Heat shock protein 70 associations with myelin basic protein and proteolipid protein in multiple sclerosis brains

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Abstract

Heat shock proteins (hsp) are known to facilitate the generation of specific immune responses by chaperoning proteins and peptides involved in T cell activation. Hsp have been shown to be strikingly elevated in multiple sclerosis (MS) lesions. The unique chaperonin properties of hsp70 have allowed identification of immunogenic proteins bound to it by the ex vivo demonstration of hsp associations with proteins implicated in the immune response. We have investigated the association of hsp70 with myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin oligodendrocyte protein (MOG) in MS and control brain tissue. In co-immunoprecipitation experiments, in all samples of MS brains examined (n = 3), but not control brain tissue (n = 3), direct association of MBP with hsp70, but not with hsp90, was found. In some MS brain samples, association between PLP and hsp70 was also seen. In similar co-immunoprecipitation experiments on brain tissue obtained from mice with experimental autoimmune encephalomyelitis (n = 5) induced by immunization with PLP peptide, specific association of hsp70 with PLP and MBP was found. Using surface plasmon resonance we demonstrated specific binding of hsp70 with MBP in vitro. Analysis of the amounts of MBP bound to hsp70 yielded a molecular ratio of MBP binding to hsp70 at 6.5:1. MBP complexed with hsp70 was taken up at significantly higher rates by antigen-presenting cells than MBP alone and enhanced MBP-specific immune responses. These results indicate that hsp70 specifically associates with MBP in MS brain tissue. This association might be relevant to the enhanced immune recognition of MBP in MS.

Introduction

Heat shock (stress) proteins (hsp) form a highly conserved group of proteins expressed by both prokaryotic and eukaryotic cells. Their expression is strongly induced by many different stress conditions (1,2). Recent data indicate that the unique chaperonin properties of hsp might contribute to the generation of specific immune responses against bound proteins and peptides (3,4). For example, studies have shown that hsp prepared from various tumors elicit protective tumor immunity in the host, whereas hsp derived from normal tissues did not (5). The immunogenic activity of hsp chaperones has also been shown in other experimental models. Hsp isolated from viral infected cells elicited a cytotoxic T lymphocyte response against viral epitopes, whereas neither hsp nor viral peptides were immunogenic by themselves (6). In a direct demonstration of hsp chaperone immunogenic activity, it was shown that hsp96 from cells transfected with the gene encoding ß-galactosidase induced a cytotoxic T lymphocyte reaction against an epitope of ß-galactosidase (7). In addition, in vitro experiments on the reconstitution of gp96 and hsp70 complexes with peptides demonstrated their ability to induce cytotoxic responses (8). Other examples include work on constructs of hsp70–ovalbumin (OVA) fusion protein (9) which confirmed that covalent binding of hsp–peptide elicited T cell cytotoxic and humoral responses.

The mechanism of enhanced immunogenicity of hsp–peptide complexes is believed to be associated with the process of antigen presentation. That antigen processing pathways are involved in hsp–peptide complex immunogeni-
city has been suggested by experiments in animals depleted of macrophages (10). Although the majority of the data on the molecular chaperone function of hsp was generated using MHC class I-restricted antigen presentation systems, an hsp-enhancing effect on MHC class II presentation was also observed. It has been shown that over-expression of hsp73, a cognate form of hsp70, in a macrophage line of the H-2k haplotype, markedly enhanced the potency of these cells to present egg lysozyme in an MHC class II-restricted manner (11). These results might implicate hsp immune chaperone function in mechanisms of autoimmune.

Over the past two decades it has been firmly established that autoimmune demyelinating diseases can be induced in many species by immunization with most myelin proteins or their peptides derivates (12,13). The analysis of expression and distribution of hsp in multiple sclerosis (MS) lesions indicated significant up-regulation of most classes of hsp both within the lesion and at the lesion edge (14,15). Previous studies on the potential role of hsp in MS focused on the possibility that hsp and myelin epitopes could cross-react, leading to the phenomenon termed 'molecular mimicry'. The present study addresses a new concept of hsp in the pathogenesis of MS—that their over-expression in the MS lesion might serve to chaperone myelin proteins, thereby contributing to enhancement or induction of the immune response to myelin antigens. We have demonstrated that hsp70 specifically associate with myelin basic protein (MBP) and proteolipid protein (PLP) in MS brains, that hsp70 and MBP complexes can be reconstituted in vitro, and that MBP complexed with hsp70 is taken up at a higher rate by antigen-presenting cells (APC).

Methods

Central nervous system (CNS) tissue

Brain tissues were obtained at early autopsy (6 h post-mortem) from four MS patients. All cases had a similar time course of the disease and represented the relapsing–remitting type of MS. None of the patients had suffered a relapse within 12 months of death. Tissue samples used for co-immunoprecipitation experiments contained MS lesions of the chronic active type. Samples were deep frozen at −70°C before use. Control CNS tissue was obtained from three stroke patients. The control CNS samples were taken from non-ischemic areas of the brain.

Immunoprecipitation and immunoblot analysis

Fragments of white matter were homogenized in hypotonic buffer (10 mM NaHCO₃, 0.2 U/ml aprotinin, 0.5 mM PMSF, pH 7.1 and BSA 1 mg/ml) by Dounce homogenization. The excess of BSA in the hypotonic buffer was added to saturate any unspecific binding properties of hsp70 in samples. After centrifugation at 14,000 r.p.m., 30 min at 4°C, the aqueous phase was collected, and 5 µl of primary antibodies against MBP (mouse monoclonal anti-MBP; Chemicon, Temecula, CA), PLP (mouse monoclonal anti-PLP; Chemicon) or myelin oligodendrocyte protein (MOG) (mouse monoclonal anti-MOG; a gift of Dr Raine) was added from a stock at a concentration of 1 mg/ml and incubated by rocking on ice for 60 min. Secondary antibodies directed against mouse IgG conjugated to agarose (50 µl anti-mouse IgG; Sigma, St Louis, MO) were added for an additional 60 min incubation and rocking on ice. The homogenates were washed by centrifugation at 14,000 r.p.m., 4 × 15 s at 4°C. The supernatants were discarded and the immunoprecipitates dissolved in SDS sample buffer (0.1 Tris–HCl, 4% SDS, 20% glycerol, 0.05% bromophenol blue and 5% 2-mercaptoethanol), and resolved on 12% PAGE–SDS and subjected to electrophoresis (25 mA, 60 min at room temperature). The sample was then transferred to a PVDF membrane (Millipore Corp., Bedford, MA) and the proteins were probed with anti-MBP, anti-PLP or anti-MOG mAb. After antibody stripping with stripping buffer (2% SDS, 0.1 M 2-mercaptoethanol and 62.5 mM Tris–Cl, pH 6.8), 30 min at 60°C, proteins were probed with anti-hsp antibodies, anti-hsp70 (recognizing both cognate and inducible forms, clone N27F3-4, IgG1) and anti-hsp90 (human, clone 16F1, IgG2a), both from StressGen (Victoria, BC, Canada). For control purposes, blots were immunoblotted with antibodies against other CNS and immune system-related proteins, anti-CNPase (Sigma-Aldrich, St Louis, MO); and the common δ chain of TCR γδ (BD Pharmingen, San Diego, CA). As a standard, 20 ng of human recombinant hsp70 protein (StressGen) was run in a separate lane (at a concentration of 200 ng/100 µl).

The same experiments were performed also in the reverse order. The homogenates were immunoprecipitated with antibodies to hsp and the immunoprecipitates subjected to SDS–PAGE, transferred to PVDF membranes and blotted for myelin proteins.

In vitro complexing

To investigate the putative binding of hsp with myelin proteins, we attempted to reconstitute complexes in vitro. Two strategies were applied for this purpose. The first assessed the binding of bovine MBP protein (Sigma) to immobilized hsp70. The hsp70 (kindly obtained from Dr M. Zylicz, UNESCO Institute, Warsaw, Poland), at a concentration of 150 ng/well in 100 µl of PBS, was coated by incubation at room temperature for 1 h onto 96-well flat-bottomed plates (ImmuNoPlate Maxisorp Surface; Nunc, Roskilde, Denmark). In control experiments we have shown that 150 ng/well allowed the amount of protein needed to bind to the well. Subsequently, the immobilized hsp70 was exposed to MBP. MBP was added in increasing amounts up to 800 ng/well in 100 µl of binding buffer [25 mM HEPES, 150 mM KCl, 25 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM dithiothreitol, 5% (v/v) glycerol and 0.05% Triton X-100] supplemented with 0.2% BSA. After washing the unbound MBP, the amount of MBP bound to hsp70 was determined by ELISA. The primary antibody against MBP (MAB 388; Chemicon) was added at dilution of 1:1500 for 60 min followed by secondary antibody (goat anti-mouse IgG) conjugated with peroxidase (Sigma), at a dilution of 1:20,000. The color reaction was carried out with OPD peroxidase substrate (Sigma-Aldrich) and the amount of bound MBP was detected by measuring at 492 nm.

The second strategy involved hsp–protein binding in solution. The hsp70 was mixed with MBP in molecular ratios of 1:0.1, 1:1 and 1:10 in 200 µl of binding buffer and incubated for 10 min at 37°C followed by 30 min at room temperature. The
unbound protein was removed by cut-off centrifugation on a microfilter 50,000 (Amicon, Beverly, MA). The binding was confirmed by co-immunoprecipitation assay. MBP was immunoprecipitated in a sample volume of 200 µl with 5 µl of anti-MBP mAb (MAB382; Chemicon), on ice for 1 h, followed by incubation with 50 µl of goat anti-mouse IgG–Agarose (Sigma-Aldrich) for another 1 h. After washing 3 times in TBS and once with 0.05 M Tris–HCl (pH 6.8) the immunoprecipitates were subjected to SDS–PAGE electrophoresis. The presence of MBP and hsp70 was confirmed by Western blot, as above.

Surface plasmon resonance (SPR)

Protein binding was examined with a Biacore X system (Biacore, Uppsala, Sweden) with the use of a CMS sensor chip (research grade). This system utilizes SPR technology. MBP protein, at a concentration of 40 µg/ml in HBS buffer (Amersham Pharmacia Biotech, Uppsala, Sweden), was immobilized on the Fc2 channel by standard amine coupling chemistry, according to the manufacturer’s instructions. About 3000 resonance units were coupled in this channel. Channel Fc1 was predicted for observation of non-specific protein–sensor surface interaction. Hsp70 or BSA proteins, at a concentration of 10 µg/ml, were injected onto the sensor in 20 µl volumes with the flow rate of 2 µl/min. The source curves obtained were aligned to the initial values using injection time and the initial resonance value as normalizing points. For inhibition analysis, anti-MBP or irrelevant antibody was injected on the sensor chip with immobilized MBP prior to injection of hsp70. The same experiments were also performed in reverse order, when hsp70 was immobilized on the sensor surface, and MBP protein and BSA were injected onto the sensor in 20 µl volumes at a flow rate of 2 µl/min.

MBP internalization assay

MBP uptake by APC was measured by flow cytometry of MBP–FITC-labeled murine fibroblast cell line L cells (APC line) as well as freshly isolated, unprimed SJL/J splenocytes and freshly isolated human peripheral blood mononuclear cells (PBMC) from a healthy individual. MBP–FITC was prepared by labeling bovine MBP protein with FITC (Sigma), according to the manufacturer’s protocol. MBP–FITC was separated from unbound FITC by column chromatography. Freshly prepared MBP–FITC was used for the flow cytometry. MBP complexes with either human recombinant hsp70 (StressGen) or OVA (Sigma) were prepared by co-incubation of the MBP–FITC with hsp70 or OVA in the mass ratio 2:1 for 10 min at 37 °C in the dark followed by 30 min incubation at room temperature in the dark. APC (L cells, murine splenocytes and human PBMC) were co-incubated with MBP–FITC complexed with either hsp70 or OVA (10 µg of MBP–FITC plus 5 µg of hsp70 or OVA per 10^6 cells) for 10 min at 4 °C in the dark followed by extensive cell washing, further incubation at 37 °C in the dark and serial flow cytometry analysis of cells. MBP uptake was expressed as the mean fluorescence intensity of MBP–FITC-labeled cells as assayed using FACSCalibur flow cytometry (Becton Dickinson, San Diego, CA); data were analyzed using CellQuest software (Becton Dickinson). Human PBMC were co-labeled with phycoerythrin-conjugated anti-human HLA-DR antibody (clone 555561; BD PharMingen).

Fig. 1. Co-immunoprecipitation of hsp70 with MBP and immunoblotting of hsp70 in MS and control brain tissue. MS (A) and control CNS (B) tissue homogenates were immunoprecipitated with non-immune mouse IgG (lane 1) or with anti-MBP mouse mAb (lanes 2–5). All samples were probed with anti-MBP mAb (lanes 2–5), and, after antibody stripping, with anti-hsp70 (lane 2), anti-hsp90 (lane 3), anti-CNPase (lane 4) and anti-TCRδ (lanes 5) mAb. The other bands at ~25 and 50 kDa represent subunits of IgG of the primary antibody used for immunoprecipitation. (C) Quantification of hsp70 by immunoblotting analysis with anti-hsp70 mAb of MS (lane 2) and control (lane 3) brain tissue samples used for a co-immunoprecipitation of hsp70. Equal volumes of MS and control brain samples (3 µl) were run on the gel. The hsp70 standard (lane 1) was loaded at 20 ng in 10 µl (concentration 200 ng/100 µl).
**Experimental autoimmune encephalomyelitis (EAE)**

Five female SJL/J mice, 6–8 weeks of age, were immunized with PLP peptide 139–151 (HSLGKWLGHPDKF) in complete Freund’s adjuvant (CFA). On day 0, each mouse received 0.25 ml of a mixture of 0.15 mg PLP peptide dissolved in 0.1 ml of double-distilled H2O and 0.75 mg of Mycobacterium tuberculosis, in 0.15 ml CFA, injected s.c. in two abdominal sites. On the day of immunization and on day 3 post-immunization, 0.6 $\times$ 10^10 heat-inactivated Bordetella pertussis organisms were injected in 0.4 ml of diluent into a tail vein. Onset of clinical signs occurred 8–12 days post-immunization. Animals were scored clinically in a blinded fashion daily on a scale of 0–5 in a blinded fashion, according to published criteria (16).

**MBP immunization and proliferation assay**

Five female SJL/J mice, 6–8 weeks of age, were immunized with MBP protein in CFA. Each mouse received 0.25 ml of a mixture of 0.8 mg of MBP protein dissolved in 0.1 ml of double-distilled H2O and 0.75 mg of M. tuberculosis, in 0.15 ml CFA, injected s.c. in two abdominal sites. On the day 12 the splenocytes were isolated from immunized mice and plated on 96-well round-bottomed plates with either MBP protein or MBP protein complexed with hsp70 in the mass ratio 2:1. After 48 h of incubation cultures were pulsed with 1 mCi/well [3H]thymidine and harvested 16 h later. [3H]Thymidine incorporation was determined in a Wallac Betaplate liquid scintillation counter (Perkin Elmer Life Sciences, Wellesley, MA). Results are expressed as a proliferation indices calculated by dividing the sample c.p.m. by the c.p.m. of the unstimulated culture.

**Statistical analysis**

Multivariate ANOVA test was applied to test the significance of the data. P values <0.05 were considered as a significant.

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**Results**

**Co-immunoprecipitation of hsp and myelin proteins in MS brain tissue**

To determine whether complexes of hsp with myelin proteins were present in MS brain tissue, we performed co-immunoprecipitation analysis. In these experiments, equal quantities of CNS tissue samples were sequentially immunoprecipitated with primary mAb anti-MBP, anti-PLP and anti-MOG, followed by incubation with secondary antibodies directed against mouse IgG conjugated to agarose. The results showed that a protein of 70 kDa was retained by anti-MBP and anti-PLP. After SDS–PAGE and sequential immunoblotting with anti-hsp70, anti-hsp90, anti-MBP and anti-PLP, the presence of hsp70, MBP and PLP was detected (Figs 1 and 2). We have found that during immunoprecipitation with anti-MBP antibody, co-immunoprecipitation with hsp70 was obtained in samples from all three MS brains. Similar results were obtained with co-immunoprecipitation of PLP with hsp70, in two out of three MS brain tissue samples. In contrast, none of the MS samples immunoprecipitated with anti-MBP or anti-PLP showed evidence of interaction with hsp90 (Figs 1 and 2). In three control brain samples immunoprecipitated with anti-MBP and anti-PLP, co-immunoprecipitation of hsp70 was not detected. We were also unable to demonstrate co-immunoprecipitation of any hsp with anti-MOG mAb in MS and control samples (data not shown). The specific nature of the hsp70 association with MBP and PLP was confirmed by control experiments in which MBP brain tissue was immunoprecipitated with antibodies to MBP, and the presence of other CNS or immune system-related proteins in the immunoprecipitates was assessed. The immunoprecipitation of MS brain samples with anti-MBP or anti-PLP mAb did not retain CNPase and TCR8 (Figs 1 and 2). Additionally, we performed co-immunoprecipitation experiments in reverse order. MS brain tissue samples were immunoprecipitated with primary antibodies to hsp70 followed by secondary antibodies directed against mouse IgG conjugated to agarose. In agreement with the immunoprecipitation of MS tissue samples with anti-MBP and anti-PLP mAb, SDS–PAGE and immunoblotting analysis revealed that MBP and PLP were retained with antibodies to hsp70 (Fig. 3). To quantify the amounts of the hsp70 in the samples used for the hsp70 immunoprecipitation, 3 ml of both MS and control brain lysates was run on the gel together with hsp70 standard and immunoblotted with anti-hsp70 mAb (Fig. 1C). Hsp70 content in both MS and control samples was much higher than 200 ng/100 ml in the reference lane. Therefore immunoprecipitation experiments were all done in the plateau part of the hsp70–protein binding curve as found by the ELISA protein-binding assay (see below).

The data obtained from these experiments pointed to a close association between hsp70 with MBP and PLP in MS brain.

**Co-immunoprecipitation of hsp and myelin proteins in EAE CNS tissue**

To further determine the specificity of the association between hsp and myelin autoantigens in CNS tissue in an immune-mediated demyelinating disease, we performed co-immunoprecipitation experiments using CNS tissue.
obtained from animals with EAE induced by immunization with PLP peptide. At the height of clinical expression of disease (day 14), animals were perfused under anesthesia with ice-cold PBS. Brains and spinal cords were removed, sonicated and processed for co-immunoprecipitation similar to the MS brain tissue samples. We were able to demonstrate co-immunoprecipitation of hsp70 with PLP and with MBP in all samples from EAE brain tissue (Fig. 4). With brain tissue from normal mice similar to control human brain tissue, we were unable to document co-immunoprecipitation of hsp70 with MBP or PLP. These results suggest similarities in the ability of hsp70 to associate with myelin proteins in both MS and EAE which might correspond to the observed association of hsp70 with immunogenic proteins in these two diseases.

Complexing of hsp70 to MBP in vitro
To assess whether hsp70 formed complexes with MBP in vitro, we applied two techniques—the binding of MBP to immobilized hsp70 combined with ELISA and the co-immunoprecipitation of pre-incubated hsp70 with MBP in solution. As shown in Fig. 5(A), hsp70 exhibited high-affinity binding to MBP. The binding was not dependent on elevated temperature and required only a short incubation time. These results indicate that hsp70 exhibits inherent abilities to bind to MBP in native form. Since the ATP/ADP balance ratio was shown to control the release of hsp70 from protein substrates, we measured how ATP and ADP affected hsp70 binding to MBP. While ADP slightly increased the affinity of hsp70 binding to MBP, the addition of ATP induced dissociation of hsp70 from MBP. The stability of the hsp70–MBP complexes was examined by sequentially assessing the presence of the complex. The hsp70–MBP complexes were relatively stable for up to 12 h (data not shown).

Fig. 3. Co-Immunoprecipitation of MBP with hsp70. MS tissue homogenates were immunoprecipitated with non-immune mouse IgG (lane 1) or anti-hsp70 (lanes 2 and 3). The samples were probed with anti-hsp70 (lanes 2 and 3) antibodies. After antibody stripping from the membrane, it was re-probed with anti-MBP mAb (lane 2) and anti-PLP mAb (lane 3).

Fig. 4. Co-immunoprecipitation of hsp with PLP and MBP in EAE brain tissue. EAE (A and C) and normal (B) mouse brain tissue homogenates were immunoprecipitated with non-immune mouse IgG (lane 1) or with anti-MBP mAb (lanes 2–5) for (A) and anti-PLP (lanes 2–5) for (C). All samples were probed with anti-MBP mAb (lanes 2–5) for (A) or with anti-PLP (lanes 2–5) for (C), and, after antibody stripping, with anti-hsp70 (lane 2), anti-hsp90 (lane 3), anti-CNPase (lane 4) and anti-TCRβ (lane 5) mAb. The other bands at ~25 and 50 kDa represent subunits of IgG of the primary antibody used for immunoprecipitation.
To confirm the ability of hsp70 to form complexes with MBP in vitro, experiments were performed in which hsp70 and MBP were preincubated in solution, then assessed for the presence of complexes. After cut-off filtration to remove the unbound proteins, the presence of hsp70–MBP complexes was determined by co-immunoprecipitation. We have shown that after 10 min incubation at 37°C, anti-hsp70 antibody retained MBP (Fig. 5B). The hsp70 binding to MBP was enhanced by the presence of Ca\(^{2+}\) and Mg\(^{2+}\).

Fig. 5. Reconstitution of hsp70–MBP complexes in vitro. (A) The amount of MBP that bound to hsp70 was determined by ELISA as described in Methods. The upper curve represents MBP binding to hsp70 and demonstrates increased binding of MBP over BSA control binding shown by the lower curve. (B) Hsp70 was mixed with MBP in a molecular ratio of 1:1 in binding buffer and the protein association was assessed by a co-immunoprecipitation assay, as described in Methods. The sample of hsp70 mixed with MBP was immunoprecipitated with non-immune mouse IgG (lane 2) or with anti-MBP mAb (lane 3). Both samples were probed with anti-MBP mAb (lanes 2 and 3) and, after antibody stripping, with anti-hsp70 (lane 2 and 3). Lane 1 represents the same sample probed with anti-hsp70 without prior immunoprecipitation.

Binding of hsp70 to MBP

To determine whether hsp70 specifically binds to MBP, we used SPR. As shown in Fig. 6(A), specific binding of hsp70 flowing over immobilized MBP protein is depicted by the upper curve. Non-specific interaction of hsp70 with the sensor surface and BSA is represented by the lower curves on the graph. Sensograms were obtained with a Biacore X instrument equipped with a CM5 sensor chip (research grade). MBP protein was immobilized on the Fc2 channel, whereas the Fc1 channel was used for control of non-specific interactions. Flow rate: 2 μl/min; applied sample volume: 20 μl. (B) Inhibition of binding of hsp70 with MBP in the presence of anti-MBP mAb (MAB382). Hsp70 was injected on MBP immobilized on a sensor after pre-injection of anti-MBP mAb or without pre-injected antibody. Difference in resonance units (RU) represents inhibition of specific binding between MBP and hsp70.
To determine the effect of hsp70 on the uptake of MBP by APC, we incubated freshly isolated murine splenocytes, freshly isolated human PBMC or murine fibroblast APC line (L cells) with MBP–FITC in complex with hsp70 or OVA. To analyze the uptake of MBP by APC, we measured the mean fluorescence intensity (MFI) of APC by flow cytometry. We found that for all tested APC MFI values were significantly higher when MBP–FITC was complexed with hsp70 in comparison to MBP–FITC complexed with OVA (Fig. 7A for murine spleen cells, P < 0.01; B for APC line, P < 0.04; C for human HLA-DR+ PBMC, P < 0.01). This difference was sustained in the extended kinetics analysis and was observed as long as 40 min from the beginning of the incubation period. Therefore we concluded that complexing MBP with hsp70 increased the uptake of MBP into APC and possibly increased the availability of MBP antigen for presentation in the context of MHC class II. The functional significance of the MBP complexing with hsp70 was tested using a presentation assay with MBP splenocytes from mice immunized with MBP protein (Fig. 7D). We found that complexing in vitro MBP protein with hsp70 leads to increased proliferative responses of MBP-sensitive splenocytes. Preincubation of splenocytes with hsp70 and subsequent presentation of MBP protein did not lead to increased responses to MBP protein (data not shown). These suggested a direct role of hsp70–MBP complexes in augmenting the responses to MBP.

**Discussion**

The results of this study have shown that hsp–myelin protein complexes could be derived from CNS tissue with inflammatory/demyelinating pathology and that these complexes could also be generated in vitro. The specificity of hsp binding to myelin proteins was established by the demonstration of an association between hsp70 and MBP and PLP in CNS tissue from MS and EAE, but not in control CNS tissue, by the lack of association between other CNS and immune related proteins with MBP and PLP, and by the physical binding of hsp70 and MBP assayed by SPR, ELISA and co-immunoprecipitation.

Although the etiology of MS remains unknown, it is widely accepted that an immune response directed against components of the myelin sheath contributes to the demyelinating process (17). Studies in animals have shown that many different myelin proteins may be targets of the immune system and that in animals immunized with myelin antigens, responses to different myelin proteins develop over the course of the disease, a phenomenon known as epitope spreading (18). The cumulative data indicate that once damage to the CNS has occurred, sensitization to additional antigens can develop that may contribute to the chronic disease. Alternatively, or in parallel, responses to non-myelin antigens that are expressed in damaged areas of the CNS may also contribute by promoting a pro-inflammatory environment or by functioning as direct targets of the immune response (19).

A role for components of the heat shock, or stress response, has been strongly implicated in immune responses. Hsp have been found to function as targets of the immune system (20), to facilitate antigen presentation to T cells (21) and to induce accessory cells to produce cytokines involved in immune activation (22). These properties led to concept that hsp function as 'molecular adjuvants'. Our results have indicated that in MS brain tissue, complexes of MBP and PLP with hsp70 were present, whereas in non-MS brain tissue, such complexes could not be detected. Similarly, in EAE, an animal inflammatory/demyelinating disease induced by immunization with PLP, complexes of PLP and MBP with hsp70 were present. These results indicate that process of inflammatory demyelination favors the physical association of hsp with myelin proteins. The failure to demonstrate hsp–myelin protein complexes in CNS tissue lacking pathologic changes pointed...
to the specificity of hsp–myelin protein complexes in MS. The mechanism underlying the generation of hsp–myelin protein complexes in MS and EAE is not clear. Hsp70 binds predominantly to unfolded proteins, recognizing short 7–9mer peptide segments with a net hydrophobic character on the surface of the extended configuration (23). It has been also shown that during CNS demyelination, MBP and other proteins are released from myelin sheaths (24). Myelin proteins are also very sensitive to denaturation and structural disturbance during inflammatory conditions. The occurrence of proteins with disturbed secondary and tertiary structures in MS and EAE is feasible. On the other hand, hsp expression has been shown to be significantly up-regulated in inflammatory and autoimmune diseases (25). We and others have shown that hsp expression in MS lesions is higher than in unaffected brain tissue. Analysis of the expression and distribution of hsp in MS lesions indicated significant up-regulation of most classes of hsp, both within the lesion and at the lesion edge (26). In early active and chronic active lesions, immunoreactivity for hsp70 was strongly positive on reactive astrocytes and some macrophages at the lesion edge (27). The small hsp, αB crystallin, a member of the hsp27 family, has also been proposed as a candidate antigen in MS (28). Using in vitro cultures, it has been shown that several inflammatory mediators can induce hsp expression in glial cells (29). These conditions make feasible a scenario in MS whereby hsp70 binds myelin proteins released from the myelin sheath in an attempt to prevent MBP and PLP degradation. The SPR analysis has proven strong and selective abilities of hsp70 binding to MBP. The unusual physical property of solubility of PLP in organic solvents (30) did not allow us to perform similar binding experiments with this protein with hsp70. In the bound form, hsp70 with myelin proteins may be targeted to APC and in an adjuvant-like fashion mechanism enhance an immune reaction to myelin antigens. Accordingly, in EAE, we demonstrated hsp70 complexes with PLP which were used for immunization and we also saw hsp70 complexes with MBP. The significance of MBP association with hsp70 in PLP-induced EAE might be relevant to the phenomenon of antigen spreading known to occur in EAE (18). In agreement with the assumption of the enhancing effect of hsp on antigen recognition, we have shown that hsp70 added to MBP significantly enhanced the uptake of MBP by APC. Hsp70 have been also shown to stimulate immune cells to produce cytokines and chemokines which activate APC (22). Enhanced immune recognition of immunogenic or even cryptic epitopes chaperoned by hsp70 and hsp90 has been documented in tumor and virally infected cells (31,32). The lack of an association of hsp90 with myelin proteins in MS might point to the specific function of hsp70 in autoimmune pathology. The presentation pathway is proposed to be initiated by interaction of hsp–protein complex with hsp receptors on the surface of APC (33). In agreement with this hypothesis, we found increased APC uptake of MBP complexed with hsp70 over pure MBP. Recently, CD14 and Toll-like receptor 4 have been shown to bind hsp60 and hsp70 (34–36), and CD91 (ω2-macroglobulin receptor) binds hsp70, hsp90, gp96 and calreticulin (37). Wang et al. (37) identified CD40 as a receptor for prokaryotic hsp70 on monocyte-derived cell lines and blood monocytes. Also, intracellular transfer of proteins and peptides to MHC involves hsp. Hsp70 and hsp90 chaperone proteins into the cytosol and peptides in the endoplasmic reticulum, and facilitate antigen processing and presentation by MHC (39). All these events may contribute to the initiation or perpetuation of the immune response in MS.

These current findings on the association between hsp70 with MBP and PLP support a new role for hsp in the pathogenesis of MS that the over-expression of hsp in the MS lesion might serve to chaperone myelin proteins and peptides and/or create a pro-inflammatory environment that might enhance or elicit the immunogenic potential of these myelin antigens. Such a concept may have important therapeutic implications.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
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<td>hsp</td>
<td>heat shock protein</td>
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<td>MBP</td>
<td>myelin basic protein</td>
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<td>MOG</td>
<td>myelin oligodendrocyte protein</td>
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<td>MS</td>
<td>multiple sclerosis</td>
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<td>OVA</td>
<td>ovalbumin</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<td>PLP</td>
<td>proteolipid protein</td>
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<td>SPR</td>
<td>surface plasmon resonance</td>
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References

Heat shock protein complexes in multiple sclerosis


