Hsp60 Peptide Therapy of NOD Mouse Diabetes Induces a Th2 Cytokine Burst and Downregulates Autoimmunity to Various β-Cell Antigens

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A peptide of the human 60-kDa heat-shock protein (hsp60), designated p277, was found to be useful as a therapeutic agent to arrest the autoimmune process responsible for diabetes in nonobese diabetic (NOD) mice. The effectiveness of peptide treatment was associated with the induction of peptide-specific antibodies of the IgG1 but not of the IgG2a isotype, suggesting the possibility that a Th2-type response may have been induced. We now report that the effectiveness of p277 treatment is associated with the transient activation of anti-p277 splenic T-cells that produce the Th2 cytokines interleukin-4 (IL-4) and IL-10. The Th2 response to p277 was associated with reduced Th1-type autoimmunity to hsp60 and to two other target antigens associated with diabetes: GAD and insulin. The Th2 shift appeared to be relatively specific; spontaneous T-cell reactivity to a bacterial antigen peptide remained in the Th1 mode in the p277-treated mice. Moreover, treatment with the bacterial peptide did not induce a change in cytokine profile, and it did not affect progression of the disease. Thus, effective peptide treatment of the diabetogenic process associated with the induction of antibodies may be explained by selective and transient activation of Th2 autoimmune reactivity.
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The spontaneous autoimmune process resulting in diabetes in the nonobese diabetic (NOD) mouse is first detectable as mild insulitis beginning at about 1 month of age. In most female mice, insulitis progresses to a penetrating intra-islet infiltrate that leads to β-cell damage and overt IDDM that surfaces at about 4–5 months of age (1). Adoptive transfer experiments have led to the conclusion that autoimmune T-cells of both $CD4^+$ and $CD8^+$ subsets are involved in the disease process (2). However, $CD4^+$ T-cells on their own have been reported to be able to trigger diabetes (3). Two characteristics of the autoimmune $CD4^+$ T-cells seem to be important: the type of cytokines produced by the T-cells and the antigens they recognize. Mouse $CD4^+$ T-cells can be divided into two functional groups by the cytokines they secrete when activated (4): Th1 cells secrete interleukin-2 (IL-2), which induces T-cell proliferation, and proinflammatory cytokines such as γ-interferon (IFN-γ), which mediates tissue inflammation and stimulates B-cells to produce IgG2a antibodies; Th2 cells, in contrast, secrete cytokines such as IL-4 and IL-10 that can downregulate Th1 cells. IL-4 helps B-cells secrete antibodies of the IgG1 isotype and suppresses the production of Th1 proinflammatory cytokines (5). IL-10 indirectly inhibits Th1 activation by affecting antigen-presentation and proinflammatory cytokine production by macrophages (6). Because Th1-type, but not Th2-type, T-cell clones can transfer diabetes (7,8), it has been suggested that the diabetogenic autoimmune process might be aborted by inducing a shift in the relevant autoimmune T-cell activity from the Th1 type to the Th2 type (9).

With regard to target antigens, three defined antigens (GAD, insulin, hsp60) have been reported to influence the diabetogenic process (10). The administration of GAD by intrathymic injection (11), nasal inhalation (12), or intravenous injection at 3 weeks of age (13) was found to inhibit the development of T-cell reactivity to GAD and to other self-antigens and to prevent diabetes. Administration of insulin to young NOD mice was also reported to affect the development of disease (14). In addition to these antigens, we have shown that autoimmunity to hsp60 has a functional role in diabetes: NOD mice spontaneously develop T-cells responsive to the hsp60 peptide p277, and these T-cells can adoptively transfer diabetes or, when attenuated, can vaccinate mice against diabetes (15). A single, subcutaneous administration of peptide p277 in oil either early at 4–6 weeks of age (15) or very late at 12–17 weeks in the autoimmune process can arrest the disease (16,17).

Peptide p277 was also found to influence toxin-induced diabetes. Mice of the C57BL/*KsJ* strain can be induced to develop a type of autoimmune diabetes about 3 months after administration of a very low dose of the β-cell toxin, streptozotocin (18). This form of diabetes could also be treated with peptide p277 administered after the toxic insult. In contrast to p277 treatment, the treatment of mice with an immunogenic GAD peptide failed to arrest the development

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cpm, counts per minute; Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; hsp60, 60-kDa heat-shock protein; IL, interleukin; IFA, incomplete Freund's adjuvant; IFN-γ, γ-interferon; mAb, monoclonal antibodies; MT, *Mycobacterium* tuberculosis; NOD, nonobese diabetic; OD, optical density; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β.

of diabetes (19). Interestingly, the effectiveness of p277 was associated with the induction of specific antibodies of the IgG1 and IgG2b isotypes, suggesting the possibility of a shift in the cytokine profile (19).

The present study was done to investigate whether a change in T-cell cytokine secretion was indeed associated with the response to p277 treatment, and whether the effect of p277 treatment might spread to the responses to other autoantigens.

RESEARCH DESIGN AND METHODS

Mice. Inbred NOD/Lt mice were raised and maintained under specific pathogen-free (SPF) conditions at the animal breeding center of this institute from a breeding nucleus originally provided by Dr. E. Leiter (Jackson ImmunoResearch Laboratories, Bar Harbor, ME). The onset of clinical IDDM in the female mice in this colony begins at about 3.5 months of age and reaches a cumulative incidence of 80% or greater by 8 months of age. Female mice were used in these studies.

Peptides and antigens. Peptides were synthesized by standard Fmoc using an automated ABIMED synthesizer AMS422 (Langenfeld, Germany) as described (16,17). The peptides were purified by reverse-phase high-performance liquid chromatography (HPLC), and their compositions were confirmed by amino acid analysis. The sequence of p277 used in the experiments shown here was VLGGGVALLRVIPALDSLTPANED. This peptide is substituted at positions 6 and 11 with valine (Val) in place of the cystein (Cys) in the native sequence. Substitution of the two Cys residues by Val enhances greatly the stability of the peptide without affecting its immunological activity: the V-substituted peptide is completely cross-reactive with the native peptide by T-cell and antibody assays, and both peptides have the same therapeutic effect on diabetes (D.A., A.M., V.A., O.S.B., P.C., S.K.-W., I.R.C., unpublished observations). All the effects shown in this paper have been repeated with either variant of p277. The sequence of *Mycobacterium* tuberculosis (MT)-p278 is EGDEATGANIVKVALEA. The sequence of GAD peptide 34 (residues 500–528; GADp34) is IPPSLRTLEDNEERMSRLSK (11). Bovine insulin was purchased from Sigma Chemical (St. Louis, MO). Recombinant hsp60 (15) and recombinant GAD65 (13) were prepared as described. The GAD65 gene was kindly supplied by Dr. D. Kaufman, (UCLA, Los Angeles, CA). Concanavalin A (Con A) was purchased from Sigma Israel Chemicals.

Cytokine assays. At various time points, groups of five treated or naïve mice were killed, their spleens were removed, and the spleen cells were pooled. The spleen cells were incubated in triplicate with medium alone or with peptides (10 μg/ml) as described (15). Supernatants were collected at 24 h (for IL-2 and IL-4 secretion) and at 72 h (for IL-10 and IFN-γ secretion). The presence of the cytokines in the culture supernatants was quantitated by enzyme-linked immunosorbent assay (ELISA) using Pharmingen paired antibodies (purchased from Pharmingen, San Diego, CA), according to the Pharmingen cytokine ELISA protocol. Pharmingen recombinant mouse cytokines were used as standards for calibration curves. Briefly, flat-bottom 96-well microtiter plates were coated with rat anti-mouse cytokine monoclonal antibodies (mAbs) for 18 h at 4°C, and the culture supernatants or recombinant mouse cytokines were added for 18 h at 4°C. The plates were washed, and biotinylated rat anti-mouse cytokine mAbs were added for 45 min at room temperature, then extensively washed, and avidin-alkaline phosphatase was added for 30 min. The plates were washed, a chromogen substrate was added, and samples were read at 405 nm in an ELISA reader (Anthos Labtec, Salzburg, Austria). The concentrations of cytokines are shown as the mean picogram per milliliter or nanogram per milliliter derived from calibration curves using recombinant cytokines as standards. The lower limits of ELISA sensitivity for the reagents used in the experiment shown in Fig. 4 were as follows: IL-4, 0.25 pg/ml; IL-2, 1 pg/ml; IL-10, 1 pg/ml; IFN-γ, 100 pg/ml. The lower limits of sensitivity for the reagents used in Table 3 were as follows: IL-4, 0.2 ng/ml; IL-10, 1 ng/ml. The differences in sensitivity between the experiments were due to biologic differences in the different cytokine standards used in these experiments.

Peptide treatment and T-cell proliferation. Groups of NOD mice were treated at the age of 12 weeks with 100 μg of peptides p277 or MT-p278 emulsified in oil (incomplete Freund's adjuvant [IFA]; Difco, Detroit, MI) or with phosphate-buffered saline (PBS) emulsified in oil as described (16,17). Five weeks later, the spleens of some of the mice were removed and the T-cell proliferative responses were assayed in vitro to the T-cell mitogen Con A (1.25 μg/ml; Sigma) or to various peptides (10 μg/ml) using a standard assay (20). Dose-response curves were done using concentrations of peptides up to 25 μg/ml (not shown). The concentration of 10 μg/ml was chosen to illustrate the results because this concentration produced the optimum response. The T-cell responses were detected by the incorporation of [^{3}H]thymidine added to the wells in quadruplicate cultures for the last 18 h of a 3-day culture. The stimulation index (SI) was computed as the ratio of the mean counts per minute (cpm) of antigen-containing wells to control wells cultured without antigens or Con A. The standard deviations from the mean cpm were always <10%. Background cpm, in the absence of antigens, was 800–1,500 cpm. Some of the treated mice were scored for the development of diabetes by 8 months of age as described (16).

Antibody assays. Groups of NOD mice, 12 weeks old, were treated with p277 in oil or with PBS in oil. Five weeks later, the mice were bled individually and their sera were diluted up to 1:500 and tested for antibodies to recombinant GAD65 (13), to recombinant human hsp60 (20), to bovine insulin, or to peptide p277 in an ELISA assay as described (20). Briefly, 10 μg of the various antigens were applied to assay plates (Maxisorp, Nunc, Røskilde, Denmark) suitable for the binding of peptides, and the plates were incubated with the test sera. The binding of antibodies to the adherent antigens was detected using alkaline phosphatase conjugated anti-mouse IgG + IgM or isotype-specific anti-mouse IgG1, IgG2a, or IgG2b (Jackson ImmunoResearch Laboratories, West Grove, PA). A significant amount of antibody was defined as an optical density (OD) 405 nm reading of >0.25, which is 3 SD over the mean ELISA reading obtained in the sera of 10 normal BALB/c mice. The figures show the results obtained at the 1:50 dilution because this was the serum dilution that produced the highest incidence of positive antibodies for all of the antigens tested.

TABLE 1
Peptide p277 treatment of NOD diabetes is specific

Peptide treatment	Incidence of diabetes at 8 months of age	
	Normoglycemic	Hyperglycemic
None	3	97
PBS	10	90
MT-p278	17*	83*
p277	53†	47†

Data are %. Groups of 30 NOD female mice were injected subcutaneously at 12 weeks of age with peptides (100 μg) MT-p278 or p277, or with PBS emulsified in IFA. The mice were examined monthly and scored for the development of hyperglycemia by 8 months of age using a glucose analyzer as described (17). Blood glucose concentrations in the hyperglycemic mice at 8 months were all >35 mmol/l. *P = 0.71 compared to PBS-treated mice; †P = 0.006 compared to MT-p278–treated mice.

RESULTS

Specificity of p277 treatment. Table 1 shows the results of treating NOD female mice with peptides p277 or MT-p278 in IFA, or with PBS in IFA. The mice were followed until the age of 8 months and marked for the development of hyperglycemia. As we have reported earlier, the administration of p277 was associated with a significant decrease in the cumulative incidence of mice developing diabetes (47%), compared with the control-untreated or PBS-treated mice (90–97%). Treatment with the immunogenic peptide MT-p278 did not lead to a significant reduction in disease (83%). Thus, the inhibitory effect of p277 peptide treatment on the spontaneous development of diabetes was specific.

Effect of p277 treatment on autoantibodies. The development of diabetes in NOD mice has been associated with spontaneous autoantibodies to self-antigens such as hsp60 (20), GAD (13), and insulin (20). We therefore wished to see the effect of peptide p277 on these spontaneous autoantibodies and on the induction of antibodies to p277. Figure 1 shows the incidence of mice with autoantibodies in the sham-treated and p277-treated groups. The control-treated NOD mice mani-

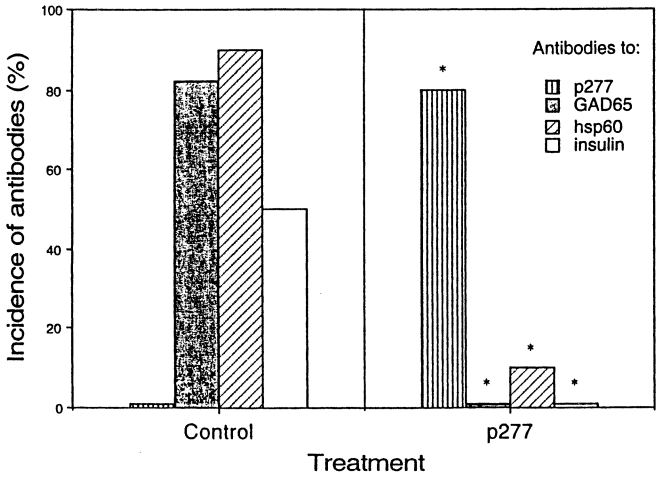

FIG. 1. Treatment with p277 induces antibodies to p277 and reduces antibodies to GAD, to insulin, and to intact hsp60. Groups of 10 NOD mice, 3 months old, were treated with p277 in oil (*A*) or with PBS in oil (*B*). Five weeks later, the mice were bled individually, and their sera, diluted 1:50, were tested for antibodies to recombinant GAD, to recombinant human hsp60, to bovine insulin, or to peptide p277 in an ELISA assay. A significant amount of antibody was defined as an OD 405-nm reading of >0.25, which is 3 SD over the mean ELISA reading obtained in the sera of 10 normal BALB/c mice. The results are shown as the incidence of mice positive for antibodies to the various antigens. A sample of actual OD 405-nm readings can be seen in Fig. 2. *$P < 0.01$ by χ^2 test.

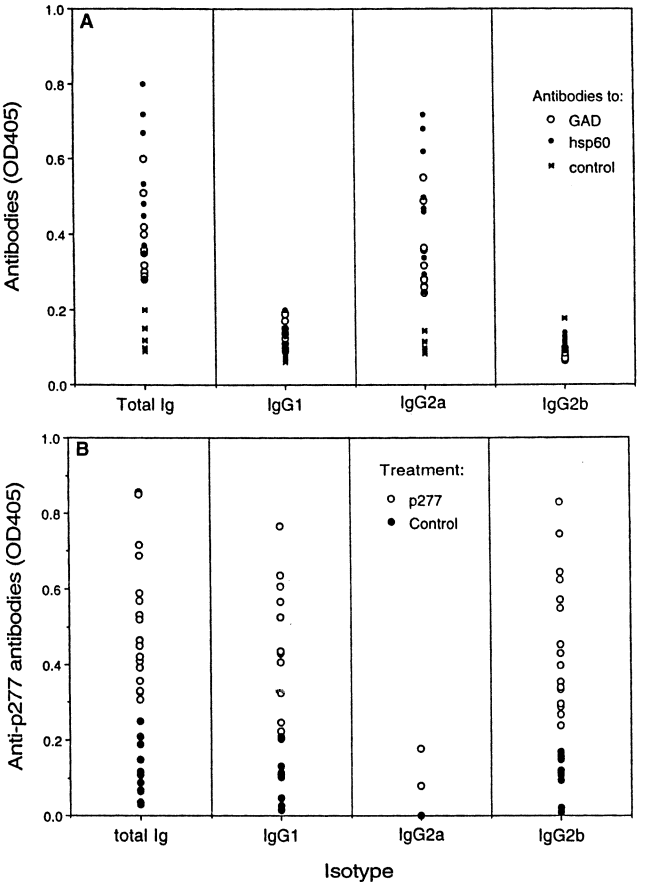

FIG. 2. Antibody isotypes before and after p277 therapy. Groups of NOD mice, 3 months old, were treated with p277 or with PBS in oil (control). The sera of individual mice in each treatment group were assayed 5 weeks after treatment for the isotypes of their antibodies to intact hsp60 or to GAD before treatment (*A*) or for the isotypes of their antibodies to p277 after treatment (*B*) (12–15 mice per group). The antibody isotypes were detected using an ELISA assay with isotype-specific developing antibody reagents. Because of superimposition of the circles, the three circles in the IgG2a column in Fig. 2*B* represent 12 mice.

fested a high incidence of antibodies to GAD (80%) and to hsp60 (90%), and a moderate incidence of anti-insulin antibodies (50%); there were no spontaneous antibodies to peptide p277. Similar results were obtained in untreated control NOD mice (not shown). In contrast, the p277-treated mice showed a marked increase (80%) in the incidence of antibodies to p277 ($P < 0.01$). Nevertheless, the p277-treated mice showed a significant reduction in the incidence of autoantibodies to GAD, whole hsp60, and insulin ($P < 0.01$).

Analysis of the isotypes of the antibodies produced before and after p277 therapy were done to extend the observation that effective treatment might be associated with certain antibody isotypes (19). Figure 2*A* shows that the spontaneous autoantibodies to GAD and to whole hsp60, which were present before treatment with p277, were of the IgG2a class, antibodies dependent on T-cells of the Th1 type that secrete IFN-γ (21). None of the anti-GAD or anti-hsp60 antibodies were of the IgG1 isotype associated with the Th2 cytokine IL-4 (21). In contrast, analysis of the antibody isotypes of the anti-p277 antibodies induced by treatment (Fig. 2*B*) showed them to be mainly of the IgG1 and IgG2b classes. There were significantly fewer Th1-type IgG2a antibodies to p277 induced by p277 therapy ($P < 0.01$).

Effects of p277 treatment on T-cell proliferation. To test the effects of p277-peptide treatment on T-cell proliferation, spleen cells were obtained from parallel groups of p277-treated and sham-treated mice and tested at 17 weeks of age (5 weeks after treatment) for their T-cell proliferative responses to peptide p277 of hsp60, to peptide p34 of GAD (11), or to peptide MT-p278 of mycobacterial hsp60. Figure 3 shows that the sham-treated NOD mice manifested spontaneous reactivity to all three peptides and to Con A. Untreated control NOD mice manifested similar spontaneous T-cell reactivities (not shown). In contrast, the p277-treated NOD mice showed a specific fall ($P < 0.01$) in their T-cell proliferative responses to the hsp60 and GAD peptides. The control responses to MT-p278 and to Con A remained intact.

Effects of p277 treatment on cytokines. The above results indicated that p277 treatment, which modulates the autoimmune process, was associated, on the one hand, with induction of IgG1 and IgG2b peptide-specific antibodies and, on the other hand, with inhibition of spontaneous IgG2a antibodies and T-cell proliferation to other antigens. The question, therefore, was whether these immunological changes could be explained by a change in cytokine profile induced by p277.

Experiments were done to document the natural history of representative Th1 and Th2 cytokines produced by anti-p277 T-cells during progression of the autoimmune process and to detect any effects of p277-peptide or sham treatment on the cytokines produced by these spontaneously appearing anti-p277 T-cells. Table 2 shows the concentrations of a key Th1 cytokine, IFN-γ, and of two Th2 cytokines, IL-4 and IL-10, pro-

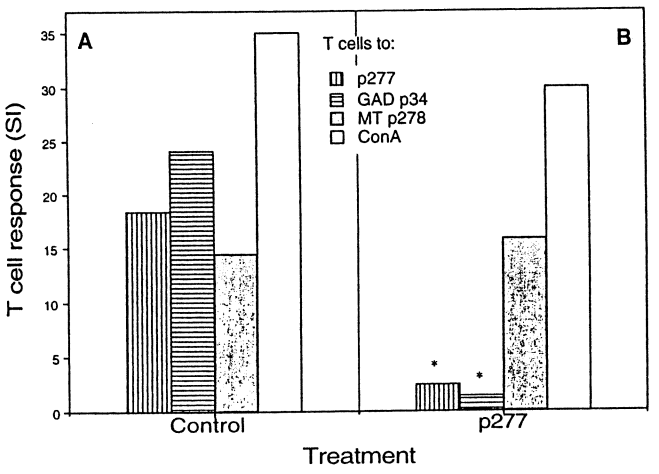

FIG. 3. Spontaneous T-cell proliferative responses to GAD and hsp60 peptides are specifically reduced by p277 therapy. Groups of NOD female mice were treated at the age of 3 months with 100 μg of peptide p277 (*A*) emulsified in mineral oil or with PBS emulsified in mineral oil (*B*) as described (16,17). Five weeks later, the spleens of the mice were removed and the T-cell proliferative responses were assayed in vitro to the T-cell mitogen Con A or to three different peptides.

TABLE 2
Cytokines produced by anti-p277 T-cells before and after treatment with peptide p277 at 12 weeks of age

	IFN-γ	IL-4	IL-10
Before treatment			
5 weeks	6	0	0
12 weeks	80	0	0
After treatment			
17 weeks			
None	60	0	0.35
Sham	50	0	0.5
p277	0.9*	12.8*	7*
28 weeks			
None	17	0	1
Sham	14	0	1.2
p277	0.6*	0.7	1.6

Data are in nanograms per milliliter. Groups of five female NOD mice were killed at various ages, and the cytokines produced in response to incubation in vitro with peptide p277 were measured. Some of the groups of mice were treated with peptide p277 (100 μg) or with PBS in IFA at the age of 12 weeks. The SE were <10% of the mean in all groups. "0" signifies a concentration below the lower level of detection; 10 p277-treated and 10 sham-treated mice were followed up to the age of 32 weeks to determine the incidence of hyperglycemia and mortality resulting from diabetes. The sham-treated mice manifested an incidence of hyperglycemia of 9/10, and 7/10 of the mice died of severe diabetes. In contrast, the p277-treated mice manifested an incidence of diabetes of 3/10 and an incidence of death of 1/10 ($P < 0.05$).*$P < 0.01$ compared with untreated or sham-treated mice.

duced by spleen cells activated in vitro in response to incubation with peptide p277. Groups of mice were examined for these anti-p277 cytokine responses at ages 5 weeks (onset of insulitis), 12 weeks (advanced insulitis), 17 weeks (onset of clinical diabetes), and 28 weeks (far-advanced clinical diabetes). We treated the mice at 12 weeks of age, just before the outbreak of overt diabetes. Table 2 includes the concentrations of cytokines produced by anti-p277 T-cells both before and after treatment.

Before treatment, the NOD mice manifested an appreciable level of IFN-γ responsiveness to p277 at 5 weeks, which rose sharply at 12 weeks. Sham-treated mice at 12 weeks, like untreated NOD mice, showed a decline in their anti-p277 IFN-γ production at the onset of diabetes (17 weeks) and late in the disease (28 weeks). In the absence of p277 treatment, there was no appreciable IL-4 activity produced by the anti-p277 T-cells; a rise in IL-10 activity was seen at 17 and 28 weeks. After treatment with peptide p277 at 12 weeks, however, there was an abrupt fall in IFN-γ secretion at 17 weeks and 28 weeks. In contrast to the fall in IFN-γ, there appeared sharp increases in IL-4 and IL-10 responsiveness at 17 weeks. The p277-treated mice, free of diabetes at 28 weeks of age, manifested a continued low IFN-γ response but showed a fall in their IL-4 and IL-10 responses (Table 2). Thus, arrest of the diabetogenic process induced by p277-peptide therapy was marked by a burst of Th2-type reactivity detected 5 weeks after treatment that later reverted spontaneously.

Specificity of cytokine modulation. The immunological specificity of T-cell cytokines was measured at 17 weeks (5 weeks after treatment) by comparing the cytokine profiles of the responses to p277 with those induced by peptide MT-p278 of mycobacterial hsp60. Our NOD mice show spontaneous T-cell reactivity to peptide MT-p278, but administration of the MT-p278 peptide does not affect diabetes (Table 1), so this peptide can serve as a convenient T-cell specificity control. Figures 4*A* and 4*B* show that the spleen cells of sham-treated mice secreted both IL-2 and IFN-γ upon incubation with either p277, MT-p278, or the T-cell mitogen Con A. In contrast, the p277-treated mice produced significantly less IL-2 and IFN-γ in response to incubation with peptide p277 ($P < 0.01$). This reduction in Th1 cytokines was specific; the p277-treated mice maintained their IL-2 and IFN-γ cytokine responses to MT-p278. Figures 4*C* and 4*D* show the amounts of IL-10 and IL-4 produced by the spleen cells of the mice. The sham-treated mice produced negligible amounts of IL-4 in response to p277, MT-p278, or Con A, and only a small amount of IL-10 in response to p277. In contrast, there was a significant increase in IL-10 and IL-4 in response to p277 in the p277-treated mice ($P < 0.01$). A decrease in IL-2 and IFN-γ coupled with an increase in IL-10 and IL-4 indicates that the shift from Th1-like behavior to Th2-like behavior in response to p277 was immunologically specific, relative to the response to MT-p278.

To confirm the specificity of the Th2-like response to peptide p277, groups of NOD female mice were treated with PBS or with peptides MT-p278 or p277, in IFA. Five weeks later, the splenic T-cells of the mice in each group were stimulated in vitro by incubation with MT-p278 or p277 and the media were assayed for the secretion of IL-4 and IL-10. Table 3 shows that the mice that had been treated with MT-p278 showed no increase in the secretion of IL-4 or IL-10 over that of PBS-treated mice when the spleen cells were incubated with MT-p278. In contrast to the mice that had been treated with PBS or with MT-p278, the mice that had been treated with p277 showed a marked increase in IL-4 and IL-10 induced by incubation with p277. Thus, the rise in Th2 cytokine reactivity was specific for the p277 peptide.

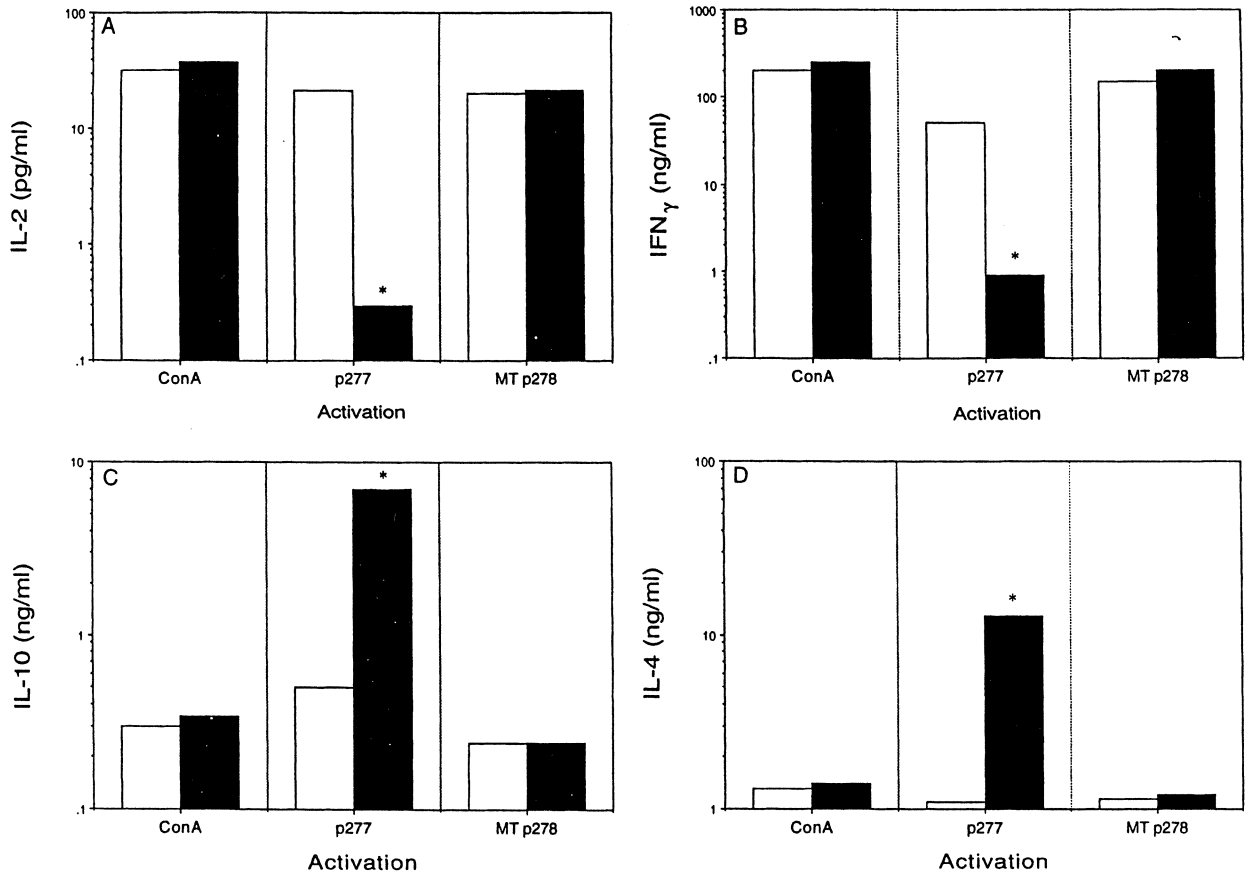

FIG. 4. Peptide p277 therapy induces a specific switch in the cytokine profile. Groups of 10 NOD mice, 3 months old, were treated with p277 in oil (solid bars) or with PBS in oil (open bars). Three weeks later, the spleens of the mice were removed and the spleen cells were pooled. The spleen cells were incubated in triplicate with Con A, p277, or MT-p278 for 24 h (for IL-2 and IL-4 secretion; [*A*, *D*]) or for 48 h (for IL-10 and IFN-γ secretion [*B*, *C*]). The presence of the cytokines in the culture supernatants was quantitated by ELISA. The concentrations of cytokines are shown as mean picogram per milliliter derived from calibration curves using recombinant cytokines as standards. The SE was <10% of the mean in each group. *$P < 0.01$ by Student's *t* test.

DISCUSSION

The experiments described here were done to learn whether the effectiveness of hsp60 peptide treatment of the autoimmune diabetogenic process in NOD mice might be associated with a change in cytokine secretion. This study was suggested by our observations that effective treatment with peptide p277 induced high titers of specific antibodies of the IgG1 isotype, thought to be induced by IL-4 (21), while immunogenic peptides that were not effective in arresting diabetes did not induce such antibodies (19). Treatment with p277 also induced peptide-specific antibodies of the IgG2b isotype (Fig. 2 [19]). The cytokines required for induction of antibodies of the IgG2b isotype are controversial; although small amounts of the "suppressor" cytokine transforming growth factor-β (TGF-β) may be required for the secretion of all IgG isotypes (22), it appears that IgG2b is induced primarily by TGF-β (21,23). However, we have yet to obtain direct evidence for the secretion of TGF-β; thus the possible involvement of this cytokine in the effect of p277 must be viewed with caution. Be that as it may, our results are compatible with the association of IgG2b with downregulation of autoimmune damage.

Autoimmune Th1 cells are thought to be the pathogenic agents in autoimmune IDDM, and Th2 cells, in contrast, are thought to be innocuous or even beneficial in halting the diabetogenic autoimmune process (9). Indeed, young NOD mice have been treated by direct injection of Th2 cytokines (24–26). Healey et al. (8) and Katz et al. (7) found that lines of IFN-γ-secreting Th1 cells could transfer diabetes, but that Th2 cell lines could not transfer the disease. However, the direct therapeutic value of Th2 cells is still not clear. Adoptive transfer of a Th2 clone was reported not to protect against diabetes (7). Moreover, a transgenic mouse hyperexpressing IL-10 in the islets was reported to develop IDDM (27). Therefore, not all Th2 cells that are targeted to the islets are beneficial, and persisting secretion of a Th2 cytokine may actually damage β-cells.

The studies reported in this paper relate to the p277 peptide of the human hsp60 molecule, which can serve as a target of diabetogenic T-cells in the NOD mouse (15). This peptide differs from the analogous hsp60 sequence in the mouse by 1 amino acid at position 455. However, the mouse and human p277 peptides appear to be completely cross-reactive for the same clone of T-cells, and administration of either peptide can halt the development of diabetes (28). Thus, the response to the human p277 sequence, even with further substitution of the two cystein residues, functions as does the self-mouse peptide.

TABLE 3
Induction of Th2 cytokines is specific for p277 treatment

Stimulation in vitro	Peptide treatment in vivo	IL-4	IL-10
MT-p278	PBS	0	0
	MT-p278	0	0
p277	PBS	0	0
	p277	13.7 ± 0.5*	8.5 ± 0.5*

Data are means ± SD and nanograms per milliliter. Groups of 10 female NOD mice, 12 weeks old, were injected subcutaneously with peptides (100 μg) MT-p278 or p277 in IFA, or with PBS in IFA. Five weeks later, the spleen cells were stimulated by incubation in vitro with peptides (10 μg/ml) MT-p278 or p277, and the culture media were assayed for the secretion of IL-4 and IL-10. "0" signifies a concentration below the lower level of detection. *$P < 0.001$ compared with mice treated with PBS or MT-p278.

Peptide p277 administered in different contexts can have different effects. Immunization to p277 covalently conjugated to an immunogenic carrier protein such as ovalbumin or bovine serum albumin emulsified in oil was found to induce hyperglycemia and insulitis in various strains of mice, including mice not known to be prone to diabetes such as C57BL/6 and C3Heb mice (29). Immunization with whole hsp60 in oil is also diabetogenic (20). In contrast to the diabetogenic potential of p277 peptide conjugates, the administration of peptide p277 unconjugated to a carrier was found to induce arrest of the spontaneous autoimmune diabetogenic process in NOD mice (15–17). It is not yet clear how the context of administration can influence disease. Intravenous injection of p277 was reported not to induce resistance to diabetes in NOD mice (11), but inspection of the report shows that the authors injected a truncated peptide of only 12 of the 24 amino acids; this truncated peptide was found by us to have reduced effectiveness upon subcutaneous inoculation (17). In contrast to p277, administration of a strongly immunogenic mycobacterial peptide, MT-p278, did not influence the disease (Table 1). A GAD65 peptide immunogenic in NOD mice, GADp35, was also not effective in arresting the disease (D.A., A.M., V.A., O.S.B., P.C., S.K.-W., I.R.C., unpublished observations). Thus, the effect of p277 was relatively specific. In adoptive transfer experiments, T-cells from p277-treated NOD mice were able to suppress the diabetogenic activity of preformed effector cells (17). This suggested that p277 peptide treatment might influence the production of inflammatory cytokines produced by T-cells responsive to relevant antigens in the treated mice. The present study makes three points about NOD disease and p277 therapy.

First, successful treatment of the advanced autoimmune process by administration of p277 is associated with the induction of IL-4 and IL-10 responsiveness to p277 accompanied by a sharp fall in IFN-γ. The IL-4 response can explain the association of the effective peptides with the induction of IgG1 (Th2) antibodies. Thus, a pathogenic Th1-type response to p277 is replaced by a Th2-type response to p277. Since Th1 but not Th2 T-cells are diabetogenic (7,8), the shutdown of the Th1 response constitutes therapy. We find that a clone of anti-p277 T-cells can transfer diabetes when the T-cells act in a Th1 mode and produce IFN-γ, but variants of the clone that produce IL-4 rather than IFN-γ are not pathogenic and can even protect (D.A., A.M., V.A., O.S.B., P.C., S.K.-W., I.R.C., unpublished observations).

Second, the IL-4 and IL-10 responses to p277 therapy do not persist, and long-term arrest of the disease process is associated with reinstatement of a "resting" state characterized by low level production of IFN-γ by splenic anti-p277 T-cells. We do not know the mechanism of the spontaneous decline of the IL-4 and IL-10 responses to p277 some months after the peptide therapy, but it is conceivable that the IL-4 and IL-10 responses end for lack of activation after the peptide is metabolized or cleared from the body. Thus, p277 peptide therapy seems to avoid the potential danger of chronic Th2-type autoimmunity (27).

Third, resetting the cytokine response to a single epitope, such as p277, can spread to existing Th1-type antibody and T-cell responses to other autoantigens involved in the disease, such as GAD and insulin. Note that the regulation of the T-cell response phenotype was relatively specific; the spontaneous T-cell response to a bacterial peptide, MT-p278, remained in the Th1 mode in the treated mice.

The mechanism by which a single antigen may regulate the response to other antigens needs investigation. It is conceivable that GAD and the p277 peptide can mutually regulate T-cell responses by a type of "bystander suppression" in which an antigen that triggers the production of Th2 anti-inflammatory cytokines at the site of inflammation can shut off the Th1 response of other T-cells to adjacent antigens (30). Such bystander suppression could account for the ability of T-cells from p277-treated mice to suppress the adoptive cotransfer of diabetogenic T-cells (17).

The ability of hsp60 autoimmunity to regulate NOD diabetes has been confirmed in an hsp60 transgenic model in which we engineered hyperexpression of hsp60 in the thymus and elsewhere by combining transgenic mouse hsp60 with an major histocompatibility complex (MHC) class II promoter (31). The transgenic NOD mice manifested downregulation of their spontaneous proliferative responses both to p277 and to the GAD peptide GADp34, but maintained their spontaneous responses to MT-p278. The transgenic mice were resistant to the development of diabetes (31). We are presently investigating the cytokine profiles of these mice. In contrast to our results with hsp60 transgenic mice, intrathymic injection of hsp60 at 3 weeks was reported not to be effective in preventing diabetes in NOD mice (13).

In summary, p277 peptide treatment not only is associated with induction of specific IgG1 isotype antibodies, but also affects both the cytokine profile and the antigen reactivity of the collective of the T-cells involved in the process. Further work will be needed to understand in molecular terms how administration of a peptide recognized by Th1 T-cells can activate anti-peptide T-cells of the Th2 type. Be that as it may, the ultimate transition from the Th2 burst to a baseline cytokine state suggests that the potential for physiological regulation of destructive autoimmunity is programmed within the immune system (32,33); it need only be activated by a suitable signal.

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