The hsp60 Peptide p277 Arrests the Autoimmune Diabetes Induced by the Toxin Streptozotocin

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The development of autoimmune diabetes in the NOD strain of mice (H-2^{g7}) is marked by the presence of T-cells reactive to the p277 peptide of the 60-kDa heat shock protein (hsp60). We have found that the p277 peptide can be used as a therapeutic vaccine to arrest NOD diabetes. Recently, we found that T-cell autoimmunity to p277 also develops spontaneously in C57BL/KsJ mice (H-2^{d}) during the induction of autoimmune diabetes by a very low dose of the β-cell toxin streptozotocin (STZ). We now report the inhibition of STZ toxin-induced autoimmune diabetes by p277 peptide therapy. Administration of two doses each of 100 μg of peptide p277 in mineral oil given 1 week after toxin induction and 85 days later was most effective. The effect of p277 on STZ toxin-induced diabetes was marked by a shift in p277 autoimmunity from a T-cell proliferative response to the production of anti-p277 antibodies. The anti-p277 antibodies were predominantly of the IgG1 and IgG2b isotypes, known to be regulated by Th2 type cytokines; IgG2a antibody, known to be dependent on interferon (IFN)-γ, was induced to a much lesser degree. Peptide p277 therapy was specific: treatment of the mice with an immunogenic peptide from the sequence of another antigen, GADp34, failed to prevent the development of diabetes. The GADp34 peptide induced lower titers of specific antibodies, and the antibodies were predominantly of the IgG2a class. Thus, p277 peptide therapy, marked by the induction of Th2-type antibodies, can be effective in toxin-induced autoimmune diabetes.
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We recently reported that it was possible to induce remission of advanced insulitis, even after the clinical onset of overt diabetes, by treating NOD mice with a peptide from the sequence of the human 60-kDa heat shock protein (hsp60) (1,2). This peptide, designated p277, is composed of the 24 amino acids spanning positions 437–460 of hsp60. Treatment of NOD diabetes using peptide p277 was found to downregulate spontaneous T-cell reactivity to the p277 portion of hsp60 (3), a T-cell reactivity that can cause diabetes (4). Thus, a peptide containing an epitope targeted by diabetogenic T-cells can be used as a therapeutic agent to turn off a spontaneous diabetogenic process.

We have found that a form of autoimmune diabetes can be induced in the C57BL/KsJ strain of mice by the administration of a very low dose of the β-cell toxin streptozotocin (STZ) (5). Whereas the standard low dose of STZ of 40 mg/kg administered daily for 5 days usually induces clinical diabetes within 3 weeks, the administration of 30 mg/kg for 5 days induces clinical diabetes only after a lag period of ~3 months. This model of induced diabetes is marked by spontaneous autoimmunity to hsp60 and to its p277 peptide and to insulin (5). Thus, the lower-than-standard low dose of STZ appears to trigger an autoimmune process immunologically similar to that observed in the spontaneous diabetes developing in NOD mice (3,6). Because treatment with peptide p277 was effective in reversing the diabetogenic process in NOD mice, we investigated whether p277 treatment might also modify the autoimmune response and thereby abort the development of the diabetes induced by the very low dose of STZ. The present paper demonstrates this to be the case.

RESEARCH DESIGN AND METHODS

Induction of diabetes. Male mice of the C57BL/KsJ strain, 4 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) and used after 2 weeks of acclimatization to our animal house. The mice were treated with five daily doses of 30 mg/kg of STZ i.p., purchased from Boehringer Mannheim (Mannheim, Germany), to induce diabetes as described (5). Blood glucose was measured at regular intervals using a Beckman Glucose Analyzer (Palo Alto, CA) to determine the development of diabetes. In these studies, significant hyperglycemia was judged to be a blood glucose concentration >15 mmol/l.

Peptides and treatment. Peptide p277 was synthesized by standard Fmoc chemistry using an automated ABIMED synthesizer (Langenfeld, Germany) as described (1,2). The sequence of p277 is VLGGGCALLRCIPALDSLTPANED. The p277 peptide in its native sequence tends to be chemically unstable, probably because of the two cystein (C) residues. To obtain a form of p277 that is more chemically stable, we have substituted the two cysteins with two valine (V) residues to produce a peptide that is immunologically the equivalent of the native sequence (D.E., I.R.C., unpublished observations). The studies reported here have been done using both forms of p277 with equivalent results. Figures 1 and 2 present results with the native sequence, and Figures 3–6 show results using the V-substituted peptide. The p277 peptides were purified by reverse-phase high-performance liquid chromatography, and the composition was confirmed by amino acid analysis. Three peptides of the 65-kDa isotype of the antigen GAD65 were prepared and purified as above and included peptide 17 (GADp17) (residues 247–266) NMYAMMIARIKMFPEVKEKG; peptide 34 (GADp34) (residues 509–528) IPPSLRTLEDNEERMSRLSK; and peptide 35 (GADp35) (residues 524–543) SRLSKVAPVIKARMMEYGTT (7). Mice received a subcutaneous inoculation of 100 μg of p277 or of GADp34 in phosphate-buffered saline (PBS) emulsified in an equal volume of incomplete Freund's adjuvant (IFA; mineral oil with emulsifying agent) purchased from Difco (Detroit, MI). A total volume of 0.1 ml was injected subcutaneously under the skin

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ELISA, enzyme-linked immunosorbent assay; hsp60, 60-kDa heat shock protein; IFA, incomplete Freund's adjuvant; IFN, interferon; IL, interleukin; OD, optical density; PBS, phosphate-buffered saline; STZ, streptozotocin; SI, stimulation index; TGF, transforming growth factor.

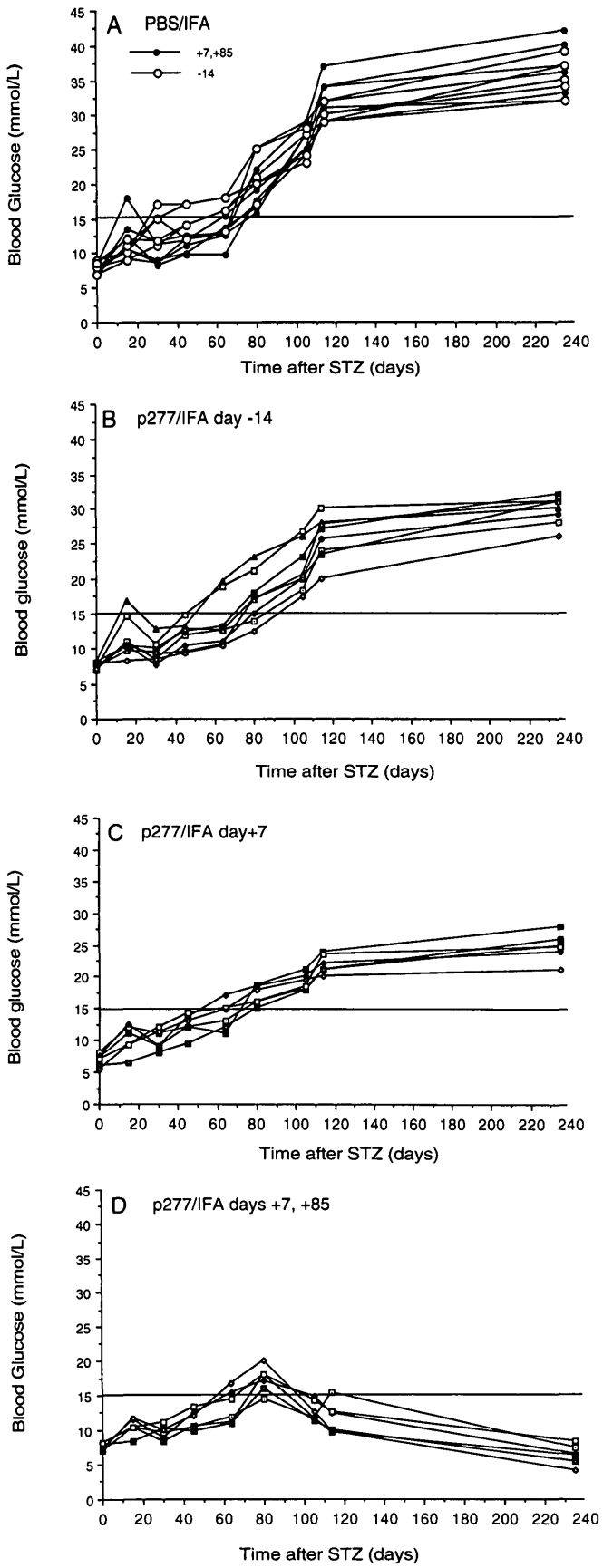

FIG. 1. Treatment of STZ-induced diabetes. Individual C57BL/ksJ male mice were treated with 100 μg p277 in an IFA emulsion on day −14 (*B*), day +7 (*C*), or days +7 and +85 (*D*). The combined control groups were treated either on day −14 or on days +7 and +85 with an emulsion of PBS and IFA (*A*). STZ was administered at 30 mg/kg for 5 consecutive days to induce diabetes. Mice were bled, and glucose concentrations in the sera were tested. Statistical significance was determined using the Wilcoxon's rank-order test. The differences between each of the test groups and the controls was significant at 240 days ($P < 0.01$).

of the back. Control treatment consisted of an emulsion of PBS in IFA without p277 or GADp34. Three different treatment schedules were tested: one treatment 14 days before STZ induction (day −14), one treatment 7 days after onset of STZ induction (day +7), and two treatments given 7 and 85 days after onset of STZ induction (days +7, +85).

T-cell proliferation. To investigate the specific immunogenicity of peptides p277 and GADp34 in C57BL/KsJ strain mice, groups of five mice were immunized in the hind foot pads with either peptide (100 μg) emulsified in 0.1 ml IFA. Ten days later, the draining lymph node lymphocytes were tested for their proliferative responses to either peptide, as described below. To assay the effects of STZ induction and peptide treatment on the proliferative response, spleens were removed on day 58 from mice inoculated with five daily doses of 30 mg/kg of STZ and treated with p277 in IFA on day 7 after induction by STZ. Control mice were treated with an emulsion of PBS and IFA. Five mice of each group were killed and their spleens tested separately in a T-cell proliferation assay, as described (3,4). Briefly, splenocytes were seeded in quadruplicate wells in microtiter plates, 0.2×10^6 cells in 0.2 ml of Dulbecco's modified Eagle's medium supplemented with 1% autologous serum for 72 h. Antigens were added at a concentration of 10 μg/ml. The antigens tested were peptide p277 and the three peptides of GAD65. The wells were pulsed with [^{3}H]-labeled thymidine for the last 18 h of culture, the cells were harvested, and the incorporated radioactivity was counted in a β-counter. The stimulation index (SI) was defined as the ratio of the antigen-driven thymidine incorporation to the background incorporation in the absence of antigen. Background counts per minute were in the range of 1,000–2,000. Standard deviations were <10% of the mean.

Antibodies. Antibodies to p277 or GADp34 peptides were assayed in the sera of treated or control mice bled 100 days after the administration of STZ. A standard enzyme-linked immunosorbent assay (ELISA) was used, as described (5,6). Briefly, 10 μg of the peptides were applied to assay plates (Maxisorp, Nunc Roskilde, Denmark) suitable for the binding of peptides, and the plates were incubated with the test sera. The binding of antibodies to the adherent peptides was detected using alkaline phosphatase conjugated anti-mouse IgG + IgM or isotype-specific anti-mouse IgG1, IgG2a, or IgG2b (Jackson ImmunoResearch, 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.

RESULTS

Treatment with p277. Treatment of NOD mice with peptide p277 was found to be effective both when administered early during the course of disease before appreciable insulitis (3) and when administered late after the initiation of the diabetogenic process (1,2). We therefore administered p277 to C57BL/KsJ mice both before and after we induced a diabetogenic process by exposing the mice to the very low dose of STZ (30 mg/kg × 5). Figure 1*A* shows the course of hyperglycemia developing in the control groups of mice given PBS in the IFA vehicle either 14 days before STZ induction (day −14) or at days 7 and 85 (days +7, +85) after induction. It can be seen that by day 240, all of the mice were diabetic with blood glucose concentrations between 32.5 and 42.5 mmol/l. In contrast, the mice treated with p277 14 days (day −14) before STZ induction showed a significantly decreased degree of hyperglycemia; range 25–32 mmol/l, $P = 0.01$ (Fig. 1*B*). In addition, we treated mice with p277 after the induction of the diabetogenic process. Figure 1*C* shows that p277 administered 7 days after the beginning of the 5-day course of STZ (day +7) also lowered the final level of hyperglycemia ($P = 0.003$ compared with Fig. 1*A*). Since moderate hyperglycemia was observed on day 80 in all mice treated, we administered a second dose of p277 on day 85. Figure 1*D* shows that this second dose did in fact turn off the

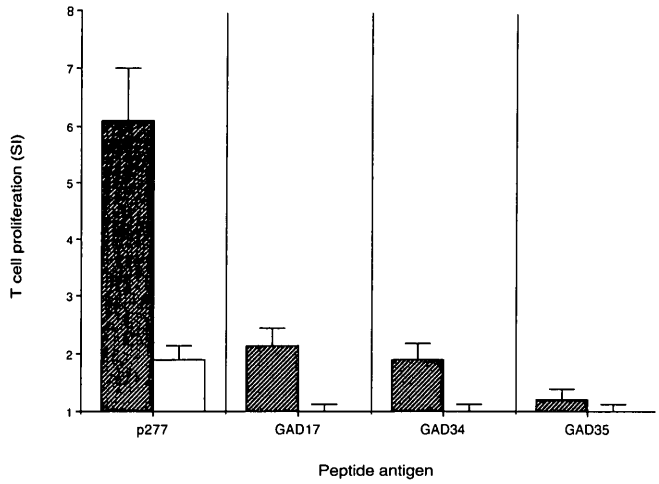

FIG. 2. Peptide p277 treatment downregulates spontaneous T-cell reactivity. Five C57BL/ksJ male mice were inoculated with 30 mg/kg of STZ for 5 consecutive days. Fifty-eight days later, the mice were killed and their spleens removed for a T-cell proliferation assay using the peptide antigens p277 and three GAD peptides. The differences between the test (□) and control groups (■) were statistically significant, determined using the Student's t test ($P < 0.01$).

diabetogenic process: the mice treated with p277 on days 7 and 85 manifested a transient hyperglycemia around day 80 that remitted; all the mice were normoglycemic 240 days after STZ induction.

T-cell proliferation. The success of peptide p277 in treating the spontaneous diabetes of NOD mice was associated with a decrease in T-cell proliferative reactivity to hsp60 and to the p277 peptide itself (2,3). Figure 2 shows that p277 treatment of the test C57BL/KsJ mice was similarly associated with downregulation of their spontaneous T-cell proliferative response to p277. T-cell responses to three peptides of the GAD65 antigen have been reported to occur spontaneously in NOD mice (7), so we tested the T-cell responses of the mice to the GAD65 peptides. Figure 2 shows that the STZ administration induced only negligible T-cell responses (SI = ~2) to the GADp17 and GADp34 peptides.

To test whether peptide GADp34 was indeed a specific T-cell epitope in C57BL/KsJ mice, we immunized naive mice against either p277 or GADp34 and tested the proliferative responses. Figure 3 shows that the C57BL/KsJ mice were able to manifest specific responses to each peptide, although the response to GADp34 was somewhat lower than the response to p277. Thus, GADp34 is immunogenic for T-cells in these mice. Because GAD65 was found to be functional in treating NOD mice (7), we tested whether therapy with the GADp34 peptide, like p277 therapy, might be effective in the STZ model.

Treatment with p277 compared with GADp34. Groups of C57BL/KsJ mice received STZ (30 mg/kg × 5) to induce autoimmune diabetes, and the mice were treated with either p277 or with GADp34 peptide in IFA on days 7 and 85. The levels of blood glucose were tested on day 100. Figure 4 shows that the mice treated with GADp34, like the control mice treated with PBS, were markedly hyperglycemic; there was no significant difference between these two groups. In contrast, 6 of the 10 mice treated with p277 manifested blood glucose concentrations <15 mmol/l, two additional mice were just over the 15 mmol/l borderline, and only two mice were frankly hyperglycemic, one of them markedly so. As a whole, the mean blood glucose concentration of the p277-

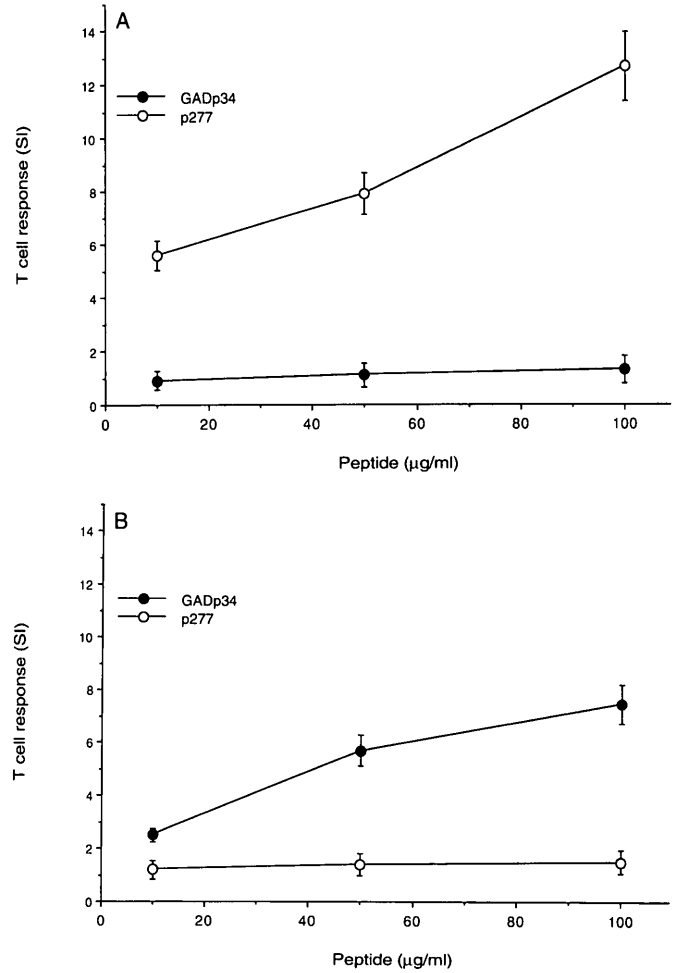

FIG. 3. Peptides p277 and GADp34 are immunogenic. Naive C57BL/KsJ mice were immunized (100 μg) in the hind foot pads with either p277 (*A*) or GADp34 (*B*) in IFA, and the proliferative responses of the draining lymph node T-cells were assayed 10 days later.

treated group was significantly lower than that of the other two groups of mice. Thus, therapy with peptide p277 was specific.

Peptide p277 induces antibodies. It has been suggested that a shift from an effector T-cell response (Th1-type) to an antibody response (Th2-type) might be therapeutic in autoimmune diabetes (8). Indeed, Fig. 5 shows that high levels of

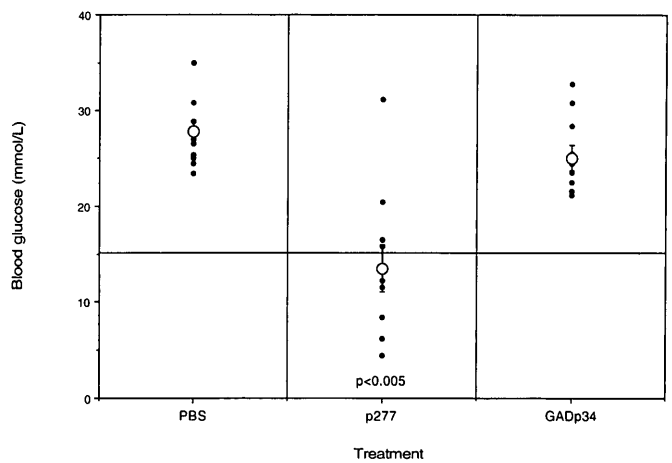

FIG. 4. Treatment of STZ-induced diabetes with p277 or GADp34 peptides. The blood glucose concentrations of individual mice were measured on day 100 after the induction of diabetes and treatment with PBS, p277, or GADp34 on days 7 and 85.

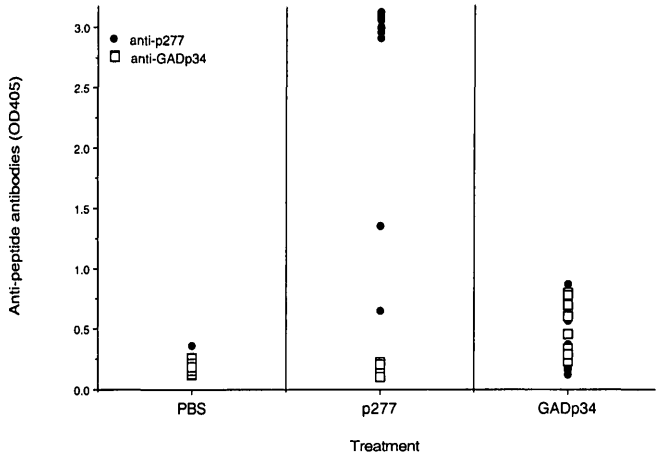

FIG. 5. Antibodies to p277 or GADp34 developing after peptide therapy. Mice were treated with p277, GADp34, or PBS, as described in Fig. 4, and the titers of antibodies to either peptide were measured in the sera on day 100.

anti-p277 antibodies were induced by p277 treatment in the STZ model. The anti-p277 antibodies were specific; the sera from the p277-treated mice showed no significant binding to peptide GADp34. Note that the two p277-treated mice that made low levels of anti-p277 antibodies were the two mice that developed gross hyperglycemia (20 mmol/l or greater) in the p277 group (Fig. 4). Treatment with peptide GADp34 also induced antibodies to GADp34 and to p277 in most of the treated mice (OD 405 nm >0.25), but the amounts of antibodies appeared to be lower than those induced by treatment with p277.

Antibody isotypes. Th2 type antibodies can be identified by their Ig isotypes; interleukin (IL)-4 is thought to induce antibodies of the IgG1 isotype (9) and transforming growth factor (TGF)-β is thought to induce IgG2b antibodies (10). In contrast, the Th1 cytokine IFN-γ induces antibodies of the IgG2a isotype (9). Figure 6 shows the isotypes of the anti-p277 antibodies in the p277-treated mice. It can be seen that the anti-p277 antibodies in the p277-treated mice were predominantly of the IgG1 and IgG2b isotypes. In contrast, the anti-GADp34 antibodies in the GADp34-treated mice were predominantly of the IgG2a isotype. Thus, p277 peptide therapy appears to induce antibodies that reflect a predominant Th2-type response. Peptide GADp34, which was not effective, induced lower titers of antibodies, mainly of the Th-1 type.

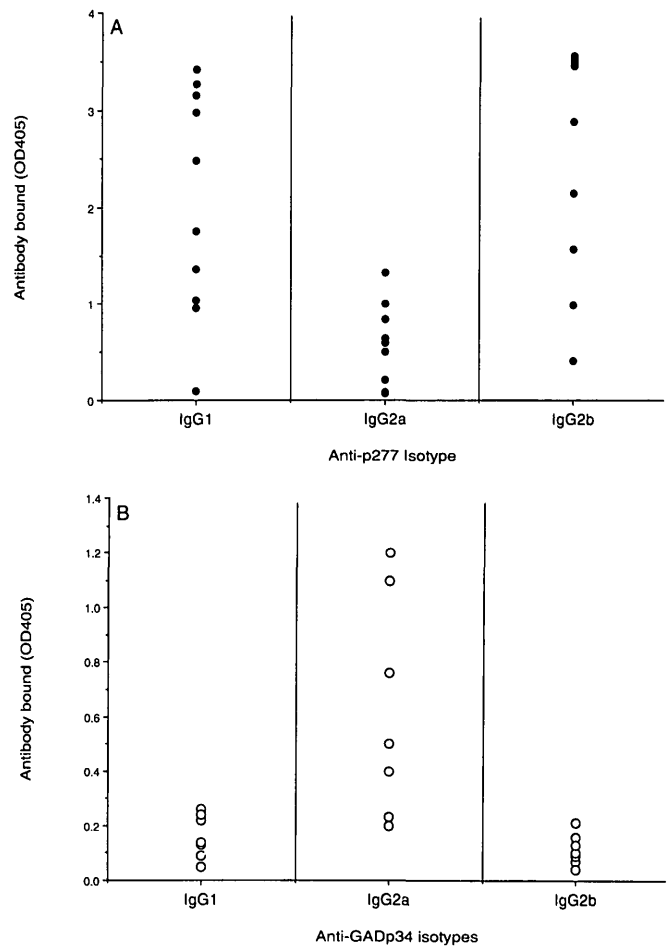

FIG. 6. Isotypes of anti-peptide antibodies. The antibodies to p277 (*A*) or GADp34 (*B*) of the IgG1, IgG2a, or IgG2b isotypes were measured using an ELISA assay of the sera in the experiment described in Fig. 4.

DISCUSSION

The effectiveness of p277 peptide therapy in STZ toxin-induced diabetes raises two general questions: what is the role of p277 immunity in this model of diabetes, and how does administration of the p277 peptide arrest the disease? We can consider these questions in the light of studies of the NOD model of autoimmune diabetes where more information about the role of p277 is available. The sequence of the p277 peptide used in these studies was derived from the human hsp60 molecule, and the human p277 peptide differs from the mouse p277 analog by one amino acid substitution, K for T at position 455 (11). Nevertheless, the human and mouse variants of p277 are immunologically cross-reactive, and the mouse p277 peptide is as effective as the human analog in treating NOD diabetes (11). Thus, the human p277 sequence probably functions in mice as if it were a part of the mouse hsp60 self-antigen.

It is reasonable to suppose that the stress caused by exposure to STZ can alter the amount and/or the nature of the presentation of hsp60 in β-cells. Indeed, we find that a β-cell tumor line (NIT-1) responds to STZ in vitro by upregulating the surface expression of hsp60; an increase in surface hsp60 in response to STZ is not shown by cells originating from other tissues (A. Meilin, D.E., I.R.C., unpublished observations). Thus, STZ might induce a special expression of hsp60 in β-cells. However, this by itself cannot explain the association of STZ-induced diabetes with hsp60 autoimmunity, and more research is needed to uncover the molecular mechanisms involved in p277 as a target of autoimmune T-cells in diabetes (5).

The role of p277 in diabetes is complicated by the fact that forms of hsp60 autoimmunity also may be involved in the pathogenesis of other immunological diseases, including arthritis (12) and encephalomyelitis (13). But whatever the role of hsp60 autoimmunity in other diseases may be, autoimmunity to hsp60 seems to be involved in β-cell damage. We have created transgenic NOD mice that hyperexpress mouse hsp60 in the thymus and elsewhere directed by a mouse major histocompatibility complex class II promoter, IE α (14). These mice demonstrate a form of tolerance to hsp60 manifested by the absence of the spontaneous T-cell proliferative response to p277 present in wild type NOD mice. Interestingly, the hsp60 transgenic mice develop peri-islet insulitis, but the inflammation does not proceed to the stage

of intra-islet insulitis and clinical diabetes (14). Thus, T-cell reactivity to p277 may be critical for β-cell destruction.

The present finding that p277 therapy led to deviation of p277 autoimmunity from proliferating T-cells to anti-p277 antibodies supports the notion that arrest of β-cell damage might be brought about by inducing a switch from a Th1 effector T-cell response to autoimmunity of the Th2 type (8). The observation that p277 therapy induced a predominance of IgG1 and IgG2b antibodies to p277 can be seen as functional evidence for the role of IL-4 and TGF-β in the response to p277 therapy. We are currently developing methods to document the quantities of these anti-inflammatory cytokines in the islets, and direct proof for the induction of a switch to Th2 cytokines awaits this methodology. However, compatible with the induction of anti-inflammatory suppresser cytokines is our finding that activated T-cells from p277-treated NOD mice could suppress diabetes in an adoptive transfer experiment (2). Thus, protection from the development of diabetes need not require the deactivation or deletion of anti-p277 autoimmunity but rather the activation of a shift in the type of autoimmunity (15–17).

It is interesting that immunization with peptide GADp34 did not cure the diabetic process. Peptide GADp34 is clearly immunogenic in C57BL/KsJ mice; a single injection of 100 μg in IFA sufficed to induce a significant T-cell proliferative response (mean SI = 7; Fig. 3), and treatment with GADp34 in the STZ model did induce significant titers (OD 405 nm > 0.25) of IgG2a antibodies in most mice (Fig. 6). Because GADp34 represents an immunogenic self-peptide, it may be viewed as a control for p277 peptide treatment. Despite the immunogenicity of GADp34 for C57BL/KsJ mice, however, the spontaneous T-cell response to GADp34 associated with STZ induction of diabetes was very low (Fig. 2). This suggests that the GADp34 peptide may not be a target in the model. In contrast, induction of diabetes by the administration of STZ was associated with spontaneous activation of a strong anti-p277 T-cell response (Fig. 2) (5); hence, immunity to p277 is intrinsic to the model. Thus, the mice may have already been primed to respond to p277 by the disease process itself, laying the foundation for the positive response to p277 peptide therapy (15). The prominent immunogenicity of p277 notwithstanding, it is intriguing that the failure of GADp34 compared with the success of p277 might be correlated with the ability of GADp34 to induce specific antibody predominantly of the IgG2a isotype associated with aTh1-type response.

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