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Identification of CD4\(^+\) T Cell-Specific Epitopes of Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein: A Novel \(\beta\) Cell Autoantigen in Type 1 Diabetes\(^1\)

Rinee Mukherjee,* Danielle Wagar,* Tracey A. Stephens,* Edwin Lee-Chan,* and Bhagirath Singh\(^2\,\*\)

Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) has been identified as a novel CD8\(^+\) T cell-specific autoantigen in NOD mice. This study was undertaken to identify MHC class II-specific CD4\(^+\) T cell epitopes of IGRP. Peptides named P1, P2, P3, P4, P5, P6, and P7 were synthesized by aligning the IGRP protein amino acid sequence with peptide-binding motifs of the NOD MHC class II (I-A\(^{\beta7}\)) molecule. Peptides P1, P2, P3, and P7 were immunogenic and induced both spontaneous and primed responses. IGRP peptides P1-, P2-, P3-, and P7-induced responses were inhibited by the addition of anti-MHC class II (I-A\(^{\beta7}\)) Ab, confirming that the response is indeed I-A\(^{\beta7}\) restricted. Experiments using purified CD4\(^+\) and CD8\(^+\) T cells from IGRP peptide-primed mice also showed a predominant CD4\(^+\) T cell response with no significant activation of CD8\(^+\) T cells. T cells from P1-, P3-, and P7-primed mice secreted both IFN-\(\gamma\) and IL-10 cytokines, whereas P2-primed cells secreted only IFN-\(\gamma\). Peptides P3 and P7 prevented the development of spontaneous diabetes and delayed adoptive transfer of diabetes. Peptides P1 and P2 delayed the onset of diabetes in both these models. In summary, we have identified two I-A\(^{\beta7}\)-restricted CD4\(^+\) T cell epitopes of IGRP that can modulate and prevent the development of diabetes in NOD mice. These results provide the first evidence on the role of IGRP-specific, MHC class II-restricted CD4\(^+\) T cells in disease protection and may help in the development of novel therapies for type 1 diabetes. * The Journal of Immunology, 2005, 174: 5306–5315.

Type 1 diabetes (T1D)\(^3\) has been well established as a T cell-mediated autoimmune disease that specifically destroys the insulin-producing \(\beta\) cells in the islets of Langherans within the pancreas. Islet cell-specific autoantigens are picked up and processed by APCs, either dendritic cells or macrophages, which migrate to the draining lymph nodes. In the microenvironment of the lymph nodes, APCs can present autoantigens to autoreactive T cells, which then escape negative selection in the thymus and have evaded peripheral tolerance. These processes arm autoreactive T cells, which then undergo clonal expansion and migrate to the pancreas, where they are able to specifically attack islet \(\beta\) cells (1). Infiltration of T cells and other lymphocytes leads to an inflammatory response and specific destruction of islet \(\beta\) cells (2). Both CD4\(^+\) and CD8\(^+\) T cell subsets have been implicated in the pathogenesis of T1D (3). These T cell subtypes have different cytokine profiles and cellular functions. It has been well established that CD4\(^+\) T cells are sufficient to transfer diabetes from NOD mice to NOD/SCID recipients who do not spontaneously develop disease due to the absence of endogenous lymphocytes. It has also been shown that an injection of anti-CD4 Abs protects NOD mice from disease, and CD4\(^+\) T cell-deficient NOD mice do not develop diabetes (4). Genetic susceptibility to diabetes in both humans and NOD mice is correlated with certain MHC class II alleles, which are responsible for the presentation of peptides to CD4\(^+\) T cells, specifically, DQ8 and DQ2 MHC class II genes in humans and the homologous I-A\(^{\beta7}\) molecule in NOD mice (5).

To understand how to control or prevent the development of T1D, it is important to determine which autoantigen is responsible for the activation of the autoreactive T cell population. Several candidate autoantigens have been identified, including insulin, glutamic acid decarboxylase, IA-2, and heat shock protein 60 (4, 6, 7).

Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) has been investigated initially as a possible islet-specific glucose-6-phosphatase that had long been postulated as a key component of a glucose substrate cycle and control of energy metabolism in the \(\beta\) cells of the pancreas (8). Recently, IGRP has been identified as an autoantigen recognized by a prevalent population of pathogenic CD8\(^+\) T cells in NOD mice (9, 10).

IGRP is expressed in the pancreas of both humans and NOD mice. IGRP expression is restricted to the islet \(\beta\) cells and, to a lesser extent, the islet \(\alpha\) cells, which would allow it to act as a specific molecular target in these cells. IGRP has 50% sequence homology to the catalytic subunit of liver islet-specific glucose-6-phosphatase and is predicted to be an endoplasmic reticulum membrane protein (8). IGRP is thought to have a role in islet \(\beta\) cell metabolism; however, its exact function has not yet been defined. In addition, the human IGRP gene maps to the diabetes susceptibility locus on chromosome 2, IDDM7 (11). Although IGRP is abundant in NOD mouse and human islets, it is not expressed in the pancreas of BB rats (6).

Although the CD8\(^+\) T cell responses to IGRP peptides have been characterized, the CD4\(^+\) T cell response has yet to be examined. Therefore, because MHC class II I-A\(^{\beta7}\)-restricted CD4\(^+\) T
cells play a significant role in disease pathogenesis and prevention, it is important to identify CD4+ T cell peptide-binding epitope(s) within IGRP. In this study, we identified and synthesized peptides from murine IGRP based on MHC class II I-Aβ-binding motifs. These peptides were tested for their effect on T cell responses, and their effect on disease progression has been investigated in NOD mice.

Materials and Methods

Mice

NOD/LtJ and NOD/SCID mice were bred in the animal facility at the John P. Robarts Research Institute and the University of Western Ontario. BALB/c mice were purchased from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions and used in accordance with institutional animal guidelines. The animal studies were reviewed and approved by University Council on Animal Care, University of Western Ontario.

Peptide selection and synthesis

Known NOD MHC class II (I-Aβ) peptide-binding motifs (12–15) were used to identify potential I-Aβ-binding sites within the murine IGRP amino acid sequence using the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). Seven peptides designated as P1, P2, P3, P4, P5, P6, and P7 were identified. Peptide P3 is a five-amino acid N-terminal extension of peptide P1. The sequences of these peptides are shown in Table I. Throughout this study, the insulin B chain (B:9-23) peptide, with alanine substitution at position 19 was used as a positive control because it has been determined previously to be immunogenic in NOD mice (16). The class II-associated invariant chain peptide (CLIP) (81-102) peptide was used as a negative control because it has been determined previously to be nonimmunogenic in NOD mice (17). The class II-I-Aβ (24-36) peptide, and the CLIP (81-102) peptide or saline (vehicle) emulsified in IFA (Sigma-Aldrich) in the hind footpads. After 10 days, draining popliteal lymph nodes were removed, and a single-cell suspension was prepared. The cells were then cultured in 96-well flat-bottom plates at 2 x 10^5 cells/well in the presence or absence of the respective IGRP peptide or control peptides at a concentration of 100, 50, 10, 1, or 0.1 μg/ml at 37°C for 72 h. To subside CD4+ and CD8+ T cell populations from popliteal lymph node cells, we used FACS sorting. Purified T cells (1 x 10^6) were cultured with relevant IGRP peptides and irradiated spleen cells (5 x 10^5) as APCs. For Ab-blocking studies, IGRP peptides P1-, P2-, P3-, and P7-primed T cells were cultured with the respective peptides (100 μg/ml) in the presence or absence of anti-MHC class II I-Aβ Ab or isotype-matched control Ab at a concentration of 1 μg/ml. After 3 days, cultures were pulsed with 1 μCi/well [3H]thymidine (PerkinElmer) for 16–20 h. Incorporation of [3H]thymidine was measured using a liquid scintillation counter (LKB Instruments).

Primed T cell responses

Four-week-old female NOD mice were immunized with 100 μg of various IGRP peptides, the control insulin B:9-23 peptide, and the CLIP (81-102) peptide or saline (vehicle) emulsified in IFA (Sigma-Aldrich) in the hind footpads. After 10 days, draining popliteal lymph nodes were removed, and a single-cell suspension was prepared. The cells were then cultured in 96-well flat-bottom plates at 2 x 10^5 cells/well in the presence or absence of the respective IGRP peptide or control peptides at a concentration of 100, 50, 10, 1, or 0.1 μg/ml at 37°C for 72 h. To subside CD4+ and CD8+ T cell populations from popliteal lymph node cells, we used FACS sorting. Purified T cells (1 x 10^6) were cultured with relevant IGRP peptides and irradiated spleen cells (5 x 10^5) as APCs. For Ab-blocking studies, IGRP peptides P1-, P2-, P3-, and P7-primed T cells were cultured with the respective peptides (100 μg/ml) in the presence or absence of anti-MHC class II I-Aβ Ab or isotype-matched control Ab at a concentration of 1 μg/ml. After 3 days, cultures were pulsed with 1 μCi/well [3H]thymidine (PerkinElmer) for 16–20 h. Incorporation of [3H]thymidine was measured using a liquid scintillation counter

Cytokine assay

Cell culture supernatants from the T cell proliferation assays were collected after 72 h and assayed for the presence of IL-2, IL-4, IL-10, and IFN-γ using cytokine-specific ELISA kits (BD OptEIA ELISA sets; BD Pharmingen). TGF-β content was measured using TGF-β Duo Set ELISA Development kit (R&D Systems). Plates were washed with PBS-Tween (PBS, 0.5% Tween (Sigma-Aldrich)) and read at 450 nm using a Bio-Rad ELISA plate reader. Standard curves were obtained using recombinant cytokines supplied with the ELISA sets.

Modulation of diabetes by IGRP peptides

Adoptive transfer.

Draining popliteal lymph nodes were isolated from NOD mice immunized with 100 μg of IGRP peptide, the insulin B:9-23 peptide, or saline (vehicle) in IFA, and a single-cell suspension was prepared. The cells were then cultured at 2 x 10^5 cells/well in the presence of the respective IGRP peptide or the insulin B:9-23 peptide for 72 h at 37°C. After 72 h, peptide-primed cells (10 x 10^6) were mixed with splenocytes (10 x 10^6) from diabetic NOD mice and injected i.v. into NOD/SCID recipients. Each group consisted of 10 mice. Mice were monitored for the development of diabetes by measuring urine glucose with Diastix strips (Bayer) twice a week. Mice were also monitored for glycosuria and regarded as overtly diabetic based on two consecutive positive (>11.5 mmol) glycosuria tests. The study was terminated 56 days postinjection.

Spontaneous model.

Four-week-old female NOD mice were immunized i.p. with an IGRP peptide (100 μg), the control peptide (24-36) peptide, and saline (vehicle) emulsified in IFA. Proinsulin group consisted of 10 mice, whereas the other groups consisted of 15 mice. After 4 wk, mice were given a booster of the same peptide i.p. Mice were monitored for the development of diabetes by measuring urine glucose with Diastix strips twice a week. Mice were also monitored for glycosuria and regarded as overtly diabetic based on two consecutive positive (>11.5 mmol) glycosuria tests. The study was terminated when the mice reached 30 wk of age.

Statistical analysis

Pooled data from proliferation experiments were computed as mean ± SEM, and significant differences between groups were calculated using the Student’s t test. Diabetes-specific and diabetes-free survival curves were analyzed using the product limit method of Kaplan and Meier, and the diabetes onset data were compared using the log-rank test to assess differences in disease onset among the various treatment groups. All among the various treatment groups. All pairwise comparisons were two-sided; a value of p < 0.05 was considered statistically significant. Kaplan and Meier and the log-rank test were performed using GraphPad Prism version 4.00 for Windows (GraphPad).

Table I. Amino acid sequences of peptides used in this study

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
<th>Location</th>
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<tr>
<td>Proinsulin 1</td>
<td>TAALSYTISMEESVTL</td>
<td>128-145</td>
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<td>IGRP peptide P1</td>
<td>HLRSGVLHILQDEYRT</td>
<td>4-22</td>
</tr>
<tr>
<td>IGRP peptide P2</td>
<td>WYVMTAALSYSMEESVTL</td>
<td>123-145</td>
</tr>
<tr>
<td>IGRP peptide P3</td>
<td>PAGLTLFVHGLFLRFASM</td>
<td>256-274</td>
</tr>
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<td>IGRP peptide P4</td>
<td>MLFLRSCQGENGTKPSFRL</td>
<td>277-294</td>
</tr>
<tr>
<td>IGRP peptide P5</td>
<td>QFTTTCTGPGPSGAMGSS</td>
<td>100-120</td>
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<tr>
<td>IGRP peptide P6</td>
<td>HTVPVGMASLVLKTVFNL</td>
<td>195-214</td>
</tr>
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<td>Insulin B chain Aβ</td>
<td>SHLVEALVLAGERG</td>
<td>B:9-23</td>
</tr>
<tr>
<td>CLIP</td>
<td>EKFVQSMRATFLLRPM</td>
<td>85-101</td>
</tr>
<tr>
<td>Proinsulin 1</td>
<td>24FTYFKSRREVE</td>
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</table>
Results

Selection and generation of IGRP peptides

To identify MHC class II-specific CD4+ T cell epitopes, the mouse IGRP protein amino acid sequence was aligned with defined peptide-binding motifs of the NOD MHC class II (I-Ag7) molecule. Based on the resultant matches, seven peptides named P1, P2, P3, P4, P5, P6, and P7 were chosen as primary candidates for investigating the role of IGRP in MHC class II-restricted CD4+ T cell activation. The P3 peptide is a five-amino acid N-terminal extension of peptide P1, whereas the P7 peptide incorporates the MHC class I-binding motif from amino acid 206-214 (9). The sequences of these peptides and their location within murine IGRP are shown in Table I.

IGRP peptides are immunogenic in NOD mice

To characterize the responses induced by IGRP peptides, 4-wk-old female NOD mice were injected with 100 μg of IGRP peptides P1, P2, P3, P4, P5, P6, or P7, the insulin B:9-23 peptide, or saline emulsified in IFA. Lymph nodes were harvested 10 days later, and the cells were then cultured at 2 × 10^6 cells/well in the presence or absence of the respective IGRP peptide at a concentration of 100, 50, 10, 1, or 0.1 μg/ml for 72 h at 37°C. Cultures were pulsed with [³H]thymidine (1 μCi/well) and incubated for another 16 h. Proliferation was assayed by measuring the [³H]thymidine incorporation as described in Materials and Methods. Results are expressed as mean counts per minute from three independent experiments ± SD.

**FIGURE 1.** In vitro proliferation of IGRP peptide-primed NOD T cells. Four-week-old female NOD mice were injected with 100 μg of IGRP peptides P1, P2, P3, P4, P5, P6, or P7, the insulin B:9-23 peptide, or saline emulsified in IFA. Lymph nodes were harvested 10 days later, and the cells were then cultured at 2 × 10^6 cells/well in the presence or absence of the respective IGRP peptide at a concentration of 100, 50, 10, 1, or 0.1 μg/ml for 72 h at 37°C. Cultures were pulsed with [³H]thymidine (1 μCi/well) and incubated for another 16 h. Proliferation was assayed by measuring the [³H]thymidine incorporation as described in Materials and Methods. Results are expressed as mean counts per minute from three independent experiments ± SD.
**FIGURE 2.** MHC class II (I-A^d^) Ab inhibits IGRP peptide-primed proliferation of NOD T cells. IGRP peptides P1, P2, P3, and P7, the insulin B:9-23, or the CLIP (81-102) peptide-primed lymph node cells (2 x 10^5 cells/well) from 4-wk-old NOD mice were cultured with the respective peptides (100 μg/ml) in the presence or absence of purified anti-MHC class II I-A^d^ mAb (10-2.16) and isotype control Ab at a concentration of 100, 10, 1, 0.1, or 0.01 μg/ml for 72 h at 37°C. Cultures were pulsed with [3H]thymidine (1 μCi/well) and incubated for another 16 h. Proliferation was assayed by measuring the [3H]thymidine incorporation as described in Materials and Methods. Results are expressed as mean counts per minute from duplicate experiments ± SD.
The results presented in Fig. 4 show that cells primed with IGRP peptides P1, P3, and P7 secreted low levels of IL-2 and high levels of IFN-γ after they were cultured in the presence of the respective IGRP peptide at a concentration of 100 μg/ml for 72 h at 37°C. For Ab responses (B, D, F, H, and J), CD4+ T cells were cultured with the respective peptides (100 μg/ml) in the presence or absence of purified anti-MHC class II I-Ak Ab or isotype-matched control Ab at a concentration of 1 μg/ml for 72 h at 37°C. Irradiated spleen cells were used as APCs (5 × 10⁵/well). Cultures were pulsed with [³H]thymidine (1 μCi/well) and incubated for another 16 h. Proliferation was assayed by measuring the [³H]thymidine incorporation as described in Materials and Methods. Results are expressed as mean counts per minute from three independent experiments ± SD.

**Spontaneous proliferation of NOD T cells in response to IGRP peptides**

To determine the relevance of the selected IGRP peptides in the T cell-mediated pathogenesis of T1D, spontaneous T cell responses were assayed. Splenocytes from young NOD (4 wk) and diabetic NOD mice were isolated, and single-cell suspensions were prepared. The cells were then cultured in the presence of IGRP or insulin B:9-23 peptides at a concentration of 100, 50, 10, 1, or 0.1 μg/ml, and the proliferation was assayed as described in Materials and Methods. The results presented in Fig. 5 show that similar to primed response only peptides P1 (p = 0.00004, young; p = 0.00003, diabetic), P2 (p = 0.03, young; p = 0.0001, diabetic), P3 (p = 0.0005, young; p = 0.00003, diabetic), and P7 (p = 0.0026, young; 0.0015, diabetic) induced a significant dose-dependent proliferation of T cells from both young and diabetic NOD mice. Response to the peptides was stronger in diabetic mice, suggesting that T cells specific for these peptides may be involved in the pathogenesis of diabetes. Although the IGRP peptides induce spontaneous proliferation in NOD mice, these peptides did not
induce proliferation of cells from BALB/c mice (data not shown). These results suggest that the spontaneous response to IGRP peptides is related to autoimmunity in NOD mice and may lead to the activation of effector T cells contributing to the pathogenesis of T1D.

**Effect of anti-MHC class II Ab on the spontaneous proliferation of T cells**

Additional experiments were performed to confirm that the spontaneous CD4^{+} T cell response to IGRP peptides was MHC class II restricted. Spleen cells from young (4-wk-old) and diabetic NOD mice were cultured with various IGRP peptides in the presence or absence of anti-MHC class II Ab and isotype control Ab. Proliferation was measured as described in Materials and Methods. The results presented in Fig. 6 show that addition of the anti-MHC class II Ab significantly inhibited the response of T cells to the peptides from both young and diabetic NOD mice compared with the isotype-matched control Ab. Addition of anti-I-A^{b7} Ab (1 μg/ml) to the culture inhibited the proliferation of T cells from 4-wk-old NOD mice to IGRP peptide P1 by 53% (p = 0.0002) and T cells from diabetic NOD by 69% (p = 0.0001), as shown in Fig. 6, A and B, as compared with isotype-matched control Ab. Proliferation induced by IGRP peptide P2 in 4-wk-old NOD (Fig. 6C) was inhibited by anti-I-A^{b7} Ab by 38% (p = 0.0004) and diabetic
NOD (Fig. 6D) by 73% (p = 0.0001) as compared with isotype-matched control Ab. Similarly, anti-I-A<sup>g7</sup> Ab significantly inhibited proliferation induced by P3 by 72% (p = 0.00004) in T cells from 4-wk-old NOD (Fig. 6E) and 78% (p = 0.00008) in T cells from diabetic NOD (Fig. 6F) as compared with isotype-matched control Ab. P7 peptide-induced proliferation was inhibited by anti-I-A<sup>g7</sup> Ab by 64% in young NOD (p = 0.00007) and by 78% in diabetic NOD (p = 0.00004) as compared with isotype-matched control Ab for the respective peptides (Fig. 6G and H). These results suggest that spontaneous T cell response to the IGRP peptides P1, P2, P3, and P7 both in young and diabetic NOD mice is predominantly CD4<sup>+</sup> T cell mediated and MHC class II restricted.

**FIGURE 6.** Anti-MHC class II Ab inhibits the spontaneous proliferation of T cells. Spleen cells (4 × 10<sup>5</sup>) from 4-wk-old (A, C, E, and G) and diabetic (B, D, F, and H) NOD mice were cultured with the respective peptides (100 µg/ml) in the presence or absence of purified anti-MHC class II I-A<sup>g7</sup> Ab or isotype-matched control Ab at a concentration of 1 µg/ml for 72 h at 37°C. Irradiated spleen cells were used as APCs (5 × 10<sup>5</sup>). Cultures were pulsed with [<sup>3</sup>H]thymidine (1 µCi/well) and incubated for another 16 h. Proliferation was assayed by measuring the [<sup>3</sup>H]thymidine incorporation as described in Materials and Methods. Results are expressed as mean counts per minute from three independent experiments ± SD.

**IGRP peptide-specific cells delay the adoptive transfer of diabetes**

To determine whether IGRP peptides P1-, P2-, P3-, and P7-activated CD4<sup>+</sup> T cells have the ability to modulate disease progression in NOD mice, adoptive transfer experiments were done as described in Materials and Methods. Cells cultured with peptides (10 × 10<sup>6</sup>) were mixed with the diabetic splenocytes (10 × 10<sup>6</sup>) and injected into NOD/SCID recipients via i.v. route. The data presented in Fig. 7 indicates a delay in diabetes development in mice injected with IGRP peptides P1-, P2-, P3-, and P7-primed cells. Particularly, the group of mice that received diabetogenic cells mixed with IGRP peptides P2- and P3-primed cells because these mice did not begin to develop diabetes until 11 days after the control group, which was injected with diabetic splenocytes alone. However, the groups of mice injected with P1- and P2-primed cells had a diabetes incidence approximately equal to the control group by the termination of the study (60 and 100%, respectively, p = 0.127 to −0.658). Partial protection was observed when cells from P3 and P7 peptide-primed mice were injected with diabetogenic cells as only 40% of mice developed diabetes. A statistically significant difference was observed in survival between the control group of mice injected with diabetic splenocytes vs mice treated with IGRP peptides P3- or P7-primed cells mixed with diabetic splenocytes (p < 0.01). The group of mice that received diabetogenic cells mixed with insulin B:9-23 peptide-primed cells did not develop diabetes (p = 0.001) as reported previously (19). These results suggest that although IGRP peptides P1-, P2-, P3-, and P7-activated CD4<sup>+</sup> T cells have an effect on disease pathogenesis in NOD/SCID recipients, they were not sufficient to completely prevent disease in this model.

**IGRP peptides protect/delay the development of spontaneous diabetes**

As the results of the adoptive transfer assay suggested that IGRP peptide-primed cells could play a role in disease progression, NOD (4 wk) mice were immunized with IGRP peptides P1-, P2-, P3-, and P7, control peptide proinsulin (24-36), and saline to determine whether this would have an effect on the development of spontaneous diabetes as described in Materials and Methods. The results
Materials and Methods

Seven predicted MHC class II-restricted peptide epitopes spanning the sequence of IGRP were identified and synthesized based on alignment with MHC class II (I-A^d) binding motifs. Interestingly, only four of the peptides, P1, P2, P3, and P7, induced both spontaneous and primed proliferative responses. The presence of a spontaneous response to these peptides suggests that they may be involved in the activation of IGRP-reactive T cells. The response to these IGRP peptides seems to be related to autoimmunity as cells from BALB/c mice did not proliferate in response to these IGRP peptides.

Discussion

The present study was undertaken to characterize the CD4^+ T cell response induced by a newly identified candidate autoantigen, IGRP, in the context of T1D. It has been reported that an IGRP peptide epitope corresponding to the amino acid region 206-214 is a major target of CD8^+ T cells in NOD mice (9, 10). In this study, we identified the epitopes on IGRP for CD4^+ T cells and investigated their role in the regulation of T1D. IGRP is an excellent candidate autoantigen because its expression is restricted to the islet cells of the pancreas. It is expressed in humans and NOD mice, and it maps to a human diabetes susceptibility locus (20, 21).

Seven predicted MHC class II-restricted peptide epitopes spanning the sequence of IGRP were identified and synthesized based on alignment with MHC class II (I-A^d) binding motifs. Interestingly, only four of the peptides, P1, P2, P3, and P7, induced both spontaneous and primed proliferative responses. The presence of a spontaneous response to these peptides suggests that they may be involved in the activation of IGRP-reactive T cells. The response to these IGRP peptides seems to be related to autoimmunity as cells from BALB/c mice did not proliferate in response to these peptides. IGRP is an intracellular endoplasmic reticulum membrane protein of β cells, and therefore, it must be released from the cells to be presented by APCs to T cells. Both viral-induced damage and FAS-FAS ligand-mediated apoptosis have been suggested as initial causes of β-islet destruction; however, a clear mechanism is currently unknown (8, 22–24). The release of IGRP and its subsequent presentation by APCs may be an important step in the activation of IGRP peptide-reactive diabeticogenic CD4^+ T cells. The absence of a response to peptides P4, P5, and P6 suggests that these peptides are not immunogenic. Alternatively, this can also be explained on the basis of differences in processing and presentation. Our spontaneous data show that cells specific for IGRP peptides P1, P2, P3, and P7 exist in vivo, suggesting that these peptides contain some epitopes that may be naturally processed and presented. Additionally, to determine whether there are any differences in the binding of these peptides to MHC class II, we aligned these peptides with known I-A^d-binding motifs and compared presence of position specific anchor residues at position 6 and 9 (Table II; Refs. 25–27). However, we observed that binding of the peptides to I-A^d may not necessarily correlate with the activation of T cells because peptides P4, P5, and P6 did not induce any significant response.

Because only four peptides induced response in NOD mice after immunization, only the responses induced by peptides P1, P2, P3, and P7 were further characterized. The responses induced by the group immunized with IGRP peptide P2, mice gradually became diabetic, and by 26 wk postinjection, 66% were diabetic. A statistically significant difference in diabetes onset was found between saline or proinsulin (24-36) peptide-treated and the IGRP peptide P2-treated groups (p < 0.003). IGRP peptide P3 significantly delayed the development of diabetes until week 30, and only 33% of mice developed diabetes. Mice immunized with IGRP peptide P7 were completely protected from the development of diabetes. Differences in diabetes incidence between saline and proinsulin (24-36) peptide-treated vs the IGRP peptide P3- and P7-treated groups were statistically significant (p = 0.0001). A significant difference between the diabetes onset of IGRP peptide P1- and P2-treated groups vs the IGRP peptide P7-treated groups was also observed (p = 0.0001). These results suggest that peptides P3 and P7 are able to activate a T cell population within the NOD mouse, which is able to protect these mice from developing diabetes.

FIGURE 7. IGRP peptide-primed cells delay the adoptive transfer of diabetes into NOD/SCID mice. Draining lymph node cells (10^6) from NOD mice primed with IGRP peptides P1, P2, P3, and P7 or the insulin B9-23 peptide and cultured with the respective peptide (100 μg/ml) for 72 h at 37°C were cotransferred with diabetogenic splenocytes (10^6) into NOD/SCID recipients via the i.v. route. Mice were monitored for the development of diabetes as described in Materials and Methods. Each group consisted of 10 mice. Results represent cumulative data from two independent experiments. A statistically significant difference was observed in diabetes onset using the product limit method of Kaplan and Meier between theses two groups (p < 0.01).

FIGURE 8. Immunization of NOD mice with IGRP peptides prevents or delays the development of spontaneous diabetes. Four-week-old female NOD mice were immunized i.p. with IGRP peptides P1, P2, and P3, control proinsulin (24-36) peptide or saline. After 4 wk, mice were given a booster injection i.p. of the respective peptide in IFA at the same dose. Mice were monitored for the development of diabetes as described in Materials and Methods. Proinsulin group consisted of 10 mice while the other groups consisted of 15 mice each. Difference in diabetes incidence between saline and proinsulin (24-36) peptide treated vs the IGRP peptide P3- and P7-treated groups were statistically significant (p = 0.0001) using the product limit method of Kaplan and Meier.
peptides were predominantly CD4\(^+\) T cell mediated and were inhibited by anti-MHC class II Ab. This is an important step in understanding the role of IGRP peptide-specific CD4\(^+\) T cells in T1D.

Analysis of the cytokine profile of CD4\(^+\) T cells generated in response to these peptides is critical to elucidate their role in T1D in NOD mice. Th1 helper T cells are a pathogenic subset of CD4\(^+\) T cells, which, through cytokine secretion, activate macrophages, NK cells, and cytotoxic CD8\(^+\) T cells, leading to inflammation and tissue injury and development of T1D. Th2 helper T cells are a regulatory subset of CD4\(^+\) T cells that are implicated in protection from T1D (20, 28, 29). These IGRP peptides showed a complex array of Th responses. Peptide P1 induced a Th1-biased response. Peptides P3 and P7, in contrast, induced secretion of high levels of IFN-\(\gamma\), IL-10, and low levels of IL-4 with little IL-2, suggesting generation of both Th1- and Th2-type responses. It has been suggested that the same cell may simultaneously secrete IFN-\(\gamma\) and IL-10 (30, 31). It has also been shown using human CD4\(^+\) cells that the presence of IL-12 induces production of high levels of both IFN-\(\gamma\) and IL-10 (32, 33). We also observed secretion of IL-12 by all the cells primed with the four IGRP peptides (data not shown). Moreover, a population of regulatory T cells (Tr1 cells) has been described that produces both IL-10 and IFN-\(\gamma\) (34–36). Because these IGRP peptide-primed cells secrete both IL-10 and IFN-\(\gamma\), they may represent the regulatory T cell population that inhibits the pathogenic effects of CD8\(^+\) T cells resulting in the inhibition of disease transfer.

The difference observed between P1 and P2 peptides in both studies may be due to differences in the cytokine profiles of the peptide primed cells. The secretion of small quantities of IL-10 and IL-4 by P1-primed cells, which are absent in P2-primed cells, may also be sufficient to provide partial protection. These results confirm that immunization with MHC class II-restricted IGRP peptides can modulate the immune response by inducing IL-10-secreting CD4\(^+\) Tr1-type cells. Tr1 cells have been shown to prevent the development of Th1-mediated autoimmune diseases via secretion of IL-10 (36, 38, 39). P3 and P7 peptide-primed cells also secrete high levels of IFN-\(\gamma\). The presence of IFN-\(\gamma\) may be representative of the ongoing autoimmune pathogenic response, whereas the peptide-specific regulatory cells may secrete the IL-4 and IL-10 to restore the immunological balance. It has been shown recently that IFN-\(\gamma\) may play a significant role in Th2 priming both in vivo and in vitro (40). In addition, transfer of IFN-\(\gamma\)-stimulated dendritic cells into NOD mice has been reported to protect against the development of diabetes (21). Other studies have shown that injection of recombinant IFN-\(\gamma\) causes a dose-dependent inhibition of diabetes in NOD mice (41, 42).

In the adoptive transfer model of diabetes, all four peptides delayed the development of disease. Peptides P1 and P3 have identical sequences, except that P3 has five additional amino acids in the N-terminal region. It seems that these additional five amino acids play a critical role in the binding of the peptide to MHC class II and the induction of T cell responses leading to the differences observed in the protection studies. Because no peptide treatment was involved in the adoptive transfer model, it is possible that the CD4\(^+\) T cells generated in response to the IGRP peptides downregulated the pathogenic CD8\(^+\) T cells. A population of regulatory T cells (Tr1 cells) have been described that produces both IL-10 and IFN-\(\gamma\) (34–36). Because these IGRP peptide-primed cells secrete both IL-10 and IFN-\(\gamma\), they may represent the regulatory T cell population that inhibits the pathogenic effects of CD8\(^+\) T cells resulting in the inhibition of disease transfer.

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response and modulating the immune response to provide protection from diabetes. Future studies are also needed to identify the naturally processed IGRP epitopes that play a role in disease process. However, we postulate that such epitopes are likely to contain many of the peptides we have identified in this study.

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Disclosures

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References