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Heat Shock Protein 70 Is Able to Prevent Heat-Shock-Induced Resistance of Target Cells to CTL

Ralf Dressel, Leslie Elsner, Thomas Quentin, Lutz Walter, and Eberhard Günther

Heat shock or transfection with heat shock protein 70 (Hsp70) genes has been shown to protect tumor cell lines against immune mechanisms of cytotoxicity. We have reported previously that heat shock confers resistance to CTL in the rat myeloma cell line Y3 that is Hsp70 defective. Evidence is now presented that Hsp70 is able to prevent the induction of the resistant phenotype. In Con A-stimulated lymphocytes and in lymphocyte × Y3 somatic cell hybrid clones a severe, non-Hsp70-inducing heat shock elicits resistance to CTL in contrast to a heat shock that results in Hsp70 expression. Thus, Hsp70 expression appears to be negatively associated with the development of resistance. Furthermore, loading of Y3 cells with recombinant Hsp70 protein before heat shock is able to prevent resistance. Because apoptosis induced in Y3 cells by heat shock is not affected, Hsp70 appears to interfere selectively with the CTL-induced lethal pathway that is found to be calcium but not caspase dependent. It is suggested that after heat shock Hsp70 enhances the CTL-induced apoptotic pathway by chaperoning certain proteins in the target cell that are involved in the execution of cell death. Thus, although shown to confer protection against many cytotoxic mechanisms, Hsp70 does not appear to be generally cytoprotective. This observation could also be of relevance when interpreting the effectiveness of tumor immunity. The Journal of Immunology, 2000, 164: 2362–2371.

In several cell lines heat shock has been shown to induce resistance to cytotoxic immune mechanisms mediated by TNF-α (1–5), CTL (4, 6), or monocytes (1). Heat shock-induced resistance has been assigned to the expression of heat shock proteins (Hsps)1 and explained by their function as molecular chaperones. In the TNF-α model resistance could be mimicked by transfection with Hsp27 or Hsp70 genes (5, 7), and an anti-apoptotic function of Hsp70 has been localized downstream of caspase 3 activation (8). Suppression of glucose-regulated protein 78 (Grp78), a member of the Hsp70 family, has been demonstrated to eliminate stress-induced resistance of a fibrosarcoma cell line to CTL (9) and also to inhibit tumor progression in vivo (10). We have described heat shock-induced resistance to CTL in the rat myeloma cell line Y3 (6). Y3 cells are unable to express the major heat shock-inducible Hsp70 protein encoded by the MHC-linked Hsp70-1 and Hsp70-2 genes, indicating that Hsp70 is not indispensable for heat shock-induced resistance to occur. This cell model was used to further analyze the role of Hsp70 in heat shock-induced resistance to CTL. Two experimental approaches were followed: 1) the use of somatic cell hybrids between Y3 cells and lymphocytes, and 2) loading of Y3 cells with recombinant Hsp70.

It will be shown that Hsp70, instead of being protective, is able to prevent heat-induced resistance.

Materials and Methods

Lymphocytes, cell lines, hybrid cell clones, and heat shock conditions

The rat myeloma cell line 210-RCY3-Ag1.2.3 (Y3) derived from LOUC rats (RT1+1) (11) and hybrid cell clones generated thereof (see below) were maintained in NaHCO3-buffered DMEM (Biochrom, Berlin, Germany) supplemented with 10% FCS (Biochrom), pyruvic acid (110 mg/L), penicillin (100000 U/L), and streptomycin (100 mg/L) in petri dishes (Sarstedt, Nümbrecht, Germany) at 37°C in a 10% CO2 atmosphere. Rat lymphocytes were prepared from lymph nodes as described (12). For mitogenic stimulation 5 μg/ml Con A (Amersham Pharmacia Biotech, Freiburg, Germany) were added to the cultures. Lymphocytes were used as targets in cytoxicity experiments after 4–5 days of mitogenic stimulation. Somatic cell hybrids between Y3 cells and normal syngeneic (LOUC/Gun, RT1+) or allogeneic (BUF/Gun, RT1+) lymph node lymphocytes were generated by cell fusion with polyethylene glycol according to common fusion protocols used for hybridoma production (13), selection in HAT medium (DMEM containing 1 × 10−4 M hypoxanthine, 4 × 10−7 M aminopterin, 1.6 × 10−7 M thymidine), and cloning by limiting dilution. For induction of a heat shock response, 5 × 105 cells in 5 ml medium were incubated in 13-ml polypropylene tubes in a fine-regulated water bath (Julabo, Schutt, Göttingen, Germany) usually for 1 h at 42°C or 30 min at 44°C. For recovery, cells were cultured as described above.

Recombinant Hsp70

The rat Hsp70-1 gene (14) was amplified from LEW.1W rat (RT1+) DNA using specific primers including BamHI or HindIII restriction sites, respectively, in the 5′ regions (5′-ATTGAATCCGCCGCAAGAAACACGGCATTGC GCA-T3′ and 5′-CCAAGGCTTCTATAACCCTCTTCGATGGTTG-3′). The PCR product was cloned in the pQE30 vector (Qiagen, Hilden, Germany) making use of the BamHI and HindIII restriction sites. The open reading frame starts with a 5′-terminal His codons. Correctness of the construct was confirmed by sequencing.

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3 Abbreviations used in this paper: Hsp, heat shock protein; β-gal, β-galactosidase; Gpr, glucose-regulated protein; Hsc, heat shock cognate protein; HAT, hypoxanthine, aminopterin, thymidine; MIF, mean intensity of fluorescence; PI, propidium iodide; X-Gal, 5-bromo-4-chloro-3-indolyl-β-galactosidase; Z-DEVD-FMK, Z-Val-Ala-Asp(OMe)-CH2F; Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; Z-DEVD-FMK, Z-Asp(OCH3)-Glu(OCH3)-Val-Asp(OCH3)-FMK

4 The PCR product was cloned in the pQE30 vector (Qiagen, Hilden, Germany) making use of the BamHI and HindIII restriction sites. The open reading frame starts with a 5′-terminal His codons. Correctness of the construct was confirmed by sequencing.

5 Escherichia coli bacteria (strain M15, Quiguen) were transformed with this construct and cultured until an OD of 0.6–0.8 was reached, when the expression of the recombinant Heat Shock was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Biomol, Hamburg, Germany) for 5 h. Recombinant Heat Shock was present in inclusion bodies and purified by using a Ni-NTA-agarose column (Qiagen) according to the manufacturer’s instructions. Fractions containing recombinant Heat Shock were dialyzed against PBS at 4°C for 14–16 h. For certain experiments recombinant Heat Shock was FITC-labeled using the
FluoReporter FITC labeling kit (F-6434, Molecular Probes, Mo Bi Tec, Göttingen, Germany) according to the manufacturer’s instructions. Bacteria containing recombinant His-tagged dynein were obtained from Dr. J. Neesen (Division of Human Genetics, University of Göttingen) and purified as above. Recombinant Hsp70 of human origin was purchased from StressGen (SPP-755, StressGen, Biomol). β-Galactosidase (β-gal) from E. coli (G-6008, Sigma, Deisenhofen, Germany) was used as further control protein.

Loading of cells with proteins by electroporation
For protein loading (15), 0.5 ml of HEPES-buffered DMEM/1% FCS containing 5 × 10^5 LEW.11/Gun (RT1^b) spleen cells. Ten to 14 days later lymphocytes from regional lymph nodes were resuspended in vitro for 5 days by cocultivation at 0.75 × 10^5 responder cells/well of round-bottom microtiter plates (Nunc, Wiesbaden, Germany) with 0.75 × 10^5 irradiated (30 Gy) lymph node cells from LEW.11/Gun rats in 200 μl of NaHCO3-buffered DMEM, supplemented with 10% FCS, pteryric acid, penicillin, streptomycin, 10^-7 M 2-ME, and 50 μl of supernatant from Con A-stimulated rat lymphocytes.

Chromome release assay
Target cells were labeled by incubating 1 × 10^6 cells in 350 μl HEPES-buffered DMEM containing 5% FCS and 50 μl 10^-3 M O2 (ICN, Eschwege, Germany) for 1 h at 37°C and washed three times with HEPES-buffered DMEM. Effector cells were added to 10^-3 Cr-labeled target cells in triplicate at ratios of 100:1 to 1:1 in round-bottom microtiter plates in 200 μl HEPES-buffered DMEM/10% FCS per well, centrifuged for 5 min at 40 × g, and incubated at 37°C for 4 h. After centrifugation for 5 min, radioactivity was determined in supernatant and sediment separately for each well using a MicroBeta Trilux counter (Wallac, Freiburg, Germany). Percentage of specific lysis was determined by subtracting percent spontaneous 3^-Cr release, which is between 10 and 15%. The SD of specific lysis in triplicate cultures was usually below 5%. In inhibition experiments anti-CD8b mAb (clone 341, mouse IgG1) was added to the cytotoxicity test, with anti-yt6TCR mAb (clone V65, mouse IgG1) serving as isotype control. Both mAbs were used as 1:20 or 1:100 diluted hybridoma supernatants (Dr. T. Herrmann, Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany). In some experiments 2 mM EGTA and 4 mM MgCl2 were added to inhibit calcium-dependent killing. Furthermore, the following inhibitors of caspases were used: Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM. Furthermore, the following inhibitors of caspases were used: Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM (Z-Val-Ala-Asp(OMe)-CH2 F; inhibitor of caspase-1-like proteases, 40 μM. Inhibition experiments were usually performed in triplicate and presented as percentage of specific lysis.

Flow cytometry
Intracellular Hsp70 was determined by flow cytometry after cytoplasmic staining as described previously (16) and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using Lys II or CellQuest software.

MHC class I cell surface expression was determined with anti-RT1.A^* mAb (clone NR3/31, rat IgG2a, MCA12, Serotec, Biozol, Eching, Germany) at a dilution of 1:50 and anti-RT1.A^* mAb (clone B5, rat IgM, 22371D, Pharmingen, Hamburg, Germany) at a concentration of 1 μg/10^6 cells. Cell surface expression of adhesion molecules CD54 (ICAM-1) and CD11a (LFA-1) was determined by mAbs 1A29 (mouse IgG, MCA773, Serotec, Biozol) and WT.1 (mouse IgG, MCA774, Serotec, Biozol), respectively, at a concentration of 1 μg/10^6 cells. Cells were washed twice with PBS in 5-ml polystyrene tubes, resuspended in 200 μl PBS containing the primary Ab, and incubated for 1 h at 4°C. After washing with PBS, cells were resuspended in 200 μl PBS containing as secondary reagents 2 μl of FITC-conjugated goat anti-mouse IgG (115-095-062, The Jackson Laboratory, Bar Harbor, ME; Dianova, Hamburg, Germany) or biotin-conjugated goat anti-rat IgG 1:500 (112-095-068, The Jackson Laboratory; Dianova). After incubation at 4°C for 1 h in the dark cells were washed twice with PBS, resuspended for 15 min in 500 μl PBS containing 10 μg/ml propidium iodide (PI) and analyzed. Cells not treated with Abs or with secondary reagent only served as controls. PI-positive dead cells were excluded from analysis.

The DNA content of hybrid cell clones and the rate of apoptotic cells appeared in the sub G 1 peak of DNA histograms were determined as described (17). In some experiments the TUNEL test was performed in parallel to assess DNA fragmentation (APO-BrDU, Pharmingen) according to the manufacturer’s instructions. A very good correlation of the TUNEL technique and sub-G 1 peak determination was observed.

Exposure of phosphatidylserine as a membrane parameter of apoptosis was determined by staining cells in binding buffer (110 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2) with 5 μl annexin V-FITC or annexin V-PE (Pharmingen) in combination with PI or 7-aminocinomycin D to distinguish apoptotic from already dead cells.

Sorting of Hsp70-loaded cells
Cells loaded with FITC-labeled Hsp70 were separated into Hsp70-positive and a Hsp70-negative fractions by FACS (FACS Vantage, Becton Dickinson).

Separation of apoptotic and nonapoptotic cells
Apoptotic cells were separated magnetically from nonapoptotic cells after staining with annexin V microbeads using the apoptotic cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Positively selected apoptotic cells and the negative fractions containing nonapoptotic cells were used as targets in the chromat cell release assay. Efficiency of selection was tested by flow cytometry as described above.

Immunoblot
Supernatant of cell lysates were prepared and separated by SDS-PAGE as described (16). Proteins were transferred to nitrocellulose (Schleicher & Schüll, Dassel, Germany) and stained with one of the following mAbs diluted 1:2000 in PBS/0.5% Tween 20: anti-Hsp70 (clone C29F3A-5, mouse IgG, SAP-810, StressGen, Biomol), anti-Hsp70/Hsc70 (clone N27F3-3, mouse IgG1, SAP-820, StressGen, Biomol), anti-Grp75 (clone 30A5, mouse IgG1, SPA-825, StressGen, Biomol), anti-Hsp60 (clone KL-1, mouse IgG1, SPA-806, StressGen, Biomol), anti-RGS-His (mouse IgG1, 34610, Qiagen), and anti-β-actin (clone AC-15, mouse IgG1, A-5441, Sigma). Subsequently blots were incubated with goat anti-mouse IgG Ig (115-005-003, The Jackson Laboratory; Dianova) and peroxidase-conjugated rabbit anti-goat IgG Ig (305-035-045, The Jackson Laboratory; Dianova) at dilutions of 1:10,000. The substrate reaction was conducted with 0.05% 3,3′-diaminobenzidine/0.03% H2O2 in PBS/0.5% Tween 20.

Gene probes
Hybridization probes specific for MHC-linked rat Hsp70-1 (positions +2875 to +3070; GenBank accession no. X77207) and Hsp70-2 (positions +3174 to +3322; GenBank accession no. X77208) were derived from the flanking limited region of the respective genes by genomic PCR amplification. A probe containing the coding part of a human Hsp70 gene (18) and a Hsp70-specific probe (19) have been described previously. The human β-actin cDNA was purchased from Clontech (Heidelberg, Germany).

Northern blot analysis
RNA was prepared according to Chomczynski and Sacchi (20), separated electrophoretically on denaturing 1.6% agarose gels, transferred onto nitrocellulose (Schleicher & Schüll), and cross-linked by UV irradiation. Blots were hybridized under stringent conditions with [32P]CTP-labeled probes (16) and exposed to Hyperfilm MP (Amersham Pharmacia) at −70°C. Before rehybridization, blots were washed with 0.1% SDS at 95°C until complete removal of the probe.

Histochemical detection of β-gal
Cells were fixed in 2% paraformaldehyde/PBS for 10 min, washed with PBS, and incubated for 14–18 h with 1 mg/ml 5-bromo-4-chloro-3-indolyl β-galactosidase (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl2 in PBS (21). At least 200 cells were counted microscopically to determine the percentage of blue-stained β-gal-positive cells.

Results
Heat shock-induced resistance to CTL in Hsp70-defective Y3 cells
The effect of heat shock on the susceptibility of Y3 myeloma cells to allospecific CTL was tested in a 51Cr release assay (Fig. 1A). A
representative experiment is shown in Fig. 1. Heat shock of 1 h at 42°C or of 30 min at 44°C followed by a 6-h recovery period induced resistance to lysis, confirming data reported earlier (6). Y3 cells are Hsp70 defective and do not express Hsp70-1 and Hsp70-2 transcripts or protein after both heat shock protocols, whereas the activity of the constitutively expressed Hsc70 gene can be detected at the RNA and protein level (Fig. 1, C and D). Lysis of Y3 target cells by cytotoxic cells could be inhibited by anti-CD8b mAb (Fig. 2A). Thus, the predominant cytotoxic effector cells are CD8\(^{+}\) CTL.

Because cytotoxic activity could also be inhibited almost completely by EGTA (Fig. 2B), killing is calcium dependent. Caspase inhibitors Z-VAD-FMK, Ac-YVAD-CHO, and Z-DEVD-FMK had no effect (Fig. 2B). Thus, the granzyme/perforine, but not the Fas, pathway seems to be predominant in killing Y3 cells.

A strong non-Hsp70-inducing heat shock leads to resistance of Con A-stimulated lymphocytes

Normal and Con A-stimulated rat lymphocytes express Hsp70 after a heat shock of 1 h at 42°C, of 30 min at 43°C, or of 10 min at 44°C when assayed after recovery periods of 4–10 h by immunoblot analysis with mAb C92 specific for inducible Hsp70 (data not shown). Surprisingly, rat lymphocytes did not express Hsp70 following a heat shock of 30 min at 44°C (Fig. 3A). Therefore, lysisability of Con A-stimulated lymphocytes by CTL was compared after a Hsp70-inducing heat shock (1 h at 42°C) and a non-Hsp70-inducing heat shock (30 min at 44°C). Resistance to CTL occurred after the non-Hsp70-inducing heat shock (Fig. 3B).
A non-Hsp70-inducing heat shock results in resistance of Y3 × lymphocyte somatic cell hybrids

To study further the effect of Hsp70 expression on heat shock-induced resistance, somatic cell hybrid clones between Y3 cells and syngeneic (LOU/CGun, RT1u) or allogeneic (BUF/Gun, RT1b) lymphocytes were established. The hybrid nature of the clones was proven by HAT resistance, increased DNA content, and, in the case of allogeneic hybrids, by the expression of partner-derived MHC Ags. The DNA content of hybrid cell clones was usually higher compared with parental Y3 cells (Table I). The presence of the MHC of the fusion partner is especially informative, because the two major heat-inducible Hsp70 genes Hsp70-1 and Hsp70-2 are localized in the MHC (22).

Two types of hybrids could be distinguished: hybrids that fail to express Hsp70 in general, and hybrids that express Hsp70 protein following a heat shock of 1 h at 42°C, but not after a heat shock of 30 min at 44°C (Table I). It is noteworthy that most hybrids showed Hsp70 expression at the mRNA level also after severe heat shock at 44°C (Table I). Thus, translation of Hsp70 mRNA appears to be more sensitive to severe heat shock than transcription.

The hybrid cell clones were tested for their susceptibility to allospecific CTL directed against the RT1u gene products. Each hybrid clone was susceptible to lysis by CTL and became resistant to CTL-mediated killing after a non-Hsp70-inducing heat shock (Table I). The most informative result was obtained with hybrid cell clones that express Hsp70 after a 42°C heat shock, but not after a 44°C heat shock. These cells developed no or only weak resistance after the Hsp70-inducing 42°C heat shock, but were clearly less susceptible to CTL after a heat shock of 44°C that did not induce Hsp70 (Table I). Hybrids that failed to express Hsp70 after a 42°C heat shock became resistant like Y3 cells (Table I). Thus, heat shock-induced resistance to CTL-mediated lysis appears to be inversely correlated with the presence of Hsp70 in the target cells. Other heat shock proteins such as Hsc70, Grp75, or Hsp60 were present to a similar degree in controls as well as after both types of heat shock treatment (data not shown).

Table I. Analysis of Y3 × rat lymphocyte hybrid cell clones for hybrid status (HAT resistance, DNA content, RT1.A cell surface expression), heat-induced Hsp70 RNA and protein expression, and heat-induced resistance to CTL

<table>
<thead>
<tr>
<th>Clone</th>
<th>HAT Resistance</th>
<th>DNA Contenta (G1 peak channel)</th>
<th>RT1.Aa</th>
<th>RT1.Ab</th>
<th>Hsp70 RNAb</th>
<th>Hsp70 Proteinb</th>
<th>Resistance to CTLb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y3</td>
<td>−</td>
<td>100</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>111 B9b</td>
<td>+</td>
<td>107 (9)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>111 C10b</td>
<td>+</td>
<td>91 (5)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>111 D9</td>
<td>+</td>
<td>178 (1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>111 E4</td>
<td>+</td>
<td>179 (10)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>111 E11</td>
<td>+</td>
<td>185 (15)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>111 G12</td>
<td>+</td>
<td>178 (12)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>137 1F12</td>
<td>+</td>
<td>152 (7)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>137 2B2</td>
<td>+</td>
<td>172 (14)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>137 2H4</td>
<td>+</td>
<td>149 (13)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>137 A1</td>
<td>+</td>
<td>135 (7)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NT†</td>
</tr>
<tr>
<td>137 A2</td>
<td>+</td>
<td>157 (6)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NT†</td>
</tr>
<tr>
<td>137 A4</td>
<td>+</td>
<td>126 (5)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NT†</td>
</tr>
<tr>
<td>137 A5</td>
<td>+</td>
<td>143 (4)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NT†</td>
</tr>
</tbody>
</table>

a The DNA content was determined by flow cytometry as described in Materials and Methods. The G1 peak channel of Y3 cells was adjusted to 100. Mean and SD of three independent experiments are given.

b Heat shock conditions were 1 h at 42°C or 30 min at 44°C followed by a recovery period of 6 h at 37°C.

† Fusion 111 was between Y3 cells (RT1u) and LOU/CGun lymphocytes (RT1u) and fusion 137 between Y3 cells (RT1u) and BUF/Gun lymphocytes (RT1b).

For the hybrid cell clones printed in bold the Hsp70 expression and CTL resistance phenotypes were assayed in at least three independent experiments with essentially the same results.

† NT, not tested.
recovery cells were labeled with $^{51}$Cr for 1 h, washed, and exposed to CTL for 4 h (Fig. 4A). Y3 cells loaded with the control proteins β-gal (Fig. 4, B and D) or His-tagged dynein (Fig. 4C) became resistant to CTL, whereas Y3 cells loaded with rat Hsp70 were almost as susceptible as non-heat-shocked cells (Fig. 4, B and C). The same effect was obtained with human instead of rat recombinant Hsp70 (Fig. 4D). Thus, prevention of heat shock-induced resistance is a specific effect of Hsp70. Efficient loading of Y3 cells with Hsp70 was proven by flow cytometry after intracellular staining (Fig. 5A) and immunoblot with Hsp70 (Fig. 5, B and C) or His-tag specific mAb (Fig. 5B). Loading of dynein protein was detected by immunoblot with anti-His-tag mAb (Fig. 5B), and
β-gal loading was monitored histochemically by cleavage of the substrate X-Gal (Fig. 5D).

Indirect intracellular staining of Hsp70 (Fig. 5A) indicated that not all cells electroporated with Hsp70 might be loaded sufficiently. To separate loaded from unloaded cells by FACS sorting, FITC-labeled recombinant Hsp70 was used for loading. The fraction of positive cells was between 61 and 92% in six experiments. Positive and negative cells were sorted (Fig. 6A) in two independent experiments. Hsp70-negative cells developed resistance whereas Hsp70-positive cells remained fully susceptible to CTL after heat shock (Fig. 6B). The use of FITC-labeled Hsp70 appeared to result in a higher percentage of loaded cells (61–92%) than the use of unlabeled Hsp70 (42–59%, see Fig. 5A). This discrepancy is most likely only apparent and due to the fact that in experiments where Hsp70 was detected indirectly the secondary Ab reacts strongly with Y3 cells. Therefore, the number of loaded
cells is underestimated and in fact presumably in these experiments in the range of 80%. This assumption is supported by the efficiency of β-gal loading being in the 80% range (Fig. 5D).

**Hsp70 loading does not affect expression of MHC class I and adhesion molecules**

Heat shock for 30 min at 44°C lead to a slight decrease of MHC class I molecules (Table II). Expression of adhesion molecules CD11a (LFA-1) and CD54 (ICAM-1) remained unaltered after heat shock (Table II). Loading with Hsp70 or β-gal did not affect MHC class I, CD54, and CD11a expression. Notably, the slight decrease of MHC class I cell surface expression occurring after severe heat shock (30 min at 44°C) was not reversed by Hsp70 loading (Table II).

**Hsp70 loading does not protect against heat shock-induced apoptosis**

The percentage of dead cells monitored by PI or trypan blue staining was low after both types of heat shock treatment (Table II). Heat shock of 30 min at 44°C leads to more apoptotic cells than heat shock of 1 h at 42°C, as is evident from annexin V staining (Table II), sub-G₁ analysis (Table II), and TUNEL test (data not shown). Loading of Y3 cells with Hsp70 or β-gal did not affect the occurrence of heat shock-induced apoptosis when tested 6 h after protein loading and 4 h after heat shock. Thus, the recombinant Hsp70 does not confer resistance to heat shock-induced apoptosis in Y3 cells.

**Heat shock-induced resistance occurs also in nonapoptotic Y3 cells**

Annexin V-positive (apoptotic) and annexin V-negative (nonapoptotic) Y3 cells were separated magnetically after heat shock (Fig. 7, A and B) and used as target cells. Heat shock-induced resistance to CTL clearly occurred in Y3 cells depleted of apoptotic cells (Fig. 7C). Heat-shocked cells that were enriched for apoptotic cells became also more resistant to CTL than annexin V-positive, nonheat-shocked cells, but these experiments were less reliable due a high spontaneous ⁵¹Cr release of up to 35% (data not shown). Thus, heat shock-induced apoptosis and subsequent selective survival of nonapoptotic cells was not responsible for the heat shock-induced resistance to CTL.

**Table II. Analysis of cell surface molecule expression and apoptosis in Y3 cells after heat shock and protein loading**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>MHC Class I</th>
<th>CD11α*</th>
<th>CD54*</th>
<th>PI Positive</th>
<th>An pos/PI neg</th>
<th>Sub-G₁ DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIF (SD)b</td>
<td>% (SD)c</td>
<td>n²</td>
<td>MIF (SD)</td>
<td>n</td>
<td>MIF (SD)</td>
</tr>
<tr>
<td>37°C 1 h at 42°C</td>
<td>114.7 (17.5)</td>
<td>88 (7)</td>
<td>6</td>
<td>6.5 (2.0)</td>
<td>3</td>
<td>82.1 (14.0)</td>
</tr>
<tr>
<td>30 min at 44°C</td>
<td>89.5 (19.4)</td>
<td>86 (6)</td>
<td>6</td>
<td>10.3 (3.2)</td>
<td>3</td>
<td>80.3 (12.9)</td>
</tr>
<tr>
<td>β-gal, 37°C</td>
<td>117.8 (15.3)</td>
<td>90 (2)</td>
<td>6</td>
<td>14.7 (6.4)</td>
<td>3</td>
<td>93.9 (15.5)</td>
</tr>
<tr>
<td>β-gal, 30 min at 44°C</td>
<td>87.2 (12.7)</td>
<td>85 (4)</td>
<td>6</td>
<td>11.8 (2.4)</td>
<td>3</td>
<td>92.0 (12.1)</td>
</tr>
<tr>
<td>Hsp70, 37°C</td>
<td>115.6 (17.8)</td>
<td>89 (5)</td>
<td>6</td>
<td>11.8 (3.8)</td>
<td>3</td>
<td>84.7 (11.7)</td>
</tr>
<tr>
<td>Hsp70, 30 min at 44°C</td>
<td>83.5 (18.2)</td>
<td>84 (6)</td>
<td>6</td>
<td>12.1 (4.4)</td>
<td>3</td>
<td>86.2 (10.1)</td>
</tr>
</tbody>
</table>

* Analysis was performed 6 h after begin of the treatment according to the experimental schedules depicted in Figs. 1A and 4A, respectively.

b Mean intensity of fluorescence (MIF).

c Percentage of positive cells.

² Number of independent experiments.

d The percentage of positive cells was in the range of 55% for all treatments.

e The percentage of positive cells was in the range of 90% for all treatments.

f Annexin V-positive PI-negative apoptotic cells.

g Cells appearing in the sub-G₁ peak of DNA histograms due to apoptotic DNA fragmentation.
Cells of the rat myeloma line Y3 are defective in the expression of the major heat shock-induced Hsp70 genes that are encoded in the MHC. After heat shock, these cells become resistant to the cytotoxic effect of alloreactive CTL (6). We now show that Hsp70 is able to prevent the induction of the resistant phenotype. The evidence for this conclusion is threefold. 1) In Con A-stimulated lymphocytes and 2) in somatic cell hybrids between Y3 cells and lymphocytes. In both systems, lymphocytes and somatic cell hybrids, inducibility of resistance to CTL by heat shock, and absence of Hsp70 expression were reproducibly correlated. It is noteworthy that in human lymphocytes Hsp70 induction could be observed under each of the heat shock conditions tested for rat lymphocytes including 30 min at 44°C (our unpublished data). However, after a more severe heat shock, e.g., 1 h at 44°C or 45°C, also in human lymphocytes Hsp70 induction was not observed any longer when assayed by flow cytometry (24).

To investigate whether indeed Hsp70 prevents the development of resistance to CTL Y3 cells were loaded with recombinant Hsp70 by electroporation before heat shock treatment. Furthermore, Y3 cells were sorted into Hsp70-positive and Hsp70-negative fractions after loading with FITC-labeled Hsp70. Also, in these experiments the presence of Hsp70 abrogated the development of heat shock-induced resistance to CTL. The recombinant Hsp70 used for supplementation is encoded by the MHC-linked Hsp70-1 and Hsp70-2 genes that are not expressed in Y3 cells. The supplementation effect is not due to loading with proteins in general and is not caused by the His-tag of the recombinant Hsp70 or by contaminating proteins, because control proteins used for loading by electroporation did not affect the development of heat shock-induced resistance. Recombinant His-tagged dynemin, which was prepared by the same procedure as recombinant His-tagged Hsp70 and β-gal protein did not affect the resistance phenotype. On the other hand, human Hsp70 that is 97% identical to rat Hsp70 at the amino acid level (22) and does not contain a His-tag had the same effect as rat Hsp70. Thus, it can be concluded that the products of the MHC-linked heat inducible Hsp70 genes are involved in regulating lysability of target cells by CTL.

It is unlikely that the slight decrease of MHC class I expression on Y3 cells after severe heat shock (30 min at 44°C) is responsible for the resistance phenomenon. Hsp70 loading prevented resistance after heat shock without reverting the slightly decreased MHC class I expression. In addition, in cold target inhibition experiments Y3 cells made resistant by heat shock were as effective as nonresistant cells (Ref. 6 and our unpublished observations). A decrease of MHC class I molecules has been described to occur in some human melanoma cell lines after long and severe heat shock treatment without effect on susceptibility to NK cell cytotoxicity (25). Furthermore, adhesion molecules CD11a and CD54 are not responsible for resistance of Y3 cells after heat shock, because their cell surface expression was not changed.

The lysis-promoting effect reported here for Hsp70 is at variance with the commonly described protective effect of heat shock proteins in general and of Hsp70 in particular when cells are exposed to cytotoxic mechanisms (8, 26–28). However, some reports show that heat shock can also increase susceptibility to lysis by NK or LAK cells (29, 30). A death-promoting effect of Hsp70 itself has been described after TCR/CD3 or CD95 activation in Hsp70-transfected Jurkat cells (31). In acute myeloid leukemia cells apoptosis correlated with the intracellular Hsp70 level (32). Thus, the finding reported here is not without precedent. Together, these data challenge the view that Hsp70 is generally a cell death-preventing protein (8). A particular mechanism of how Hsp70 can increase target cell lysis has been described for some tumor cell lines that express Hsp70 on their cell surface. Recognition and lysis by NK or LAK cells is then improved (33). The target cells studied here did not express Hsp70 on the cell surface and were not susceptible to NK cell cytotoxicity (our unpublished data).
The allospecific cytotoxic cells that mediate lysis of the Y3 cells and somatic cell hybrids used in this study are CTL as is evident from inhibition by anti-CD8 mAb. The main mechanism of killing appears to be mediated by the granule exocytosis pathway, because it is calcium dependent and not affected by caspase inhibitors. Exocytosis of CTL granules is generally thought to induce apoptosis in the target cell (34). Granzymes are assumed to activate late phases of apoptosis signaling. The pathway that actually leads to cell death appears to be independent of caspases, but the proteins involved are not yet known (35). Granzyme B has been shown to cleave directly several downstream caspase substrates (36), and granzyme A induces a distinct pathway of apoptosis in target cells (37, 38).

We hypothesize that Hsp70 interferes with apoptotic pathways that are induced in the target cells by CTL due to granule exocytosis. Functioning as a molecular chaperone Hsp70 might “protect” certain proteins that are necessary for granule-mediated killing of the target cell. If Hsp70 is lacking, the respective protein might loose its function due to heat shock-mediated denaturation. Killing by CTL is then impaired, so that the resistant phenotype occurs. It is of interest that granzyme A has been shown to be bound by Hsp27 and Hsp70 (39), indicating that Hsp70 indeed is involved in CTL-induced cell death. In Y3 cells Hsp70 does not protect against apoptosis that is induced by heat shock, as is shown in our experiments (Table II), but only against apoptosis that is additionally elicited by CTL. This observation implies that the pathway of CTL-induced cell death should differ from that of heat shock-induced apoptosis. In additional experiments relevant target proteins of Hsp70 during CTL-induced cell death will have to be identified.

Our findings may be relevant for the interpretation of some experimental tumor models. Because of its protective role, Hsp70 is expected to favor tumor growth, as has indeed been shown (40). On the other hand, Hsp70 expression was found associated with enhanced tumor immunogenicity and regression in vivo (41-43). Furthermore, Hsp70, among other chaperones, has been shown to bind antigenic peptides and to elicit tumor immunity after vaccination (44). The chaperoned peptides can be channeled into the endogenous class I presentation pathway of APC (45), which are then able to prime CD8+ CTL. Thus, tumors might elicit a specific immune response by HspS released from dying tumor cells. One might expect that a cytotoxic effect of Hsp70 would counteract the effectiveness of the CTL response. This apparent contradiction may be resolved by the data reported here, showing that Hsp70 need not confer protection against the CTL-mediated cytotoxicity but instead can improve lysisability of target cells after stress.

Acknowledgments

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References


