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Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes

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Abstract The increasing interest in stress/heat shock proteins (Hsps) as markers of exposure to environmental stress or disease requires an easily applicable method for Hsp determination in peripheral blood cells. Of these cells, monocytes preferentially express Hsps upon stress. An appropriate fixation/permeabilization procedure was developed, combined with immunofluorescence staining and flow cytometry for the detection of the inducible, cytosolic, 72 kDa Hsp (Hsp70) in human monocytes. Higher relative fluorescence intensity was observed in cells exposed to heat shock (HS), reflecting a higher expression of Hsp70 in these cells as compared with cells kept at 37°C. The heat-inducible increased Hsp70 expression was temperature- and time-dependent. Expression of Hsp70 was not uniform within the monocyte population, indicating the presence of subpopulations expressing variable levels of Hsp70 in response to HS. Simultaneous measurements of intracellular Hsp70 and membrane CD14 expression revealed that the higher Hsp70 inducibility coincided with the higher CD14 expression. Comparisons performed with biometabolic labelling, Western blotting, immunofluorescence and immunoperoxidase microscopic analysis, showed a high concordance between these different methods; however, cytometry was more sensitive for Hsp70 detection than Western blotting. Flow cytometric detection of intracellular Hsp70 is a rapid, easy and quantitative method, particularly suited for the determination of protein levels in individual cells from an heterogeneous population such as peripheral mononuclear blood cells, and applicable to cohort studies.

INTRODUCTION

The increasing interest in stress/heat shock proteins (Hsps) as markers of exposure to environmental stress or diseases requires a generally applicable method for Hsp determination, preferably in easily accessible cells such as peripheral blood cells. Classical methods evaluate Hsps at the protein level by biometabolic labelling with radiolabelled amino acids, followed by polyacrylamide gel electrophoresis (PAGE) separation and/or Western blot analysis with Hsp-specific antibodies. Immunofluorescence and immunoperoxidase microscopic analysis are commonly used to determine Hsps at the cellular or tissue levels and more recently, reverse transcriptase-polymerase chain reaction (RT-PCR) procedures have been developed to analyse Hsp expression at the mRNA level (R. Morimoto, personal communication). However, these methods often lack the necessary sensitivity and in addition to the hazard of working with radioisotopes they have cumbersome and time-consuming protocols. Furthermore, none of these methods allows for concomitant analysis of other markers of given cell subpopulations.

Peripheral blood cells are easily accessible and their Hsp expression levels have been examined in a number

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of diseases as diverse as lupus erythematosus and sleep apnea syndrome (Norton et al 1989; Noguchi et al 1997), in all of which Hsps are thought to play a role. Among these peripheral blood cells, monocytes preferentially express Hsps upon stress, much more so than either lymphocytes, or neutrophils (Mariéthoz et al 1994; Polla et al 1995). Monocytes thus appear to be the ideal cells to examine and follow Hsp expression and inducibility in humans, whether in cohort studies or in a given disease. Therefore an appropriate fixation/permeabilization procedure was developed, combined with immunofluorescence staining and flow cytometry for the detection of the inducible, cytosolic, 72 kDa Hsp (Hsp70) in human monocytes. The successful development of this method depended on the selection of suitable fixative and cell-permeabilizing agents which maintain the cell morphology in optimal conditions and provide access to the intracellular antigens. The fixative paraformaldehyde and the cell permeabilizing agent saponin, which have been reported as satisfying these criteria (Sander et al 1991; Jung et al 1993), were employed in this protocol. Subsequent indirect immunofluorescence staining was performed with monoclonal antibodies and the $F(ab')_2$ fluorescent-conjugate of the corresponding isotype. This methodology was used to evaluate the levels of Hsp70, the most abundant and highly inducible Hsp, in human monocytes. The synthesis and expression of Hsp70 are significantly induced in these cells by heat shock (HS) and other cellular stresses, including exposure to reactive oxygen species, heavy metals or selective invasive micro-organisms (Polla 1988; Garbe 1992). Data obtained by flow cytometry revealed a good correlation with previous observations obtained by biometabolic labelling, Western blotting, immunofluorescence and immunoperoxidase microscopic analysis, although cytometry was more sensitive than Western blotting. Furthermore, simultaneous measurements of intracellular Hsp70 and membrane antigens by flow cytometry, allow the phenotypic characterization of distinct monocyte subpopulations with variable levels of Hsp70 expression.

Analysis of Hsp expression and inducibility in peripheral blood cells by flow cytometry should provide a useful tool with large-scale applicability for studying the contribution of Hsps to stress or stress-related diseases, or as markers of individual or population environmental exposures.

MATERIALS AND METHODS

Reagents and antibodies

Paraformaldehyde, Triton X-100 and saponin were purchased from Sigma (St Louis, MO, USA). Ethanol was from Merck (Darmstadt, Germany). Culture medium (RPMI 1640), fetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin-EDTA (0.05% trypsin, 0.02% EDTA), L-glutamine, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer and bovine serum albumin (BSA, fraction V) were from ICN Biomedicals (Orsay, France). Lactate dehydrogenase (LDH) viability determination kit was from Boehringer (Mannheim, Germany). The monoclonal antibody directed against the inducible form of Hsp70 (mouse IgG1, SPA-810) was from StressGen (Victoria, Canada). In some experiments the monoclonal antibody RPN 1197 (Amersham, Buckinghamshire, UK) was used against the inducible form of Hsp70. The $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (Ig)G conjugated to fluorescein isothiocyanate (FITC) used as secondary antibody, was from Dako (Glostrup, Denmark). Anti-CD14 phycoerythrin (PE) conjugate was also purchased from Dako (mouse IgG2a kappa, R0864). [^{35}S]methionine for metabolic labelling was from Amersham (specific activity > 1000 Ci/mmol). Acrylamide, nitrocellulose membrane (0.45 μ) and different reagents for polyacrylamide gel electrophoresis separation were from BioRad (Hercules, CA, USA). Bromophenol blue, anti-mouse IgG conjugated to horseradish peroxidase (IgG fraction of antisera, A5278), H_2O_2 and 4-chloro-1-naphtol, were from Sigma. Autoradiographs were performed using X-Omat films (Kodak Scientific, Rochester, NY, USA). Immunoperoxidase staining was performed with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and Sigma Fast DAB (3,3'-diamino-benzidine). Mayer's hemalun solution was from Merck.

Cell preparation

Mononuclear cells were isolated by density gradient centrifugation from buffy coats obtained from healthy donors, as described previously (Polla et al 1987). Mononuclear cells were resuspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine and 25 mM HEPES, and adjusted to a concentration of 10×10^6/ml. Aliquots of the cell suspension (1 ml) were plated in 35 mm diameter Petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) and allowed to adhere for 45 min in a CO_2 incubator. Non-adherent cells were removed and the remaining monolayers were incubated overnight in supplemented RPMI medium.

Heat shock procedure

Adherent cells were exposed to a thermal challenge (38–44°C, for 30 min or 1 h) in Petri dishes surrounded with a parafilm band, floating in a thermostatically regulated water bath (Julabo PC, Seebach, Germany).

Constant pH was assured by the presence of 25 mM HEPES in the culture medium. Following HS, adherent cells were allowed to recover for different intervals of time (0.5–20 h) at 37°C in a CO_2 incubator. At the end of the recovery period, cells were detached from Petri dishes with 0.5 ml trypsin-EDTA, washed in PBS and transferred to 5 ml disposable polypropylene tubes (Greiner, Strasbourg, France) adapted to the flow cytometer cap.

Fixation

Fixation was performed with a paraformaldehyde solution freshly prepared as follows: 0.3 g paraformaldehyde was solubilized in 10 ml PBS by heating the mixture at 70°C. After cooling the solution to room temperature the pH was checked and adjusted to 7.4. Cells ($1–2 \times 10^6$) were resuspended in 100 µl paraformaldehyde solution, incubated for 10 min at room temperature and washed by centrifugation (470 ×g, 3 min) in 1 ml PBS supplemented with 1% BSA (PBS-BSA). In some experiments, comparisons were performed with ethanol fixation (70%, 1 h at 4°C).

Permeabilization, Hsp70 detection and staining

Permeabilization and incubation with the primary antibody were performed simultaneously. After the fixation step, cells were resuspended in 50 µl of 0.6% saponin (stock solutions of 6% in PBS were stored at −20°C) and 50 µl of the anti-Hsp70 antibody diluted to 1:100 in PBS-BSA. Incubation was carried out for 10 min at room temperature and cells were washed in 1 ml PBS-BSA. Staining was performed by resuspending cells in 50 µl of saponin solution and 50 µl of the secondary antibody (FITC-conjugated $F(ab')_2$ fragment of rabbit anti-mouse IgG) diluted to 1:30 in PBS-BSA and further incubation for 10 min in the dark at room temperature. Comparative experiments were also performed with Triton X-100 (0.005–0.05%) instead of saponin. In negative controls for background staining, paraformaldehyde-fixed cells were incubated with saponin and the secondary antibody alone. For double staining experiments to determine Hsp70 and CD14 expression simultaneously, monocytes stained for Hsp70 as previously defined were incubated with 50 µl anti-CD14-PE-conjugated antibody (diluted to 1:100 in PBS-BSA) for 10 min at 4°C. Cells were washed in PBS-BSA, resuspended in 1 ml PBS-BSA and kept at 4°C in the dark until flow cytometry analysis.

Flow cytometry analysis

Cells were analysed with an Epics Elite fluorescence activated cell sorter (Coulter Miami, FL, USA). The FITC and PE dyes were excited with 15 mW of the 488 nm from an argon laser. A total of 5000 cells were analysed in list-mode for green fluorescence through a 525 nm filter and for red fluorescence through a 575 nm filter. The fluorescence histograms were gated on the forward-angle light scatter signal to measure fluorescence only from the monocyte population. The purity of the monocyte cluster was determined by reacting cells with the monoclonal antibody anti-CD14-PE conjugate. Generally at least 85% of the monocyte cluster reacted positively and no more than 2.5% of the lymphocyte cluster reacted. The fluorescence signals were analysed using a 4 decade logarithmic amplifier. The Hsp70-related immunofluorescence was quantified by calculating the mean fluorescence channel from histograms of logarithmic fluorescence intensity. All data were analysed with the Elite software version 4.02. Cells could be stored at 4°C in the dark for at least 1 week before flow cytometry analysis without significant alterations in the light scatter or the fluorescence staining pattern.

Metabolic labelling, PAGE and Western blotting

For metabolic labelling, cells were exposed for 90 min to 9 µCi/ml [^{35}S]methionine in supplemented RPMI medium without methionine. Cells were harvested, counted and lysed in 2% sodium dodecyl sulphate (SDS) buffer containing 4% 2-mercaptoethanol and 5% bromophenol blue. Samples corresponding to equal cell numbers were resolved by one-dimensional SDS-PAGE (10% acrylamide) as described by Laemmli (1970) and revealed by autoradiography with X-Omat film. For Western blot analysis, samples corresponding to equal cell numbers were electrophoresed and transferred to nitrocellulose membranes (0.45 µ). The membrane was saturated with casein containing buffer for 2 h and then exposed to the monoclonal antibody against the inducible form of Hsp70 (SPA 810). Bound antibody was revealed with the appropriate antibody (horseradish peroxidase-conjugated anti-mouse IgG) in the presence of H_2O_2 and 4-chloro-1-naphthol.

Analysis of Hsp70 by immunofluorescence and immunoperoxidase

For immunofluorescence, monocytes were purified by adhesion to glass cover slides for 45 min at 37°C. Control cells (maintained at 37°C) or cells exposed to 44°C for 30 min and allowed to recover for 4 h were fixed, permeabilized and stained for Hsp70 visualization as described for flow cytometry analysis. Photographs were produced with an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany).

For immunoperoxidase, control cells or cells exposed to 44°C for 30 min and allowed to recover for 4 h were

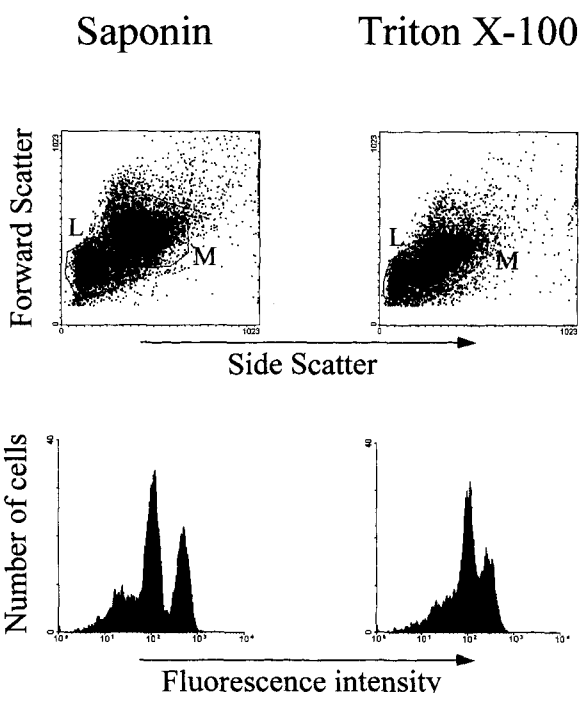

Fig. 1 Comparative procedures for evaluating Hsp70 by flow cytometry. Forward scatter *versus* side scatter dot plots (upper panels) and fluorescence intensity histograms (lower panels) of human monocytes subjected to heat shock (44°C, 30 min followed by 4 h recovery period). In dot plots gates are set around lymphocytes (L) or monocytes (M). Paraformaldehyde-fixed cells were permeabilized either with saponin (0.3%) or with Triton X-100 (0.05%), and labelled as described (see Materials and methods and also legend to Fig. 3). Single parameter histograms show Hsp70-FITC fluorescence of cells within the scatter gate set on the monocyte population.

detached from Petri dishes with trypsin-EDTA, washed in PBS and fixed with paraformaldehyde (3%) for 10 min at room temperature. Immunoperoxidase staining was performed in cytospin preparations (Shandon, Pittsburg, PA, USA). Slides were incubated with the anti-Hsp70 antibody SPA 810 (diluted to 1:100 in PBS-BSA containing 0.3% saponin), and with the Vectastain Elite ABC kit that employs a biotinylated secondary antibody and a preformed avidin biotinylated radish peroxidase complex. Colour development was carried out with Sigma Fast DAB (one tablet dissolved in 15 ml Tris-HCl 0.05 M, pH 7.6). The reaction was stopped with Tris-HCl 0.01 M. Some slides were counterstained with Mayer's haemalun solution. Photographs were produced with a Leitz Diaplan microscope (Wetzlar, Germany).

RESULTS

Comparative procedures for evaluating Hsp70 by flow cytometry

Three fixation/permeabilization methods were compared: i) 70% ethanol at 0°C for 1 h; ii) paraformaldehyde (3%, 10 min at room temperature) followed by Triton X-100 (0.005–0.05%, 10 min); iii) paraformaldehyde (3%, 10 min at room temperature) followed by saponin (0.3%, 10 min). Experiments with ethanol revealed several drawbacks, including high levels of cell aggregation and altered recognition of the cell surface antigen, CD14. The best results were obtained using a brief fixation with paraformaldehyde and subsequent permeabilization with saponin. Examples of these experiments are shown in Figure 1. When Triton X-100 was tested as permeabilizing agent in paraformaldehyde-fixed cells, lower levels of fluorescence intensity were found along with higher alterations in light scatter properties of human monocytes, as compared with saponin (Fig. 1).

Heat shock-induced levels of Hsp70 as a function of the recovery time after heating monocytes at 44°C for 30 min

Experiments performed with biometabolic labelling followed by one-dimensional SDS-PAGE and Western blotting were compared with flow cytometry. Biometabolic labelling experiments depicted in Figure 2A show Hsp70 expression in human monocytes heated at 44°C for 30 min and labelled with [^{35}S]methionine following various recovery periods (30 min–8 h). Maximal Hsp70 synthesis was observed 4 h after HS (lane 6, Fig. 2A) and progressively decreased thereafter, indicating decreased de novo synthesis of Hsp70. Similar kinetics of appearance for Hsp70 expression were observed in the corresponding Western blot (Fig. 2C) although the accumulation of Hsp70 remained stable along the total recovery period of 8 h. Figure 2D shows the relative levels of Hsp70 detected by flow cytometry in FITC-labelled monocytes after different recovery periods (30 min–20 h). Hsp70 synthesis reached maximal levels between 4 and 6 h after HS, indicating approximately the same kinetics of appearance as observed with biometabolic labelling and Western blotting. As depicted in Figure 2D, accumulation of Hsp70 decreased 8–20 h after HS.

Hsp70 levels in monocytes exposed to increasing temperatures

The HS-induced Hsp70 expression in control monocytes and in cells exposed to various temperatures for 30 min or 1 h is shown in Figure 3. After exposure to the indicated temperatures, cells were allowed to recover for 4 h at 37°C. The smallest positive response was detected in cells exposed for 1 h to a thermal challenge as low as 39°C and this response increased gradually as a function of the temperature to reach a maximum at exposure to 44°C for 30 min. Levels of Hsp70 expression also increased in human monocytes as a function of the

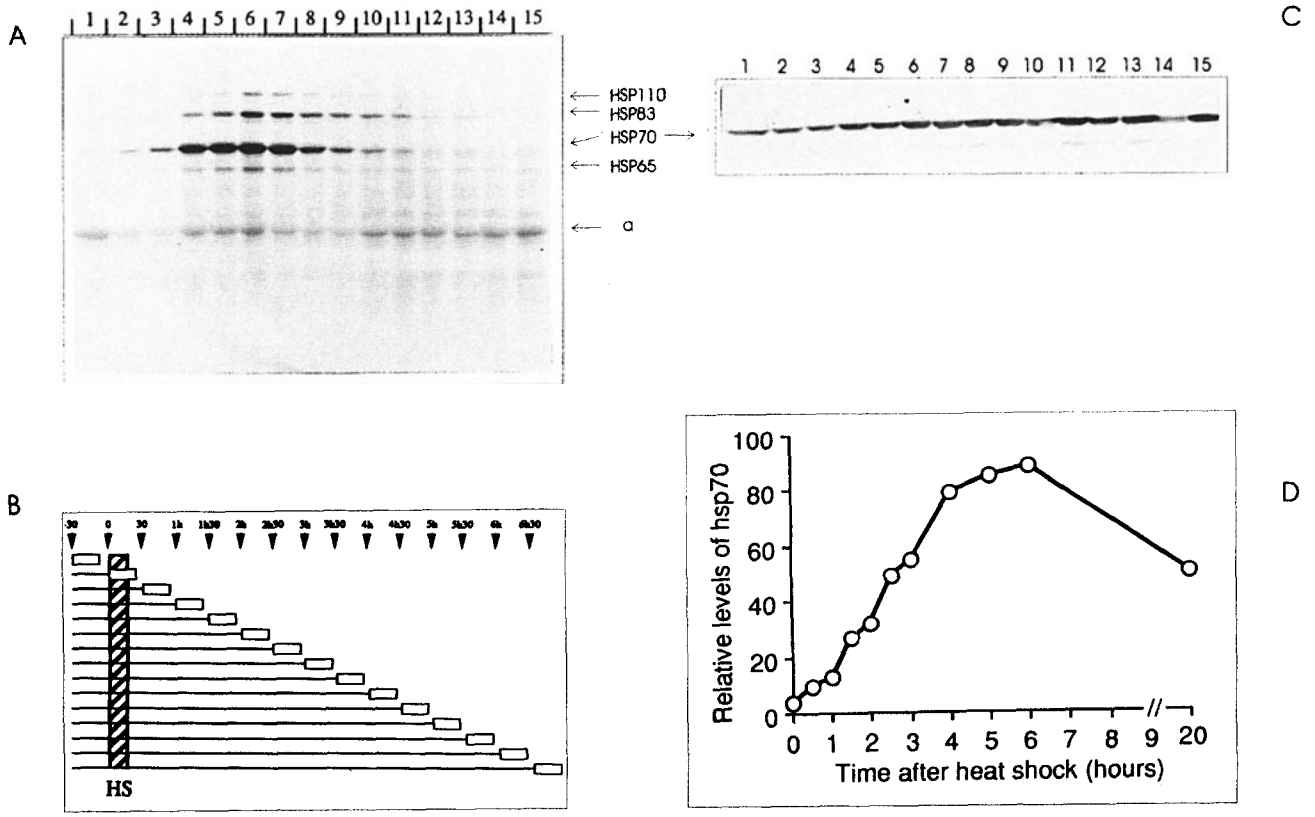

Fig. 2 Heat shock-induced levels of Hsp70 as a function of the recovery time after heating. (A) SDS-PAGE analysis of [^{35}S]-labelled proteins from monocytes as a function of increasing time recovery periods after heat shock (44°C, 30 min). Monocytes were allowed to recover for 30 min–8 h after heat shock, and then labelled with [^{35}S]methionine and prepared for SDS-PAGE (10% acrylamide) as described in Materials and methods. Proteins were revealed by autoradiography. (B) Schematic representation of the experimental protocol. After heat shock (HS) cells were incubated at 37°C for different time periods as indicated by the upper arrows. Rectangles at the end of each line represent the 90 min labelling period. For example, in lane 6 corresponding to maximal levels of Hsp70 synthesis, monocytes were allowed to recover for 2.5 h and then labelled for 90 min, thus the total recovery period was 4 h. (C) Western blot analysis of monocyte lysates as a function of the recovery time after HS. Monocytes were allowed to recover for 30 min–8 h after HS (44°C, 30 min). Cell lysates subjected to SDS-PAGE were transferred to nitrocellulose and immunoblotted with the monoclonal antibody directed against the inducible form of Hsp70. Samples were applied as depicted in (B). (D) Flow cytometry analysis of Hsp70 expression as a function of increasing recovery periods after HS (44°C, 30 min). Monocytes were labelled following 30 min–20 h recovery period as described (see Materials and methods and also legend to Fig. 3). Values represent the mean channel obtained in the corresponding histograms of fluorescence intensity.

duration of the exposure to the thermal challenge, for temperatures ranging between 39 and 43°C, with a higher expression always observed in cells exposed for 1 h as compared with 30 min. At 44°C, lower levels of Hsp70 expression were observed after 1 h as compared with 30 min exposure to thermal challenge, conceivably indicating reduced viability and protein synthesis under these highly stressful conditions. Control cells (maintained at 37°C) exhibited detectable Hsp70 expression, indicating that the monoclonal antibody directed against the inducible 72 kDa protein also recognized the constitutive 73 kDa Hsc70, in agreement with previous reports (Barazzone et al 1996). The present study did not detect any positive signal in non-permeabilized cells, indicating that Hsp70 was not expressed at the cell membrane either in control or in heated monocytes from normal donors, which is in contrast to recent observations in tumor cells (Multhoff et al 1995; Multhoff and Hightower 1996). Lack of expression of cell surface Hsp70 was similarly observed with the monoclonal antibody RPN 1197 (from Amersham), even when incubation was carried out at 0°C to avoid eventual internalization (data not shown).

Intercellular heterogeneity in Hsp70 expression

Hsp70 appeared to be differentially expressed in human monocytes, because at least two peaks with distinct fluorescence intensity were generally distinguished. Differences in viability did not account for these differences in Hsp70 expression, as no significant differences were observed in the release of LDH (control *versus* heat shocked monocytes: 12.3 ± 1.3% *versus* 14.6 ± 0.9% of total LDH, $n = 4$). The largest increase in Hsp70 was

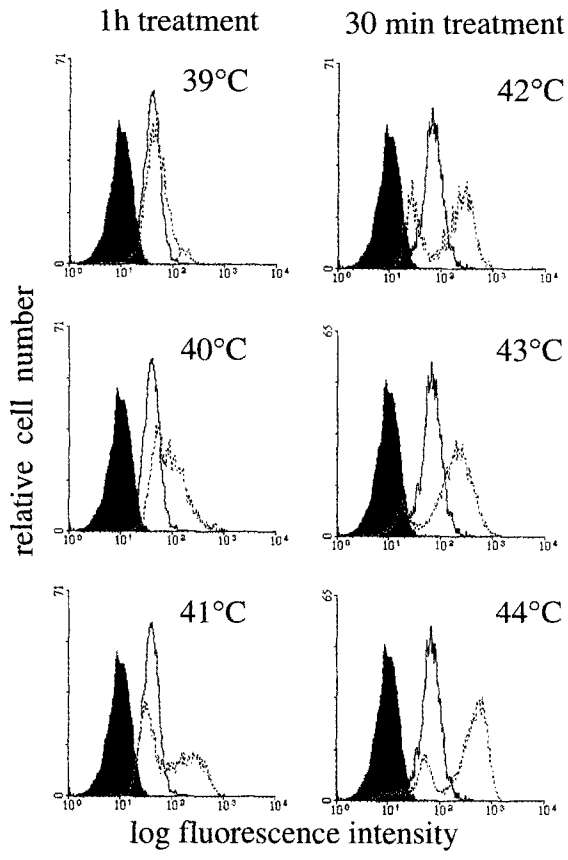

Fig. 3 Hsp70 levels in monocytes exposed to increasing temperature. Flow cytometry analysis was performed in human monocytes exposed to increasing temperature for 1 h (39–41°C) or 30 min (42–44°C) and allowed to recover for 4 h. The analysis was performed in paraformaldehyde-fixed/saponin permeabilized cells labelled with the monoclonal antibody directed against the inducible form of Hsp70 (SPA 810) and with the isotype-specific FITC-conjugated secondary antibody. Increasing fluorescence intensity is plotted on the horizontal axis in log fluorescence intensity units *versus* cell numbers on the vertical axis. Dark shaded curves represent negative control peaks, plain line open curves represent control monocytes and dashed lines open curves represent monocytes exposed to heat shock.

associated with the greatest degree of heterogeneity in Hsp70 expression. Cell numbers corresponding to the monocyte subpopulation expressing maximal levels of Hsp70 increased as a function of the temperature. Thus, the increased expression of Hsp70 observed as a function of cell exposure to increasing temperatures was due to both an increase in Hsp70 expression levels and to an increase in the number of cells expressing Hsp70. These results were then compared with those obtained with both immunofluorescence and immunoperoxidase (Fig. 4). Both procedures revealed a diffuse mild cytoplasmic staining under control conditions. After HS (44°C, 30 min followed by 4 h recovery period), Hsp70 translocated to the nuclear compartment and monocytes displayed a more intense staining. No membrane staining was observed in either control or heated monocytes, again in agreement with the results obtained with flow cytometry. The variable intensity of the staining pattern observed from one cell to another was also consistent with the heterogeneity observed in flow cytometry experiments with respect to the levels of Hsp70 expression.

Alternative experiments demonstrated that the differential capacity of human monocytes to produce Hsp70 in response to the thermal challenge was associated with the expression of CD14, the lipopolysaccharide receptor in monocytes, whose expression increases with increasing maturation/differentiation of these cells (Wright et al 1990). The highest Hsp70 expression coincided with the highest CD14 expression, as shown in Figure 5.

DISCUSSION

The purpose of this study was to develop a simple and sensitive method to evaluate Hsp expression and inducibility in human monocytes. Recent advances in flow cytometry technology have expanded the use of these techniques for direct assessment of intracellular proteins such as cytokines, at a single cell level (Sander et al 1991; Jung et al 1993). To detect intracellular proteins, cells have to be stabilized and permeabilized to allow antibodies to penetrate through the cell membrane, preserving the cell morphology and intracellular antigenicity, with minimal cell aggregation and cell loss. These criteria were satisfied for Hsp70 measurements in human monocytes, by a combination of a brief paraformaldehyde (3%) fixation and a subsequent permeabilization of the cell membrane by the mild detergent-like saponin (0.3%). Furthermore, the procedure does not alter cell surface antigens in monocytes or lymphocytes (Jacob et al 1991), thereby permitting simultaneous detection of intracellular Hsp70 and membrane antigens such as CD14 in fixed/permeabilized cells. In agreement with previous studies on intracellular cytokines is the observation that saponin must be present during the incubation with antibodies to make the staining procedure successful. An explanation for this finding is that saponin intercalates in the cell membrane at the place of cholesterol and permeabilizes cells in a reversible way by solubilizing cholesterol (Willingham and Pastland 1985; Sander et al 1991).

Other authors have recently reported flow cytometric procedures for the evaluation of intracellular Hsp (Hjelstuen and Davies 1994; Hang et al 1995; Hang and Fox 1995). Both DNA content and Hsp70 levels were measured in these studies in several mammalian cell lines, through the cell cycle, using methanol (80%) or ethanol (70%) that simultaneously fix and permeabilize cells. Our initial attempts to measure Hsp70 in human monocytes with these procedures were disappointing,

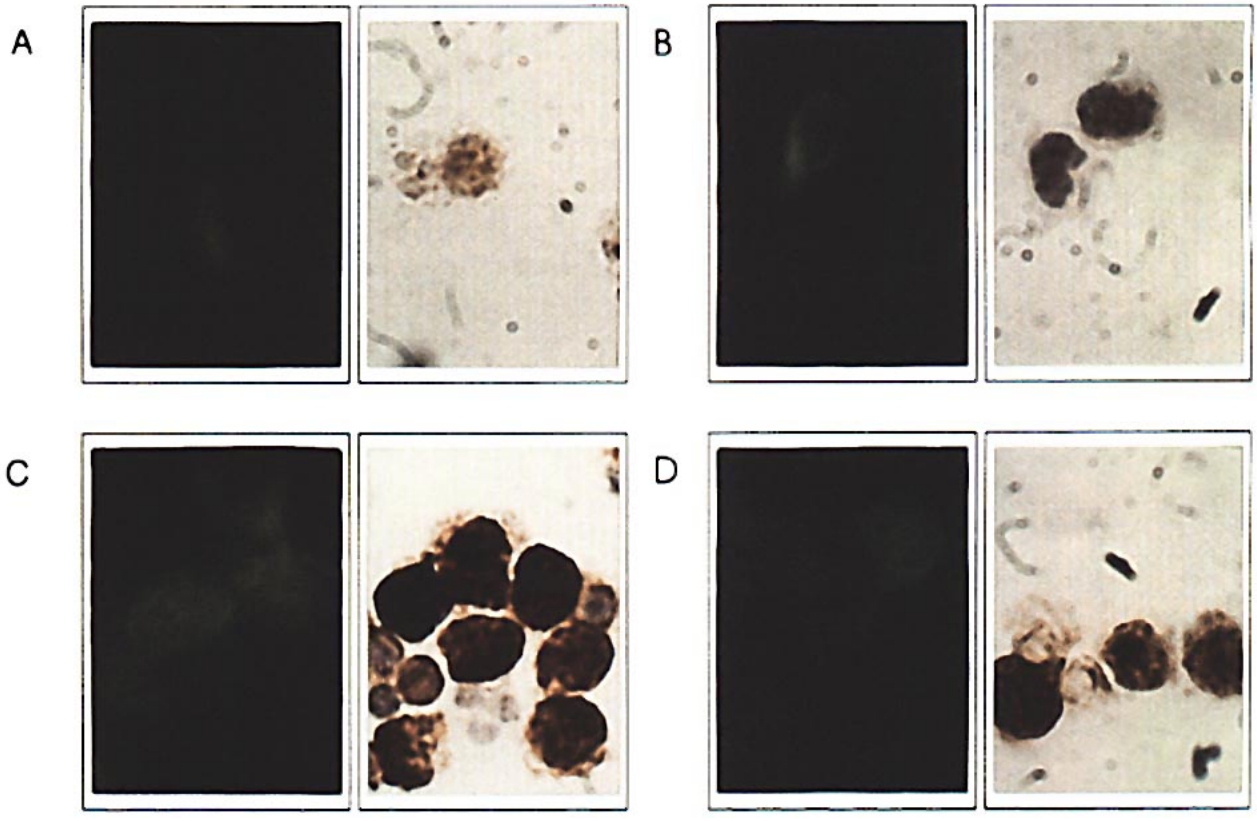

Fig. 4 Analysis of Hsp70 expression in fixed/permeabilized human monocytes by immunofluorescence and immunoperoxidase. Monocytes were labeled with the monoclonal antibody directed against the inducible form of Hsp70 and the FITC-conjugated corresponding isotype for immunofluorescence or the Vectastain Elite ABC kit and Sigma Fast DAB for immunoperoxidase. (A,B) Control monocytes express low diffuse Hsp70. (C,D) Monocytes after heat shock (44°C 30 min followed by 4 h recovery period) express higher and heterogeneous Hsp70 staining in both the cytoplasmic and in the nuclear compartment. In (B) and (C) monocytes stained by immunoperoxidase were counterstained with Mayer's hemalun solution.

because of cell loss, aggregation and lower fluorescence intensity signals, unlike data reported for cell lines. Previous studies on Hsp expression by flow cytometry indicate that different cell types might have distinct optimal fixation/permeabilization procedures. In addition, fixation/permeabilization procedures should be individually characterized for each antigen before quantification by flow cytometry.

In combination with paraformaldehyde fixation, permeabilization of the monocyte membranes with saponin was preferred to Triton X-100 because of a stronger positive immunofluorescence signal accompanied by less changes in cell scatter properties, as previously reported for both monocytes and lymphocytes (Jacob et al 1991). The temperature-dependent increase in Hsp70 expression observed in human monocytes by flow cytometry is in good agreement with numerous previous studies. The pattern of Hsp70 expression after HS and different recovery periods was analysed by different methods, including biometabolic labelling followed by SDS-PAGE separation, Western blotting or flow cytometry with similar results, indicating a high correlation between the three detection methods. Those experiments showed that the heat-induced synthesis of Hsp70 in human monocytes is a transient process that is maximal by 4–8 h after heating and decays thereafter. However, no heat-induced Hsp70 expression has been detected so far in cells exposed to a mild HS such as 39–40°C for 30 min–1 h, demonstrating the high sensitivity of flow cytometry. This might be of significant interest as those temperatures occur in vivo during inflammation and fever. The observed localization of Hsp70 within the nuclear region during the 4 h recovery period after HS is consistent with previous reports (Welch and Suhan 1986) and might be related to its involvement in the repair of nuclear lesions, as well as in the recovery of the normal translational activity.

Monocyte populations split into at least two subpopulations with distinct levels of Hsp70 after HS. To our knowledge, the existence of subpopulations of human monocytes differentially expressing Hsp70 in response to HS has not been described so far and this study demonstrates that the increased Hsp70 displayed by monocyte subpopulations is linked to the expression of CD14. The lipopolysaccharide receptor CD14 is known to be

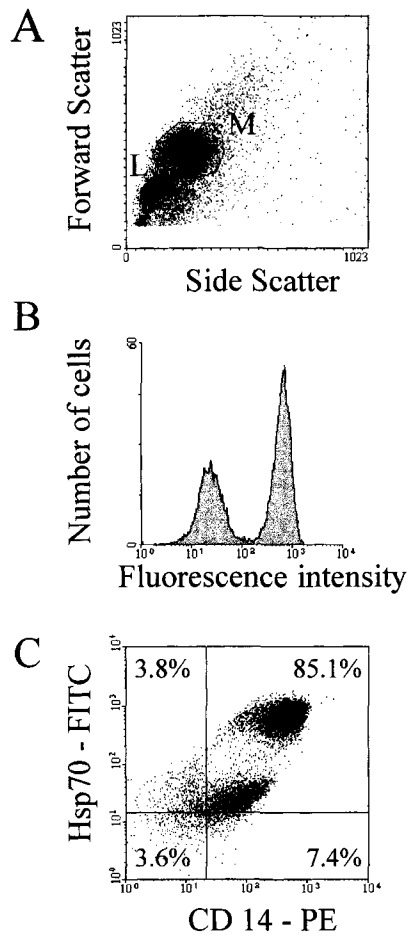

Fig. 5 Flow cytometry analysis of Hsp70 and CD14 expression in human monocytes. (A) Forward scatter *versus* side scatter dot plot of fixed/permeabilized mononuclear cells (lymphocytes, L and monocytes, M). (B) Single parameter histogram of monocytes exposed to heat shock (44°C, 30 min followed by 4 h recovery period) and labeled with anti-Hsp70 and the FITC-conjugated antibody as described in Materials and methods. (C) Double parameter histogram of monocytes exposed to heat shock and stained with both Hsp70 and CD14 antibodies conjugated to FITC or PE, respectively, as described in Materials and methods. The number of gated cells as a percentage of the total population is shown in the selected quadrants.

upregulated as a function of the maturation stage of monocytes and has been shown to be induced by $1,25(OH)_2$-vitamin D3 (Zhang et al 1994) that also induces Hsp70 expression in monocytes (Polla et al 1987). Furthermore, both constitutive and inducible levels of Hsp70 have been shown to be higher in differentiated monocytes such as alveolar macrophages, both under basal conditions and after HS (Kantengwa and Polla 1993), indicating that Hsp70 expression is dependent on the maturation state of these cells. The precise mechanisms linking monocyte differentiation, CD14 expression and Hsp70 inducibility are currently being investigated in this laboratory. In contrast, no correlation was found with other surface antigens/differentiation markers such as CD16.

Another aspect of this study was to investigate whether stained cells could be stored before flow cytometry analysis. In these experiments, monocytes were analysed over a 1 week period and fluorescence histograms remained essentially identical. This should be of considerable value in vivo for investigators who have limited access to flow cytometry facilities. For example, investigations undertaken in this laboratory have demonstrated the potential of this technique for studying Hsp70 in monocytes from patients suffering from adult respiratory distress syndrome (ARDS) (Durand et al, manuscript in preparation), as well as in smokers (Kreps et al 1997). These results indicate that both basal expression and Hsp70 inducibility should be analysed in follow-up studies.

Most of the work supporting a role for Hsp70 in infectious, inflammatory or autoimmune diseases results from observations of the stress response in human monocytes/macrophages. Therefore the availability of a rapid assay to measure Hsp70 in human monocytes has a great number of potential clinical applications. This report describes a system that greatly reduces total assay time, while increasing the sensitivity with easily obtained reproducibility. A significant advantage of this method is that cell isolation procedures and the determination of the precise number of cells per sample are not required. Although analysis may be performed in an heterogeneous cell population, it does allow discrimination between distinct phenotypical populations, which is not the case for any other method, apart from the non-quantitative immunofluorescence or immunoperoxidase procedures. Moreover, simultaneous multifactorial analysis may be performed in the chosen population and Hsp evaluation may be carried out in parallel with cell surface markers.

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