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Treatment of NOD Diabetes with a Novel Peptide of the hsp60 Molecule Induces Th2-type Antibodies

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A peptide from the sequence of hsp60 molecule, designated p277, has been shown to be functionally involved in modulating the development of autoimmune diabetes in the NOD mouse: administration of p277 to NOD mice can arrest the diabetogenic autoimmune process, even when far advanced. Is p277 the only hsp60 peptide able to modulate the disease? We mapped T cell responses to peptides spanning the mouse hsp60 molecule and identified an immunogenic peptide, designated p12, that is also functional in arresting NOD diabetes. Although no spontaneous T cell reactivity to p12 could be detected in NOD mice, subcutaneous administration of 100 μg of p12 in mineral oil to 10-week-old female NOD mice, similar to treatment with p277, significantly prevented progression of the disease. Administration of other immunogenic peptides was not effective. A peptide from the glutamic acid decarboxylase (GAD65) sequence, GADp35, and a peptide from the mycobacterial hsp60 molecule did not influence the development of diabetes. The effectiveness of hsp60 peptides p12 and p277 was associated with the induction of antibodies to the peptides of the IgG1 and IgG2b isotypes, antibodies which appear to be regulated by anti-inflammatory cytokines. There was a negative correlation between the amounts of antibodies induced by the hsp60 peptides and the level of blood glucose. Thus, more than one peptide of the hsp60 molecule can be used to inhibit the development of NOD diabetes, and the effect of peptide therapy appears to be associated with the induction of specific antibody isotypes.

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Introduction

Insulin-dependent diabetes mellitus (IDDM) developing spontaneously in female NOD mice has been associated with immune reactivity to a variety of self-antigens (reviewed in [1]). Notable among these antigens is the p277 peptide from the sequence of the mammalian 60 kDa heat shock protein (hsp60) molecule, residues 437–460. Prediabetic NOD mice manifest spontaneous, diabetogenic T cell responses to hsp60 and to the human [2] or mouse variants of the p277 peptide [3]. The mouse and human peptides differ by one amino acid and are immunologically cross-reactive [3]. Some non-diabetes-prone strains of mice, such as C57BL/6, develop transient hyperglycemia and insulitis when immunized to p277 covalently conjugated to a foreign immunogenic carrier molecule [4]. Also, mice of the C57BL/KsJ strain develop spontaneous T cell responses to hsp60 and to p277 after treatment with a very low dose of the β-cell toxin streptozotocin (STZ), that induces autoimmune diabetes [5].

In addition to being involved in the expression of the disease, peptide p277 appears to be functional in arresting the autoimmune process: subcutaneous administration of p277 in incomplete Freund's adjuvant (IFA; mineral oil) led to arrest of disease progression in young NOD mice [2] or in 12–17-week-old NOD mice with advanced insulitis [6, 7]. Both the human [6, 7] and mouse [3] variants of p277 were effective. NOD mice transgenic for the mouse hsp60 gene on an MHC class II promoter showed down-regulation of their spontaneous T cell proliferative response to p277 and a significant proportion of the mice were spared the development of diabetes [8]. Moreover, administration of p277 to C57BL/KsJ mice aborted the development of autoimmune diabetes in mice that had earlier received a very low dose of STZ; treatment of these mice with an immunogenic peptide of the GAD65 molecule was not effective [9]. The response to treatment with p277 in the STZ model was associated with the induction of antibodies to p277 of the IgG1 and IgG2b isotypes [9]. Since mouse antibodies of the IgG1 isotype are induced by the cytokine IL-4 [10], these findings are compatible with the

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hypothesis that the development of type 1 diabetes may be influenced by the Th1-Th2 balance of the autoimmune response [11]. Indeed, effective treatment of 12-week-old NOD mice with peptide p277 was associated with the induction of antibodies of the IgG1 and IgG2b isotypes, which appeared to mark a shift in the cytokine profile of the anti-p277 T cell population from Th1-type secretion of IFN-γ to Th2-type secretion of IL-4 and IL-10 [12].

The arrest of murine diabetes, spontaneous and STZ-induced, by treatment with p277 raises a number of questions. Is p277 the only peptide of hsp60 that can be used to treat the disease process? Is the effectiveness of p277 in arresting the disease related to the existence of spontaneous T cell reactivity to p277? Will any peptide for which there is spontaneous T cell reactivity be effective in NOD diabetes, irrespective of whether the peptide is self- or foreign? Will other effective peptides also induce Th2-type antibodies in NOD mice? To investigate these questions, we mapped the T cell proliferative response of NOD mice to overlapping peptides spanning mouse hsp60 and selected a peptide, designated p12 (residues 166–185), that was immunogenic for NOD T cells. In contrast to p277, however, we could not detect spontaneous T cell proliferation to p12 in prediabetic NOD mice. We treated 10-week-old NOD mice with p12, p277, an immunogenic GAD65 peptide, GAD-p35, or with the foreign mycobacterial hsp60 peptide, MT-p278, for which our NOD mice manifest spontaneous T cell proliferation [12]. Only the hsp60 peptides p12 and p277 were effective in inhibiting diabetes, and only these peptides induced high titers of specific antibodies of the IgG1 and IgG2b isotypes. Thus we may conclude that more than one domain of hsp60 can be effective in arresting NOD diabetes, that pre-existing spontaneous T cell reactivity to a peptide may not be a prerequisite for a therapeutic response, and that other self- or foreign peptides immunogenic for NOD T cells may not be effective.

Table 1. *Overlapping peptides of the mouse hsp60 molecule used for screening*

Peptide number	Position	Sequence
p1	1–20	MLRLPTVLRQMRPVSRALAP
p2	16–35	RALAPHLTRAYAKDVKFGAD
p3	31–50	KFGADARALMLQGVDLLADA
p4	46–65	LLADAVAVTMGPKGRTVIIE
p5	61–80	TVIIEQSWGSPKVTKDGVTV
p6	76–95	DGVTVAKSIDLKDKYKNIGA
p7	91–110	KNIGAKLVQDVANNTNEEAG
p8	106–125	NEEAGDGTTTATVLARSIAK
p9	121–140	RSIAKEGFEKISKGANPVEI
p10	136–155	NPVEIRRGVMLAVDAVIAEL
p11	151–170	VIAELKKQSKPVTTPEEIAQ
p12	166–185	EEIAQVATISANGDKDIGNI
p13	181–199	DIGNIISDAMKKVGRKGVI
p14	195–214	RKGVITVKDGKTLNDELEII
p15	210–229	ELEIIEGMKFDRGYISPYFI
p16	225–244	SPYFINTSKGQKCEFQDAYV
p17	240–259	QDAYVLLSEKKISSVQSIVP
p18	255–275	QSIVPALEIANAHRKPLVIIA
p19	271–290	LVIIAEDVDGEALSTLVLNR
p20	286–305	LVLNRLKVGLQVVAVKAPGF
p21	301–320	KAPGFGDNRKNQLKDMAIAT
p22	316–335	MAIATGGAVFGEEGLNLNLE
p23	331–350	NLNLEDVQAHDLGKVGEVIV
p24	346–365	GEVIVTKDDAMLLKGKGDKA
p25	361–380	KGDKAHIEKRIQEITEQLDI
p26	376–395	EQLDITTSEYEKEKLNERLA
p27	391–410	NERLAKLSDGVAVLKVGGTS
p28	406–425	VGGTSDVEVNEKKDRVTDAL
p29	421–440	VTDALNATRAAVEEGIVLGG
p30	436–455	IVLGGGCALLRCIPALDSLK
p31	451–470	LDSLKPANEDQKIGIEIIKR
p32	466–485	EIIKRALKIPAMTIAKNAGV
p33	481–500	KNAGVEGSLIVEKILQSSSE
p34	496–515	QSSSEVGYDAMLGDFVNMVE
p35	511–530	VNMVEKGVIDPTKVVRTALL
p36	526–545	RTALLDAAGVASLLTTAEAV
p37	541–560	TAEAVVTEIPKEEKDPGMGA
p38	556–573	PGMGAMGGMGGGMGGGMF

Materials and Methods

Mice

Female NOD/Lt mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The onset of diabetes occurs at about 13–15 weeks and the incidence of diabetes in these mice is 80% or greater by the age of 32 weeks, provided that the mice are maintained under specific pathogen-free conditions. Inbred male NOD mice, 8–10 weeks of age, were supplied by the animal-breeding center of this institute. The breeding nucleus was the gift of Dr E. Leiter of the Jackson Laboratory.

Antigens

Peptides were synthesized in the Department of Organic Chemistry of the Weizmann Institute of Science using an automated multiple peptide synthesizer (Abimed model AMS 422; Langenfeld, Germany) following the company's protocols for N-a-fluorenylmethoxycarbonyl (Fmoc) synthesis. Peptides were purified by reversed phase HPLC on a semi-preparative C_8-column (Lichrosorb RP-8, 7 µm, 250×10 mm, Merck, Darmstadt, Germany). Elution of peptides was achieved by linear gradients established between 0.1% trifluoracetic acid in water and 0.1% trifluoracetic acid in 75% acetonitrile in water (v/v). The purity of the single peptide products was ascertained by analytical reversed-phase HPLC and amino acid analysis. Table 1 shows the sequences of the 38 overlapping 20 mer peptides, with overlaps of five amino acids, that we used to span the mouse hsp60 sequence. Peptide p277 corresponds to the 437–460 sequence of mouse hsp60 and is not represented intact in the 20 mer spanning peptides. Peptide p277 is substituted at positions 6 and 11 with valine (V) in

Table 2. *Amino acid sequences of peptides used for treatment*

Peptide	Sequence
p277	VLGGGVALLRVIPALDSLTPANED
p12	EEIAQVATISANGDKDIGNI
MT-p278	EGDEATGANIVKVALEA
GAD-p35	SRLSKVAPVIKARMMEYGTT
p38	PGMGAMGGMGGGMGGGMF

place of the cysteine (C) in the native sequence [7, 9, 12]. Substitution of the two C residues by V greatly enhances 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 (in preparation). Peptide GAD-p35 is from the GAD65 molecule (524–543). A non-immunogenic peptide, p38, from hsp60 (556–573) was used as a negative control. Peptide MT-p278 is from the sequence of mycobacterial hsp60 (431–447). NOD mice housed in our facilities manifest spontaneous T cell proliferative responses to MT-p278 [12]. The amino acid sequences of the peptides used in the functional studies are shown in Table 2.

Peptide mapping and T cell proliferation

For peptide mapping, the 38 peptides covering the mouse hsp60 sequence (Table 1) were used to immunize groups of three male NOD mice with pools of four peptides, 25 µg each, emulsified in 0.1 ml of complete Freund's adjuvant (CFA: Difco Laboratories, Detroit, MI) and injected into the hind footpads. After 10 days, the draining popliteal lymph nodes were removed and T cell proliferative assays were carried out to detect responses to each of the peptides separately as described [2; and see below].

To assay the immunogenicity of selected individual peptides, male NOD mice were immunized by injection into the hind footpads of 25 µg of peptide emulsified in incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, MI). Draining inguinal lymph nodes were collected and pooled 10 days later. T cell proliferation assays were done in 96-U well plates, (2×10^5 cells/well) using complete DMEM media (200 µl/well) supplemented with 1% NUTRIDOMA-SP (Boehringer Mannheim) and 5–50 µg/ml of peptide or antigen in triplicate wells. The cultures were pulsed with [*methyl*-^{3}H]thymidine (1 µCi/well) in the last 12 h of 72 h culture, as described [2]. The results are shown at the optimal peptide concentration of 20 µg/ml as the stimulation index (SI): the ratio of the mean test cpm to the mean control cpm without antigen. SDs were always less than 10% of the mean CPM.

Peptide treatment

Peptides, 100 µg in PBS, were emulsified with an equal volume of IFA and injected subcutaneously into 10-week-old female NOD mice as described [7]. Control mice received an equal volume of PBS emulsified in IFA. The mice were monitored monthly for non-fasting blood glucose at 10:00 hours using the Blood Glucose Sensor (MediSense. Inc., Waltham, MA). Mice with a blood glucose greater than 11.1 mM were considered to be diabetic; this concentration of glucose was greater than 3 SD above the mean blood glucose concentration measured in non-diabetic mice [7].

Serum antibodies

Mice were bled monthly to detect antibody responses. The ELISA assay was done as previously described [2]. Briefly, flat-bottom Maxi-sorp plates (Nunc, Roskilde, Denmark) were coated with 100 µl per well of peptide in PBS, at a concentration of 10 µg/ml, for 2 h at RT followed by overnight incubation at 4°C. After incubation with peptide, the plates were washed and blocked for 2 h at 37°C with 7% BSA (Sigma) in PBS. Sera were diluted 1:50 then added for 2 h at 37°C, followed by incubation for 2 h with 100 µl per well of goat isotype-specific anti-mouse IgG (gamma chain Fc-specific) conjugated to alkaline phosphatase (Jackson, Philadelphia, PA). After washing, the plates were incubated with the substrate p-nitrophenyl/phosphate (P104; Sigma) and read using an ELISA reader at 405 nm. The results at 7 months of age are shown.

Statistics

Statistical analyses were done using the chi-square test and the Student's *t*-test where appropriate.

Results

T cell proliferative responses

Spontaneous T cell responses in prediabetic NOD mice have been detected to the p277 peptide [2, 3, 12] and to larger fragments of the mouse hsp60 molecule that contain the p277 sequence [3]. To detect other T cell epitopes on the mouse hsp60 molecules, we immunized NOD mice with pools of peptides overlapping the hsp60 sequence and found that all mice showed strong responses to p12, and lesser responses to some other hsp60 peptides; a response to p277 is shown for reference (Figure 1). To confirm the immunogenicity of p12 alone, male NOD mice were immunized with p12, p277, or other peptides immunogenic for NOD mice, the MT-p278 peptide (residues 431–447 in the mycobacterial hsp60 molecule), and GAD-p35 (residues 524–543 in the GAD65 molecule). Figure 2 shows that p12 was immunogenic, as were p277 and MT-p278; GAD-p35 was also immunogenic, but less so. Hsp60 peptide p38 was not immunogenic. None of the peptides were cross-reactive; the T cell proliferative responses were limited to the immunizing peptide (not shown).

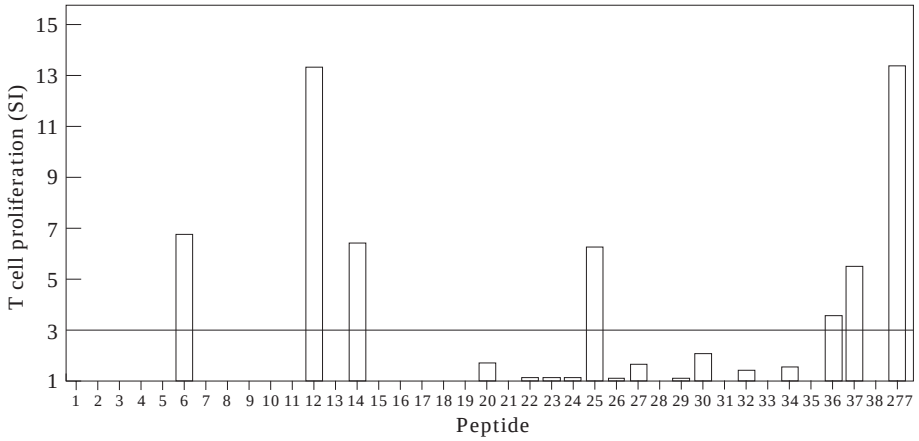

Figure 1. Detection of hsp60 peptides immunogenic for NOD mice. Groups of male NOD mice were immunized in the hind footpads with pools of four peptides in CFA containing 25 µg of each peptide. After 10 days, the draining popliteal lymph node cells were assayed *in vitro* for T cell proliferative responses to each of the peptides in the pool. Peptide p277 is included among the 38 overlapping peptides (see Table 1). The medium control cpm were 2,000 in each group. Two additional experiments produced a similar pattern of reactivities. The horizontal line at SI=3 is included as a reference for significant T cell proliferation

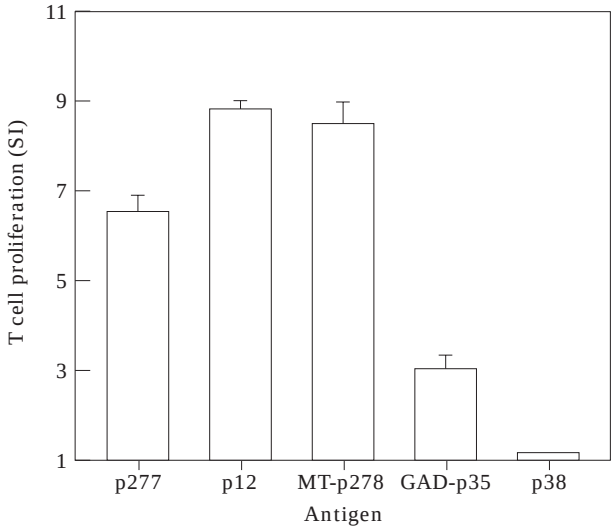

Figure 2. T cell proliferative responses induced to peptides. Groups of three to five male NOD mice were immunized with peptides p12, p277, p38, GAD-p35, or MT-p278 in IFA and the draining lymph nodes were assayed for T cell proliferative responses to the peptides. The following cpm values were obtained in the medium controls without peptide: p12, 881; p277, 1243; MT-p278, 698; and GAD-p35, 1430. The SD values are indicted by the bars.

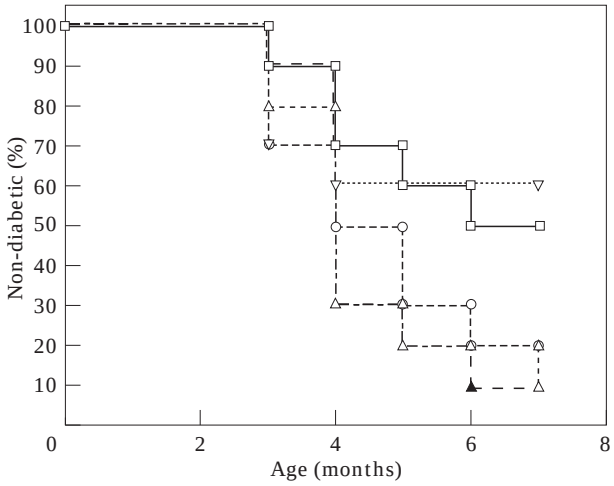

Figure 3. Effect of peptide administration on diabetes. Groups of 10–20 NOD mice were treated at 10 weeks of age with 100 µg of p12, p277, p35-GAD, or MT-p278 in IFA, or IFA alone. The mice were bled monthly and followed for the onset of hyperglycemia. As compared to the IFA-treated control group, the mice treated with p12 and p277 were significantly protected, $P<0.05$. □, p12; ▽, p277; △, MT-p278; ○, GAD-p35; ▲, IFA.

A longitudinal study of female NOD mice at age 3–16 weeks showed no spontaneous T cell proliferative responses to p12 in their spleens (not shown), although responses to p277 and to whole hsp60 were seen as described [2, 3, 12]. Thus we had in hand four immunogenic peptides: p12 and p277 from the mammalian hsp60 molecule, GAD-p35 from the diabetes-associated GAD65 molecule, and MT-p278, a foreign immunogen. Of these, spontaneous responses were detected to only p277 and MT-p278 [12].

Peptide treatment

Following a protocol shown to be effective with p277 [2, 6, 7, 12], groups of 10-week-old female NOD mice were treated by a single subcutaneous injection of each peptide (100 µg) emulsified in IFA. The mice were observed for the development of diabetes up to 7 months of age. Figure 3 shows that peptides p277 and p12 were both effective in inhibiting the development of diabetes; 60 and 50% of the mice, respectively, were free of hyperglycemia at 7 months of age ($P<0.05$). In contrast, treatment with peptides

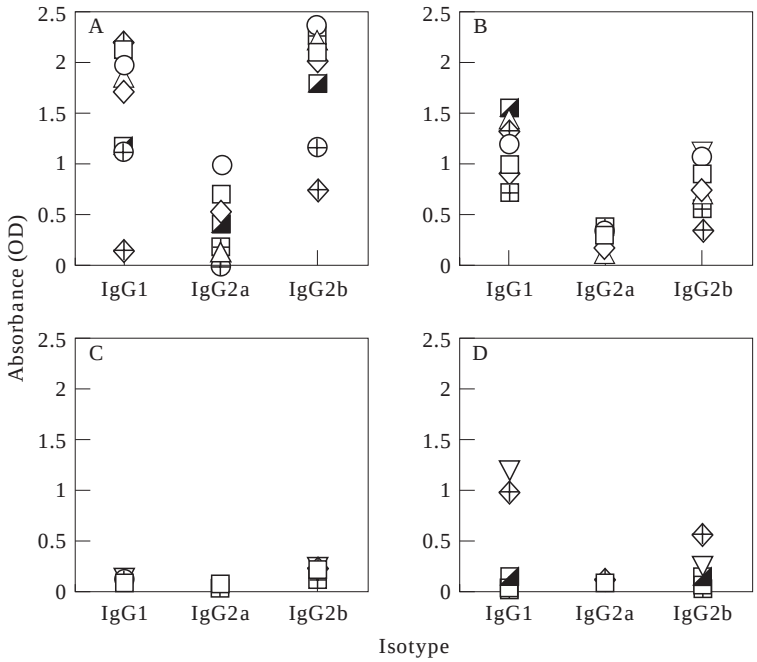

Figure 4. Antibody isotypes in response to peptide treatment. Mice, 10 per group, were treated as described in the legend to Figure 2. Individual mice were analysed monthly for antibodies to (A) p12; (B) p277; (C) GAD-p35; and (D) MT-p278, of the IgG1, IgG2a and IgG2b isotypes. The results are shown at 7 months of age. The level of significance of the prevalence of IgG1 and IgG2b antibodies in groups A and B compared to IgG2a is $P<0.001$. The differences between the levels of IgG1 and IgG2b antibodies compared to the IgG2a antibodies in groups A and B were significant ($P<0.001$).

MT-p278 or GAD-p35 was no different from treatment with IFA alone; 90% of the mice manifested hyperglycemia. Three repeated experiments showed essentially the same results.

Antibodies

Successful treatment of STZ-induced diabetes [9] or of spontaneous NOD diabetes [12] with peptide p277 was associated with the appearance of anti-peptide antibodies predominantly of the IgG1 and IgG2b isotypes. We therefore examined the peptide-treated NOD mice for their serum antibodies at 7 months of age. Figure 4 shows that the two peptides effective in arresting diabetes, p12 and p277, were also effective in inducing strong antibody titers of the IgG1 and IgG2b isotypes that were significantly greater than the IgG2a antibody titers in these groups ($P<0.001$). The lower amounts of IgG2a antibodies were not a technical artefact because we could readily detect the predominance of IgG2a antibodies to other antigens in NOD mice (see [12]). The mice treated with peptides MT-p278 or GAD-p35 did not respond as strongly; none of the GAD-p35-treated mice produced specific IgG1 antibodies and only two of the 10 MT-p278-treated mice produced antibodies of the IgG1 isotype. The mice treated with MT-p278 or GAD-p35 showed significantly lower titers of IgG1 and IgG2b antibodies ($P<0.001$). Similar results were obtained in two additional experiments. There was no cross-reactivity between any of the antibodies (not shown). Thus, effectiveness in inhibiting diabetes was associated with the induction of an antibody response mainly of the IgG1 and IgG2b isotypes.

The relationship between an effective therapeutic response and the titer of antibody was confirmed by a comparison of the concentration of blood glucose with the concentration of IgG antibodies in individual mice at 7 months of age. Figure 5 shows that mice with higher titers of IgG anti-p12 antibodies tended to have lower blood glucose concentrations; conversely, the p12-treated mice that produced little antibody to p12 tended to have high blood glucose ($P<0.004$).

Discussion

In this investigation, we screened NOD mice for their T cell proliferative responses to a set of peptides overlapping the sequence of the mouse hsp60 molecule. A number of peptides appeared to be immunogenic: p6, p12, p14, p25, p36, and p37 (Figure 1). These peptides probably do not exhaust the T cell epitopes in mouse hsp60 because, for practical reasons, the immunizations used peptide pools and competition between peptides in the pools could have obscured their immunogenicity. Indeed, p30, which was very poorly immunogenic in the pool immunization, contains most of the naturally immunogenic p277 sequence (see Tables 1 & 2). Thus, other potentially immunogenic hsp60 peptides could also have been missed in this screening. Nevertheless, we succeeded in identifying p12, which is an effective T cell immunogen. In fact, p12 is strongly bound by the NOD MHC class II molecule and served as a reference

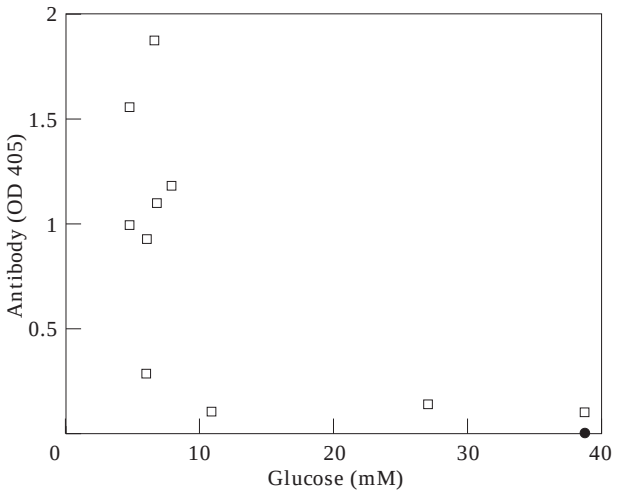

Figure 5. Inverse relationship between antibodies and blood glucose. Groups of female NOD mice were treated with p12 (10 mice) or with IFA alone (nine mice) as described in the legend to Figure 2. The amount of anti-p12 specific antibody is plotted together with the blood glucose concentration measured at 7 months of age. The degree of negative correlation between high antibodies and blood glucose was significant using the Spearman rank correlation: $r = -0.73$, $P = 0.0004$. □, p12 IFA; ●, IFA.

peptide to analyse the peptide-binding motif of the IA^{g7} molecule [13]. The results presented here indicate that peptide p12 of the mouse hsp60 molecule, like peptide p277, can be effective in treating mice close to the outbreak of overt hyperglycemia. In contrast to p277, we did not observe spontaneous T cell proliferative responses to p12 in the spleens of prediabetic NOD mice. Thus, a spontaneous anti-peptide proliferative response detectable in the periphery is not a requirement for a peptide to be effective in blocking the diabetic autoimmune process. There is no way of knowing, as yet, whether anti-p12 T cells are present in the islets.

The finding that peptide p277 is not the only hsp60 peptide that can modulate NOD diabetes is significant. It was conceivable that the involvement of hsp60 in NOD diabetes could have come about by mimicry between the p277 peptide of hsp60 and some unknown molecule more specific for β-cells [3, 14]. However, the effectiveness of a second hsp60 peptide, p12, supports the conclusion that the hsp60-like molecule functional in diabetes is probably hsp60 itself [3].

The failure of peptides MT-p278 and GAD-p35 to arrest the development of diabetes indicates that not any self-antigen or spontaneously reactive T cell antigen can be used to abort the autoimmune process. It is interesting that MT-p278 failed to induce high titers of antibodies or to protect, despite the fact that this peptide binds IA^{g7} strongly [13] and is strongly immunogenic for NOD T cells (see Figure 2 and [12]). Moreover, the induction of antibodies of any specificity does not necessarily affect NOD diabetes; treatment of NOD mice with BSA, which induces high titers of antibodies as well as strong T cell responses (not shown), does not affect the development of diabetes [6]. Although GAD-p35 was not found by us to be as strongly immunogenic for T cells as were the other peptides (Figure 2), NOD mice have been reported to manifest spontaneous T cell responses to this peptide [15]. The administration of the whole GAD65 molecule in IFA was reported to arrest the disease [16]. Thus, the administration of the GAD-p35 peptide alone may not have provided an adequate therapeutic stimulus. It is interesting that treatment with p277 was found to downregulate autoimmunity to GAD65 epitopes [12], and vice versa, treatment with whole GAD65 was reported to downregulate the spontaneous T cell reactivity of NOD mice to p277 [16]. The spontaneous responses to p277 [2] and to GAD-p35 [15] may be explained by the involvement of these peptides in the autoimmune process. Why p12 differs from p277 in not eliciting spontaneous T cell reactivity *in vitro* is not known; perhaps p12 is more cryptic [17] than p277. The spontaneous responses to MT-p278 can be explained by colonization of the mice with normal mycobacterial flora that may express a similar peptide sequence.

Finally, the association of effective treatment with induction of antibody specific to the peptide suggests that the therapeutic effects of p12, like those of p277 [12], might be related to the activation of Th2-like T cells responsible for helping the induction of specific IgG1 antibodies, antibodies regulated by the production of IL-4 [18]. Such T cells could suppress the Th1 T cells thought to be responsible for damaging the β-cells [11, 12, 16, 19]. Although peptides p277 and p12 also induced peptide-specific antibodies of the IgG2a isotype, thought to be dependent on the Th1-type cytokine IFN-γ [20], the amounts of these antibodies were significantly less than the amounts of the IgG1 antibodies. Thus, the cytokine balance was weighted more on the Th2 side of the scale. The cytokine required for the induction of IgG2b antibodies appears to be TGF-β, a cytokine with known suppressive effects [20, 21]. Further studies are needed to confirm directly the involvement of particular cytokines. It remains to be seen whether the antibodies to the hsp60 peptides induced by peptide treatment can actually affect the disease process or only serve to mark the cytokine shift [12]. Be that as it may, the predominance of peptide-specific antibodies bearing the IgG1 and IgG2b isotypes appears to be an indicator of a beneficial response to the peptides.

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