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Vaccination with Empty Plasmid DNA or CpG Oligonucleotide Inhibits Diabetes in Nonobese Diabetic Mice: Modulation of Spontaneous 60-kDa Heat Shock Protein Autoimmunity[1]

Francisco J. Quintana, Asaf Rotem, Pnina Carmi, and Irun R. Cohen[2]

Nonobese diabetic (NOD) mice develop insulitis and diabetes through a process involving autoimmunity to the 60-kDa heat shock protein (HSP60). Treatment of NOD mice with HSP60 or with peptides derived from HSP60 inhibits this diabetogenic process. We now report that NOD diabetes can be inhibited by vaccination with a DNA construct encoding human HSP60, with the pcDNA3 empty vector, or with an oligonucleotide containing the CpG motif. Prevention of diabetes was associated with a decrease in the degree of insulitis and with down-regulation of spontaneous proliferative T cell responses to HSP60 and its peptide p277. Moreover, both the pcDNA3 vector and the CpG oligonucleotide induced specific Abs, primarily of the IgG2b isotype, to HSP60 and p277, and not to other islet Ags (glutamic acid decarboxylase or insulin) or to an unrelated recombinant Ag expressed in bacteria (GST). The IgG2b isotype of the specific Abs together with the decrease in T cell proliferative responses indicate a shift of the autoimmune process to a Th2 type in treated mice. These results suggest that immunostimulation by bacterial DNA motifs can modulate spontaneous HSP60 autoimmunity and inhibit NOD diabetes. ***The Journal of Immunology,* 2000, 165: 6148–6155.**

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The nonobese diabetic (NOD)[3] mouse spontaneously develops insulin-dependent diabetes mellitus (IDDM) as a consequence of an autoimmune process that leads to destruction of the insulin-producing β-cells of the pancreas (1). Several Ags have been identified as targets for diabetogenic T cells, including β-cell-specific proteins such as insulin, non-β-cell-restricted Ags such as glutamic acid decarboxylase (GAD), and even ubiquitous Ags such as 60-kDa heat shock protein (HSP60) (1). It has been shown that the onset of diabetes is preceded by an increase in T cell reactivity toward HSP60 and to an HSP60 peptide contained between aa 437 and 460 named p277 (2). In contrast to the early T cell reactivity, Abs to HSP60 and p277 can only be detected late in the natural history of the disease, months after the onset of clinical diabetes when the destructive process has terminated (3). Peptide p277 administered to NOD mice in IFA can arrest the development of diabetes (4). Furthermore, p277 treatment is able to induce remission of advanced insulitis even after the clinical onset of hyperglycemia (5). Successful treatment is associated with down-regulation of spontaneous T cell reactivity to p277 and the induction of Abs to p277; these Abs have Th2-associated isotypes (IgG1 and IgG2b), otherwise not found in young NOD mice (6, 7).

However, peptide therapy is only one way to modulate an ongoing autoimmune process. DNA vaccination is also an efficient approach to induce protection against infectious pathogens (8) and cancer (9) and to modulate autoimmune processes (10). It has been shown that after i.m. injection of a naked expression vector, plasmid DNA is taken up by muscle cells and maintained episomally, allowing the expression of the encoded Ag (11). Thus, after single or repeated injections of DNA, cellular and/or humoral immune responses to the encoded protein are mounted, and long-lived memory lymphocytes are induced (12). These memory cells may have regulatory functions and, therefore, might serve as tools for the modulation of autoimmune conditions.

To explore the potential of a DNA-based therapy of diabetes, we set out to investigate whether immunization with a DNA construct encoding for the HSP60 protein could modulate autoimmunity and prevent the onset of the disease. Surprisingly, not only the HSP60-containing construct but also the empty vector (pcDNA3) were capable of reducing the incidence of diabetes. Indeed, the CpG oligonucleotide motif present in the construct could by itself be used to inhibit the development of NOD diabetes. Despite the absence of HSP60, effective treatment was associated with specific immune effects on HSP60 autoreactivity: down-regulation of the spontaneous T cell proliferation to HSP60 and p277 and the induction of specific Abs to these molecules.

Materials and Methods

Mice

Female mice of the NOD/LtJ strain were raised and maintained under pathogen-free conditions in the Animal Breeding Center of this institute from breeders supplied by Dr. E. Leiter (The Jackson Laboratory, Bar Harbor, ME). These mice manifest insulitis beginning at about 1 mo of age, which progresses to overt hyperglycemia beginning at about 3 mo of age. The cumulative incidence of IDDM rises to 85% or greater by 6 mo of age. Female BALB/c mice were also raised in this Institute.

Construction of DNA vaccine

The full-length cDNA of human the *hsp60* gene was cloned into the pcDNA3 vector (Invitrogen, Leek, The Netherlands) under the control of the human CMV promotor. In brief, *hsp60* cDNA in pGEM was amplified using specific oligonucleotides containing restriction sites for the enzyme *Bam*HI or *Hin*dIII. The amplicon and the pcDNA3 vector were purified and digested with *Bam*HI/*Hin*dIII. The digested PCR product coding for HSP60 and the linearized pcDNA3 vector were ligated using T4 DNA

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[3] Abbreviations used in this paper: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; HSP60, 60-kDa heat shock protein; GAD, glutamic acid decarboxylase.

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ligase according to the standard protocol provided by the manufacturer. The ligated plasmid was transformed into *Escherichia coli* and later sequenced to confirm correct insertion of the cDNA (data not shown).

Plasmid preparation and injection

Large-scale plasmid DNA preparations were produced by the alkaline lysis method using Qiagen Plasmid Mega Prep (Qiagen, Santa Clarina, CA). DNA was ethanol-precipitated 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 found to be under acceptable levels for in vivo use (<0.02 EU/μg DNA).

Eight-week-old NOD or BALB/c females were injected with 100 μl of 10 mM cardiotoxin (Sigma, Rehovot, Israel) into the tibialis anterior muscle using a sterile 27-gauge syringe, witted with a plastic collar to limit needle penetration to 2 mm. Five, 12, and 19 days later the mice were injected with 100 μl (1 μg/μl) of the desired DNA vaccine or with PBS as controls.

Phosphorothioaete oligonucleotides were synthesized at the Oligonucleotide Synthesis Unit of the Weizmann Institute of Science (Rehovot, Israel). One hundred microliters (1 μg/μl) of each preparation was injected as described above following the same time schedule. The oligonucleotide CpG contains two 9-mer segments, which are present in the pcDNA3 ampicillin resistance gene. The control oligonucleotide GpC displays the same nucleotides with an inverted motif: oligonucleotide CpG, 5′-TCCATA A<u>CG</u>TTGCAAA<u>CG</u>TTCTG-3′; and oligonucleotide GpC, 5′-TCCATA A<u>GC</u>TTGCAAA<u>GC</u>TTCTG-3′.

Blood glucose

Hyperglycemia was defined as a blood glucose level exceeding 13 mM, tested using a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA).

Peptides and Ags

Peptides were synthesized by a standard F-moc procedure as previously described (5). The peptides were purified by reverse phase HPLC, and their compositions were confirmed by amino acid analysis. The p277 peptide used in this study was VLGGGVALLRVIPALDSLTPANED (2). Insulin and GAD were purchased from Sigma (Rehovot, Israel). Recombinant HSP60 was prepared as described previously (2). Con A was purchased from Sigma.

T cell proliferation

Groups of 8-wk-old female NOD mice received three weekly injections of PBS, pcDNA3, or pHSP60 as described. Four weeks after the last dose the spleens were removed, and the T cell proliferative responses were assayed in vitro in response to the T cell mitogen Con A, the p277 peptide, or HSP60 (13). Dose-response curves were made to establish optimal doses (not shown). The concentration of 10 μg/ml was chosen for the HSP60 protein, 1 μg/ml was chosen for p277, and 1.25 μg/ml was chosen for Con A to illustrate the results, because these concentrations produced the optimum response. T cell responses were detected by the incorporation of [*methyl*-^{3}H]thymidine added to the wells in quadruplicate cultures for the last 18 h of a 72-h culture. The stimulation index was computed as the ratio of the mean counts per minute of Ag- or mitogen-containing wells to that of control wells cultured without either. The SD from the mean counts per minute was always <10%. Background counts per minute in the absence of Ags ranged from 800-1500.

Cytokine assays

Spleen cells were prepared from 10-wk-old NOD females. The spleen cells were incubated in triplicate with medium alone or with increasing concentrations of CpG or GpC oligonucleotide. Supernatants were collected at 48 h. Cytokines in supernatants were detected by ELISA using PharMingen paired Abs (PharMingen, San Diego, CA), according to the PharMingen cytokine ELISA protocol. PharMingen recombinant mouse cytokines were used as standards for calibration curves. The concentrations of cytokines are shown as the mean nanograms per milliliter derived from calibration curves using recombinant cytokines as standards.

ELISA

Mouse sera were tested for Abs binding to the p277 peptide or HSP60 as previously described (6). Briefly, 10 μg/ml of the various Ags were applied to assay microplates (Maxisorp, Nunc, Roskilde, Denmark), and the plates were incubated with the test sera. The binding of Abs was detected using alkaline phosphatase-conjugated anti-mouse IgG or isotype-specific anti-mouse IgG1, IgG2a, or IgG2b (Jackson ImmunoResearch, West Grove, PA). A significant amount of Ab was defined as an $OD_{405\ nm}$ reading >0.25, which is 3 SD above the mean ELISA reading obtained using sera from 10 normal BALB/c mice.

Pancreas histology

Mice from each treatment group were killed at 6 mo of age, when almost all the nontreated mice or control NOD mice were sick. The pancreata were fixed in 10% buffered formalin, cut, and stained by standard hematoxylin and eosin, and the average degree of insulitis was assessed over 20 islets scored per pancreas. The islets where classified as clear when no infiltrate was detected, as mildly infiltrated when peri-insulitis or an intraislet infiltrate occupying <25% of the islet was detected, infiltrated when 25–50% of the islet was occupied by intraislet inflammatory cells, and heavily infiltrated when >50% of the islet was occupied.

Statistical significance

The InStat 2.01 program (GraphPad Software, San Diego, CA) was used for statistical analysis. Student's *t* test and the χ^2 test were conducted to assay significant differences between experimental and control groups.

Results

HSP60 DNA specifically immunizes BALB/c mice

To test whether the pcDNA3 plasmid containing human HSP60, here named pHSP60, was specifically immunogenic, female BALB/c mice were injected twice (days 5 and 23) i.m. with 100 μg of pcDNA3 or pHSP60 and assayed periodically for serum Abs. Fig. 1*A* shows that the BALB/c mice immunized with pHSP60 developed specific anti-HSP60 IgG Abs, whereas no Abs

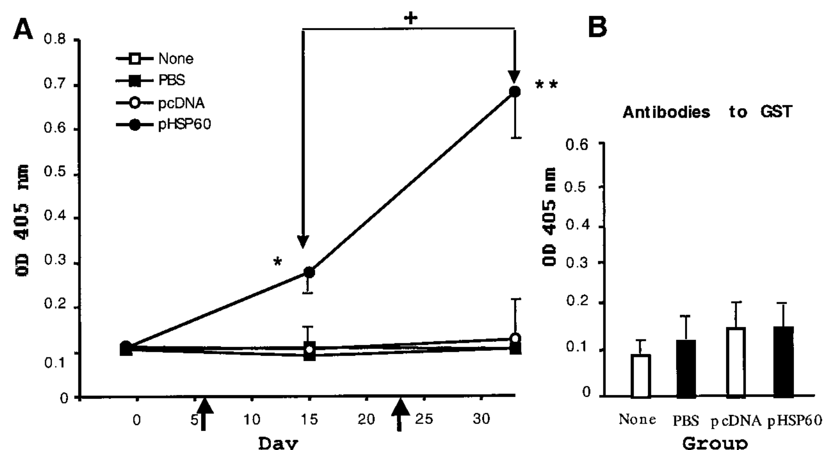

FIGURE 1. Abs to HSP60 in BALB/c mice immunized with pHSP60. Groups of five 8-wk-old female BALB/c were pretreated with cardiotoxin (day 0) and immunized i.m on days 5 and 23 with pHSP60, pcDNA3, or PBS or were left untreated. The arrows indicate the time of injections. Serum samples were taken before treatment with cardiotoxin and 10 days after each injection, and Abs (to HSP60 (*A*) and GST (*B*)) were measured by ELISA. The Abs to GST are shown 10 days after the last injection. The mean ± SD are shown. *, $p < 0.02$ compared with pcDNA3-treated mice. **, $p < 0.005$ compared with pcDNA3-treated mice. +, $p < 0.05$ compared with pHSP60-treated mice after the first dose of DNA.

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to the HSP60 protein could be detected in those animals immunized with pcDNA3. Anti-HSP60-specific Abs were detected as early as 14 days after a single DNA injection ($p < 0.02$ vs pcDNA3-vaccinated controls). A booster effect was evident 10 days after the second DNA injection ($p < 0.05$ vs the same group after the first dose; $p < 0.005$ vs pcDNA-vaccinated mice). The immune response induced by DNA vaccination with pHSP60 was specific; pHSP60 did not induce Abs to the nonrelated recombinant protein GST, as shown in Fig. 1*B*. These results demonstrate that the pHSP60 construct, but not the empty pcDNA3 vector, can induce in BALB/c mice significant amounts of specific Abs after one vaccination and increasing titers after boosting.

DNA injection inhibits the development of NOD diabetes

To test whether immunization with pHSP60 might modulate the development of spontaneous diabetes in NOD mice, we vaccinated groups of 8-wk-old female NOD mice, three times at weekly intervals, and followed their glucose levels. Fig. 2 shows the cumulative incidence of diabetes. Both untreated animals and those treated with PBS developed the expected incidence of diabetes for NOD females; ~90% of them were sick by the age of 6 mo. Those vaccinated with pHSP60 and, to our surprise, also those vaccinated with the empty pcDNA3 construct showed a significant reduction in the incidence of diabetes. Only about 41% of those treated with pHSP60 ($p < 0.002$) and 38% of those treated with pcDNA3 ($p < 0.001$) were diabetic at the age of 6 mo. Thus, DNA vaccination modulates the onset of diabetes by a mechanism that is not associated with the presence of the *hsp60* gene in the administered vector.

At the end of the observation period, when the mice where 6 mo old, pancreata were obtained for histological examination. Fig. 3 shows that 40–50% of the islets obtained from the nontreated or PBS-treated mice were heavily infiltrated, and only 5–10% of the islets were free from insulitis. In contrast, 50–70% of the islets obtained from DNA-treated mice were free from insulitis ($p < 0.001$ for both pcDNA3-injected mice and the pHSP60 group vs nontreated mice or those treated with PBS). The differences between the groups treated with pHSP60 and pcDNA3 were not significant.

Therefore, DNA vaccination, either with a vector encoding human HSP60 (pHSP60) or with an empty vector (pcDNA3), diminished the incidence of spontaneous diabetes in NOD females. This effect was accompanied by a significant increase in the number of pancreatic islets remaining free of insulitis.

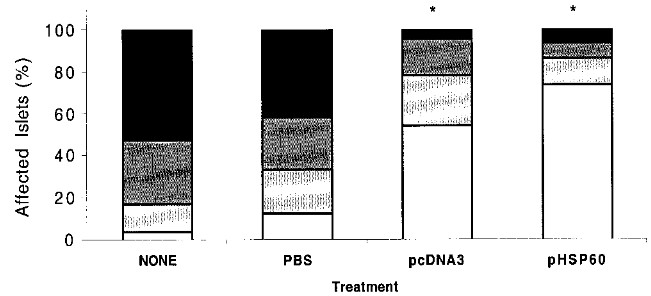

FIGURE 3. Reduction of insulitis by DNA vaccination. Eight-week-old NOD females were injected with PBS, pcDNA3, or pHSP60 as described in Fig. 2 or were left untreated, and their pancreata were removed at the age of 6 mo. The degree of insulitis was determined by scoring at least 20 islets in each pancreas. The islets are depicted as clear (open bars), peri-insulitis or an intraislet infiltrate occupying <25% of the islet (light gray bars), an intraislet infiltrate occupying 25–50% of the islet (dark gray bars), and an intraislet infiltrate occupying >50% of the islet (black bars). *, $p < 0.001$ compared with PBS-treated mice

Inhibition of T cell proliferation in response to HSP60 and p277 in DNA-vaccinated mice

The process leading to the onset of diabetes in NOD mice can be arrested by administration of peptide p277, derived from HSP60 (2). Successful treatment of NOD mice with peptide p277 is associated with the induction of specific Abs to p277 along with a decrease in the proliferation of T cells in response to HSP60 and p277 (6). We therefore assayed the splenocytes isolated from DNA-vaccinated or PBS-treated NOD mice to check their proliferative responses to p277 and HSP60. As shown in Fig. 4, PBS-treated NOD mice manifested spontaneous reactivities to HSP60 and p277. In contrast, splenocytes from the mice vaccinated with pcDNA3 or pHSP60 showed diminished reactivities to p277 ($p < 0.05$) and HSP60 ($p < 0.01$). However, T cells from both treated and nontreated mice showed similar reactivities to Con A (not shown), thus indicating that there was no general inhibition of T cell reactivity induced by DNA vaccination. These results suggested that treatment with plasmid DNA down-regulated the spontaneous proliferative response directed to HSP60 and p277 characteristic of the diabetogenic process in NOD mice.

Induction of Abs to p277 and HSP60 by DNA vaccination

The decrease in T cell proliferation in response to HSP60 and its peptide p277 observed in NOD mice protected from diabetes by

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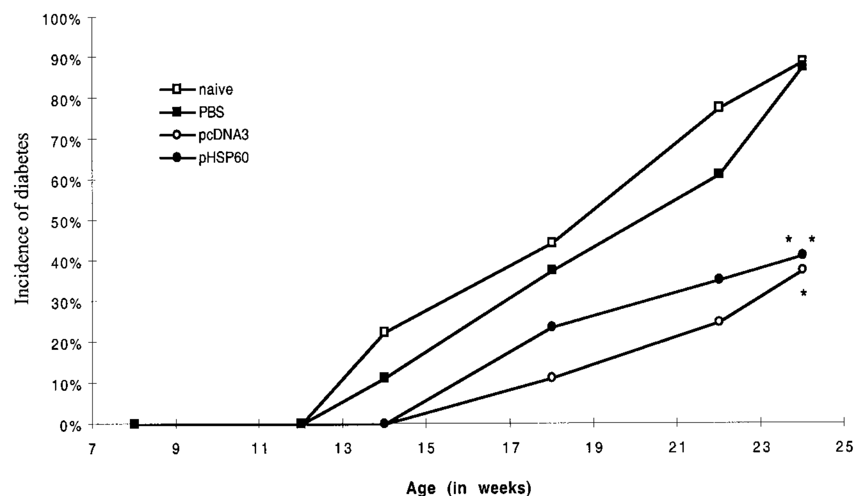


FIGURE 2. Prevention of NOD diabetes by DNA vaccination. Female NOD mice were allocated to groups of 17–18 mice each and were immunized with PBS, pcDNA3, or pHSP60. A control group was left untreated. The pcDNA3- and HSP60-vaccinated groups developed a significantly lower incidence of diabetes. *, $p < 0.001$; **, $p < 0.002$ (compared with PBS-treated mice).

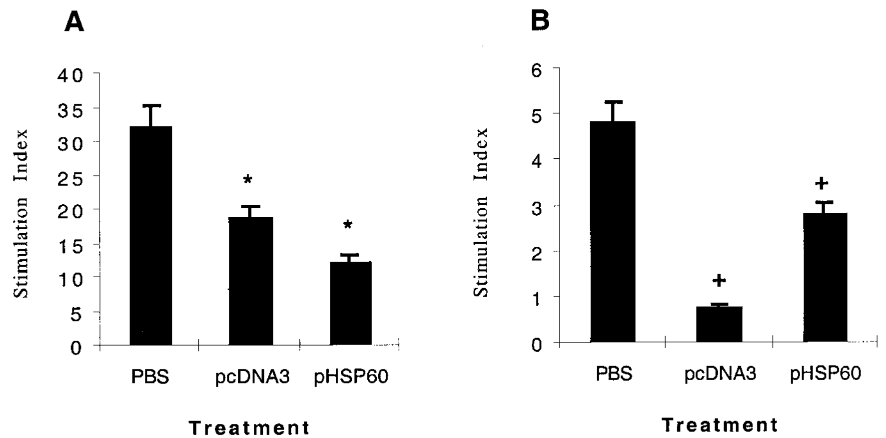
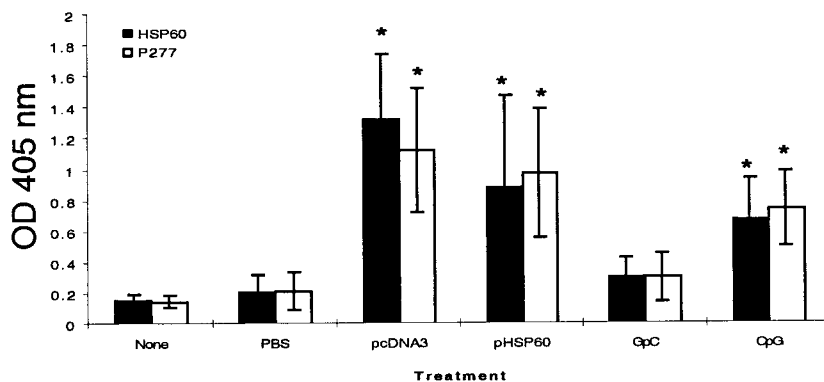


FIGURE 4. Proliferative responses to HSP60 and p277 in DNA-vaccinated mice. Groups of five 8-wk-old female NOD mice received three weekly injections of PBS, pcDNA3, or pHSP60. Four weeks later their spleens were removed, and the T cell proliferative responses were assayed after 72 h of stimulation with 10 μg/ml of human HSP60 (*A*) or 1 μg/ml of p277 (*B*). The results are expressed as the stimulation index (SI) ± SD compared with paired samples incubated with medium alone. *, $p < 0.01$; +, $p < 0.05$ (compared with PBS-treated mice).

treatment with p277 is associated with the induction of Abs directed to p277 (6). To determine whether the protective effect of DNA vaccination might be associated with the appearance of Abs to HSP60 and p277, we analyzed Ab responses in DNA-vaccinated animals 14 days after the last DNA injection, at the age of 14 wk. Fig. 5*A* shows that Abs to p277 were not detected in the sera of untreated or PBS-injected animals. The absence of Abs to p277 and HSP60 is expected in NOD mice of this age (3). We also did not detect Abs to p277 in BALB/c mice immunized with pHSP60, where the appearance of anti-HSP60 Abs was demonstrated (Fig. 7 and data not shown). However, NOD mice vaccinated with pHSP60 or pcDNA3 manifested significant levels of Abs to p277 ($p < 0.001$). Thus, inhibition of diabetes in NOD mice by DNA vaccination with either pcDNA3 or pHSP60 is associated with the induction of Abs to HSP60 and peptide p277, even though the pcDNA3 construct does not contain genetic material encoding HSP60.

CpG injection induces Abs to HSP60 and peptide p277

Bacterial DNA contains immunostimulatory sequences that are recognized by the immune system as danger signals and trigger a series of responses in cells of both the innate and adaptive immune system (14–16). The pcDNA3 vector contains the immunostimulatory CpG sequence in its ampicillin resistance gene (17). We



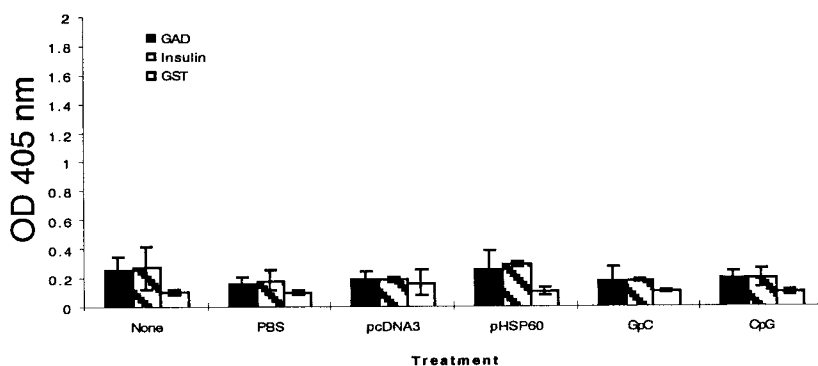


FIGURE 5. Induction of Abs to HSP60 and p277 by vaccination with plasmids or the CpG oligonucleotide. Groups of 18 NOD mice were treated with PBS, pcDNA3, pHSP60, or CpG or GpC oligonucleotides, and one group was left untreated. Two weeks after treatment individual sera were tested at a 1/100 dilution for the presence of specific Abs. *A*, Serum Abs to HSP60 and p277. *B*, Serum Abs to GAD, insulin, and GST. Data represent the mean ± SD for each group. *, $p < 0.001$ compared with PBS-treated mice

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therefore tested whether a DNA oligonucleotide with two CpG sequences could induce the production of specific Abs to HSP60 and p277 that followed vaccination with pcDNA3. As a control we used the oligonucleotide GpC, in which the CpG motifs were inverted.

Eight-week-old NOD mice were treated with oligonucleotide CpG or GpC, and Abs to HSP60, p277, GAD, insulin, and GST were assayed by ELISA at the age of 14 wk. As shown in Fig. 5*A*, treatment with the CpG oligonucleotide induced significant levels of Abs to HSP60 and p277 ($p < 0.002$). Moreover, the titer of Abs induced by CpG was also significant compared with the levels found in GpC-treated mice ($p < 0.02$). Because the GpC oligonucleotide failed to induce specific Abs to HSP60 or p277, the induction of these specific Abs by the pcDNA3 vector may be linked to the presence of the CpG motif. Thus, stimulation of the NOD immune system with an immunostimulatory sequence alone can trigger the production of specific autoantibodies to HSP60 and its peptide p277.

It was conceivable that the appearance of Abs to p277 and the HSP60 Ag reflected a polyclonal activation of IgG-secreting clones. Therefore, we assayed the sera from the different groups of mice for Abs to insulin, GAD, and the bacterial recombinant protein GST. Fig. 5*B* shows that the levels of Abs to insulin, GAD, or GST were essentially the same among the groups. Thus, administration of pHSP60, the pcDNA3 vector, or the CpG oligonucleotide induced specific Abs to HSP60 and p277. This indicates that the induction of specific Abs to HSP60 and p277 was not the result of polyclonal activation.

CpG injection inhibits NOD diabetes

To test whether administration of the CpG oligonucleotide can, like the pcDNA3 vector, modulate the development of spontaneous diabetes in NOD mice, we vaccinated groups of 8-wk-old female NOD mice three times at weekly intervals and followed their glucose levels. Fig. 6 shows the cumulative incidence of diabetes. Both untreated animals and those treated with PBS developed the expected incidence of diabetes for NOD females; about 85% of them were sick by the age of 6 mo. Furthermore, the incidence of diabetes was not affected in the group of mice vaccinated with the control oligonucleotide GpC. However, the mice vaccinated with CpG showed a significant reduction in the incidence of diabetes. Only about 40% of those treated with CpG ($p < 0.015$) were diabetic at the age of 6 mo.

Therefore, the protective effect observed after immunization with pcDNA3 could be reproduced with a DNA oligonucleotide containing CpG motifs. The mechanism involved is sequence specific, because the control oligonucleotide GpC did not have a significant effect on the incidence of the disease.

Ab isotypes

The isotype of specific serum Abs characterizes the phenotype of the immune response to an Ag; the Ab isotype reflects the in vivo integration of the complex network of cytokines that regulates the immune response. Abs of the IgG1 and IgG2b isotypes evidence a specific Th2 response, because they are dependent on IL-4 and TGF-β, respectively (18, 19). In contrast, Abs of the IgG2a isotype are IFN-γ dependent, and they reveal the existence of a Th1 response (18, 19). Therefore, we studied the isotypes of the Abs to p277 and HSP60 detected in DNA-vaccinated mice 14 days after the last injection. Fig. 7 shows that the Abs induced to HSP60 and p277 were predominantly of the IgG2b isotype ($p < 0.01$ vs IgG2a levels). There was also a slight increase in the levels of IgG1 Abs to HSP60 and p277, but this induction was significant compared with the amount of the IgG2a-specific Abs only in the group treated with the CpG oligonucleotide. Furthermore, there were no differences in the isotypes of the Abs among the pHSP60-, pcDNA3-, and CpG-treated NOD mice. Thus, the inhibition of diabetes induced by the DNA plasmids or by the CpG oligonucleotide in both cases was accompanied by the induction of Abs to HSP60 and p277 of the IgG2b isotype, characteristic of a Th2-type response.

Interestingly, there was a marked difference in the Abs induced in the BALB/c compared with the NOD mice. The BALB/c mice made Abs to HSP60 when they were vaccinated with pHSP60 but not following immunization with pcDNA3 (Fig. 1). Moreover, the Abs induced were mainly of the IgG1 subclass, and the BALB/c mice did not make Abs to p277. These results indicate strain-specific differences in the cytokine networks that regulate Ab secretion to the self-Ag HSP60.

Induction of IL-10 and IFN-γ secretion by the CpG oligonucleotide

To gain some insight into the cytokine effects of CpG, we assayed the amounts of IL-10, a Th2 cytokine, and IFN-γ, a Th1 cytokine, secreted by NOD spleen cells after CpG oligonucleotide stimulation in vitro. Because different cytokines are secreted in different physiological amounts, we included control groups of spleen cells incubated with Con A, a prototypic T cell mitogen. As shown in Fig. 8, the CpG oligonucleotide induced both IL-10 and IFN-γ

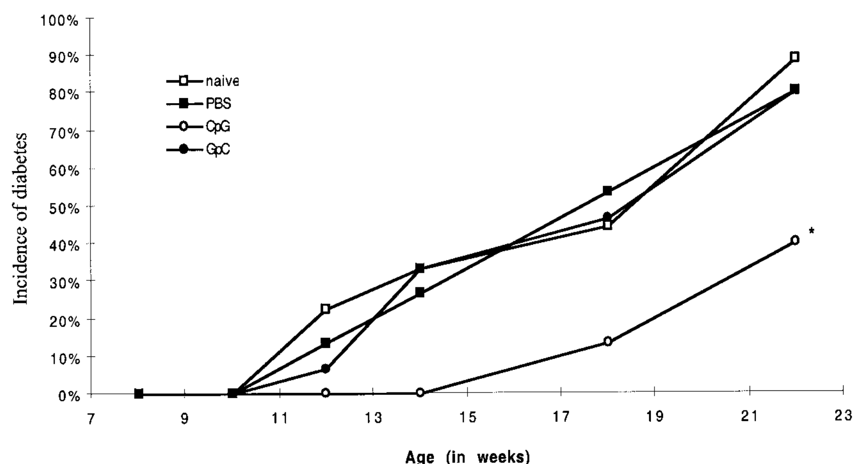

FIGURE 6. Prevention of NOD diabetes by CpG injection. Female NOD mice were allocated to groups of 15–18 mice each and were immunized with PBS, CpG, or GpC. A control group was left untreated. The CpG-vaccinated group developed a significantly lower incidence of diabetes. *, $p < 0.015$ compared with GpC-treated mice.

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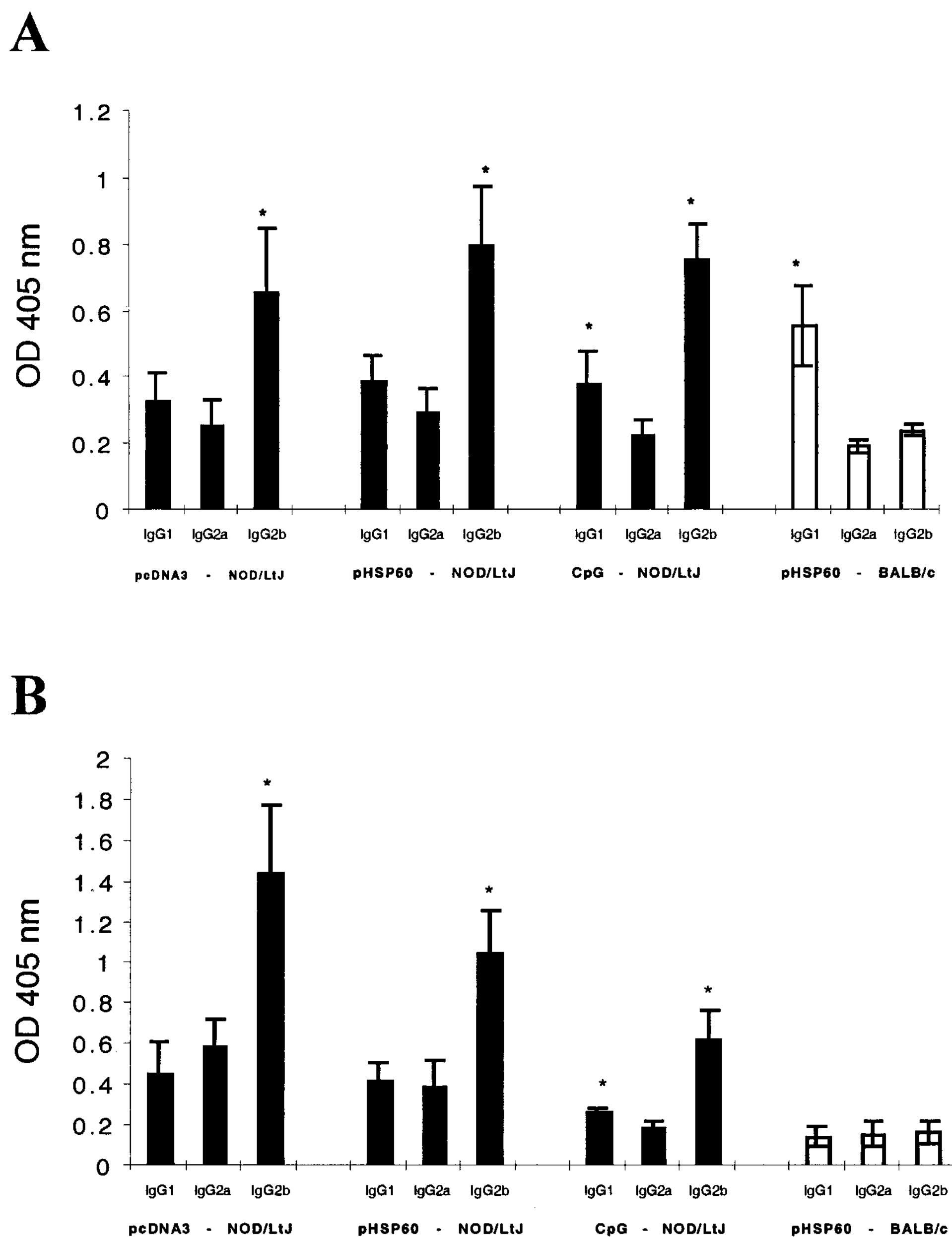

FIGURE 7. Isotypes of Abs to HSP60 and p277 induced by vaccination with plasmids or the CpG oligonucleotide. The isotypes of serum Abs to HSP60 (*A*) or p277 (*B*) from NOD (filled bars; $n = 18$) or BALB/c (open bars; $n = 5$) mice treated with pcDNA3, pHSP60, or the CpG oligonucleotide were determined 2 wk after the last vaccination. The isotypes of the Abs were tested at a 1/100 dilution of individual sera. Data are shown as the mean ± SD for each group. *, $p < 0.01$ compared with IgG2a levels in the same group.

production in NOD spleen cells in a dose-dependent manner. However, compared with the amount of cytokine released in response to Con A stimulation, the effect of CpG treatment seemed to be relatively more effective in stimulating IL-10 than in stimulating IFN-γ. CpG triggered a maximal release of IFN-γ of 7 ng/ml, about one-fourth of the IFN-γ released by Con A. In contrast, CpG induced the release of 1.5 ng/ml of IL-10, almost 10 times more than the amount induced by Con A stimulation.

Discussion

In this investigation we tested the effectiveness of DNA vaccination with HSP60 as a specific immunotherapy for NOD diabetes. We first ascertained the specific immunogenicity of the pHSP60 plasmid in BALB/c mice (Fig. 1). However, we made three unexpected observations when we used DNA treatment in NOD mice. First, the pcDNA3 plasmid, which did not contain any sequences encoding HSP60, was as effective in inhibiting the development of diabetes as was the pHSP60 plasmid (Figs. 2 and 3). Second, the pcDNA3 plasmid, despite the absence of HSP60, could still induce specific effects on the autoimmunity to HSP60 intrinsic to the NOD diabetogenic process: down-regulation of T cell proliferation and the induction of IgG2b Abs to whole HSP60 and its critical p277 peptide. Responses to other Ags implicated in NOD diabetes, GAD and insulin, were not detected (Figs. 4, 5, and 7). Third, the CpG oligonucleotide by itself could essentially reproduce the effects of the pcDNA3 plasmid on HSP60 autoimmunity and on diabetes (Figs. 5–7).

The CpG oligonucleotide is an immunostimulatory sequence present primarily in bacteria (14–16), and our results using CpG might explain one of the mechanisms by which bacterial infections

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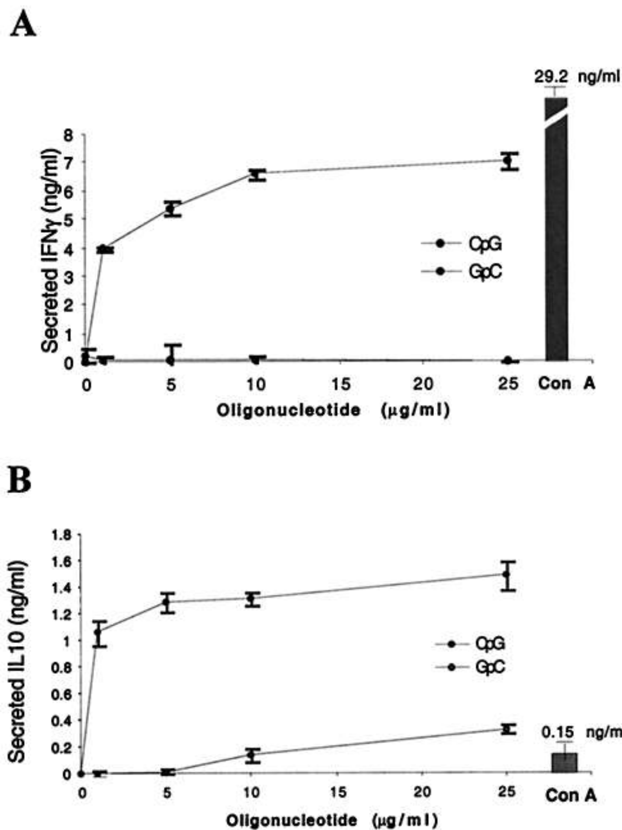

FIGURE 8. Production of IL-10 and IFN-γ in response to the CpG oligonucleotide in NOD spleen cell cultures. NOD spleen cells were incubated in triplicate with increasing concentrations of the CpG or GpC oligonucleotide for 48 h, and their supernatants were tested for the amount of IFN or IL-10 cytokine released. Control spleen cells were incubated with Con A (1.25 μg/ml) to determine the relative response magnitude. *A*, IFN-γ production. *B*, IL-10 production. The data are the mean $\pm$ SD of triplicate determinations. Three independent experiments produced similar results.

can inhibit the development of diabetes in NOD mice (20); bacterial infections may supply CpG stimulation.

It is noteworthy that the Abs to HSP60 and peptide p277 were of the IgG2b isotype (Fig. 7). The cytokine required for the production of IgG2b Abs is TGF-β, known for its suppressive effects (18, 19). TGF-β is a Th2-associated cytokine that has been shown to protect NOD mice from diabetes (21). Although DNA vaccination also induced HSP60- and p277-specific Abs of the IgG2a subclass, considered to be IFN-γ dependent, the amount of these Abs was significantly less than the amount of IgG2b Abs. Thus, the cytokine balance was weighted more toward a Th2 response, suggesting that the therapeutic effects of DNA might be related to the activation of Th2-like T cells. Activation of Th2-like T cells was also described when spontaneous diabetes of NOD was prevented by the administration of the HSP60-derived peptides p12 or p277 (6, 22). Such T cells might suppress the Th1 T cells thought to be involved in the damage to the β-cells (22). Further studies are needed to directly confirm the involvement of particular cytokines.

The origin of the Abs to HSP60 and p277 in mice protected from diabetes by pcDNA3 or treatment with the CpG oligonucleotide or induced by the CpG oligonucleotide (Fig. 5*A*) remains to be clarified. Clearly, this effect is strain specific; BALB/c mice did not produce these Abs when injected with pcDNA3 (Fig. 1). NOD mice seem to manifest a spontaneous autoimmune response to HSP60 and p277, which is depicted in Fig. 4. Immunity to HSP60 and p277 manifests as a peak of T cell reactivity before the onset of the disease (13, 23). Months after the onset of overt diabetes, Abs to HSP60 and p277 can be detected (3). After DNA treatment the T cell proliferative response was diminished and replaced by the production of Abs, mostly IgG2b. This suggests that the pre-existing autoimmune response, spontaneously arising in NOD mice, changes its phenotype after activation by bacterial DNA or CpG motifs, leading to the induction of Th2-like, IgG2b Abs. Similarly, prevention of NOD diabetes by idiotypic induction of lupus with a mAb was also associated with the induction of specific Abs to HSP60 and p277 (3). Thus, even when the induction of Abs to HSP60 and p277 does not result from specific immunization, the appearance of such Abs seems to serve as an indicator of the arrest of the diabetogenic process.

The effect of bacterial DNA on autoimmune inflammation is intriguing. Bacterial DNA contains immunostimulatory motifs consisting of a central unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines (24). CpG motifs are under-represented in mammalian genomes due to a combination of CpG suppression and CpG methylation (25). This motif stimulates Th1 responses in vivo (25). However, modulation of autoimmune conditions by bacterial DNA has been already reported. Gilkeson et al. demonstrated that immunization with bacterial DNA can modulate renal disease in autoimmune NZB/NZW mice, while calf thymus DNA was not effective (26). Furthermore, improvement in renal disease was associated with the induction of Abs to glomerular Ags immediately after immunization (26). Boccacio and her colleagues have reported that noncoding plasmid DNA can inhibit experimental allergic encephalomyelitis while activating IFN-γ in vitro (17). How could a motif classically associated with a Th1 phenotype be associated with the inhibition of diabetes, known to be Th2 mediated? This paradox has been observed repeatedly in animal models of spontaneous diabetes. PolyI:C, IFN-γ, IL-12, TNF-α, and IL-18, all of which are well-known inducers or mediators of Th1 responses, were shown to decrease insulitis and prevent diabetes (27–32). Furthermore, in the case of IL-18, protection was associated with systemic activation of Th1-type immunity together with a shift to a Th2 phenotype of the cells infiltrating the islets (29). Therefore, nonspecific stimulation of the NOD immune system, even by Th1 inducers, is able to reset the ongoing immune response to islet Ags and arrest the diabetogenic process. When we analyzed in vitro the effect of the CpG oligonucleotide on NOD spleen cells, it clearly induced IFN-γ and IL-10 in a dose-dependent manner (Fig. 8). However, when the amounts of cytokine produced by CpG were compared with those triggered by Con A stimulation, it was evident that the effect of the CpG motif favored the release of IL-10. Perhaps the prominence of IL-10 is important in modulating the diabetogenic process. Other explanations are possible, and more work must be performed to clarify the CpG effect. Nevertheless, the present results encourage the study of therapies aimed to activate pre-existing regulatory networks for the management of IDDM (13, 33).

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References

1. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell 85: 291.*
2. 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.*
3. Krause, I., Y. Tomer, D. Elias, M. Blank, B. Gilburd, I. R. Cohen, and Y. Shoenfeld. 1999. Inhibition of diabetes in NOD mice by idiotypic induction of SLE. *J. Autoimmun. 13:49.*

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4. Elias, D., and I. R. Cohen. 1995. Treatment of autoimmune diabetes and insulitis in NOD mice with heat shock protein 60 peptide p277. *Diabetes 44:1132.*
5. Elias, D., and I. R. Cohen. 1994. Peptide therapy for diabetes in NOD mice. *Lancet 343:704.*
6. 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 β-cell antigens. *Diabetes 46:758.*
7. Ablamunits, V., D. Elias, T. Reshef, and I. R. Cohen. 1998. Islet T cells secreting IFN-γ in NOD mouse diabetes: arrest by p277 peptide treatment. *J. Autoimmun. 11:73.*
8. 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.*
9. Stevenson, F. K., C. J. Link, Jr., A. Traynor, H. Yu, and M. Corr. 1999. DNA vaccination against multiple myeloma. *Semin. Hematol. 36:38.*
10. Waisman, A., P. J. Ruiz, D. L. Hirschberg, A. Gelman, J. R. Oksenberg, S. Brocke, F. Mor, I. R. Cohen, and L. Steinman. 1996. Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity. *Nat. Med. 2:899*.
11. Wolff, J. A., J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani. 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet. 1:363.*
12. Hassett, D. E., J. Zhang, M. Slifka, and J. L. Whitton. 2000. Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by conventional immunization. *J. Virol. 74:2620.*
13. Elias, D., Y. Tikochinski, G. Frankel, and I. R. Cohen. 1999. Regulation of NOD mouse autoimmune diabetes by T cells that recognize a TCR CDR3 peptide. *Int. Immunol. 11:957.*
14. Lipford, G. B., K. Heeg, and H. Wagner. 1998. Bacterial DNA as immune cell activator. *Trends Microbiol. 6:496.*
15. Krieg, A. M., A. K. Yi, J. Schorr, and H. L. Davis. 1998. The role of CpG dinucleotides in DNA vaccines. *Trends Microbiol. 6:23.*
16. Krieg, A. M. 1999. CpG DNA: a novel immunomodulator. *Trends Microbiol. 7:64*.
17. Boccaccio, G. L., F. Mor, and L. Steinman. 1999. Non-coding plasmid DNA induces IFN-γ in vivo and suppresses autoimmune encephalomyelitis. *Int. Immunol. 11:289.*
18. McIntyre, T. M., D. R. Klinman, P. Rothman, M. Lugo, J. R. Dasch, J. J. Mond, and C. M. Snapper. 1993. Transforming growth factor β1 selectivity stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *J. Exp. Med. 177:1031.*
19. Snapper, C. M., and J. J. Mond. 1993. Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today 14:15.*
20. 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.*
21. King, C., J. Davies, R. Mueller, M. S. Lee, T. Krahl, B. Yeung, E. O'Connor, and N. Sarvetnick. 1998. TGF-β1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. *Immunity 8:601.*
22. 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.*
23. 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 β-cell target antigen of autoimmune T cells. *J. Autoimmun. 9:159.*
24. Klinman, D. M., G. Yamshchikov, and Y. Ishigatsubo. 1997. Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol. 158:3635.*
25. Klinman, D. M., A. K. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg. 1996. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ. *Proc. Natl. Acad. Sci. USA 93:2879.*
26. Gilkeson, G. S., P. Ruiz, A. M. Pippen, A. L. Alexander, J. B. Lefkowith, and D. S. Pisetsky. 1996. Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J. Exp. Med. 183:1389.*
27. Campbell, I. L., L. Oxbrow, and L. C. Harrison. 1991. Reduction in insulitis following administration of IFN-γ and TNF-α in the NOD mouse. *J. Autoimmun. 4:249.*
28. Nicoletti, F., P. Zaccone, R. Di Marco, G. Magro, S. Grasso, F. Stivala, G. Calori, L. Mughini, P. L. Meroni, and G. Garotta. 1998. Paradoxical antidiabetogenic effect of γ-interferon in DP-BB rats. *Diabetes 47:32.*
29. Rothe, H., A. Hausmann, K. Casteels, H. Okamura, M. Kurimoto, V. Burkart, C. Mathieu, and H. Kolb. 1999. IL-18 inhibits diabetes development in nonobese diabetic mice by counterregulation of Th1-dependent destructive insulitis. *J. Immunol. 163:1230.*
30. Serreze, D. V., K. Hamaguchi, and E. H. Leiter. 1989. Immunostimulation circumvents diabetes in NOD/Lt mice. *J. Autoimmun. 2:759.*
31. Sobel, D. O., D. Goyal, B. Ahvazi, J. W. Yoon, Y. H. Chung, A. Bagg, and D. M. Harlan. 1998. Low dose poly I:C prevents diabetes in the diabetes prone BB rat. *J. Autoimmun. 11:343.*
32. Yang, X. D., R. Tisch, S. M. Singer, Z. A. Cao, R. S. Liblau, R. D. Schreiber, and H. O. McDevitt. 1994. Effect of tumor necrosis factor α on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J. Exp. Med. 180:995.*
33. Cohen, I. R. 2000. *Tending Adam's Garden: Evolving the Cognitive Immune Self*. Academic Press, London.

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