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T Cell Proliferative Responses of Type 1 Diabetes Patients and Healthy Individuals to Human hsp60 and its Peptides

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T cell responses to peptide epitopes of the 60 kDa heat shock protein (hsp60) have been shown to play a role in the pathogenesis of type 1 insulin-dependent diabetes mellitus (IDDM) in mice. To test whether hsp60 autoimmunity might be involved in human type 1 diabetes, we studied T cell proliferative responses (stimulation index; SI) to intact human hsp60, to hsp60 peptides and to a recall antigen (tetanus toxoid) in 25 newly diagnosed type 1 diabetes patients, in 22 type 2 (non-insulin-dependent diabetes mellitus, NIDDM) patients, and in 25 healthy blood donors. There were no significant differences between the T cell responses of the three groups to tetanus toxoid. However, the responses to hsp60 of the type 1 diabetes group (median SI=5) were significantly greater ($P<0.01$) than those of the type 2 group (median SI=1.67) and of the blood donors (median SI=1.7). Epitope mapping revealed significant responses to at least seven different peptides, with prevalent responses to the p277 peptide previously mapped in NOD mice and to peptide p32. Thus, newly diagnosed type 1 diabetes patients, similar to prediabetic and newly diabetic NOD mice, show heightened autoimmunity to hsp60 and hsp60 peptides.

Introduction

Type 1 insulin-dependent diabetes mellitus (IDDM) results from destruction of the insulin-producing β-cells of the pancreatic islets caused by autoimmune T cells [1–3]. Both the human disease and the model disease spontaneously developing in NOD strain mice appear to involve autoimmunity to a similar collective of antigens (reviewed in [4]) including proinsulin and insulin [5, 6], glutamic acid decarboxylase (GAD) [7, 8], the islet cell antigen ICA-69 [9] and the insulin secretory-granule 38 kDa protein (38 kDa) [10]. Among the autoantigens functional in NOD mouse type 1 diabetes is the 60 kDa heat shock protein (hsp60): anti-hsp60 T cells can mediate insulitis and hyperglycemia [11] and modulation of the anti-hsp60 T cell response can arrest the autoimmune destruction of β-cells [11–13]. The 437–460 peptide sequence of hsp60, designated p277, has been found to serve as a functionally important target in mouse type 1 diabetes. T cells reactive to p277 can adoptively transfer diabetes, and a single subcutaneous administration of peptide p277 (100 μg in oil), even late in the autoimmune process, can induce a shift in the cytokine profile of the spontaneous T cell response from a damaging Th1-type to an anti-inflammatory Th2 type [13], leading to the permanent arrest of β-cell damage in most NOD mice [14]. Recently, another hsp60 peptide, p12 (166–185), has been found to be effective in halting the NOD disease [15].

In view of the role of hsp60 and various hsp60 peptides in NOD diabetes, we tested whether recently diagnosed human type 1 diabetes patients might show T cell proliferative responses to human hsp60. We also mapped responses to a series of hsp60 peptides including p277, and made some preliminary observations about changes in these T cell responses over time.

Materials and Methods

Subjects

Type 1 diabetes patients

Twenty-five patients, consecutively admitted to the Department of Endocrinology of the Hadassah University Hospital, Jerusalem, Israel, were enrolled in the study. The day of diagnosis was defined as the day on which hyperglycemia was detected and insulin therapy was initiated. The mean and median elapsed time since diagnosis was 6 weeks (range 1–14

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weeks). The Department of Endocrinology is not a paediatric department, therefore the median age of the patients was relatively high (21 years) and the mean age was 26.6±15.3 years. A diagnosis of type 1 diabetes in patients younger than 21 years of age was made on the basis of hyperglycaemia (blood glucose >200 mg/dl), ketonuria, low body weight and BMI less than 23, and the absence of family history of type 2 diabetes. In patients older than 21 years of age, the diagnosis of type 1 diabetes was confirmed by the presence of anti-GAD antibodies, measured in the Endocrinology Unit of the Hadassah University Hospital, Jerusalem, Israel, using the GAD-AB kit (CIS Bio International, Gif-sur-Yvette, France); a serum sample was considered positive at >1000 U/ml. Informed consent was obtained.

Type 2 diabetes patients

Twenty-two patients previously diagnosed as having type 2 diabetes at the Department of Endocrinology were admitted to the study. In an attempt to control for the possible effects of disease severity and insulin therapy, the type 2 patients, like the type 1 patients, were all being treated with insulin to manage their hyperglycaemia. All patients were diagnosed as having type 2 diabetes due to dietary or hypoglycaemic drug control of diabetes for at least 5 years from diagnosis and prior to the need for insulin treatment. Moreover, all patients had a family history of type 2 diabetes. The median age of the type 2 diabetes patients was 64 years and their mean age was 63.9±5.9 years. The mean duration of disease was 20.1±7.7 years and the mean time they had been treated with insulin was 6.6±4.9 years. Informed consent was obtained.

Healthy blood donors

Surplus blood was obtained from the blood bank from 25 healthy donors.

T cell proliferation

Fifty milliliters of venous blood was drawn from patients or obtained from the blood bank. Anticoagulation was achieved with heparin, 10 IU/ml. PBMC were isolated by density centrifugation on Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden). The cells were washed with AIM-V serum-free culturing media (Gibco, Grand Island NY, USA), supplemented with 1% sodium-pyruvate, 1% l-glutamine (200 mM), 1% penicillin/streptomycin (10,000 U/ml/10,000 mg/ml) and 2% HEPES (1 M, pH 7.3) (Biological Industries, Kibbutz Beit Haemek, Israel).

PBMC were plated in quadruplicate in 96-well round-bottom microplates (Falcon, Lincoln Park, NJ, USA) at a cell concentration of 2×10^5 cells/well in 100 µl AIM-V media, with or without test antigens or mitogen: 0.3 mg/ml PHA (Murex Diagnostic Ltd., Dartford, UK); 5 µg/ml tetanus toxoid (Connaught Laboratory Inc., PA, USA); 10 µg/ml recombinant human hsp60 (StressGen, Vancouver, Canada); or 20 µg/ml hsp60 peptides. Epitope mapping was carried out in RPMI medium supplemented with 10% autologous serum. The plates were incubated at 37°C in a 5% CO_2 humidified incubator for 6 days. On day 5, the cells were labelled overnight with 1 µCi/well of ^{3}H-thymidine and counted in a Matrix 96 β-counter (Packard, Meriden, CT, USA).

Proliferation is presented as the stimulation index (SL); the ratio of the mean counts per minute (cpm) with antigen to the cpm without antigen. SI values of 2 or greater were considered to be positive, marked by the horizontal line in Figures 1–3. The screening of T cell responses was carried out in AIM-V serum-free medium, which produces lower and more specific cpm responses compared to autologous serum or FCS supplemented media, therefore the cut-off of 2 was considered appropriate. No responses were detected against a control preparation of *E. coli* transfected with the pGEX vector, without the hsp60 gene, as a test for possible *E. coli*-derived artifacts. Moreover, the recombinant hsp60 was sent for lippopolysaccharide (LPS) testing (Quantitative Chromogenic LAL assay, Biological Industries, Beit Haemek, Israel) and tested negative for detectable endotoxins. The range of non-antigen-stimulated ^{3}H-thymidine incorporation (background) was 606–3285 cpm (mean=1870) for the healthy blood donors and 336–4866 (mean=1570) for the patients. The intraassay coefficient of variation was 5–20%. Patients and controls were studied in the same assay.

Statistical analysis was performed using the InStat 2.01 computer program. *P* values were approximated using Kruskal–Wallis non-parametric ANOVA test.

hsp60 peptides

Thirty-eight peptides spanning the sequence of human hsp60 (Table 1) were synthesized by Ms Aviva Kapitkovsky of the Biological Services Laboratory, The Weizmann Institute of Science, Rehovot, Israel using an automated ABIMED synthesizer AMS422 (Abimed, Langenfeld, Germany). The peptides were purified by reverse phase HPLC and their compositions were confirmed by amino acid analysis. Note that peptide p277 contains an exchange of its two cys residues by val residues. This modification enhances the stability of p277 without changing its immune properties in NOD mice [13, 16].

Results

T cell responses to hsp60

Table 2 shows the T cell responses of the individual type 1 diabetes, type 2 diabetes and blood donor subjects to the recall antigen tetanus toxoid and to intact hsp60. It can be seen that there were no significant differences between the three groups in their

Table 1. *Overlapping peptides of the human hsp60 molecule used for testing T cell responses*

Peptide number	Position	Sequence
p1	1–20	MLRLPTVFRQMRPVSRVLAP
p2	16–35	RVLAPHLTRAYAKDVKFGAD
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	EEIAQVATISANGDKEIGNI
p13	181–199	EIGNIISDAMKKVGRKGVI
p14	195–214	RKGVITVKDGKTLNDELEII
p15	210–229	ELEIIEGMKFDRGYISPYFI
p16	225–244	SPYFINTSKGQKCEFQDAYV
p17	240–259	QDAYVLLSEKKISSIQSIVP
p18	255–275	QSIVPALEIANAHRKPLVIIA
p19	271–290	LVIIAEDVDGEALSTLVLNR
p20	286–305	LVLNRLKVGLQVVAVKAPGF
p21	301–320	KAPGFGDNRKNQLKDMAIAT
p22	316–335	MAIATGGAVFGEEGLTLNLE
p23	331–350	TLNLEDVQPHDLGKVGEVIV
p24	346–365	GEVIVTKDDAMLLKGKGDKA
p25	361–380	KGDKAQIEKRIQEIIEQLDV
p26	376–395	EQLDVTTSEYEKEKLNERLA
p27	391–410	NERLAKLSDGVAVLKVGGTS
p28	406–425	VGGTSDVEVNEKKDRVTDAL
p29	421–440	VTDALNATRAAVEEGIVLGG
p30	436–455	IVLGGGCALLRCIPALDSLT
p31	451–470	LDSLTPANEDQKIGIEIIKR
p32	466–485	EIIKRTLKIPAMTIAKNAGV
p33	481–500	KNAGVEGSLIVEKIMQSSSE
p34	496–515	QSSSEVGYDAMAGDFVNMVE
p35	511–530	VNMVEKGIIDPTKVVRTALL
p36	526–545	RTALLDAAGVASLLTTAEVV
p37	541–560	TAEVVVTEIPKEEKDPGMGA
p38	556–573	PGMGAMGGMGGGMGGGMF
p277	437–460	VLGGGVLLLRVIPALDSLTPANED

The table shows the sequence of 38 peptides spanning the human hsp60 molecule with five overlapping amino acids on each side. The sequence of peptide p277 was modified by exchange of its 2 cys residues by val residues

responses to tetanus toxoid. However, the type 2 diabetes subjects showed a tendency towards lower responses, probably because they were older then the others, and less likely to have received a tetanus toxoid boost in the recent past.

The T cell responses to hsp60, in contrast, were significantly elevated in the type 1 diabetes group compared to the other groups. Although each group contained persons who were clearly high responders, significantly more (P=0.006) of the type 1 diabetes group (23/25; 92%) were positive compared to the blood donors (10/25; 40%) or type 2 diabetes patients (8/22; 36%). The degree of responsiveness was also higher in the type 1 diabetes group (median SI=5) compared to the type 2 diabetes group (median SI=1.67) or to the blood donors (median SI=1.7), P<0.01 and P<0.001, respectively. Thus, recently diagnosed type 2 diabetes patients, as a group, show a heightened T cell proliferative response to hsp60.

Epitope mapping

To determine the spectrum of hsp60 peptides recognized by type 1 diabetes patients, we tested the proliferative responses of five patients showing multiple reactivities (5–7 hsp60 peptides) to the 38 overlapping peptides. Figure 1 shows that three or more of the patients each reacted to the following seven peptides: p3, p10, p18, p20, p30, p32, and p35. Peptide p30 (residues 436–455) is quite similar to p277 (residues 437–460). Thus, multiple hsp60 peptides appear to be recognized by the type 1 diabetes population.

Responses to hsp60 and peptides

Figure 2 shows the magnitude of the T cell responses of individual type 1 diabetes patients and blood donors to intact hsp60 and to two major hsp60 peptides: p277 and p32. It can be seen that 20 of the 23 type 1 diabetes patients (87%) positive to hsp60 also responded to one or both peptides; 57% to p277 and 83% to p32. Six of the blood donors responding to hsp60 also responded to one or both of the peptides. One donor, number 25, responded to p32 without a detectable response to intact hsp60.

Dynamics of responsiveness

A preliminary study of the dynamics of responsiveness was carried out on a small number of patients. It was possible to obtain three or more measurements over time of the response to intact hsp60 in five type 1 diabetes patients. Figure 3 shows that some patients manifested a rise in their responsiveness after the first weeks of diagnosis, coincident with their entry into insulin treatment and glucose homeostasis. However, the responses to hsp60 all fell from 6 weeks after diagnosis. This suggests that the IDDM patients studied 8 or more weeks after their diagnosis (see Table 2A, patients numbers 1, 13, 17, 19, 21 and 23) may have been tested after the peak of their responsiveness.

Discussion

This study shows that a significant proportion of recently diagnosed type 1 diabetes patients manifest T cell proliferative reactivity to human hsp60 and to particular hsp60 peptides. Persons with type 2 diabetes did not show a higher incidence of responsiveness to hsp60 than did healthy blood donors, suggesting that hsp60 may be a member of the

Table 2. *T cell proliferative responses to hsp60. PBMNC were isolated from 25 recently diagnosed type 1 diabetes patients (A), 25 healthy blood donors (B) and 22 type 2 diabetes patients (C), and were tested for their proliferative responses to hsp60 and to a recall antigen tetanus toxoid*

A No.	Subject	Age (years)	Disease duration (weeks)	T cell responses (SI)	
				Tetanus T.	hsp60
1	LM	32	14	5	2
2	DZ	20	3	23	6
3	SD	20	6	11	9
4	GH	60	7	14	3.6
5	FR	45	6	5.5	10
6	IS	53	2	12	4
7	TO	20	4	2.2	4
8	KK	21	4	11	2.3
9	RI	40	6	4.5	1
10	YC	20	6	3.2	5.3
11	SM	16	5	8.7	3.7
12	AS	21	2	4.8	5
13	AYM	38	8	4.2	5
14	AD	15	6	30	6
15	RD	21	6	84	24
16	FM	27	5	12	7.5
17	KO	29	12	10	4.7
18	RS	30	4	23	5.2
19	NR	24	10	10	7.8
20	MS	5	1	16	3
21	KB	5	8	3.2	4
22	BI	11	3	42	2.5
23	WI	60	8	7	5
24	BN	17	ND	ND	27
25	PA	14	5	13	1.1
Median		21	6	10.5*	5**

B Subject	T cell responses (SI)	
	Tetanus T.	hsp60
1	1.3	1.2
2	27	14
3	4	1
4	1.1	1
5	2	1
6	1.8	1
7	10	14
8	3.4	3
9	7.6	4
10	3	1
11	17	1.7
12	11	1.4
13	3	4
14	2.6	1.2
15	10	1
16	16	4
17	8	2.3
18	3.5	1
19	12	1.1
20	5.3	1.4
21	2	2.5
22	9.5	3
23	15	2.6
24	19	2.7
25	9	1.9
Median	7.6*	1.7#

Table 2. *Continued*

C Subject	Age (years)	Disease duration (years)	Insulin (years)	T cell responses (SI)	
				Tetanus T.	hsp60
HY	4	24	0.5	21	1.3
LO	64	28	10	2.5	1.3
LY	70	15	7	4	1
ER	67	34	10	6	1.5
PB	70	18	12	4	1.5
AV	62	30	20	4.5	1
JA	60	28	5	3	2.8
BR	64	20	2	10.3	3.2
BE	65	20	4	2.4	1.8
PS	63	10	2	3	1.3
FS	60	20	10	3.6	5.5
RJ	68	14	12	2.1	5.6
SA	75	30	4	1.8	1
AD	72	24	3	3.7	1.7
KY	51	10	2	16	10
TY	66	10	4	20	4
SE	50	20	6	5.7	1.6
EE	65	10	3	2.5	1.5
NM	60	23	8	17	4.4
YS	64	24	14	8.3	1.6
AR	63	25	4	1	1
YE	64	6	3	8.3	3.6
Median	64	20	4.5	4.13*	1.67#

*$P>0.05$ when compared to each of the three groups.
**$P<0.001$ compared to the blood donor group. $P<0.01$ compared to the type 2 diabetes group.
#$P>0.05$ when compared to the blood donor and to the type 2 diabetes group.
ND =not determined.
The patients are shown by their initials. Blood donors are anonymous and were consecutively numbered.

collective of self-antigens to which there is heightened T cell reactivity in type 1 diabetes [4]. A number of peptide fragments of hsp60 were recognized, notably the p277 peptide (similar to p30), p32 and 5 other peptides: p3, p10, p18, p20, and p35.

The responses to hsp60 seemed to rise following diagnosis, as noted in the five patients we could follow longitudinally (Figure 3). The response to hsp60 appeared to peak about 6 weeks after diagnosis and fell thereafter. It should be considered that the day of diagnosis of the disease depended on the day the patient came to medical attention; the actual duration of the disease in any patient is uncertain and depends on the individual clinical course. It is possible that the rise in responsiveness noted after the initial period of insulin treatment may reflect restoration of T cell competence following restoration of glucose homeostasis. The later fall in T cell reactivity to hsp60 may be similar to the decline in T cell responses in newly diagnosed type 1 patients to the β-cell antigens insulin and GAD [17]. Obviously, our observations of the dynamics of the hsp60 response need to be documented in greater detail in more patients before we can draw any conclusions. However, these preliminary findings call attention to the possibility that the response to hsp60 epitopes can vary over time.

It is interesting that type 1 diabetes patients and NOD mice appear to make T cell responses to similar hsp60 peptides, such as p12 and p277 [11, 12, 15]. The similarity in target peptides may result from the similar peptide-binding motifs of the mouse I-Ag7 [18] and the human DQ8 MHC molecules [19], both associated with susceptibility to IDDM. Moreover, the I-Ag7 and DQ8 molecules are relatively unstable compared to some of the MHC class 2 molecules associated with resistance to type 1 diabetes [20]. Nevertheless, the I-Ag7 and DQ8 molecules were found to be uniquely stabilized by the same hsp60 peptide, p12 [21]. Thus, structurally similar MHC susceptibility genes may provide a molecular basis for the immunological resemblance between type 1 diabetes in humans and in NOD mice [22]. Additional studies are needed to analyse the effect of DQ8 on responses to hsp60 and its peptides.

It is not known why autoimmunity to hsp60, GAD, 38 kDa, and other antigens not exclusively expressed in the islets should be associated with the autoimmune process leading to type 1 diabetes in humans and mice [4, 9, 23, 24]. Nevertheless, hsp60 autoimmunity does seem to have a functional role in the type 1 diabetes process, at least in mice. NOD mice manifest spontaneous T cell autoimmunity to hsp60 coincident with the development of insulitis [11, 12];

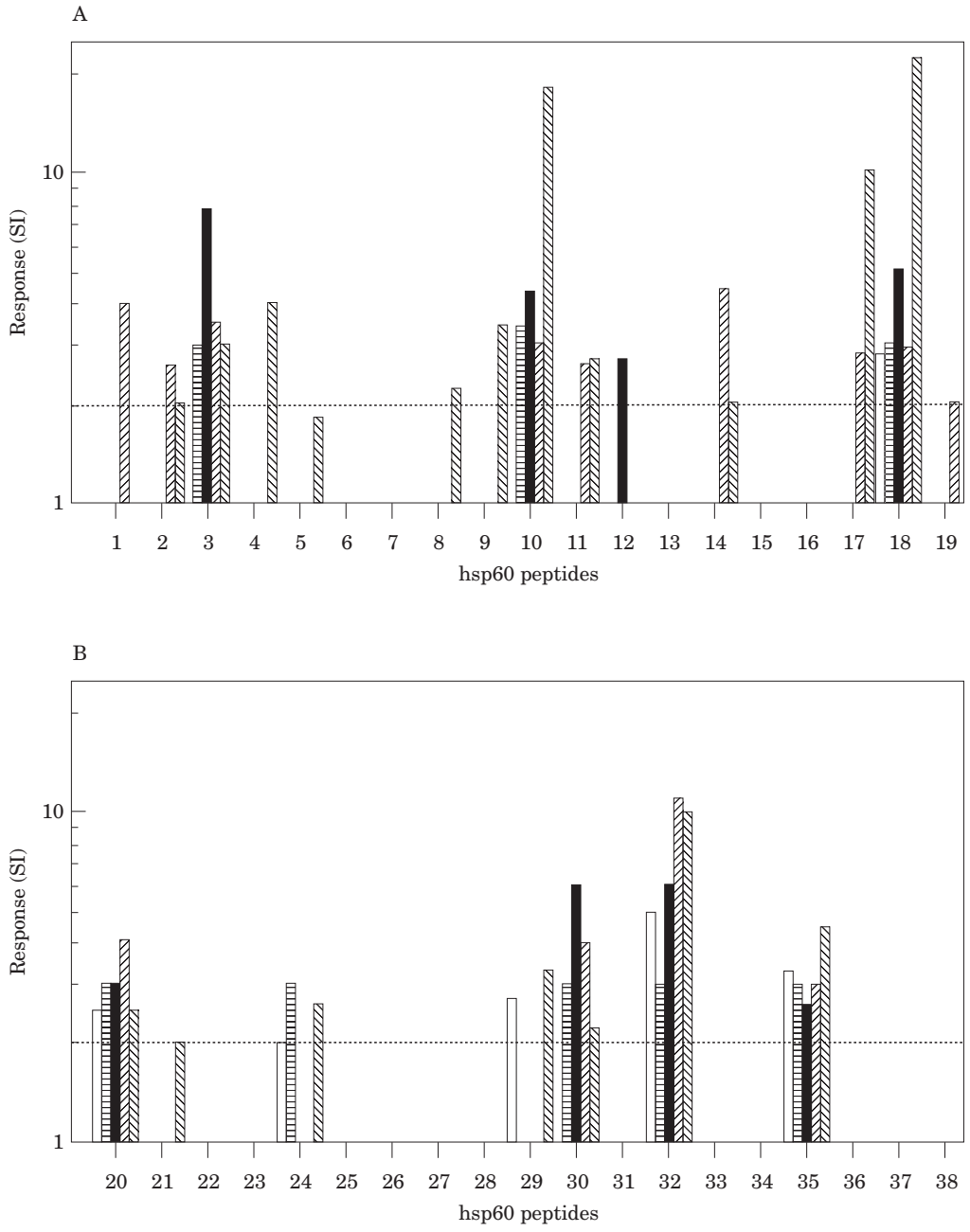

Figure 1. Peptide mapping of hsp60 epitopes. PBMNC isolated from five patients selected for their multiple reactivities to hsp60 peptides were assayed for T cell proliferative responses to the spanning hsp60 peptides 1–38 shown in Table 1. Patients: AD (□), RD, (▤), FM (■), RS (▨), NR (▧).

hsp60-specific T cells can adoptively transfer insulitis and hyperglycaemia [12]; induction of type 1 diabetes in C57BL/KsJ mice by exposure to a very low dose of the β-cell toxin streptozotocin is associated with T-cell reactivity to hsp60 [25]; and active immunization to a bacterial hsp60 [12] or to the hsp60 peptide p277 conjugated to a foreign immunogenic carrier molecule can induce transient hyperglycemia and insulitis in various strains of mice [26].

Irrespective of whether or not hsp60 autoimmunity plays a primary role in pathogenesis, it is of interest that the administration of hsp60 peptides either once or twice can halt the progression of insulitis and diabetes in NOD mice with spontaneous type 1 diabetes [14, 27] and in C57BL/KsJ mice with toxin-induced type 1 diabetes [28]. Moreover, transgenic NOD mice that hyperexpress mouse hsp60 in the thymus and elsewhere are relatively resistant to the development of type 1 diabetes [29].

Effective treatment of the diabetic process in mice with hsp60 peptides appears to involve a temporary burst of 'anti-inflammatory' Th2-like reactivity that downregulates pathogenic Th1-like reactivity to hsp60. This downregulation induced by p277 appears to spread, to downregulate the Th1-like responses to other antigens targeted in type 1 diabetes [13]. A

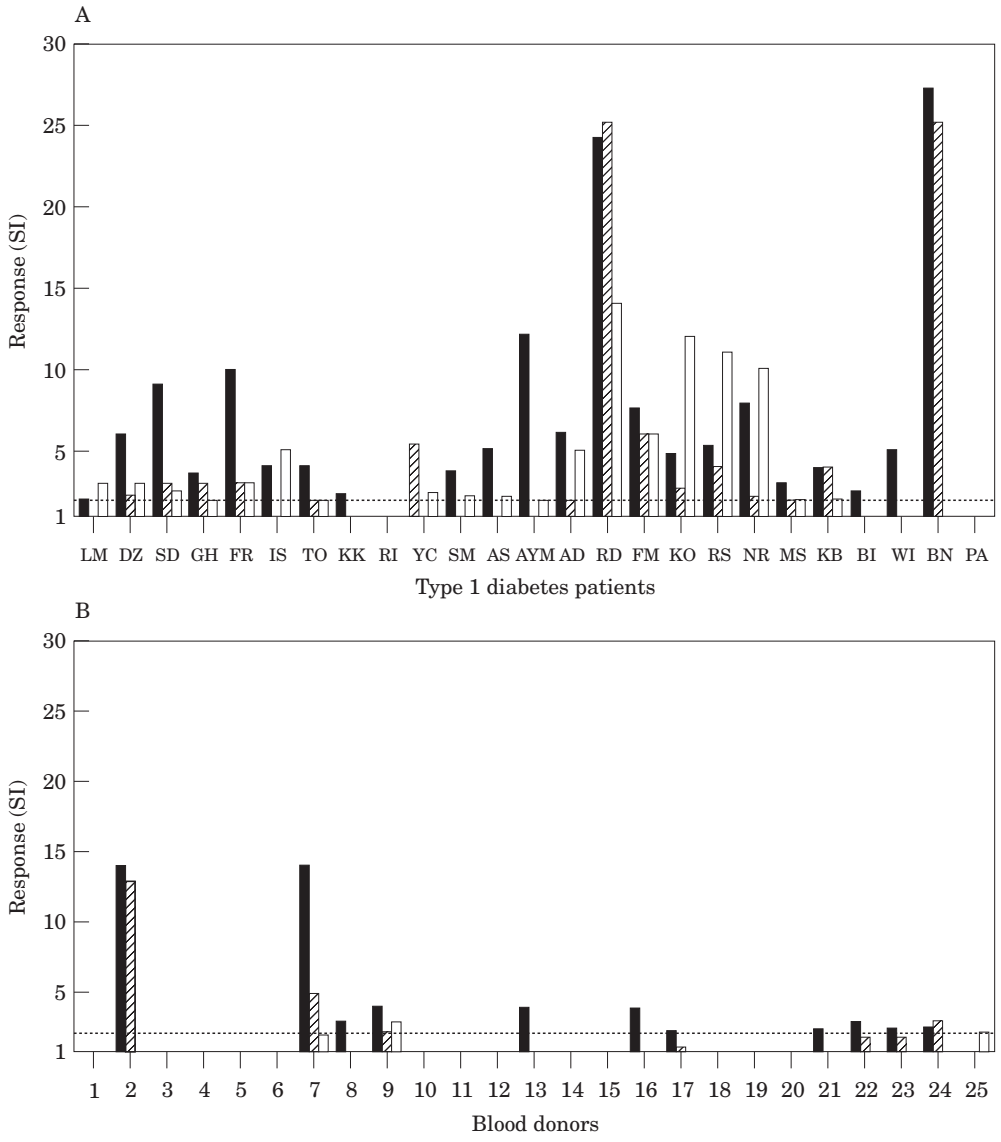

Figure 2. T cell responses of type 1 diabetes patients (A) and healthy donors (B) to the intact hsp60 molecule (■) and to peptides p277 (▨) and p32 (□). An *E. coli* pGEX lysate preparation used as a control elicited no responses (not shown).

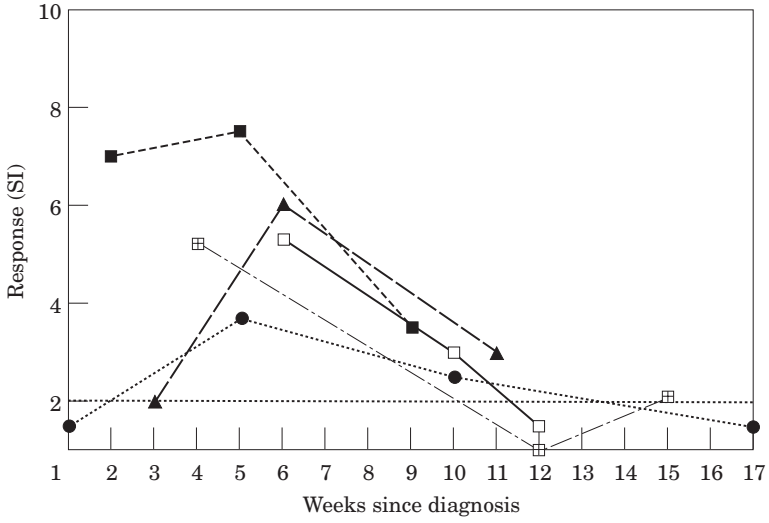

Figure 3. Dynamics of T cell proliferative responses to hsp60. Five IDDM patients were tested longitudinally for their responses to hsp60 Patients: YC (□), SM (●), AD (▲), FM (■), RS (⊞).

dichotomy between pathogenic Th1 T cells and non-pathogenic Th2 T cells has been described in the NOD mouse model [30, 31, 32]. Because of the effectiveness of hsp60 peptides in treating the diabetic process in NOD mice, the observation of hsp60 reactivity in newly diagnosed human type 1 diabetes patients raises the possibility that hsp60 peptides might be of similar benefit in humans. A phase 1 trial of peptide p277 has shown no toxicity and phase 2 trials of effectiveness are presently underway.

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