# DNA Vaccination with Heat Shock Protein 60 Inhibits Cyclophosphamide-Accelerated Diabetes[1]

**Francisco J. Quintana, Pnina Carmi, and Irun R. Cohen[2,3]**

**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-$\gamma$ 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 $\beta$ 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)[4] is a metabolic disorder caused by the autoimmune destruction of the insulin-producing $\beta$ 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 usually 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 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 $\leq$1.80. The purity of DNA preparations was confirmed on a 1% agarose gel. Endotoxin levels were checked by *Limulus* amebocyte lysate and were always under acceptable levels for in vivo use ($<$0.02 endotoxin U/$\mu$g 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′-TCCATAA**CG**TT

Department of Immunology, Weizmann Institute of Science, Rehovot, Israel.

Received for publication June 18, 2002. Accepted for publication September 6, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a grant from the Center for the Study of Emerging Diseases.

[2] I.R.C. is the incumbent of the Mauerberger Chair in Immunology and the Director of the Robert Koch-Minerva Center for Research in Autoimmune Diseases.

[3] Address correspondence and reprint requests to Dr. Irun R. Cohen, Department of Immunology, Weizmann Institute of Science, Rehovot, 76100 Israel. E-mail address: irun.cohen@weizmann.ac.il

[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.



0022-1767/02/$02.00

Table I. *Peptides used*

| Peptide | Ag | Position | Sequence | Ref. |
|---|---|---|---|---|
| p12 | Hsp60 | 166–185 | EEIAQVATISANGDKDIGNI | 30 |
| p277 | Hsp60 | 437–460 | VLGGGVALLRVIPALDSLTPANED | 11 |
| p34 | GAD | 509–528 | IPPSLRTLEDNEERMSRLSK | 6 |
| p35 | GAD | 524–543 | SRLSKVAPVIKARMMEYGTT | 6 |

GCA-AA**CG**TTCTG-3′; oligonucleotide GpC, 5′-TCCATAA**GC**TTGCA AA**GC**TTCTG-3′.

NOD females were injected with 100 $\mu$l of 10 mM cardiotoxin (Sigma-Aldrich, Rehovot, Israel) into the tibialis anterior muscle. After 5 and 12 days, the mice were injected with 100 $\mu$l (1 $\mu$g/$\mu$l) of the desired DNA vaccine, with 100 $\mu$l (1 $\mu$g/$\mu$l) of the oligonucleotides bearing CpG or GpC motifs, or with 100 $\mu$l 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 over 20 islets scored per pancreas. Each islet was classified as: clear, if no infiltrate was detected; mildly infiltrated, if peri-insulitis 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 7.5% $CO_2$. T cell responses were detected by the incorporation of [*methyl*-$^3$H]thymidine (Amersham Biosciences, Little Chalfont, U.K.; 1 $\mu$Ci/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-$\gamma$ were quantitated in the culture supernatants with ELISA, using appropriate paired Abs from BD PharMingen (San Diego, CA) (IL-4, IL-10, and IFN-$\gamma$) 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 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-$\gamma$.

### *Statistical significance*

The InStat 2.01 program was used for statistical analysis. Student's *t* test and the $\chi^2$ 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

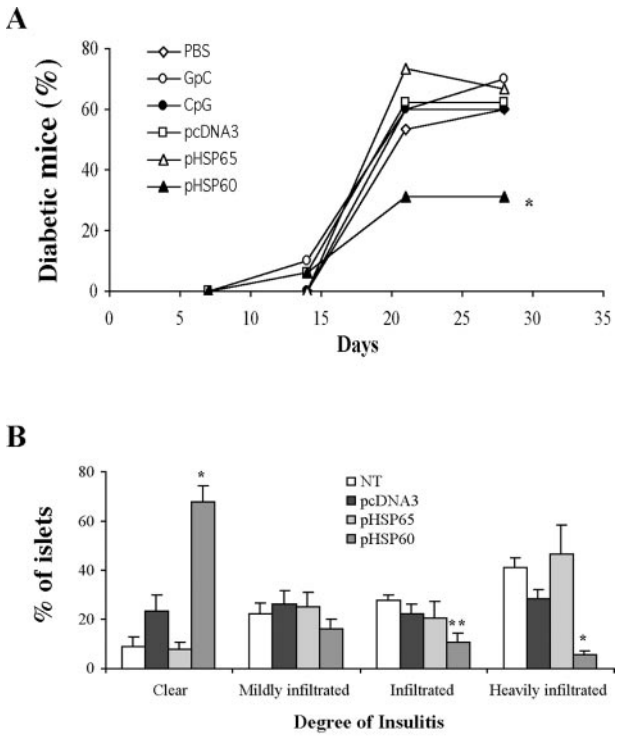


**FIGURE 1.** phsp60 inhibits CAD. *A*, Groups of fifteen 4-week-old female NOD/LtJ mice were pretreated with cardiotoxin and immunized i.m. 5 and 12 days later with pcDNA3, phsp65, phsp60, or CpG- or GpC-bearing oligonucleotides. One group was injected i.m. with PBS on the same days. Twelve days after the last vaccination, the animals received 200 mg/kg cyclophosphamide i.p., and their glucose levels were checked weekly; 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. 1*A*). Fig. 1*B* 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 $\pm$ 1.2; phsp65, 6.6 $\pm$ 1.9; phsp60, 5.5 $\pm$ 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-$\gamma$ (20); hence we followed the secretion of IFN-$\gamma$, 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-$\gamma$ secretion on stimulation with hsp60 was diminished in the phsp60-treated animals (Fig. 3*A*). This down-regulation of IFN-$\gamma$ 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*).

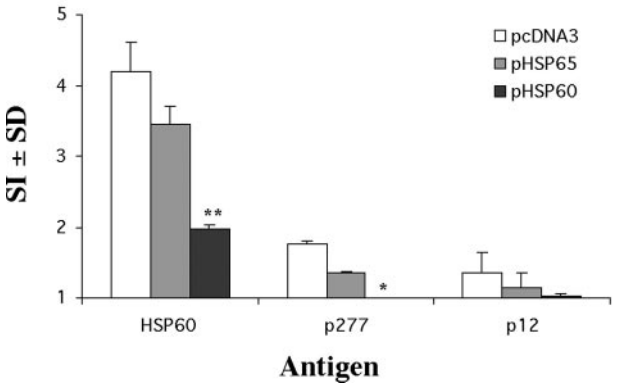


**FIGURE 2.** Proliferative responses to hsp60 in DNA-vaccinated mice. Groups of three 4-week old female NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 and treated with cyclophosphamide as described in Fig. 1. Four weeks later, their spleens were removed, and the T cell proliferative responses were assayed after 72 h of stimulation with 1.25 $\mu$g/ml Con A or 25 $\mu$g/ml hsp60, p277, p12, or OVA. Results are expressed as the SI $\pm$ SD in comparison with paired samples incubated with medium alone with counts per minute readings as follows: pcDNA3, 902 $\pm$ 200; phsp65, 1029 $\pm$ 98; and phsp60, 959 $\pm$ 62. Three independent experiments produced similar results. *, $p < 0.05$ compared with pcDNA3-vaccinated mice; **, $p < 0.01$ compared with pcDNA3-vaccinated mice.

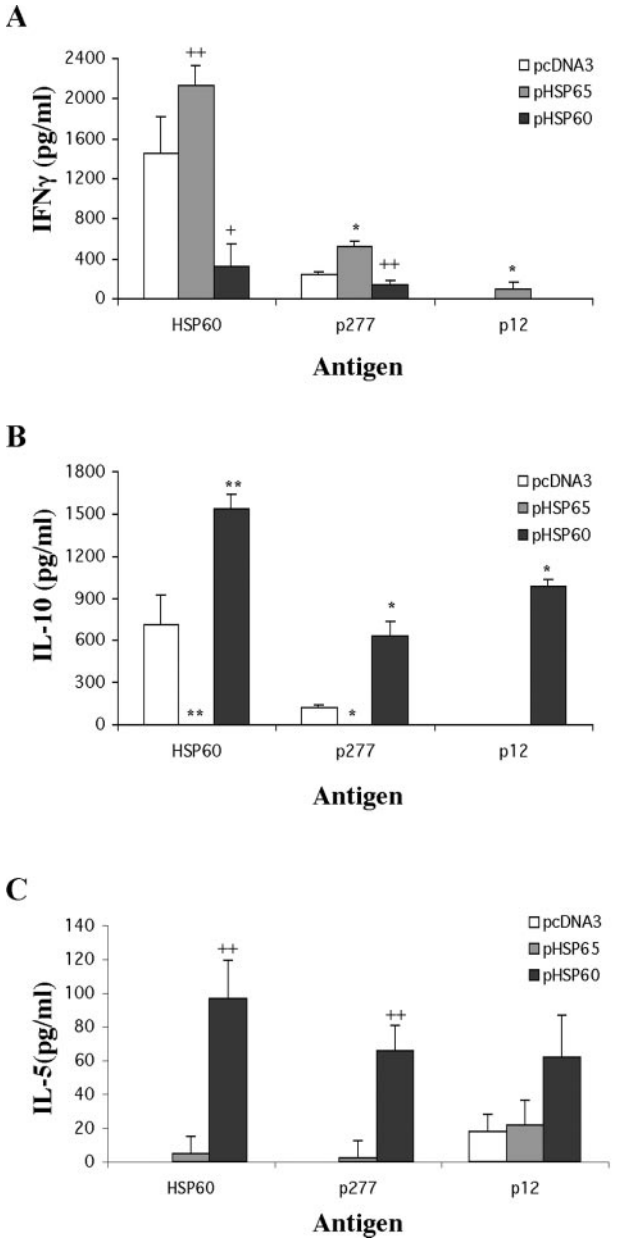


**FIGURE 3.** Cytokine release to hsp60 in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 as described in Fig. 2. Four weeks after receipt of cyclophosphamide, their spleens were removed and stimulated with 25 $\mu$g/ml hsp60, p277, or p12, and the supernatants were tested for the amounts of INF-$\gamma$ (*A*), IL-10 (*B*), or IL-5 (*C*) released. Data are shown as the mean $\pm$ SD of triplicates. IFN-$\gamma$ detected after Con A stimulation was: pcDNA3, 7079 $\pm$ 143 pg/ml; phsp65, 7021 $\pm$ 299 pg/ml; phsp60, 7343 $\pm$ 276 pg/ml. IL-10 detected after Con A stimulation was: pcDNA3, 2702 $\pm$ 429 pg/ml; phs65, 2958 $\pm$ 404 pg/ml; phsp60, 2802 $\pm$ 122 pg/ml. IL-5 detected after Con A stimulation was: pcDNA3, 81 $\pm$ 29 pg/ml; phsp65, 84 $\pm$ 14 pg/ml; phsp60, 105 $\pm$ 22 pg/ml. No IFN-$\gamma$, 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.

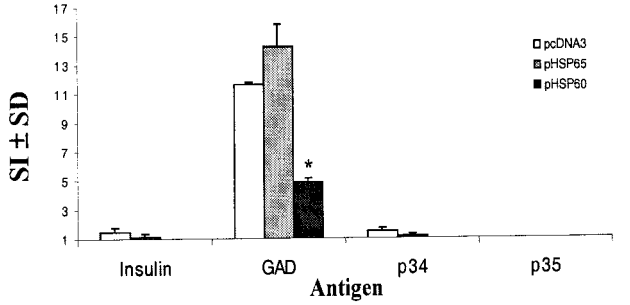


**FIGURE 4.** Proliferative responses to insulin and GAD in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 as described in Fig. 2. Four weeks after receipt of cyclophosphamide, their spleens were removed, and the T cell-proliferative responses were assayed after 72 h of stimulation with 25 $\mu$g/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; phsp65, 11 ± 0.9 ng/ml; and phsp65, 11.8 ± 0.5 ng/ml. A decrease in IFN-$\gamma$ 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 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-$\gamma$ 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 phsp60 vaccination induces a shift toward Th2 in the hsp60-specific T cell response and in the T cell responses to insulin and GAD.

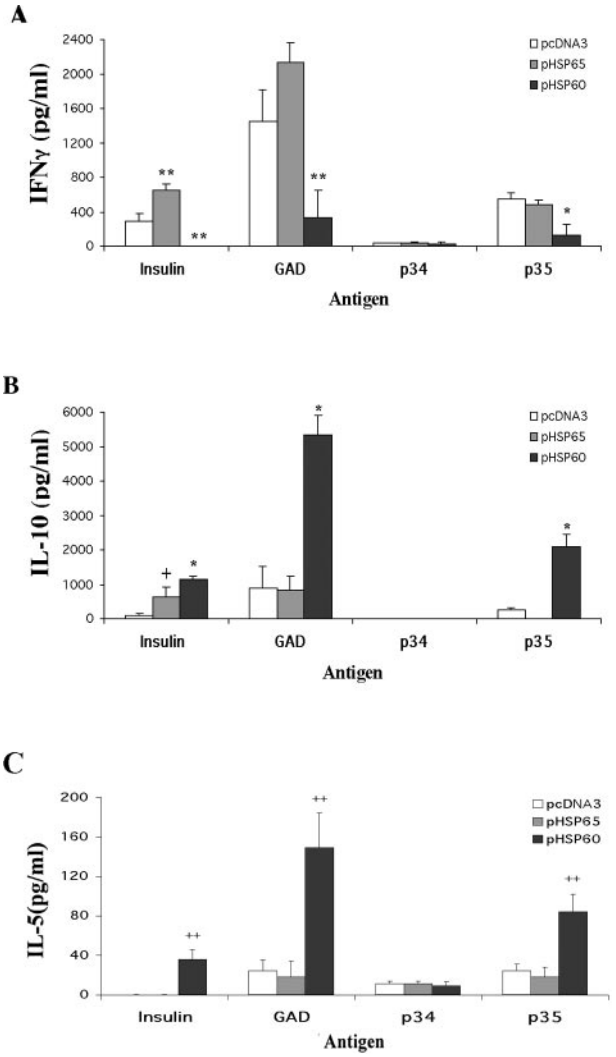


**FIGURE 5.** Cytokine release to insulin and GAD in DNA-vaccinated mice. Groups of NOD/LtJ mice were immunized with pcDNA3, phsp65, or phsp60 as described in Fig. 2. Four weeks after receiving cyclophosphamide, their spleens were removed and stimulated with 25 $\mu$g/ml hsp60, p277, or p12, and the supernatants were tested for the amounts of IFN$\gamma$ (*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.

## 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

Table II. *Comparison of human hsp60, murine hsp60,* Mycobacterium tuberculosis *hsp65, and* M. leprae *hsp65 in the regions corresponding to the p12 and the p277 sequence*[a,b]

**P12**

| | | | |
|---|---|---|---|
| *H.sapiens* | 166 | EEIAQVATISANGDKEIGNI | 185 |
| *M.musculus* | 166 | EEIAQVATISANGDKDIGNI | 185 |
| *M.tuberculosis* | 141 | EQIAATAAISA-GDQSIGDI | 159 |
| *M.leprae* | 141 | EQIAATAAISA-GDQSIGDL | 159 |

**P277**

| | | | |
|---|---|---|---|
| *H.sapiens* | 437 | VLGGGCALLRCIPALDSLTPANE | 460 |
| *M.musculus* | 437 | VLGGGCALLRCIPALDSLKPANE | 460 |
| *M.tuberculosis* | 411 | VAGGGVTLLQAAPTLDELKLEGD- | 434 |
| *M.leprae* | 410 | VAGGGVTLLQAAPALDKLKLTGD- | 433 |

[a] *H. sapiens, Homo sapiens*; *M. musculus, Mus musculus*.

[b] Residues sharing identity with the corresponding sequence of human hsp60 are shown on a black background, and conserved substitutions are shown on a gray background.

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-$\gamma$) 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 $\beta$ 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-$\gamma$ 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 $\beta$ 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-$\gamma$ (37). Furthermore, regulatory T cell clones capable of inhibiting NOD diabetes, both spontaneous and accelerated by cyclophosphamide, can secrete IFN-$\gamma$ (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.

## Acknowledgments

We thank Danielle Sabah-Israel for devoted secretarial assistance. We are grateful to Prof. Douglas Lowrie for providing the phsp65 construct and to Dorith Nathan and Baruch Shajimob for the preparation of the slides used in our studies.

## References

1. Bach, J. F., and L. Chatenoud. 2001. Tolerance to islet autoantigens in type 1 diabetes. *Annu. Rev. Immunol. 19:131*.
2. Cohen, I. R. 1997. The Th1/Th2 dichotomy, hsp60 autoimmunity, and type I diabetes. *Clin. Immunol. Immunopathol. 84:103*.
3. Cohen, I. R. 1997. Questions about NOD mouse diabetes. *Res. Immunol. 148:286*.
4. Birk, O. S., D. Elias, A. S. Weiss, A. Rosen, R. van-der Zee, M. D. Walker, and I. R. Cohen. 1996. NOD mouse diabetes: the ubiquitous mouse hsp60 is a $\beta$-cell target antigen of autoimmune T cells. *J. Autoimmun. 9:159*.
5. Tisch, R., X. D. Yang, S. M. Singer, R. S. Liblau, L. Fugger, and H. O. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature 366:72*.
6. Kaufman, D. L., M. Clare-Salzler, J. Tian, T. Forsthuber, G. S. Ting, P. Robinson, M. A. Atkinson, E. E. Sercarz, A. J. Tobin, and P. V. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature 366:69*.
7. Wegmann, D. R., M. Norbury-Glaser, and D. Daniel. 1994. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol. 24:1853*.
8. Bach, J. F. 2001. Immunotherapy of insulin-dependent diabetes mellitus. *Curr. Opin. Immunol. 13:601*.
9. Elias, D., D. Markovits, T. Reshef, R. van der Zee, and I. R. Cohen. 1990. Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. *Proc. Natl. Acad. Sci. USA 87:1576*.
10. Elias, D., T. Reshef, O. S. Birk, R. van der Zee, M. D. Walker, and I. R. Cohen. 1991. Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. *Proc. Natl. Acad. Sci. USA 88:3088*.
11. Elias, D., A. Meilin, V. Ablamunits, O. S. Birk, P. Carmi, S. Konen-Waisman, and I. R. Cohen. 1997. Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and downregulates autoimmunity to various $\beta$-cell antigens. *Diabetes 46:758*.
12. Oldstone, M. B. 1988. Prevention of type I diabetes in nonobese diabetic mice by virus infection. *Science 239:500*.
13. Oldstone, M. B. 1990. Viruses as therapeutic agents. I. Treatment of nonobese insulin-dependent diabetes mice with virus prevents insulin-dependent diabetes mellitus while maintaining general immune competence. *J. Exp. Med. 171:2077*.
14. Cooke, A., P. Tonks, F. M. Jones, H. O'Shea, P. Hutchings, A. J. Fulford, and D. W. Dunne. 1999. Infection with *Schistosoma mansoni* prevents insulin dependent diabetes mellitus in non-obese diabetic mice. *Parasite Immunol. 21:169*.
15. Bras, A., and A. P. Aguas. 1996. Diabetes-prone NOD mice are resistant to *Mycobacterium avium* and the infection prevents autoimmune disease. *Immunology 89:20*.
16. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol. 2:675*.

17. Sai, P., and A. S. Rivereau. 1996. Prevention of diabetes in the nonobese diabetic mouse by oral immunological treatments: comparative efficiency of human insulin and two bacterial antigens, lipopolysaccharide from *Escherichia coli* and glycoprotein extract from *Klebsiella pneumoniae*. *Diabetes Metab. 22:341*.
18. Tian, J., D. Zekzer, L. Hanssen, Y. Lu, A. Olcott, and D. L. Kaufman. 2001. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. *J. Immunol. 167:1081*.
19. Quintana, F. J., A. Rotem, P. Carmi, and I. R. Cohen. 2000. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J. Immunol. 165:6148*.
20. Ablamunits, V., F. Quintana, T. Reshef, D. Elias, and I. R. Cohen. 1999. Acceleration of autoimmune diabetes by cyclophosphamide is associated with an enhanced IFN-$\gamma$ secretion pathway. *J. Autoimmun. 13:383*.
21. Yasunami, R., and J. F. Bach. 1988. Anti-suppressor effect of cyclophosphamide on the development of spontaneous diabetes in NOD mice. *Eur. J. Immunol. 18:481*.
22. Atkinson, M. A., and E. H. Leiter. 1999. The NOD mouse model of type 1 diabetes: as good as it gets? *Nat. Med. 5:601*.
23. Maron, R., M. Guerau-de-Arellano, X. Zhang, and H. L. Weiner. 2001. Oral administration of insulin to neonates suppresses spontaneous and cyclophosphamide induced diabetes in the NOD mouse. *J. Autoimmun. 16:21*.
24. Charlton, B., C. Taylor-Edwards, R. Tisch, and C. G. Fathman. 1994. Prevention of diabetes and insulitis by neonatal intrathymic islet administration in NOD mice. *J. Autoimmun. 7:549*.
25. Qin, H. Y., and B. Singh. 1997. BCG vaccination prevents insulin-dependent diabetes mellitus (IDDM) in NOD mice after disease acceleration with cyclophosphamide. *J. Autoimmun. 10:271*.
26. Quinn, A., B. McInerney, E. P. Reich, O. Kim, K. P. Jensen, and E. E. Sercarz. 2001. Regulatory and effector CD4 T cells in nonobese diabetic mice recognize overlapping determinants on glutamic acid decarboxylase and use distinct V beta genes. *J. Immunol. 166:2982*.
27. Sai, P., A. S. Rivereau, C. Granier, T. Haertle, and L. Martignat. 1996. Immunization of non-obese diabetic (NOD) mice with glutamic acid decarboxylase-derived peptide 524–543 reduces cyclophosphamide-accelerated diabetes. *Clin. Exp. Immunol. 105:330*.
28. Garren, H., and L. Steinman. 2000. DNA vaccination in the treatment of autoimmune disease. *Curr. Dir. Autoimmun. 2:203*.
29. Tascon, R. E., M. J. Colston, S. Ragno, E. Stavropoulos, D. Gregory, and D. B. Lowrie. 1996. Vaccination against tuberculosis by DNA injection. *Nat. Med. 2:888*.
30. Bockova, J., D. Elias, and I. R. Cohen. 1997. Treatment of NOD diabetes with a novel peptide of the hsp60 molecule induces Th2-type antibodies. *J. Autoimmun. 10:323*.
31. Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Hacker, and H. Wagner. 2001. Endocytosed hsp60s use toll-like receptor 2 (TLR2) and TLR4 to activate the Toll/interleukin-1 receptor signaling pathway in innate immune cells. *J. Biol. Chem. 276:31332*.
32. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740*.
33. Tian, J., and D. L. Kaufman. 1998. Attenuation of inducible Th2 immunity with autoimmune disease progression. *J. Immunol. 161:5399*.
34. Tian, J., P. V. Lehmann, and D. L. Kaufman. 1997. Determinant spreading of T helper cell 2 (Th2) responses to pancreatic islet autoantigens. *J. Exp. Med. 186:2039*.
35. Raz, I., D. Elias, A. Avron, M. Tamir, M. Metzger, and I. R. Cohen. 2001. $\beta$-Cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, Phase II trial. *Lancet 358:1749*.
36. Tisch, R., B. Wang, M. A. Atkinson, D. V. Serreze, and R. Friedline. 2001. A glutamic acid decarboxylase 65-specific Th2 cell clone immunoregulates autoimmune diabetes in nonobese diabetic mice. *J. Immunol. 166:6925*.
37. Serreze, D. V., H. D. Chapman, C. M. Post, E. A. Johnson, W. L. Suarez-Pinzon, and A. Rabinovitch. 2001. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause, of diabetes resistance elicited by immunostimulation. *J. Immunol. 166:1352*.