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Chapter 55
Apoptosis and the Immune System: Flow Cytometry Techniques for Measurement of Apoptosis

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Cellular thanatology has revealed two mechanisms that ultimately lead to the death (Thanatos) of cells: programed cell death (PCD), a suicidal mechanism of death, and necrosis, which can be described as death by murder. PCD is a non-pathological process that occurs in a large number of cells during development. In contrast, necrosis is a passive process that occurs in cells killed by pathological stimuli and involves loss of cell membrane integrity, cell swelling, and eventually cell lysis. Studies by Kerr et al [1]and Wyllie et al [2] investigating the ultrastructure of mammalian cells undergoing PCD, revealed certain common morphological characteristics. This process, which was named apoptosis, involves cell shrinking, zeiosis (boiling), or blebbing of plasma membrane resulting from changes in the cytoskeleton, chromatin condensation, and DNA fragmentation. Apoptosis therefore is a morphological definition of PCD. However, it has been suggested that not all PCD occurs via apoptosis, but that some PCD occurs without the characteristic features of apoptosis [3]. Cells that have undergone apoptosis are finally phagocytized before they lyse and release their intracellular content, thus avoiding risk of inflammation to the organism [4].

From an immunologist's standpoint, apoptosis has come to define any death that shows the characteristic morphological features mentioned above, irrespective whether the inducing stimulus is a pathological one, i.e. irradiation-induced apoptosis [5]. The field has seen a rapid expansion in the last several years with the growing awareness of the involvement of apoptosis in many aspects of the immune system and disease pathogenesis.

Apoptosis of lymphocytes can be induced in a number of different ways such as glucocorticoids, activation, irradiation, growth factor deprivation, and hyperthermia [4]. Apoptosis appears to be involved in the development and regulation of T and B cells. Immature thymocytes have been shown to die by apoptosis in the thymic cortex due to lack of positive selection while negative selection results in apoptosis in the thymic medulla. In both cases, apoptotic thymocytes are engulfed by macrophages [6]. Peripheral deletion of unwanted clones also involves apoptosis in the lymph nodes. This peripheral deletion occurs as a result of activation-induced cell death (AICD), in which apoptosis is induced by signals that would otherwise induce activation and proliferation [7]. AICD probably plays an important role in the maintenance of self-tolerance and the elimination of "hyperactivated" T cells in the periphery [8]. Immature and in some instances mature B cells also have been shown to undergo apoptosis under certain conditions, with apoptosis presumably playing a role in establishing and maintaining self-tolerance [9].

The association between apoptosis and autoimmunity has also come from recent findings that some mouse strains that are prone to develop autoimmunity bear defects in Fas/Fas ligand interactions. MRL lpr/lpr and gld/gld autoimmune strains of mice bear defects of Fas antigen and Fas ligand, respectively [10, 11]. Fas/Fas ligand interactions are involved in costimulation of activation and proliferation as well as apoptosis of T cells [12–14]. Therefore, defects in these interactions affect important mechanisms of apoptosis, and may result in the rise of autoreactive T and B cell clones and the ensuing emergence of autoimmunity. In systemic lupus erythematosus increased T cell Fas expression and increased serum levels of soluble Fas have been reported, indicating a possible involvement of defective apoptosis in human autoimmunity [15, 16]. A recent report has shown another possible association of apoptosis and autoimmunity. Autoantigens have been found to be enclosed in blebs of apoptotic cells: thus it is possible during apoptosis that autoantigens are subject to modifications that render them immunogenic and result in autoreactivity [17]. It will also be of interest to determine whether target cells in organ- specific autoimmunity undergo apoptosis within the lesions.

Apoptosis is also involved in cell-mediated cytotoxicity. Cytotoxic T lymphocytes, NK cells, and lymphokine-activated killer (LAK) cells all kill their targets by apoptosis [4]. Cytotoxic CD8+ and CD4+ T lymphocytes kill their target cells using Fas/Fas ligand interactions in addition to perforin [18, 19]. This has implications in viral infections, oncology, and transplantation. Apoptosis of virally infected cells could ensure that the intracellular infectious content is not released, possibly induce fragmentation of viral DNA or RNA, and ensure virus killing by the engulfing macrophages.

Viruses can induce apoptosis of T cells in a number of viral infections affecting the immune system. During infectious mononucleosis, Epstein-Barr virus (EBV) has been shown to induce apoptosis of peripheral blood T cells, even though the virus infects B cells [20]. This transient T cell apoptosis, which results in T cell hyporesponsiveness, may serve as a mechanism of evading host response. In HIV infection, spontaneous, activation-induced, and anti-Fas-induced T cell apoptosis have been documented. This chronic apoptosis has been suggested to play an important pathogenic role in HIV infection, being responsible for early T cell defects and later T cell loss of HIV disease [21–23]. In both the above cases of viruses inducing T cell apoptosis, the mechanism appears to be an indirect one rather than due to viral infection of cells.

DNA viruses have been shown to carry genes that alter apoptosis signaling when the virus infects cells. This provides a survival advantage for these viruses, because viral infection and replication often promote apoptosis of host cells. For example, adenovirus encodes genes that promote proliferation and trigger apoptosis (such as E1A) as well as genes that rescue the host cell

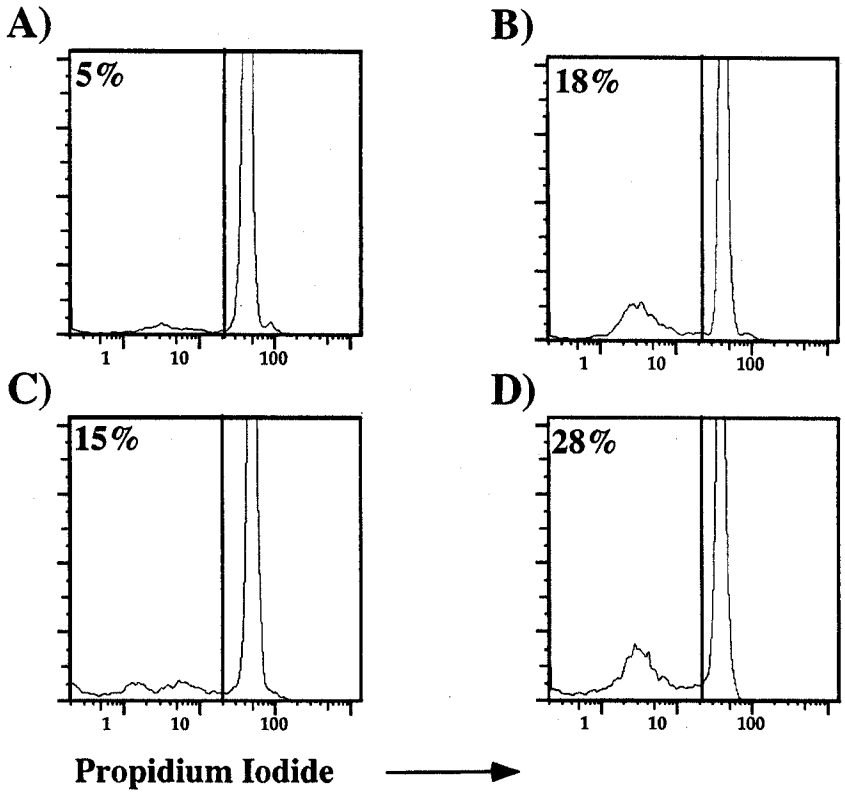

Fig. 55.1. *Hypotonic propidium iodide staining of human peripheral blood mononuclear cells (PBMC).* Twenty-four-hour culture of PBMC from an HIV- individual: **(A)** media alone and **(B)** anti-Fas antibody treated. PBMC cultures from an HIV+ individual: **(C)** media alone, and **(D)** anti-Fas antibody treated. The hypodiploid peak is to the left of the vertical line. Percent apoptotic hypodiploid nuclei shown in upper left corner.

from apoptosis (such as E1B). Another example is the EBV virus gene BHRF1, which encodes a protein homologous to bcl-2 that supresses apoptosis. BHRF1 probably plays an important role in the transformation of EBV-infected cells [24].

The increased interest in apoptosis has resulted in development of techniques for its assessment. As mentioned above, the definition of apoptosis is primarily a morphological one [1], therefore electron microscopy (EM) is the method of choice for the demonstration of apoptosis. However, EM is laborious, not quantitative, and the extent/frequency of apoptosis cannot be easily estimated. Alternatively, DNA fragmentation can be demonstrated after electrophoresis in agarose gels by the appearance of low molecular weight DNA bands differing by 180 bp (DNA laddering), and this is considered a hallmark of apoptosis [2]. Again, quantification is rather difficult. In addition, estimation of DNA fragmentation in a mixed cell population does not permit the identification of the cell type undergoing apoptosis. Finally, DNA agarose gels are labor intensive, making it difficult to analyze large numbers of samples.

These problems have led to the development of flow cytometry techniques for measuring apoptosis. These methods permit quantification and in some cases determination of apoptosis in different cellular subsets when a mixed population is analyzed. A number of flow cytometric techniques have been presented based on measurement of cellular DNA content, integrity of plasma membrane, mitochondrial transmembrane potential, function of lysosomal proton pump, or *in situ* nick translation [25].

In our laboratory we have used a number of flow cytometry methods to measure apoptosis in both human and mouse cells. These include a) hypotonic-propidium iodide (PI), b) labeling of DNA breaks, c) merocyanine 540, and d) Hoechst 33342.

Hypotonic-PI [26] can determine apoptosis in both human and mouse cells (Fig. 55.1). This method takes advantage of the DNA fragmentation observed during apoptosis, which results in decreased DNA staining by PI as a result of loss of DNA content. Cells must be left overnight at 4°C or for shorter periods at 37°C in a hypotonic solution of PI (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) which permeabilizes the cells and allows the small-sized DNA fragments to diffuse out. Apoptotic cells appear hypodiploid and can be easily discriminated from necrotic and live cells. However, surface antigen staining of cells is not possible with this method, and therefore apoptosis in different cellular subsets cannot be determined.

As mentioned above, DNA fragmentation has been considered a hallmark of apoptosis. Two methods have taken advantage of these DNA breaks to measure apoptosis. In these methods terminal deoxynucleotidyl transferase (TdT) or DNA polymerase are used to label the 3'-hydroxyl termini of the DNA breaks with either biotin- or digoxigenin-labeled dUTP [22, 27, 28]. However, it should be remembered that DNA strand breaks are not unique to apoptosis [25]. Fixing and permeabilization of cells during these treatments alters the light scatter properties of the cells, making it difficult to use the scatter changes that occur during apoptosis (decreased forward scatter and increased side scatter) to identify apoptotic cells. Simultaneous surface labeling with fluorochrome

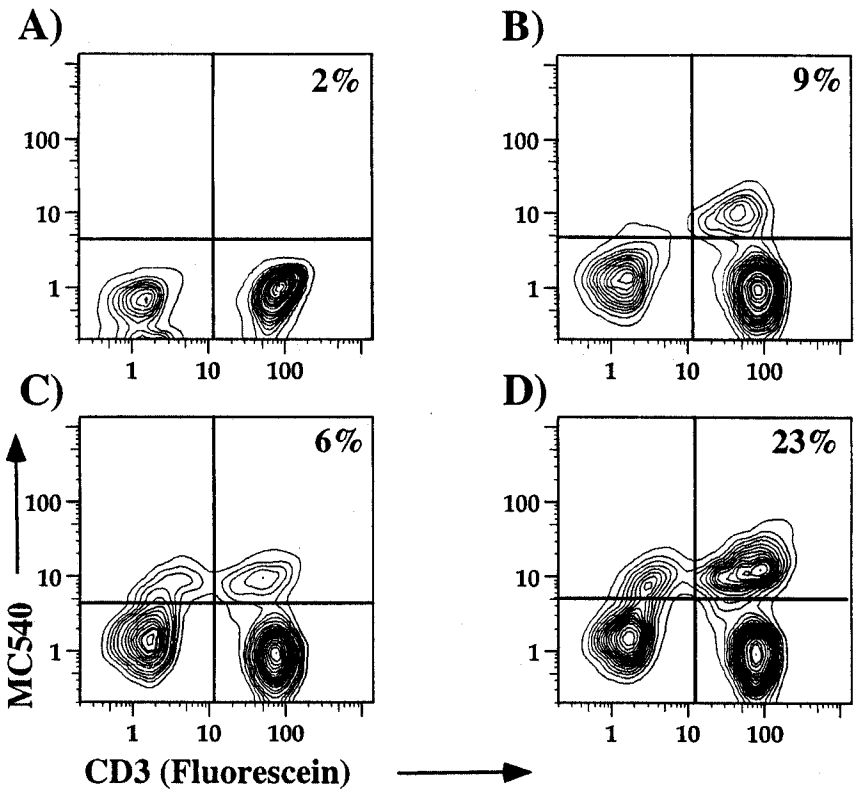

Fig. 55.2. *Multicolour analysis of PBMC using Merocyanine 540 (MC540) stain for apoptosis.* Apoptosis (MC540+ cells) of CD3+ lymphocytes shown. Twenty-four-hour cultures of PBMC. HIV- individual: **(A)** media and **(B)** anti-Fas antibody treated. HIV+ individual: **(C)** media and **(D)** anti-Fas antibody treated. Percent apoptotic cells shown in upper right corner.

conjugated monoclonal antibodies with these methods can also prove difficult.

Merocyanine 540 (MC540), which intercalates more readily in the membranes of cells undergoing apoptosis due to their more loosely packed lipid bilayer [29, 30], permits multiparameter surface staining of cells (Fig. 55.2). Loss of cell membrane asymmetry, which results in both loose membrane phospholipid packing and phosphatidylserine exposure, is an early event during apoptosis that precedes DNA fragmentation [31]. This makes MC540 a very useful stain for the study of early apoptosis. MC540 is maximally excited at 555 nm, has peak emission at 575 nm in aqueous solution that shifts to 590 nm when MC540 binds to lipids [29]. It can be measured on a flow cytometer using a 488-nm argon-ion laser for excitation and optical filters for phycoerythrin (PE) detection. One problem we encountered with MC540 was a low-level non-specific staining of monocytes. MC540 has also been reported to stain non-specifically activated cells. Recently, binding of fluorescein-conjugated annexin V to exposed phosphatidylserines of apoptotic cells has also been employed to measure apoptosis with promising results [32].

The bisbenzimide dye Hoechst 33342 (HO342) has been used for a number of years for the quantification of DNA content. HO342 is a nuclear dye that preferentially intercalates into A-T rich regions of DNA [33]. Its excitation and emission peaks are at 346 and 460 nm, respectively. HO342 is unique among nuclear dyes in that it is somewhat membrane permeable [25]. DNA staining by HO342 may reflect nuclear changes and the activity of an energy-dependent efflux pump [34, 35]. The light scatter properties of cells undergoing apoptosis are altered with forward scatter decreasing and side scatter increasing (Fig. 55.3), and these changes combined with HO342 staining have been used to quantitate apoptosis [34]. The combination of HO342 with nuclear dyes that are not cell membrane permeable, such as propidium iodide or 7-amino-actinomycin D (7AAD) has also been used with simultaneous staining with FITC- or PE-conjugated monoclonal antibodies to surface antigens, thus permitting the analysis of specific cell populations [36, 37].

We have taken advantage of these unique features of HO342 to measure apoptosis with four-color analysis of mixed cellular populations [23]. HO342 is excited with UV from an argon-ion laser (351–363 nm), and its broad emission can be detected in the blue or green (420–460 or 515–545 nm). Cells can be stained simultaneously with three 488-nm excited fluorescent reagents, thus permitting the detection and measurement of apoptosis even in small populations requiring multi-color identification. In this method cells are stained on ice with fluorochrome-conjugated monoclonal antibodies to surface antigens for 15 minutes. During the last seven minutes 1 μg/ml of HO342 is added. Both monoclonal antibody and HO342 solutions are made up in RPMI with 3% fetal calf serum (FCS) and 0.02% NaN_3. Cells are washed twice and then fixed in 0.5% of paraformaldehyde. HO342 staining of cells results in three populations of low, intermediate, and high staining intensity. The low HO342 population is live, intermediate HO342 cells are early apoptotic, and the high HO342 cells have lost membrane integrity and can be late apoptotic or necrotic [34]. Plots in Figures 55.3 and 55.4 show

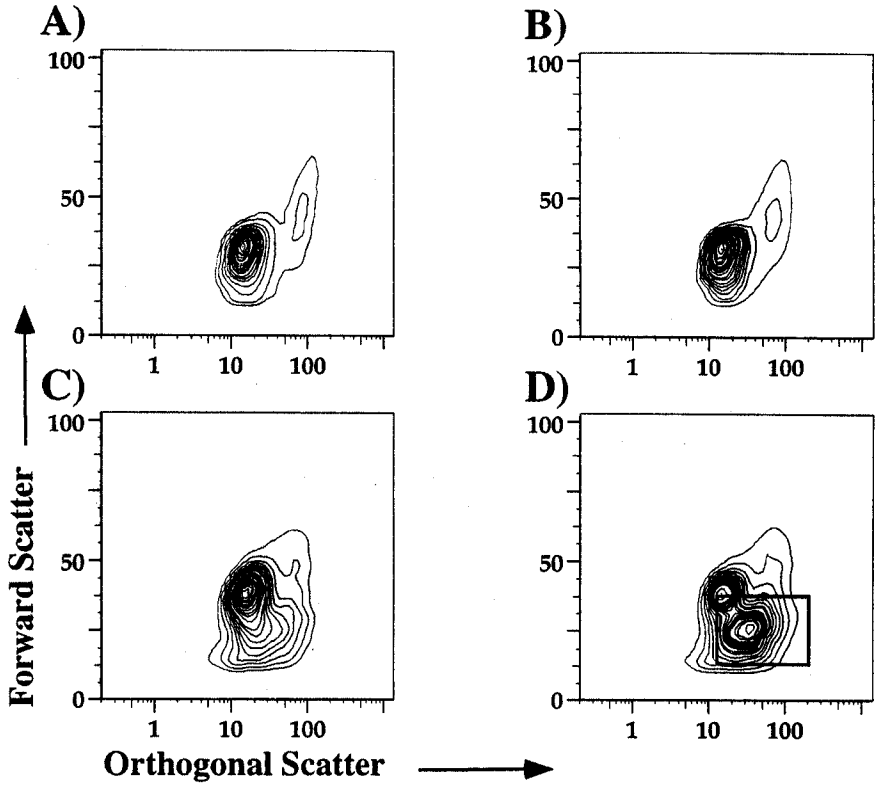

Fig. 55.3. *Forward and orthogonal scatter of PBMC cultured for 24 hours.* PBMC from HIV- individual: **(A)** media and **(B)** anti-Fas antibody treated. PBMC from HIV+ individual: **(C)** media and **(D)** anti-Fas antibody treated. Box in **D** shows apoptotic cells with reduced forward and increased orthogonal scatter. Samples were simultaneously stained for CD3, CD4, and CD8, shown in Figure 55.4.

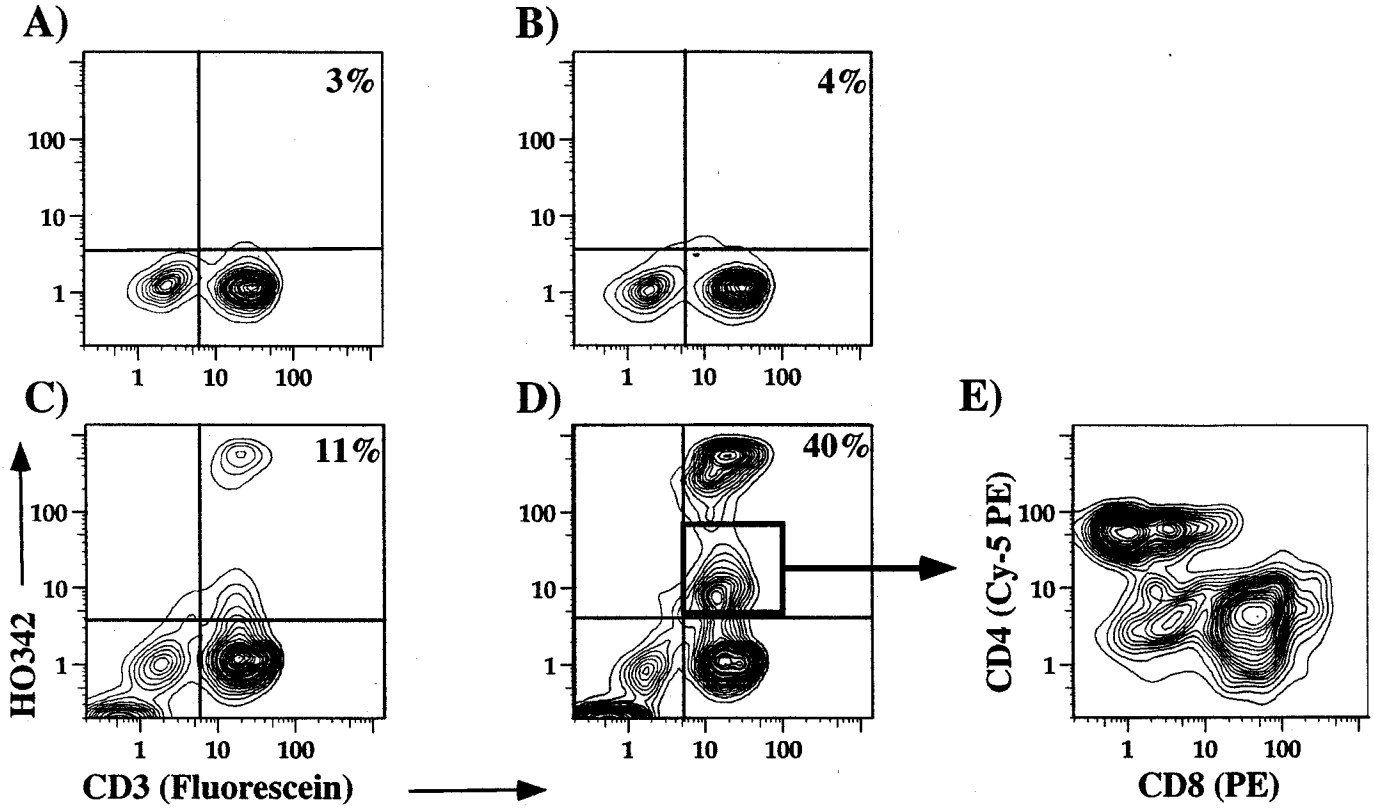

Fig. 55.4. *Apoptosis of human CD3+ lymphocytes.* Multicolor analysis of PBMC using HO342 stain for apoptosis. Cells stained with HO342, anti-CD3-FITC, anti-CD4-CyChrome (Cy-5PE), and anti-CD8-PE. Apoptosis ($HO342^{Interm}$ and $HO342^{hi}$ cells) of CD3+ lymphocytes shown. PBMC from HIV- individual: **(A)** media and **(B)** anti-Fas antibody treated. PBMC from HIV+ individual: **(C)** media and **(D)** anti-Fas antibody treated. **(E)** CD4 and CD8 expression of $HO342^{Interm}$ CD3+ lymphocytes from anti-Fas antibody treated PBMC from HIV+ individual. Percent apoptotic CD3+ lymphocytes shown in upper right quadrant.

scatter properties and HO342, CD3, CD4, and CD8 stains of human PBMC stained simultaneously with the above method. This method permits multiparameter analysis of mixed cell populations identifying apoptotic cells in different cellular subsets. It has been used successfully with human PBMC, mouse thymocytes, and tumor cell lines. This method is easy to perform and is suitable for the quantification of apoptosis in mixed populations and in large numbers of samples.

Flow cytometry methods that are quantitative, permit cell subset analysis and cell sorting, and are easy to perform are a powerful tool for the study of apoptosis. Reliable and simple flow cytometry techniques will prove important not only in the rapidly expanding studies of the role of apoptosis in the development and regulation of the immune system, but also in the understanding of the molecular events and genes involved in apoptosis. It should be noted, however, that none of the methods described above is sufficient on its own to determine apoptosis, and therefore, qualitative methods such as ultrastructure study or DNA fragmentation should be combined with flow cytometry methods.

Acknowledgments

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