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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.

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 Qiagen Plasmid Mega Prep (Qiagen, 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
GCA-AACGTTCG-3'; oligonucleotide GpC, 5'-TCCATAACCTGGCA
AAACTTCG-3'.

NOD females were injected with 100 μl of 10 mM cardiotonin (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 examina-
tions, 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 over 20 islets scored per pancreas. Each islet was
classified as: clear, if no infiltrate was detected; mildly infiltrated, if peri-
islets 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 hs60 was prepared as described (19).

T cell proliferation
Groups of 3–4 female NOD mice were sacrificed 4 wk after the acceler-
ation of diabetes with cyclophosphamide, their spleens were removed, and
the T cell-proliferative responses to Con A or test Ags were studied. Cul-
tures were incubated for 72 h at 37°C in a humidified atmosphere with
7.5% 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
(Maxisorp; 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
each 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
phsp60 inhibits CAD
We treated 4-wk-old female NOD mice with phsp60, with phsp65,
with the empty vector containing CpG motifs (pcDNA3), or with

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<tr>
<td>p277</td>
<td>Hsp60</td>
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<td>GAD</td>
<td>524–543</td>
<td>SRLKVELVIKAMMEYGTT</td>
<td>6</td>
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</tbody>
</table>

FIGURE 1. phsp60 inhibits CAD. A. Groups of fifteen 4-week-old fe-
male NOD/LtJ mice were pretreated with cardiotonin and immunized i.m.
5 and 12 days later with pcDNA3, phsp65, phsp60, or GpC- or GpC-
GpC-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 week-
ly; hyperglycemia was defined as a blood glucose level exceeding 13 mM.
The phsp60-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 phsp65-treated mice; **, p < 0.02
compared with pcDNA3- or phsp65-treated mice. NT, No treatment.
CpG or GpC oligonucleotides. 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. 3B and C).

![FIGURE 2](http://www.jimmunol.org/) Proliferative responses to hsp60 in DNA-vaccinated mice. Groups of three 4-week-old female NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 and treated with cyclophosphamide as described in Fig. 1. Four weeks later, their spleens were removed, and the T cell proliferative responses were assayed after 72 h of stimulation with 1.25 μg/ml Con A or 25 μg/ml hsp60, p277, p12, or OVA. Results are expressed as the SI ± SD in comparison with paired samples incubated with medium alone with counts per minute readings as follows: pcDNA3, 902 ± 200; phsp65, 1029 ± 98; and phsp60, 989 ± 62. Three independent experiments produced similar results. *, p < 0.05 compared with pcDNA3-vaccinated mice; **, p < 0.01 compared with pcDNA3-vaccinated mice.

![FIGURE 3](http://www.jimmunol.org/) 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 ± 49 pg/ml; phsp65, 2958 ± 404 pg/ml; phsp60, 2802 ± 122 pg/ml. IL-5 detected after Con A stimulation was: pcDNA3, 81 ± 40 pg/ml; phsp65, 84 ± 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.
Effects of phsp60 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 phsp60-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 phsp60-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 in vitro stimulation with p34 or with the control Ag OVA. Thus phsp60 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 (phsp60), but not with a construct encoding mycobacterial hsp65 (phsp65), controlled CAD (Fig. 1). The effective phsp60 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 (phsp65) did not have any significant effect on CAD progression (Fig. 1). Thus, it is unlikely that the efficacy of the phsp60 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 phsp65 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 4. Proliferative responses to insulin and GAD 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 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.

FIGURE 5. Cytokine release to insulin and GAD in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 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, hsp60, and insulin. Later Tisch et al. (36) showed that a single GAD-specific Th2 cell clone can delay the onset of diabetes, spreading a regulatory Th2-like response to non-cross-reactive Ags such as hsp60 or carboxypeptidase H. In our hands, this effect is relatively β cell specific, because we could not detect the induction of Th2 immunity to the control Ag OVA (data not shown). Also, treatment of mice (11) or humans (35) with the hsp60 peptide p277 did not interfere with Th1 immunity to bacterial Ags.

On the basis of these data, we might propose a three-step process by which phsp60 triggers inhibition of CAD: (1) anti-hsp60 T cells are shifted from Th1 to Th2 as a consequence of phsp60 vaccination, perhaps through the activation of hsp60-specific Th2 cells from a pool of undifferentiated T cells (33); (2) hsp60-specific regulatory Th2 cells meet the pathogenic T cells in the islets or in the pancreatic draining lymph nodes; and (3) the hsp60-specific regulatory Th2 cells control the pathogenic self-reactive T cells, either by direct T–T interactions involving the local release of regulatory cytokines or through indirect interactions that modify local APC function. Other explanations are also conceivable. For example, it has been reported that the inhibition of spontaneous NOD diabetes triggered by some immunostimulatory protocols is dependent on the presence of IFN-γ (37). Furthermore, regulatory T cell clones capable of inhibiting NOD diabetes, both spontaneous and accelerated by cyclophosphamide, can secrete IFN-γ (26). Cyclophosphamide enhances Th1 responses (20); therefore, it is quite possible that our treatment of CAD by DNA vaccination might also involve Th1 regulators.

The administration of the hsp60-derived peptide p277 has been recently reported to stop islet destruction in human IDDM (35). Hence, hsp60-derived DNA vaccines encoding relevant regulatory epitopes like p277 might constitute an additional method for the management of human autoimmune diabetes.

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References


