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Heat-Induced Expression of MHC-Linked HSP70 Genes in Lymphocytes Varies at the Single-cell Level

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Abstract The expression of MHC-linked heat shock protein 70 (HSP70) genes HSP70–1 and HSP70–2 has been studied in human and rat lymphocytes and Concanavalin A (con A)-induced lymphoblasts by in situ hybridization and flow cytometry. In in vitro experiments transcripts of these genes were observed only after heat shock, and mitogen stimulation per se did not lead to induction. Heat shock-induced expression of HSP70–1 and HSP70–2 mRNA varied at the single-cell level. The fraction of HSP70-positive lymphocytes increased continuously with the severity of heat shock. However, 15–35% of the cells always remained HSP70 transcript negative. After fever induction in vivo similar variation of HSP70–1 and HSP70–2 mRNA expression could also be observed. Analysis of heat shocked lymphocytes by flow cytometry demonstrated that HSP70 induction varied also at the protein level, a fraction of cells always remaining unresponsive. Thus, HSP70 induction does not appear to occur in each cell and to a similar extent when a given cell population is exposed to the same stress. J. Cell. Biochem. 72:558–569, 1999.

Key words: heat shock; stress response; apoptosis; gene expression; in situ hybridization; flow cytometry; mitogen stimulation

Heat shock proteins (HSP), a highly conserved group of proteins, are organized in families according to their relative molecular mass of about 100, 90, 70, 60, and 20 kDa, respectively. The HSP70 family includes the constitutively expressed cytosolic heat shock cognate protein 70 (HSC70), the heat-inducible cytosolic protein HSP70, the glucose-regulated protein 78 (GRP78) of the endoplasmatic reticulum, and the mitochondrial GRP75. These proteins perform essential chaperoning functions during synthesis, transport, assembly, renaturation, and degradation of proteins in the cell [Gething and Sambrook, 1992]. The most prominent proteins induced by heat shock belong to the HSP70 family. HSP70 expression after heat shock and under other stressful conditions [Li and Werb, 1982] or due to transfection of HSP70 genes [Angelidis et al., 1991; Li et al., 1991] has been correlated with the acquisition of thermotolerance, a state of transient resistance of the cell against a second more severe stress.

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Several genes are known to encode heat-inducible HSP70. In human two HSP70 genes, HSP70–1 (official designation HSPA1A) and HSP70–2 (HSPA1B), coding for identical proteins are located in the major histocompatibility complex (MHC) on chromosome 6, together with a third HSP70 gene, HSP70-HOM (HSPA1L) [Milner and Campbell, 1990], which encodes a testis-specific member of the HSP70 family [Fujimoto et al., 1992]. Also in serveral other species, including rat [Wurst et al., 1989; Walter et al., 1994] and mouse [Gaskins et al., 1990], three HSP70 genes are found in the MHC [reviewed in Günther and Walter, 1994]. In human two further heat-inducible HSP70 genes are known that are localized on chromosome 1, HSP70-B′ (HSPA6) [Leung et al., 1990] and HSP70B (HSPA7) [Voellmy et al., 1985; Leung et al., 1992]. Because of the high degree of homology, these proteins are so far not sufficiently distinguishable by monoclonal antibodies.

Although HSP70 expression has been studied extensively [reviewed in Perdrizet 1997] reports on the induction of distinct HSP70 genes and on their expression at the single-cell level are rare. In this study, expression of the three MHC-linked HSP70 genes has been investigated in normal and mitogen-stimulated lym-

phocytes of rat and human origin at the RNA level by in situ hybridization and in part at the protein level by flow cytometry.

MATERIALS AND METHODS

Cell Isolation and Culture

Human peripheral blood mononuclear cells were isolated from heparinized blood of healthy young donors by density gradient centrifugation on Ficoll-Hypaque (Biochrom, Berlin, Germany). These cells were 95% lymphocytes, as they were CD3, CD19, or CD56 positive in flow cytometric analysis. After three washes in Hepes-buffered Dulbecco's modified Eagle medium (DMEM) (Biochrom) cells were cultured in $NaHCO_3$-buffered DMEM supplemented with 10% fetal calf serum (FCS) (Biochrom) at 2×10^6 cells/ml. For induction of proliferation 5 µg/ml Concanavalin A (con A)(Pharmacia, Freiburg, Germany) were added to the cell culture. Rat lymphocytes were prepared from lymph nodes as described [Günther and Wurst, 1984] and cultured as above. Three-month-old rats of inbred strains BUF/Gun, LEW.1A/Gun, LEW/Gun, and LOU/CGun, bred in our own colony under conventional conditions, were used.

Heat Shock Conditions and Apoptosis Assays

For the induction of a heat shock response, 2×10^6 cells in 13-ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) were incubated in a fine-regulated water bath (Julabo VC, Schütt, Göttingen, Germany) for 1 h at a temperature indicated in the results section. The fraction of dead cells (trypan blue or propidium iodide positive) before start of mRNA and protein analysis, but after heat shock up to 42°C, was always below 5%. The terminal deoxytransferase-mediated deoxyuridine 5′triphosphate nick end labeling (TUNEL) technique and the annexin V/7-amino-actinomycin D (7-AAD) assay were employed to determine the fraction of apoptotic cells before and after heat shock using test kits for flow cytometry (APO-BRDU, Annexin V-PE, Pharmingen, Hamburg, Germany) according to the manufacturer's instruction. After heat shock up to 42°C, always less than 5% apoptotic cells were detected.

Gene Probes

Specific hybridization probes were derived from the 3′ untranslated region of the human HSP70–1, HSP70–2, and HSP70-HOM genes (positions 2143–2301, 2225–2407, 2902–3242, respectively) [Milner and Campbell, 1990] and of the corresponding rat genes HSP70–1, HSP70–2, and HSP70–3 (positions 2143–2301, 1927–2075, 1992–2182, respectively) [Walter et al., 1994; Rothermel et al., 1994] by genomic polymerase chain reaction (PCR) amplification and cloning of the resulting gene fragments into pCRII (Invitrogen, Heidelberg, Germany) or pSPT18 (Boehringer, Mannheim, Germany). Rat HSC70 transcripts were specifically detected by a 508-bp *Sst*I fragment subcloned from rat cosmid pKE (positions 1383–1890) [Rothermel et al., 1995]. For the sake of simplicity, also rat genes will be written in capital contrary to official nomenclature.

In Situ Hybridization

Lymphocytes were resuspended in phosphate-buffered saline (PBS) at a concentration of 2×10^7/ml and spread on glass slides precoated with poly-L-lysine (Sigma, Deisenhofen, Germany). Slides were fixed with 4% paraformaldehyde (Fluka, Neu-Ulm, Germany) for 20 min, washed in PBS, dehydrated in graded ethanol, and then stored at −70°C. Sense and antisense riboprobes were transcribed from linearized vector-insert constructs in the presence of [^{35}S]UTP (Amersham Buchler, Braunschweig, Germany) using SP6 and T7 RNA polymerases (Boehringer). The transcripts were precipitated with ammonium acetate and ethanol in order to remove free nucleotides. The ^{35}S-labeled riboprobes were diluted to a specific activity of 5×10^4 cpm/µl in a hybridization buffer containing 300 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM sodium phosphate pH 6.8, 5 mM EDTA pH 8.0, 1 × Denhardt's solution, 10% dextran sulfate, 100 mM DTT, 50% deionized formamide, 3 µg/µl tRNA (*Escherichia coli*), 10 µM α-thio-UTP and 0.5 µg/µl heparin. Hybridization was performed in a wet chamber (50% formamide, 5 × SSC) overnight at 45°C. Parts of each slide were hybridized with antisense and corresponding sense probes, respectively. Slides were washed 4 times at 45 °C for 30 min in 50% formamide, 2 × SSC, 10 mM β-mercaptoethanol and incubated for 30 min at 37°C in buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) containing 8 µg/ml RNase A. Slides hybridized with human HSP70 or rat HSC70 probes were washed 4 times at 60°C for 30 min in 2 × SSC, 10 mM β-mercaptoethanol and twice for 30 min in 0.1 × SSC, 10 mM β-mercaptoethanol. Slides hybridized with rat HSP70 probes were washed 4 times at 55°C for 30 min

in 2 × SSC, 10 mM β-mercaptoethanol and twice for 30 min in 1 × SSC, 10 mM β-mercaptoethanol. After incubation for 10 min in 0.1 × SSC at room temperature slides were dehydrated in graded ethanol containing 0.3 M ammonium acetate, air dried, and covered with photoemulsion (Kodak, Rochester, NY). After 4 weeks of exposure at 4°C slides were developed, Giemsa stained and embedded in Eukitt (Kindler, Freiburg, Germany). For quantification, the number of grains/cell of about 200–300 cells was determined from microphotographs focused to the cell nuclei and encompassing an area of twice the cell size. In sense controls, no cell was found directly associated with >3 grains. Therefore, cells associated with >3 grains after antisense hybridization were scored positive. It should be noted that in positive slides a certain "background" of grains can be regularly found due to RNA release from cells [Berger, 1986].

Patients and Induction of Fever

Induction of heat shock response was tested in patients undergoing fever therapy for metastatic melanoma in the Department of Dermatology, University Hospital Göttingen [Dressel et al., 1996]. Lymphocytes were obtained (see above) after fever induction by injection of bacterial lysates, and induction of HSP70 genes was tested by in situ hybridization as described above. Informed consent was obtained from each patient. The evaluation of HSP70 induction had been approved by the ethics committee of the Medical Faculty of the University of Göttingen.

Flow Cytometry

To analyze HSP70 expression at the protein level in single lymphocytes by flow cytometry cytoplasmic staining was performed in 5-ml polystyrene tubes (Becton Dickinson, Heidelberg, Germany). The cells were washed twice with PBS and resuspended in freshly prepared 1% paraformaldehyde/PBS at a concentration of 1×10^6 cells/ml for fixation and incubated for 10 min at room temperature before they were washed again in PBS and in 0.25% saponin/PBS (Sigma). 1×10^6 cells were resuspended in 200 µl 0.25% saponin/PBS containing 2 µg of a mouse monoclonal antibody specific for the inducible form of HSP70 (clone C92F3A-5, IgG1, SPA-810, Biomol, Hamburg, Germany). In preliminary experiments, increasing the amount of antibody from 1 µg to 2 µg was not found to increase HSP70 staining any more. After 1 h of incubation tubes were filled up with 0.25% saponin/PBS, spun down, and the pellet was resuspended in 200 µl saponin buffer with 4 µg fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (115–096–062, Dianova, Hamburg, Germany). Cells were kept for 1 h at room temperature in the dark, washed in PBS and resuspended in 500 µl before analysis. Cells not treated with antibodies or with secondary antibody only served as controls.

In some experiments with human lymphocytes intracellular staining of HSP70 was followed by cell surface staining of CD3 and CD19 molecules modifying a protocol by Assenmacher [1992]. After washing with PBS the intracellularly stained cells were resuspended in 200 µl PBS containing 1 µg of a tricolor-conjugated mouse anti-human CD3 antibody (clone S4.1, IgG2a, MHCD306, Medac, Hamburg, Germany) and/or 1 µg of a phycoerythin-conjugated mouse anti-human CD19 antibody (clone SJ25-C1, IgG1, MHCD1904, Medac). After incubation for 30 min and washing with PBS, cells were resuspended in 500 µl PBS. The analysis was performed on a FACScan flow cytometer (Becton Dickinson) using Lysis II software.

RESULTS

Expression of MHC-Linked HSP70 Genes in Human and Rat Lymphocytes In Vitro at the mRNA Level

In order to examine mRNA expression of the three MHC-linked HSP70 genes at the single-cell level rat and human lymphocytes were tested by in situ hybridization with gene-specific probes. The results of an individual experiment with human lymphocytes are shown in Figure 1. Before heat shock no HSP70 mRNA expressing cells could be identified. After heat shock up to 42°C, HSP70–1 and HSP70–2, but no HSP70-HOM transcripts, were detectable. Essentially the same results could be obtained with lymphocytes from four different individuals. Heat shock-induced HSP70–1 and HSP70–2 expression in single cells is quantified in Figure 2a. Intensity of gene expression in HSP70-positive lymphocytes determined by the number of grains per cell varied considerably between the cells. Notably, in each experiment 15–35% of heat-shocked cells did not show HSP70–1 and HSP70–2 specific autoradiographic signals (Figs. 1, 2). Rat lymphocytes from four different inbred strains showed the same expression pattern (Fig. 2b) again about 30–40% of heat-shocked lymphocytes remain-

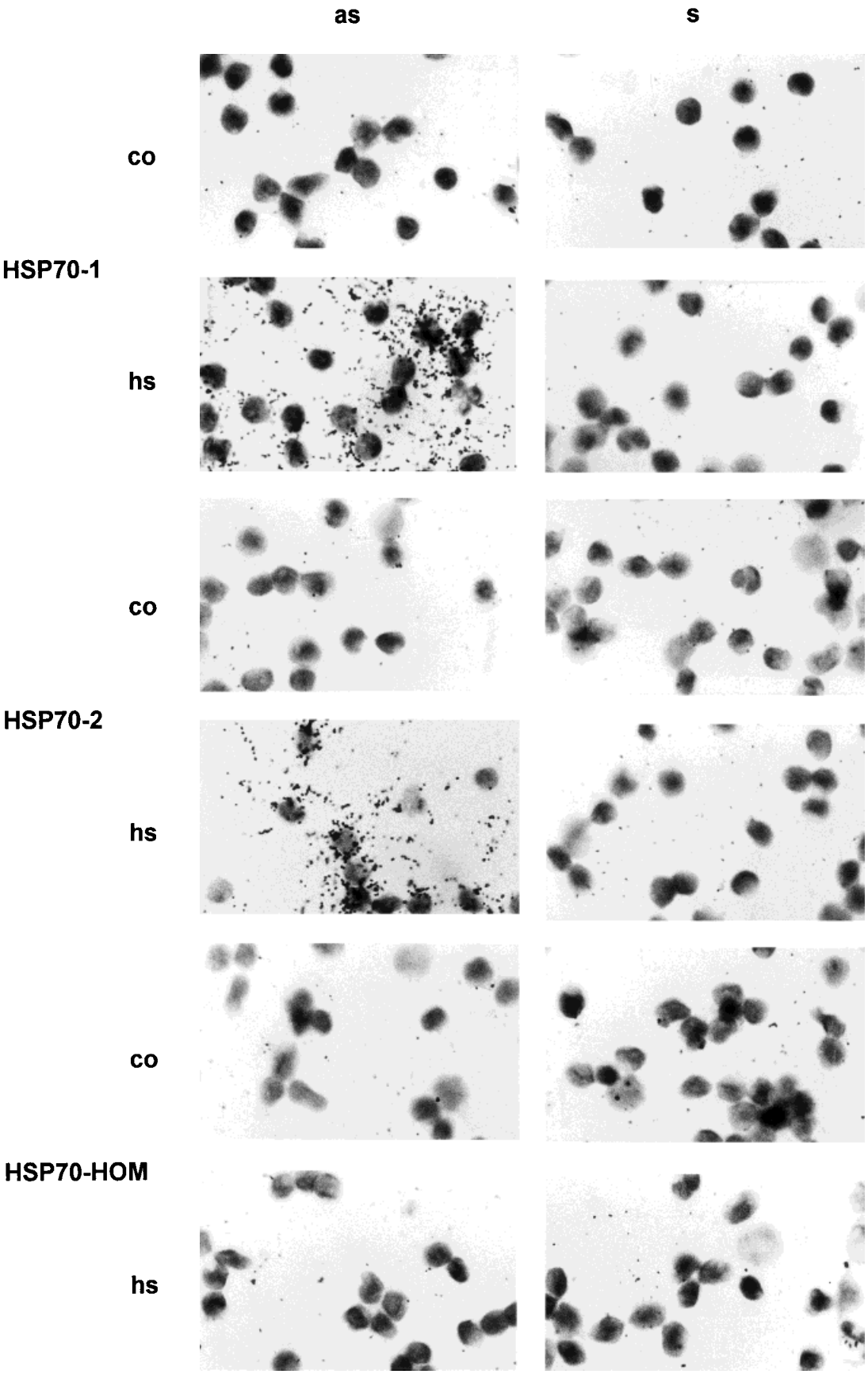


Fig. 1. Expression analysis of the three MHC-linked HSP70 genes by in situ hybridization of human lymphocytes cultivated at 37°C (co) or exposed to a heat shock for 1 h at 42°C (hs). Hybridization with antisense (as) and, as specificity control, sense (s) probes is shown. Autoradiographic signals were quantified as described under Materials and Methods. ×500.

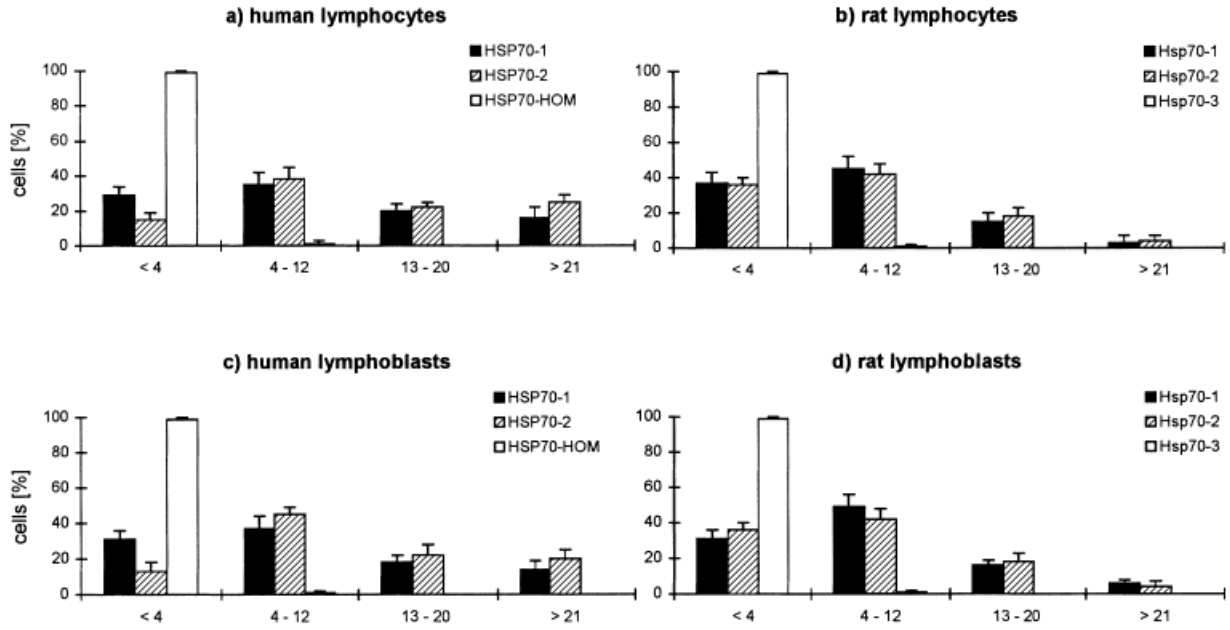


Fig. 2. Quantitative analysis of heat shock-induced HSP70 expression in human **(a,c)** and rat **(b,d)** lymphocytes (a,b) or Concanavalin a (con A)-stimulated lymphocytes (day 4; c,d), by in situ hybridization. Heat-shock conditions were as in Fig. 1. The ordinate represents the fraction of cells in each category defined with respect to the number of grains associated with the cells and indicated below the columns. Cells with <4 grains were scored negative, as these signals could also be observed in sense controls. Mean $\pm$S.D. from four experiments are shown. No grains were detected for HSP70-HOM and HSP70–3 in the 13–20 and >21 categories.

ing HSP70–1 and HSP70–2 mRNA negative. The overall level of HSP70–1 and HSP70–2 expression was slightly weaker in rat than in human lymphocytes.

HSP70 expression has been reported to be changed in lymphocytes by mitogen stimulation [Kaczmarek et al., 1987; Spector et al., 1989]. Therefore, in situ hybridization analysis was carried out after 24, 48, 72, and 96 h of con A stimulation. Induction of any of the three MHC-linked HSP70 genes could not be observed (data not shown), unless the cells were heat shocked at these time points. The results are evaluated quantitatively in Figure 2c,d, and an individual experiment is shown for rat HSP70–1 in Figure 3. HSP70-reactivity varied in the same range as observed for heat-shocked lymphocytes that were not stimulated with mitogen. The proportion of cells showing no HSP70 expression was again 15 to 35% of the heat shocked cells.

It could be argued that HSP70 negativity of a fraction of heat-shocked lymphocytes (Fig. 2) is a methodological artifact due to loss of cytoplasm during the assay procedure. This explanation is, however, ruled out by in situ hybridization results obtained with the constitutively expressed HSC70 mRNA (Fig. 3). HSC70 transcripts were detectable in each single cell, in contrast to HSP70–1 and HSP70–2 (Figs. 1, 3). Furthermore, no accumulation of grains around HSP70 negative cells was observed.

Time and Temperature Kinetics of HSP70–1 and HSP70–2 mRNA Induction in Human Lymphocytes In Vitro

The time course of HSP70–1 and HSP70–2 expression was examined in four lymphocyte samples from different individuals up to 8 h after heat shock of 42°C. Control cells, kept in parallel at 37°C, did not show any induction of HSP70–1 or HSP70–2. Among heat-shocked lymphocytes, the highest proportion of HSP70-positive cells was observed at 1–2 h after heat shock (Fig. 4a). Thereafter, the proportion of positive cells decreased, and control levels of expression were almost reached after 7 h of recovery. There was no time point when all cells reacted with HSP70 expression.

The temperature threshold of HSP70–1 and HSP70–2 mRNA was determined in four lymphocyte samples from different individuals. At 39°C, a significant proportion of cells showed reproducibly transcripts of both genes. Raising the heat shock temperature was associated with a steadily increasing proportion of positive cells. However, even the strongest heat shock applied

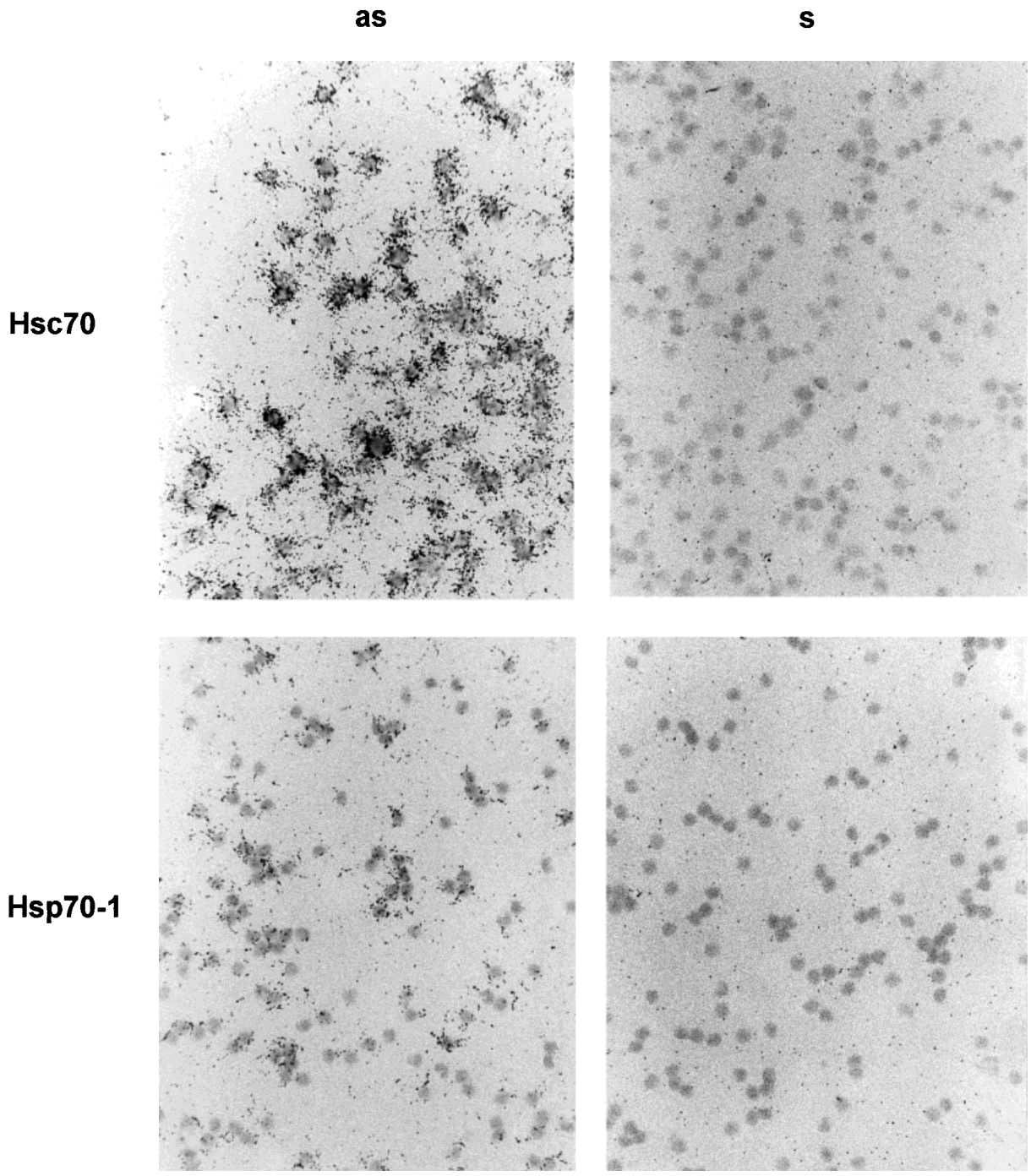
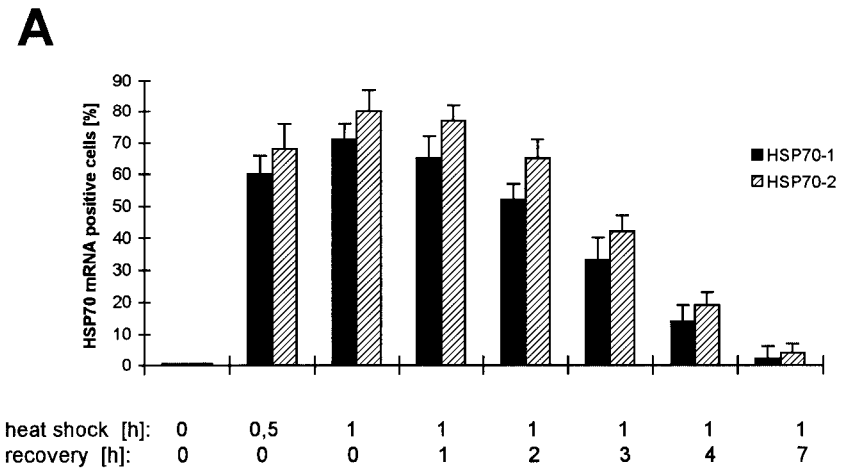


Fig. 3. In situ hybridization analysis of HSC70 and HSP70–1 expression in heat-shocked (1 h at 42°C) rat lymphocytes after 4 days of Concanavalin A (con A) stimulation. Hybridization with antisense (as) and, for specificity control, sense (s) probes is shown. ×100.

(42°C) did not lead to induction of HSP70–1 or HSP70–2 expression in each cell (Fig. 4b).

Expression of HSP70–1 and HSP70–2 mRNA After Induction In Vivo

Induction of HSP70–1 and HSP70–2 was analyzed by in situ hybridization in lymphocytes from patients undergoing fever therapy for metastatic malignant melanoma. Lymphocytes assayed immediately before fever induction were negative, but lymphocytes collected 4–6 h later, i.e., at the temperature maximum of ≥39°C, showed HSP70 induction in one-half of the patients [Dressel et al., 1996]. In positive



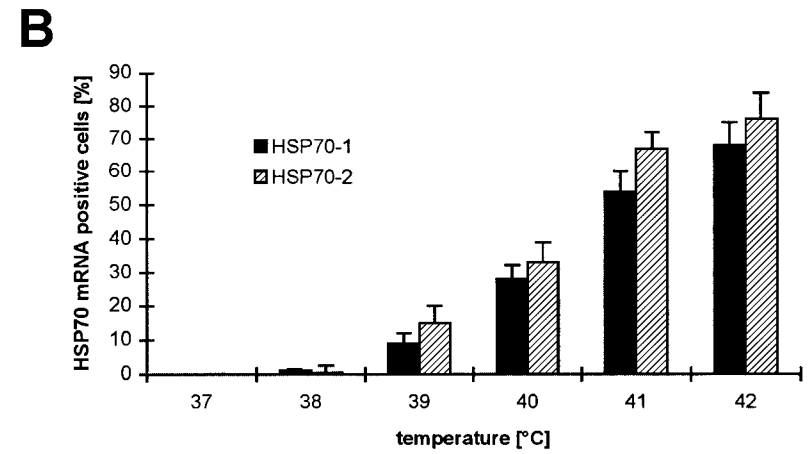


Fig. 4. Time **(A)** and temperature kinetics **(B)** of HSP70–1 and HSP70–2 induction in human lymphocytes determined by in situ hybridization. For the time kinetics experiments cells were heat shocked at 42°C for 1 h and then incubated at 37°C. HSP70–1 and HSP70–2 expression was analyzed before heat shock (0 h), after 0.5 h of heat shock, after 1 h of heat shock, and at the indicated times of recovery after an 1 h heat shock. Temperature kinetics of HSP70–1 and HSP70–2 induction (B) were determined in cells incubated at the indicated temperature for 1 h before analysis. Mean and standard deviation of four separate experiments are shown.

patients, a fraction of 50–60% of cells always remained negative for HSP70–1 and HSP70–2 mRNA (data not shown). Thus, heterogeneity of heat shock responsiveness at the single-cell level can be observed not only in vitro, but also after HSP70 induction in vivo. The proportion of cells expressing HSP70 in vivo was usually lower than after in vitro heat shock of lymphocytes from the same patients.

Analysis of HSP70-Positive Cells at the Protein Level by Flow Cytometry

In order to test whether the heterogeneity of HSP70 mRNA induction observed by in situ hybridization for the MHC-linked HSP70–1 and HSP70–2 genes can be also observed at the protein level human lymphocytes were analyzed by flow cytometry with an antibody specific for the inducible form of HSP70. Under control conditions (37°C) about 42% of the lymphocytes were found to be HSP70 positive (Fig. 5). After heat shock for 1 h at 42°C and 1 h of recovery at 37°C, the average percentage of positive cells increased to 74%. In none of the six experiments were more than 88% of the heat-shocked lymphocytes HSP70 positive. Staining of normal lymphocytes with this antibody is indeed due to the presence of HSP70 protein, since the HSP70-defective myeloma cell lines 210-RCY3-Ag1.2.3 [Galfre et al., 1979] and X63-Ag8.653 [Kearny et al., 1979] are negative with the antibody in flow cytometry (data not shown).

It could be reasoned that HSP70 is induced in certain cells only after strong heat shock that will lead to apoptosis in other cells. Therefore, human lymphocytes were subjected to heat shock of 43°, 44°, or 45°C for 1 h, followed by a recovery period of 1 h at 37°C in a series of three further experiments. Table I shows that the percentage of HSP70-positive cells decreased with heat shock strength, whereas the percentage of apoptotic, i.e., annexin V positive, 7-AAD negative cells, increased. The rate of

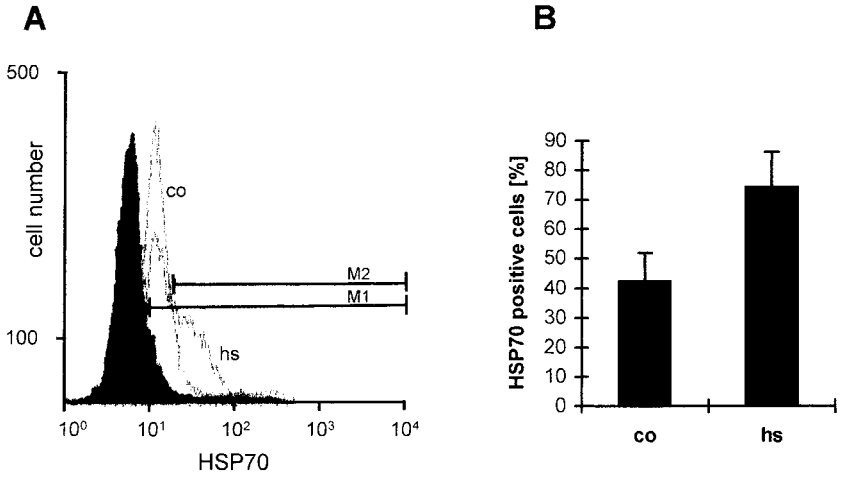


Fig. 5. Analysis of HSP70 expression at the protein level by flow cytometry. **A:** Individual experiment. The closed histogram represents heat-shocked human lymphocytes stained with the secondary antibody alone; the corresponding histogram of unshocked cells is almost identical and omitted for simplicity. The open histograms represent lymphocytes cultured at 37°C (co) or heat-shocked (hs, 1 h at 42°C, followed by 1 h of recovery at 37°C) and stained with the anti-HSP70 antibody. The percentage of HSP70 positive cells was calculated using the marker M1. The marker M2 defines cells in which HSP70 was increased after heat shock. **B:** To summarize six independent experiments, the average percentage ±S.D. of HSP70 positive lymphocytes before (co) and after heat shock (hs) are shown.

TABLE I. Effect of Heat Shock on Induction of HSP70 and Apoptosis in Human Lymphocytes[a]

	Temperature				
	37°C	42°C	43°C	44°C	45°C
HSP70 expression (mean intensity of fluorescence)	50 (15)	282 (63)	212 (54)	38 (17)	42 (13)
HSP70 positive cells (%)	45 (9)	76 (8)	67 (11)	43 (10)	35 (8)
Apoptotic cells (%) (annexin V pos./7-AAD neg.)	2 (1)	3 (2)	14 (5)	29 (8)	37 (9)
Dead cells (%) (annexin V pos./7-AAD pos.)	2 (1)	2 (1)	2 (1)	2 (1)	4 (1)

[a]Cells were heat shocked at the indicated temperature for 1 h, followed by a recovery period of 1 h at 37°C. Mean and standard deviation (in brackets) of three independent experiments are given.

dead, i.e., annexin V and 7-AAD positive cells, remained almost constant. Heat shock of 44° or 45°C failed to induce HSP70, since the proportion of HSP70-positive cells was below the control range and the mean fluorescence did not increase.

Resting B lymphocytes have been reported to be unable to synthesize HSP70 after heat shock [Spector et al., 1989; Hardy et al., 1997]. Therefore, intracellular and cell surface staining were combined in order to examine lymphocyte subpopulations separately. By triple fluorescence analysis CD3-positive T lymphocytes and CD19-positive B lymphocytes were gated and the percentage of HSP70 positive cells was determined in each population (Fig. 6). At 37°C, in the mean 39% of B lymphocytes and 44% of T lymphocytes were HSP70 positive. Heat shock of 42°C for 1 h increased the proportion of HSP70-positive B cells to 60% and of HSP70-positive T cells to 73% on average (n=3). Thus, HSP70 inducibility could be seen in T as well as in B lymphocytes, and in both subpopulations a fraction of cells remained HSP70 negative.

DISCUSSION

Although HSP70 expression has been studied extensively, only limited data are available on the expression pattern of single HSP70 genes. Nearly all reports on HSP70 mRNA expression were performed with probes crosshybridizing with transcripts of more than one HSP70 gene. An exception is the study showing expression of HSP70–2 in human lymphocytes after in vitro heat shock [Pociot et al., 1993].

Our data clearly show that the major heat shock genes HSP70–1 and HSP70–2 that map to the MHC are not constitutively expressed at

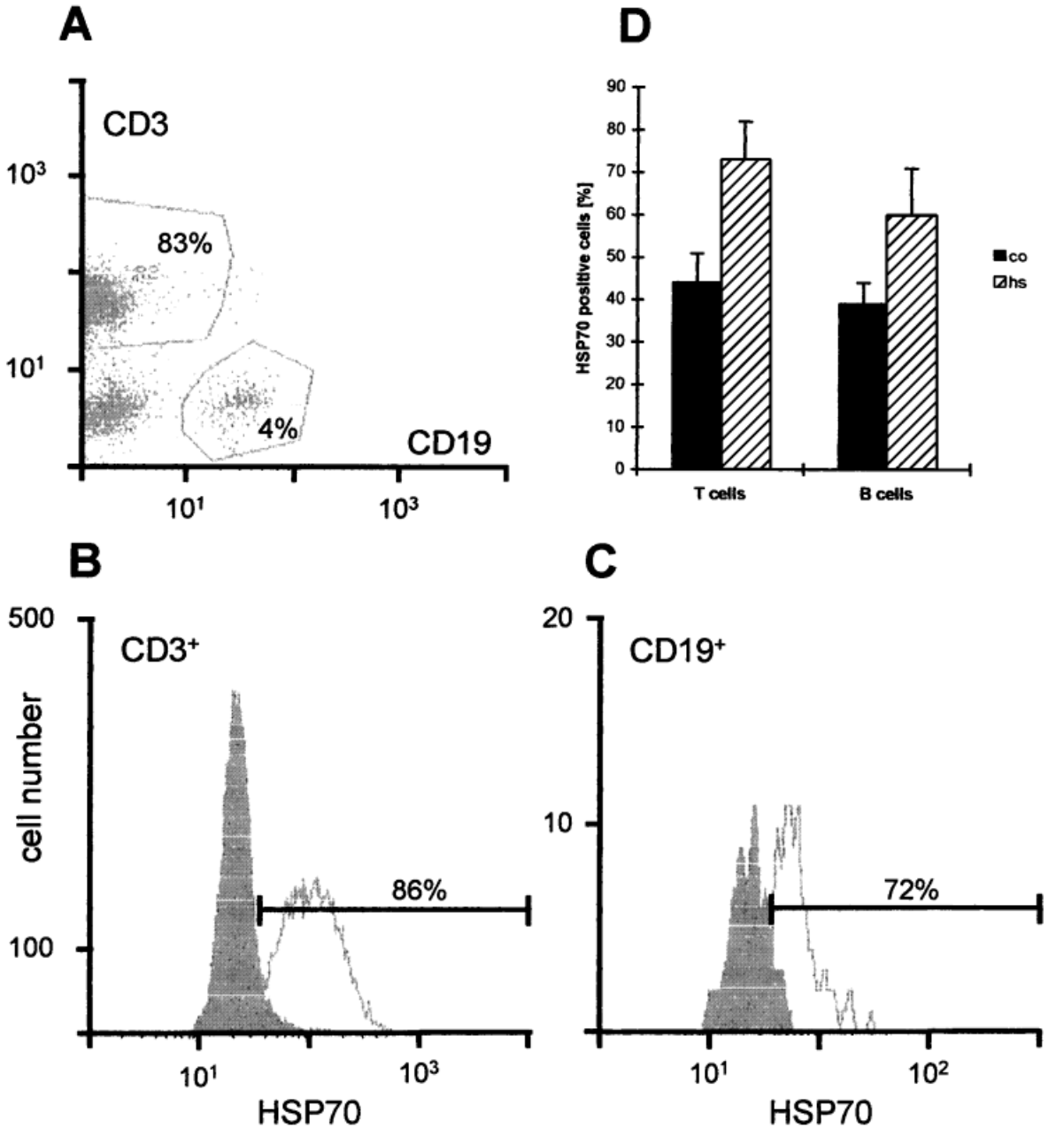


Fig. 6. Examination of HSP70 expression in human CD3-positive T lymphocytes and CD19-positive B lymphocytes by combined intracellular and cell surface staining. The CD3- and CD19-positive cells were gated **(A)** and analyzed for HSP70 expession separately **(B,C)**. The closed histograms (B,C) were obtained from heat-shocked cells (1 h at 42°C, followed by 1-h recovery at 37°C) that were treated with the secondary antibody alone; the open histograms represent cells stained with the anti-HSP70 antibody. The percentage of HSP70-positive heat-shocked cells was calculated using the markers shown. The results of three independent experiments are summarized **(D)** as the mean ±S.D. of HSP70-positive T and B lymphocytes before (co) and after heat shock (hs).

the mRNA level but highly heat shock-inducible in lymphocytes. RNA of the third MHC-linked HSP70 gene, HSP70-HOM in human and HSP70–3 in the rat, is not detectable in lymphocytes, as expected from their testis-specific expression [Fujimoto et al., 1992; own unpublished data]. Concordant expression patterns of HSP70–1 and HSP70–2 were obtained, in both human and rat.

For the human HSP70–1 and HSP70–2 genes, cell cycle dependency of expression has been described in HeLa cells using a probe which did

not differentiate between these two genes [Milarski and Morimoto 1986]. Our in situ hybridization analysis with rat and human lymphocytes did not show expression of HSP70–1 and HSP70–2 after 24, 48, 72, and 96 h of con A stimulation; we concluded that cell cycle-dependent expression of these genes is unlikely to occur in lymphocytes and thus might be characteristic of cell lines. This conclusion is in agreement with the failure to detect HSP70 expression during hepatocyte regeneration after partial hepatectomy in the rat [Krawczyk et al., 1989].

The data reported on the effect of mitogens on HSP70 expression are controversial. It has been reported that the level of HSP70 mRNA is elevated in circulating mononuclear cells and decreases within 6 h after incubation of the cells with the T-cell mitogen phytohemagglutinin [Kaczmarek et al., 1987]. Ferris and co-workers observed that the HSP70 mRNA level is not changed by stimulation with this mitogen in human T cells, but upregulated by interleukin-2 (IL-2) treatment in IL-2-dependent T lymphocytes [Ferris et al., 1988]. It has been also reported that stimulation of B lymphocytes results in the increase of HSP70 mRNA and induction of thermotolerance [Spector et al., 1989]. Our results show that HSP70–1 and HSP70–2 mRNA are not expressed in freshly prepared lymphocytes, nor are they induced by mitogen and, therefore, by cytokines secreted during mitogenic stimulation. These data are in agreement with reports showing that HSP70 synthesis is hardly enhanced in blood lymphocytes following mitogenic activation [Haire et al. 1988; Hansen et al. 1991].

In situ hybridization allows for quantitative analysis of single cells. The strength of the heat shock reaction was found to vary among individual cells, and 15–35% of the heat-shocked cells failed to respond at all. At the protein level, a similar heterogeneity of HSP70 responsiveness was found; again a fraction of about 25% of human lymphocytes remained HSP70 negative. Interestingly, no human lymphocytes are HSP70–1 or HSP70–2 mRNA positive, whereas a large proportion is HSP70 positive at the protein level under non-heat-shock conditions. This observation confirms previous data obtained by immunoblot analysis and metabolic labeling of protein synthesis [Dressel et al., 1996].

For several reasons, it is unlikely that the heterogeneity observed for HSP70 responsiveness reflects methodological artifacts due to selective loss of HSP70 mRNA and protein. Control cells as well as cells heat-shocked at $\leq$42°C always contained less than 5% dead cells before starting in situ hybridization or flow cytometry. Concordant results were obtained at the mRNA and protein level. Most importantly, in the case of the constitutively expressed HSC70 gene, mRNA was detected abundantly in each cell.

The heterogeneity of the heat shock response at the cellular level is not only an in vitro phenomenon, since it was also found in lymphocyte samples from patients undergoing fever therapy. In patients, this heterogeneity could easily be attributed to peculiarities of lymphocyte trafficking and recruitment. This interpretation is not appropriate for the in vitro data, however. In this case, heterogeneity might be explained by differential sensitivity of predefined lymphocyte subpopulations. However, the number of HSP70 mRNA positive cells and the strength of the reaction (number of grains) of individual cells are closely associated with heat shock temperature. Increasing recruitment of cells would not be compatible with the presence of a fixed preexisting and non responsive subpopulation. It has been reported that resting B lymphocytes respond to heat shock with only a slight increase of the amount of HSP70 mRNA that is already present before heat shock, and that only activated B lymphocytes develop a strong heat shock response [Spector et al. 1989; Hardy et al. 1997]. Our results cannot be explained by the differential behavior of B versus T lymphocytes, since HSP70 induction occurred in B as well as in T lymphocytes.

If HSP70 induction reflects protein denaturation in the cell and the effort of the cell to cope with this damage, the heterogeneity of HSP70 responsiveness could mean that not each cell is equally susceptible to heat-induced damage. It is conceivable that the amount of constitutively present HSC70 or HSP70 somehow determines the need of additional HSP70 chaperones after heat shock. Interestingly, about one-half of human lymphocytes were found to be HSP70 protein positive under control conditions when the mRNA of the MHC-linked HSP70 genes was not detectable.

Heat shock at higher temperature than 42°C did not lead to a greater rate of HSP70 positive lymphocytes, indicating that the severity of heat stress was not a limiting factor in rendering each cell HSP70 positive. Under conditions of a 44° or 45°C heat shock, the proportion of apoptotic cells increased, whereas no HSP70 was induced in apoptotic as well as non apoptotic cells. Thus, lymphocytes can be observed that undergo apoptosis after severe heat shock without activating their HSP70 stress response.

Irrespective of the underlying mechanisms, the HSP70 response to heat shock does not appear to occur and to be of similar magnitude in each cell, when a cell population is exposed to stress. Further experiments are needed to elucidate the critical features of lymphocytes not reacting to heat shock with HSP70 expression. Since HSP70 appears to play an important role in antigen presentation [Srivastava et al., 1998], the variable character of HSP70 inducibility in immune cells could be of particular relevance.

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