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T Cells and autoantibodies to human HSP70 in Type 1 diabetes in children

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Abstract

We studied T-cell proliferative responses (stimulation index: SI) and autoantibodies to human HSP60, HSP70 and HSP90 proteins in 25 children (mean age 10.1 ± 3.8 years) newly diagnosed with Type 1 diabetes. The control group for T cells included 25 adults and three pediatric donors without Type 1 diabetes. Controls for antibodies included 10 pediatric subjects. The T-cell responses to HSP70 of the test group (mean SI=4.5±3.1) were significantly greater than those of the control group (mean SI=1.4±0.6; $p<0.0001$); the incidence of HSP70 responders was (85%) compared to 14% in the control group. All but three of the Type 1 children who responded to HSP70 also responded to HSP60 (85%). The T-cell responses of the Type 1 group to HSP90 (mean SI=1.7±1.1) were similar to those of the control group (mean SI=1.5±0.7). We mapped HSP70 epitopes recognized by T cells in seven subjects using overlapping peptides of the molecule. Among the Type 1 subjects, IgG seropositivity was 45% to HSP60, 30% to HSP70, and 15% to HSP90. Thus, we conclude that children with newly diagnosed Type 1 diabetes manifest heightened T-cell autoimmunity to HSP70 and HSP60, but not to HSP90.

Keywords: Autoantibodies; Heat shock protein 70; T-cell Immunity; Type 1 insulin-dependent diabetes

1. Introduction

Type 1 diabetes is caused by autoimmune T cells that destroy the insulin-producing β cells of the pancreatic islets [1,2]. In humans and in NOD mice, the disease appears to involve autoimmunity to a similar collection of antigens (reviewed in Ref. [3]) including, glutamic acid decarboxylase (GAD) [4], proinsulin and insulin [5,6], the islet cell antigen ICA69 [7], the insulin secretory-granule 38 kDa protein [8], and HSP60 [9]. Anti-HSP60 T cells can mediate insulitis and hyperglycemia [10], and modulating the anti-HSP60 T-cell response can lead to the arrest of the autoimmune destruction of β cells [11]. Recently, a clinical trial of therapeutic vaccination with p277, an HSP60 peptide, has shown effectiveness in arresting beta-cell destruction in adults [12].

The HSP70 molecule appears to have a role in antigen presentation [13] and the 8.5 kDa HSP70-2 allele has been associated with diabetic haplotypes [14]. Moreover, autoantibodies against HSP70 have been found in patients suffering from autoimmune diseases such as systemic lupus erythematosus [15] and multiple sclerosis [16]. In addition, T-cell responses to other HSP's have also been suggested in diabetes [17].

In this study, we compared the T-cell proliferative responses and the IgG and IgM antibody titers to human HSP60, HSP70, and HSP90 in 25 children newly diagnosed with Type 1 diabetes and in control individuals. We found T-cell proliferative responses to HSP70 and HSP60, but not to HSP90. In addition, we tested the specific response to HSP70 peptides and mapped some major HSP70 protein epitopes.

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2. Materials and methods

2.1. Subjects

2.1.1. Type 1 diabetes patients

Twenty-five children, 15 females and 10 males, mean age; 10.1 ± 3.8 years, (range 1.8–18 years) with newly diagnosed Type 1 diabetes mellitus admitted to the Departments of Pediatrics at the Hadassah University Hospital, Jerusalem, Shaarei-Zedek Hospital, Jerusalem, Assaf Harofe Medical Center, Zerifin, and outpatient clinic at the Kupat Holim Klalit Clinic, Jerusalem, all in Israel, were enrolled in the study, with informed consent obtained from their parents. The mean time elapsed from the time of diagnosis was 3.8 ± 2.3 weeks (range 1–12 weeks). The criteria for diagnosis of Type 1 diabetes were classical clinical symptoms, including a recent history of polyuria, polydipsia, weight loss and hyperglycemia (glucose ≥ 200 mg/dl or 11.1 mM/L) with or without associated severe ketoacidosis, and ketonuria.

Ideally, it would have been highly desirable to stratify the patients and controls according to HLA. Technically, however, it was not possible to do HLA typing on the anonymous blood donors and the added blood needed from the children could not be obtained with parental consent.

2.2. Controls

2.2.1. Healthy blood donors

Samples of surplus blood were obtained from 25 healthy adult donors from the Blood Bank of Tel-HaShomer Hospital, Tel Aviv, Israel. It was necessary to use blood from healthy adults as controls because drawing relatively large amounts of blood from healthy children is ethically unjustified.

2.2.2. Pediatric T-cell donors

We were, however, able to use surplus blood from three children undergoing endocrine tests for evaluation of short stature, two female and one male, ages 3, 7 and 10 years, admitted to the Departments of Pediatrics at the Hadassah University Hospital, with parental permission, as non-Type 1 childhood controls for both T-cell and antibody tests.

2.2.3. Pediatric serum donors

Serum from 10 normoglycemic children, nine males and one female; mean age 6.5 ± 4.3 years, (range 0.5–13 years), were obtained, with parental permission, from children admitted to the Emergency Room of the Hadassah University Hospital, Jerusalem, Israel, for treatment of acute medical conditions. Since 1 ml of blood is not a sufficient amount for isolation of T cells, we could only test the sera for antibodies.

2.3. T-cell proliferation

Fifteen to twenty-five milliliters of peripheral blood were drawn from patients or were obtained from the blood bank. Anti-coagulation was obtained using heparin (10 IU/ml). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation. The cells were washed with RPMI culture medium (Biological Industries, Kibbutz Beit Haemek, Israel), 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), all from (Biological Industries, Kibbutz Beit Haemek, Israel).

PBMC were plated in triplicate or quadruplicate in 96-well round-bottom micro plates (Falcon, Lincoln Park, NJ, USA) at a cell concentration of 2×10^5 cells per well in 100 μl RPMI media, with or without test antigens: PHA (Murex Diagnostic Ltd. Dartford, England) 0.3 μg/ml; tetanus toxoid (Connaught Lab., Inc., Pennsylvania, USA) 5 μg/ml; *Candida albicans* (Hollister-Stier, Toronto, Canada), 10 μg, recombinant human HSP60, HSP70 or HSP90 (StressGen, Vancouver, Canada), 2–5 μg/ml; and HSP70 and HSP60 peptides, 5–20 μg/ml (synthesized at the Biological Services Laboratory of the Weizmann Institute of Science Rehovot, Israel, using an automated ABIMED synthesizer; model AMA422, Langenfeld, Germany). We carried out all proliferations including epitope mapping in RPMI medium supplemented with 10% autologous serum, and incubated at 37 °C in a 5% CO_2 humidified incubator for 7 days. On the sixth day, we labeled the cells overnight with 1 μCi per well of ^{3}H-thymidine and counted the radioactivity in a beta-counter (Packard model, 2000, Meriden, CT, USA). We present proliferation as the stimulation index (SI): the ratio of the mean cpm with antigen to the cpm without antigen. We considered SI values to be positive if they were greater than or equal to 2.0.

2.4. Assay of autoantibodies

Serum samples were stored at −20 °C and thawed before each assay. We detected total human IgG or IgM anti-HSP60, anti-HSP70, anti-HSP90 and anti-tetanus toxoid using an antibody capture-type enzyme immunoassay ELISA [18]. We pre-coated 96-well micrometer Nunc-Immuno™ plates (Nalge Nunc International, Roskilde, Denmark) with the test protein (10 μg/ml). Into these pre-coated wells, we pipetted test serum samples at dilutions of 1:70 to detect antibodies. After washing away unbound serum proteins, we added an alkaline phosphatase-conjugated mouse monoclonal

Table 1
T-cell proliferative response to HSP60, HSP70, and HSP90: Type 1 diabetes children

Subject	Age/sex (years)	Duration (weeks since diagnosis)	Tetanus toxoid	Candida	HSP60	HSP70	HSP90
P1	16/F	3	5.4	6.5	1.3	1.3	1.2
P2	5/F	5	24.0	19.2	3.6	2.0	1.0
P3	13/F	3	34	38	2.5	1.7	1
P4	9/F	1	13.8	7.3	1.0	4.8	1.4
P5	14/M	3	40.6	35.2	6.0	7.3	3.8
P6	14/F	4	6.7	3.9	3.3	5.6	1.0
P7	1.8/M	3	72.8	23.5	5.5	3.3	1.1
P8	9.5/M	4	10.9	13.0	1.4	2.1	1.6
P9	13/M	3	14.2	25.4	6.1	7.8	1.0
P10	5/F	3	39.3	4.3	2.1	14.3	1.5
P11	9/F	3	4.5	3.6	3.7	8.0	3.0
P12	16/M	3	7.1	1.7	1.9	1.0	1.0
P13	18/M	3	25.0	15.3	13.4	8.9	1.7
P14	10/M	3	21.4	18.5	2.4	2.3	1.6
P15	6/F	2	9.2	8.1	2	1	2.6
P16	7.5/F	3	10.1	10.5	4.6	4.9	0.8
P17	9/F	3	4.2	6.3	2.4	1.5	1
P18	9/F	4	10.2	6.3	3.7	3.7	1
P19	6.5/F	3	4.5	5.3	7.1	2	1
P20	10/M	3	17.7	13	2.7	4.4	5.1
P21	16/F	8	nd	23	1	4.9	1.9
P22	10.5/M	2	5.6	7.1	3.6	4.4	3.7
P23	7/F	12	17.4	18	3	6.2	n.d.
P24	9/M	4	5.5	9.4	4.5	4.7	1.5
P25	8/F	8	7.7	6.9	2.1	4.4	1.5
Mean ± SD	10.1 ± 3.8	3.8 ± 2.3	17.2 ± 15.9*	13.2 ± 9.6^{ns}	3.6 ± 2.6**	4.5 ± 3.1**	1.7 ± 1.1^{ns}

T-cell responses (SI) to HSP60, HSP70, and HSP90, and to the recall antigens tetanus toxoid and *C. albicans* were compared between Type 1 diabetes children and a control group of healthy blood donors and pediatric T-cell donors (see Table 2). *p* Values indicate the comparison of Type 1 responses to those of the control group. Background CPM without antigen ranged from 391 to 2110 (mean 850) for the healthy controls, and 165 to 2656 (mean 853) for the Type 1 patients.
*P=0.035; **P<0.0001; nd: not determined; ns: not statistically significant.

anti-human IgG or IgM to the wells. After an additional wash to remove any unbound anti-enzyme reagent, we added *p*-nitrophenyl phosphate (*p*NPP) solution to the wells. The intensity of the color that developed in proportion to the amount of antibody was measured using the Anthos htl ELISA reader (Anthos Analytical Apparatus, Inc., Durham, NC., USA) at 405 nm. We represent the amount of antibody detected as Units of Optical Density at 405 nm. Sera from patients and controls were tested in the same assay.

2.5. Statistical analysis

We used the InStat 2.01 computer program for statistical analysis of correlation coefficients and *p* values utilizing the two-tailed Fisher's exact test. Using the results of the pediatric serum donors, we determined the cut-off for each assay by calculating the mean and adding two standard deviations (SDs). Values above this calculated cut-off were considered positive. We present the results as percent of positives from the total tested group.

3. Results

3.1. T-cell responses

We compared the T-cell responses of the Type 1 diabetes children (Table 1) to those of the control group (Table 2) to HSP60, HSP70, and HSP90 and to the recall antigens tetanus toxoid and *C. albicans*.

The T-cell responses of the Type 1 children to HSP70 were significantly higher than those of the controls. Though there were responders in each group, there were significantly more responding Type 1 children (20 of 25; 85%) compared to the control group (four of 28; 14%; p=0.0006). The degree of responsiveness to HSP70 was also higher in the Type 1 group (mean SI=4.5±3.1) compared to the control group (mean SI=1.4±0.6, p<0.0001). Thus, as a group, recently diagnosed Type 1

Table 2
T-cell proliferative response to HSP60, HSP70, and HSP90: control group

Subject	Tetanus toxoid	Candida	HSP60	HSP70	HSP90
C1	1	16	1.9	2.1	2.5
C2	50	26	2.5	1.5	2.3
C3	13.8	18.3	2.1	1	1.8
C4	2.9	2.5	1	1	1
C5	8.7	6.5	2.6	1.3	4
C6	3	5	1.3	1.4	1.3
C7	15.7	8.5	1	1.1	1
C8	3.2	10.5	1.4	1.5	2.2
C9	10.6	12.3	2	1	1.8
C10	4.3	7.1	1.75	1	1.4
C11	11.3	17.8	2.9	1	1
C12	3.5	3.6	2.9	1.5	1
C13	5.5	11.1	2.3	1.7	1.9
C14	9	4.5	1	1	1
C15	19.3	13.9	1.3	1.6	1
C16	3	6	1	1	1.5
C17	6.6	8.8	2.5	1.5	1.2
C18	4.3	8.9	3.5	3.2	2.4
C19	2.5	5.1	1.3	1.3	2.4
C20	2.4	4.6	1	1.5	2
C21	3.4	7.3	1.9	1	1
C22	10.7	25	1	3.1	1.1
C23	2.9	2.5	1	1	1
C24	2.7	3.7	1.5	1.9	1
C25	11.1	77	11	22	11
C26*	7.3	16	1.25	1.1	1
C27*	3.5	3.6	1	1	1
C28*	2.2	2.1	1	1	1
Mean ± SD	8.0 ± 9.3	9.4 ± 6.4	1.7 ± 0.72	1.4 ± 0.6	1.5 ± 0.7

C1–C25, healthy blood donors; C26*–C28*, pediatric T-cell donors.

children manifested a T-cell proliferative response to HSP70 significantly higher than that of the controls. In addition, 20 of the 25 (85%) children responded to HSP60 (mean of SI=3.6±2.6); among them were three children (P3, P15 and P17) who responded to HSP60 and not to HSP70. Three children responded to HSP70 and not to HSP60 (P4, P8, P21).

In contrast, there were no differences between the groups in response to HSP90: five of the 24 Type 1 diabetic children (21%) responded to HSP90 (mean SI=1.7±1.1), and seven of the 28 healthy blood donors (25%) responded to HSP90 (mean SI=1.5±0.7). The two groups responded similarly to *C. albicans* antigens. We would expect that the responses to tetanus toxoid would be higher in younger subjects because they would have received a tetanus toxoid booster more recently than older subjects. In fact, the responses of the Type 1 children (17.2 ± 15.9, Table 1) were higher than those of the control group (8.0±9.3; p=0.035, Table 2). Nevertheless, responsiveness to HSP60 and HSP70 appeared to be independent of the elevated responses to tetanus toxoid because no correlation was found (correlation coefficient=0.2296 and 0.2837, respectively). As mentioned, T cells were available from only three control children C26*, C27*, C28*. Their responses to HSP60, HSP70 and HSP90 were similar to those of the healthy adult blood donors.

Table 3
Overlapping peptides of the human HSP70 molecule used for screening

Peptide Number	Position	Sequence
p1	1–20	MAKAAAVGIDLGTTYSCVGV
p2	16–35	SCVGVFQHGKVEIIANDQGN
p3	31–50	NDQGNRTTPSYVAFTDTERL
p4	46–65	DTERLIGDAAKNQVALNPQN
p5	61–80	LNPQNTVFDAKRLIGRKFGD
p6	76–95	RKFGDPVVQSDMKHWPFQVI
p7	91–110	PFQVINDGDKPKVQVSYKGE
p8	106–125	SYKGETKAFYPEEISSMVLT
p9	121–140	SMVLTKMKEIAEAYLGYPVT
p10	136–155	GYPVTNAVITVPAYFNDSQR
p11	151–170	NDSQRQATKDAGVIAGLNVL
p12	166–185	GLNVLRIINEPTAAAIAYGL
p13	181–199	IAYGLDRTGKGERNVLIFDL
p14	195–214	LIFDLGGGTFDVSILTIDDG
p15	210–229	TIDDGIFEVKATAGDTHLGG
p16	225–244	THLGGEDFDNRLVNHFVEEF
p17	240–259	FVEEFKRKHKKDISQNKRAV
p18	255–275	NKRAVRRLRTACERAKRTLS
p19	271–290	KRTLSSSTQASLEIDSLFEG
p20	286–305	SLFEGIDFYTSITRARFEEL
p21	301–320	RFEELCSDLFRSTLEPVEKA
p22	316–335	PVEKALRDAKLDKAQIHDLV
p23	331–350	IHDLVLVGGSTRIPKVQKLL
p24	346–365	VQKLLQDFFNGRDLNKSINP
p25	361–380	KSINPDEAVGYGAAVQAAIL
p26	376–395	QAAILMGDKSENVQDLLLLD
p27	**391–410**	**LLLLDVAPLSLGLETAGGVM**
p28	406–425	AGGVMTALIKRNSTIPTKQT
p29	421–440	PTKQTQIFTTYSDNQPGVLI
p30	436–455	PGVLIQVYEGERAMTKDNNL
p31	451–470	KDNNLLGRFELSGIPPAPGV
p32	466–485	PAPGVPQIEVTFDIDANGIL
p33	481–500	ANGILNVTATDKSTGKANKI
p34	496–515	KANKITITNDKGRLSKEEIE
p35	**511–530**	**KEEIERMVQEAEKYKAEDEV**
p36	526–545	AEDEVQRERVSAKNALESYA
p37	541–560	LESYAFNMKSAVEDEGLKGK
p38	556–575	GLKGKISEADKKKVLDKCQE
p39	571–590	DKCQEVISWLDANTLAEKDE
p40	586–605	AEKDEFEHKRKELEQVCNPI
p41	601–620	VCNPIISGLYQGAGGPGPGG
p42	616–635	PGPGGFGAQGPKGGSGSGPT
p43	631–640	GSGPTIEEVD

The sequence of 43 peptides spanning the molecule of human HSP70 with five overlapping peptides is shown. The sequences of peptides p27 and p35 are highlighted.

3.2. Epitope mapping of HSP70 peptides

To determine the spectrum of the HSP70 peptides that were recognized in the Type 1 diabetes children, T-cell proliferative responses to 43 overlapping HSP70 peptides (Table 3) were assayed (Fig. 1). Results of seven

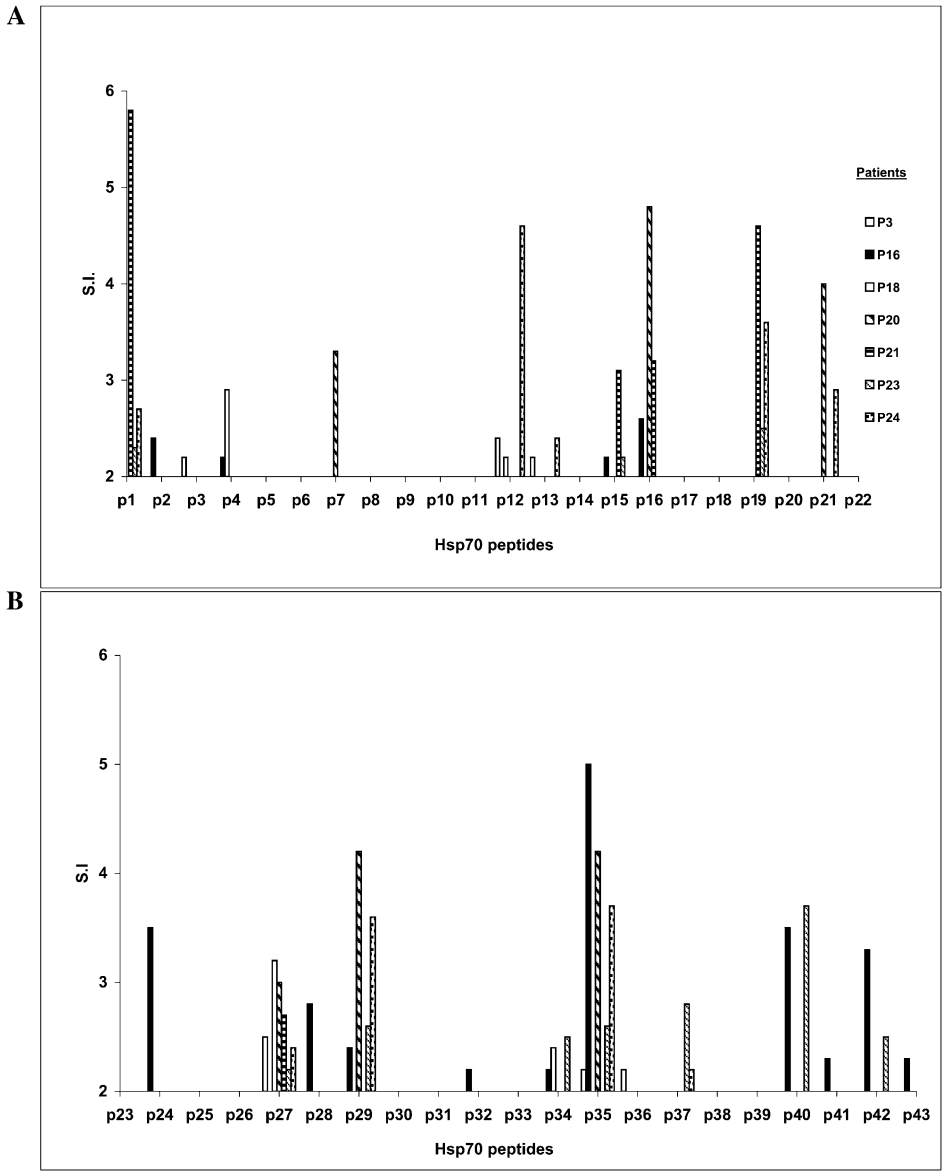

Fig. 1. Epitope mapping of the HSP70 protein. Seven Type 1 diabetes patients (P3, P16, P18, P20, P21, P23 and P24) were tested for responsiveness to multiple hsp70 epitopes that span the human hsp70 molecule (detailed in Table 3). A shows the responses to peptides p1–p22, and B shows the responses to peptides p23–p43. T-cells were activated with 20 μg/ml of each peptide.

Type 1 diabetes patients (P3, P16, P18, P20, P21, P23 and P24 from Table 1) are shown in Fig. 1. Peptides were considered to be dominant when at least three of the seven patients tested had a positive response. We found reactivity to nine of the 43 peptides: p1, p12, p15, p16, p19, p27, p29, p34 and p35 (Fig. 1). Patients P24 and P23 reacted to six and seven of these nine peptides, respectively. Two peptides could be considered major antigenic epitopes, peptides p27 (residues 391–410) and p35 (residues 511–530), because six of seven of the Type 1 diabetes subjects tested responded to p27 and five responded to p35 (see Fig. 1). Thus, multiple HSP70 peptides appear to be recognized by the Type 1 diabetes population, amongst which, two seem to harbor major antigenic sites. Note that we used five amino acid overlaps in our peptide library; this means that we may have missed a significant number of additional epitopes beyond the nine we detected.

3.3. Antibodies to HSP60, HSP70, and HSP90

Levels of IgG antibodies to HSP60, HSP70, and HSP90 were measured in the sera of 20 Type 1 diabetes children P1–P21 (patient P6 was not tested), and two control groups: 10 pediatric serum donors (normoglycemic) and 15 healthy blood donors (Fig. 2 and Tables 4–6). Of the Type 1 diabetes children, nine of 20 (45%) were positive to HSP60, six out of 20 (30%) were

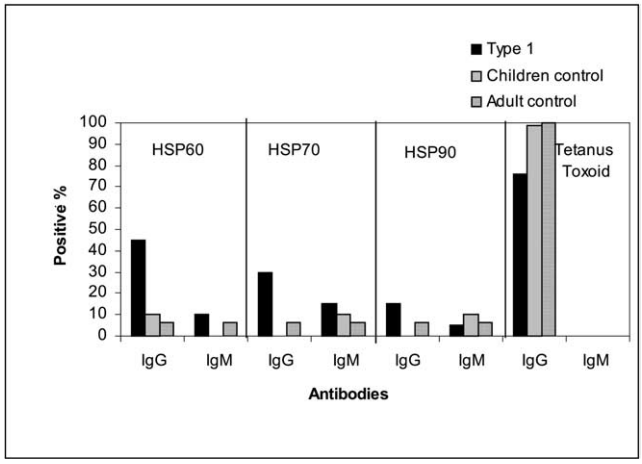

Fig. 2. Serum autoantibodies to HSP60, HSP70 and HSP90. This graph presents the levels of IgG and IgM antibodies to HSP60, HSP70 and HSP90 in the sera of 20 patients with Type 1 diabetes (Tables 4–6) in comparison with the sera of 10 normoglycemic pediatric serum donors and 15 healthy blood donors. A level of antibodies was considered positive if it was greater than the mean of the antibody levels obtained from the two control groups plus two standard deviations.

positive to HSP70, and three of 20 (15%) were positive to HSP90 (Fig. 2 and Table 4). Of the nine Type 1 diabetes children positive to HSP60, five were also positive to HSP70. In contrast, of the 10 healthy serum donors, only one (10%) was positive to HSP60, and none was positive to either HSP70 or HSP90 (Fig. 2 and Table 5). Among the healthy blood donors, only 6.6% were positive to HSP60, HSP70 and HSP90 (Table 6). Since the healthy blood donors were adults, we did not use their titers to calculate the cut-off. It is remarkable that although only 30% of the Type 1 diabetes children were positive to HSP70, none of the healthy children were positive to HSP70. Antibodies to tetanus toxoid (IgG) served as a control, and were similarly high in both Type 1 diabetes and control groups. Levels of IgM antibodies to HSP60, HSP70, and HSP90 were measured in the sera of 20 Type 1 diabetes children, 10 normoglycemic pediatric serum donors and 15 healthy blood donors. Of the Type 1 diabetes children, 10% were positive to HSP60, 15% were positive to HSP70, and 5% were positive to HSP90 (Fig. 2 and Table 4). Interestingly, patient P13, the oldest patient among the 20 tested (18 years), was positive to all three heat shock proteins. There was no correlation between the IgG antibody level and the magnitude of the T-cell response.

4. Discussion

The results presented here show that Type 1 diabetes patients in the pediatric age group, compared to controls, manifest heightened T-cell proliferative responses to HSP70 and HSP60, but not to HSP90. The mean SI of the group and the number of individuals with a SI above 2 were significantly elevated. Even if we were to limit a positive SI to 3 or greater, 16 of the 25 diabetic subjects would have been positive to HSP70 and 12 to HSP60 ($p<0.0001$, $p=0.0002$, respectively), compared to positives among the 28 control subjects of only 2 to HSP70 and 1 to HSP60 (see Tables 1 and 2). These results were relatively specific; there was no increased responsiveness to HSP90 and no correlation between the degree of response to HSP70 and the recall antigens *C. albicans* or tetanus toxoid. Heightened T-cell proliferative responses to HSP60 were reported in adult Type 1 diabetes patients [9]. The present study indicates that pediatric Type 1 diabetes patients also respond to HSP60 and adds HSP70 to the list of autoantigens targeted in Type 1 diabetes. Further work is necessary to document the role of HLA in this phenomenon. The responding T-cell populations also need to be characterized—CD4, CD8, TCR αβ, TCR γδ, and cytokine profiles.

Interestingly, the incidence of heightened T-cell autoimmunity was greater than the incidence of the corresponding autoantibodies; the percent of diabetic children positive for IgG antibodies to HSP70 or HSP60 were only 45 and 30%, respectively. But the relatively low antibody autoimmunity may be a result of the relatively high background of IgG autoantibodies to HSP70, HSP60 and even HSP90 found in the control population; the background cut-off OD values ranged from 0.53 to 0.67 for IgG and from 0.87 to 1.09 for IgM. Thus, it appears that the healthy human population includes many persons with measurable amounts of IgG antibodies to HSP70, HSP60 and HSP90. The development of T-cell proliferation to HSP60 and HSP70 in disease may be the realization of an autoimmune potential present naturally in the general population. The set of antigens to which there exists natural autoimmunity in healthy individuals has been termed the immunological homunculus [19]. We show here that many different peptides of the HSP70 molecule may harbor T-cell epitopes, although certain peptides such as p27 and p35 may be more widely recognized in disease.

Important unanswered questions are why and how autoimmune T cells get activated to HSP60 and HSP70 in Type 1 diabetes. Is such reactivity only a result of the disease, or is this reactivity a causal factor in disease pathogenesis? Even if pathogenic, is HSP60 or HSP70 autoreactivity a primary cause of disease or is it a secondary, aggravating factor? Another open question is the relationship between HSP60 and HSP70 autoimmunity; do these two chaperone molecules work together functionally in the autoimmune process? Interestingly, both HSP60 and HSP70 have been found to serve as ligands for innate Toll-like receptors that regulate the inflammatory functions of macrophages and other leukocytes [20]. Thus, such molecules may have a broad role

Table 4
Antibodies to HSP60, HSP70 and HSP90: Type 1 diabetes children

Subject	Age/sex	Diabetes duration (weeks since diagnosis)	HSP60 OD		HSP70 OD		HSP90 OD	
			IgG	IgM	IgG	IgM	IgG	IgM
P1	16/F	3	1.04	1.13	0.53	1.1	0.66	1.2
P2	5/F	5	0.86	0.5	2	0.4	0.68	0.71
P3	13/F	3	0.45	0.2	0.28	0.24	0.45	0.38
P4	9/F	1	0.67	0.67	0.37	0.64	0.45	0.58
P5	14/M	3	0.77	0.77	0.7	1.3	0.44	0.79
P7	1.8/M	3	0.29	0.24	0.26	0.37	0.35	0.49
P8	9.5/M	4	0.69	0.49	0.34	0.5	0.59	0.87
P9	13/M	3	0.57	0.56	0.3	0.7	0.37	0.67
P10	5/F	3	0.46	0.28	0.26	0.37	0.45	0.47
P11	9/F	3	0.34	0.26	0.25	0.38	0.39	0.6
P12	16/M	3	0.46	0.26	0.27	0.34	0.39	0.41
P13	18/M	3	1.25	1.08	0.75	1.5	0.82	1.6
P14	10/M	3	0.51	0.37	0.29	0.37	0.45	0.56
P15	6/F	2	0.72	0.25	0.33	0.35	0.27	0.43
P16	7.5/F	3	0.76	0.5	1.22	0.88	0.6	0.79
P17	9/F	3	0.54	0.55	0.28	0.6	0.43	0.9
P18	9/F	4	0.49	0.86	0.29	0.6	0.37	0.65
P19	6.5/F	3	0.35	0.22	0.23	0.3	0.38	0.42
P20	10/M	3	0.39	0.48	0.88	0.48	0.33	0.71
P21	16/F	8	0.73	0.73	0.44	0.9	0.56	1
Mean ± SD	10.1 ± 4.1	3.3 ± 1.2	0.6 ± 0.23	0.52 ± 0.27	0.5 ± 0.41	0.62 ± 0.33	0.47 ± 0.13	0.71 ± 0.28
Cut-off			0.67	0.87	0.53	1.09	0.64	1.32
Positive			45%	10%	30%	15%	15%	5%

Levels (OD) of IgG and IgM antibodies to HSP60, HSP70 and HSP90 in the sera of 20 patients P1–P21 with Type 1 diabetes.

Table 5
Antibodies to HSP60, HSP70 and HSP90: pediatric control serum donors

Subject	Age/sex	HSP60 OD		HSP70 OD		HSP90 OD	
		IgG	IgM	IgG	IgM	IgG	IgM
C1	7/M	0.61	0.53	0.41	0.8	0.41	0.8
C2	3/M	0.39	0.4	0.34	0.4	0.54	0.5
C3	12/M	0.35	0.42	0.45	0.36	0.47	0.55
C4	13/M	0.28	0..36	0.26	0.5	0.41	0.68
C5	10/M	0.47	0.55	0.47	1.09	0.56	1.4
C6	0.5/M	0.39	0.01	0.35	0.01	0.3	0.03
C7	3/M	0.43	0.43	0.27	0.4	0.3	0.55
C8	2/M	0.5	0.42	0.37	0.29	0.55	0.43
C9	4/F	0.4	0.54	0.17	0.47	0.28	0.59
C10	10/M	0.67	0.84	0.44	0.8	0.5	0.91
Mean ± SD	6.45 ± 4.3	0.45 ± 0.11	0.46 ± 0.2	0.35 ± 0.09	0.51 ± 0.29	0.43 ± 0.1	0.64 ± 0.34
Cut -off		0.67	0.87	0.53	1.09	0.64	1.32
Positive		10%	0	0	10%	0	10%

Levels (OD) of IgG and IgM antibodies to HSP60, HSP70 and HSP90 in the sera of 10 pediatric serum donors C1–C10.

in autoimmune and other types of inflammation through their multiple roles in signaling both innate receptors on leukocytes and antigen-specific receptors on lymphocytes.

Administering an HSP60 peptide (p277) to NOD mice was found to arrest diabetes progression associated with down-regulation of autoimmunity to other autoantigens targeted in Type 1 diabetes such as GAD and insulin [11]. A clinical trial of such peptide treatment in human Type 1 diabetes patients produced similar results [12]. Future research will indicate whether HSP70 too could be used as a therapeutic vaccine.

Table 6
Antibodies to HSP60, HSP70 and HSP90: healthy blood donors

Subject	HSP60 OD		HSP70 OD		HSP90 OD	
	IgG	IgM	IgG	IgM	IgG	IgM
C1	0.53	0.45	0.30	0.4	0.42	0.69
C2	0.57	0.42	0.35	0.6	0.45	0.67
C3	0.76	0.33	0.70	0.39	0.41	0.45
C4	0.75	0.46	0.50	0.4	0.74	0.50
C5	0.57	0.66	0.35	0.57	0.47	0.65
C6	0.38	0.27	0.26	0.4	0.31	0.43
C7	0.52	0.42	0.44	0.7	0.49	0.67
C8	0.62	0.65	0.41	0.7	0.45	0.92
C9	0.63	0.41	0.46	0.6	0.49	0.70
C10	0.89	1.1	0.52	0.9	0.61	1.1
C11	0.58	0.53	0.38	0.4	0.44	0.65
C12	0.52	0.67	0.35	0.64	0.3	0.61
C13	0.59	0.62	0.5	0.7	0.47	0.72
C14	0.64	0.48	0.52	0.5	0.40	0.63
C15	0.67	0.39	0.41	0.58	0.49	0.88
Mean ± SD	0.61 ± 0.12	0.52 ± 0.19	0.43 ± 0.1	0.56 ± 0.14	0.46 ± 0.1	0.68 ± 0.17
Cut-off	0.85	0.91	0.64	0.85	0.67	1.02
Positive	6.6%	6.6%	6.6%	6.6%	6.6%	6.6%

Levels (OD) of IgG and IgM antibodies to HSP60, HSP70 and HSP90 in the sera of 15 healthy blood donors (adults) C1–C15.

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References

[1] Bach JF. Insulin-dependent diabetes mellitus is an autoimmune disease. Endocr Rev 1994;15:516–42.
[2] Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. New Engl J Med 1994;331:1428–36.
[3] Roep BO. Perspective in diabetes: T-cell responses to auto-antigens in IDDM. The search for the Holy Grail. Diabetes 1996; 45:1147–56.
[4] Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 1992;339:458–9.
[5] Harrison LC, De Aizpurua H, Loudovaris T, Campbell IL, Cebon JS, Tait BD et al. Reactivity to human islets and fetal pig pro islets by peripheral blood mononuclear cells from subjects with preclinical and clinical insulin-dependent diabetes. Diabetes 1991;40(9):1128–33.
[6] Rudy G, Stone N, Harrison LC, Colman PG, McNair P, Brusic V et al. Similar peptides from two beta cell autoantigens, proinsulin and glutamic acid decarboxylase, stimulate T cells of individuals at risk for insulin-dependent diabetes. Mol Med 1995;1:625–33.
[7] Roep BO, Duinkerken G, Schreuder GM, Kolb H, de Vries RRP, Martin S. HLA-associated inverse correlation between T cell and antibody responsiveness to islet autoantigen in recent-onset insulin-dependent diabetes mellitus. Eur J Immunol 1996;26(6):1285–9.
[8] Roep BO, Kallan AA, Hazenbos WL, Bruining GJ, Bailyes EM, Arden SD et al. T-cell reactivity to 38 kD insulin-secretory-granule protein in patients with recent-onset Type 1 diabetes. Lancet 1991;337(8755):1439–41.
[9] Abulafia-Lapid R, Elias D, Raz I, Keren-Zur Y, Atlan H, Cohen IR. T-cell proliferation responses of Type 1 diabetes patients and healthy individuals to human HSP60 and its peptides. J Autoimmunity 1999;12:121–9.
[10] Elias D, Reshef T, Birk OS, Van Der Zee R, Walker M, Cohen IR. Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. Proc Natl Acad Sci USA 1991;88:3088–91.
[11] Elias D, Meilin A, Ablamunits V, Birk OS, Carmi P, Konen-Waisman S et al. HSP60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and down-regulates autoimmunity to various beta-cell antigens. Diabetes 1997;46(5):758–64.
[12] Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset Type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomized double-blind phase II trial. Lancet 2001;24:358 (9295):1749–53.
[13] Kaufman SH. Heat shock proteins and immune response. Immunol Today 1990;11:129–36.
[14] Van Buskirk A, Crump BL, Margoliash E, Pierce SK. A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. J Exp Med 1989;170:1799–809.
[15] Minota S, Cameron B, Welch WJ, Winfield JB. Autoantibodies to the constitutive 73-kD member of the HSP70 family of heat shock proteins in systemic lupus erythematosus. J Exp Med 1988; 168(4):1475–80.

[16] Salvetti M, Ristori G, Buttinelli C, Fiori P, Falcone M, Britton W et al. The immune response to mycobacterial 70-kDa heat shock proteins frequently involves autoreactive T cells and is quantitatively disregulated in multiple sclerosis. J Neuromimmunol 1996; 65(2):143–53.
[17] Durinovic-Bello I, Hummel M, Zigler AG. Cellular immune response to diverse islet cell antigens in IDDM. Diabetes 1996; 45:795–800.
[18] Engvall E, Perlman P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 1971;8(9):871–4.
[19] Cohen IR. The cognitive paradigm and the immunological homunculus. Immunol Today 1992;13(12):490–4.
[20] Wallin RP, Lundqvist A, More SH, von Bonin A, Kiessling R, Ljunggren HG. Heat-shock proteins as activators of the innate immune system. Trends Immunol 2002;23(3):130–5.