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Research Article

Conformational dynamics of the β_2-microglobulin C terminal in the cell-membrane-anchored major histocompatibility complex type I

M. Massa[a], P. Mangione[a,b], P. Pignatti[c], M. Stoppini[b], G. Zanotti[d], P. Arcidiaco[e], G. Merlini[a,b], G. Ferri[b] and V. Bellotti[a,b,*]

[a]Biotechnology Research Laboratory, IRCCS Policlinico S. Matteo, Pavia (Italy)
[b]Department of Biochemistry, University of Pavia, via Taramelli 3b, I-27100, Pavia (Italy),
Fax + 39 382 423108, e-mail: vbellot@unipv.it
[c]Department of Pediatrics, University of Pavia (Italy)
[d]Institute of Organic Chemistry, University of Padova (Italy)
[e]Centro Grandi Strumenti, University of Pavia (Italy)

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Abstract. We have recently described an anti-β_2-microglobulin (β2-m) monoclonal antibody (mAb 14H3) capable of recognizing the epitope 92–99 of the protein in the monomeric native state as well as in the fibrillar polymeric state, but not in the major histocompatibility complex type I (MHCI) anchored to the cell membrane. In the present study, we investigated the molecular basis for the inaccessibility of the C-terminal end of β2-m in the MHCI complex, and demonstrated that mAb 14H3 binds the soluble fraction of the MHCI complex with a K_d of 0.3 μM. An interaction between the complex and the membrane protects β2-m from immunological recognition at the MHCI level. This protection from antibody recognition can be weakened by procedures such as heat shock or γ irradiation that perturb the membrane structure and commit the cell to the apoptotic pathway. mAb 14H3 can recognize MHCI in a transient state that most likely precedes β2-m shedding and may be proposed as a useful tool for dynamic analysis of MHCI conformational modifications.

Key words. Amyloidosis; major histocompatibility antigen type I; apoptosis; β_2-microglobulin; monoclonal antibody.

Class I MHC (MHCI) molecules are expressed on the cell surface of most mammalian cells as a heterotrimeric complex that consists of the HLA heavy chain, β_2-microglobulin (β2-m) and the bound peptide [1]. The role of the MHCI in antigen presentation and modulation of cell proliferation has been extensively investigated, and structural and thermodynamic studies have shown that β2-m plays an active role in the assembly and stability of MHCI quaternary structure [2]. The physiological process of MHCI catabolism is also associated with β2-m shedding from the complex, inducing a transitory persistence of the free heavy chain on the cell membrane, followed by its internalization into the cytoplasm and a lysosomal degradative process [3].

The dynamic interaction between the MHCI heavy chain, β2-m and peptides is under extensive investigation because elucidation of the association kinetics of the three MHCI constituents could shed light on the

* Corresponding author.

mechanisms of the immune response [4]. Furthermore, the kinetics of the formation and catabolism of the MHCI complex are particularly relevant in dialysis-related amyloidosis, where the amyloid fibrils are created by an excess of circulating monomeric β2-m. In this type of amyloidosis, the MHCI can be considered the storage component of the amyloid fibril precursor and its synthesis and dissociation kinetics are most likely associated with progression of the disease. We have recently described a monoclonal antibody (mAb 14H3) produced against the C-terminal end of β2-m (sequence 92–99) that recognizes β2-m as a monomer or as a fibrillar polymer, but which is apparently unable to detect folded β2-m in the MHCI anchored to the intact cell membrane [5].

In this study, we show that following in vitro cell damage of both the Jurkat T cell line and peripheral blood cells from normal subjects, the 92–99 epitope of β2-m becomes accessible to the 14H3 mAb, possibly as a consequence of conformational changes in the MHCI.

Materials and methods

Materials. The following materials were obtained from the commercial source given in parentheses: reagents used for immunofluorescence analysis (Tecnogenetics, Becton Dickinson, Pharmingen, Clontech and Dako); mouse isotyping kit and reagents for electrophoresis (Bio-Rad); reagents for cell culture and enzyme immunoassay (ICN, Gibco BRL, Hyclon). mAb 14H3 was prepared as previously described [5]. The recombinant MHCI complex, produced as described by Springer et al. [4] was kindly supplied by K. Doering and recombinant human β2-m was prepared as described by Reid et al. [6]. All other reagents were from Sigma.

Electrophoresis and immunoblotting. The electrophoresis was performed in 0.8% agarose gels in 75 mM sodium barbital buffer pH 8.6 at the constant current of 100 mA for 45 min. The transfer to polyvinylidene difluoride sheets was accomplished by overnight diffusion and the immunodetection was performed as previously described [7] using the anti-β2-m mAb 14H3. Immunological typing of the antibody was accomplished with peroxidase-conjugated rabbit anti-mouse immunoglobulins. The purification of mAb 14H3 from cell culture medium was carried out as previously described [5] by affinity chromatography on a protein G-conjugated Sepharose column.

Scatchard analysis. The K_d for the binding of mAb 14H3 to native β2-m and soluble MHCI was determined as described by Friguet et al. [8] from data obtained in competitive enzyme-linked immunosorbent assay (ELISA). The MHCI used in the competitive ELISA was previously submitted to gel filtration in 0.1 M phosphate buffer to exclude the presence of free β2-m in the protein solution. Equimolar concentrations of β2-m and MHCI were incubated separately with a constant amount of mAb 14H3 (1–0.7 nM) for 4 h at 20 °C in 0.1 M sodium phosphate, pH 7.4. Quantification of free and bound antibody was carried out by testing the antibody/protein mixture on microtiter plates coated with 1 µg/well of β2-m. The data obtained were introduced in the Scatchard equation to calculate the K_d values.

Size exclusion chromatography of recombinant MHCI. The analysis was performed in an FPLC system using a BioPrep SE 1000/17 in a 20 mM phosphate buffer pH 8.0.

Induction of cell damage. Jurkat cells, a human leukaemic T cell line, were cultured in RPMI 1640 complete medium with 10% fetal calf serum at 37 °C in a humidified atmosphere at 5% CO_2. Alternatively, 3 ml of whole blood from three healthy subjects were anticoagulated with ethylenediamine tetraacetic acid; 50 µl/sample was washed twice in saline, and peripheral blood cells (PBCs) resuspended in RPMI complete medium. To damage cells, they were: (i) cultured at 37 °C for 24 h at a concentration of 2.5×10^6 cells/ml; (ii) exposed to heat shock at 42 °C for 1 h and incubated for 2 h at 37 °C [9]; (iii) γ irradiated (20 Gy) and incubated for 24 h at 37 °C [10]. All the experiments were performed at least five times, unless otherwise indicated.

Immunofluorescence staining. Jurkat cells (3×10^5/sample), or 50 µl of PBCs were washed twice with 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 6.46 mM $Na_2HPO_4 \cdot 12H_2O$, pH 7.0 (NaCl/P_i), before starting the staining procedure. Ten microlitres of a purified murine mAb 14H3 (IgG1 isotype, 100 µg/ml), 1 µl of a murine anti-MHCI mAb (IgG1 isotype, 500 µg/ml), 10 µl of a rabbit purified anti-β2-m polyclonal antibody (200 µg/ml), or 5 µl of annexinV-biotin (20 µg/ml in the presence of 1.8 mM $CaCl_2$) were added to the cells for 30 min at 4 °C; the cells were then washed twice with NaCl/P_i, and stained with 30 µl of goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated (25 µg/ml) $F(ab')_2$, or 1 µl of big anti-rabbit FITC-conjugated $F(ab')_2$. Phycoerythrin (PE)-conjugated streptavidin (0.4 µg/ml) was used to reveal the annexin-V-biotin. After 30 min at 4 °C, the cells were washed twice and resuspended in 300 µl of NaCl/P_i or propidium iodide (PI) at 5 µg/ml in NaCl/P_i. A negative control was performed using 10 µl of an irrelevant purified antibody (mouse IgG1, 100 µg/ml), followed by the FITC-conjugated secondary antibody. Cells incubated only with PE-conjugated streptavidin were considered negative controls for the staining with annexin-V-biotin. Red PBCs were lysed at the end of the staining procedure by 1 ml of lysing reagent (Ortho Diagnostic, Raritan, N.J.) added to the pellet of PBCs and washed after 15 min. A total of 30,000 events were acquired from each sample and

analysed with a cytofluorimeter (FACScan, Becton Dickinson). Results were expressed as percentage of positive cells or as mean fluorescence intensity (MFI) calculated as mean fluorescence of the sample/mean fluorescence of the isotype control.

Microscopy immunofluorescence staining. After induction of cell damage, Jurkat cells were washed and incubated with mAb 14H3 or with an irrelevant purified antibody (IgG1) as described above, and then stained with 10 µl of the secondary antibody [goat anti-mouse FITC-conjugated $F(ab')_2$ fragment] (1 mg/ml). After 30 min at 4 °C, the cells were washed twice and the nuclei stained with diaminophenylindole (DAPI) (0.2 µg/ml) for 10 min at 4 °C. After spreading the cells on a slide, pictures were taken with an epifluorescence microscope (Zeiss) equipped with a high-performance CCD camera controlled by an Apple Macintosh computer. Grey-scale source images were captured separately with filters set for DAPI and fluorescein. Although the positive cells were always clearly visible to the eye through the microscope, grey-scale images were pseudocoloured and merged using computer software (PSI).

Statistical analysis. Data were analysed using the Wilcoxon test for paired samples and a P value ≤ 0.05 was considered statistically significant.

Results

Binding of mAb 14H3 to soluble MHCI. The ability of mAb 14H3 to recognize β2-m in the MHCI was previously investigated using MHCI integrated in the cell membrane [5]. To explore the interaction between this mAb and soluble MHCI, we used a soluble form of the recombinant MHCI complex constituted by the extracellular portion of the heavy chain (residues 1–284) and full-length recombinant human β2-m (residues 1–99). The immunoblot (fig. 1B), stained with mAb 14H3 after electrophoretic separation in native conditions of both monomeric β2-m and soluble MHCI (fig. 1A), shows that β2-m is specifically recognized by this antibody in soluble MHCI (fig. 1B). The cathodal portion of the MHCI band recognized by mAb 14H3 has identical electrophoretic mobility of monomeric β2-m and we cannot exclude the possibility that this could represent a minor β2-m component that dissociates from the complex under the electrophoretic conditions; however, the main portion of the band immunodetected by mAb 14H3 displays a faster mobility than free β2-m and corresponds to the electrophoretic mobility of soluble MHCI (fig. 1A). Investigation of the affinity of mAb 14H3 toward β2-m and the MHCI established a K_d of $2.8 \pm 0.4 \times 10^{-8}$ M for β2-m and of $3 \pm 0.3 \times 10^{-7}$ M for MHCI. The affinity of mAb 14H3 for recombinant β2-m is consistent with a previous determination for natural β2-m [5] and its affinity for soluble MHCI is one order of magnitude lower.

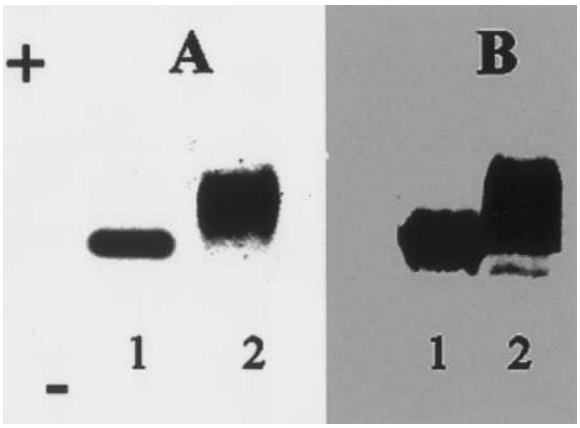

Figure 1. Agarose gel electrophoresis stained with Coomassie Blue (*A*) and immunoblotting stained with mAb 14H3 (*B*). Lane 1, recombinant human β2-m; lane 2, recombinant human soluble fraction of MCHI.

Evaluation of mAb 14H3 binding to β2-m following cell damage. Since the 14H3 mAb can detect β2-m in a soluble or fibrillar form, but not as an MHCI complex on the cell surface of PBMCs from healthy subjects [5], we investigated whether cell damage induced accessibility of the 14H3 epitope on the cell surface. To study a homogenous cell population, we first used the Jurkat cell line. In agreement with previous data on PBMCs [5], a very low percentage of Jurkat cells bound 14H3 mAb above background level (percentage of 14H3-positive cells: 0.5 ± 0.2). Jurkat cells were damaged by culture at a high cell density, by exposure to non-lethal heat shock, or by γ irradiation. Each of these cell-damage-inducing procedures resulted in a modest increase in the percentage of 14H3-positive cells (table 1). However, analysis on the basis of cell volume (forward scatter, FSC) and granularity (side scatter, SSC) [11] showed that following induction of damage, cells with a decreased FSC showed a marked and significant increase (fig. 2A, B). Moreover, cells presenting decreased FSC values also showed increased PI uptake (data not

Table 1. Percentage ($\pm$SD) of 14H3+ Jurkat cells evaluated before (baseline) and after the induction of cell damage.

	Number of experiments	14H3+ cells (%)
Baseline	5	0.5 ± 0.2
High cell density	7	0.68 ± 0.87
Heat shock	5	2.75 ± 1.93
γ irradiation	5	4.92 ± 0.87

Table 2. Percentage ($\pm$SD) of 14H3+ cells evaluated in gate R1 before (baseline) and after the induction of cell damage.

	Number of experiments	14H3+ cells in gate R1 (%)		
		Baseline	Following cell damage	*P* value*
Increased cell density	7	1.8 ± 0.5	10.7 ± 5.3	0.02
Heat shock	5	3.7 ± 2.5	13.9 ± 8.2	0.04
γ irradiation	5	1.8 ± 0.2	9.8 ± 1.4	0.04

* Evaluated with the Wilcoxon test for paired samples.

shown). When we evaluated the binding of the 14H3 mAb on the gated cells, we found that all the 14H3-positive cells had low FSC values (table 2; fig. 2C, D for one representative experiment).

To evaluate whether experimental conditions inducing exposure of the 14H3 epitope on β2-m were related to changes in the cell membrane charge, we tested damaged cells for their capacity to bind annexin-V, an early marker of apoptosis that recognizes the negatively charged phosphatidylserine once it is exposed outside the cellular membrane [12]. We found that 67% of the cells with low FSC values bound annexin-V. Double-staining analysis showed that 40% of the annexin-V-positive cells were also recognized by 14H3 mAb (fig. 2E). It is worth pointing out that 14H3 staining was absent in annexin-V-negative cells. Similar results were obtained with heat-shocked or high-density-cultured Jurkat cells (data not shown). These data show that binding of 14H3 occurs only in cells entering or undergoing apoptosis, as shown by annexin-V binding.

Immunofluorescence microscopy of γ-irradiated Jurkat cells confirmed the specific immunostaining by the 14H3 mAb of a damaged cell population characterized by condensed chromatin or fragmented nuclei typical of apoptotic cells (fig. 3B, C), while no staining was observed with control isotype antibody (fig. 3D) or on cells without apoptotic morphological changes (fig. 3A). Similar results were obtained with heat-shocked or high-density-cultured Jurkat cells (data not shown). In summary, we found that cell damage induced by a variety of methods causes exposure of the 14H3 epitope in a cell population characterized by low FSC, increased PI uptake, annexin-V binding and condensed chromatin, all signs of cells entering or undergoing apoptosis.

Interestingly, induction of cell damage was also followed by a 30% decrease in expression of the MHCI complex, as monitored by evaluation of the MFI of staining with both anti-heavy chain (baseline MFI: 24.1 ± 1.3, after heat shock: 16.5 ± 1.4) and anti-β2-m (baseline MFI: 6.0 ± 1.6, after heat shock: 3.9 ± 1.0) polyclonal antibodies.

We applied the same cell-damage-inducing procedures to PBCs from healthy subjects. In the entire population, the percentage of 14H3-positive cells increased following γ irradiation (4.77% vs 0.63% in the non-irradiated sample). Granulocytes, monocytes and lymphocytes were distinguished according to standard FSC and SSC characteristics, and the binding of the 14H3 mAb and annexin-V was evaluated on gated cells. The 14H3-positive annexin-V-positive cells did not exceed 0.8% in the non-irradiated sample (fig. 4A lymphocytes; panel 4B monocytes; 4C granulocytes). Similarly to the findings for Jurkat cells, following cell damage, a population of cells, predominantly granulocytes, binding both 14H3 and annexin-V appeared (fig. 4D–F). 14H3 binding was limited to annexin-V-positive cells, while 14H3 staining was absent in annexin-V-negative cells. These data suggest that the binding of the 14H3 mAb to β2-m on PBCs, as on Jurkat cells, is on damaged cells, possibly as a consequence of conformational changes in the MHCI, or in its interaction with a perturbed cell membrane.

Discussion

The contribution of the physiological environment (i.e. the cell membrane) to the structure, folding and function of the natural and complete form of the MHCI complex is difficult to investigate, because most of the biophysical techniques used to analyse protein structure are precluded. mAbs recognizing well-determined epitopes of the MHCI subunits may represent useful tools in this area of research. We have recently described a mAb that was considered unable to recognize β2-m in the MHCI complex because human cells expressing the MHCI on their membrane were not detected by mAb 14H3 using flow cytometry [5]. The results reported here demonstrate that this mAb can in fact recognize, through the epitope 92–99 of β2-m, the soluble form of the MHCI. We found that in conditions of cell injury, the MHCI becomes accessible to this antibody, suggesting that dynamic modification of the protein-membrane contacts allows this antibody to bind to β2-m.

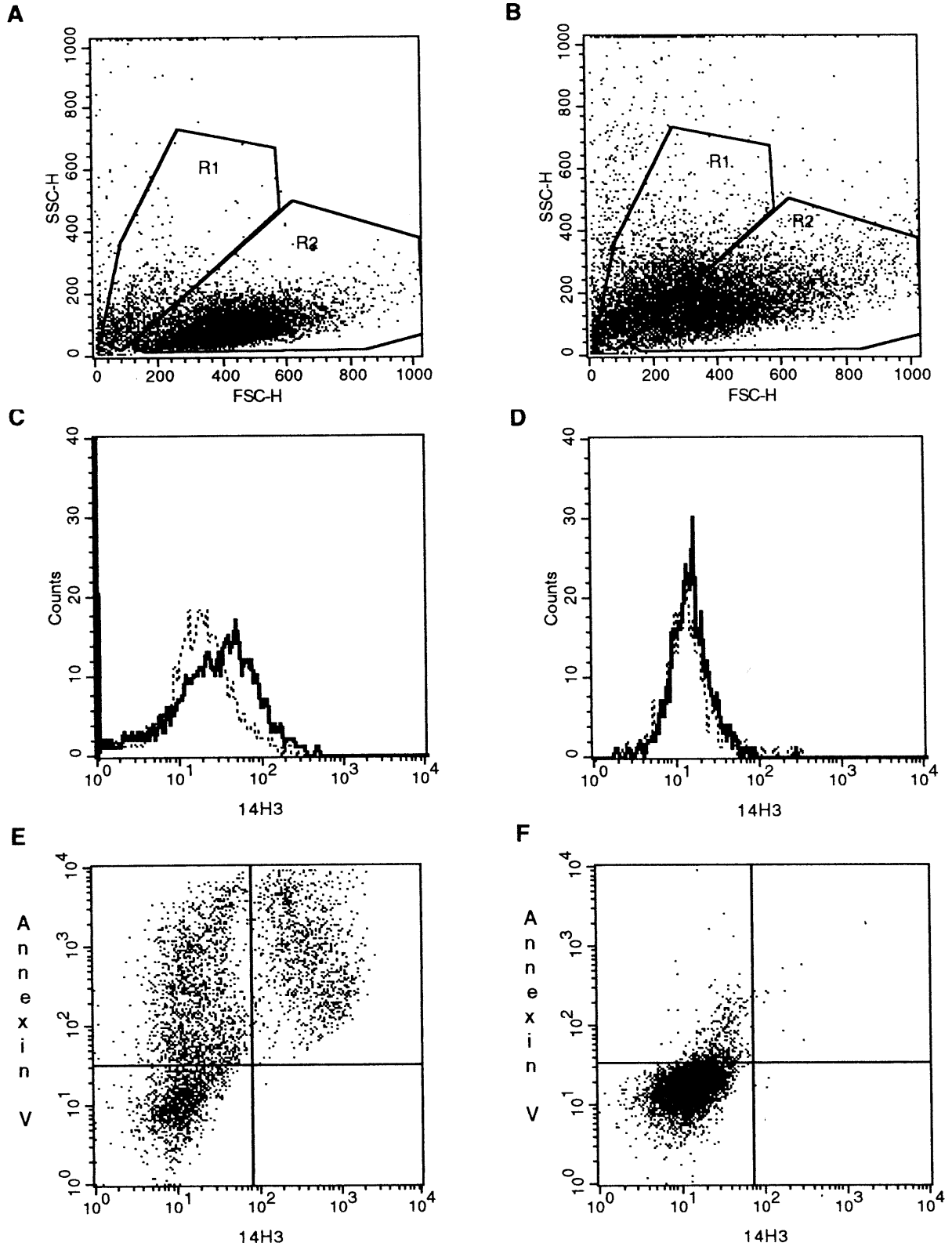

Figure 2. Resting (*A*) or damaged (γ-irradiated) (*B*) Jurkat T cells gated according to forward scatter (FSC) and side scatter (SSC) values. Cytofluorimetric analysis of damaged Jurkat cells with low FSC values (gate R1) (*C*) or with high FSC values (gate R2) (*D*) stained with the 14H3 mAb (thick line), or with the irrelevant isotypic control (dotted line). (*E*, *F*) Upper right quadrants show results of Jurkat cells with low FSC values (*E*) or high FSC values (*F*) double-stained with annexin-V and mAb 14H3. γ-irradiated Jurkat cells double-stained with PE-conjugated streptavidin, and an irrelevant murine isotypic control were used as negative control.

The cell population recognized by mAb 14H3 on both Jurkat cells and PBCs from healthy subjects has the phenotype of damaged cells committed to the apoptotic pathway. Their membrane appears deeply perturbed and exposes to the external surface negatively charged molecules of phosphatidylserine easily recognized by annexin-V [12].

Conformational modifications of the MHCI have already been demonstrated as a consequence of changes in the membrane depolarization state [13] and a large body of literature exists concerning the effects of membrane composition and fluidity on the activity of integral membrane enzymes [14]. The modifications in the extent and strength of the interaction between the plasma membrane and the MHCI molecules required for mAb 14H3 binding can be schematically depicted in the model presented in figure 5, which illustrates two possible scenarios. In figure 5A, the MHCI is oriented with its long axis nearly perpendicular to the positively charged phospholipid layer and the peptide-binding site is opposite to it. In this orientation, peptide 92–99 of β2-m is hidden between the heavy chain and the membrane surface and is not exposed for interaction with the aqueous phase and therefore with the antibody; moreover, the proximity of the charged surface of the membrane may prevent the antibody from approaching the epitope. In figure 5B, a simple rotation of the flexible peptide that anchors the MHCI to the membrane produces a reorientation of the complex with respect to the membrane, making epitope 92–99 accessible to the antibody. The evidence that a limited number of cells undergoing apoptosis are 14H3 positive suggests that the antibody is able to identify a small percentage of cells in which β2-m is still bound to the heavy chain but epitope 92–99 is exposed to the solvent. In 14H3-positive cells, the compactness of the MHCI complex and its interaction with the membrane is most likely weakened. In this phase, the MHCI could go through an intermediate state along the degradation pathway in which the exposure of the C-terminal portion of β2-m to the solvent may represent a very initial and transient phenomenon during β2-m shedding from the membrane.

Fluctuations in the strength of the MHCI-membrane interaction and in the amplitude of movement of the protein complex over the phospholipid layer are likely

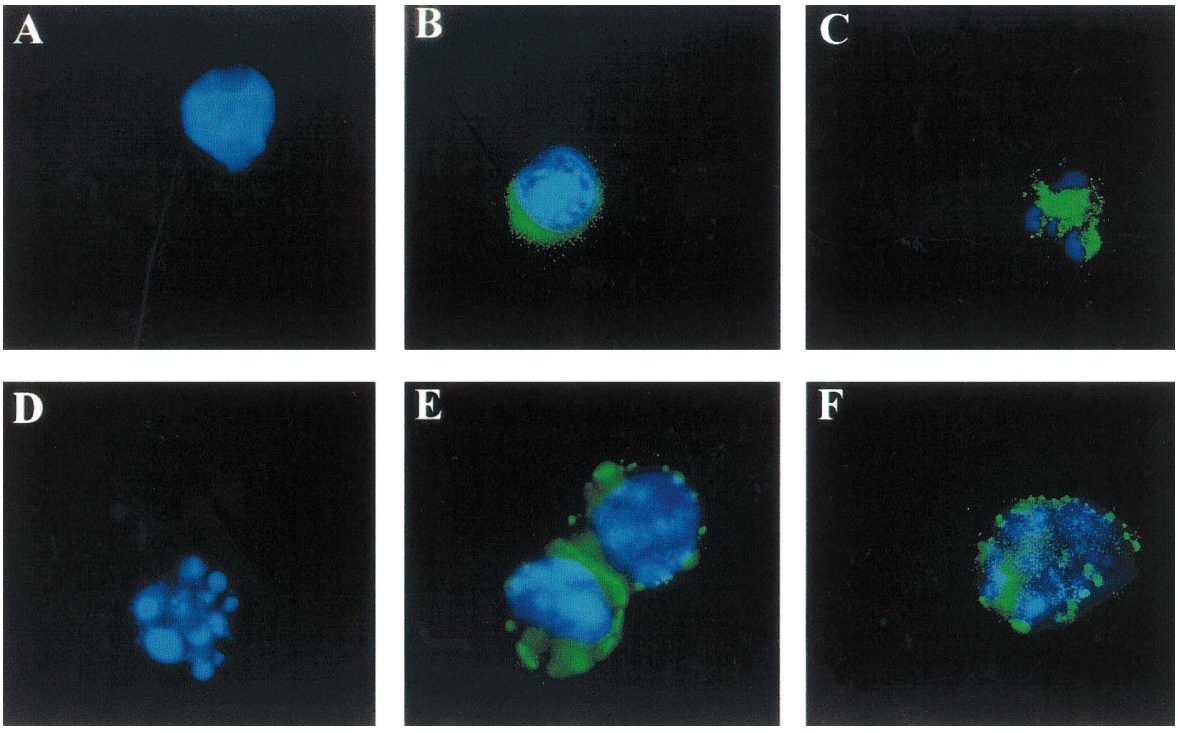

Figure 3. (*A–C*) Immunofluorescence analysis of irradiated Jurkat cells stained with the blue DNA marker (DAPI) and the purified murine mAb 14H3 revealed with a FITC-conjugated goat anti-mouse secondary antibody (green fluorescence). (*A*) A cell with no evidence of apoptosis (14H3 negative). (*B*) An apoptotic cell presenting condensed chromatin in the nucleus (14H3 positive). (*C*) An apoptotic cell presenting a totally fragmented nucleus (14H3 positive). (*D*) A cell with a fragmented nucleus stained with DAPI, and an irrelevant murine isotypic control revealed with a secondary FITC-conjugated goat anti-mouse antibody. (*E*, *F*) Cells stained with DAPI and a FITC-conjugated anti-MHCI antibody. (*E*) Non apoptotic cells. (*F*) An apoptotic cell with fragmented nucleus.

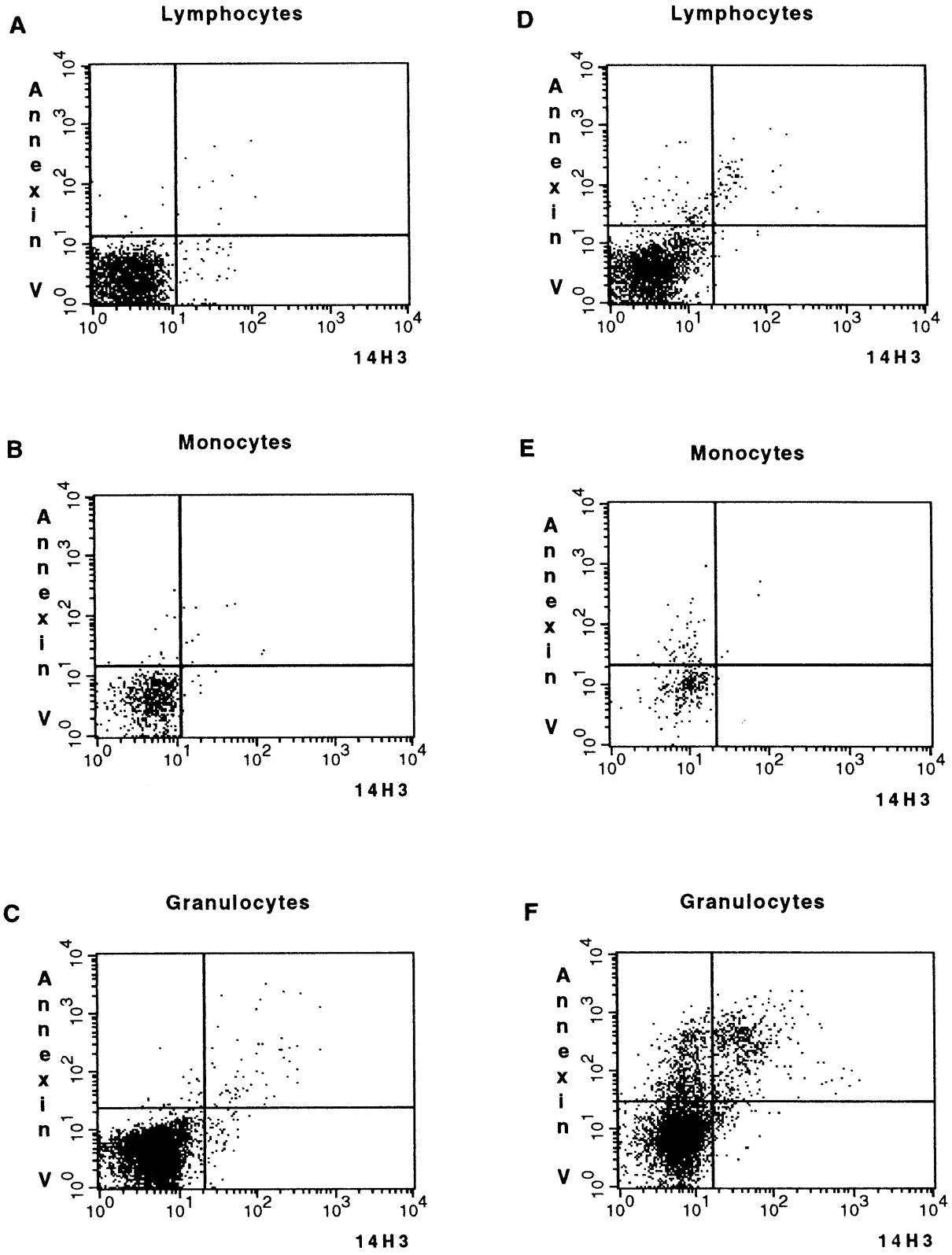

Figure 4. Cytofluorimetric analysis of PBCs before (*A–C*) and after (*D–F*) γ irradiation, gated according to standard FSC and SSC characteristics in lymphocytes (*A*, *D*), monocytes (*B*, *E*) and granulocytes (*C*, *F*). The results of one representative experiment out of three are shown.

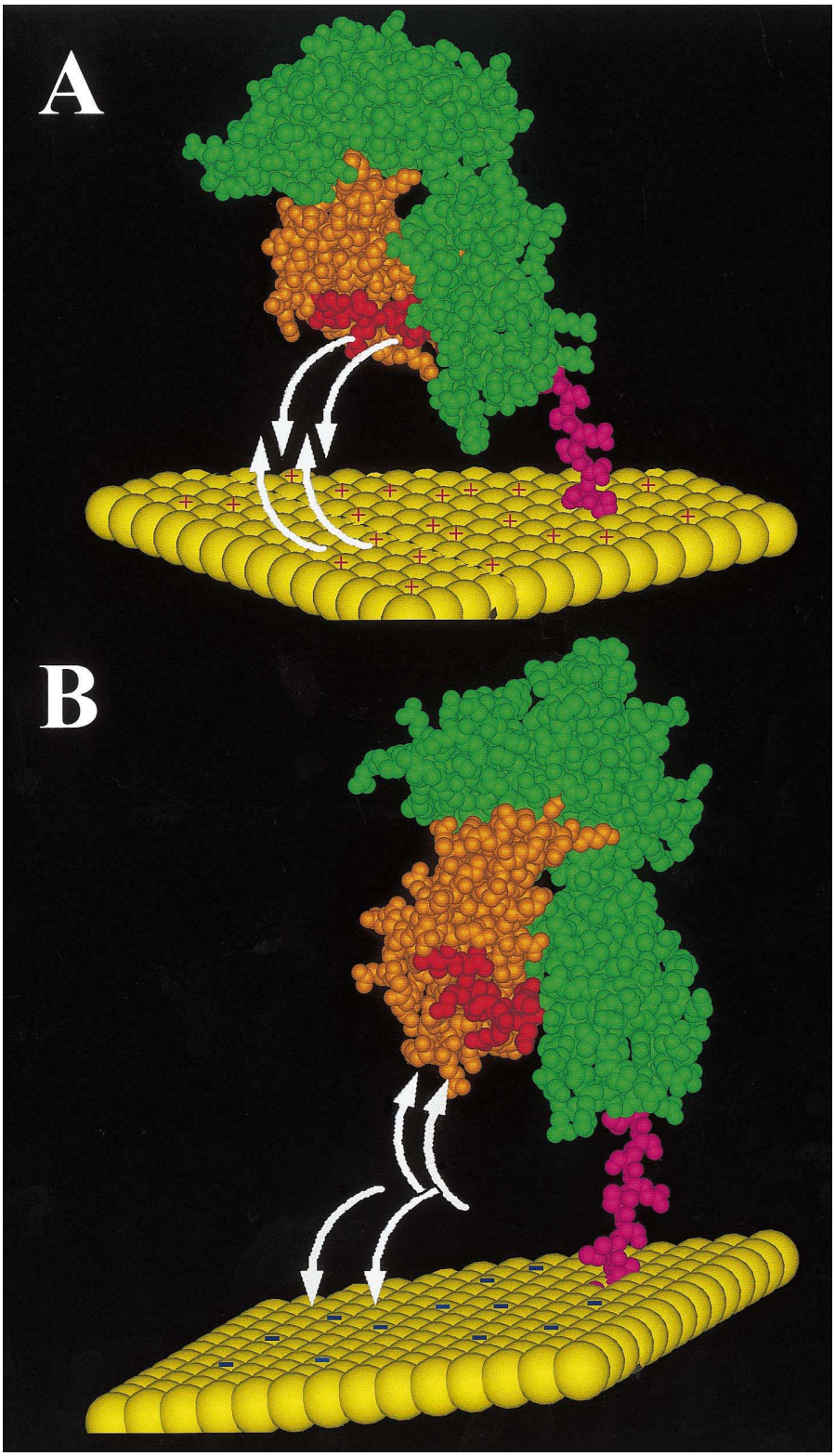

Figure 5. (*A*, *B*) A hypothetical model of the MHCI complex bound to a cell membrane. Yellow spheres represent the polar heads of the phospholipids, while the MHC heavy-chain atoms are coloured green, the peptide connecting the heavy chain to the membrane is violet, β2-m is orange and the antigenic peptide 92–99 is red. Atomic coordinates of the MHC complex were taken from the Brookhaven Protein Data Bank, set 3HLA [1], whereas the connecting peptide (amino acids 271–284 according to the numbering system of the crystal structure) was built as a β strand in an extended conformation.

to be physiologically possible. In the stress conditions we created, we probably amplified this phenomenon and stabilized a state of partial membrane-MHCI misassembly suitable for the identification of β2-m by the mAb 14H3.

Possible investigation of the process of MHCI unfolding on PBCs appears particularly promising for evaluating the biocompatibility of dialytic membranes during the haemodialytic procedure, in which the contact of PBCs with the dialytic membranes can increase synthesis and release of β2-m [15]. The properties displayed by the mAb 14H3 appear particularly relevant for the attempt to evaluate possible conformational changes allowing the exposure of the 92–99 epitope that would precede β2-m shedding, which is responsible for the osteoarticular amyloidosis that represents one of the most severe and inevitable complications of haemodialysis [16].

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