HEAT SHOCK PROTEINS IN AUTOIMMUNE DISEASES

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Heat shock proteins (hsp's) are among the most conserved proteins in evolution. They have been identified as important pathogen-related antigens as well as autoantigens suitable for construction of novel vaccines. The high evolutionary homology of hsp's has raised the question about the safety of such vaccines. Experimental and clinical observations have confirmed that hsp proteins are involved in the regulation of some autoimmune disease such as autoimmune arthritis, type 1 diabetes mellitus, atherosclerosis, multiple sclerosis, and other autoimmune reactions. It has been shown in experimental animals that some hsp proteins (especially hsp60, hsp70, and hsp10) can either induce or prevent autoimmune reactions depending on the circumstances.

This article discusses the involvement of hsp proteins in the etiology of autoimmune diseases and it presents promising experimental data on the effects of immunization with hsp proteins in the prevention and therapy of autoimmune diseases.

ABBREVIATION

AA, adjuvant arthritis; APC, antigen-presenting cells; hsp, heat shock protein; GAD, glutamic acid decarboxylase; GALT, gut-associated lymphoid tissues; grp, glucose regulating protein; IDDM, insulin-dependent diabetes mellitus; IFN-γ, interferon-γ; IL, interleukin; MHC major histocompatibility complex; NOD, non-obese diabetic mice; TcR, T cell receptor; TGFβ, transforming growth factor β; TLR, Toll-like receptor.

HSP INSIDE OF THE CELLS

Heat shock proteins (hsp's) comprise a very heterogeneous group of proteins that can be divided into 6 families according to molecular weight: small hsp's (m.w. 12–43 kDa), hsp40, hsp60, hsp70, hsp90, and large hsp's (m.w. 100–110 kDa)[1]. Heat shock proteins belong to super family of stress proteins, which also comprise glucose regulating proteins (grp), ubiquitine and lectin chaperons – calnexin and calreticulin. The characteristic sign of the stress proteins is their overexpression during cellular stresses (high temperature, osmotic stress, UV, inflammation). Hsp's act as chaperons of nascent proteins (hsp60, hsp70, hsp40), protecting them from aggregation and improper folding, or as chaperons of intracellular signal proteins (hsp90, hsp40, hsp70), keeping them in an unstable substrate-grabable state[2]. Other members of hsp's proteins are participate in the protein degradation pathway (ubiquitin, hsp104), driving damaged, abnormal, or abundant proteins to proteasome degradation[3]. Calreticulin, calnexin, and grp's are involved in the transport of antigens between TAP proteins (transporter associated with antigen processing) and major histocompatibility complex (MHC)(ref.[4,5]). Some SPs such as hsp90 and calreticulin have been confirmed to be tightly involved in morphogenesis[6,7].

HSP AS A "DANGER SIGNAL"

An indispensable role of hsp proteins in intracellular processes explains their high evolutionary conservancy, which together with their overexpression during cellular stress makes them an important part of immune system recognition. Following their release from stressed microorganisms or from autologous inflamed or necrotizing tissue (tumors, target tissue of autoimmune diseases), hsp's may be recognized by the surface receptors of the cells of the host immune system (Toll-like receptor 4 – TLR-4, TLR-2, CD14, CD91, CD94, LOX-1, SR-A) or as an immunodominant antigens[8-11]. With the contribution of hsp proteins, the immune system is informed about the presence of internal pathologic processes. The high evolutionary conservancy of the bacterial hsp's and their overexpression during inflammation make them important antigens for host immune system[4,11,12]. Bacterial hsp-specific immune reaction (based mainly on T cell recognition) is rapid and highly effective and is permanently modulated by the presence of bacteria (especially normal gut flora)(ref.[12-14]). Thus any deviation in bacterial feedback signaling can be one of the factors involved in the etiology of autoimmune diseases. This article summarizes data from animal models of autoimmune diseases where hsp's play an important role in the induction of autoimmunity and/or setting of immune tolerance.

The host immune response to microorganisms such as *Borrelia burgdorferi, Chlamidia trachomatis, Helicobacter pylori, Mycobacterium leprae, M. tuberculosis, Yersinia enterocolitica, Leishmania donovani, Toxoplasma gondii, P. falciparum, Candida albicans, Trichophyton mentagrophytes* and others) is associated with recognition of pathogen's hsp's[15-26]. During the inflammative immune response stressed host cell expressing hsp on their surface can be crossrecognized by the immune system. This association is described retrospectively in human medicine and thus it is not clear whether cross reactivity takes place in induction of autoimmune reaction[12, 27].

HSP AND AUTOIMMUNITY

In some autoimmune models immunization of experimental animals with killed bacteria or hsp peptides broke immune tolerance and indeed induced autoaggression. Immunization of mice with mycobacterial hsp60 induced crossreactive CD8 α/β T cells. These T cells recognized hsp60 peptides on MHC molecules *in vitro* and after transfer to α/β T cell-deficient mice these cells induced autoimmune disease with severe damage of the gut epithelium[28]. Experimental infection of mice with bacterium *M. bovis* BCG induced production of hsp60 reactive CD8 α/β T cells[27]. After stimulation with mycobacterium hsp60 peptides *in vitro* these hsp60-specific CD8 T cell recognized and lysed host cell exposed to thermal stress. Recognition and lysis was prevented when hsp60 expression was blocked in target cells with antisense nucleotides[29]. This experiment confirmed that the host immune system is able to induce autoreactive CD8 α/β T cell recognizing self hsp60 peptides[27, 29].

Involvement of hsp's in the pathogenesis of autoimmune diseases has been studied using animal models such as: artificially induced arthritis (pristane, adjuvant arthritis, avridine induced arthritis, arthritis induced by streptococci cell wall and collagen type II induced arthritis)(ref.[12, 30-32]) and insulin-dependent diabetes mellitus model (IDDM) in non-obese diabetic mice (NOD)(ref.[33, 34]). The adjuvant arthritis (AA) model showed, for first time, that hsp60-specific T cells are not only proarthritogenic because the same epitope-recognizing T cells are also important for the setting of immune tolerance.

Animal models studying role of hsp's in autoimmune arthritis. AA is used as a model for rheumatoid arthritis or juvenile idiopathic arthritis since Pearson's first AA experiments[35]. Similarities between AA and rheumatoid arthritis comprise: 1. Synovial histopathological abnormalities, 2. Erosive polyarthritis. Similarities between AA and oligoarticular juvenile idiopathic arthritis comprise: 1. Synovial histopathological abnormalities, 2. Induction by a microbial agent, 3. Selfremitting course of disease, 4. Presence of uveitis, 5. Protective role of T cell reactivity to self hsp[36-38].

The polyarthritis can be induced following a single intracutaneous injection with heat-killed *Mycobacterium tuberculosis* in incomplete Freund's adjuvant[35] to susceptible rodents, such as Lewis, Buffalo, Sprague-Dawly and Wistar rats[40]. Interestingly, very young and old animals seem to be less susceptible to AA[41]. AA can be transferred from diseased animals to healthy naive animals with a single T cell clone specific for a particular sequence of *M. tuberculosis* hsp65. This finding supports the 'hit and run' hypothesis: "a microbial agent may pull the trigger and initiate a selfperpetuating autoimmune process in a susceptible individual"[42]. Detailed study of the T cell response has revealed two hsp-specific clones designated A2b and A2c. T cell clone A2b was strongly arthritogenic, while A2c was protective in AA. A2b and A2c recognize the same epitope: a sequence derived from a *M. bovis* BCG hsp60, namely amino acid 180–188 (TFGLQLELT)(ref.[36, 37]). The mechanism of the protective T cell response is probably associated with crossrecognition of autologous hsp peptides. Hsp is readily available for synovial antigen-presenting cells (APC) which present hsp fragments on MHC to the specific T cells population. At present it seems that the self crossreactive antigen of the arthrogenic A2b T cell is rather than self hsp60 some still unknown epitope of the cartilage proteoglycan[36, 43]. Exploring the whole human nonredundant protein databases (GenBank CDS translations, PDB, SwissProt, PIR, PRF) using BLAST-p software (http://www.ncbi.nlm.nih.gov) fail as well.

Experimental results indicate that the immune response against xenogenous hsp's is associated with induction of the protective as well as autoaggressive clones of specific T cell and the severity of disease is determined by interaction of both clones and many other factors. Concerning potential therapeutic benefits, the majority of experiments have been focused on induction of tolerance after immunization with hsp.

Protective effects of immunization with mycobacterium hsp65 was evaluated on CBA/Ig[h] mouse model of pristane induced arthritis[44]. Pristane arthritis is autoimmune arthritis induced by i.p. application of pristane oil (2,6,10,14-tetramethyl-penta-decan). Pristane-induced arthritis is characterized by dominancy of Th1 response (elevated level of IFN-γ and IL-2) after *in vitro* restimulation of arthritic mice splenocytes with mycobacterial hsp60. Mycobacterial hsp60 preimmunized mice were protected against Pristane arthritis. Isolated splenocytes response toward mycobacterial hsp65 stimulation *in vitro* by production of Th1 (IFN-γ and IL-2) as well as Th2 (IL-4, IL-5 and IL-10) cytokines. Furthermore, in the same experiment the authors used IL-12 in a group of mice as an inductor of Th1 response. Application of the IL-12 abrogates protective effect of mycobacterial hsp60 immunization in Pristane arthritis. Furthermore, mycobacterial hsp60 immunization of mice induced preferentially IgG1 isotype of hsp60-specific antibody (Th2), in contrast to nonimmunized arthritogenic mice group with dominancy of IgG2a isolype (Th1)(ref.[44]).

In another experiment immunization with entire mycobacterial hsp65 protects against collagen type II-induced arthritis, aviridine induced arthritis, and streptococcal cell wall-induced arthritis[32, 44, 45].

Apart from the above mentioned mycobacterial hsp60 peptide 180 – 188 another protective peptide of mycobacterial hsp60 has been identified namely amino acid 256 – 270 (ALSTLVVNKIRGTFK)(ref.[46]). Immunization of Lewis rats with mycobacterial hsp60 peptide M256 – 270 induced strongly autoproliferative but not cytotoxic T cells. This T cell clone after restimulation with peptide M256 – 270 responded to naive syngeneic APC *in vitro* by increased proliferation and production of IL-4, IL-10 and IFN-γ. The reaction was MHC II restricted. Exaggerated proliferation was observed when APC were heat-shocked before testing. Furthermore T cell expressed the cell-surface molecule B7.2. Contrary to high autoreactivity, these T cell clone induced reduction of AA elicited by i.d. application of heat killed mycobacteria (H37Ra) in incomplete Freund's adjuvant after transfer to mice. Increased expression of B7.2 molecule is associated with down regulation of specific T cell response due to interaction with another T cell-surface molecule CTLA-4. The suppressive interaction B7.2 – CTLA-4 is believed to be one of the most important protective mechanisms of autoreactive T cell clones in autoimmune diseases[12, 41, 47, 48]. Along with the peptide 256 – 270 a highly evolutionary conserved core hsp60 peptide has been identified: mycobacerial 256 – 265 (ALSTLVVNKI), compare to rat hsp60 peptide 256 – 265 (ALSTLVLNRL)(ref.[46]). Concerning the protective effect of T cell clones crossrecognizing autologous hsp60 the question arises as to whether other highly conserved proteins, especially other hsp proteins, may act as protective antigens.

The mycobacterial hsp70 and hsp10 have been most studied[49-52]. Immunizations of experimental rodents with highly conserved peptides of the mycobacterial hsp70 in the DDA adjuvant (dimethyl dioctadecyl ammonium bromide) did not confer assumed protective effects in the AA model[53]. However isolated T cells responding *in vitro* to the conserved *M. tuberculosis* hsp70 peptide namely amino acid 111 – 125 (ITDAVITTPAYFNDA) produced elevated levels of IL-10[53]. The same T cell clone responded to rat hsp70 peptide 111 – 125 (VTNAVITVPAYFNDS). Recently DNA vaccination expressing human hsp70 or hsp90 has been shown to inhibit AA in Lewis rats [51-52].

Mucosal intranasal low dose administration of protein antigen is generally associated with induction of T cells producing IL-10. Thus, the authors preimmunized the rats four times i.n. with the mycobacterial hsp70 peptide 111 – 125 and indeed achieved protection against subsequent induction of AA(ref.[53]).

IDDM and hsp's. Insulin-dependent diabetes mellitus is another example of hsp60 involvement in autoimmune diseases. IDDM is the clinical consequence of pancreas β-cell destruction by T cells. Infection of non-obese diabetic mice with *M. avium* prevents the development of diabetes[54]. Similarly immunization of NOD mice with mycobacterial hsp60 significantly reduces the incidence of IDDM[33]. Furthermore adoptive transfer of a CD4 T cell clone specific for the mycobacterial hsp60 accelerated the onset of IDDM[55]. Although controversial, taken together with the fact that the β-cells of NOD mice showed elevated expression of hsp60 protein, hsp60 becomes a candidate autoantigen[56, 57]. An epitope of the human hsp60 namely amino acid 277 – 300 (VLGGGCALL RCIPALDSLTPANED) was recognized by CD4 T cell clones isolated from NOD mice. After adoptive transfer, T cell clones produced profound insulitis in mice, but when gamma irradiated, the same T cells protected NOD mice against IDDM(ref.[55]). In another experiment human hsp60 peptide 277 – 300 treatment of NOD mice induces a Th2 cytokine burst[58]. Peptide 277 – 300 binds only to the TLR-2 on the T cells. Thus administering the peptide 277 – 300 causes a specific anti-inflammatory T cell response without the induction of a pro-inflammatory macrophage response mediated through binding of hsp60 to TLR-4(ref.[38, 39]). Recently, several studies have reported that the host immune response against *M. tuberculosis* hsp60 take place in chronic phase of infection and is described to be Th2 associated, contrary to early immune Th1 response specific to secreted bacilli proteins[59]. The above experiments suggest that hsp60 plays a principal role in the etiology of IDDM, but continuous research favors a 65 kDa protein, glutamic acid decarboxylase (GAD) as a self antigen in both NOD mice and humans IDDM[60, 61]. NOD mice after immunization with GAD were protected against IDDM development[61]. GAD epitopes are recognized by T cell of NOD mice[62]. Three immunodominant GAD epitopes have been found namely amino acid 78 – 97 KPCSCSKVDVNYAFLHATDL, 202 – 221 TNMFTYEIAPVFVLLEYVTL, and 217 – 236 EYVTLKKMREIIGWPGGSGD. After administration to NOD mice the epitopes 78 – 97 and 202 – 221 induced Th1 response whereas epitope 217 – 236 induced Th2 response. Amino acid analysis of *M. tuberculosis* hsp60 and insulin motives presented on MHC II molecule, I-Ag7 (NOD homologue of HLA-DQB1) confirmed that GAD peptide 202 – 221 contains the same anchor consensus V/YXVXXE[62]. Although undoubtedly involved in IDDM etiology, the above-mentioned antigens such as GAD, hsp60 and insulin cannot cause IDDM sensitive or resistant phenotype. Recently Tian et al demonstrate on NOD mice that the IDDM sensitive phenotype is determined by the amino acid composition of MHC II molecule that may affect the ability of this molecule to mediate the negative selection of autoreactive T cells during ontogenesis[63].

NON α/β T CELLS MECHANISMS OF HSP'S INVOLVEMENT IN AUTOIMMUNE DISEASES

Not only the α/β T cells are involved in target tissue destruction. Other mechanisms such as γ/δ T cells, Natural killer cells, APC, and specific antibodies have been confirmed to participate in autoimmune diseases[27, 46, 64-67]. γ/δ T cells were found in autoimmune plaques in multiple sclerosis and they have been colocalized with oligodendroglial cells expressing hsp. These γ/δ T cells act as cytotoxic[68].

Crossreacting hsp-specific antibodies were confirmed in the sera of patients with chronic granulomatous bowel disease (m. Crohn). Human hsp60-recognizing antibodies crossreact with mycobacterial hsp60 analog. Mycobacteria, especially low pathogenic species, are considered as an inductive factor in Crohn's disease[69]. Species-specific hsp60-recognizing antibodies have been confirmed during infection with *B. burgdorferi, C. trachomatis*. Antibodies crossrecognized human hsp60[28, 65]. Anti-hsp70 antibodies have been found in the sera of patients with malaria[70]. In patients with lupus erythematodus increased expression of the hsp90 on the surface of peripheral lymphocytes and monocytes as well as increased level of serum anti-hsp90 antibodies were detected[11, 71, 72].

The positive role of the hsp-specific immune crossreactivity, which is probably evoked by low pathogenic or gut commensal bacteria, may enable the already prepared immune system to react quickly before the immune response to more pathogen-specific antigens develops[28, 73].

THREE MECHANISMS HOW HSP-SPECIFIC T CELLS CONTRIBUTE TO IMMUNE TOLERANCE

Hsp-specific T cells contribute to long-lasting autotolerance and prevent autoimmune diseases by several mechanisms:

1) **Bystander antiinflammatory effects of lymphocytes educated in GALT mainly through IL-10.** Gut-associated lymphoid tissues (GALT) are continuously confronted with components of food and gut microflora to which long-term immune tolerance establishment is essential[74]. Antiinflammatory cytokines, such as IL-10 and TGF-β are produced not only by cells of the adaptive immune system but also, as in the case of IL-10, by intestinal epithelial cells[41]. Subsequently, whenever confronted with the homologues present in mammalian hsp overexpressed in the inflamed joint (or other organ) immigrating GALT cells control inflammation through bystander suppressive effects[12, 41].

Another mechanism – oral tolerance – is effective in inhibiting adjuvant arthritis induction in rats. Low doses of antigen administration favor the induction of active cellular regulation, e.g. by release of the antiinflammatory cytokines TGF-β, IL-4, and IL-10, whereas higher doses favor clonal anergy or deletion[75]. In animal models, feeding Lewis rats with mycobacterial hsp60 in the presence of soybean trypsine inhibitor reduced significantly the severity of ongoing AA. Moreover, isolated splenocytes respond *in vitro* to hsp60 stimulation by IL-10 production[76]. More recently, atherosclerosis and inflammation have been shown to be reduced by mucosal administration of mycobacterial hsp60 in mice[77, 78]. Experimental allergic encephalomyelitis can be prevented in the mouse by prior administration of myelin basic protein or copolymer 1, which simulates myelin basic protein immunologically[75, 79]. In human trials, the administration of autoantigens has not yet yielded successful therapy[79]. The concomitant

generation of antigen-specific cytotoxic T cells possibly accounts for this failure. Choosing the adequate antigen, the adequate dose, and manipulation of the antigen-presenting dendritic cells are strategies that can be used to overcome the remaining obstacles[79].

2) **The low affinity TcR recognition of self hsp peptide leads to suppression of inflammatory T cell response through B7.2 – CTLA-4 interaction.**
The cells can present peptides derived from hsp60 through MHC I or MHC II. Such cells may be target for cytotoxic α/β T cells[28, 80]. Cytotoxic T cells are considered main ethio-pathologic factor, although, many experiments confirmed that hsp-specific α/β T cells are involved only in advanced disease[28]. Crossreacting hsp70-specific T cells were found in humans with tuberculosis and leprosis[81]. In IDDM patients, the hsp60 reactivity of the α/β T cells is considered as the secondary effect of the autoreactivity against the dominant autoantigen, the GAD(ref.[62]). In *Y. enterocolitica*-triggered reactive arthritis, hsp60-specific synovial CD4 α/β T cells crossreact with human hsp60 protein, heat stressed APC, and synovial mononuclear cells *in vitro*[82]. Moreover autologous hsp60-specific T cells were identified in Bechcet disease[83, 84], rheumatoid arthritis[85], juvenile rheumatoid arthritis, and juvenile chronic arthritis[82, 86].

Only a partial homology is required for the α/β T cell TcR crossrecognition of peptides presented on the MHC I molecule. In contrast, the amino acid motifs involved in anchoring the peptides in particular MHC I groove have to be identical in the full spectrum of corresponding MHC I presented peptides[29]. The amino acid differences between microbial and self hsp may play a crucial role. In thymic areas involved in positive selection hsp's are expressed among others. Positively selected T cells show a low affinity for self, including self-hsp60. Those low affinity T cells that recognize conserved epitopes, could expand after contact with microbial homologues and T cells with a relatively high affinity for the microbial homologues prevail. Arising T cells pool can still recognize the self hsp epitopes with relatively low affinity. T cells from Lewis rats (immunized with mycobacterial hsp60 peptide M256 – 270) upregulate B7.1 and B7.2 after *in vitro* stimulation with mycobacterial hsp60 peptide (256 – 265) in dose dependent manner. In case of low peptide concentration only B7.2 expression was observed. Contrary, the same T cells after stimulation with rat 256 – 265 peptide (as an altered peptide ligand) responded by selective upregulation of only B7.2 molecule independently on peptide concentration used[46, 47]. Thus high affinity peptide recognition (in high dose conditions) is coupled with proinflammatory (Th1) T cell response (situation during infection) whereas lowered affinity recognition (independently on dose), promote a response in the direction of a Th2 or regulatory response[41, 47]. The described antiinflammatory phenotype is probably linked with the selective B7.2 – CTLA-4 interaction of T cells-expressed B7.2 molecule which interacts only with CTLA-4 but not with CD28, contrary to B7.2 on APC(ref.[46,47]). Observed selectivity is probably linked with the different glycosyla-

tion of B7.2. The antiinflammatory feedback associated with the action of altered peptide ligands (such as hsp) is permanently induced by the presence of low virulent bacteria probably by mechanism of low dose stimulation observed in the mycobacterial hsp60 256 – 265 peptide[37, 47]. Thus paradoxically, long-lasting germ free conditions can contribute to development of the autoimmune process[87].

Conserved regions of the hsp's comprise the target antigens also for γ/δ T cells whose participation in early phase of infections caused by *Listeria monocytogenes, Plasmodium yoelii* and *Leishmania major*[27]. In newborn mice were confirmed thymic γ/δ T cells recognizing mycobacterial hsp60 peptides as well as homologous mammalian hsp60 peptides[13, 14]. Those γ/δ T cells may contribute to autoimmunity[28]. Experiments with minimized mycobacterial hsp60 peptides recognized by γ/δ TcR did not confirm cross reactivity with mammalian analogous peptides[88]. Furthermore, *in vitro* experiments confirmed, that all γ/δ T cells expressing Vγ1 chain recognizes the same minimal peptide. The hsp peptides are supposed to bind conserved regions of Vγ1 chain and not hypervariable Vγ1 domains. The meaning of these *in vitro* observations remains to be elucidated. Otherwise hsp70-specific γ/δ T cells elicited during protozoan and/or intracellular bacterial infections showed weak interspecies hsp70 crossreactivity and occasionally hsp70-specific γ/δ T cells recognized unique species-specific hsp70 epitope. Beside the γ/δ T cells, another cell type – dendritic cells – use pathogen hsp to fight against infection. Dendritic cells recognize the pathogen hsp through surface receptors (TLR-4)(ref.[8, 89]).

3) **Nonprofessional APC presentation of hsp epitopes without costimulatory molecules drives T cell to anergy.**

The ubiquitous low level of constitutive hsp expression in every cell of the body will guarantee that T cells will notice the presence of self hsp epitopes also on nonprofessional APC that lack the costimulatory molecules needed to induce the T cell response. Recognition in the absence of proper costimulation is known to drive T cells into a state of anergy[12, 41].

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