A role of Hsp60 in autoimmune diabetes: Analysis in a transgenic model
(transgenic mice/nonobese diabetic mouse)

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ABSTRACT A pathogenic role for self-reactive cells against the stress protein Hsp60 has been proposed as one of the events leading to autoimmune destruction of pancreatic β cells in the diabetes of nonobese diabetic (NOD) mice. To examine this hypothesis, we generated transgenic NOD mice carrying a murine Hsp60 transgene driven by the H-2Eα class II promoter. This would be expected to direct expression of the transgene to antigen-presenting cells including those in the thymus and so induce immunological tolerance by deletion. Detailed analysis of Hsp60 expression revealed that the endogenous gene is itself expressed strongly in thymic medullary epithelium and islet cells (as well as other sites), or have the capability for self-tolerance.

For the majority of spontaneous autoimmune diseases, the primary target autoantigens are not known. Attention in various diseases has focused on the stress protein Hsp60/65 as a possible candidate for T-cell recognition. Hsp60 molecules are highly immunogenic components in the response to infection yet they are highly conserved in evolution: mycobacterial Hsp65 is 48% homologous with human Hsp60 (1). This conservation is presumed to reflect a vital chaperone role in the correct folding, assembly, and compartmentalization of intracellular proteins (2). Responses to Hsp60 have been investigated in several autoimmune diseases. For example, adjuvant arthritis of Lewis rats is transferable by T-cell clones specific for the 180–188 epitope of mycobacterial Hsp65, which is apparently cross-reactive with self tissues in the rat (3). The search for the primary target autoantigen in insulin-dependent diabetes mellitus has focused on T-cell responses to islet components in patients and on anti-self T cells that spontaneously develop early in pathogenesis and/or have the ability to transfer disease in the nonobese diabetic (NOD) mouse disease model. Glutamic acid decarboxylase (GAD) meets many of the criteria for a candidate autoantigen since it is expressed in pancreatic β cells (as well as other sites), insulin-dependent diabetes mellitus patients respond to disease-specific GAD epitopes, NOD mice spontaneously develop responses to GAD epitopes early in the pathogenic process, and induction of tolerance to GAD prevents disease (4–6). Another candidate is a β-cell granule antigen, the target of a T-cell clone capable of transferring diabetes to prediabetic NOD mice. Expressing the T-cell receptor of this clone as a transgene greatly accelerated diabetes (7, 8). Elias and colleagues (9, 10) proposed Hsp60 as an early antigen in the triggering of insulin-dependent diabetes mellitus: antibody and T-cell responses to Hsp60 precede disease. Hsp60-reactive T cells can transfer disease to prediabetic NOD mice, and vaccination with Hsp65 in PBS blocks disease induction. Murine Hsp60 was cloned and the expressed cDNA clone was used in conjunction with a panel of synthetic peptides to show that the dominant epitope is within amino acid residues 437–460 (a peptide termed p277) (10). Vaccination with p277 blocks the development of disease and can halt ongoing disease (11, 12). Moreover, immunization to peptide p277 conjugated covalently to an immunogenic protein carrier could induce hyperglycemia and insulin in some standard strains of mice not prone to spontaneous diabetes (13). Thus, the p277 peptide of Hsp60 is functionally involved in murine diabetes. The target antigen in any tissue-specific autoimmune disease should presumably have distribution limited largely to that tissue, whereas Hsp60 is thought to be ubiquitously inducible. However, heat shock proteins show different levels of constitutive expression and can be locally induced in vivo, for example, after tissue injury or fever (14, 15). Furthermore, there may be tissue-specific differences in the Hsp60 epitopes generated for presentation to T cells.

Experiments show that expression of a transgene in professional thymic antigen-presenting cells leads to clonal deletion of T cells specific for that transgene product, the archetypal promoter for this analysis being the mouse class II Eα promoter (16). For example, thymic expression of self H/K ATPase, a candidate autoantigen in autoimmune gastritis, induces immunological tolerance and abrogates the disease (17). We have investigated the role of Hsp60 as a candidate autoantigen by overexpressing the gene in the thymus to manipulate self recognition by T cells and so alter pathogenesis. Transgenic expression of murine Hsp60 has allowed us to approach its role as a target in autoimmunity, analyzing various diseases to assess differences in pathology.

MATERIALS AND METHODS

Hsp60 Transgenic Mice. A construct consisting of the Xba I–Nru I linear fragment from the pDOI-5 vector (supplied by D. Mathis, Institut National de la Santé et de la Recherche Antigénique) was inserted in reverse orientation (18) downstream of the H-2Eα II promoter (16). For example, thymic expression of self H/K ATPase, a candidate autoantigen in autoimmune gastritis, induces immunological tolerance and abrogates the disease (17). We have investigated the role of Hsp60 as a candidate autoantigen by overexpressing the gene in the thymus to manipulate self recognition by T cells and so alter pathogenesis. Transgenic expression of murine Hsp60 has allowed us to approach its role as a target in autoimmunity, analyzing various diseases to assess differences in pathology.

Abbreviations: NOD, nonobese diabetic; GAD, glutamic acid decarboxylase; r, recombinant.

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Médicale, Université Louis Pasteur, Strasbourg, France) (18), carrying the full-length mouse Hsp60 cDNA, was microinjected into the pronuclei of fertilized NOD oocytes that were transferred into pseudopregnant (C57BL/10 × CBA)F1 foster mothers. NOD mice were obtained from the Clinical Research Centre (Harlow, U.K.); details of the diabetes incidence and immunological characterization of this colony have been published in detail (19, 20). Litters were genotyped by Southern blot or PCR analysis of genomic DNA from tail biopsies.

DNA and RNA Analysis of Transgenic Mice. PCR of genomic DNA from litters utilized a sense oligonucleotide primer located in the H-2Ea² promoter sequence [corresponding to bp −92 to −70 in the published upstream sequence (21)] and an antisense oligonucleotide primer in the Hsp60 cDNA insert [corresponding to bp 40–60 in the cDNA sequence; O.S.B., unpublished results]. All PCR amplifications used 30 cycles and an annealing temperature of 58°C. PCR products were analyzed on 2% agarose gels. RNA was purified from the tissues of transgenic mice or nontransgenic littermates by using the acid phenol method (RNAzol B; Biogenesis, Bourne-
mouth, U.K.). For Northern blot analysis, total RNA (10 μg per lane) was electrophoresed through denaturing agarose/formaldehyde gels and then blotted onto Hybond-N membranes (Amersham) for hybridization with murine Hsp60 or actin cDNA probes. Membranes were then exposed to Kodak XAR-5 film for 48 h. In some cases cDNA was synthesized from total RNA for reverse transcription-coupled PCR, as described in detail elsewhere (22).

Immunohistochemistry of Tissues. Cryosections (5 μm) of thymus, spleen, and pancreas from transgenic mice or nontransgenic littermates were fixed in ice-cold acetone and stained with the following antibodies: LK1 (biotinylated), mouse anti-human Hsp60-(383–447) monoclonal antibody, which cross-reacts with all mammalian but not bacterial Hsp60 (23) (gift of C. Boog and W. van Eden, University of Utrecht, Utrecht, The Netherlands); A575, rabbit anti-cytokeratin 19 polyclonal antibody (Dako). These were probed, respectively, with streptavidin-Texas red (Vector Laboratories) and mouse-adsorbed donkey F(ab')² anti-rabbit IgG (heavy and light chains) conjugated to fluorescein isothiocyanate (Stratech Scientific). Staining was analyzed with a Leica IR confocal scanning microscope.

T-Cell Proliferation Assays. Spontaneous reactivity to antigens was analyzed by culture of freshly isolated splenocytes at 4 × 10⁴ cells per flat-bottom well in H1L medium supplemented with penicillin, streptomycin, glutamine, and 2-mercaptoethanol, as described (24). In some cases responses were measured in popliteal lymph node cells 10 days after priming in both hind footpads. The antigens used were recombinant (r) human Hsp60 as a β-galactosidase fusion construct expressed from plasmid pRH710 (23), mouse rHsp60 residues 448–573, and rat rGAD67 residues 332–584. The mouse Hsp60 and GAD fusion proteins were generated after ligation of GAD or Hsp60 EcoRI cDNA fragments into the pGEX2 vector; induction and purification of recombinant glutathione S-transferase fusion proteins were done as described (24, 25). The negative control fusion protein for experiments with human rHsp60 was β-galactosidase expressed from pEX2 and for murine rHsp60, fusion proteins from other regions of the molecule and glutathione S-transferase fusion protein preparations from pGEX vector without a cDNA insert. The peptides p277 [Hsp60-(437–460)] and p278 [Hsp60-(458–474)] were synthesized as described (10).

Disease Susceptibility. Transgenic NOD mice and nontransgenic littermates were compared for development of insulitis, diabetes, and sialitis. Parallel disease studies were undertaken in the original London transgenic colony and in a colony maintained at the Animal Breeding Center of the Weizmann Institute. For assessment of insulitis and sialitis, groups of age-matched transgenic and nontransgenic mice were sacrificed at 3 and 5.5 months and pancreata and salivary glands were fixed in formalin, embedded in paraffin, and stained with hematoxylin/eosin. Pancreas sections in each of two levels of the organ were scored blindly for islet pathology as follows: 1 = perislet infiltration; 2 = mild intraislet infiltration; 3 = severe intraislet infiltration; 4 = loss of islet architecture. When different scores were recorded at each of the two tissue levels, the mean score was used. Mice were tested biweekly for glucosuria by using Diastix (Bayer Diagnostics, Basingstoke, U.K.). Blood glucose was measured by using a Glucometer GX blood glucose meter (Bayer Diagnostics, Hampshire, U.K.) or a Glucose Analyzer (Beckman). Mice with blood glucose of >11 mmol·l⁻¹ were scored as diabetic, subject to confirmation from histopathology, since this value was found to be 2.5 SD above the mean blood glucose readings from disease-protected NOD-Ea transgenic mice in the same facility (19). Salivary gland sections at each of two tissue levels were scored blindly as follows: 1 = periductal infiltration; 2 = mild infiltration; 3 = severe infiltration. When different scores were recorded at each of the two levels, the mean was used.

RESULTS AND DISCUSSION

Hsp60 Expression in Control and Transgenic Mice. The full-length cDNA for the murine Hsp60 sequence was subcloned into a modified pDOI-5 expression vector by a series of EcoRI partial digests and ligations (Fig. 1A). A number of NOD transgenic lines shown by Southern blot analysis to carry the transgene were generated and one line was chosen for further study. Positive offspring were subsequently identified by using a construct-specific PCR (Fig. 1B) with a sense primer in the H-2Ea promoter and an antisense primer in the Hsp60 cDNA sequence. Reverse transcription-coupled PCR of cDNA from tissues of transgenic or nontransgenic mice showed Hsp60 transcripts in all tissues examined, including brain, thymus, and pancreas (data not shown). Irrespective of the method chosen for detection of mRNA, the kidney was found to express a very large amount of Hsp60 and the pancreas was found to express relatively little. By using Northern blot analysis, we obtained preliminary evidence that the constitutive expression of Hsp60 was up-regulated in the

![Fig. 1. Generation and characterization of Ea-Hsp60 NOD transgenic mice.](image-url)
Fig. 2. Thymic expression of Hsp60 in transgenic mice. Thymic cryosections from nontransgenic (A–C) (images of a single field) or transgenic (D–F) (images of a single field) littermates were stained with the epithelial cell marker A575 (A and D, green label) and the Hsp60 mAb LK1 (B and E, red label). Colocalization of Hsp60 and epithelial cells is shown in yellow (C, F, and G). The thymic medulla (M) and cortex (C) are indicated. (G) High-power field of transgenic cortex showing LK1 staining of both epithelial cells (yellow) and nonepithelial bone marrow-derived cells (red).

Fig. 3. Severity of insulitis but not sialitis is reduced in Eα-Hsp60 transgenic mice. Female transgenic mice and negative female littermates were sacrificed at 3 or 5.5 months for analysis of islet (A) and salivary gland (B) histopathology in hematoxylin/eosin-stained paraffin sections.
transgenic mice (Fig. 1C): comparison of nontransgenic and transgenic spleen RNA (lanes 2 and 6) showed substantially increased expression in the transgenic spleen, as was expected since the transgene was linked to a strong class II promoter.

Immunohistochemical analysis permitted a more detailed comparison of expression in transgenic mice and nontransgenic littermates (Fig. 2). Fig. 2 A-C shows, respectively, staining of wild-type NOD thymus with an epithelial marker, with anti-Hsp60, and with the two overlaid. In Fig. 2B it can be seen that Hsp60 is strongly expressed in thymic medullary epithelium and more sparsely in cortical epithelium. In contrast, LK1 staining of transgenic thymus revealed strong staining of both medullary and cortical epithelium such that the two regions cannot be delineated on the basis of Hsp60 expression (Fig. 2 D-F). Furthermore, many of the LK1+ cells are A575+ (cells appearing as red only, in Fig. 2F and shown at higher magnification in Fig. 2G), suggesting the strong expression of transgenic Hsp60 in large nonepithelial bone marrow-derived cells, probably dendritic cells. This enhanced thymic expression was confirmed by comparing the LK1 staining of disaggregated permeabilized thymic epithelial cells from transgenic and control mice by flow cytometry analysis (data not shown). Hsp60 expressed by thymic epithelium was accessible to T cells since disaggregated thymic cells could stimulate an Hsp60-specific T-cell line (data not shown). All thymocytes from transgenic mice and negative littermates were weakly positive for Hsp60 by confocal imaging, although this is not evident at the low laser voltage used to image accurately the brighter thymic epithelial and bone marrow-derived cells. The relatively high baseline of Hsp60 expression in the thymocytes and stromal cells probably explains why by using Northern blot analysis, where no distinction can be made between different thymic compartments, Hsp60 transcripts in the organ as a whole did not appear to be obviously up-regulated. Protein expression by Western blot analysis and immunohistochemistry in various other tissues was in line with the findings from mRNA analysis. Hsp60 protein expression in pancreas was weak in both transgenic and control mice.

It is noteworthy that Hsp60 is normally expressed by medullary thymic epithelium (as well as by some cortical cells) at a level equivalent to that of thymic major histocompatibility complex class II expression. Nevertheless, anti-self Hsp60 T cells are not deleted and could be easily detected in all species examined (3, 11, 26, 27). In the Eα–Hsp60 transgenic thymus, Hsp60 is overexpressed by major histocompatibility complex class II+ cells known to be critical for positive and negative selection of the T-cell receptor repertoire. It was therefore anticipated that despite the failure of endogenous Hsp60 to induce tolerance, transgenic mice may show altered T-cell responses to Hsp60 and, in turn, an altered phenotype in any disease process in which anti-Hsp60 T cells are involved.

**Insulitis and Sialitis in Hsp60 Transgenic Mice.** Transgenic mice and negative littermates were assessed to determine the incidence of insulitis, diabetes, and sialitis. Hemizygous transgenic mice were mated with wild-type NODs and the female offspring were separated into transgene positive (+/−) and negative (−/−) groups. Studies were carried out in two colonies, in London and in Rehovot: wild-type mice in the latter colony showed about 90% diabetes at 6 months, whereas the disease in the former colony was delayed so that 40–45% of the mice show disease at 6 months. For the analysis of the disease in the former colony was delayed so that 40–45% of the mice show disease at 6 months, whereas the latter colony showed about 90% diabetes at 6 months, whereas the incidence in nontransgenic (open symbols) and transgenic (solid symbols) mice.

In the group of 14 transgenic mice, only three showed intraislet infiltration, while histological findings in the remaining 11 mice ranged from periislet infiltration in 5 mice to normal or very mildly affected pancreata in 6 mice. Thus, 16 of 24 nontransgenic and 3 of 23 transgenic mice displayed insulitis. Thus, there was a pronounced protective effect of Hsp60 transgene expression on the development of insulitis. However, the fact that a number of transgenic mice did display periislet infiltration suggests that the immunological modulation may have affected the ability to invade islets rather than the development of self reactivity per se.

Although the pathogenic events in the pancreas and salivary glands of NOD mice are concomitant, they are under different immunological and genetic controls (28, 29). We observed no protection from sialitis in Eα–Hsp60 transgenic mice: indeed the salivary glands of transgenic mice were...
significantly more severely infiltrated than those of nontransgenic controls (Fig. 3B).

**Clinical Diabetes.** The protection from insulitis observed histologically was mirrored by the incidence of overt diabetes assessed by serum glucose levels (Fig. 4). Eo-Hsp60 transgenic mice were substantially protected from the development of full-blown diabetes. Four out of 15 mice in the transgenic group became diabetic by 9 months (3 of these 4 showing serum glucose concentrations only marginally above normal levels) compared with 16 of 21 diabetic mice in the wild-type controls.

**T-Cell Responses in Hsp60 Transgenic Mice.** The changes in disease phenotype suggested that thymic overexpression of Hsp60 may have indeed affected T-cell recognition of the antigen. In several other transgenic studies, thymic expression of transgenes induced tolerance to the transgene product, albeit to different degrees (30). T-cell tolerance was observed by Heath et al. (31) in rat insulin promoter–Eκ transgenic mice as a consequence of weak thymic expression of the transgene at a level detectable only by reverse transcription-coupled PCR (31). In rat insulin promoter–simian virus 40 tumor antigen transgenic mice, weak thymic expression was sufficient to induce partial tolerance (32). The situation in this case is somewhat different: our system involves retargeting of a self-antigen already expressed in mice, which despite high expression in all tissues including thymus, does not naturally induce clonal deletion. We compared the T-cell responses of transgenic mice and nontransgenic littermates by using a number of antigenic preparations (Fig. 5). To obtain a broad view of T-cell responses to the self antigen and closely related sequences in these groups, we analyzed proliferation of splenocytes from unimmunized 11- to 12-week female mice to human rHsp60, murine rHsp60-(448–573), Hsp60 peptide p277, Hsp60 peptide p278 [an epitope to which NOD mice do not respond spontaneously (10)], and rat rGAD65-(332–584) (Fig. 5A–E, respectively). Spontaneous T-cell proliferation to islet antigens in NOD mice has been described (5, 24). The responses precede disease onset in NOD mice, although conventional strains can recognize self-Hsp60 if immunized. Spontaneous T-cell responses to each of the recombinant Hsp60 preparations including the murine Hsp60-(448–573) antigen were at least as strong in the transgenic as in the nontransgenic group. However, responses to p277, the previously characterized immunodominant Hsp60 epitope, were much reduced in transgenic mice (Fig. 5E). Responses to GAD65 were strong in Eo–Hsp60 transgenic mice despite their relative resistance to diabetes. Recall responses after immunization to a number of other antigens including *M. tuberculosis* Hsp65-(431–447), purified protein derivative, and oxazolone (by delayed-type hypersensitivity) were identical in transgenic and nontransgenic mice.

As there was no evidence of tolerance to *in vitro* challenge with recombinant Hsp60 protein preparations and yet responses to the p277 epitope were reduced, it is possible that a shift in immunodominant epitopes occurred in the transgenic mice. Nevertheless, the p277-responsive T cells were not completely deleted since an anti-p277 response could be induced by footpad immunization to p277 peptide in incomplete Freund’s adjuvant (background without added p277, 850 cpm, and in the presence of p277, 2890 cpm). There is a precedent for a shift in immunodominance causing a change in disease phenotype: in the Lewis rat model of adjuvant arthritis, preimmunization with Hsp65 prevents disease, an effect shown to correlate with a shift in T-cell recognition from the 180–188 epitope to a protective epitope, 256–270 (33). Furthermore, natural regulation of responses to self-Hsp60 is implied by the finding that T-cell reactivity is not necessarily linked with pathogenesis: when peripheral T cells from healthy humans are stimulated with mycobacterial Hsp65, the majority of cells recognize epitopes cross-reactive with the human homologue (26). Similarly, in healthy mice mounting a nonspecific inflammatory response to mineral oil, cells at the inflammatory site recognize both mycobacterial and mammalian Hsp60/65 epitopes (27). A hypothesis compatible with our findings and those of others is that T cells responding to different epitopes have differing cytokine profiles that skew responses to disease or protection.

In summary, we have shown that overexpression of Hsp60 in major histocompatibility complex class II-positive cells of transgenic mice results in altered self-recognition of the p277 peptide of this antigen. Development of diabetes in transgenic NOD mice is largely blocked at the stage of periislet infiltration, before islet penetration and destruction occurs. This suggests that Hsp60-reactive T cells play a significant role in the final penetration and destruction of β cells in NOD mice.

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**Fig. 5.** Spontaneous T-cell responses in Eo–Hsp60 transgenic mice. Individual 11-week-old female transgenic mice (solid symbols) or nontransgenic littermates (open symbols) were tested for spontaneous proliferation of splenocytes to the antigens human rHsp60 (A), mouse rHsp60 residues 448–573 (B), synthetic peptide p277 [Hsp60-(437–460)] (C), p278 [Hsp60-(458–474)] (D), and rat rGAD65 residues 332–584 (E). T-cell proliferation in the presence of negative control fusion proteins, mouse rHsp60 fusion proteins from other regions of the molecule and GST fusion protein preparations from pGEX vector without a cDNA insert, used in the range 5–25 μg/ml, was always <7000 cpm. For example, mean proliferation in response to the control β-galactosidase fusion protein is indicated by the open square in A. Background cpm in the presence of medium was always <3000 cpm.
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