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Nonobese diabetic (NOD) mice spontaneously develop diabetes as a consequence of an autoimmune process that can be inhibited by immunotherapy with the 60-kDa heat shock protein (hsp60), with its mycobacterial counterpart 65-kDa (hsp65), or with other Ags such as insulin and glutamic acid decarboxylase (GAD). Microbial infection and innate signaling via LPS or CpG motifs can also inhibit the spontaneous diabetogenic process. In addition to the spontaneous disease, however, NOD mice can develop a more robust cyclophosphamide-accelerated diabetes (CAD). In this work, we studied the effect on CAD of DNA vaccination with constructs encoding the Ags human hsp60 (phsp60) or mycobacterial hsp65 (phsp65). Vaccination with phsp60 protected NOD mice from CAD. In contrast, vaccination with phsp65, with an empty vector, or with a CpG-positive oligonucleotide was not effective, suggesting that the efficacy of the phsp60 construct might be based on regulatory hsp60 epitopes not shared with its mycobacterial counterpart, hsp65. Vaccination with phsp60 modulated the T cell responses to hsp60 and also to the GAD and insulin autoantigens; T cell proliferative responses were significantly reduced, and the pattern of cytokine secretion to hsp60, GAD, and insulin showed an increase in IL-10 and IL-5 secretion and a decrease in IFN-γ secretion, compatible with a shift from a Th1-like toward a Th2-like autoimmune response. Our results extend the role of specific hsp60 immunomodulation in the control of β cell autoimmunity and demonstrate that immunoregulatory networks activated by specific phsp60 vaccination can spread to other Ags targeted during the progression of diabetes, like insulin and GAD. The Journal of Immunology, 2002, 169: 6030–6035.

Insulin-dependent diabetes mellitus (IDDM) is a metabolic disorder caused by the autoimmune destruction of the insulin-producing β cells of the pancreas (1). Nonobese diabetic (NOD) mice can develop two types of autoimmune diabetes, spontaneous diabetes and cyclophosphamide-accelerated diabetes (CAD).

Spontaneous autoimmune diabetes is hereby detectable as overt hyperglycemia in female NOD mice, first appearing after the third month of life (2, 3), and is characterized by increased Th1 cell responses to several autoantigens, including the 60-kDa heat shock protein (hsp60) (4), glutamic acid decarboxylase (GAD) (5, 6), and insulin (7). Ag-specific therapies can halt the progression of spontaneous NOD diabetes (8); among them are treatment with the mycobacterial 65-kDa heat shock protein (hsp65) (9) or the mammalian hsp60 (10, 11). Spontaneous NOD diabetes is also inhibited by viral (12, 13), parasitic (14), or bacterial (15) infection or by the administration of bacterial molecules known to signal, directly or indirectly, through Toll-like receptors (TLRs) (16), such as LPS (17, 18) or immunostimulatory CpG DNA motifs (19).

A single injection of cyclophosphamide can synchronize and accelerate IDDM in NOD mice through a mechanism that involves the death of various cell populations (20), including regulatory cells (21). When compared with spontaneous NOD diabetes, CAD stands as a stronger variant of autoimmune diabetes; from the large list of agents that modulate the spontaneous disease (22), only a few of them can affect CAD (23–27).

In this work, we studied the effect of DNA vaccination (28) on CAD with constructs encoding the antigens hsp60 (phsp60) or hsp65 (phsp65). Animals treated with phsp60 were protected from CAD; treatment with phsp65, an empty vector, or CpG-positive oligonucleotides did not protect. The phsp60-treated mice showed a modulation of the T cell responses to hsp60, GAD, and insulin, suggesting that phsp60 vaccination activates regulatory networks that control the autoimmune response against other Ags associated with diabetes, besides hsp60 itself.

Materials and Methods

Mice

Female mice of the NOD/LtJ strain were raised from breeders kindly supplied by Dr. E. Leiter of The Jackson Laboratory, and maintained under pathogen-free conditions in the Animal Breeding Center of this institute. Experiments were conducted under the supervision and guidelines of the Animal Welfare Committee. The mice were 1 mo old at the start of the experiments.

DNA plasmids and CpG

The pcDNA3 (Invitrogen, Leek, The Netherlands) vectors encoding human hsp60 (phsp60) (19) or Mycobacterium leprae hsp65 (phsp65) (29) have been previously described and shown to be immunogenic in mice. Dr. D. Lowrie (Medical Research Council, London, U.K.) kindly provided the phsp65 construct. Plasmid DNA was prepared in large scale using the alkaline lysis method of Quagen Plasmid Mega Prep (Quagen, Santa Clara, CA). Plasmid DNA was precipitated with ethanol and resuspended in sterile PBS. Spectrophotometric analysis revealed 260:280 nm ratios ≥1.80. The purity of DNA preparations was confirmed on a 1% agarose gel. Endotoxin levels were checked by Limulus amebocyte lysate and were always under acceptable levels for in vivo use (<0.02 endotoxin U/µg DNA).

The phosphorothioate-stabilized oligonucleotides used in these studies were synthesized in the Oligonucleotide Synthesis Unit of this institute as previously described (19). Oligonucleotide CpG, 5’-TCCATAACGTT

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4 Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; hsp60, 60-kDa heat shock protein; hsp65, 65-kDa heat shock protein; GAD, glutamic acid decarboxylase; CAD, cyclophosphamide-accelerated diabetes; phsp60, DNA vaccine encoding hsp60; phsp65, DNA vaccine encoding hsp65; TLR, Toll-like receptor; SI, stimulation index.

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GCA-AACGTTCGT-3', oligonucleotide GpC, 5'–TCCATAAAGCTTGGCA AACGTTCGT-3'.

NOD females were injected with 100 μl of 10 mM cardiotoxin (Sigma-Aldrich, Rehovot, Israel) into the tibialis anterior muscle. After 5 and 12 days, the mice were injected with 100 μl (1 μg/μl) of the desired DNA vaccine, with 100 μl (1 μg/μl) of the oligonucleotides bearing CpG or GpC motifs, or with 100 μl of PBS as controls. Diabetes was accelerated by a single injection of 200 mg/kg cyclophosphamide (Sigma-Aldrich) given 12 days after the last injection of DNA, at the age of 8 wk.

**Hyperglycemia**

Blood glucose was measured weekly. A mouse was considered diabetic when its blood glucose level was >13 mM on two consecutive examinations, tested using a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA).

**Pancreas histology**

Mice from each treatment group were killed 1 mo after the injection of cyclophosphamide, at the age of 12 wk. The pancreata were fixed in 10% buffered formalin, cut, and stained by standard H&E; the average degree of insulitis was assessed on 20 islets scored per pancreas. Each islet was classified as: clear, if no infiltrate was detected; mildly infiltrated, if peripherally infiltrated or an intra-islet infiltrate occupied <25% of the islet; or infiltrated or heavily infiltrated, if 25–50% or >50% of the islet was occupied by inflammatory cells.

**Peptides and Ags**

The peptides used in this study are listed in Table I. The peptides were synthesized by a standard F-moc procedure and purified by reverse phase HPLC, and their compositions were confirmed by amino acid analysis. Peptide p277 was stabilized by substituting two cysteines at positions 442 and 447 for valines. These substitutions do not affect the immunological properties of p277 (11). Insulin, GAD, OVA, and Con A were purchased from Sigma-Aldrich. Recombinant hsp60 was prepared as described (19).

**T cell proliferation**

Groups of 3–4 female NOD mice were sacrificed 4 wk after the acceleration of diabetes with cyclophosphamide, their spleens were removed, and the T cell-proliferative responses to Con A or test Ags were studied. Cultures were incubated for 72 h at 37°C in a humidified atmosphere with 5% CO2. T cell responses were detected by the incorporation of 3H-thymidine (Amersham Biosciences, Little Chalfont, U.K.; 1 μCi/well) added to the wells for the last 18 h of incubation. The stimulation index (SI) was computed as the ratio of the mean cpm of Ag- or mitogen-containing wells to control wells cultured with medium alone.

**Cytokine assays**

 Supernatants were collected after 72 h of stimulation with test Ags, Con A, or medium alone. Murine IL-4, IL-5, IL-10, and IFN-γ were quantitated in the culture supernatants with ELISA, using appropriate paired Abs from BD PharMingen (San Diego, CA) (IL-4, IL-10, and IFN-γ) or Endogen (Woburn, MA) (IL-5) with some modification. Briefly, ELISA plates (Maxisorb; Nunc, Roskilde, Denmark) were coated overnight at 4°C with anti-mouse cytokine monoclonal capture Abs. Nonspecific binding was blocked by incubation with 1% BSA for 1 h at room temperature, and culture supernatants or recombinant cytokines were incubated overnight at 4°C. After the plates were washed, biotinylated detection Abs were added for 1 h at room temperature, then extensively washed, and incubated with streptavidin conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature. The plates were washed, Sigma-Aldrich alkaline phosphatase substrate was added, and samples were read at 405 nm after 30 min of incubation at room temperature. Cytokine levels in supernatants are expressed as picograms per milliliter based on calibration curves constructed using recombinant cytokines as standards. The lower limits of detection for the experiments described in this paper were 15 pg/ml for IL-5, IL-10, and IFN-γ.

**Statistical significance**

The InStat 2.01 program was used for statistical analysis. Student’s t test and the χ2 test were conducted to assay significant differences between experimental and control groups.

**Results**

*pshp60 inhibits CAD*

We treated 4-wk-old female NOD mice with pshp60, with pshp65, with the empty vector containing CpG motifs (pcDNA3), or with

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**Table I. Peptides used**

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<th>Position</th>
<th>Sequence</th>
<th>Ref.</th>
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<td>EIAQVATISANGDKDIGN</td>
<td>30</td>
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<tr>
<td>p277</td>
<td>Hsp60</td>
<td>437–460</td>
<td>VGGLGVALRVPALDSLTPANED</td>
<td>11</td>
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<tr>
<td>p34</td>
<td>GAD</td>
<td>509–528</td>
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<tr>
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<td>GAD</td>
<td>524–543</td>
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<td>6</td>
</tr>
</tbody>
</table>

**FIGURE 1.** pshp60 inhibits CAD. A. Groups of fifteen 4-week-old female NOD/LtJ mice were pretreated with cardiotoxin and immunized i.m. 5 and 12 days later with pcDNA3, pshp65, pshp60, or GpC- or CpG-bearing oligonucleotides. One group was injected i.m. with PBS on the same days. Twelve days after the last vaccination, the animals received 200 mg/kg cyclophosphamide i.p., and their glucose levels were checked weekly; hyperglycemia was defined as a blood glucose level exceeding 13 mM. The pshp60-vaccinated group developed a significantly lower incidence of diabetes. Three independent experiments produced similar results. #, p < 0.02 compared with the other groups. B. The degree of insulitis was determined by scoring at least 20 islets in each pancreas at day 28 after the injection of cyclophosphamide. The islets were scored as clear, mildly infiltrated (peri-insulitis or an intra-islet infiltrate occupying <25% of the islet), infiltrated (an intra-islet infiltrate occupying 25–50% of the islet), or heavily infiltrated (an intra-islet infiltrate occupying >50% of the islet). *, p < 0.0003 compared with pcDNA3- or pshp65-treated mice; **, p < 0.02 compared with pcDNA3- or pshp65-treated mice. NT, No treatment.
A control group was treated with PBS. Each group consisted of 15 mice. Cyclophosphamide was injected 12 days after the last injection of DNA, and glucose levels were measured at weekly intervals.

Vaccination with phsp60 led to significant protection from CAD. In contrast, the progression of diabetes after administration of cyclophosphamide was the same in the mice treated with PBS, pcDNA3, phsp65, or CpG- or GpC-bearing oligonucleotides (Fig. 1A). Fig. 1B depicts the results obtained on histological examination of the pancreas; phsp60 vaccination led to a significant increase in the number of islets free of insulitis 30 days after the injection of cyclophosphamide, together with a significant decrease in the numbers of infiltrated and heavily infiltrated islets.

**Effects of phsp60 on T cell response to hsp60**

Increasing spontaneous T cell reactivity to hsp60 has been previously related to the progression of diabetes (4, 10), and modulation of the hsp60-specific immune response was associated with the control of the diabetogenic process (11). We therefore studied the proliferative T cell response to hsp60 and to two of its peptides, p277 and p12, by splenocytes isolated from DNA-vaccinated mice 30 days after the administration of cyclophosphamide. When compared with phsp65- or pcDNA3-vaccinated mice, phsp60-treated animals showed significantly reduced T cell-proliferative responses to hsp60 and p277 (Fig. 2). The responses to p12 were too low to be considered significant (SI < 2). Nevertheless, the responses to Con A were of the same magnitude in all the groups (pcDNA3, 6.8 ± 1.2; phsp65, 6.6 ± 1.9; phsp60, 5.5 ± 0.7), suggesting that the inhibition of T cell responses to hsp60 and to p277 were Ag specific and not due to a general down-regulation of the immune response.

The progression of CAD has been previously shown to be associated with T cell secretion of IFN-γ (20); hence we followed the secretion of IFN-γ, IL-4, IL-5, and IL-10 as indicators of the Th1/Th2 phenotype. The different experimental groups did not differ in their responses to Con A, and no stimulation of cytokine secretion was detected on stimulation with the control Ag OVA. Nevertheless, IFN-γ secretion on stimulation with hsp60 was diminished in the phsp60-treated animals (Fig. 3A). This down-regulation of IFN-γ secretion was associated with an increase in IL-10 and IL-5 secretion in response to stimulation with hsp60, and also with the hsp60-derived peptides p12 and p277 (Fig. 3, B and C).

**FIGURE 3.** Cytokine release to hsp60 in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 as described in Fig. 2. Four weeks after receipt of cyclophosphamide, their spleens were removed and stimulated with 25 μg/ml hsp60, p277, or p12, and the supernatants were tested for the amounts of INF-γ (A), IL-10 (B), or IL-5 (C) released. Data are shown as the mean ± SD of triplicates. IFN-γ detected after Con A stimulation was: pcDNA3, 7079 ± 143 pg/ml; phsp65, 7021 ± 299 pg/ml; phsp60, 7343 ± 276 pg/ml. IL-10 detected after Con A stimulation was: pcDNA3, 2702 ± 429 pg/ml; phsp65, 2958 ± 404 pg/ml; phsp60, 2802 ± 122 pg/ml. IL-5 detected after Con A stimulation was: pcDNA3, 81 ± 29 pg/ml; phsp65, 85 ± 14 pg/ml; phsp60, 105 ± 22 pg/ml. No IFN-γ, IL-10, or IL-5 release was detected on activation with OVA. Three independent experiments produced similar results. *, p < 0.001 compared with pcDNA3-vaccinated mice; **, p < 0.005 compared with pcDNA3-vaccinated mice; +, p < 0.01 compared with pcDNA3-vaccinated mice; +++, p < 0.05 compared with pcDNA3-vaccinated mice.
FIGURE 4. Proliferative responses to insulin and GAD in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, pshp65, or pshp60 as described in Fig. 2. Four weeks after receipt of cyclophosphamide, their spleens were removed, and the T cell-proliferative responses were assayed after 72 h of stimulation with 25 μg/ml insulin, GAD, p34, or p35. Results are expressed as the SI ± SD in comparison with paired samples incubated with medium alone. Three independent experiments produced similar results. *, p < 0.001 compared with pcDNA3-vaccinated mice.

No IL-4 was detected on Ag stimulation; however, high levels of IL-4 were found in the supernatants of Con A-stimulated cultures: pcDNA3, 11.9 ± 0.6 ng/ml; pshp65, 11 ± 0.9 ng/ml; and pshp65, 11.8 ± 0.5 ng/ml. A decrease in IFN-γ together with an increase in IL-10 and IL-5 in response to hsp60 stimulation is compatible with a shift of the anti-hsp60 T cell response from what has been termed a pathogenic Th1 to a regulatory Th2 phenotype, in an Ag-specific manner.

Effects of pshp60 on the T cell response to GAD and insulin

The autoimmune process leading to overt diabetes targets other Ags besides hsp60, such as GAD (5, 6) and insulin (7). We therefore studied T cell reactivity to insulin, to GAD, and to two GAD-derived peptides, p34 and p35 (6), by splenocytes isolated from DNA-vaccinated mice 30 days after the administration of cyclophosphamide. The pshp60-treated animals showed a significant decrease in their spontaneous proliferative response to GAD. The proliferative responses to GAD-derived peptides p34 and p35, or to insulin were too low to be evaluated (Fig. 4). However, splenocytes taken from pshp60-treated mice secreted significantly lower amounts of IFN-γ and higher amounts of IL-10 and IL-5 on stimulation with insulin, GAD, or p35 (Fig. 5). Again, IL-4 was not detected after Ag-specific stimulation. We did not detect any cytokine secretion on in vitro stimulation with p34 or with the control Ag OVA. Thus, pshp60 vaccination induces a shift toward Th2 in the hsp60-specific T cell response and in the T cell responses to insulin and GAD.

Discussion

Earlier studies conducted in this laboratory demonstrated that spontaneous NOD diabetes could be treated by vaccination with hsp65, hsp60, or peptides p277 or p12 (4, 9, 10, 30). In addition, it has been shown that treatment of NOD mice with molecules that stimulate the innate immune system, such as CpG DNA (19) or LPS (17, 18), can also inhibit spontaneous NOD diabetes. Thus, the spontaneous development of diabetes in NOD mice can be arrested by activation of the immune system by both innate ligands and vaccination with specific Ags.

In this paper, we studied the susceptibility of CAD to modulation by DNA vaccination. DNA vaccination with a construct encoding human hsp60 (pshp60), but not with a construct encoding mycobacterial hsp65 (pshp65), controlled CAD (Fig. 1). The effective pshp60 vaccine contained two kinds of signals: a specific Ag associated with diabetes autoimmunity, hsp60 (2), and ligands for receptors that stimulate the innate immune system; hsp60, through TLR-2 and TLR-4 (31); and CpG motifs through TLR-9 (32). However, CAD, in contrast to spontaneous NOD diabetes, did not respond to treatment with pcDNA3 (empty vector) or a CpG-containing oligonucleotide (Fig. 1). In addition, although hsp65 too has been shown to stimulate the innate immune response via TLR-4 and TLR-2 (31), the plasmid encoding mycobacterial hsp65 (pshp65) did not have any significant effect on CAD progression (Fig. 1). Thus, it is unlikely that the efficacy of the pshp60 vaccine could be based solely on its activity on innate receptors; it is more likely that regulatory epitopes present in the hsp60 molecule are also needed. Indeed, the failure of pshp65 vaccination could be explained by the lack in the hsp65 molecule of the two major hsp60 T cell epitopes found to control spontaneous autoimmune diabetes when administered as peptides (10, 30); p12 and

FIGURE 5. Cytokine release to insulin and GAD in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, pshp65, or pshp60 as described in Fig. 2. Four weeks after receiving cyclophosphamide, their spleens were removed and stimulated with 25 μg/ml hsp60, p277, or p12, and the supernatants were tested for the amounts of IFNγ (A), IL-10 (B), or IL-5 (C) released. Data are shown as the mean ± SD of triplicates. Three independent experiments produced similar results. *, p < 0.001 compared with pcDNA3-vaccinated mice; **, p < 0.01 compared with pcDNA3-vaccinated mice; +, p < 0.02 compared with pcDNA3-vaccinated mice; ++, p < 0.05 compared with pcDNA3-vaccinated mice.
p277 are not conserved in mycobacterial hsp65 (Table II). This hypothesis does not rule out the possibility that the effects of phsp60 in controlling CAD might involve signals contained within the hsp60 molecule for both the adaptive and innate arms of the immune system. Study of the functions of innate receptors in the NOD mouse is needed to explore this issue; our results certainly do not exclude the possibility that under different conditions TLR-mediated stimulation might control CAD.

In NOD mice, the balance between the Th1 and Th2 autoimmune responses can lead, respectively, to the progression or control of autoimmune diabetes (2). Proinflammatory Th1 responses (characterized by the production of IFN-γ) are a feature of the diabetogenic attack, whereas regulatory Th2 responses (characterized by the secretion of IL-4, IL-5, and IL-10) have been associated with the inhibition of β cell destruction (2). In our experiments, protection from CAD by phsp60 vaccination was associated with a significant reduction of T cell proliferation and of IFN-γ secretion by T cells responding to hsp60 or its T cell epitope p277 (Figs. 2 and 3). Although we could not detect Ag-specific release of IL-4, an increase in the release of IL-5 and IL-10 was detected (Fig. 3), indicating that phsp60 vaccination shifted the diabetogenic Th1 autoimmune attack more into the direction of a regulatory Th2 response. We (11) and others (33, 34) have previously associated a shift in the T cell reactivity to hsp60 with the control of the diabetogenic response in NOD mice and in newly diagnosed human IDDM patients treated with p277 (35). Remarkably, the immune response to insulin, GAD, and GAD-derived peptides showed reduced proliferation and was also shifted towards Th2 (Figs. 4 and 5), suggesting a role for Th2-like spreading in the control of CAD by phsp60 vaccination. Tian et al. (34) demonstrated the occurrence of Th2 spreading to diabetes-associated Ags in NOD mice using a panel of autoantigens made up of GAD, human 65-kDa heat shock protein. Proc. Natl. Acad. Sci. USA 87:1576.

References


