Journal of Autoimmunity (1996) **9,** 159–166

NOD Mouse Diabetes: The Ubiquitous Mouse Hsp60 is a β-Cell Target Antigen of Autoimmune T Cells

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Received 27 November 1995
Accepted 8 December 1995

Key words: hsp60, autoimmunity, diabetes mellitus

In the NOD mouse, the onset of β-cell destruction is associated with spontaneous development of T-lymphocytes reactive to members of the 60 kDa heat shock protein (hsp60) family, including the Mycobacterial (MT) and the human (H) hsp60 molecules. Diabetes in the NOD mouse is a spontaneous tissue-specific autoimmune disease occurring without prior immunization. Therefore, it has been suggested that the anti-hsp60 T cells involved in the autoimmune diabetes of NOD mice might reflect molecular mimicry between MT-hsp60 and a β-cell tissue specific molecule sharing similar T cell epitopes, the p277 peptide of hsp60 in particular. We cloned and expressed the mouse hsp60 cDNA from a β-cell tumour. This mouse β-cell hsp60 cDNA was found to be identical in sequence to the hsp60 of mouse fibroblasts. We further report that NOD spleen cells and an NOD diabetogenic T cell clone C9 responded to the recombinant mouse hsp60 and to its peptide M-p277 to the same extent as to H-hsp60 and H-p277. Splenocytes of mice of other strains did not respond to p277. Moreover, treatment of 3 month old NOD mice with the non-modified self M-p277 peptide was as efficient as H-p277, from which it differs in one amino acid, in halting progression of the disease. Thus, anti-H-p277 T cells modulating diabetes in the NOD mouse are autoreactive, and are targeted at the mouse β-cell hsp60, which is not tissue specific. These findings raise the question of how a non-tissue specific molecule may be a target of a tissue-specific autoimmune disease.

Introduction

Insulin-dependent diabetes is an autoimmune disease mediated by T-cells [1–3]. The non-obese diabetic (NOD) strain of mice serves as a model for the disease [1]. We initially reported [4] that the onset of β-cell destruction in the NOD mouse was associated with the spontaneous development of anti-mycobacterial hsp60 (MT-hsp60) immunity. Early on, MT-hsp60 cross-reactive antigen was detectable in the sera of pre-diabetic mice, accompanied by anti-MT-hsp60 antibodies and T cells. We subsequently demonstrated that in pre-diabetic NOD mice, the spontaneous T-cell reactivity was much stronger to human hsp60 derived from monocytes (H-hsp60) than it was to MT-hsp60 [5]. Moreover, a diabetogenic T-cell cone, C9, isolated from NOD mice was found to react strongly to a 24 amino acid peptide from the H-hsp60 molecule, termed peptide H-p277 [5]. We further demonstrated that NOD diabetes could be prevented by treating pre-diabetic mice either with MT-hsp60 in PBS [4], or with the H-p277 epitope of H-hsp60 in oil [5]. The H-p277 peptide was also effective in treatment of the early stages of clinically overt diabetes in NOD mice [6]. Prevention of overt diabetes could also be obtained by vaccinating mice with anti-H-p277 T cells attenuated by irradiation [5]. Thus, immunity to H-hsp60 seems to be functionally involved in the diabetes of NOD mice: T cells responsive to the H-p277 epitope of H-hsp60 can mediate disease, and down-regulation of this response by vaccination can abort the disease.

But why should T cells targeted at MT-hsp60 or at H-p277 be involved in autoimmune diabetes? Since IDDM is a relatively tissue-specific process, it was reasoned that MT-hsp60 and H-p277 might mimic a mouse tissue-specific pancreatic beta-cell antigen. This could be either a molecule other than hsp60 [7] harbouring a p277-like epitope, or a tissue-specific variant of mouse hsp60. In fact, antigenic mimicry between MT-hsp60 and a joint-specific proteoglycan was suggested to play a role in autoimmune arthritis [8]. The aim of the experiments reported here was to identify the mouse β-cell molecule cross-reactive with hsp60 and H-p277. We now report that the β-cell target of anti-H-p277 T cells of the NOD mouse is the

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0896-8411/96/020159+08 $18.00/0

mouse hsp60 molecule. This antigen does not appear to be specific for β-cells. These findings raise the question of how a ubiquitous molecule may be a target in a tissue-specific autoimmune disease.

Materials and methods

Mice

NOD/Lt and CD1 mice were raised in the animal facilities of the Weizmann Institute of Science. The NOD breeding nucleus was a gift of Dr E. Leiter of Jackson Laboratories. In our colony of NOD mice, more than 90% of the females develop insulitis by 4 weeks of age and overt diabetes by 6 months of age. SJL, C57BL/6 and BALB/c mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Antibody preparation

Polyclonal antibodies were raised by immunizing a rabbit with purified recombinant MT-hsp60 emulsified in incomplete Freund's adjuvant [4]. Nitrocellulose was soaked (4°C, 4 h) with MT-hsp60 [9] (200 μg/2 ml in buffered saline) and the polyclonal rabbit antibodies were affinity-purified using the nitrocellulose-bound MT-hsp60 as described by Snyder *et al.* [10].

Western blot analysis

Mouse sera (1 μl per lane) or β-cell lysates (100 μg of protein per lane) were fractionated on SDS-10% polyacrylamide gels. Western-blot analysis was done as in Towbin *et al.* [11], using the affinity-purified polyclonal anti-MT-hsp60 antibodies, and the ECL detection system (Amersham).

Library screening

βTC1 cells are a line of insulin-secreting cells derived from a pancreatic endocrine tumour that developed in a transgenic mouse [12]. A cDNA library [13] constructed from βTC1 cells was screened using standard procedures [14] with a 1.5 kb radioactive DNA probe prepared from the H-hsp60 gene [15] by a random priming procedure (Amersham, UK). Several positive clones were identified and subcloned onto the pBS-KS vector (Stratagene, Cambridge, UK). Plasmids were sequenced on both strands using the Sequenase kit (US Biochemicals, Cleveland, Ohio, USA). Oligonucleotide primers were prepared that corresponded to the flanking sequences of the plasmid polylinker or to regions within the hsp60 cDNA insert. The primers were synthesized by Dr Ora Goldberg of the Biological Services Laboratory of the Weizmann Institute.

Fusion protein production and purification

Fragments of the cloned mouse β-cell hsp60, spanning the entire protein coding region of the cDNA (Figure 2), were ligated into pEX [16] expression vectors: F1 and F2 into the EcoRI sites of pEX2 and pEX1, respectively, and F2i and F2r into the PstI and EcoRl sites of pEX1 and pEX2, respectively. Competent *E. coli* (HB101) were transformed with each of the resulting constructs, or with the control pEX2 vector, and protein production was induced as in Stanley *et al.* [16]. The resulting β-galactosidase fusion proteins were isolated as insoluble inclusion bodies; the *E. coli* cells were lysed by treatment with lysozyme followed by freezing, thawing and sonication. The lysate was centrifuged at 12,000 rpm for 10 min at 4°C and the pellet was resuspended in NaCl 1M, Tris-HCI 100 mM pH 8, EDTA 50 mM, sonicated and spun as before. This step was repeated three times. The final pellet was dialysed against PBS.

Peptides

Peptides were prepared with an automated multiple peptide synthesizer (Abimed model AMS 422; Langenfeld, Germany) using the company's protocols for N-α-fluorenylmethoxycarbonyl (F-moc) synthesis, and purified on HPLC by reverse phase chromatography. Sequences were confirmed by amino acid analysis. Sequences of the 24 amino-acid hsp60 epitope peptides H-p277 of H-hsp60, and M-p277, of M-hsp60, are shown in Table 1. The sequence of control H-hsp60 peptide p278 is NEDQKIGIEIIKRTLKI. MT-hsp70 and MT-hsp60 were prepared as previously described [4].

T-cell proliferation assay

Cells of clone C9 and spleen-cell suspensions obtained from female mice were assayed for T-cell proliferation as described previously [4, 5]. Briefly, clone cells, 2×10^4/ml, or splenocytes, 2×10^5/ml, were incubated in quadruplicate for 72 h in 0.2 ml of culture medium in microtiter wells in the presence of absence of various antigens at 5 μg/ml. Proliferation was measured by the incorporation of [^{3}H] thymidine into DNA during the final 12 h of incubation. The results were computed as the stimulation index: the ratio of the mean test cpm in the presence of antigen to the mean background cpm in the absence of antigen. Standard deviations between quadruplicates were always <10% of the mean cpm. The background cpm was <1000 in splenocyte experiments and <200 with clone C9 cells. The spleen of each mouse was tested separately. Results of each group of mice are shown as the mean ± SD.

NIT cells, hsp60 cDNA analysis

NIT cells (transformed NOD β-cells) were the gift of Dr E. Leiter [17]. RNA was prepared using RNAzol^{TM}, and cDNA using reverse transcriptase (Promega). The PCR was done (Vente^{TM} Taq polymerase, annealing temperature 55°C) using two primers of the mouse hsp60 sequence and NIT cDNA as templates. The NIT hsp60 cDNA fragment obtained was cloned into Bluescript plasmid and sequenced.

Peptide therapy

Peptides M-p277 or H-p277 at a concentration of 2 mg/ml in phosphate buffered saline (PBS) were emulsified with an equal volume of incomplete Freund's adjuvant (IFA). Female 3-month old NOD mice were injected subcutaneously with 0.1 ml of emulsion containing 100 μg of either peptide. Control mice were injected with an emulsion of PBS and IFA. Mice were bled every 4 weeks and blood glucose levels were determined with a Beckmann Glucose Analyser II. Mice were considered diabetic if their blood glucose concentration was greater than 11 mmol/L because this concentration of glucose is greater than 3 SD above the mean glucose concentration measured in 100 healthy mice (not shown). Each group consisted of 10 mice.

Statistical analysis of the association between peptide treatment and the occurrence of disease or mortality by 6 months of age (as compared to the PBS treated control group) was done using Fisher's exact test to determine one-sided *P* values.

Results

Western blot analysis of NOD sera and pancreatic β-cells

Our first aim was to identify a mouse β-cell self-molecule cross-reactive with the MT-hsp60 and H-p277 molecules found earlier to be functional in NOD diabetes. We previously demonstrated by radioimmunoassay that a MT-hsp60 cross-reactive molecule was present in the sera of NOD mice and that its level was elevated some weeks before disease onset [4]. To identify this MT-hsp60 mimicking molecule, mouse sera were fractionated on 10% SDS-PAGE and blotted onto nitrocellulose. Using affinity-purified polyclonal anti-MT-hsp60 antibodies, a single band of ~60 kD was detected strongly in the sera of NOD mice and weakly in the sera of control C57BL/6 and BALB/c mice (Figure 1A). Using the same antibodies, a single 60 kD band was also detected in extracts of beta-cell tumour cells (βTC1) (Figure 1B) and rat insulinoma cells (not shown). Moreover, a computer search through the Embl and GenBank databases revealed no molecule with a major similarity to the H-p277 sequence other than members of the hsp60 family. Thus, it appeared that the mouse β-cell target antigen mimicked by hsp60 might be mouse hsp60 itself. Therefore, we set-out to clone mouse β-cell hsp60.

Cloning and sequencing of mouse β-cell hsp60

Screening of the βTC1 cDNA library yielded four partially overlapping cDNA fragments of lengths ~420, 700, 750 and 1500 bp (Figure 2). Complete sequence identity was seen in the areas of overlap. The inserts were aligned and were found to span a sequence of 2230 bp. Complete sequence information was then obtained for both strands using primers flanking the insert and internal primers synthesized according to the determined sequence. An open reading frame corresponding to 573 amino acids was observed, beginning with an ATG codon in a sequence context compatible with initiation of translation. The encoded protein was similar to that described for

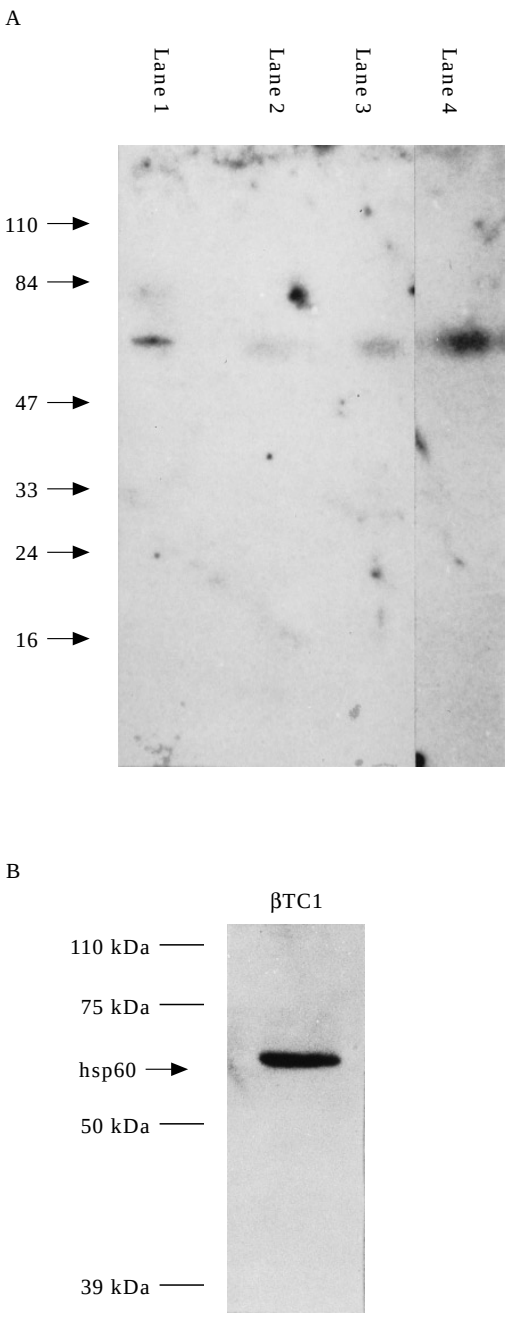

Figure 1. (A) Western-blot analysis of hsp60 in mouse sera. Sera of 4 month old mice of various strains (1 μl/lane) were run on a 10% polyacrylamide-SDS gel. Nitrocellulose transfer and Western-blot analysis were done using affinity-purified rabbit anti-MT-hsp60 antibodies. Lane 1=1 μg recombinant MT-hsp60. Lane 2=C57BL/6 mouse. Lane 3=BALB/c mouse. Lane 4=NOD mouse. (B) Western-blot analysis of hsp60 in β-tumour cell (βTC1) lysate. βTC1 lysate (100 μg protein per lane) was run on a 10% polyacrylamide-SDS gel. Nitrocellulose transfer and western-blot analysis were done using affinity-purified rabbit anti-MT-hsp60 antibodies.

Table 1. *Comparison of M-hsp60 and H-hsp60 in the 24 amino-acid region of the p277 sequence. Dots represent positions of sequence identity with the mouse β-cell hsp60 sequence.*

M-hsp60	437 V L G G G C A L L R C I P A L D S L K P A N E D
H-hsp60	437 . . . . . T . . . . .

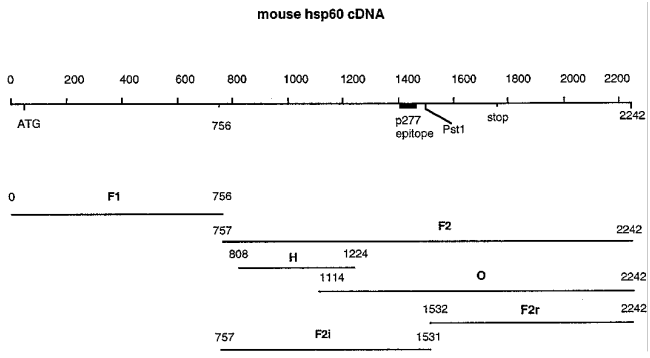

Figure 2. Map of cloned cDNA fragments of mouse β-cell hsp60.

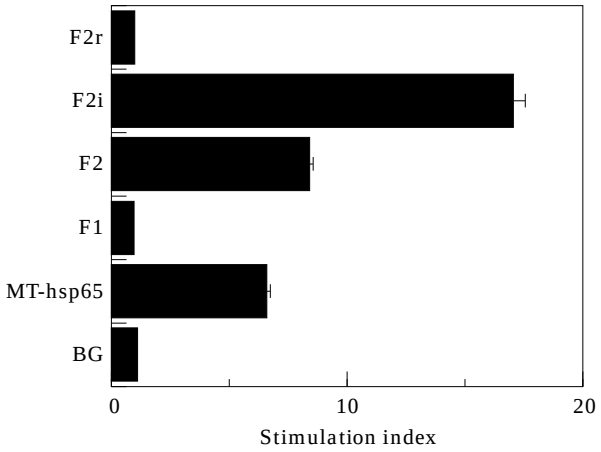

Figure 3. NOD spleen T cell proliferation to recombinant protein fragments spanning the M-hsp60 molecule. Spleen T-lymphocyte proliferative responses were measured to 5 μg/ml β-galactosidase fusion proteins of mouse β-cell hsp60 fragments (F1, F2, F2i, F2r) and to recombinant MT-hsp60. Background (BG) refers to T cell proliferation to β-galactosidase protein purified in the same manner from parental pEX transformed *E. coli.* Three female 3 month old NOD mice were tested separately, and the results are shown as the mean stimulation index±SE.

hamster hsp60 [18] from CHO cells (99% identity) and to human hsp60 [15] from HL60 monocyte cells (97% identity); the protein was only ~50% identical to MT-hsp60 [9]. The sequence of the β-cell hsp60 was identical to that reported for mouse fibroblast hsp60 [19]. Within the 24 amino acid sequence constituting the H-hsp60 p277 T-cell epitope [5], conservation between mouse hsp60 cloned from βTC1 and human monocyte hsp60 is very high (Table 1). Partial sequence analysis of the NIT (NOD β-cell) hsp60 cDNA demonstrated full identity to the βTC1 cloned hsp60 in the p277 region. Thus, NOD β-cell p277 is identical to the β-cell p277 sequence of the BALB/c strain.

NOD T-cell proliferation to mouse hsp60 fragments

The results of the above investigation indicated that the mouse self-molecule M-hsp60 was very similar to H-hsp60. However, in the p277 epitope region, there is a single, yet major difference in the amino acid sequence between the mouse and the human: threonine in place of lysine. Even single amino acid changes are known to cause changes in T-cell recognition [20]. Would M-hsp60 and M-p277 manifest the immunological properties detected for H-hsp60?

To test whether the M-hsp60 cloned from βTC1 was recognized by diabetogenic T-cells, we expressed the cDNA and tested the spontaneous T cell responses of NOD mice. In our previous work, maximal T-cell proliferation was seen in 3-month old female NOD mice at a concentration of 5 μg/ml of MT-hsp60 or H-hsp60 [4, 5]. Therefore, this was the age group of mice and antigen concentration we used to test the spontaneous T-cell reactivity to self M-hsp60. Figure 3 shows the splenic T-cell proliferative response to fragments spanning the M-hsp60 molecule. The response to M-hsp60 fragment F2 was somewhat greater than that stimulated by MT-hsp60. The response was even more marked to F2i, a smaller fragment of mouse-hsp60 harbouring the M-p277 sequence. In contrast, hardly any T-cell proliferation was seen in response to either the control β-galactosidase or to fragments F1 and F2r of M-hsp60 which do not contain the M-p277 region. We conclude that self M-hsp60 can function as an antigen for spontaneously occurring NOD T cells. Moreover, self M-hsp60 stimulates the T cells better than does the foreign MT-hsp60. The results also indicate that there may be no major epitopes in the F1 and F2r regions of the molecule detected by spontaneous NOD T-cell reactivity.

T-cell proliferation to the M-p277 peptide

To ascertain whether the M-hsp60 molecule is an autoantigen in NOD IDDM, we compared T-cell proliferation to peptide H-p277 and to the corresponding mouse peptide M-p277. Figure 4 demonstrates that T cells of female NOD spleens proliferated to the M-p277 peptide to the same extent as to the H-p277 peptide. There was no response to the control peptide p278.

The above results were confirmed using clone C9, a diabetogenic NOD T-cell clone that responds to H-p277 [5]. Figure 5 shows that C9 cells proliferated to the M-p277 peptide to the same extent as to H-p277. Therefore, it appears that the native self M-hsp60 and

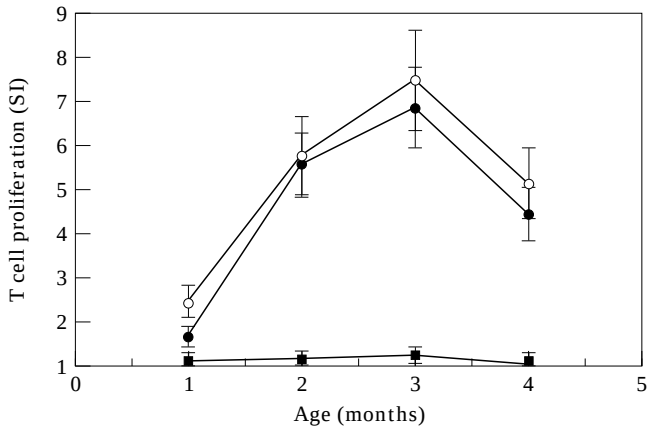

Figure 4. NOD spleen T cells proliferate to mouse hsp60 peptide M-p277 and H-p277. T-cell proliferation was measured in response to M-hsp60 epitope M-p277 (●), H-p277 (○), and control peptide p278 (■). The spleens of five female NOD mice were tested in each age group. The results are shown as the mean stimulation index±SE.

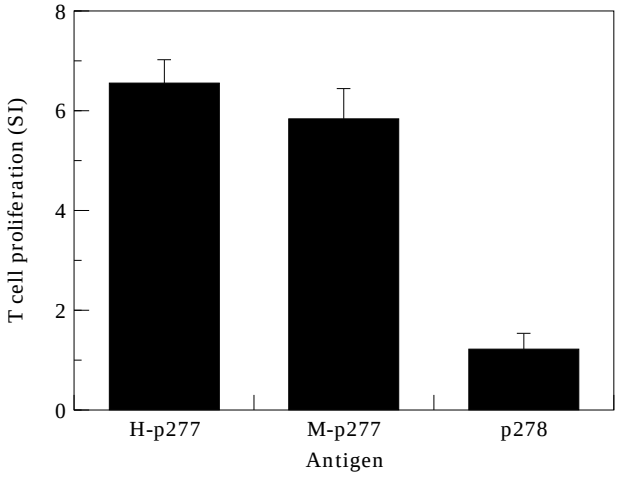

Figure 5. Clone C9 proliferate in response to M-p277 and to H-p277. T cells of the diabetogenic clone C9 were assayed in a proliferative response in the presence of self mouse β-cell hsp60 peptide M-p277 or H-p277.

its M-p277 peptide can function as target molecules for diabetogenic NOD T cells. Moreover, M-p277 and H-p277 are wholly cross-reactive at the T-cell level, despite a non-conserved amino acid substitution.

Spontaneous T-cell proliferation to p277 in various mouse strains

It was reported that human cord blood manifests a high frequency of T cells reactive to MT-hsp60 [21] and that healthy humans may respond to homologous peptides present in MT-hsp60 and in H-hsp60 [22]. These findings raised the question of whether the spontaneous response to M-p277 was unique to NOD mice. We therefore tested the T-cell responses of additional strains of mice.

Unlike splenocytes of female 3 month old NOD mice, splenocytes of naive age-matched female mice of the strains did not proliferate in the presence of p277 or of MT-hsp60 (Figure 6). Hence, a spontaneous response to M-p277 is not a general property of all mouse strains.

M-p277 therapy of diabetes

We previously reported that the advanced insulitis present in 3 month old female NOD mice could be arrested by treating the mice with a single injection of H-p277; the treated mice developed a lower incidence of clinically overt diabetes and were spared from death resulting from severe hyperglycaemia [6]. Because of the single amino acid difference, H-p277 can be viewed as a modified homologue of M-p277 [23]. It has been reported recently that EAE could be reversed by treatment with a peptide variant of a myelin basic protein epitope [24]. Therefore, it was important to investigate whether peptide therapy could be achieved using the native unmodified M-p277 sequence. We treated 3 month old female NOD mice with 100 μg of either H-p277 or M-p277 in IFA; control mice were sham treated with PBS in IFA. Table 2 shows the results. At 6 months of age, 90% of the control mice were overtly hyperglycaemic and 60% had died of diabetes. In contrast, treatment with either H-p277 or M-p277 prevented both diabetes (a 40% and 50% incidence, respectively) and death (a 10% and 20% incidence, respectively), with no significant difference between the two p277 peptides. Thus, the mouse self peptide M-p277 seems to be as effective as the foreign H-p277 in the therapy of advanced insulitis.

Discussion

T-cells specific for mycobacterial and human hsp60 have been found in the target lesions of human chronic arthritis [25], adjuvant arthritis [26], multiple sclerosis [27] and EAE (experimental autoimmune encephalomyelitis) [28]. Thus, naturally occurring T cells targeted at variants of hsp65 may be involved in various tissue-specific autoimmune diseases. How can this be explained? Two approaches have been suggested:

(a) There might exist tissue-specific molecules different from hsp60 that contain epitopes similar to hsp60. For example, a cartilage glycoprotein has been implicated as a tissue-specific molecule mimicking MT-hsp60 in adjuvant arthritis [8]. In IDDM, cross-reactivity between hsp60 and GAD has been suggested [29]. In fact, a recent theoretical review [7] suggested molecular mimicry between hsp60 and various tissue-specific molecules as a mechanism by which anti-hsp60 T cells might modulate various autoimmune disease.

(b) There may be tissue-specific variants of hsp60. Thus, T cells targeted at one epitope of hsp60 will attack only cells containing hsp60-variants harbouring this epitope. Although discussed in theory, these possibilities have not been tested experimentally in depth.

The present results in the NOD mouse model of IDDM suggest that neither of the above explanations

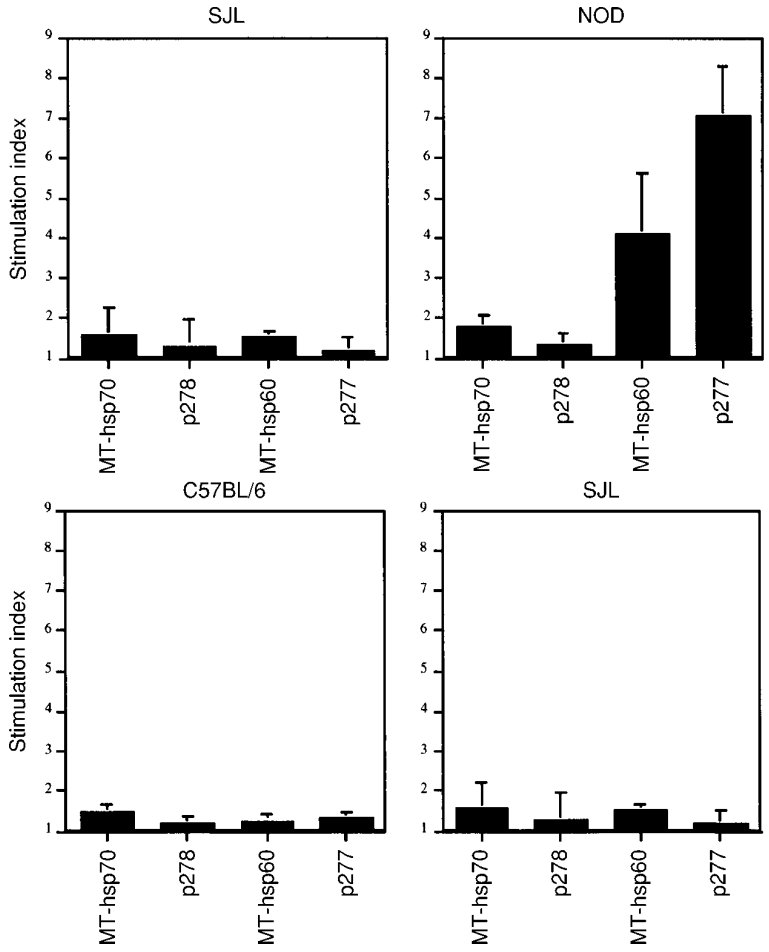

Figure 6. T cell proliferation assays of spleen cells of naive mice of various strains. The spleens of five 3 month old female mice of each strain were tested with the indicated antigens.

Table 2. *Treatment of diabetes with mouse or human p277*

Treatment at 3 months	Diabetes at 6 months	
	Hyperglycemia (%)	Mortality (%)
H-p277	40*	10*
M-p277	50†	20†
PBS	90	60

Groups of 10 female NOD mice were treated at the age of 3 months with 100 µg of M-p277, H-p277 or PBS emulsified in IFA. The incidence of hyperglycaemia and death from diabetes was scored at 6 months of age.
*P=0.03 compared to PBS control
†Not significant compared to H-p277.

is very plausible. Diabetogenic anti-H-hsp60 T cells recognize mouse pancreatic β-cell hsp60, which is identical in sequence to its fibroblast homologue, and is apparently present in all tissues. Therefore, there is no evidence in favour of a tissue-specific hsp60 variant or mimic bearing a specific epitope. Thus, the T cells are autoimmune, and their target is not tissue-specific. How, then, can they produce or modulate a tissue-specific disease? This question is not new. Both the pyruvate dehydrogenase antigen in primary biliary cirrhosis [30] and glutamate decarboxylase in IDDM [31–33] are considered target antigens in autoimmune diseases, yet are expressed not only in the target organs of those diseases [34]. Several suggestions have been proposed to account for the specific targeting of hsp60 in IDDM:

(a) Tissue-specific role or location. hsp60 is present in the mitochondria of all cells, but it is claimed to be present uniquely in secretory vesicles of β-cells [35]. Since these vesicles fuse with the cell membrane when insulin is secreted, it is conceivable that β-cells might present hsp60 on their membranes or even secrete hsp60, differently from other cells, even in the absence of stress.

(b) Cell-specific post-translational processing of hsp60. Venner *et al*. [36] have shown that there are 8–12 genes for hsp60 in the vertebrate genome, yet sequencing demonstrates all but one to be pseudogenes. This is in line with our results indicating that the β-cell hsp60 transcript is not tissue-specific. Ross *et al*. [37] isolated hsp60 from human kidney cell membranes and demonstrated amino acid sequence identity to the human mitochondrial hsp60 molecule, extending 24 amino acids from the N-terminus, but lacking the leader sequence. Preliminary experiments in our laboratory

demonstrate that C9 cells injected into NOD mice migrate preferentially to the pancreas and kidney (in preparation). These experiments taken together, suggest the possibility that there may be tissue-specific post-translational processing of hsp60.

(c) Tissue-specific hsp60 processing. C9 cells proliferate in the presence of β-cells even in the absence of other antigen-presenting cells (Elias *et al.*, in preparation). Thus, β-cells are capable of presenting their own hsp60 to T cells. It is plausible that different cell types might process antigens in different ways and β-cell processing of endogenous hsp60 might yield specific immunogenic peptides, such as p277.

(d) Tissue-specific vulnerability. In comparison to other cells, β-cells are more easily damaged by oxidants and other toxic agents [38]. Therefore, T cells targeted at a non-tissue specific molecule may preferentially damage the β-cells, causing cell destruction and release of true tissue-specific antigens, and these antigens could generate a cascade of events leading to tissue-specific destruction.

(e) Environmental tissue-specific trigger. The development of autoimmune diabetes mellitus is associated with certain susceptibility genes [39]. However, the concordance of only about 30–35% among identical twins suggests a major role for environmental factors. The possibility of a viral trigger has been debated in the last decades with no clear conclusions [40] and chemical and nutritional stressants have also been implicated in IDDM [40]. The route by which the environment might trigger an autoimmune disease is as yet unclear. T cells targeted at epitopes of 'self' hsp60 exist in healthy individuals [22]. In eukaryotic cells, viral infection and γ-interferon can augment expression of heat-shock proteins, rendering such cells susceptible to lysis by autoimmune anti-hsp60 T cells [41, 42, 43]. High concentrations of T cells targeted at hsp60 have been demonstrated in autoimmune lesions in multiple sclerosis [27] and in rheumatoid arthritis [25]. Preliminary evidence suggests this may be true also for NOD mouse diabetes. Our data, taken together with those cited above, can support the following possible model: an environmental tissue-specific trigger (viral or other) augments the local expression of hsp60 (via γ-interferon? other intermediates?) rendering the pancreatic β-cells targets for naturally existing anti-self hsp60 T cells. If this is true, non-tissue-specific self-hsp60 may serve to link environmental factors to the initiation of autoimmune diabetes.

Irrespective of how hsp60 might function as a target in IDDM, the present results show that a native, unmodified self-peptide sequence can be used as a therapeutic agent to arrest the autoimmune process. Thus, there is no universal requirement for modifying the sequence of a self peptide to have it function therapeutically.

Acknowledgements

O. S. Birk is a recipient of the Foulkes Foundation Scholarship. I. R. Cohen is the incumbent of the Mauerberger Chair of Immunology and the Director of the Robert Koch-Minerva Centre for Research in Autoimmune Diseases. The work was supported by grants from the Minerva Foundation, The Tauro Foundation, Mr Rowland Schaeffer and Portman Pharmaceuticals, Inc.

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