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A Self MHC Class II β-Chain Peptide Prevents Diabetes in Nonobese Diabetic Mice

Pratibha Chaturvedi, Babita Agrawal, Marc Zechel, Edwin Lee-Chan, and Bhagirath Singh

We explored T cell responses to the self class II MHC (I-A\textsuperscript{g7}) β-chain-derived peptides in diabetic and prediabetic nonobese diabetic (NOD) mice. We found that one of these immunodominant epitopes of the β-chain of I-A\textsuperscript{g7} molecule, peptide 54–76, could regulate autoimmunity leading to diabetes in NOD mice. T cells from prediabetic young NOD mice do not respond to the peptide 54–76, but T cells from diabetic NOD mice proliferated in response to this peptide. T cells from older nondiabetic mice or mice protected from diabetes do not respond to this peptide, suggesting a role for peptide 54–76-specific T cells in pathogenesis of diabetes. We show that this peptide is naturally processed and presented by the NOD APCs to self T cells. However, the peptide-specific T cells generated after immunization of young mice regulate autoimmunity in NOD mice by blocking the diabetogenic cells in adoptive transfer experiments. The NOD mice immunized with this peptide are protected from both spontaneous and cyclophosphamide-induced insulin-dependent diabetes mellitus. Immunization of young NOD mice with this peptide elicited T cell proliferation and production of Th2-type cytokines. In addition, immunization with this peptide induced peptide-specific Abs of IgG1 isotype that recognized native I-A\textsuperscript{g7} molecule on the cell surface and inhibited the T cell proliferative responses. These results suggest that I-Aβ\textsuperscript{g7}(54–76) peptide-reactive T cells are involved in the pathogenesis of diabetes. However, immunization with this peptide at young age induces regulatory cells and the peptide-specific Abs that can modulate autoimmunity in NOD mice and prevent spontaneous and induced diabetes. The Journal of Immunology, 2000, 164: 6610 – 6620.

Insulin-dependent diabetes mellitus (IDDM)\textsuperscript{3} is a T cell-mediated autoimmune disease characterized by the destruction of insulin-producing β cells in the islets of Langerhans (1, 2). Islet β cell-specific T cells play an important role in the pathogenesis of IDDM (3). MHC class II molecules are critical self molecules playing central role in the induction and regulation of an immune response (4). They are also present in the thymus at the time of negative and positive selection, required for the acquisition of T cell repertoire during development. The class II MHC molecules have been implicated in the pathogenesis of diabetes mellitus and other autoimmune diseases (5). Susceptibility to diabetes is strongly associated with the expression of class II β-chain that lacks the usual acidic aspartate residue at position 57 (6). The expression of transgenic class II β-chain with aspartate at position 57, transgenic I-A\textsuperscript{4}, and I-E has been shown to protect mice from developing diabetes (7–9). Self tolerance in the immune system is essential for the self/nonself discrimination and maintenance of integrity of self. The general mechanisms proposed for achieving self tolerance are clonal deletion (10–12) or clonal anergy (13–16) of self-reactive T cell clones. These mechanisms may operate at various stages of T cell development. The self Ags involved in tolerance induction are combinations of self peptides/MHC molecules and self proteins that require the processing and presentation in the context of self MHC molecules. Direct evidence for the existence of self peptides/self MHC molecule complexes comes from functional studies (17, 18) as well as from studies in which naturally processed peptides were acid eluted from affinity-purified class II molecules (19). Although self tolerance is necessary to prevent autoimmunity, overwhelming T cell deletion or clonal anergy has to be avoided to provide a functionally diverse T cell repertoire.

Using a number of peptides from the α-helical and β-pleated region of the Ag-binding groove of the MHC class II (I-A) molecules, we and others have examined the T cell responses to self I-A molecules (20, 21). It has also been found that exogenously added peptides of self Ags are processed into forms that are recognized by self T cells (22). MHC class II (I-A\textsuperscript{g7})-derived synthetic peptides from NOD mice bind to syngeneic and allogeneic MHC class II molecules (23). Based on these studies, we used a number of peptides from the third hypervariable region of I-A\textsuperscript{g7} β-chain and tested the response of NOD mice toward these self MHC peptides. We found that one of the peptides corresponding to the region 54–76 did not stimulate proliferation of T cells from young NOD mice. However, this peptide induced proliferative response in unprimed old diabetic NOD mice, suggesting a breakdown of tolerance to this self MHC peptide with age and disease status and role of peptide-specific cells in pathogenesis of diabetes. The peptide I-Δβ\textsuperscript{7}(54–76) represents an immunodominant region on I-A\textsuperscript{g7} molecule. This region has also been implicated in contacting the TCR in the recognition of MHC-peptide complex (24). We found that immunization of NOD mice at a young age with this peptide was associated with production of IgG1 Ab and Th2 responses and protected mice from diabetes. However, spontaneous breakdown of tolerance to this peptide in older mice leads to a Th1 dominant response that may contribute to the development of diabetes in NOD mice. Therefore, immunization with this self MHC peptide at young age alters the natural history of the disease,

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3 Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; Cy, cyclophosphamide; NOD, nonobese diabetic mice; NOR, nonobese diabetes-resistant mice; PPD, purified protein derivative.

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Table I. Synthetic peptides used in this study

<table>
<thead>
<tr>
<th>Synthetic Peptide</th>
<th>Sequence of Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-A(^{b})((1–14))</td>
<td>GDSEHRFVQFPGGE</td>
</tr>
<tr>
<td>I-A(^{b})((48–60))</td>
<td>RAVTELGPHSAEY</td>
</tr>
<tr>
<td>I-A(^{b})((54–76))</td>
<td>GHRSAEYYNKLTERAELDTA</td>
</tr>
<tr>
<td>I-A(^{b})((82–95))</td>
<td>EETEVPTSLRLEQ</td>
</tr>
<tr>
<td>Proinsulin((24–36))</td>
<td>FFYTPKSRRED</td>
</tr>
<tr>
<td>OVA((323–339))</td>
<td>IQAVHAHAEINEAGR</td>
</tr>
</tbody>
</table>

modulates the autoimmune responses, and prevents the development of IDDM.

Materials and Methods

Mice

Female NOD/Lt and NOR mice were bred in the animal facility at the John P. Robarts Research Institute and the University of Western Ontario (London, Ontario, Canada). Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antigens

Peptide Ags used in this study were synthesized in this laboratory, as previously described (20), using the Merrifield solid-phase peptide synthesis technique on a ABI 431A peptide synthesizer (Applied Biosystems, Mississauga, Ontario, Canada). The crude peptides were purified by reverse-phase HPLC on a semipreparative synchropak RP-P C18 (250 \(\times\) 10 mm ID) column using a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile (1% of the second solvent/min). Peptide purity and composition were confirmed by amino acid analysis. For functional assays, peptides were dissolved in saline by adjusting the pH with 0.1 M NaOH and were sterilized by filtration through a 0.22-\(\mu\)m filter.

The sequences of the peptides used in this study are presented in Table I. Purified protein derivative of tuberculin (PPD) was obtained from Statens Serum Institute (Copenhagen, Denmark) and Con A from Sigma (St. Louis, MO).

Immunization and T cell proliferation assay

For T cell proliferation, 3- to 4-wk-old female NOD mice were immunized s.c. with 50 \(\mu\)g peptide in 25 \(\mu\)l saline, emulsified with 25 \(\mu\)l CFA (Sigma) in each hind footpad. After 10 days, draining popliteal lymph nodes were harvested and T cells were purified using nylon wool. T cells (\(\times\) \(10^6\)) were cultured for 4 days with 1-A\(^{b}\)\((54–76)\) peptide (50 \(\mu\)g/ml) in presence of irradiated spleen cells as APCs (1 \(\times\) \(10^6\)). Cells were washed and incubated in medium alone at 37°C for 7 days. This process was repeated twice. Cells were collected and dead cells were removed using lympholyte M (Cedarlane, Hornby, Ontario, Canada). T cells (\(\times\) \(10^6\)) were cultured with the peptide (50 \(\mu\)g/ml) in presence of irradiated APCs. Two days later, cells were diluted in 100 ml medium and expanded in presence of 15 U/ml IL-2 (Becton Dickinson). After 10 days, cells were used for the experiments or restimulated to maintain the cell line. For control, a GAD 67-specific T cell line was used.

Cytokine assay

Female NOD mice (3–4 wk old) were immunized with I-A\(^{b}\)\((54–76)\) (50 \(\mu\)g/footpad) emulsified in CFA. After 10 days, popliteal lymph nodes were harvested and cells (\(\times\) \(10^6\)) were cultured in presence of I-A\(^{b}\)\((54–76)\) peptide (50 \(\mu\)g/ml) or PPD (40 \(\mu\)g/ml) in 24-well plates (Becton Dickinson). Culture supernatants were collected after 24 h and assayed for the presence of IL-2, IL-4, and IFN-\(\gamma\) using cytokine-specific ELISA. Briefly, ELISA plates (Becton Dickinson) were coated with 1 \(\mu\)g/ml anti-cytokine Ab (PharMingen Canada, Mississauga, Ontario) overnight at 4°C. Plates were washed and blocked with 5% BSA for 2 h at room temperature. Supernatants (100 \(\mu\)l) from different groups were added to the plates and incubated overnight at 4°C. Plates were washed and incubated further with 1 \(\mu\)g/ml biotinylated anti-cytokine Ab (PharMingen Canada) for 2 h at room temperature. After washing, streptavidin-alkaline phosphatase (1:1000) was added to the wells and incubated for an additional 30-min incubation. Plates were washed and developed using p-nitrophenyl phosphate substrate (Sigma). Plates were read at 405 nm using a Bio-Rad (Richmond, CA) ELISA plate reader. Standard curves were obtained using recombinant cytokines.

Immunization of mice for Ab production

Female NOD mice (3–4 wk old) were immunized s.c. with 25 \(\mu\)l (50 \(\mu\)g) of I-A\(^{b}\)\((54–76)\) peptide emulsified with equal volume of IFA in one hind footpad. Our previous studies have shown that CFA immunization protects NOD mice from diabetes (25). Therefore, IFA was used for immunization. Two weeks later, mice were immunized with 50 \(\mu\)g of the same peptide in IFA i.p. Serum was collected 2 wk after the second injection and tested for the presence of peptide-specific Abs. Sera from mice immunized with saline or OVA(323–339) peptide emulsified with IFA and sera from diabetic NOD mice were used as controls.

Detection of peptide-specific Abs

Peptide-specific Abs were detected using ELISA assays. Briefly, the I-A\(^{b}\)\((54–76)\) peptide (1 \(\mu\)g/ml) was immobilized in 96-well flat-bottom ProBind plates (Becton Dickinson) by overnight incubation at 4°C, followed by washing with PBS containing 0.1% BSA, 0.05% Tween 20 (PBS-B). Plates were washed with PBS followed by addition of 100 \(\mu\)l of serum to each well. Plates were washed and developed using p-nitrophenyl phosphate substrate (Sigma). Anti-isotype-specific Abs (IgG1) were used in an ELISA to determine the isotype of the Abs generated. Plates were read at 405 nm.

Flow-cytometric analysis

Spleen cells (\(\times\) \(10^6\)) from NOD female mice (3–4 wk old) were incubated with sera from I-A\(^{b}\)\((54–76)\) peptide-immunized mice, washed, and stained with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). mAb 10.2.16, which cross-reacts with I-A\(^{b}\), was used as positive control. Sera from CFA saline- or IFA saline-immunized, or diabetic NOD mice were used as controls for these studies.

Induction of diabetes in NOD mice by cyclophosphamide treatment

Female NOD mice, 4–6 wk of age, were injected with a single dose of cyclophosphamide (Cy) (Sigma) i.p. at 200 mg/kg of body weight. Mice were then randomly divided into four groups. One group was injected with the I-A\(^{b}\)\((54–76)\) peptide (100 \(\mu\)g/mouse) emulsified in IFA after 3 days of injection. For control, one group was injected with saline or OVA(323–339) peptide emulsified in IFA. Mice were monitored three times per week for glycosuria and regarded as overtly diabetic based on two consecutive positive (>11.5 mmol) glycosuria tests.

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Adoptive transfer of spleen cells for disease modulation

Female NOD mice (3–4 wk old) were immunized i.p. with 100 μl (100 μg) of I-Aβ7(54–76) peptide emulsified with equal volume of IFA. For control, mice were immunized with saline emulsified with IFA. After 10 days, spleens were harvested and a single cell suspension was prepared. Spleen cell suspension was also prepared from spontaneously diabetic NOD mice. Splenocytes (10 × 106) from I-Aβ7(54–76) peptide or saline-immunized mice were mixed with splenocytes (10 × 106) from diabetic mice (1:1) and injected at 20 × 106 cells per mouse into 4- to 6-wk-old NOD-SCID mice. Control mice were injected with splenocytes (10 × 106) from diabetic NOD mice alone. Recipient mice were then monitored three times per week for glycosuria and regarded as overtly diabetic based on two consecutive positive (>11.5 mmol) glycosuria tests.

Histology

Mice were sacrificed when they developed diabetes or at the end of the study. Pancreata were removed, fixed in 10% Formalin, and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin. The severity of lymphocytic infiltration in islets was determined by light microscopy. Seven to eleven islets were examined for each section and scored as follows: 0 = no infiltration; 1 < 25% infiltration; 2 = 25–50% infiltration; 3 = 50–75% infiltration; 4 > 75% infiltration.

Statistical analysis

Results were analyzed using Mann-Whitney Rank Sum test or ANOVA on Ranks test, in which p value of 0.05 or less was considered significant.

Results

Response of NOD T cells to self I-Aβ7-derived peptides

We have reported earlier that a number of peptides from self MHC class II molecules induce proliferation of T cells (20). It has also been shown that I-Aβ7 peptides 1–16 and 52–77 bind to self-I-Aα6 molecules (23). Therefore, experiments were conducted to examine the proliferative response of T cells from NOD mice to various self-I-Aβ7 peptides. Splenic T cells from unprimed young NOD female (3–4 wk) mice were cultured with various I-Aβ7(54–76) peptides and proliferation was assayed. The results show that the I-Aβ7(54–76) peptides corresponding to regions 1–14, 48–60, 54–76, and 82–95 did not induce proliferation of T cells from young unprimed NOD mice (Fig. 1A). T cells from unprimed diabetic NOD female (>6-mo-old) mice did not proliferate in response to syngeneic I-Aβ7 peptides 1–14, 48–60, and 82–95, but there was a considerable response to peptide 54–76 (Fig. 1B). These results suggest that the young mice are tolerant to self-I-Aβ7 peptides, but the tolerance to the peptide corresponding to region 54–76 is broken at an older age.

Furthermore, to find out whether the immunization with these peptides will induce a response in young NOD mice, 3- to 4-wk-old female NOD mice were immunized with various peptides, and proliferation was assayed. Immunization with the peptide I-Aβ7(1–14) induced a weak proliferative response, whereas I-Aβ7(48–60) and 82–95 were nonimmunogenic. However, immunization of young NOD mice with peptide 54–76 induced a strong proliferative response, suggesting that tolerance to this peptide can be broken at an early age by immunization (Fig. 1C).

Response of T cells from nondiabetic NOD mice to I-Aβ7(54–76) peptide

Additional experiments were done to determine the role of peptide 54–76-induced T cells in the pathogenesis of IDDM. Proliferative response of T cells from NOD mice protected from diabetes by CFA and insulin B (9–23) peptide treatment to I-Aβ7(54–76) peptide was measured. The results presented in Fig. 2A show that T cells from NOD mice protected from diabetes did not proliferate in response to peptide 54–76. Response of T cells from older nondiabetic male and female NOD and female NOR mice to peptide 54–76 was also measured. The data presented in Fig. 2B show that T cells from these nondiabetic mice did not proliferate in response to peptide 54–76 either. The absence of T cell response to peptide 54–76 in nondiabetic older mice and mice protected from diabetes suggests that T cells responding to self-I-Aβ7(54–76) peptide contribute to the pathogenesis of diabetes. Lack of response in NOR mice further confirmed that the T cells from nondiabetes-prone mice with same MHC as NOD do not respond to this peptide.
Immunization with I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide induces Th2-like response

To determine the subset of Th cells generated in response to the I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide, young NOD mice were immunized with the peptide. After 10 days, draining lymph nodes were collected and single cell suspension was prepared. Cells were then cultured in the presence of peptide, and supernatants were tested for the presence of IL-2, IL-4, and IFN-γ. The cytokine profile of spleen cells from unimmunized diabetic NOD female mice was also tested, as described in Materials and Methods. Results are presented as mean units of cytokine in triplicate cultures ± SD.

Ab response to self I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide

To investigate whether immunization with I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide induces an Ab response, mice were immunized with peptide emulsified in IFA for Ab production, and peptide-specific Abs were detected using ELISA. Results presented in Fig. 4 show that immunization with the peptide 54–76 induced a strong peptide-specific Ab response. Furthermore, isotype of the Abs generated in response to the peptide was determined. It was also observed that immunization with the I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide induced IgG1 isotype of Abs (Fig. 4). The serum obtained from mice immunized with IFA-saline, IFA-OVA(323–339), and diabetic mice showed no reactivity to I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide in both the assays. This suggested that Abs generated were specific for the I-A<sup>b</sup>g<sup>7</sup> (54–76) peptide.
Ab response of NOD mice to I-\(\beta\)\(^{7}(54–76)\) peptide. NOD mice were immunized with the I-\(\beta\)\(^{7}(54–76)\) peptide (50 \(\mu\)g) emulsified in 50 \(\mu\)l IFA in one hind footpad. After 2 wk, a second injection of the peptide (50 \(\mu\)g) emulsified in IFA (50 \(\mu\)l) was given i.p. Serum was collected after 2 wk and tested for \(\alpha\)-specific Ab using ELISA. As control, sera from IFA-saline-immunized, IFA-OVA(323–339) peptide-immunized, and diabetic NOD mice were used.

Recognition of I-A molecules by anti-I-\(\beta\)\(^{7}(54–76)\) peptide Ab on NOD spleen cells

Additional experiments were done to investigate whether anti-I-\(\beta\)\(^{7}(54–76)\) peptide-specific Abs would recognize the native I-A molecules on the cell surface. It was found that the peptide-specific Abs bound to 37.9% of the NOD spleen cells, while Ab 10.2.16 stained 44.5% of the spleen cells. In contrast, serum from IFA-saline-immunized mice stained only 9.7% of the cells, and serum from diabetic NOD mice stained 12.1% cells (Fig. 5). The peptide-specific Ab did not stain the spleen cells obtained from BALB/c mice (data not shown). These results show that anti-I-\(\beta\)\(^{7}(54–76)\) peptide-specific Ab can recognize the native I-A molecules on the cell surface.

In vitro inhibition of Ag presentation to T cells by anti-I-\(\beta\)\(^{7}(54–76)\) peptide-specific Abs

Additional experiments were done to find out whether anti-I-\(\beta\)\(^{7}(54–76)\) Ab can inhibit the proliferative response of T cells to recall Ags. NOD mice were immunized with 50 \(\mu\)l of Mycobacterium tuberculosis preparation of CFA emulsified with saline in both the hind footpads. After 10 days, popliteal lymph nodes were collected and single cell suspension was prepared. Cells were cultured with recall Ag PPD with or without anti-sera collected from I-\(\beta\)\(^{7}(54–76)\) peptide-immunized or IFA-saline-immunized NOD mice. Ab 10.2.16 was used as positive control in these experiments. The results presented in Fig. 6 show that anti-I-\(\beta\)\(^{7}(54–76)\) Ab inhibited the response of T cells to PPD Ag, while serum from IFA-saline-immunized mice did not inhibit the response. This suggests that anti-I-\(\beta\)\(^{7}(54–76)\) Ab binds to native I-A molecules and inhibits the presentation of Ag to T cells in dose-dependent fashion.

Prevention of Cy-induced diabetes by I-\(\beta\)\(^{7}(54–76)\) peptide

Because immunization with this peptide shifted the T cell response toward Th2 type, additional experiments were done to determine its effect on the development of diabetes in NOD mice. Cy at a dose of 200 mg/kg of body weight precipitates overt diabetes in NOD mice, which is similar to spontaneous diabetes. As shown in Fig. 7, a single dose of 200 mg/kg Cy caused a rapid onset of diabetes in young NOD females, and 80% mice became diabetic after 25 days of Cy treatment. The incidence of diabetes in mice treated with Cy, followed by an injection of OVA peptide (323–339) or IFA-saline emulsion, was similar, indicating that IFA or the control OVA peptide did not influence the onset of disease. On the other hand, none of the mice treated with Cy followed by an injection of the I-\(\beta\)\(^{7}(54–76)\) peptide developed diabetes (Fig. 7). Histology of the pancreas showed a significant decrease in the islet infiltration after treatment with the peptide compared with saline treatment (Table II). The infiltration seen in some islets in peptide-treated group is similar to that observed in NOD mice protected from diabetes by other treatments such as CFA (25) and probably represents immunoregulatory Th2-like cells.

Prevention of spontaneous diabetes by immunization with I-\(\beta\)\(^{7}(54–76)\) peptide

Young NOD female (3–4 wk) mice were given two injections (2 wk apart) of the peptide emulsified in IFA and observed for the development of overt diabetes. For control, mice were injected with saline or a control proinsulin B (24–36) peptide emulsified in IFA. The proinsulin B (24–36) peptide induces T cell responses and has been implicated in the pathogenesis of IDDM (26). This peptide bears marked similarity to GAD 65 (506–518) peptide (26). The data presented in Fig. 8 show that 80% of mice in the IFA-saline group developed diabetes by 12–14 wk after injection, while the incidence reached 100% by 16–18 wk after injection in mice treated with control proinsulin (24–36) peptide. The disease incidence in the I-\(\beta\)\(^{7}(54–76)\) peptide-treated group was 30% by 20 wk and 40% by 40 wk after injection. The histology of pancreas from saline-treated mice showed massive infiltration of islets, but the pancreas from I-\(\beta\)\(^{7}(54–76)\) peptide-treated mice showed only periinsulitis (Fig. 9). Histology of the pancreas showed a significant decrease in the islet infiltration after treatment with the peptide compared with saline or proinsulin (24–36) treatment (Table III). This suggests that islet-infiltrating cells are regulatory cells generated in response to I-\(\beta\)\(^{7}(54–76)\) peptide, as observed in NOD mice protected with other treatments such as CFA immunization (25). Furthermore, our data presented in Fig. 5 showed that I-\(\beta\)\(^{7}(54–76)\) peptide-specific Abs were detectable in these mice as late as 9 mo after peptide immunization. These Abs bind to the MHC class II molecules (Fig. 5) and inhibit the presentation of autoantigens (Fig. 6), resulting in the down-regulation of immune responses leading to protection from disease.

Adaptive transfer of diabetes

An adoptive transfer protocol was used to determine whether cells generated in response to I-\(\beta\)\(^{7}(54–76)\) peptide immunization are able to inhibit the transfer of diabetes by diabetogenic splenocytes. It was observed that cotransfer of splenocytes from I-\(\beta\)\(^{7}(54–76)\)
peptide-immunized mice with diabetogenic splenocytes significantly delayed the onset of diabetes as compared with splenocytes from saline-injected mice. After 12 wk, all the mice in control group (diabetogenic spleen cells plus splenocytes from saline-injected mice) became diabetic, whereas only 40% of mice cotransferred with peptide-specific cells plus diabetogenic splenocytes tested positive for diabetes after 12 wk of cell transfer (Fig. 10). These results suggest that cells generated in response to

**FIGURE 5.** Recognition of native I-A<sup>g7</sup> molecules by I-A<sub>b</sub><sup>g7</sup>(54–76) peptide-specific Abs. Spleen cells from normal NOD mice were stained for flow-cytometric analysis with sera from I-A<sub>b</sub><sup>g7</sup>(54–76) peptide immunized mice collected at various time points. As control, sera from IFA-saline-immunized, CFA-saline-immunized, and diabetic NOD mice were used. As positive control, 10.2.16 mAb was used. Cells were analyzed by flow cytometry, as described in Materials and Methods.

**FIGURE 6.** Inhibition of in vitro proliferative response by I-A<sub>b</sub><sup>g7</sup>(54–76) peptide-specific Ab. Mice were immunized with CFA-saline emulsion and lymph nodes were harvested after 10 days. Cells were cultured with PPD (40 μg/ml), as recall Ag present in CFA, in the presence or absence of serum from IFA-I-A<sub>b</sub><sup>g7</sup>(54–76) peptide-immunized mice, IFA-saline-immunized mice, or 10.2.16 Ab (supernatant from 10.2.16 B cell hybridoma). Proliferation was assayed by measuring [<sup>3</sup>H]thymidine uptake, as described in Materials and Methods. Results represent mean cpm of triplicate cultures ± SD.
I-Aβ\(\gamma\)\(^{(54–76)}\) peptide down-regulate the pathogenic cells and thus inhibit the induction of diabetes by the diabetogenic cells.

**Peptide I-Aβ\(\gamma\)\(^{(54–76)}\) is naturally processed and presented in vivo**

To determine whether this peptide fragment is naturally processed and presented in vivo, an I-Aβ\(\gamma\)\(^{(54–76)}\) peptide-specific T cell line was generated and used for proliferation assays. T cells from young female NOD (4 wk old) and diabetic NOD mice were also used. T cells (5 × 10\(^5\)) were cultured with irradiated splenocytes (1 × 10\(^8\)) from 12-wk-old NOD female mice, and proliferation was assayed. The data presented in Fig. 11 show that T cells from diabetic NOD mice and I-Aβ\(\gamma\)\(^{(54–76)}\) peptide-specific T cell line proliferated when cultured with splenocytes in absence of exogenously added peptide. The peptide-specific T cell line also proliferated in response to splenocytes from NOR mice. T cells from diabetic NOD mice also proliferated when cultured with splenocytes from NOR, NOD-SCID, and male NOD mice (data not shown). However, peptide 54–76-specific T cells did not proliferate when cultured with BALB/c splenocytes, suggesting that the response is specific. T cells from young (4-wk-old) NOD mice did not proliferate when cultured with splenocytes in absence of peptide. A GAD 67-specific T cell line did not proliferate when cultured with splenocytes from NOD mice in absence of GAD Ag, suggesting that peptide 54–76-specific T cell line responds to peptide 54–76 presented by APCs specifically. These results suggest that the I-Aβ\(\gamma\)\(^{(54–76)}\) peptide is naturally processed and presented by APCs in NOD mice. These APCs may activate peptide 54–76-specific T cells that contribute to the pathogenesis of diabetes.

**Discussion**

The process of thymic selection during T cell ontogeny ensures the deletion/inactivation of potentially autoreactive T lymphocytes (10, 11, 27). Therefore, it is generally expected that all potential self determinants or self peptides that bind to MHC molecules with high affinity would be nonimmunogenic due to clonal deletion or inactivation of potentially autoreactive T lymphocytes. However, exogenously added and internalized self proteins give rise to immune response to such peptides (22). We and others have reported (20, 21) the presence of self MHC peptide-reactive T cells in syngeneic mice. Our results further suggested that syngeneic T cells could be primed by self MHC peptides presented by APC in vivo in naive mice. In this study, we have found that a number of peptides from the self I-Aβ\(\gamma\)\(^{(54–76)}\) did not induce T cell proliferation in young NOD mice. However, a considerable response to a self I-Aβ\(\gamma\)\(^{(54–76)}\) peptide corresponding to region
54–76 was found in T lymphocytes isolated from old mice (>6 mo old). The response to this peptide was absent in T cells from old nondiabetic (male NOD, female NOD, and NOR mice) and NOD mice protected from disease using CFA and insulin B (9–23) peptide treatment. These results suggest a possible role for peptide 54–76-specific T cells in the pathogenesis of type I diabetes.

To determine the relevance of I-A\textsuperscript{b}g(54–76) peptide in vivo, we investigated whether this peptide is naturally processed and presented by NOD APCs. T cells from 4-wk-old NOD mice did not proliferate when cultured with splenocytes from 12-wk-old NOD mice, but proliferation was observed with T cells from diabetic mice and I-A\textsuperscript{b}g(54–76) peptide-specific T cell line. The I-A\textsuperscript{b}g(54–76) peptide-specific T cell line also proliferated when cultured with splenocytes from NOR mice, but not in response to splenocytes from BALB/c mice. GAD 67-specific T cell line did not proliferate when cultured with splenocytes alone, suggesting

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Table III. Immunization with I-A\textsuperscript{b}g(54–76) peptide prevents insulitis

<table>
<thead>
<tr>
<th>Groups&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Severity of Insulitis&lt;sup&gt;b&lt;/sup&gt; (% of islets infiltrated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>IFA-saline treatment</td>
<td>17.7</td>
</tr>
<tr>
<td>IFA-proinsulin(24–36) peptide treatment</td>
<td>15.9</td>
</tr>
<tr>
<td>IFA-I-A\textsuperscript{b}g(54–76) peptide treatment</td>
<td>71.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> NOD mice were treated with self I-A\textsuperscript{b}g(54–76) peptide (100 \( \mu \)g) emulsified in IFA i.p. as described in Materials and Methods. For control, one group of mice was injected with saline emulsified in IFA and another group was injected with proinsulin peptide emulsified in IFA. Mice were then observed for the development of diabetes. Mice were sacrificed when they became diabetic (control groups) or 10 mo after the injection (peptide-treated group). Pancreata were removed, fixed in 10% formalin, and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin. The severity of lymphocytic infiltration in islets was determined by light microscopy.

<sup>b</sup>The severity of insulitis was defined as: 0 = no infiltration, 1 = <25% infiltration, 2 = 25–50% infiltration, 3 = 50–75% infiltration, and 4 = >75% infiltration.
that response is specific for peptide 54–76-specific cell line. T cells from diabetic mice and peptide-specific T cell line also proliferated in response to spleenocytes from older nondiabetic female NOD, NOD-SCID, and male NOD mice (data not shown). Possibly, presentation of this peptide by APCs primes the small population of T cells in young NOD mice. During the course of disease, release of cross-reactive autoantigen may cause the expansion of these peptide-specific T cells, resulting in the observed proliferation.

The relevance of a T cell response to self I-A\(\beta\)(54–76) peptide in autoimmune condition may be indirect and could be related to other genetic factors because autoimmune diabetes is a multigenic disease (33). A direct link between class II MHC molecules and autoantigens is also possible. It has been reported that NOD mice have Abs directed against a 58-kDa islet Ag identified as peripherin (34). Possibly, during the course of the disease, peripherin is released from damaged islets and already existing small population of self cross-reactive autoantigens is also possible. It has been reported that NOD mice have Abs directed against a 58-kDa islet Ag identified as peripherin, which cross-reacts with MHC class II gene products (34). Possibly, during the course of the disease, peripherin is released from damaged islets and already existing small population of self I-A\(\beta\)(54–76) peptide-reactive T cells becomes activated due to cross-reactivity and contributes to the pathogenesis of diabetes. We also found that T cells from NOD mice protected from diabetes and the nondiabetic (male NOD, female NOD, and NOR) mice did not proliferate in response to I-A\(\beta\)(54–76) peptide. APCs from male NOD and NOR mice induced proliferation of peptide-specific T cell line and T cells from diabetic mice, suggesting that this peptide is naturally processed and presented in these mice as well. Because \(\beta\) cells in these mice are not destroyed, cross-reactive autoantigens are not released and peptide-specific T cell population does not expand. These results again confirm the involvement of peptide-specific cells in the pathogenesis.

There have been reports indicating that self-reactive T cells are causally involved in the pathogenesis of type I diabetes (35, 36). However, all the T cells infiltrating the islets are not destructive (37). A number of transgenic mice in which \(\beta\) cells express certain cytokines constitutively show extensive lymphocytic infiltration without overt diabetes (38–40). These studies suggest the possibility that a proportion of T cells present within an islet downregulates the anti-self immune response, as opposed to causing its destruction. This balance in the immune response may be maintained by the ratio of Th1 to Th2 cells. Th1 cells producing IFN-\(\gamma\) and IL-2 induce the cytotoxic response, and Th2 cells producing IL-4, IL-6, and IL-10 appear to promote humoral responses (41, 42). There is evidence for the importance of this balance in IDDM (1, 2, 37). Immunization of young NOD mice with the I-A\(\beta\)(54–76) peptide induced a Th2-like response with secretion of little or no IL-2 and IFN-\(\gamma\) and large amounts of IL-4. Furthermore, peptide-specific Abs were found to be of IgG1 isotype, suggesting the generation of Th2-like response. The peptide-specific Abs recognized native I-A molecules on the cell surface and inhibited the activation of T cells. However, the spontaneous T cell response to this peptide in diabetic mice is of Th1 type. This difference in the outcome of response to the same Ag depending on the age of mice and disease status could be due to the Ag concentration and increased number of peptide 54–76-specific cells. During the course of disease, a number of autoantigens are released after \(\beta\) cell destruction, and peptide-specific cells may expand due to the cross-reactivity. These T cells may recognize more than one cross-reactive Ag presented by APCs shifting the response toward protective Th2. The peptide-specific Abs may inhibit the activation of Th cell response and may be induced due to the absence of cross-reactive autoantigens as most of the autoantigens are released after \(\beta\) cell destruction. This may also result in the decreased ligand density on the surface of APCs shifting the response toward protective Th2. Indeed, it has been reported that there is a difference in optimal ligand density on APC for Th1 and Th2 activation. High ligand
density on APCs activates Th1 cells, while Th2 cells are activated by low ligand density on the APCs (43, 44).

Thus, immunization of young NOD mice with the I-\textalpha\textbeta\textg7 (54–76) peptide may prevent onset of diabetes by inducing protective Th2-like response, and peptide-speciﬁc Abs that bind to I-\textalpha\textg7 and inhibit presentation of autoantigens to T cells. We used this peptide to prevent onset of diabetes in NOD mice using spontaneous as well as Cy-accelerated diabetes model. In the spontaneous model, only 40% of mice became diabetic after immunization with the I-\textalpha\textbeta\textg7 (54–76) peptide. The peptide-speciﬁc Ab response declined with age, although was still detectable as late as 9 mo after injection. When mice became diabetic, the Ab titer was much reduced. The peptide-speciﬁc Abs bind to I-\textalpha\textg7 molecules and inhibit the presentation of autoantigens to T cells, resulting in down-regulation of pathogenic response. Possibly, to completely protect mice from diabetes, a high Ab titer is needed. Indeed, injection of anti-

class II (anti-I-\textalpha\textbeta\textg7) Ab has been shown to block IDDM in NOD mice (45). The presence of I-\textalpha\textbeta\textg7 (54–76) peptide-speciﬁc Ab does not seem to compromise the health of mice, as evident from our spontaneous disease protection studies. Mice were healthy up to the age of 10 mo, when they became diabetic.

A single injection of I-\textalpha\textbeta\textg7 (54–76) peptide 3 days after Cy treatment also protected mice from Cy-accelerated diabetes. Cy treatment increases Th1 cells, IFN-\gamma production, and NO production in NOD mice (46). We postulate that regulatory cells (Th2) are induced when these mice are immunized with the I-\textalpha\textbeta\textg7 peptide and block the generation of Th1 cells. The perilsinitis observed in the pancreas of mice protected with I-\textalpha\textg7 peptide in both Cy-accelerated and spontaneous models of diabetes may be due to the accumulation of these regulatory cells. Similar perilsinitis is observed in a number of systems using synthetic peptides.

The generation of an immune response to self MHC peptides has been observed in a number of systems using synthetic peptides from MHC class I and II molecules (20, 21, 47). The incomplete tolerance to self components may not be normally pathogenic. However, genetic and environmental factors or age-dependent changes may lead to an altered autoreactive T cell repertoire. Our results show that age of mice and disease status can lead to generation of responses to a self MHC class II peptide that is pathogenic. We suggest that down-regulation of anti-self responses using self I-\textalpha\textg7 peptides at a younger age may be one way of modulating the immune system and preventing autoimmune diseases.

References


