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Therapeutic Alteration of Insulin-Dependent Diabetes Mellitus Progression by T Cell Tolerance to Glutamic Acid Decarboxylase 65 Peptides In Vitro and In Vivo

Stephen S. Wilson, † Todd C. White,* and Dominick DeLuca²*

We have reported previously that nonobese diabetic (NOD) fetal pancreas organ cultures lose the ability to produce insulin when maintained in contact with NOD fetal thymus organ cultures (FTOC). Initial studies indicated that exposure to glutamic acid decarboxylase (GAD65) peptides in utero resulted in delay or transient protection from insulin-dependent diabetes mellitus (IDDM) in NOD mice. We also found that exposure of young adult NOD mice to the same peptides could result in acceleration of the disease. To more closely examine the effects of early and late exposure to diabetogenic Ags on T cells, we applied peptides derived from GAD65 (GAD AA 246–266, 509–528, and 524–543), to our “in vitro IDDM” (ivIDDM) model. T cells derived from NOD FTOC primed during the latter stages of organ culture, when mature T cell phenotypes are present, had the ability to proliferate to GAD peptides. ivIDDM was exacerbated under these conditions, suggesting that GAD responsiveness correlates with the ivIDDM phenotype, and parallels the acceleration of IDDM we had seen in young adult NOD mice. When GAD peptides were present during the initiation of FTOC, GAD proliferative responses were inhibited, and ivIDDM was reduced. This result suggests that tolerance to GAD peptides may reduce the production of diabetogenic T cells or their capacity to respond, as suggested by the in utero therapies studied in NOD mice. The Journal of Immunology, 2001, 167: 569–577.

Type I (insulin-dependent) diabetes mellitus (IDDM) is the clinical result of immune-mediated β islet cell destruction. Because IDDM is due, in part, to one’s genetic background (1), a cure may be affected by manipulation of the genome. However, this approach is not heritable and may cause serious harm to the patient via as yet unknown mechanisms. A potentially safer approach might be to remove (or severely dampen) the capacity of a predisposed individual’s T cells to respond to the pancreatic Ag(s) that are most directly involved in the initiation and/or chronic activation that leads to the destruction of islet cells. Several candidate islet Ags have already been identified. Given more extensive research, IDDM-related immune responses to a limited number of these Ags may be correlated with the specific MHC backgrounds found in given individuals (2, 3). This information has been used to develop a strategy for preventing the induction of IDDM by treating patients with diabetogenic Ags before their disease had fully manifested itself (4–8). However, in some initial clinical studies, IDDM onset was not altered by diabetogenic Ag treatment, and evidence suggested the possibility that such treatment may accelerate disease induction (9).

Because of their similarities to patients with human IDDM (10), nonobese diabetic (NOD) mice have been studied to determine what islet-associated Ags are recognized early in disease. In two studies, the earliest spontaneous responses in preclinical mice were directed against proteins or peptides of human glutamic acid decarboxylase (GAD65) (11, 12). The fact that mice spontaneously developed reactivity to these peptides before the development of reactivity to other islet-associated components led some researchers to hypothesize that GAD65 may play a role in disease initiation (11, 12) through the process of determinant spreading (13). More recently, additional immunogenic epitopes from GAD have been described (13); their role in disease pathogenesis remains unclear, but they appear to be responsible for a large part of the T cell response to GAD65. Neonatal mice treated with tolerizing doses of GAD65 protein were protected from spontaneous IDDM induction (11). Further, NOD mice were protected from IDDM by administration of GAD at 3 wk of age (12). Together, these studies suggest that autoimmunity reactivity to GAD65 is an important step in the progression toward clinical disease in NOD mice.

One potential problem when using the NOD model is the ability of general immune stimulation to block efficient IDDM induction. Reports have demonstrated IDDM protection by treatment of NOD mice with LPS (14) or poly(I:C) (15). Indeed, NOD mice that are not kept in largely pathogen-free environments succumb to IDDM at significantly reduced rates (10). Thus, a second interpretation of GAD65-induced IDDM modulation could be that the high dose of Ag used to stimulate the immune system prevents animals from becoming clinically diabetic in a manner independent of the specific determinant. Some researchers, for example, have found that whereas GAD peptide immunization could not prevent IDDM in NOD mice, diphtheria-tetanus toxoid-acellular pertussis alone or insulin B chain peptide could mitigate IDDM (16). Furthermore, recent work also suggests that intrathymic injection of NOD mice...
with whole pancreas tissue or insulin B chain peptides could prevent IDDM but that certain GAD peptides accelerate disease (17).

To explore the potential role of GAD65 reactivity in the inhibition or acceleration of NOD IDDM, several experiments were performed using peptides from GAD65 to affect the generation of diabetogenic T cells in vivo as well as in the more controlled NOD fetal thymus organ cultures (FTOC) in vitro IDDM (ivIDDM) system.

NOD mice exposed only in utero to GAD peptides developed IDDM at a significantly lower rate. This limited protection is postulated to be due to increased intrathymic presentation of GAD peptides, leading to a reduction in the initial number of GAD-autoreactive T cells that would reach maturity. Thus, experiments to directly measure the effects on islet responsiveness after early T cell tolerance to GAD were performed. Previously, we had shown that FTOC that has been precultured for 14 days can inhibit the production of insulin in subsequently added fetal pancreas, as well as provide T cells which migrate into the cocultured fetal pancreas (FP) causing insulitis-like lesions (18). This system allows for the evaluation of the effects of immunomodulators on the development of diabetogenic T cells without the involvement of peripheral regulatory mechanisms. The ivIDDM activity of these cultures can be attenuated by coculturing the developing FTOC while in contact with NOD fetal pancreas organ cultures from the first day of culture. Coculturing presumably induces tolerance by exposing pre-T cells to high doses of islet Ags, leading to their inactivation or deletion. To determine whether GAD65 peptides could confer the same protective effects (and mimic those of in utero exposure), FTOC were treated with increasing doses of soluble peptide from the initiation of culture. Those T cells that were cultured in the presence of GAD65 peptides were compared with those from NOD FTOC that were cultured with control peptides or in the absence of high dose peptides to determine whether GAD65 treatment could alter the ability of NOD FTOC to inhibit insulin production by the subsequently added FP. Indeed, FTOC that are normally ivIDDM diabetogenic were rendered benign by early exposure to certain GAD peptides, suggesting that this specificity is critical to efficient T cell involvement in recognition and islet cell destruction.

A similar approach was taken to evaluate the acceleration of IDDM in NOD mice treated with GAD peptides as young adults. In this case, NOD FTOC were treated with GAD peptides after the production of mature T cells, increasing their ability to react with these Ags. When ivIDDM was subsequently performed, those FTOC that had been primed to GAD peptides demonstrated an increased induction of this in vitro correlate to diabetes.

Materials and Methods

Peptides

GAD65246–266, GAD65509–528, GAD65524–543, SRLSKVAPVIKARMMEYGT, prostatic-specific antigen (PSA) 95–109, myelin basic protein (MBP) 123–137, and hen egg lysozyme (HEL) 11–25 (AMKRHGLNRYGYS) peptides were synthesized by Biosyn Biotechnology and Research Genetics using standard and proprietary Fmoc chemistry (12), and a purity of >80% was established by HPLC and mass spectrometer measurements. All peptides were dissolved in HPLC grade water before addition to FTOC in sterile organ culture DME (in vitro). The GAD peptides and the control HEL peptides were recognized by the IA<sup>+</sup> and I-E<sup>+</sup> MHC class II molecules expressed in NOD mice, whereas the PSA and MBP control peptides are not.

Mice

Breeding pairs of NOD/Lt mice were obtained as a gift from the laboratory of Dr. E. Leiter at The Jackson Laboratory (Bar Harbor, ME). Our colony was maintained in a specific pathogen-free vivarium at the University of Arizona Central Animal Facility and propagated by brother-sister mating. Mice are allowed free access to standard breeder chow (S-2335 irradiated breeder chow; Harlan Teklad, Madison WI) and autoclaved drinking water. The incidence of IDDM in NOD/Lt females in our colony at the University of Arizona is ≈80–90% by 40 wk of age. NOD/Lt mice were then bred to produce timed-pregnant females. The fetuses were removed from pregnant females at the indicated time points (plug date = day 0). We consistently found that our animals were variable with regard to their stage of development, even though they had been vaginally plugged on the same day. We therefore restaged the pups that we obtained based on their developmental characteristics (such as digit separation on the paws) as given in Ref. 19.

Fetal thymus/pancreas organ culture

The organ culture methods used have been described in detail by our laboratory and others (20). Briefly, at least 6 thymus lobes and/or equal numbers of pancreata (usually 10) dissected from 13- to 16-day gestation fetal mice were placed on the surface of Millipore (25 mm thick, 0.45 mm pore size) filters supported in 3 ml medium in 10 × 55 mm plastic petri dishes, DMEM (4.5 g/L n-glucose), supplemented with 20% FBS (HyClone Laboratories, Logan, UT) was used. The medium also contained streptomycin (100 mg/ml), penicillin (250 mg/ml), gentamicin (10 mg/ml), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (2 × 10<sup>-5</sup> M), as well as 3.4 g/L sodium bicarbonate. The cultures were grown in a fully humidified incubator in 5% CO<sub>2</sub> in air at 37°C. Cells were harvested from FTOC and fetal pancreas organ cultures using collagenase digestion, as has been reported previously (20), or by manual dissociation by teasing tissue from the Millipore strips in HBSS plus 5% FBS solution. Viability was consistently high (>90%) for both thymic and pancreatic tissue, as determined by trypan blue exclusion.

Supernatant recovery and insulin RIA

At the specified days of culture, 300 μl culture supernatant were removed from each 3-ml culture dish for insulin RIA. MicroMedic insulin RIA kits were obtained from ICN MicroMedic Systems (Horsham, PA), used to measure the quantity of insulin in culture supernatants as directed by the manufacturer’s instructions, and standardized to bovine insulin. After the amount of insulin was measured in a 300-μl sample, the amount of insulin in the 3-ml culture was determined. This value was divided by the number of FTOC to give the amount of insulin secreted per pancreas (μU insulin per pancreas). The total amount of insulin in each culture was measured without regard to the degradation of insulin in culture and represents the total accumulation of insulin during the time interval reported.

Proliferation assays

NOD FTOC were cultured for 14 days in standard FTOC, as previously reported (20), to produce phenotypically mature populations of TCR-bearing T cells (18). Except for studies of spontaneous responses, after the initial culture period of 14 days either in the presence from day 0 (‘tolerance’-inducing) or absence (in vitro ‘priming’) of synthetic peptides (see Results), FTOC were treated with a mixture of soluble GAD peptides (10 μg/ml), the control peptide (10 μg/ml), or fresh medium. After a final addition of 48 h in organ culture, cells were removed from FTOC, dissociated, counted, and plated in triplicate at 2–4 × 10<sup>5</sup> cells/well in 96-well plates. Cells were then immediately challenged in 10 μg/ml GAD65 peptides 246–266, 509–528, and 524–543 or the control peptide (10 μg/ml) for 48 h. After this stimulation period, FTOC cells were pulsed with [3<sup>H</sup>]thymidine for an additional 24 h, mechanically harvested, and counted using a Packard β scintillation counter. In addition to GAD65 peptides and the nonimmunogenic PSA peptide (10 μg/ml), identical FTOC populations were also challenged with Con A (2.5 μg/ml) as a positive control. Stimulation index (SI) was calculated as the test divided by the background control standard (as defined in the text).

Peptide treatment of pregnant and young adult mice and assessment of IDDM in vivo

Pregnant NOD/Lt mice, at 14–15 days gestation, received a single injection (i.p.) consisting of 50 μg peptide in 250 μl sterile PBS. Treatment consisted of one of the following peptides or peptide combinations: 1) GAD65246–266; 2) GAD65509–528; 3) GAD65524–543; 4) GAD65509–528 and GAD65524–543 combined (50 μg of each peptide per mouse); 5) GAD65524–543, and GAD65509–528 combined (50 μg of each peptide per mouse); 6) HEL<sub>11–25</sub>; 7) MBP<sub>123–137</sub>; 8) MBP<sub>23–137</sub> and PSA<sub>95–109</sub> combined (50 μg of each peptide per mouse). Injection groups 1–5 consisted of autoreactive GAD<sub>19</sub> binding peptides. Injection was performed on day 0 of gestation. This literature suggests that pregnancy is critical to efficient T cell involvement in recognition and islet cell destruction.
group 6 consisted of HEL11–25, an IAβ2-binding peptide known to produce proliferative responses in NOD mice. Injection groups 7 and 8 consisted of peptides unable to be bound by IAβ2.

Age-matched 3- to 4-wk-old littermate prediabetic female NOD mice were injected once IP with a mixture of GAD65509–528 and GAD65524–543 at a low (0.5 μg of each peptide per mouse, n = 5) or high (50.0 μg of each peptide per mouse, n = 5) dose, or 50 μg/mouse of an irrelevant control peptide (PSA95–109 n = 5) in PBS.

Mice were assessed for the development of IDDM by testing every 10–14 days (after 10 wk of age for the offspring of pregnant mice and after 15 wk for young adults) for glycosuria. Those mice with a urine glucose reading of >200 mg/dl (GlucOSix) were removed from the colony and tested for hyperglycemia with a digital blood glucose monitor. Those mice with a confirmed blood glucose level >250 mg/dl were considered diabetic.

Statistical analysis

Data were analyzed using StatView 4.5 from Abacus Concepts (Berkeley, CA). Experimental differences in IDDM incidence in treatment groups were assessed by Kaplan-Meier life table analysis using the log rank (Mantel-Cox) test for significance. An unpaired Student t test was used for final determination of the significance of the effects of treatments given to organ cultures. p < 0.05 was considered statistically significant for all statistical analyses.

Results

Low dose immunization with GAD65 peptides increases IDDM incidence in NOD mice

Early reports have established that GAD65 responsiveness is found in 3-wk-old NOD mice (11, 12) and that tolerance to GAD65 protein is protective (11). However, some reports suggest that GAD peptides can accelerate IDDM (17). Thus, to determine whether IDDM severity or onset in NOD mice could be increased or accelerated, GAD65 peptides were given in tolerogenic and stimulatory doses to prediabetic mice.

Age-matched 3- to 4-wk-old littermate female NOD mice were injected once i.p. with either 50 μg each of a mixture of GAD65 peptides or 50 μg of the control peptide in saline. Mice were then followed for the development of hyperglycemia (Fig. 1). Prediabetic female NOD mice treated with a 0.5-μg dose of GAD65 peptides (n = 5) developed IDDM at an accelerated rate as compared with control peptide (n = 5)-treated groups (p = 0.0466). The high dose treatment appeared to have protective effects on treated NOD mice which, in stringent analysis, was nearly statistically significant in Kaplan-Meier survival analysis (p = 0.0845).

Offspring of GAD-treated pregnant NOD mice display a significantly delayed onset of IDDM

Because the initiation of IDDM seemed so critically dependent on the responsiveness to GAD65 initially, we examined the ability of individual and mixtures of GAD peptides 246–266, 509–528, and 524–543 to alter the clinical effects of IDDM in NOD mice at the earliest possible time point.

At 14–15 days gestation, three groups of pregnant NOD mice were injected once i.p. with 50 μg GAD65246–266, GAD65509–528, or GAD65524–543 (GAD65 p17, p34, or p35 single); 50 μg of a mixture of GAD65509–528 plus 50 μg GAD65524–543 (GAD65 p34 plus p35 mix); or 50 μg of a mixture of GAD65246–266, 50 μg GAD65509–528, and 50 μg GAD65524–543 (GAD65 p17 plus p34 plus p35 mix). Controls consisted of 50 μg HEL11–25, a tight binding peptide to IAβ2 that elicits a strong immune response), 50 μg MBP123–137 (control MBP single, which does not bind to IAβ2) and a mixture of 50 μg MBP123–137 plus 50 μg PSA95–109 (control MBP plus PSA mix). After weaning, male NOD mice were culled, and female offspring mice were monitored for the development of hyperglycemia (see Materials and Methods). This protocol was chosen to examine the potential benefits of using a wider range of GAD determinants for tolerance induction and disease prevention as compared with single peptides alone.

As compared with offspring of NOD mothers treated midgestation with either a single control peptide (the IAβ2-binding HEL11–25 or the nonbinding MBP123–137) or a mixture of control peptides (PSA95–109 plus MBP123–137), offspring of mice given some GAD65 peptide treatment regimens clearly had a reduced incidence of IDDM (Fig. 2). NOD offspring treated with GAD65246–266 singly (p = 0.035, n = 14) developed IDDM at a reduced rate when compared with HEL control peptide. Offspring treated with the GAD65509–528–GAD65524–543 mixture (p = 0.003, n = 10) developed IDDM to a reduced overall level, and at a significantly reduced rate of onset, as compared with age-matched control (PSA plus MBP) mix-treated animals. However, offspring of mice treated with GAD65524–543 (p = 0.202) or GAD65524–543 singly (p = 0.388) were not significantly protected from disease when compared with animals treated with HEL. Offspring of mice treated with a mixture of GAD65246–266, GAD65509–528, and GAD65524–543 were also not significantly protected (p = 0.226, n = 9) when compared with animals from dams treated with MBP plus PSA. Of interest, however, is that the initial incidence of IDDM in the three-peptide-treated group is similar to the controls at 18 wk, but this value failed to climb until week 37, and then only slightly. Animals given the mix with two peptides or single peptides developed disease more slowly, but after 27 wk the incidence increased rapidly to a value similar to that of the three-peptide-treated animals. Clearly, the rapid rise of disease incidence in the three-peptide-treated animals is responsible for the lack of significance of disease protection in this group. However, some animals in all GAD peptide-treated groups never developed disease. The GAD65509–528–GAD65524–543 mixture group has been conducted for >45 wk with
were cultured to produce phenotypically mature populations of TCR-bearing T cells. After this initial culture period, cells were removed from FTOC and placed in 96-well plates. Cells were then challenged to proliferate in response to GAD65 peptides 246–266, 509–528, and 524–543 (10 μg/ml). In addition to GAD65 peptides, identical FTOC populations were also challenged with a nonimmunogenic peptide (PSA95–109) and Con A.

NOD FTOC-derived cells did not proliferate in response to GAD65246–266, GAD65509–528, or GAD65524–543, significantly (p > 0.3) above the level of the nonimmunogenic control peptide PSA (SI = 1.0) or FTOC cells left unchallenged (not shown). The cultures did respond to Con A stimulation (SI = 4.5 ± 0.5), and were thus considered competent to respond in a receptor-mediated manner. The response to Con A, which is lower than that of mature splenic T cells, is typical of the immature cells produced by organ cultures (21), and the response is roughly equivalent to those made by thymocytes during the first month after birth (22).

T cells in NOD FTOC can be primed to GAD65 peptides

Based on data described above, it appeared that a significantly enhanced spontaneous response to GAD65 peptides was lacking in FTOC. We wished to determine whether mature phenotype NOD FTOC indeed produced self-reactive cells in low abundance and could be enriched or activated/primed to produce GAD65-responsive T cells, given that this might normally occur in the NOD periphery.

NOD FTOC were cultured for 14 days in standard FTOC, as described previously (18) to produce phenotypically mature populations of TCR-bearing T cells. Peptide solutions of either a mixture of GAD65 peptides 246–266, 509–528, and 524–543; fresh cultures (21), and the response is roughly equivalent to those made in organ cultures. We wished to determine whether mature phenotype NOD FTOC indeed produced self-reactive cells in low abundance and could be enriched or activated/primed to produce GAD65-responsive T cells, given that this might normally occur in the NOD periphery.

As shown in Fig. 3, NOD FTOCs that were primed using the mixture of GAD65 peptides were significantly more responsive to GAD65509–528 than cultures that were present in control peptide-primed cultures. The range of cpm responses used to calculate the average SI in these experiments was 3,371–48,765 cpm.
were not primed \( (p = 0.045) \). In contrast, the response to GAD65524–543 was not significantly enhanced compared with control peptide \( (p = 0.51) \) or untreated cultures \( (p = 0.19) \). Cultures primed with PSA and then challenged remained unresponsive to PSA \( (p = 0.10) \), whereas the Con A response was positive.

**FTOC that is primed specifically to GAD65 524–543 displays greater ivIDDM activity**

To determine the functional impact of specific autoantigen stimulation on the ivIDDM model system, FTOC were primed with synthetic GAD65 peptides after the development of phenotypically mature T cells, at the initiation of coculture with FP (usually 14 days of culture). Peptide solutions of either a mixture of the three GAD65 peptides at 10 or 100 \( \mu g/ml \) or 100 \( \mu g/ml \) control PSA peptide were then added to FTOC, as was done previously to prime FTOC. This treatment increases FTOC-proliferative responses to GAD65 peptides 509–528 and 524–543 (Fig. 3). At this time, freshly procured NOD FP were added to each of the GAD5-treated and control FTOC. At 7, 14, and 21 days poststimulation/coculture, supernatants were removed from the cultures and used to determine the effect of stimulated and unstimulated FTOC on insulin production in the FP in coculture.

As shown in Table I, at the peak of ivIDDM (day 21), priming NOD FTOC to GAD65 peptides 524–543 increased the ivIDDM activity (bold type). Interestingly, this “priming” protocol did not produce an enhanced ivIDDM effect when using GAD65 246–266 or GAD65 509–528, even though the latter was clearly an antigenic peptide for the FTOC-derived cells (Fig. 2). Indeed, in one experiment, both 10 and 100 \( \mu g/ml \) of GAD65 246–266 and 100 \( \mu g/ml \) GAD65 509–528 prevented ivIDDM at 21 days of FP coculture (italics). This effect, however, was modest and not reproducible. These data suggest that there can be a parallel between the ivIDDM system and the diabetogenic response in vivo, because both can show an increase in disease if T cells are activated to diabetogenic Ags before exposure to pancreatic tissue.

**NOD FTOC can be rendered specifically unresponsive to GAD65 peptides**

In parallel with the priming experiments detailed above and in Fig. 3, we wished to determine whether priming and recall to GAD65 peptides could be specifically inhibited by treatment with GAD65 peptides during the initial development of NOD FTOC. These studies are of particular interest in light of recent reports that propose the use of peptides to induce protective tolerance to early autoantigens by treatment of prediabetic individuals (23, 24).

NOD fetal thymi (FT) were placed in standard FTOC, and some cultures were immediately treated \((\text{day } = 0)\) with peptide solutions of either a mixture of GAD65 509–528 and GAD65 524–543, the control PSA peptide at 10 \( \mu g/ml \), or standard organ culture medium. FTOC was then cultured for 14 days to produce phenotypically mature populations of TCR-bearing T cells. During the final 48 h of FTOC, as with primed cultures above (Fig. 3), FTOC were treated with a mixture of GAD65 peptides (10 \( \mu g/ml \)), control peptide (10 \( \mu g/ml \)), or fresh medium. At the end of this period, the cells were removed from FTOC and assayed for specific proliferative capacity in response to GAD65 and control peptide challenge.

A representative experiment is shown in Fig. 4. NOD FTOC that develops in the presence of GAD65 peptides is no longer reactive to peptides 524–543 and 509–528 of GAD65. In summary of these experiments, the response to GAD65 524–543 \((\text{SI } = 1.11 \pm 0.11, n = 3)\) was much less in GAD65 peptide-precultured FTOC after priming than in cultures that had not been precultured with GAD65 peptide mixtures \((\text{SI } = 2.64 \pm 0.20, n = 5)\), or as compared with FTOC challenged with control peptide \((\text{SI } = 1.1 \pm 0.04, p > 0.3, n = 3 \text{ for all peptides})\). As a positive control of responsiveness (25), Con A stimulation of identically treated cultures remained significantly higher than all peptide-challenged FTOC \((\text{SI } = 2.23 \pm 0.42, p < 0.05, n = 3)\).

**Unresponsiveness to GAD65 peptides prevents ivIDDM**

Reports from in vivo studies have indicated that selective tolerance to GAD65 protein prevents the onset of IDDM in NOD mice (11, 12). We wished to determine whether rendering FTOC unresponsive by peptide treatment during the development of mature T cells would ablate ivIDDM.

NOD FT were placed in standard FTOC and then immediately treated with peptide solutions of either a mixture of GAD65 peptides \((\text{see Materials and Methods})\) at a total of 0.1–100 \( \mu g/ml \) or 100 \( \mu g/ml \) control PSA peptide. FTOC was then cultured for 14 days in standard FTOC to produce phenotypically mature populations of TCR-bearing T cells (in the presence of GAD65 and control peptides). At this time, freshly procured NOD FP were added to each of the treated and untreated control FTOC. At 21 days of coculture, supernatants were removed from the cultures and used to determine the effect stimulated and unstimulated FTOC had on insulin production in the FP in coculture.

Fig. 5 shows two experiments in which there was a dose-dependent reduction in ivIDDM caused by early “tolerogenic” treatment of FTOC with GAD65 peptides.

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**Table I. Addition of GAD65 peptide 524–543 at the time of FP coculture induces a greater degree of ivIDDM activity from NOD FTOC**

<table>
<thead>
<tr>
<th>Type</th>
<th>Priming Peptide (µg/ml)</th>
<th>Insulin (µIU/pancreas)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>FP alone</td>
<td>INS. control (none)</td>
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</tr>
<tr>
<td>FT/FP</td>
<td>IDDM control (none)</td>
<td>192.1</td>
</tr>
<tr>
<td>FT/FP</td>
<td>509–528 (100)</td>
<td>212.1</td>
</tr>
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<td>FT/FP</td>
<td>509–528 (10)</td>
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</tr>
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<td>FT/FP</td>
<td>524–543 (100)</td>
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<td>FT/FP</td>
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<td>FT/FP</td>
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</tr>
<tr>
<td>FT/FP</td>
<td>246–266 (10)</td>
<td>ND</td>
</tr>
</tbody>
</table>

\* NOD FTOC were cultured 14 days to produce phenotypically mature populations of TCR-bearing T cells. Peptide solutions of either a mixture of the three immunodominant GAD65 peptides at 10 or 100 µg/ml or the control PSA peptide at 100 µg/ml were then added to FTOC. At this time, freshly procured NOD FP were added to each of the GAD65-treated and control FTOC. At 21 days poststimulation/coculture, supernatants were removed from the cultures and used to determine the effect of stimulated and unstimulated FTOC on insulin production in the FP in coculture. GAD_{535–543} induced greater ivIDDM as shown by a decreased production of insulin by the cultures (underlined and bold). GAD_{509–528} (at the high dose) and GAD_{246–266} (at both doses) slightly inhibited ivIDDM, as indicated by an increased production of insulin (italics).
FIGURE 4. Mature NOD FTOC treated since day 0 (□) of culture are no longer responsive to challenge when compared with untreated cultures (■). NOD FTOC was treated with 10 µg/ml of either a mixture of GAD65 peptides or a control peptide or were left untreated at the initiation of culture. NOD FTOC were cultured for 14 days and then primed in vitro with a mixture of GAD65 peptides. After an additional 48 h of culture, FTOC cells were dissociated and challenged with 10 µg/ml of each peptide, a control peptide (PSA), left unchallenged, or challenged with 2.5 µg/ml Con A (shown is a representative experiment of n = 3). The responses of cultures to GAD65:509–528 and GAD65:524–543 that are specifically reduced by pretreatment with the relevant peptide are circled.

Discussion

Experiments designed to test the efficacy of GAD or GAD peptides to prevent IDDM in NOD mice have given mixed results. Regimens that diminish GAD responsiveness in NOD mice prevented the development of IDDM (11, 12, 26). Those results suggest that the therapeutic effect mediated by the in utero treatment in the experiments presented here is due to Ag-specific immune tolerance at a very early developmental stage. However, T cell clones specific for GAD peptides 35 and 17 (the latter being very similar to Coxsackie virus (27)) did not respond to islet cells nor did they accelerate IDDM onset when transferred into NOD mice (28), thus disputing the need for specific T cell activity directed to particular GAD Ags. In addition, recent work using intrathymic injections of whole GAD65 into young NOD mice showed that IDDM could be retarded, but similar treatment with some GAD65 peptides accelerated IDDM (17). It is imperative that we determine the role and capacity of the earliest T cells specific to islet Ags such as GAD65 that are released into the periphery if they are to be targets for IDDM remediation. This concern is heightened by recent data in clinical trials in which treatment with islet cell Ags has the potential to accelerate, rather than prevent, IDDM (9). Our results using the ivIDDM model suggest that indeed tolerance to GAD peptides has immediate effects on the most primary population of T cells and the resultant immune capacity to inhibit islet function.

We have previously reported on the use of a modified in vitro FTOC system for the study of the development of diabetogenic T cells in NOD mice (18). Here, we wished to determine whether the ability of our in vitro system to decipher how the response to GAD peptides might alter IDDM in NOD mice treated in utero as well as those in vivo systems in which GAD protein was therapeutic, as reported by others. If substantial correlations exist between in vitro systems in which GAD protein was therapeutic, as those in vivo systems in which GAD65 peptides accelerated IDDM, the same peptides that delay IDDM in vivo to prevent IDDM onset when given during early thymic development in vitro suggest that these peptides may be used to mitigate disease. From our in utero data and those from other studies in which GAD65 peptides accelerated IDDM in young adult NOD mice (17), we would postulate that protection and acceleration of IDDM are both age and dose dependent. Thus, a fetal NOD mouse given a high dose of GAD65 peptide may very well be protected during the early part of its life when it is using the repertoire established during prenatal development. As an adult, the same NOD mouse that is now more immunologically advanced and that has continued to produce T cells that are no longer under specific tolerogenic pressures may be more resistant to disease prevention with the same peptide regimen. Indeed, such a mouse may become diabetic at an increased rate when given the same peptide therapy, especially if a relatively low dose of peptide were delivered. Dose-dependent tolerance induction to islet Ags has already been reported (29). In the experiments reported here, we have found that injection of single peptide preparations or a mixture of GAD509–528 and GAD524–543 into 15-day gestation pregnant NOD mice can significantly delay IDDM onset in the pups. The mixture of GAD509–528 plus GAD524–543 was considerably better in preventing IDDM than the single peptides given alone, suggesting that treatment with a broader range of epitopes of GAD could alter the course of IDDM, although the mixture of all three GAD peptides did not seem to do any better. In contrast, the same treatment given to young adult NOD mice can either have a marginal inhibitory effect or accelerate disease, depending on the dose of GAD peptides used. The
latter results confirm earlier reports suggesting that some GAD treatment regimens can, indeed, induce disease (30). This result is consistent with the clinical data mentioned above.

It is of interest that in the in utero studies the peptides apparently were effectively retained in the pups long enough to alter the production of diabetogenic T cells. Presumably, the delay in the induction and overall incidence of IDDM in mice treated in utero with GAD peptides, rather than complete inhibition of disease, is due to the continued production of new T cells by the thymus after the introduced high dose of peptides have been degraded and lost. It would seem likely that treatment with higher (or multiple) doses of peptide over the entire late gestation and early neonatal period will improve protection against disease. These experiments are currently under way.

We found that NOD FTOC could be induced to respond to a nominal Ag (GAD565524–543) at a level ~3 times greater than background proliferation levels. This is of interest because reports have demonstrated the presence of allogeneic CTL precursors in FTOC (31, 32) and the ability of FTOC to respond in MLC and IL-2 production assays (31, 33) but not the ability of these cells to be specifically reactive to nominal Ag. As compared with spleen cell preparations from adult mice, a 3-fold proliferation index, as shown in Fig. 3, is low. However, our results compare quite favorably with FTOC studies that showed a 2- to 28-fold (usually 3–5 times control) response in allogeneic MLC (33, 34). The finding that cells recovered from FTOC (that are roughly equivalent in function and gestational age to 1 wk after birth) are able to respond to nominal Ag is in agreement with a report that suggests that neonatal immune populations are responsive to antigenic stimulation (35). In fact, in our proliferation assay system, 10 μg/ml were used to challenge and restimulate NOD FTOC; a level one-half to one-tenth that of other reported proliferation assays. Using greater amounts of peptide Ag (or >2.5 μg/ml Con A) caused uniformly depressed proliferation index values (not shown).

We found that NOD FTOC could not be effectively primed to the immunogenic GAD65246–266 vs a control nonimmunogenic peptide (Fig. 3). The response to GAD509–528 was also weak. Other work showing early reactivity to GAD246–266, GAD509–528, and GAD524–543 from 3-wk-old NOD mice (11, 12) suggests that these mice have been primed to these peptides at an early age. The lack of a broad response to GAD peptides in NOD FTOC may indicate an inherent limitation in the response diversity or precursor frequency of the cultures during early fetal development. We chose the 14-day time point because the maximum number of cells in FTOC are found at roughly 14 days of culture and because longer cultures, to our present knowledge, do not produce cells with a more mature phenotype. It is of obvious importance to determine whether responses to other nominal Ags at similar levels can also be generated by FTOC cells, as well as to determine which Ag FTOC is capable of responding to, as compared with similarly aged NOD mice (1 wk old). However, this report now indicates that 14-day gestation fetal thymus lobes cultured in FTOC for an additional 14 days are capable of responding to at least one early GAD Ag (524–543) to a level of statistical significance and that doses of Ag that are normally benign to adult T cell populations are inhibitory to FTOC-derived responder populations.

The need for priming to obtain a proliferative response to GAD peptides by organ culture-derived cells requires consideration as to how the ivIDDM response is achieved. It may be that extrathyMIC events are critical in the induction of IDDM in NOD mice. Thus, in vivo, an event that causes the damage of islet cells with release of islet cell Ags may allow for the stimulation of GAD-specific T cells that have escaped deletion in the thymus due to poor activation machinery in NOD mice (36). This idea gains support from data that show that the GAD65 peptides that we used have been reported to be presented in a class II-restricted manner (24), downstream of a more primary (possibly class I-restricted) event (37). Such events may be pancreas damage due to occult pancreas infections, or infections with viruses such as coxsackie B4 which may possess epitopes that are identical with some GAD peptides; thus inducing immunoreactivity to GAD by cross-reaction (molecular mimicry) (27, 38–40). This endogenous priming may also be a result of a generalized inflammatory defect which is adjunct to viral infections (41). In either case, an early event (preceding the generation of a response to GAD65) may lead to up-regulation of processing and presentation of otherwise cryptic peptides. This process of up-regulation of presentation leading to exposure of previously cryptic peptide determinants on a protein has been extensively characterized in other autoimmune systems such as the experimental autoimmune encephalomyelitis model of multiple sclerosis (42–44). It is of some interest, however, that we were able to generate an ivIDDM response in our system in the present study and in earlier work (18) without overt islet damage in the organ cultured pancreas. The necessary priming of diabetogenic T cells in our system must occur by some process other than acute viral infection or inflammation, possibly by culture-induced release of pancreatic Ags.

We found that the priming of FTOC with GAD peptides in the manner required to induce a response to GAD524–543 resulted in an increase in ivIDDM activity, but an increased response to GAD509–528 did not increase ivIDDM. This result suggests that a peptide that is capable of eliciting a T cell response can cause diabetes in our system. Thus, peptides that have been shown to be antigenic in NOD FTOC (GAD509–528 and GAD524–543) were differentially capable of increasing ivIDDM. This result is important because it shows that peptides that are antigenic for NOD FTOC are not necessarily capable of affecting ivIDDM. These peptides are internal controls for one another in this experiment, which also suggests that the mere activation of T cells by a peptide Ag in this system is not responsible for the increase in ivIDDM activity. In the present study, we used a mixture of GAD peptides to accelerate IDDM in young adult mice, but GAD524–543 alone can accelerate IDDM in these animals (17), suggesting that the ivIDDM system can parallel results obtained in vivo.

However, our in utero data suggest that GAD246–266 was capable of preventing IDDM, even though it was not recognized by FTOC-derived T cells. This peptide could not exacerbate ivIDDM either. Therefore, FTOC is not a completely accurate indicator of the range of responsiveness of NOD mice in vivo. Perhaps, as discussed above, the response to GAD246–266 matures later in the development of the thymus than can be determined in FTOC, preventing its involvement in ivIDDM. However, the apparent persistence of peptide in the pups of mice treated in utero may allow for the induction of unresponsiveness by GAD246–266 in these experiments.

We also found that a relatively low dose (10 μg/ml in vitro) of a mixture of GAD peptides could mitigate ivIDDM if they were given to NOD FTOC at the beginning of the culture period, before the development of mature T cells. Thus, peptides that could induce ivIDDM could also prevent ivIDDM if they were given under conditions that could prevent their ability to induce a response (immune tolerance (Figs. 4 and 5)). This result would be expected if GAD peptides are responsible for some of the immune recognition of pancreas tissue, given that preculture of NOD FTOC with the whole pancreas is also capable of preventing ivIDDM against another challenge with pancreas tissue in organ culture (18).
The in utero data suggest that mixtures of GAD peptides were the same as single peptides in preventing IDDM, if the final percentage of deceased mice is measured. It is interesting, however, that the induction of disease in mice treated with different combinations of peptides was different. For the GAD$_{246-266}$ single peptide-treated mice and the mice treated with all three peptides, a relatively large number of animals became diabetic at the same time as the controls, but after ~20 wk few mice treated with the three-peptide mixture became diabetic. The GAD$_{246-266}$-treated mice and the GAD$_{509-528}$ plus GAD$_{524-543}$-treated mice eventually reached the same overall disease incidence, although the induction of disease in the latter was delayed. It is possible that a later induction of GAD$_{246-266}$ responsiveness in the developing pups may be responsible for late disease induction in adult progeny (e.g., >25 wk in Fig. 2). Animals treated with this peptide may only be partially tolerant for this response, and those treated with GAD$_{509-528}$ and GAD$_{524-543}$ would not be tolerant at all. These mice would develop disease later, and this response may account for the delayed disease induction seen in some of the treated mice. Presumably, the response to GAD$_{509-528}$ and GAD$_{524-543}$ matures earlier in the developing pups, and these responses could be more easily prevented by treatment with these peptides in utero with the protocol used in our studies, especially if the peptides were used together. Apparently, however, mixing all three peptides diluted out the ability of GAD$_{509-528}$ and GAD$_{524-543}$ to induce tolerance to the early IDDM response, because this response occurred in the groups of mice treated with all the peptides. These data are an indication of the complexity of peptide treatment to prevent IDDM, even with a genetically identical population of animals. These results suggest that caution should be exercised in the use of these treatments in a clinical setting.

Other researchers, using in vivo systems, have shown that protection from IDDM by injection of insulin (16, 45, 46) or intranasal administration of GAD (24) involves “immune diversion” from a Th1 to a Th2 response. A similar mechanism has been proposed for the protection of NOD mice by intrathymic injection of islet cells, whole insulin B chain, and whole GAD65 (17). Although our data using the ivIDDM model do not preclude Th1 to Th2 diversion as the mechanism of protection by pretreatment of the FTOC with GAD peptides, the decrease in the ability of the cells from treated FTOC to respond to these peptides in proliferation assays is also consistent with clonal deletion or efficient regulation/anergy of GAD-responsive cells. Regardless, the loss of these cells through deletion caused by therapeutic high peptide concentrations during T cell development may prove an effective method for prevention of IDDM, should the critical initiating Ags be found.

Overall, our data suggest that GAD is an important target Ag in IDDM and that it may be a trigger or required component of the T cell response cascade that results in IDDM. Work with other islet-associated Ags such as insulin (45, 46) or insulin peptides (16), and heat shock protein 60 peptides (47) to prevent IDDM are also promising. Recent clinical trials (9), however, support work shown here and elsewhere (17, 48) that these Ags administered to adult NOD later in development, when peripheral regulatory responses have matured, can exacerbate disease. The present data showing the efficacy of inducing tolerance to GAD peptides during fetal development in FTOC or in utero, suggest that IDDM may be prevented with the appropriate immunotherapy given early in T cell development. Our results also suggest that the ivIDDM model may be useful in the rapid screening of candidate Ags in various combinations for peptide immunotherapy to prevent IDDM. Promising combinations of peptides could then be tested in the longer term in vivo models to establish the best time to treat with these Ags before use in clinical trials.

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References
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