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Induction of diabetes in standard mice by immunization with the p277 peptide of a 60-kDa heat shock protein

We previously reported that immunity to the p277 peptide of the human 60-kDa heat shock protein (hsp60) was a causal factor in the diabetes of non-obese diabetic (NOD) mice, which are genetically prone to develop spontaneous autoimmune diabetes. The present study was done to test whether immunization with the p277 peptide could cause diabetes in standard strains of mice. We now report that a single administration of the p277 peptide conjugated to carrier molecules such as bovine serum albumin or ovalbumin can induce diabetes in C57BL/6 mice and in other strains not genetically prone to develop diabetes. The diabetes was marked by hyperglycemia, insulitis, insulin autoantibodies, glucose intolerance and low blood levels of insulin. The diabetes could be transferred to naive recipients by anti-p277 T cell lines. Similar to other experimentally induced autoimmune diseases, the autoimmune diabetes remitted spontaneously. After recovery, the mice were found to have acquired resistance to a second induction of diabetes. Susceptibility to induced diabetes in C57BL/6 mice was influenced by sex (males were much more susceptible than were females) and by class II genes in the major histocompatibility complex (B6.H-2^{bm12} mice with a mutation in the MHC-II molecule were relatively resistant). Other strains of mice susceptible to induced diabetes were C57BL/KSJ, C3HeB/FeJ, and NON/Lt. BALB/c and C3H/HeJ strains were relatively resistant. Immunization to p277-carrier conjugates could also induce transient hyperglycemia in young NOD mice, but upon recovery from the induced diabetes, the NOD mice were found to have acquired resistance to later development of spontaneous diabetes. Thus, T cell immunity to the p277 peptide can suffice to induce diabetes in standard mice, and a short bout of induced diabetes can affect the chronic process that would otherwise lead to spontaneous diabetes in diabetes-prone NOD mice.

1 Introduction

It is now generally agreed that type I diabetes mellitus, also called insulin-dependent diabetes mellitus, is caused by an autoimmune process in which T cells invade the pancreatic islets and destroy the insulin-producing β cells [1]. However, controversy surrounds the identity of the target self antigen to which the diabetogenic T cells are directed. A number of different antigens have been identified using antibodies or T cells found in human patients or in the non-obese diabetic (NOD) strain of mouse [2]. Some of these self antigens may be bystanders in the disease process. The question is whether immunity to a particular antigen is both necessary and sufficient to produce the disease.

The p277 peptide is composed of a 24-amino-acid sequence of the 60 kDa heat shock protein (hsp60) of the human [3]. This peptide bears at least one T cell epitope important to the development of diabetes. NOD mice spontaneously developing diabetes manifest progressive T cell reactivity to p277 beginning at the onset of insulitis. T cell clones reactive to p277 can produce diabetes in 1-month-old pre-diabetic NOD mice within 7–10 days of adoptive transfer, administration of the p277 peptide to pre-diabetic NOD mice leads to down-regulation of their spontaneous anti-p277 T cell reactivity and prevents the development of diabetes [3], and treatment with p277 can even arrest the progression of clinically overt diabetes [4]. Spontaneous T cell reactivity to hsp60 in pre-diabetic NOD mice has also been documented by others who reported that T cell immunity to the enzyme glutamic acid dehydroxylase (GAD) might precede the reactivity to hsp60 [5, 6]. These findings raise the question of whether immunity to the p277 peptide of hsp60 is merely a secondary manifestation of islet inflammation or an idiosyncrasy of the NOD genetic program. Can p277 immunity by itself suffice to trigger diabetes? The objective of the present study was to test whether immunity to the p277 peptide could cause diabetes in standard strains of mice such as C57BL/6 that are not prone to develop diabetes and are seemingly free of β cell abnormalities. As administration of the p277 peptide by itself induces resistance to spontaneous diabetes in NOD mice [3, 4], we conjugated the peptide to foreign protein carriers, BSA or OVA, to render it more immunogeneic and tested the ability of the p277-carrier conjugates to induce disease. We here describe the experimental induction of transient autoimmune diabetes mellitus.

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Abbreviations: **hsp60:** 60-kDa heat shock protein **GAD:** Glutamic acid decarboxylase **SI:** Stimulation index **MMTV:** Mouse mammary tumor virus **EDC:** 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

Key words: Autoimmunity / Type I diabetes / T cells / hsp60 / Insulitis

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2 Materials and methods

2.1 Mice

Inbred mice of the C57BL/6, C57BL/KSJ, BALB/c, C3HeB/FeJ and C3H/HeJ strains were purchased from Jackson Laboratories, Bar Harbor, ME. Mice of the NOD/Lt and NON/Lt strains were raised at the animal facilities of the Weizmann Institute of Science from breeding stock kindly supplied by Dr. E. Leiter of the Jackson Laboratories.

2.2 Reagents and immunization

Male or female mice, 2 months old, were immunized s.c. on the back with 50 μg of various antigens in 0.1 ml PBS emulsified in 0.1 ml IFA (Difco, Detroit, MI). OVA and BSA were purchased from Sigma (St. Louis, MO). The peptides p277 and p278 of human hsp60 were synthesized and purified as described [3]. The sequence of p277 is VLGGGCALLRCIPALDSLTPANED, and that of p278 is NEDQKIGIEIIKRTLKI. Conjugation of peptides to OVA or to BSA was done using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [7]. Briefly, a molar part of OVA or BSA was mixed with 40 molar parts of peptide and 400 molar parts of EDC and incubated at room temperature for 18 h, dialyzed for 3 days with three daily changes against 100 volumes of double-distilled H_2O, and lyophilized. The number of conjugated peptides per carrier molecule was determined by amino acid analysis of the conjugates, and was 18:1 for the p277-BSA, 10:1 for the p277-OVA, and 12:1 for the p278-OVA conjugates. The immunization with non-conjugated p277 and BSA was carried out by mixing the peptide and the carrier at the same molar ratio as the conjugate. Human hsp60 and mycobacterial hsp65 were kindly supplied by Dr. Ruurd van der Zee of the University of Utrecht. Two weeks after immunization, the mice were bled at 10:00 AM without fasting for determinations of serum antibodies and blood glucose concentrations.

Persons wishing to carry out experiments with p277 should note that the peptide is prone to oxidation, aggregation and other processes that affect its biological activity, probably because of the two cysteine residues in its sequence. In solution, p277 loses biological activity within 1–2 months, even when kept at −20 °C.

2.3 Blood glucose

Blood glucose was measured using a Glucose Analyzer 2 (Beckman Instruments, Brea, CA) and hyperglycemia was defined by a blood glucose concentration of 11.1 mmol/l or greater, as this value is 3 standard deviations above the mean value of blood glucose determined in 200 naive mice (not shown).

2.4 Antibodies

Antibodies to insulin and to the unconjugated peptides p277 or p278 were measured in serum diluted 1:50 using an ELISA as described [3]. Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were used because they were suitable for high absorption of the peptides.

2.5 T cell proliferation

Mice were immunized s.c. into the hind foot pads with 50 μg antigen in 0.1 ml PBS emulsified in 0.1 ml IFA. After 10 days, the proliferative responses of cells from the draining popliteal lymph nodes were assayed as described [3] using 5–20 μg/ml of various antigens in quadruplicate, in microtiter wells, each well containing 2×10^5 cells in 0.2 ml medium. The results are shown as the cpm or the stimulation index (SI): the ratio of the mean test cpm to the mean control cpm without antigen. The SD of the means were no greater than 10 % of the mean.

2.6 T cell lines

Lines of T cells were developed to p277 or to OVA by immunizing C57BL/6 mice with p277 conjugated to OVA in IFA as above. The lines were obtained by culturing the draining lymph node cells with either the p277 peptide or with OVA (5 μg/ml) for three cycles of stimulation; each cycle consisted of 3 days of culture with antigen and irradiated (3000 rad) syngeneic splenocytes as APC followed by rest for 7 days in medium supplemented with 10 % IL-2 without added antigen or APC as described [3]. The lines manifested immunologic specificity: the line selected with p277 showed proliferative responses to p277 of 16762 ± 987 cpm and to OVA of 3750 ± 236 cpm, with a background of 3930 ± 387 cpm; the line selected with OVA showed proliferation responses to OVA of 7850 ± 503 cpm and to p277 of 2495 ± 197 cpm, with a background of 2520 ± 211 cpm. To test the function of the line cells *in vivo*, naive mice were inoculated i.p. with 5×10^6 T cells line blasts obtained 3 days after stimulation with either p277 or OVA and added APC as described [3]. The mice were then scored for hyperglycemia.

2.7 Glucose tolerance test

Male C57BL/6 mice, 7 weeks old, were immunized with p277-OVA in IFA or IFA alone and were tested for basal blood glucose 14 days later. Sixteen days after immunization, the mice received 40 mg glucose i.p. (0.2 ml of a 20 % glucose solution). The mice were then bled at 20 min, 40 min, 60 min and 90 min, and the sera of individual mice were assayed for their glucose concentrations as described above.

2.8 Insulin concentration

Serum insulin in immunized and control mice was measured by radioimmunoassay as described [8], using an insulin radioimmunoassay kit (Sorin Biomedica, Saluggia, Italy). To dissociate any complexes between insulin and anti-insulin antibodies in the test sera, we incubated the test sera for 18 h with the labeled insulin and the insulin antibodies of the kit. The calibration curve was made using rat insulin (Novo-Nordisk, Denmark) as a standard. The

Table 1. Induction of diabetes in C57BL/6 male mice by immunization to p277 conjugates[a)]

	Hyperglycemia in immunized mice			Anti-insulin antibodies	Anti-p277 antibodies	Anti-p278 antibodies
Immunogen (50 μg)	Sex	% Incidence	No. of mice	A_{405} (mean ± SD)		
OVA	male	0	20	0.22 ± 0.03	0.35 ± 0.05	0.21 ± 0.03
BSA	male	0	20	0.21 ± 0.02	0.31 ± 0.06	0.25 ± 0.04
p277 + BSA (unconjugated)	male	0	30	0.25 ± 0.03	0.27 ± 0.05	0.22 ± 0.04
p277-BSA	male	67*	99	1.32 ± 0.22*	1.42 ± 0.32*	0.31 ± 0.03
p277-OVA	male	83*	89	1.25 ±0.20*	1.71 ± 0.41*	0.28 ± 0.03
p277-OVA	female	0	25	0.29 ± 0.04	1.23 ± 0.25*	0.33 ± 0.04
p278-OVA	male	0	26	0.25 ± 0.03	0.45 ±0.1	0.57 ± 0.11*

a) C57BL/6 mice, 2 months old, were immunized s.c. with 50 μg OVA or BSA or peptide carrier conjugates in IFA. Two weeks later, the individual mice were bled and tested for hyperglycemia and for antibodies to insulin, to peptide p277, and to peptide p278, regardless of whether or not they were hyperglycemic.

* $p < 0.01$ compared to controls immunized with OVA alone.

detection limit of the assay was 0.03 ng/ml insulin, and the working range of the curve (20–80 % binding) was between 0.4 and 40 ng/ml.

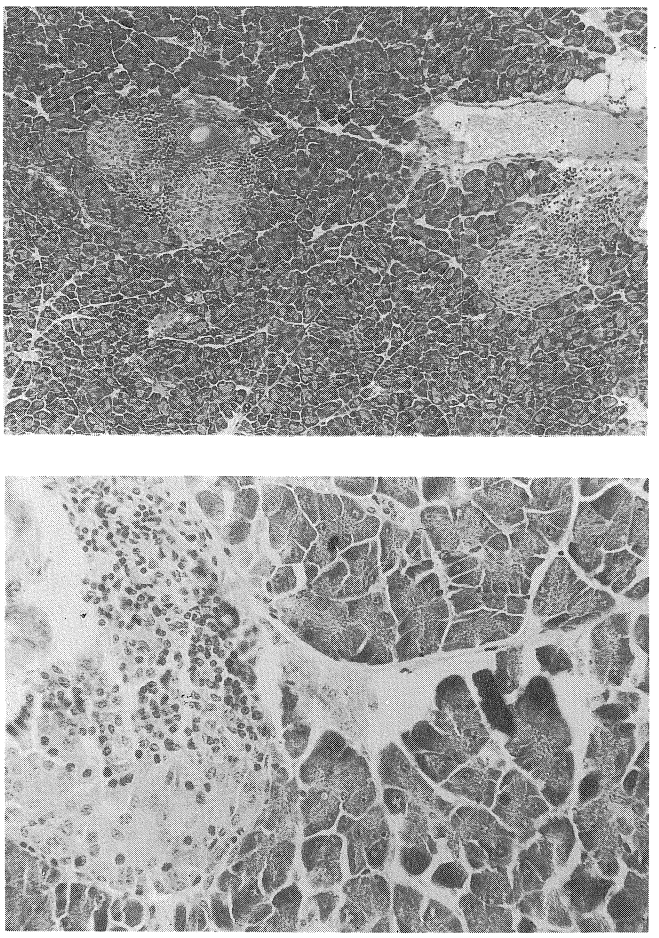

Figure 1. Insulitis induced by immunization of male C57BL/6 mice with the p277-BSA conjugate. Histology was done 3 weeks after immunization when the blood glucose concentration was 14.5 mmol/l (Bouin fixation, hematoxylin and eosin staining). The upper panel shows a low magnification view (×80) of three islets manifesting a predominately peri-islet insulitis. The lower panel shows a higher magnification view (×340) of an islet manifesting partial intra-islet penetration of the infiltrate.

2.9 Statistical significance

Student's *t*-test and the Chi square test were used where appropriate to assay significant differences between experimental and control groups.

3 Results

3.1 Immunization to p277 conjugates induces diabetes

Table 1 shows the effect of various immunizations of both sexes of C57BL/6 mice on the development of hyperglycemia and of antibodies to insulin, to the p277 peptide and to a control hsp60 peptide, p278 [3]. Administration of OVA, BSA, or a mixture of p277 and BSA did not cause hyperglycemia. However, p277 conjugated to OVA or to BSA induced hyperglycemia in a high proportion of the male mice. Hyperglycemia was accompanied by antibodies to p277 and to insulin. Fig. 1 shows that the mice developing hyperglycemia also manifested insulitis. It can be seen that the degree of infiltration was generally mild; most of the affected islets showed only peri-islet infiltrates. Immunohistochemical staining of the pancreas showed that the affected islets still contained insulin (not shown). The low level of insulitis and the persistence of insulin are compatible with the clinical course of the disease (see below). The female mice immunized to p277-OVA produced anti-p277 antibodies but did not develop hyperglycemia, had no insulitis (not shown), and made no antibodies to insulin. The control peptide p278 conjugated to OVA failed to induce diabetes in either the male or female C57BL/6 mice. We conclude, therefore, that p277 immunity, influenced by sex, can induce diabetes, that antibodies to p277 alone do not cause diabetes, and that anti-insulin antibodies may be a manifestation of the β cell damage associated with diabetes.

Class II genes in the major histocompatibility complex (MHC) can also influence susceptibility to induction of diabetes by p277-OVA in C57BL/6 mice: diabetes developed in only 2/11 of MHC class II-mutant male B6.H-2^{bm12} mice ($p < 0.01$) compared to 74/89 of male C57BL/6 mice (Table 1). Male B6.H-2^{bm1} mice bearing an

MHC class I mutation [9] showed the same high incidence of diabetes (8/10) as males of the wild-type C57BL/6 strain.

3.2 Induced diabetes remits

Fig. 2 illustrates the time course and the magnitude of hyperglycemia in male C57BL/6 mice. It can be seen that the disease was self-limited: the mice became hyperglycemic within 2 weeks of immunization to p277-OVA, but by 6 weeks, the mice had fully recovered (Fig. 2, upper panel). Moreover, after their recovery, the mice were found to be resistant to a second attempt to induce diabetes by immunization to p277, even when conjugated to a different carrier (BSA instead of OVA, Fig. 2, lower panel). Control mice immunized with p277-BSA for the first time did develop diabetes. Thus, diabetes induced by active immunization of non-diabetic mice to p277 conjugates is similar to other experimental autoimmune diseases such as EAE in rats which tends to be a monophasic, transient disease and in which spontaneous recovery is marked by the acquisition of resistance to further attempts to induce the disease [10].

3.3 T cell responses to p277

Fig. 3 illustrates that immunization to p277-OVA activated T cell responses to both the p277 peptide and to the carrier antigen. Note that the p277 peptide was highly immunogenic: the single primary immunization to the conjugate induced a response to the p277 peptide that was greater ($p < 0.01$) than the response measured against the large OVA antigen with its many foreign epitopes. Also note that the T cells activated by immunization to p277-OVA responded to the human hsp60 molecule to a much greater degree ($p < 0.01$) than they responded to mycobacterial hsp65 [11]; the mycobacterial version of the p277 epitope appears to be only somewhat cross-reactive [3]. Thus, p277 conjugates are highly immunogenic for T cells as well as inducers of antibodies. Immunization to unconjugated OVA induced T cell responses to OVA alone.

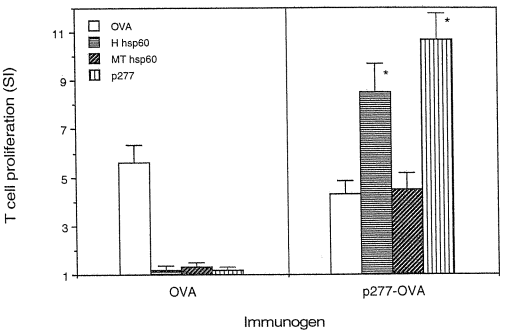

Figure 3. Immunodominance of peptide p277. The T cell proliferative responses to OVA, to p277, to human hsp60 (H hsp60), or to mycobacterial hsp65 (MT hsp60) [11] in male C57BL/6 mice that had been immunized with p277-OVA or with unconjugated OVA. Immunization was done as described in the legend to Table 1, except that the injections were into the hind foot pads. After 10 days, the proliferative responses from the draining popliteal lymph nodes were assayed [3] using 5 μg/ml of the antigens. The background cpm values (cpm without added antigens) were 4520 ± 233 and 4044 ± 301 for the mice immunized with OVA or with p277-OVA, respectively; the responses to mitogen stimulation with concanavalin A (1.25 μg/ml; Sigma) were 89460 ± 5320 cpm and 85615 ± 4019 respectively. * $p < 0.01$ compared to the responses to OVA or to MT hsp60.

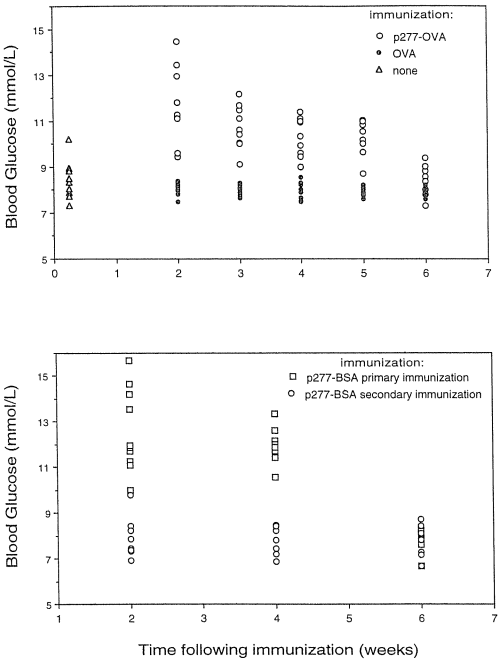

Figure 2. Time course of hyperglycemia and resistance to challenge induced by p277 conjugates. The upper panel shows the concentrations of blood glucose in individual mice immunized with p277-OVA (open circles) or with OVA (closed circles). Unimmunized mice are indicated by the open triangles. The lower panel shows the effects of challenging the mice with p277-BSA 4 weeks after their recovery from hyperglycemia induced by the p277-OVA (open circles). Control mice (open squares) which had not been immunized previously with p277-OVA, were also immunized with p277-BSA.

3.4 T cell lines mediate hyperglycemia

To test the pathogenicity of anti-p277 T cells, lines of T cells specific either for p277 or for OVA were raised

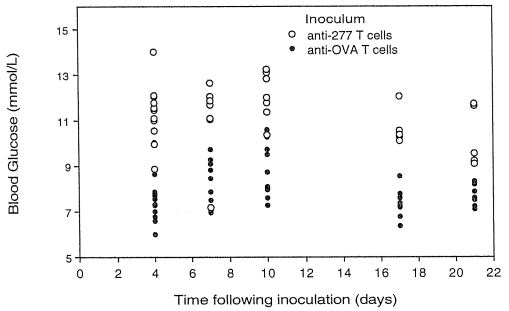

Figure 4. Adoptive transfer of hyperglycemia by anti-p277 T cells. Blood glucose was measured in individual, naive male C57BL/6 mice that had received T cell lines responsive to OVA (closed circles) or to p277 (open circles). The lines had been isolated from the draining popliteal lymph nodes of mice immunized with p277/OVA as described in Fig. 3. Each mouse received 5×10^6 antigen-activated line cells intraperitoneally.

from mice immunized with p277-OVA. The activated line cells (5×10^6) were inoculated into naive C57BL/6 mice. Fig. 4 shows that the recipients of the p277-reactive T cells developed hyperglycemia within 1 week. Histologic examination demonstrated insulitis similar to that shown in Fig. 1. The anti-OVA T cells failed to cause hyperglycemia or insulitis. Thus, the hyperglycemia and insulitis induced by immunization to p277-OVA could be attributed to the activities of T cells responding to p277.

3.5 Central glucose intolerance

Since the magnitude of the hyperglycemia and the degree of insulitis induced by immunization were much lower than that observed in NOD mice at the peak of their chronic progressive disease, we felt obliged to confirm the diagnosis of diabetes clinically. The hallmark of type I diabetes is intolerance to glucose caused by a deficiency of β-cell secretion of insulin. We therefore measured the insulin concentrations in the sera of mice immunized with p277-OVA or with OVA. Blood was taken 2 weeks after immunization from groups of five unfasted mice at 10:00 AM and assayed for insulin levels. The OVA-immunized mice had a mean insulin level of 3.7 ± 1.2 ng/ml, while the mice immunized with p277-OVA had a mean insulin level of 0.65 ± 0.24 ng/ml ($p < 0.01$). Thus, the hyperglycemia induced by p277-OVA immunization was associated with hypoinsulinemia. Similar results were obtained in three consecutive experiments.

To confirm the effect of immunization to p277 conjugates on glucose tolerance, we immunized male C57BL/6 mice with p277-BSA, and 2 weeks later, when the mice were hyperglycemic, we challenged the immunized and control mice with an i.p. injection of 40 mg glucose. The mice were bled at regular intervals and the blood was tested for glucose concentrations. Fig. 5 shows that the control mice, injected with IFA alone, manifested a peak of hyperglycemia at 20 min that did not exceed 28 mmol/l. Thereafter, glucose concentrations in control mice fell to baseline levels. The p277-BSA immunized mice, in contrast, manifested grossly abnormal responses to the glucose challenge. Mean peak glucose concentrations were abnormally high: 39 mmol/l, and the hyperglycemia persisted for at least 90 min.

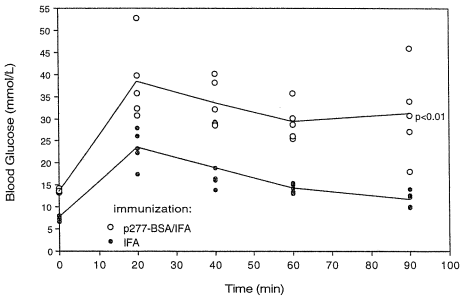

Figure 5. Abnormal glucose tolerance test results in p277-immunized mice. Male C57BL/6 mice were immunized with p277-BSA in IFA (12 mice; open circles) or IFA alone (8 mice; closed circles). After 14 days, all the mice were bled for determination of their basal glucose serum levels. The mice were challenged 2 days later with 40 mg glucose i.p. To avoid excess bleeding, one half of the mice of each group were bled individually at the alternate time points and the sera were assayed for glucose concentration. The results of the glucose determinations are shown for the individual mice; the solid line indicates the means for the two groups.

Table 2. Susceptibility of various mouse strains to hyperglycemia induced by immunization with p277 conjugates[a)]

Immunized mice				Peptide conjugate	Development of hyperglycemia (%)
Strain	MHC	Sex	No.		
C57BL/6	H-2^b	M	184	p277	75.5*
		F	35		0
		M	42	p278	2.3
		F	15		0
NON/Lt	H-2^b	M	45	p277	28.8*
		F	16		62.5*
		M	20	p278	0
		F	6		0
C57BL/KSJ	H-2^d	M	40	p277	80*
		F	10		0
		M	20	p278	0
		F	10		0
BALB/c	H-2^d	M	16	p277	0
		F	30		26.6*
		M	10	p278	0
		F	15		0
C3HeB/Fej	H-2^k	M	20	p277	60*
		M	20	p278	0
C3H/Hej	H-2^k	M	10	p277	0
		M	10	p278	0

a) Male (M) and female (F) mice of the indicated strains were immunized with p277 or p278 conjugated to OVA or to BSA. Two weeks later, the mice were bled and the individual sera were studied for hyperglycemia.

* $p < 0.01$ compared to control mice immunized with p278 conjugates.

We also investigated whether anti-p277 immunity, in addition to insulitis, might cause peripheral resistance to insulin. Using a lipogenesis assay [12], we found that the fat cells derived from the hyperglycemic mice did not differ from the control groups in their sensitivity to insulin; insulin binding was estimated to be 11 fmol/10^6 cells and the insulin ED_{50} was 0.15–0.18 ng/ml, values which are in the physiological range [12]. Therefore, no peripheral insulin resistance could be detected. Thus, the hyperglycemia was probably a result of a central defect in insulin secretion caused by the T cells.

3.6 Various mouse strains are susceptible to induced diabetes

To learn whether additional strains of mice are susceptible to diabetes, we immunized them with p277-OVA and measured hyperglycemia developing 2 to 3 weeks later. Table 2 shows that mice of the C57BL/KSJ, C3HeB/FeJ, and NON/Lt strains were relatively susceptible. BALB/c and C3H/HeJ mice showed lower incidences of hyperglycemia. Note that male C57BL/KSJ mice, like male C57BL/6 mice, were more susceptible than females. In contrast, females were more susceptible in the NON/Lt and BALB/c strains. Thus, diabetes inducible by p277-carrier immunization is not limited to C57BL/6 mice.

Table 3. Transient diabetes induced in young NOD mice prevents the development of spontaneous diabetes[a)]

Sex	Induced hyperglycemia in pre-diabetic 4-week-old NOD mice			Spontaneous diabetes at 8 months
	Immunogen	% Incidence	no. of mice	
Female	None	0	25	100
	OVA	0	30	100
	p277	0	40	20*
	p277-OVA	82*	50	22*
Male	None	0	30	57
	OVA	0	30	47
	p277	0	30	10*
	p277-OVA	55*	40	12*

a) Four-week-old pre-diabetic NOD mice were immunized, and two weeks later hyperglycemia was determined as described in the legend to Table 1. The mice were also assayed for hyperglycemia developing spontaneously at the age of 8 months.

* $p < 0.01$ compared to unimmunized control mice.

3.7 Induced diabetes protects against spontaneous diabetes

As we have seen above, immunization to p277 conjugates induces diabetes in standard mice; what would such immunization do to NOD mice prone to develop diabetes spontaneously? Table 3 shows that prediabetic NOD mice immunized to p277-OVA developed hyperglycemia and insulitis in 2 weeks. The females showed a somewhat higher incidence of diabetes than did the males, consistent with the higher incidence of spontaneous diabetes in NOD females in our colony [11]. However, both the females and male NOD mice spontaneously recovered from the induced disease after several weeks. Note that the bout of induced diabetes was followed by increased resistance to the development of spontaneous diabetes: at the age of 8 months, only 22 % of female NOD mice and 12 % of male NOD mice were diabetic compared to 100 % of control female NOD mice and 47–57 % of control male NOD mice ($p < 0.01$). In fact, all of the individual mice that did not develop hyperglycemia after p277-OVA immunization were also not protected later against spontaneous diabetes (not shown). Thus, induced diabetes effectively endowed the NOD mice with resistance to spontaneous diabetes (Table 3) just as it produced resistance in C57BL/6 mice to a second bout of diabetes (Fig. 2). As we reported earlier [3, 4], administration of the p277 peptide unconjugated to a carrier also induced resistance to the development of spontaneous diabetes. However, the unconjugated p277 does not induce a bout of hyperglycemia before it induces resistance.

4 Discussion

The results presented here show that the p277 peptide of hsp60 can induce a diabetogenic process in otherwise healthy strains of mice; the involvement of p277 in diabetes is not a peculiarity of the spontaneous disease of the NOD mouse. Note that the transient disease induced by immunization to p277 conjugates is quite unlike the classical diabetes expressed in NOD mice, which is marked by progressively destructive insulitis and the total loss of β cells and insulin production ending, untreated, in death. Nevertheless, the induced disease satisfies a definition of immunological diabetes in that it is caused by a specific immunization, is mediated by specific T cells, and is expressed pathologically by a form of islet inflammation and manifested clinically by hypoinsulinemia, hyperglycemia and glucose intolerance. Although all of these manifestations seem mild when compared to the NOD disease, the disease manifestations, nevertheless, are clearly pathological when the p277-conjugate immunized mice are compared to the control immunized mice. Actually, the disease induced by p277 conjugate immunization progresses much more rapidly to its peak than does the NOD disease: the induced hyperglycemia takes 2 weeks to be expresed, while the NOD hyperglycemia may take months to appear after the onset of insulitis. The causes for the differences between NOD diabetes and the immunological diabetes described here remain to be investigated, but it is likely that the dozen or so different susceptibility genes carried by the NOD mouse [13] will be found to contribute significantly.

The sequence of the p277 peptide is from the human variant of hsp60, and differs by one amino acid from the homologous mouse peptide (T for K at the sixth residue from the carboxy-end of the peptide; [14]). However, mouse hsp60 is also recognized by the diabetogenic mouse T cells that respond to p277 (in preparation). This indicates that the T cell response to p277 is an autoimmune response.

The functional association of p277 autoimmunity with diabetes is unexpected: the hsp60 molecule is a mitochondrial protein [15] expressible in all the cells in the body and is not specific to β cells. However, as shown by Brudzinsky et al. [16], hsp60 is expressed in mouse β cells not only in the mitochondria, but also in the mature insulin-containing secretory granules. Therefore, the physiology of hsp60 in the β cell may be unique, but how this unique expression could account for the role of hsp60 in diabetes is a question for further research. Although GAD is another antigen implicated in autoimmune diabetes which is expressed in the secretory granules of β cells [17], it too is not specific for β cells, and is also expressed in the nervous system [18]. Perhaps tissue specificity may be related to the nature of an antigen's expression and not merely to its expressibility. In any case, there seems to be a functional relationship between T cell autoimmunity to the p277 peptide of hsp60 and diabetes. Such immunity is present in the spontaneous diabetes of NOD mice [3] and, as we show here, the induction of such immunity in various standard strains of mice can lead to diabetes. Moreover, we have recently found that anti-p277 and anti-insulin autoimmunity accompanies the development of autoimmune diabetes induced in C57BL/KSJ mice by administration of a low dose of the β cell toxin streptozotocin [19]. Thus, p277 autoimmunity is a feature of mouse diabetes regardless of whether the disease arises spontaneously [3], is induced by specific immunization, or results from a toxic insult [19].

It is interesting that immunity to the p277 peptide is observable in these various forms of diabetes appearing in genetically diverse strains of mice. NOD mice do share H-$2K^d$ and H-$2D^b$ class I molecules with the p277-susceptible strains C57BL/6 (H-2^b), NON/Lt (H-2^d) and C57BL/KSJ

($H\text{-}2^d$). However, the MHC class II molecules of these strains differ from that of NOD. Moreover, the susceptible C3HeB/FeJ strain is $H\text{-}2^k$. Thus, epitopes on the p277 peptide may be presentable by different MHC alleles [20].

Note that the C3H/HeJ strain was resistant to diabetes and the C3HeB/FeJ was susceptible (Table 3). These two strains bear the same $H\text{-}2^k$ genotype on a similar genetic background. However, the resistant C3H/HeJ strain expresses a mouse mammary tumor virus (MMTV) that the susceptible C3HeB/FeJ strain does not [21]. It is known that MMTV encodes a superantigen that eliminates from the repertoire T cells expressing certain Vβ gene segments in the T cell receptor repertoire [22]. Hence it is conceivable that the resistance of the C3H/HeJ strain could be caused by the deletion of T cells responsible for diabetes.

With regard to the effect of sex on susceptibility to diabetes, sex differences in immune responses are usually attributable to the different effects of sex hormones on the immune system [23]. However, it is not known why these differences should affect susceptibility differently in different strains.

The questions of the transience and termination of diabetes also are in need of further study. It is generally considered that the outbreak of clinical diabetes occurs only after about 90% of the β cells have been destroyed [1]. Our results demonstrate, however, that there is a stage of β cell injury inflicted by T cells that is reversible; this damage is possibly mediated by cytokines [24]. However, the process that arrests the autoimmune T cells is not yet clear. The induction of regulatory T cells, possibly anti-idiotypic T cells [25], has been observed to accompany the resistance to EAE that develops following recovery from a bout of the disease [26].

Note that the induction of diabetes in pre-diabetic NOD mice by immunization to a p277 conjugate also led to resistance to the spontaneous diabetes programmed to appear later in life. This suggests that an episode of acute autoimmunity may activate or strengthen regulatory mechanisms that are able to cope with the chronic autoimmunity characteristic of spontaneous diabetes. In any case, it is clear that the context in which the p277 peptide is administered affects the nature of the immune response [27, 28]: unconjugated p277 peptide induces arrest of the diabetogenic process [3, 4] while, as we show here, conjugated p277 activates the diabetogenic process, albeit transiently.

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