes-free exhibited intra- and intermolecular determinant spreading of the Th2 cell response which is indicative of bystander suppression. Second, adoptive transfer of diabetes to neonate recipients was effectively suppressed by CD4+ T cells purified from nondiabetic NOD mice immunized with the appropriate GAD65-specific peptides. Third, NOD mice deficient in IL-4 expression and typical Th2 effector cell function develop diabetes despite peptide immunizations that protect wild-type NOD mice. Induction of Th2 effector cell function may in part be attributable to the use of IFA. This adjuvant has previously been reported to preferentially induce Th2 cell reactivity. Indeed, we have found that systemic administration of soluble GAD65-specific peptides is ineffective in suppressing progression to overt IDDM in NOD mice that have already established extensive β cell autoimmunity. On the other hand, the development of overt IDDM can be effectively blocked in such mice by immunizing with p217 and 290 in alum, an adjuvant known to elicit strong Th2 cell responses (data not shown). Therefore, the use and presumably type of adjuvant are key factors in determining the efficacy of GAD65-specific peptide immunotherapy.

The induction of regulatory Th2 effector cells, and subsequent prevention of overt IDDM is GAD65 dependent. Immunizing NOD mice with the candidate β cell autoantigen CPH or the foreign Ag OVA prepared in IFA failed to elicit detectable Th2 cell function and protection from overt IDDM (Fig. 1, Table I). The data presented here and in previous studies clearly demonstrate that the identity of the target β cell autoantigen is critical for eliciting Th2 effector cell function in vivo. This may reflect the relative importance of a given β cell autoantigen in the diabeticogenic response and/or the frequency of the specific T cell clonotypes. Currently, the precise role that GAD65 plays in IDDM remains undefined. Indirect evidence suggests that GAD65 may be a key target promoting the progression of perinsulitis to intrainsulitis (4). Experiments involving the adoptive transfer of GAD65-specific Th1 clones into NOD recipient mice support this notion (R. Tisch, unpublished data). Recently, two studies have demonstrated that the progression of IDDM can be modulated in NOD mice expressing a GAD65 transgene. In one study, the disease process was exacerbated in NOD mice in which the GAD65 transgene was ubiquitously expressed via an MHC class I promoter (31). In the second study, the frequency of IDDM was reduced in NOD mice when GAD65 transgene expression was targeted to β cells through the use of a rat insulin promoter (32). The opposing effects of GAD65 in the respective studies may result from differences in the level and/or tissue specificity of transgene expression. These observations support the notion that GAD65 has a key role in IDDM.

Interestingly, the NOD.IL4null mice used in this study and in a recently published report (29) exhibit a similar onset and frequency of IDDM relative to wild-type NOD mice (Fig. 3A). Furthermore, virtually identical β cell autoantigen-specific Th2 cell reactivity is seen in spleen cell cultures prepared from age-matched NOD.IL4null or wild-type NOD mice (Fig. 4A). The apparent lack of an obvious effect of the IL-4 gene mutation on IDDM may reflect already low levels of endogenous IL-4 that are produced in wild-type NOD mice. These basal levels may normally be insufficient to regulate IDDM, unless IL-4 secretion is induced after immunization, for example.

One of the major findings of this work is that GAD65-specific peptides differ in their capacity to induce effective Th2 effector cell function and that these differences become more apparent when attempting to suppress progression to overt IDDM at a late preclinical stage of disease. Whereas the entire panel of GAD65-specific peptides tested induced sufficient Th2 cell reactivity in young NOD mice to prevent IDDM, only administration of a mixture of p217 and p290 effectively suppressed disease development in older mice exhibiting more advanced β cell autoimmunity. A similar protective effect may also be mediated by other GAD65-specific peptides identified as T cell epitopes but not included in this study (33, 34). The binding properties of the peptides and/or frequency of the relevant T cell clonotypes may explain the disparity within the panel to induce sufficient Th2 cell function in the context of established Th1 effector cells. In support of the latter notion, we have found that the majority of GAD65-specific T cell clones established from unimmunized 4- or 12-wk-old female NOD mice recognize either the p217 or p290 epitopes. In contrast, we have yet to establish p247- or p524-specific T cell clones (R. Tisch, unpublished data). The marginal IFN-γ response to p247/p524 in cultures prepared from unimmunized NOD mice may also be indicative of a low frequency of these clonotypes. However, IFN-γ secretion in response to p217 and p290 clearly does not equal the response to intact GAD65, and other GAD65-specific T cell epitopes have been identified (p78, p206, p535) (33, 34).
Treatment of NOD mice with various GAD65 peptides at an age before the onset of insulitis, but not after β cell autoimmunity had been established, effectively prevented insulitis and diabetes. These results demonstrate that under our conditions, regulatory Th2 cells can be readily induced in the absence of significant β cell-specific Th1 cell reactivity. Once established, the Th2 phenotype appears to be maintained long term. The conditions to block progression to overt IDDM at a late preclinical stage of disease development, on the other hand, are more stringent, requiring multiple immunizations with high doses of the appropriate combination of GAD65-specific peptides. This leads to deletion of GAD65-specific Th1 cells (Fig. 4, B, C, and E), and concomitant induction of Th2 effector cell function (Fig. 4D). Indeed, various studies have reported that Th1 cells are more susceptible than Th2 cells to Ag-specific induced clonal deletion and/or anergy (35, 36). Inhibition of Th1 cell reactivity to GAD65 may be necessary, but not sufficient to suppress progression of established β cell autoimmunity to overt IDDM, because NOD.IL4null mice continued to develop IDDM (Fig. 3B), despite the loss of such Th1 cell reactivity after treatment with the combination of p217 and p290 (Fig. 4, B and C). This may be explained by the observation that the in vitro hyporesponsiveness to intact GAD65 is transient. Significant levels of IFN-γ secretion and Th1 cell proliferation in response to intact GAD65 can be detected in cultures prepared from 22-wk-old NOD.IL4null mice immunized with p217/p290 at 12 wk of age (Fig. 4F). Induction of GAD65-specific hyporesponsiveness therefore appears to facilitate differentiation of Th2 effector cell function by presumably altering the extracellular cytokine milieu. As a result, Th2 cells via secretion of IL-4 mediate long term protection by down-regulating development of pathogenic Th1 cells responding to GAD65 and other β cell autoantigens, in addition to propagating intra- and intermolecular determinant spreading of β cell-specific Th2 cell reactivity (Fig. 2, E and F). Intramolecular determinant spreading within GAD65, and among Th2 cells has previously been reported (9). Interestingly, this determinant spreading was readily detectable in mice immunized with either p217 or p290 but not with p247/p524 (Fig. 2E). The relative capacity of a peptide(s) to mediate intramolecular determinant spreading may in turn determine the general immunotherapeutic efficacy of the peptide(s). Because cellular infiltration is significantly reduced in mice immunized either before the onset or after significant levels of insulitis have developed, GAD65-specific Th2 cells may in fact down-regulate β cell-specific Th1 cells primarily in the periphery (such as in the pancreatic lymph nodes), thereby inhibiting T cell migration directly into the pancreas. A similar observation was made in a study in which NOD mice received repeated i.p. injections of IL-4 (15).

Based on this study and the work of others, there appears to be a distinct hierarchy of β cell autoantigens and corresponding peptide epitopes with the capacity to effectively induce regulatory Th2 cells and in turn prevent progression to overt IDDM in NOD mice at various preclinical stages of disease development. We argue that this is in large part due to the frequency of the corresponding T cell clonotypes found in the periphery. Therefore, the efficacy of peptide-based immunotherapy in suppressing IDDM development appears to vary depending on the preclinical stage of disease at which it is initiated, the adjuvant used, and the frequency of the relevant β cell-specific T cell clonotypes being targeted. These findings may be of importance in designing prophylactic therapies for humans included in diabetes prevention trials, because such individuals are currently selected on the basis of having already developed significant levels of β cell autoimmunity.

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References


