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Brain-Derived Heat Shock Protein 70-Peptide Complexes Induce NK Cell-Dependent Tolerance to Experimental Autoimmune Encephalomyelitis

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Heat shock proteins (Hsp) are markedly up-regulated at sites of inflammation during autoimmune diseases like experimental autoimmune encephalomyelitis (EAE). In this study, we show that Hsp70-peptide complexes (pc) isolated from brains of mice with EAE prevented the development of EAE clinically and pathologically when administered before proteolipid protein 139–151 (PLP139–151) immunization. In contrast, pure Hsp70 or Hsp70-pc derived from brains of healthy mice or other inflamed tissue did not modulate the expression of EAE. In animals in which EAE had been suppressed by Hsp70-pc, lymphocytes showed increased cell death in response to PLP139–151 that correlated with elevated IFN-γ and NO production. Coculture of spleen cells from Hsp70-pc immunized mice with spleen cells from untreated EAE mice, in addition to depletion experiments, showed that NK cells reduced reactivity to PLP139–151. Transfer of NK cells from Hsp70-pc-immunized mice to recipients sensitized for EAE abolished disease development. Thus, we propose that Hsp70 demonstrate the ability to bind to peptides generated during brain inflammation and to induce a regulatory NK cell population that is capable of preventing subsequent autoimmunization for EAE. The Journal of Immunology, 2006, 176: 1588–1599.

H
eat shock proteins (Hsp) are among the most abundant and most conserved proteins in cells. Apart from functioning as a major protective mechanism against several noxious environmental factors, Hsp have been implicated in the generation of immune responses. As part of the innate immune response, Hsp have been shown to interact with specific cell surface receptors and to induce the expression of inflammatory cytokines (1, 2) and chemokines (3, 4). Hsp have also been shown to enhance the maturation of dendritic cells (DC) (5), and to activate other immune cells (6, 7), leading to Ag-independent immunemediated protection. Hsp also demonstrate the unique potential to bind to a wide array of endogenous and exogenous peptides. Hsp have been shown to be critical factors in directing peptides to APCs, and in chaperoning peptides along the Ag-presentation pathway (8). This makes Hsp a potential source from which peptides involved in the generation of an immune response may be derived, characterized, and used for immunotherapy. In the last decade, it has been shown that Hsp isolated from tumor tissue can elicit strong immune responses against autologous tumors (9–11), an activity found to be dependent upon the association of Hsp90 with endogenous peptides, because Hsp or peptides alone induced no immune response (9, 11, 12). These results formed the basis for the present hypothesis that complexes of Hsp with peptides might represent a unique and efficient way to induce immunity. Consistent with this notion is the observation that the amount of peptide within the Hsp complex required for the generation of an immune response is several hundred-fold lower than that required for immune responses induced with peptide alone (13). However, the multipotential function of Hsp in the immune system is underscored by the demonstration that Hsp can also down-regulate an immune response, an observation with implications for the generation of autoimmune diseases (14). Hsp have been implicated in a number of autoimmune processes in a variety of ways. Hsp have been shown to accumulate at sites of pathology in rheumatoid arthritis (15), and in the NOD mouse (16). Furthermore, Hsp-induced down-regulation of the immune response has been shown to inhibit rheumatoid arthritis in rats (17) and diabetes in mice (18). The mechanism proposed for the immunoregulatory function of Hsp involved the generation of immune cell subsets that mediated the inhibition of immunologic function. These immunoregulatory cells secreted IL-10 and were Ag-specific (19, 20).

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease induced with myelin Ags (21, 22) and is considered an animal model of the human demyelinating disease, multiple sclerosis (MS). Within the inflammatory lesions in the CNS of animals with EAE, significant up-regulation of Hsp has been detected (23, 24), and when injected before EAE induction, Hsp60 has been shown to prevent the development of EAE (25). However, the role of Hsp up-regulation within the CNS during EAE has not been determined. In this study, we have investigated the effect of a pure Hsp70 fraction alone and an Hsp70 fraction associated with peptide complexes (pc), isolated from the CNS of control healthy animals or animals with EAE, on the subsequent development of EAE. We show that Hsp70-pc isolated...
from the CNS of animals with EAE, but not from the CNS of normal animals, inhibited the development of EAE. Furthermore, in this inhibition, the data further implicated a role for NK cells and IFN-γ. Thus, the mechanism of Hsp70-pc-induced inhibition of EAE is different from the suggested IL-10-dependent Hsp-induced immunoregulation.

Materials and Methods

Animals

Female SJL/J mice were obtained from The Jackson Laboratory, and were maintained by the Animal Care Institute at the Medical University of Lodz. Mice were 6–8 wk old when sensitized for EAE.

Materials

The following primary Abs were used: anti-Hsp70/hsc70 mouse mAb (SPA-822), anti-Hsp40 mouse mAb (SPA-450; StressGen Biotechnologies), anti-Hsp70 mouse mAb (W27), anti-hsc70 mouse mAb (B-6), and anti-Hsp70 goat polyclonal Ab (K-20) (Santa Cruz Biotechnology). For detection of primary Abs, the following secondary Abs conjugated with HRP were used: anti-mouse IgG-HRP (sc-2005), and anti-goat IgG-HRP (sc-2020), (Santa Cruz Biotechnology), and purified hamster anti-mouse IFN-γ mAb (neutralizing Ab; BD Pharmingen). Mouse Abs used were: FITC-conjugated mAb anti-IFN-γ, R-PE-conjugated mAb anti-pan TCRv6, R-PE-conjugated mAb anti-CD3, R-PE-conjugated rat mAb anti-CD4/80/pan-NK (DX5 Ab), R-PE-conjugated rat mAb anti-CD4, R-PE-conjugated rat mAb anti-CD8, and appropriate isotype controls were purchased from BD Pharmingen. Proteolipid protein 139–151 (PLP139–151; TCR-α/β), R-PE-conjugated mAb anti-CD3, R-PE-conjugated rat mAb anti-CD4/80/pan-NK (DX5 Ab), R-PE-conjugated rat mAb anti-CD4, R-PE-conjugated rat mAb anti-CD8, and appropriate isotype controls were purchased from BD Pharmingen. The proteins were dissolved in PBS and quantified using a Brad- ford assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad).

Purification of Hsp70 preparations

Hsp70-pc and pure Hsp70 were isolated from CNS tissues of normal mice and mice sensitized for EAE 14 days previously. CNS tissue, 10 g, was homogenized in 40 ml of hypotonic buffer (30 mM NaHCO3 and 0.5 mM PMSF (pH 7.5), 20 mM NaCl, 3 mM MgCl2, 0.5 mM PMSF, and 15 mM 2-ME). For purification of Hsp70 preparations, the eluant was loaded onto an ADP-agarose column with ethyl ether and livers were obtained for histological examinations. The proteins were dissolved in PBS and quantified using a Bradford assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad). All chemical reagents, PD10 columns, ADP and ATP agarose were purchased from Sigma-Aldrich. All proteins used in this study were tested for endotoxin content by a quantitative Endotoxin Assay (Bio-Rad).

Histopathologic examination of CNS

On day 15 after induction of EAE, mice (n = 5, from two separate experiments), were deeply anesthetized and perfused intracardially with 2.5% glutaraldehyde in phosphate buffer (pH 7.2). The brains and spinal cords were removed and thin slices were made from 10 levels of the neocortex, postfixed in cold 1% osmium tetroxide for 1 h, dehydrated, and embedded in epoxy resin (Epon 812). One-micrometer sections of epoxy-embedded tissue were stained with toluidine blue and examined by light microscopy under code by a blinded observer. An inflammation score of 0–4 was determined based on the density of inflammation in the meningeal, perivascular, and parenchymal compartments of the CNS, in a blinded fashion according to established criteria (28). The demyelinating score ranged from 0 to 4+ and was determined according to the number and extent of denuded axons (28).

Liver inflammation induction

Six-week-old SJL/J female mice were injected i.p. with LPS dissolved in pyrogen-free PBS, in a concentration range from 12.5 to 100 μg/mouse (29). At 0, 12, 24, and 48 h after LPS injection, mice were anesthetized with ethyl ether and livers were obtained for histological examinations. The livers were treated with 10% buffered paraformaldehyde and embedded in paraffin. Sections (3 μm) were prepared and stained with H&E (Sigma-Aldrich).

Cell proliferation assays

Spleen cells (SC) were isolated from mice preinjected with Hsp70 preparations and control EAE animals on days 2, 7, and 14. SC, 2 × 105 cells/well, were cultured in triplicate with 50 μg/ml PCL40–51 for 96 h in 200-μl flat-bottom microtiter well plates. For the final 18 h, 1 μCi/well [3H]thymidine (Tdr; Amersham Biosciences), was added, and uptake was measured using a liquid scintillation beta counter (Pharmacia). Results were expressed as cpm.

Cytokine ELISA

Cytokines produced by SC were detected in culture supernatants by an ELISA kit for IFN-γ, IL-2, IL-4, and IL-10 (BD Pharmingen). SC were

Gel electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE according to the Laemmli protocol (27), followed by Coomassie brilliant blue staining and Western blot analysis using specific Abs. Gels were blotted onto polyvinylidene fluoride membranes (Millipore), and incubated in blocking buffer containing 5% nonfat milk in TBST buffer (20 mM Tris-base, 137 mM NaCl (pH 7.6), and 0.05% Tween 20; Sigma-Aldrich), overnight at 4°C, followed by incubation in the appropriate primary Ab (0.1 μg/ml), for 1 h at room temperature. After washing five times in TBST buffer, blots were incubated for 1 h at room temperature in secondary conjugated Ab (0.1 μg/ml). The blots were developed with the ECL substrate (ECL-plus; Amersham Biosciences), according to the manufacturer’s protocol. The stripped peptides from Hsp70-pc preparations were resolved on ready precast minipoly-
isolated from mice on day 14 after induction of EAE, stimulated with 50 µg/ml PLP139–151, and harvested at 72 h. Supernatants were collected, and frozen or tested immediately for the presence of cytokines, according to the manufacturer’s instructions. Results were reported as the mean obtained from three experiments ± SD.

**Measurement of NO production**

The determination of accumulated nitrite (NO₂⁻) in cell-free supernatant was detected by the colorimetric Griess reaction. One hundred microliters of supernatant and a series of dilutions of a NaNO₂ standard solution were added to prevent nonspecific binding. After incubation for 10 min at room temperature, the OD of the reaction product was measured at 550 nm on a microplate reader (Bio-Rad), and the amount of accumulated nitrite in the samples was quantified using the standard curve obtained with NaNO₂.

**Flow cytometry**

The analysis of IFN-γ production by subpopulations of SC was performed by the three-color flow cytometry with FACS Calibur equipment (BD Biosciences). For this purpose, SC isolated from EAE or Hsp70-pc/EAE mice were cultured for 12 h in culture medium supplemented with 50 µg/ml PLP139–151. For the last 4 h of culture, 10 µg/ml Brefeldin A (Sigma-Aldrich) was added to culture medium to stop cytokine secretion. After the culture period, cells were harvested, washed for 10 min, centrifuged at 300 x g, resuspended (5 x 10⁷/100 µl) in staining buffer (PBS, 0.1% sodium azide, 1% bovine albumin; Sigma-Aldrich), and incubated with PE- and/or PerCP-conjugated mAbs specific for cell surface Ags (CD4, CD8, Dx5; 10 µl/10⁶ cells), for 30 min in 4–8°C under light protection. To each sample, Fl Beck Reagent (20 µl/10⁶ cells; BD Pharmingen), was added to prevent nonspecific binding. After staining of surface Ags, cells were fixed and permeabilized using the Fix & Perm Permeabilization kit (Caltag Laboratories), according to the manufacturer’s protocol, and stained with monoclonal FITC-conjugated anti-mouse IFN-γ Ab. As a control for binding specificity, isotype-matched control mAbs were used. The staining was analyzed with CellQuest software (BD Pharmingen).

**Depletion of CD4⁺, CD8⁺, γδ, and NK cells**

SC were isolated from mice with EAE preinjected with Hsp70-pc or non-preinjected. The depletion procedure was performed by SC incubation with anti-CD4⁺, anti-CD8⁺, anti-NK1.1, or anti-pan TCR mAb, respectively, for 45 min at 4°C. The SC were then washed and incubated with rabbit complement for 60 min at 37°C (30). As a control, SC were incubated with rabbit complement alone. In addition, in several experiments for γδ and NK cell depletion, we used negative selection with the MACS sorting system and anti-CD49b (Dx5; Miltenyi Biotec), and GL3 Ab mAb (Caltag Laboratories). NK cells for in vivo transfer were isolated by depletion of non-NK cells using the NK Cell Isolation kit (Miltenyi Biotec). The efficiency of cell depletion was confirmed by flow cytometry using a FACSCalibur (BD Biosciences).

**In vitro cell culture**

SC from mice preinjected with Hsp70-pc were isolated on day 15 and were cocultured with PLP-reactive SC isolated from EAE mice on day 14 post-PLP139–151 immunization at the following ratios: 1:30, 1:15, 1:6, and 1:3, with or without PLP139–151 (50 µg/ml). Similarly, SC from Hsp70-pc preinjected mice depleted of given cell populations (see above) were cocultured with SC from EAE mice at a ratio of 1:6. After 72 h of coculture, supernatants were taken for quantitation of IFN-γ secretion and a proliferation assay was performed as described above.

**In vivo cell transfer**

SC and NK cells from mice preinjected with Hsp70-pc and Hsp70 were isolated on day 15 postimmunization, suspended in sterile PBS at concentrations of 2 x 10⁶ or 10 x 10⁶ for SC and 3 x 10⁶ for NK cells in a volume of 200 µl, and injected into the tail vein of mice on the day of sensitization for EAE with PLP139–151 in CFA. Mice were observed daily for the presence of neurological signs of EAE and scored clinically.

**SC death assessment**

SC were isolated from EAE mice preinjected with Hsp70-pc and from non-preinjected mice, cultured for 3 days, stimulated with PLP139–151 peptide (50 µg/ml). Cell death was assessed each day based on fractional DNA content in the cells as described before (31). Briefly, cells were collected, spun down, and fixed in 70% ethanol. Then, the cells were washed and resuspended in DNA staining solution containing propidium iodide (20 µg/ml) and DNase-free RNase A (0.2 mg/ml; Sigma-Aldrich), and incubated for 30 min at room temperature. After that, cells were assessed by flow cytometry. DNA content was measured in FITC fluorescence.

**Statistical methods**

Data were expressed as means and SD. For multiple comparison measures, the Student t test, two-way ANOVA, and the Mann-Whitney U test were applied.

**Results**

**Isolation of Hsp70-pc and Hsp70 from CNS tissue**

Western blotting of brain eluates separated by affinity chromatography using either an ADP- or an ATP-agarose column, demonstrated the presence of Hsp70 in both eluates (Fig. 1A). To demonstrate that the ADP-purified Hsp70 was complexed with endogenous peptides, we electrophoresed the samples from brains of mice with EAE on a polyacrylamide gel gradient with Tris-tricine buffer. Coomassie blue staining of the gel demonstrated a band that migrated with a molecular mass <6.5 kDa only from the fraction eluted from the ADP-agarose column (Fig. 1B, lane 1), but not from the ATP-agarose column (Fig. 1B, lane 2). Using both ADP- and ATP-agarose columns, we isolated 0.8–1.2 mg of Hsp70 from 10 g of CNS tissue.

**Hsp70-pc isolated from EAE and normal CNS tissue showed a different peptide profile**

To address the issue of whether Hsp70 bound the same peptides in inflamed or control healthy brains, we performed chromatographic analysis of the peptide profile from both Hsp70-pc preparations. The results demonstrated significant differences in the chromatogram patterns obtained with peptides stripped from Hsp70-pc derived from EAE brain tissue and from healthy brain tissue (Fig. 1C). To exclude the possibility of the presence of PLP peptide (used for EAE induction) in the Hsp70-pc preparation, we performed Western blot analysis and immunoprecipitation with anti-PLP and found no signal for PLP. In addition, we performed mass spectrometry analysis of the Hsp70-pc preparation and found no sequences homologic to PLP or any other myelin peptides (data not shown).

**Hsp70-pc, but not Hsp70, prevents clinical EAE in SJL/J mice**

To assess the effects of Hsp70-pc and pure Hsp70 on the development and severity of EAE, we preinjected SJL/J mice with Hsp70-pc or Hsp70 preparations twice, at weekly intervals, 1 wk before sensitization for EAE with PLP139–151. We compared the effect of Hsp70-pc and Hsp70 obtained from the CNS of normal mice with Hsp70-pc and Hsp70 isolated from the CNS of EAE mice. The results showed that prior injection with Hsp70-pc isolated from the CNS of mice with EAE inhibited the subsequent development of disease and also significantly reduced the severity of disease, p < 0.001 (Fig. 2A). In contrast, preinjection with pure Hsp70 resulted in a slight reduction of EAE severity only (Fig. 2A). Interestingly, preinjection with Hsp70-pc or pure Hsp70 isolated from the CNS of normal mice did not influence the subsequent development of EAE (Fig. 2B). These results showed that only Hsp70-pc derived from the CNS tissues of mice with EAE had tolerogenic activity toward EAE. The protective effect of prior injection with Hsp70-pc on the development of EAE was dose-dependent, because the inhibitory effect of Hsp70-pc increased when a higher Hsp70-pc dose was used for preinjection (Fig. 2C). Because the PLP-induced model of EAE in SJL mice is protracted and involves secondary relapses, mice were observed for 50 days
and no delayed EAE occurrence in Hsp70-pc preinjected mice were recorded.

To determine whether resistance to EAE depended on peptides associated with Hsp70, these peptides were stripped from Hsp70-pc by incubation with ATP. Next, the pure peptide fraction was used for injection before EAE induction. In experiments when peptides were injected without adjuvant, no effect on EAE development was observed. However, in mice injected with peptide fraction mixed with CFA, a reduction in the severity of EAE was detected \( (p < 0.005; \text{Fig. 2D}) \). The injection of mice with CFA alone or CFA mixed with Hsp70 had no effect on the subsequent induction of EAE (Fig. 2D). These data indicate that peptides isolated with Hsp70 from brains of EAE mice are essential for the tolerogenic effect on subsequent EAE induction. In another set of experiments when we use IFA instead to CFA, we have not seen reduction in EAE expression (data not shown).

Hsp70-pc from other inflamed tissues did not prevent EAE

To assess whether Hsp70-pc isolated from other inflamed tissues might affect the development of EAE, we induced inflammation in the livers of mice using LPS (29). After a single i.p. injection of LPS, with an optimal concentration of 50 \( \mu \)g/mouse, we observed severe inflammatory changes in liver with hepatocyte destruction and numerous inflammatory cell infiltrates by 48 h. Hsp70-pc were...
FIGURE 2. Preinjection with Hsp70-pc isolated from EAE brain inhibits the development of EAE. Hsp70 preparations were injected s.c. twice on days 0 and 7 into SJL/J female mice. One week after the last injection, EAE was induced with PLP_{139–151} peptide. Each point represents the mean of the clinical course obtained from five to eight animals in each group in 6–15 experiments. SD was below 15%. A. Preinjection with Hsp70-pc (0.6 mg/kg), isolated from the CNS of mice with EAE, inhibited development of EAE (p < 0.001). Mice preinjected with the same amount of Hsp70 did not prevent EAE. B. Preinjection with Hsp70-pc isolated from the CNS of healthy mice did not influence the development of EAE. C. The protective effect of preinjection with Hsp70-pc on the development of EAE was dose-dependent, with increasing protection noted with doses from 0.1 to 1.2 mg/kg. D. Preinjection with peptides stripped from Hsp70-pc derived from CNS tissues of EAE animals with CFA, protected against EAE (p < 0.005). Peptides without CFA, CFA alone, and Hsp70 with CFA, did not protect from EAE. E. Preinjection with Hsp70-pc (0.6 mg/kg), isolated from the inflamed and control liver of mice, did not influence the development of EAE. F. Light micrographs of 1-μm epoxy sections from lumbar spinal cord stained with toluidine blue. Mouse preinjected with Hsp70. Note that numerous inflammatory cells and several demyelinated axons are present; ×400. G. Mouse preinjected with Hsp70-pc. Note the well preserved myelin and lack of inflammatory cells; ×400.
isolated as for the CNS and used at the same concentration for the preinjection of mice before EAE induction. However, in contrast to Hsp70-pc isolated from the CNS, we observed no inhibitory effect of liver-derived Hsp70-pc on the induction of EAE (Fig. 2E).

**Hsp70-pc prevents CNS inflammation and demyelination in EAE**

The brain and spinal cords of mice preinjected with Hsp70-pc isolated from the CNS of mice with EAE followed by sensitization for EAE suppressed the development of EAE. Slides from these animals were read under code and showed significantly reduced inflammation and demyelination (Table I). There were no inflammatory cells whatsoever in all examined spinal cord regions (C7, Th2, L2, L5, L6, and S1) of the Hsp70-pc preinjected mice (Fig. 2G). In contrast, control mice preinjected with Hsp70, showed an abundance of inflammatory infiltrates typical of EAE (Fig. 2F). One Hsp70-pc preinjected mouse showed inflammation in the brain, but this did not correlate with any clinical activity. Similarly, in Hsp70-pc preinjected mice, there was no evidence of demyelination (Fig. 2G), whereas in mice preinjected with Hsp70, abundant evidence of demyelination was always present (Fig. 2F). These findings correlated with the reduced clinical signs in mice preinjected with Hsp70-pc and suggested a tolerogenic effect on EAE, because preinjection with Hsp70-pc prevented the development of inflammation in the CNS.

**T cell proliferation to PLP<sub>139–151</sub> is suppressed in mice preinjected with Hsp70-pc**

To determine the effect of preinjection of Hsp70-pc on the subsequent recognition of Ag used for induction of EAE, we assessed the proliferation of SC isolated from mice at 2, 7, and 14 days postsensitization for EAE. Proliferation of cells stimulated with PLP<sub>139–151</sub> was 2.5-fold lower in the group of mice preinjected with Hsp70-pc than in control EAE animals, (p < 0.01), or in mice preinjected with the pure Hsp70 preparation (p < 0.01; Fig. 3A). We present data with the PLP<sub>139–151</sub> dose of 50 μg/ml which was shown to be most effective in the dose (0–100 μg/ml) defining experiments (data not shown). The reduced responsiveness of SC to PLP<sub>139–151</sub> was not overcome by the addition of IL-2 (data not shown). Importantly, SC obtained from mice pretreated with Hsp70-pc did not respond to PLP<sub>139–151</sub>, indicating that in the Hsp70-pc fraction, no PLP peptides were present (data not shown).

**IFN-γ is significantly increased by PLP<sub>139–151</sub> stimulation in mice preinjected with Hsp70-pc**

To assess the role of cytokines in the mechanism of EAE suppression in mice preinjected with Hsp70-pc, we measured cytokine production by SC sampled 14 days after induction of EAE and restimulated with PLP<sub>139–151</sub>. Interestingly, we detected significantly greater production of IFN-γ by SC from Hsp70-pc preinjected mice than in mice with EAE preinjected with pure Hsp70 (p < 0.001) or mice preinjected with the peptide fraction (p < 0.001; Fig. 3B). Also, PLP<sub>139–151</sub>-induced IL-4 production by SC was higher in Hsp70-pc preinjected mice than in controls (Fig. 3B). IL-10 was detected in the Hsp70-pc preinjected mice, but at low levels only. These results strongly suggest that the inhibition of EAE and the suppression of PLP<sub>139–151</sub>-induced proliferation of T cells in Hsp70-pc preinjected mice correlated with a burst of IFN-γ production and an increase in IL-4. The instrumental role of IFN-γ in the Hsp70-pc-induced decreased PLP response was confirmed by the demonstration that IFN-γ neutralization with anti-IFN-γ mAb resulted in the restoration of PLP reactivity (Fig. 3C).

**NO is highly up-regulated in response to PLP<sub>139–151</sub> in mice preinjected with Hsp70-pc**

Immune tolerance dependent on IFN-γ secretion has frequently been linked with increased NO production (32). Because we detected high IFN-γ secretion in PLP-reactive cells in mice preinjected with Hsp70-pc; we also assessed the accumulation of NO in cultures of SC stimulated with PLP<sub>139–151</sub>. NO levels in supernatants of SC derived from Hsp70-pc preinjected mice stimulated with PLP<sub>139–151</sub> were very high compared with levels in control EAE (p < 0.001) and mice preinjected with pure Hsp70 (p < 0.001), where no increase in NO in response to PLP<sub>139–151</sub> stimulation was observed (Fig. 3D).

**IFN-γ showed higher expression in CD4<sup>+</sup> and NK cells in Hsp70-pc induced inhibited EAE**

To assess which cell population expressed high IFN-γ in Hsp70-pc-induced tolerance to EAE, we performed intracellular staining of SC with anti-IFN-γ mAb. Compared with regular EAE, two populations showed high IFN-γ expression, CD4<sup>+</sup> cells and DX5<sup>+</sup> cells (Fig. 3E). These results indicate that PLP-reactive CD4<sup>+</sup> cells under the tolerance-permissive condition induced by Hsp70-pc showed higher IFN-γ expression than in control EAE mice. In addition, cells expressing DX5 which do not respond directly to PLP also produce IFN-γ.

**Hsp70-pc and Hsp70 immunization equally induced cells producing IL-10**

To investigate the mechanisms of Hsp70-pc-dependent prevention of EAE, we determined cell proliferation and cytokine profile in Hsp70-pc- and Hsp70-immunized mice without subsequent sensitization for EAE. SC isolated from mice immunized with Hsp70-pc showed strong proliferation when Hsp70-pc was added to the SC culture (Fig. 4A). SC proliferation to pure Hsp70 was significantly lower (p < 0.01). However, the SC response to the peptide fraction stripped from the Hsp70-pc preparation was negligible, indicating that simultaneous presence of both Hsp70 and...
FIGURE 3. PLP139–154-induced responses of SC isolated from Hsp70-pc preinjected and control mice subsequently sensitized for EAE. SC were isolated 14 days post-PLP139–154 immunization and were stimulated for 72 h. A, Proliferation of SC as measured by [3H]thymidine uptake. Note the diminished proliferation of SC from animals preinjected with Hsp70-pc isolated from EAE brains (*, \( p < 0.01 \)), but not in mice preinjected with Hsp70 or Hsp70-pc isolated from healthy mouse brain. Bars represent mean cpm (± SD) values from 12 experiments and five-eight animals per group. B, Cytokine profile of SC measured by ELISA. Note the high levels of IFN-\( \gamma \) in response to PLP139–154 in animals preinjected with Hsp70-pc, but not in mice preinjected with Hsp70 (*, \( p < 0.001 \)), EAE mice (**, \( p < 0.001 \)), or mice preinjected with the peptide fraction (***, \( p < 0.001 \)). Bars represent mean ± SD from 12 experiments and five to eight animals per group. C, IFN-\( \gamma \) neutralization with anti-IFN-\( \gamma \) mAb resulted in the restoration of PLP reactivity. Bars represent mean cpm (± SD) values from three experiments and five to seven animals per group. D, NO production by SC. Note the high NO production in SC from mice preinjected with Hsp70-pc but not with Hsp70 (*, \( p < 0.001 \)) or control EAE mice (**, \( p < 0.001 \)). The results represent the mean level (±SD) from three experiments with five to six animals in each group. E, CD4+ and NK cells expressed increased IFN-\( \gamma \) in Hsp70-pc preinjected mice restimulated with PLP. SC isolated from EAE or Hsp70-pc preinjected mice with subsequent induction of EAE were cultured for 12 h with PLP139–151. For the last 4 h of culture, 10 μg/ml brefeldin A was added. After culturing, intracellular staining for IFN-\( \gamma \) SC was performed, as described in Materials and Methods. One of five representative experiments is shown.
the peptide fraction was required for SC proliferation. The proliferative response to pure Hsp70 of SC isolated from mice immunized with Hsp70-pc or Hsp70 in response to Hsp70-pc, Hsp70, and peptide fractions, SC were isolated 14 days after the second injection of Hsp70-pc or Hsp70 preparations and stimulated for 72 h with Hsp70-pc, Hsp70, and peptide fractions. A and B. Proliferation responses to Hsp70-pc or Hsp-70, respectively. C and D, The cytokine profile of SC production was measured by ELISA from mice immunized with Hsp70-pc and Hsp70, respectively. Note the equally high production of IL-10 in response to Hsp70 and Hsp70-pc in both groups of animals (preinjected with Hsp70-pc and with Hsp70). The results represent the mean ± SD from three experiments with five to six animals in each group.

**FIGURE 4.** Proliferation and cytokine profile of SC from mice immunized with Hsp70-pc or Hsp70 in response to Hsp70-pc, Hsp70, and peptide fractions. SC were isolated 14 days after the second injection of Hsp70-pc or Hsp70 preparations and stimulated for 72 h with Hsp70-pc, Hsp70, and peptide fractions. A and B, Proliferation responses to Hsp70-pc or Hsp-70, respectively. C and D, The cytokine profile of SC production was measured by ELISA from mice immunized with Hsp70-pc and Hsp70, respectively. Note the equally high production of IL-10 in response to Hsp70 and Hsp70-pc in both groups of animals (preinjected with Hsp70-pc and with Hsp70). The results represent the mean ± SD from three experiments with five to six animals in each group.

Coculture of SC from Hsp70-pc-immunized mice diminishes PLP reactivity of SC from EAE mice

To assess further the mechanism of Hsp70-pc-induced EAE tolerance, we investigated the potency of SC from mice immunized with Hsp70-pc to influence PLP reactivity in EAE animals. For this, we performed experiments in which PLP-reactive SC from EAE were cocultured with SC from Hsp70-pc-immunized mice. SC from Hsp70-pc–, but not Hsp70–immunized, mice reduced PLP139–151–induced proliferation of SC from EAE mice in a dose-dependent manner (p < 0.01; Fig. 5A). Most interestingly, SC from Hsp70-pc–, but not Hsp70, immunized mice also induced production of IFN-γ by EAE SC stimulated with PLP139–151, which correlated with inhibition of their proliferation (Fig. 5B). Thus, the results of these in vitro coculture experiments corresponded well with inhibition of proliferation and high IFN-γ production by SC in animals preinjected with Hsp70-pc and tolerized for EAE.

**Cells with NK markers induce inhibition of PLP-reactive CD4+ T cells**

To assess the population of immune cells from Hsp70-pc–preinjected mice that induced reduction in response of PLP-reactive cells from EAE mice, we performed coculture experiments as above but with ablation of several cell types. These results showed that when SC from Hsp70-pc–immunized mice were deprived of cells expressing CD49b, a pan-NK marker, with mAb DX5 or cells expressing NK1.1 marker, the inhibitory effect of SC on PLP139–151 proliferation was reversed. In contrast, the ablation of CD4+, CD8+, or γδ T cells did not restore PLP reactivity (Fig. 5C).
FIGURE 5. Coculture of SC from Hsp70-pc and Hsp70 immunized mice with PLP139–151-reactive cells from EAE animals. A, A dose-dependent inhibitory effect of SC is seen from Hsp70-pc, but not Hsp70, immunized mice on the PLP139–151-induced proliferation of SC from EAE mice. B, A dose-dependent enhancing effect of SC from Hsp70-pc-immunized, but not Hsp-70-immunized mice, on PLP139–151-simulated (Figure legend continues)
Transfer of NK cells from Hsp70-pc-immunized mice prevents EAE

The DX5 and anti-NK1.1 mAbs used for ablation experiments recognize both NK and NK T cells. To directly demonstrate an instrumental role for NK cells in induction of EAE tolerance, we performed transfer experiments in which NK cells isolated from Hsp70-pc-immunized mice were injected i.v. into mice immunized for EAE. NK cells were isolated from mice that had been injected with Hsp70-pc 15 days earlier and these cells were injected i.v. coincidently with immunization for EAE. First, the results showed a dose-dependent reduction in the development of EAE with injection of SC from Hsp70-pc but not Hsp70-immunized mice (p < 0.01; Fig. 5D). However, most importantly, when NK cells were transferred from Hsp70-pc, but not from Hsp70, preinjected mice into EAE mice, the disease was significantly abolished to the level achieved with transfer of the whole SC population (p < 0.01; Fig. 5E). The SC population from Hsp70-pc-immunized mice deprived of NK cells lost their protective effect on EAE (Fig. 5E). T cells from the protected mice after in vivo SC or NK cell transfer produced more IFN-γ upon PLP stimulation (data not shown).

Increased death of PLP-reactive cells in mice preinjected with Hsp70-pc

EAE inhibition induced with Hsp70-pc correlated with loss of proliferative response to PLP139–151 and increased IFN-γ expression by PLP-reactive cells. This suggested activation-induced cell death as the cellular mechanism responsible for the inhibition of EAE. To verify this hypothesis, we assessed cell death of PLP-reactive cells from mice preinjected with Hsp70-pc. Using DNA staining with propidium iodide (PI) and flow cytometry, we showed that cells from mice preinjected with Hsp70-pc undergo apoptosis in response to restimulation with PLP139–151 in vitro.

Discussion

In this study, we present evidence that immunization of mice with Hsp70 associated with a peptide fraction (Hsp70-pc) derived from the CNS of animals with EAE led to down-regulation of subsequent induction of EAE. The mechanism of Hsp70-pc-induced tolerance to EAE depends on an NK cell-mediated increase in IFN-γ production and enhanced apoptotic death of autoreactive T cells.

Hsp70 is one of the major peptide-binding proteins that has been implicated as a chaperone in the formation of immunogenic complexes (10, 12, 33). Although most of the data that have examined the contribution of Hsp70 peptide interactions have addressed immune responses that involve an MHC class I-dependent pathway (34, 35), more recent results from these (36) and other laboratories (37) indicate that Hsp70 may also assist Ag presentation in an MHC class II-dependent pathway. In this study, we used an experimental model, EAE, which is an inflammatory/demyelinating disease generally considered to be dependent on MHC class II-restricted CD4+ T cells (38). Our results are the first to show that Hsp70 complexed with an endogenous peptide can down-regulate the immune process in an MHC class II-dependent disease.

A striking finding in our experiments was the induction of high levels of IFN-γ production in PLP139–151-stimulated SC derived from mice that had been preinjected with Hsp70-pc and in which both clinical and pathological expression of EAE had been suppressed. Although IFN-γ is considered one of the major proinflammatory cytokines, there is ample evidence that IFN-γ can also down-regulate T cell responses (39–41). We also noted an increase in IL-4 production in response to PLP139–151 in SC from mice preinjected with Hsp70-pc, but high IFN-γ and low IL-10 production by SC makes it unlikely that a TH2 shift could contribute to the EAE prevention induced with Hsp70-pc. The instrumental role of IFN-γ in prevention of EAE was confirmed in our experiments by the demonstration that IFN-γ neutralization restored PLP reactivity. IFN-γ has been shown to be critically involved in the control of EAE. In mice deficient for the ligand-binding chain of the IFN-γ receptor, severe EAE was observed.
following active immunization (41). The inhibitory effect of IFN-γ on EAE has been shown to be dependent on NO production (42). In line with these observations, we observed that SC from mice with Hsp70-pc-induced EAE inhibition produced large amounts of NO. NO inhibits lymphocyte proliferation (43) by interfering with intracellular kinase-dependent signaling (44).

To further address the mechanism involved in EAE inhibition by preinjection with Hsp70-pc, we investigated the immune cell populations generated in Hsp70-pc-immunized mice without subsequent EAE induction. SC obtained from these mice showed vigorous proliferation and increased IL-10 production in response to the Hsp70-pc preparation. These data might have suggested the generation of Hsp70-pc-induced regulatory cells, similar to that reported earlier in mice immunized with pure Hsp70 (19, 20). However, the immunization with pure Hsp70 preparation was not able to prevent EAE, although Hsp70 was still able to induce IL-10 production. Thus, there have to be substantial differences in the nature of the regulatory cell function induced with Hsp70-pc vs pure Hsp70 with regard to control of the development of EAE. To validate this concept, we performed coculture experiments in which we showed that direct interaction of SC from Hsp70-pc, but not from Hsp70, immunized mice with PLP139–151-specific cells, suppressed the response of the latter to PLP. More importantly, the suppression of PLP139–151 reactivity correlated with high IFN-γ production. To obtain definitive proof that Hsp70-pc immunization led to the generation of regulatory cells, we performed in vivo transfer of SC cells derived from Hsp70-pc-prime mice shortly after immunization with PLP139–151 and demonstrated a significant reduction in the expression of EAE. To characterize the phenotype of regulatory cells in the SC population from Hsp70-pc-immunized mice, we performed depletion experiments which showed that cells with NK markers were responsible for reduced PLP reactivity. To directly address the role of NK cells, in transfer experiments, we showed that negatively isolated pure NK cells from Hsp70-pc-immunized mice induced tolerance to EAE induction. The involvement of NK cells in the regulation of adoptive immunity has only recently been discovered. It has been shown that CD16-activated NK cells costimulate TCR-induced IFN-γ production by CD4+ T cells (45) and NK cells can also directly activate mature DC (46). TCR stimulation leads to T cell apoptosis (47) which is the major mechanism limiting the expansion of the immune response and plays an essential role in both central and peripheral immune tolerance. TCR-induced cell death requires IFN-γ activity (48). The concordance of decreased NK cell responses with the emergence of Ag-specific T cells implies a reverse correlation between NK cell function and TCR stimulation (49). NK cells were shown to inhibit T cells (50) and, more recently, it has been shown that activated T cells can be recognized and killed by syngeneic NK cells (51). Our findings indicate that NK cells generated in Hsp70-pc-immunized mice induced high IFN-γ production which correlated with enhanced apoptotic death of PLP-reactive T cells. We were not able to explore whether DC are involved in NK-mediated death of autoreactive cells because DC are required for PLP responses during the induction of EAE. The results of our experiments are in line with an interesting observation on the regulatory role of NK cells in EAE and MS (52–55) which demonstrated that high levels of activated NK cells correlated with lower reactivity to myelin basic protein.

An inhibitory effect on EAE was observed only when the Hsp70 fraction was derived from the CNS of mice with EAE and not from healthy CNS tissue. This observation suggests the existence of substantial differences in the peptide(s) that bind Hsp70 in pathological and normal CNS tissues, and chromatographic analysis of both peptide profiles stripped from Hsp70 supported this conclusion. Recently, the important role of DC and macrophages in NK cell activation has been emphasized (56). The inhibition of the development of EAE only with Hsp70-pc preparation isolated from inflamed CNS, but not from inflamed liver, might point to molecules generated specifically by inflammatory stimuli in nervous tissue. Using mass spectroscopy analysis, in ongoing studies, we derived several peptides from Hsp70-pc (data not shown). However, none of these corresponded to major myelin Ags (including PLP) and MHC peptides (which have been shown to associate with Hsp70 and to induce down-regulation of immune responses (57); data not shown). The hypothesis on the instrumental role of peptides bound to Hsp70 in induction of EAE tolerance was supported by the results showing that the peptide fraction stripped from Hsp70 also prevented EAE development when injected with CFA, whereas the pure peptide fraction was inefficient in EAE prevention. These data suggest that Hsp70 serves as a natural adjuvant following the injection of the Hsp70-pc fraction into mice before EAE induction. Further studies will be aimed at defining molecules from Hsp70-pc leading to NK cell activation. These molecules might have potential therapeutic effect in EAE and MS.

In conclusion, we have demonstrated that peptides derived from inflamed CNS and bound to Hsp70 are able to induce a novel regulatory circuit involving NK cells inhibiting autoreactive T cells. These findings might further contribute to our understanding of Hsp immunoregulatory function in autoimmune diseases.

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Disclosures

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References


