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Fluorescein Isothiocyanate-Dextran can Track Apoptosis and Necrosis Induced by Heat Shock of Peripheral Blood Mononuclear Cells and HeLa Cells

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Abstract: Dextran does not penetrate the living cells. However when the plasma membrane becomes permeable, it is concentrated in the cells. This is why we used fluorescein isothiocyanate-conjugated dextran (FITC-Dextran, MW 4000) to identify apoptotic and necrotic cells by flow cytometry. Heat shock was used to induce cell apoptosis or necrosis. To induce apoptosis cells were heated to 43.5°C for 1 hour and then incubated at 37°C. Thereafter the cells were stained with FITC-Dextran or propidium iodide (PI), and analyzed by flow cytometry. FITC-Dextran stained the cytoplasm and/or the nucleus of 80% of the HeLa cells, while annexin V-FITC stained 43% of cells and PI stained the nucleus of 18% of cells. When heated at 50°C and 60°C, the percentages of necrotic cells increased proportionally to heat treatment, 41.7% and 77% of the cells were stained by FITC-Dextran, while 39.5% and 70.3% were stained by PI. FITC-Dextran was selectively internalized across plasma membrane after moderate heat shock. In contrast, in the necrotic cells, the permeability of the membrane was not selective and the percentage of cells stained with FITC-Dextran or PI, was equivalent. Our results indicate that FITC-Dextran can be used as a marker to reveal the cellular damage induced by heat shock and to study the early as well as the late stages of apoptosis.

Keywords: Apoptosis, flow cytometry, fluorescein labeled dextran, heat shock, lethal hyperthermia, necrosis, propidium iodide.

1. INTRODUCTION

Necrosis "passive death" is caused by cell injury induced by stress. It generates the alteration of the permeability of the plasma membrane resulting in the release of cytoplasmic components of the cell, which triggers the inflammatory process. In contrast, apoptosis "active death" is programmed to remove unwanted cells, such as cancer cells or infected cells [1, 2]. Apoptosis involves the activation of specific genes and is accompanied by an active nuclear fragmentation by endonucleases. It is characterized by cytoplasmic granularity, cell shrinkage and puffiness of the plasma membrane causing apoptotic bodies [3, 4].

Flow cytometry is widely used in detecting apoptotic cells [5]. Apoptosis can be detected by various molecular probes, however each probe identifies apoptosis in the early stages or at an advanced stage, but not both. To follow all the steps, it is necessary to combine several markers [6, 7]. The translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer layer, is an early event of apoptosis. It can be detected by the binding of a protein with high avidity for phosphatidylserine, annexin V, to the plasma membrane [8]. In spite of this early event, the integrity of the plasma membrane is preserved and most functions of the membrane remain unchanged. Apoptotic cells in the early stages exclude viability testing dyes such as trypan blue or propidium iodide (PI), a DNA binding dye, while late stage apoptotic or necrotic cells take up PI which stains the nucleus [9]. Plasma membrane integrity seems to contrast with mitochondrial membrane depolarization, alteration of membrane permeability and the release of proteins such as cytochrome c, which are early apoptotic events of the intrinsic pathway [10].

To work on apoptosis and necrosis we used peripheral blood mononuclear cells and HeLa cells. The latter is an immortal cell line derived from cervical cancer, which is easily cultured in vitro, multiply quickly and have excellent cell viability [9, 11, 12]. We stained with FITC-Dextran and PI, heat shock stressed cells. After a mild heat shock to induce apoptosis, FITC-Dextran was selectively internalized through the plasma membrane though PI was excluded in a majority of the cells. However in necrotic cells, membrane permeability was not selective and the percentage of cells stained by FITC-Dextran or PI was equivalent. Our findings

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show that FITC-Dextran can be used as a label to analyse cell damage induced by heat shock and to examine the early as well as the late phases of apoptosis.

2. MATERIALS AND METHODS

2.1. Cells Preparation for Apoptosis

To induce apoptosis we worked with HeLa cells (lined cells), very commonly used in laboratories (a kind gift from Professor Jean-Claude Nicolas, Virology Laboratory, Hospital Rothschild and Hospital Tenon, AP-HP). HeLa cells were maintained in RPMI medium (Gibco, Cergy Pontoise, France) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HeLa cells at a concentration of 5.10^5 cells/ml set in culture dishes, are incubated at 37°C for 48 hours in 5% CO2 and 95% air; check HeLa cells to less than 2% cell death was made by blue trypan. Cells resist and do not fit easily into apoptosis in culture, competence apoptotic cell lines vary in resistance (< 3%) to hypersensitivity (> 96%); to induce apoptosis HeLa cells are incubated in a water bath for 1 hour at 43.5°C, then for about 8 hours at 37°C in 5% CO2 and 95% air; control HeLa cells were incubated for 9 hours at 37°C in 5% CO2 and 95% air.

2.2. Cells Preparation for Necrosis

Peripheral blood mononuclear cells (PBMC) donors were healthy subjects (n=16); those are quiescent cells in G0 phase. The study protocol was in accordance with the local ethical committee guidelines. Peripheral blood mononuclear cells were isolated from EDTA anti-coagulated blood (Vacutainer 7 ml plastic tubes, Beckton Dickinson, Le Pont de Claix, France) by centrifugation at 500g for 20 minutes at 20°C on a layer of Ficoll (d=1.077) (Eurobio, Les Ulis, France), then washed with RPMI medium; check cells to less than 2% cell death was made by blue trypan. To induce necrosis, peripheral blood mononuclear cells or HeLa cells at a concentration of 5.10^5 cells/ml, were suspended in the culture medium RPMI, and then incubated in a water bath for about 1 hour at 50°C or 60°C; control cells were incubated for 1 hour at room temperature.

2.3. Labeling of Cells

Treated HeLa cells, control HeLa cells, treated PBMC and control PBMC were washed with 3 ml of RPMI medium and centrifuged for 10 minutes at 500g. The cells were re-suspended in 100 µl of RPMI medium and mixed in Q-prep tubes with 10 µl of PI (Sigma, Saint-Quentin Fallavier, France), 10 µl of FITC-Dextran (MW 4000 daltons, reference 46944), or 5 µl of Annexin V-FITC (Sigma-Aldrich reference A9210). The final concentration of PI and FITC-dextran was 7.5 µM and 1.13 µM, respectively. The cells were then incubated for 25 minutes at room temperature in the dark. The labeled cells are taken with 3 ml of RPMI medium and centrifuged for 10 minutes at 500g. The cells are then taken with 1 ml of RPMI medium and analyzed by flow cytometry or fluorescence microscopy.

2.4. Microscopy and Photography

Immunofluorescence and cell images were acquired on Zeiss Axioplan microscope equipped with the filter set 09 (Excitation 450-490, Beam splitter FT 510, Emission LP 515), the filter set 15 (Excitation BP 546, Beam Splitter FT 580, Emission LP 590) and the camera MC80DX.

2.5. Analysis by Flow Cytometry

The sample was analyzed on COULTER EPICS XL flow cytometry equipped with single argon ion laser (15 mW, 488 nm). PI is excited at 500 nm and has an emission at 600 nm; FITC-Dextran is excited at 495 nm and has an emission at 525 nm. The photomultiplier (PMT) captures the fluorescence passing through the filters; and it generates electrical signals by amplifying and increases tension. Band Pass filters select the photons entering the PMT, avoiding the overlapping emission spectra of the fluorochromes. Compensation subtracts the interference due to leakage of fluorescence captured by sensors in the PMT, according to an equation in which we subtract a percentage of the green fluorescence from FITC-Dextran (FL1) to the orange fluorescence from PI (FL2) "FL2 compensated = FL2 uncompensated – z% FL1"; this was taken into consideration in our labeling experiments simultaneously with FITC and PI, due to an overlap of emission spectra. Photomultiplier tube voltage and spectral compensation were also set by using cells stained with only a single dye Either FITC or PI.

Fluorescent beads (Flow-CheckTM Beckman Coulter) were used to control the values of fluorescence. For each sample, a region for apoptotic and necrotic cells was defined; lyophilized human lymphocytes (CYTOTROL Beckman-Coulter) were used to control the results. To analyze cells by flow cytometry we used four histograms; a first histogram bi-parametric wherein the cell size (FS log) depending on the cell structure (SS log), cell population stained with PI or FITC-Dextran was gated on the basis their forward and side light scatter with any cell debris excluded from analysis; cells will be analyzed in a second histogram bi-parametric of FS log versus fluorescence markers, the numbers in a parenthesis is the mean percentage of dead cells FITC-Dextran$^+$ in case C2 or PI$^+$ in case E2; a third histogram bi-parametric of fluorescence FITC-Dextran versus fluorescence PI, the numbers in a parenthesis is the mean percentage of dead cells FITC-Dextran$^+$ / PI$^+$ in case E2; a fourth histogram mono-parametric, wherein the number of cells is a function of the fluorescence markers, the numbers in a parenthesis is the mean percentage of dead cells FITC-Dextran$^+$ at cursor I or PI$^+$ at cursor F.

3. RESULTS

Living HeLa cells and human PBMC were unstained by PI, annexin V-FITC or FITC-Dextran (Fig. **1** and Table **1**). After induction of apoptosis by heating for 1 hour at 43.5°C and incubating at 37°C, the nucleus of some cells was brightly stained by PI and the staining was proportional to DNA content of cells. FITC-Dextran stained cells were distributed among three peaks corresponding to unstained, dim stained and bright stained cells (Fig. **2**). In dim stained cells, FITC-Dextran was present only in the cytoplasm; but

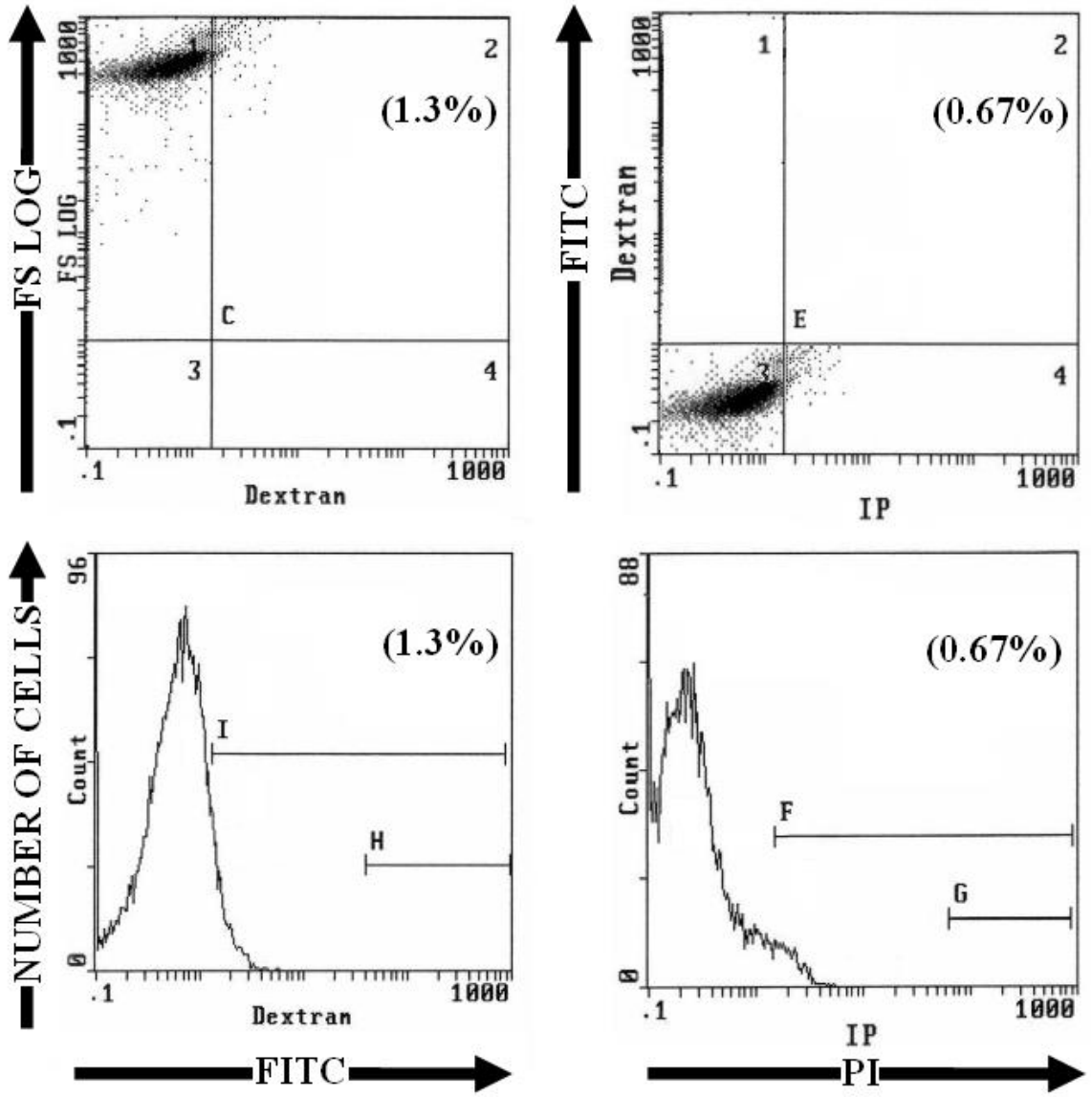

Fig. (1). Analysis by flow cytometry of live HeLa cells incubated with FITC-Dextran or PI. These dyes do not penetrate into living cells. On the histograms the background noise of the fluorescence is visible. The cells were targeted by scatters (FS, SS).

Table 1. Staining with FITC-Dextran, PI or Annexin V-FITC apoptotic cells induced by a heat shock.

Cells*	% stained cells			
	FITC-Dextran	PI	Annexin V	Statistics (Student t test)
HeLa control	1.3±0.83	0.67±0.22	1.8±0.9	
HeLa heated	79.6±5.5 %	18.2±1	42,6±3.8	p<0.001
PBMC control	1.42±0.86%	1.03±0.32	2±0.7	
PBMC heated	70.3±6.2%	15±1.8%	38.9±4.5	p<0.001

*Cells were incubated at 43,5°C for 1 hour and at 37°C for 8 hours to induce apoptosis; then they were labelled with Annexin V-FITC, FITC-Dextran and PI, then analysed by flow cytometry. Control cells were unheated. The results represent the means ± S.D of 16 different samples, each tested twice.

in bright stained cells, FITC-Dextran was present in cytoplasm and nucleus (Fig. **3**). The fraction of cells stained with FITC-Dextran, was three times more than those stained by PI or by Annexin V-FITC ($p<0.001$) (Table **1**). This suggests that FITC-Dextran stains different stages of cell damage induced by heat shock, from early stages to late stages of apoptosis with fragmentation of the nucleus and very late stage with loss of nucleus. While PI marks apoptotic cells still having a nucleus even if it is fragmented.

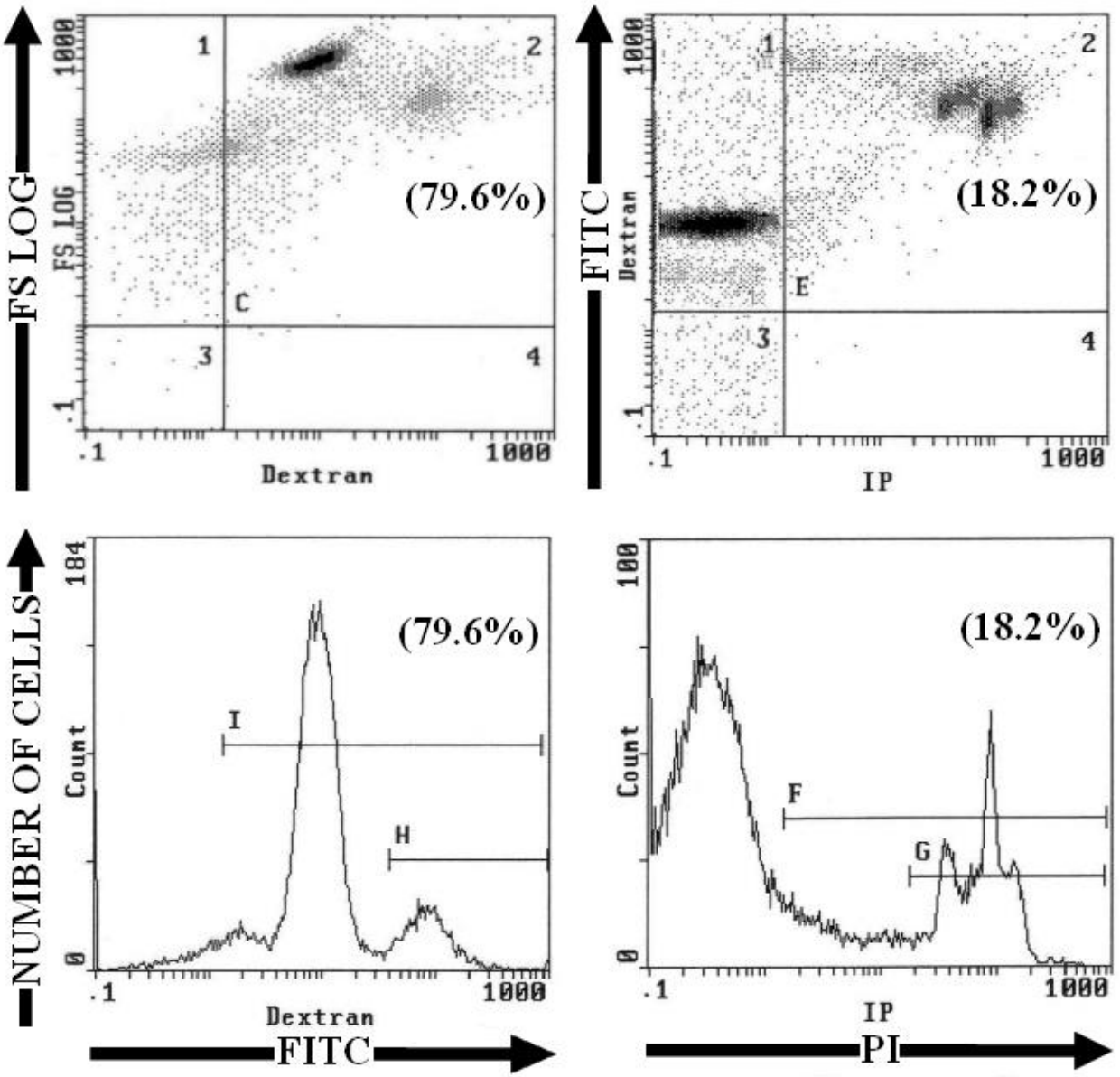

Fig. (2). Analysis by flow cytometry of apoptotic HeLa cells stained with FITC-Dextran and PI. Apoptosis was induced by incubating the cells at 43.5°C for 1 hour and at 37°C for 8 hours. Then the cells were labeled with PI or FITC-Dextran. The results are typical of those seen with the 16 different samples, each tested twice.

FITC-Dextran stained cells were distributed among three peaks of frequency corresponding to unstained, dim stained and bright stained cells. The population dim stained with FITC-Dextran was unstained by PI, in contrast to the bright stained one which was also stained by PI. Staining by PI was proportional to the DNA content of the cells, so they were distributed among several peaks. Because HeLa cells were actively proliferating cancer cells, when harvested they were at different stages of the cell cycle G1, G2 or S, or were aneuploid, so they contain varying amounts of DNA; some cells had fractioned nucleus and having lost a part of the DNA due to apoptosis; some cell aggregates were also present.

In the left histogram, cursor I underlines the peak of dim stained cells with FITC-Dextran, cursor H underlines the bright stained cells. In the right histogram, the cursor G underlines three peaks of bright PI stained cells (dead cells), cells in G1 of cell cycle, those in G2 and the polyploid or agregated cells. The dim stained cells, at the left of cursor G and underlined by cursor F, are at the different stages of apoptosis.

To induce cell necrosis HeLa cells and PBMC were heated at 50°C or 60°C for 1 hour, then incubated with FITC-Dextran and or PI. In single staining, FITC-Dextran and or PI stained roughly the same percentages of cells (the difference was not statistically significant). Double staining confirmed that all dead cells were stained both with FITC-Dextran and with PI. In cells heated at 50°C, FITC-Dextran stained cells were distributed over several peaks of fluorescence intensity. Each peak could be related to the intensity of necrosis, the most affected cells are more permeable to staining. In contrast in cells heated at 60°C, cells were stained brightly by FITC-Dextran in one large peak (Fig. (**4**) for PBMC); and FITC or PI stained cells were aproximately twice as many than in cells heated at 50°C ($p<0.001$; $n = 16$) (Table **2**).

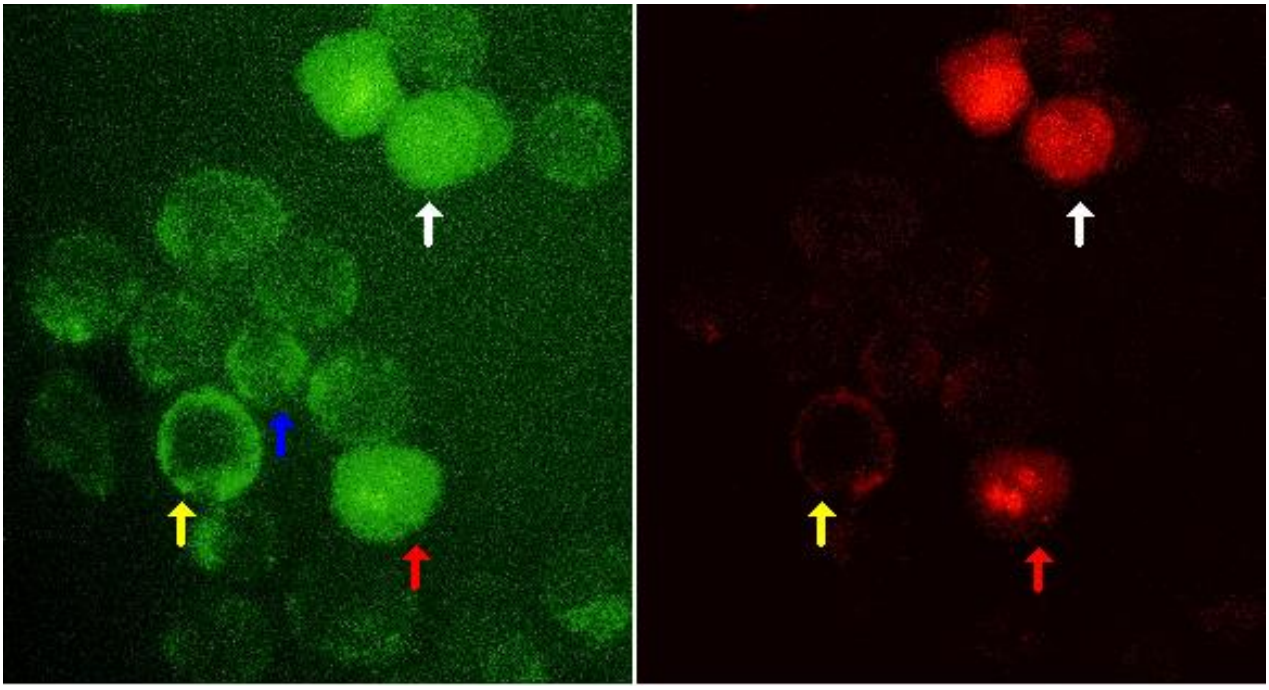

Fig. (3). Microphotography of HeLa cells undergoing apoptosis stained by FITC-Dextran and PI (x1000). HeLa cells were incubated at 43,5°C for 1 hour and at 37°C for 8 hours then labeled with FITC-Dextran and PI and analyzed by fluorescence microscopy. The left micrograph shows the green fluorescence (FITC), and the right micrograph shows the red fluorescence (PI). Because FITC-Dextran stained the early and late stages of apoptosis, the cells were labeled with FITC-Dextran more than PI. The white arrow shows apoptotic cells with plasma membrane rupture, the red arrow indicates the apoptotic cells with nucleus fragmentation, the yellow arrow shows apoptotic cells with nucleus alteration, and the blue arrow indicates apoptotic bodies. The living cells impermeable to FITC-Dextran aren't stained.

Table 2. FITC-Dextran or PI labelling of necrotic cells induced by a heat shock.

Cells*	% stained cells		
	FITC-Dextran	PI	Statistics (Student t test)
PBMC control	1±0.42	0.47±0.32	
PBMC heated at 50°C	41.7±11.2	39.5±10.6	NS
PBMC heated at 60°C	77±17	70.3±11.6	p<0.2

*Cells were heated to 50°C or 60°C for 1 hour and then stained with PI and FITC-Dextran, and analyzed by flow cytometry. Control cells were unheated. PBMC were obtained from healthy donors and results represent the means ± S.D of 16 donors tested twice.

4. DISCUSSION

FITC-Dextran did not penetrate living cells, but during apoptosis or necrosis it was internalized and concentrated by binding to cellular proteins. In contrast to PI, FITC-Dextran was internalized in the majority of the heat shock stressed cells and therefore it could be used as a marker to identify cells in the early as well as in the late stages of apoptosis. PI marks apoptotic cells having a nucleus, binds and marks DNA, but it is unable to detect the different phases of apoptosis [13].

In early apoptosis, phosphatidylserine translocates from the inner leaflet of plasma membrane to the outer leaflet. This phenomenon can be detected in flow cytometry by the binding to the phosphatidylserine of the outer leaflet of FITC-conjugated Annexin V [14, 15]. Larger changes in the integrity of the plasma membrane has not been described [8]. Apoptotic cells in the early phase exclude viability testing dyes such as trypan blue or propidium iodide (PI). PI, penetrates in apoptotic cells at a late stage or in necrotic cells and stains the nucleus [16]. The integrity of the plasma membrane seem to contrast with the depolarization and the alteration of the permeability of the mitochondrial membrane with consequent release of proteins such as cytochrome c, which are early apoptotic events of the intrinsic pathway [10].

Different methods are used to assess cell death or mitochondrial dysfunction [17]: detection of activated enzymes such as caspases [18], alteration of the asymmetry of the membrane as the flip-flop of phosphatidylserine [8], DNA damage by staining with dyes as PI or Hoechst 33342 [19, 20]. These markers can detect apoptosis, but they are not able to label a very late stage of apoptosis.

During the late stage of apoptosis, cell DNA is fragmented by endonucleases. The TUNEL technique

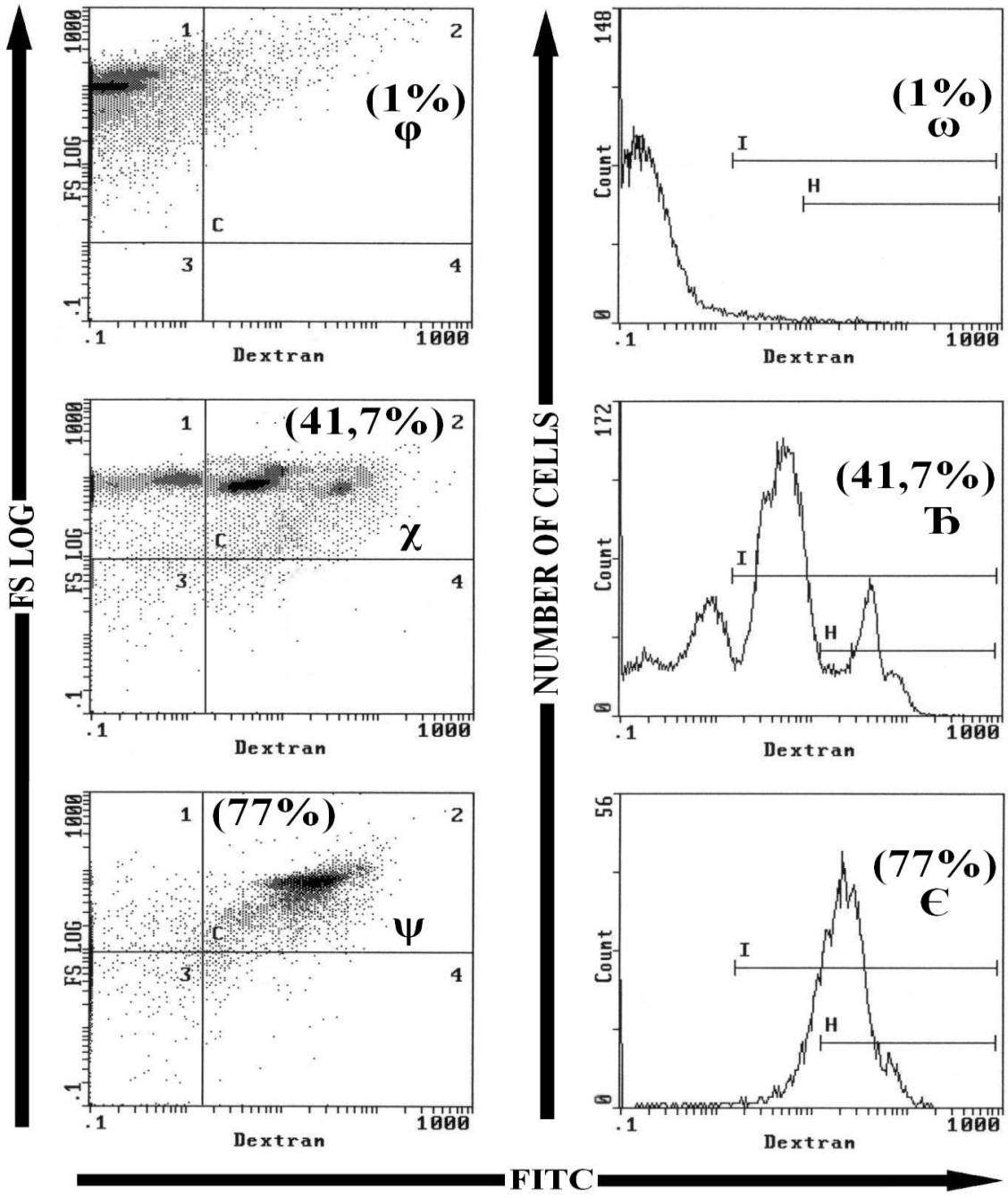

Fig. (4). Flow cytometric analysis of necrotic PBMC stained with FITC-Dextran. Healthy donors PBMC were heated at 50°C or 60°C for 1 hour, stained with FITC-Dextran and analyzed by flow cytometry. The numbers in parenthesis are the mean percentage observed with 16 healthy donors; each one tested two times.

The histograms at the top show the background staining observed with the live control cells (1% dead cells targeted by the cursor I), histograms located in the middle show the cells heated at 50°C, histograms at the bottom show the cells heated to 60°C. The left histograms (φ, χ, ψ) are bi-parametric, log FS versus green fluorescence (FITC-Dextran). The right histograms (ω, Ъ, Є), are mono-parametric and show the percentage of cells with various fluorescence intensity after staining with FITC-Dextran.

On the histogram Ъ each peak corresponds to different stages of necrosis, on the histogram Є there is a single peak corresponding to dead cells.

(terminal uridine nucleotide end labeling) using TdT (terminal deoxynucleotidyl transferase), detects these DNA fragments by fixing dUTP (deoxyribonucleotide triphosphate) coupled to a label to the free 3'OH ends. TUNEL technique allows to detect apoptotic cells in late stage [21]. The commonly used tracers vary in molecular size and the chemical nature, the combination of techniques is required to track the various stages of apoptosis.

We tested FITC-Dextran to investigate alterations of plasma membrane permeability in apoptosis and necrosis.

FITC-Dextran has the advantage of being stable, water soluble, non-toxic to cells and available in different molecular weight from 2000 to 15000. The different experiments in this work were performed by Fluorescein Isothiocyanate Dextran MW 4000. Dextran is a polysaccharide consisting of linear chains of D-glucose linked by α (1-6) glycosidic bonds and branched by α (1-3) glycosidic linkages. It is generally extracted from the bacteria type Leuconostoc mesenteroide or streptococcus; it binds to proteins in the physiological pH and ionic strength [22]; it has no spontaneous fluorescence.

FITC-dextran was already used to study the permeability of membranes [23, 24]. It has been widely used to characterize the blood-brain barrier and to quantify the alteration of its permeability during ischemic stroke, trauma, infarction, inflammation and brain tumors. Studies on vascular permeability were performed using FITC-Dextran in deep tissues, intestine, kidney, brain oedemas, and peripheral nerves [25, 26]. FITC-Dextran was also used to determine the mechanisms of the transport of molecules across vascular endothelial cells, and it has been suggested that the rate of transport through inter-cellular junctions depends on the molecular size of FITC-dextran [27]. FITC-dextran, can not passively enter the cells, it penetrates through endocytosis. Thus it was used as a marker to study the encapsulation process and endocytosis of substances by erythrocytes [28, 29], the pinocytosis by leukocytes [30], and the vesicular pH in a variety of cell types including hepatocytes, kupffer cells and parenchymal cells [31, 32]. During trauma of the plasma membranes, FITC-Dextran diffuses through passages in the membrane of about 3-5 nm, causing intense staining of the cytoplasm. This phenomenon has been confirmed in a variety of cells including epidermal cells, intestinal cells and fibroblasts [33, 34]. Thus, FITC-Dextran was used as a molecular marker to measure the absorption of proteins in living cells by electroporation [35], to study its distribution in CHO cells by sonoporation [36], and to detect the permeability of the plasma membranes of dead cells [37].

Apoptosis can be induced by various techniques including hyperthermia. Indeed hyperthermia between 41.5°C to 46°C is widely used to kill cancer cells. However certain cancer cells such as prostate cells are necrotic at temperatures above 46°C [38]. By gradually increasing the temperature from 37°C to 46°C for 1 hour incubation, apoptosis was increased gradually to a maximum at 43°C, but above 46°C cell death was due to necrosis [39-43]. It has been suggested that heat stress on intestinal epithelial cells IEC-6 between 40°C and 43°C for 2-5 hours produces 11.9% to 85% apoptotic cells against 3.6% in non heated controls [44]. These results are similar to our results obtained from HeLa cells hyperthermia at 43.5°C for 1 hour, followed by incubation at 37°C for 8 hours.

Many studies have been carried out on several types of cells to study the induction of apoptosis by hyperthermia. It appears that the induction and regulation mechanisms vary among different cell types. If activation of the mitochondrial pathway is usually accepted, however the results are conflicting on the initiator. With Jurkat T-lymphocytes an essential role for caspases was shown. The activation of initiator caspase 9 is essential in the execution of apoptosis induced by heat shock. Mitochondrial outer membrane permeability and cytochrome c release occur downstream of initial caspase 9 activation [45]. In contrast, it was observed that caspase 9 as well as other known initiator caspases, were not essential for the activation of the mitochondrial pathway. However activation requires a new apical protease with caspase-like activity [46]. Heat shock alters other physiological functions of these cells in reducing the splicing of pre-mRNA, U2AF35, SF3a3 and snRNP [47].

Thermal shock causes the denaturation and aggregation of cell proteins, but cells respond to hyperthermia by synthesizing molecular chaperones hsps (heat shock proteins) that protect against necrosis and apoptosis. Hsps bind to cell proteins exposed to stress and maintain their conformation [48]. It has been reported that thermal shock of PC12 cells induces the expression of apoptotic proteins such as JNK (c-Jun-N-terminale kinase), but the anti-apoptotic protein Hsp70 binds JNK to protect cells against heat stress [49]. A work mentioned that tobacco smoke induces cell apoptosis and necrosis, while the anti-apoptotic proteins Bcl-2 and Hsp70 offset this induction by protecting cells against oxidative damage, lipid peroxidation and ROS (reactive oxygen species) [50]. Other studies have indicated that hyperthermia of testicles in cryptorchidism, varicocele or chronic fever induced the expression of p53 apoptotic protein. This protein is co-regulated by MTA1 (Metastasis-associated protein 1) which protects cells against heat stress [51]. In HeLa cells, which we have used in our work, it was shown that hyperthermia induces apoptosis by ROS production and through receptors such as FAS [52].

To determine whether the FITC-Dextran internalization by a cell is due to an alteration of the permeability of the membrane caused by heat only, we compared the internalization of FITC-Dextran at various temperatures. After induction of HeLa cells apoptosis by heating at 43.5°C, the percentage (79.6%) of cells stained by FITC-Dextran, was higher than those observed at 50°C (41.7%) and 60°C (77%). It seems that the internalization of FITC-Dextran was more pronounced during apoptosis or by steps before apoptosis than the combined effect of heating and necrosis. In fact, after cells were subjected to a thermal stress at 43.5°C for 1 hour, they were incubated 8 hours at 37°C to induce apoptosis. During this incubation, the cells are also trying to repair the damage due to thermal shock and different processes will occur such as endocytosis of macromolecules (this is the case of polysaccharide) or overexpression of hsp that interacts with cell membranes to ensure homeostasis or cell survival [53, 54]. Similarly, it has been shown that endocytosis is a reversible process occuring in the early phase of apoptosis [55]. At this stage FITC-Dextran may be internalized by endocytosis.

Once it has entered the cell, FITC-Dextran is concentrated by binding to cytoplasmic or nuclear molecules. After being subjected to fatal hyperthermia, apoptotic cells and necrotic cells had internalized FITC-dextran proportionally to their damage. The percentage of apoptotic cells stained with FITC-Dextran was three times higher than those stained with PI. It remains to show, if apoptosis goes to completion in all the cells that internalize

FITC-Dextran. Reversibility of apoptosis was observed in several cell types including HeLa cells. In the early phase of apoptosis, some cells can restore the exposure of phosphatidylserine on their membrane, repair mitochondrial damage or stop caspase activation [56, 57]. In the advanced stage of apoptosis corresponding to DNA damage, some cells can survive by becoming clonogenic [58, 59]. Staining of cells with FITC-Dextran is useful in the study of apoptosis or necrosis by flow cytometry in various pathologies such as infectious diseases, cellular aging, cancer, lethal hyperthermia, inflammation and autoimmune diseases.

ABBREVIATIONS

AP-HP	=	Assistance Publique des Hôpitaux de Paris
BP	=	Band pass
CD	=	Cluster of Differentiation
DL	=	Dichroic Long-pass
dUTP	=	deoxyribouridine Triphosphate
FAS	=	TNF Receptor Superfamily, Member 6
FL	=	Fluorescent Light
FITC-Dextran	=	Fluorescein isothiocyanate coupled to dextran
FS	=	Forward Scatter
hsp	=	heat shock protein
JNK	=	c-Jun-N-terminale kinase
PBMC	=	Peripheral Blood Mononuclear Cells
PI	=	Propidium iodide
ROS	=	Reactive Oxygen Species
SF3a3	=	Splicing Factor 3A subunit 3
SS	=	Side Scatter
snRNP	=	small nuclear ribonucleoproteins
TdT	=	Terminal deoxynucleotidyl Transferase
TUNEL	=	Terminal Uridine Nucleotide End labeling
U2AF35	=	Splicing factor U2AF U2 (small nuclear RNA auxiliary factor) 35 kDa subunit
htpR	=	heat shock regulatory gene - heat-shock promoters

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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