

S. Hoornaert*, E. Hanon, J. Lyaku, and P.-P. Pastoret

Department of Immunology/Vaccinology, Faculty of Veterinary Medicine, University of Liège, B43bis, B-4000 Liège, Belgium

*corresponding author (e-mail : s912432@student.ulg.ac.be)

The Use of Annexin for Concomitant Detection of Apoptosis and Cellular Phenotype

Introduction

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished on the basis of differences in morphological and biochemical characteristics. Under the electron microscope, cells undergoing apoptosis display cell shrinkage, apoptotic body formation, and chromatin condensation. Biochemically, the apoptotic process is characterized by fragmentation of DNA into oligonucleosomal fragments. Furthermore, during the early stages of apoptosis, changes also occur at the cell surface membrane (1,3,4). One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part to the outer layer of the plasma membrane (11), thus exposing PS at the external surface of apoptotic cells, where it can be specifically recognized by macrophages (4).

Annexin V, a Ca^{2+} -dependent phospholipid-binding protein, possesses high affinity for PS (11) and can thus be used for detecting early apoptotic cells (6,10,11,12). Since Annexin V can also detect necrotic cells as a result of the loss of membrane integrity, apoptotic cells have to be differentiated from these necrotic cells by the use of propidium iodide (PI). Indeed, PI selectively labels necrotic, but not apoptotic, cells.

Several studies have revealed a correlation between apoptosis and cell phenotype (2,8). The investigation of this relationship ideally requires techniques that permit the concomitant detection of apoptosis and cell phenotype analysis at a single-cell level. In this report, we describe the development of a procedure that permits concomitant detection of apoptosis and cell phenotype characterization by flow cytometry.

Materials and Methods

Cells and culture conditions

Blood from steers was collected by jugular venipuncture and mixed with heparin. The peripheral blood mononuclear cells (PBMC) were then isolated on Ficoll® Hypaque (Pharmacia) density gradient (density = 1.077 g/ml) and washed three times with phosphate-buffered saline (PBS). The PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin in a polystyrene round-bottomed tube (Becton Dickinson) at a density of 2×10^6 cells/tube for 48 hours. To induce the proliferation of PBMCs, the culture medium was supplemented with 0.5 µM Ionomycin (Sigma) and 10 nM Phorbol 12,13-dibutyrate (Sigma) (IONO/PDB).

Virus

The BHV-1 Cooper strain was kindly provided by Dr. J. T. van Oirschot (Lelystad, The Netherlands). The virus was multiplied on Madin Darby Bovine Kidney cells (MDBK) (American Type Culture Collection CCL22) and purified as previously described (5). Purified virus was resuspended in RPMI 1640 (Gibco) supplemented with 2 mM glutamine (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco), and stored at -70°C until use.

Annexin V-FITC/phenotype double staining

After harvesting, PBMCs were washed twice with PBS and incubated for 30 min with the appropriate predetermined concentration of primary mAb 1H4, which is

specific for bovine cell surface IgM present on B lymphocytes (7). The cells were washed twice with PBS containing 5% FCS, and further incubated with PE-conjugated $F(ab')_2$ goat anti-mouse IgG (H+L chains) (Dako) for 30 min. After an additional wash with PBS containing 5% FCS, the cells were labeled with Annexin V-FITC and PI as described in the Annexin V-FLUOS pack insert (Boehringer Mannheim). Cells were analyzed by flow cytometry for green (FITC), orange (PE), and red (PI) fluorescences.

Flow cytometry

Flow cytometric analysis was performed using a FACStar^{PLUS}™ Becton Dickinson fluorescence activated cell sorter equipped with an argon laser (ILT air cooled with 100 mW excitation lines at 488 nm). Debris was excluded from the analysis by the conventional scatter gating method. The cells or the nuclei doublets were excluded from the analysis by using the pulse processor boards (Becton-Dickinson). Green fluorescence detection of FITC was detected at 530 nm (DF 530/30 dichroic bandpass filter); PE fluorescence was measured at 575 nm (DF 575/26); and PI was measured at 610 nm (LP 610). Crossover of FITC fluorescence into the PE detection window and of PE into the PI detection window was electronically compensated for analogue subtraction at the preamplifier stage. Ten thousand events per sample were collected in a list mode, stored, and analysed by the Consort™ 32 System (Becton-Dickinson).

Results and Discussion

In this study, PBMCs were mock-infected or infected with BHV-1 in order to induce apoptosis in mitogen-stimu-

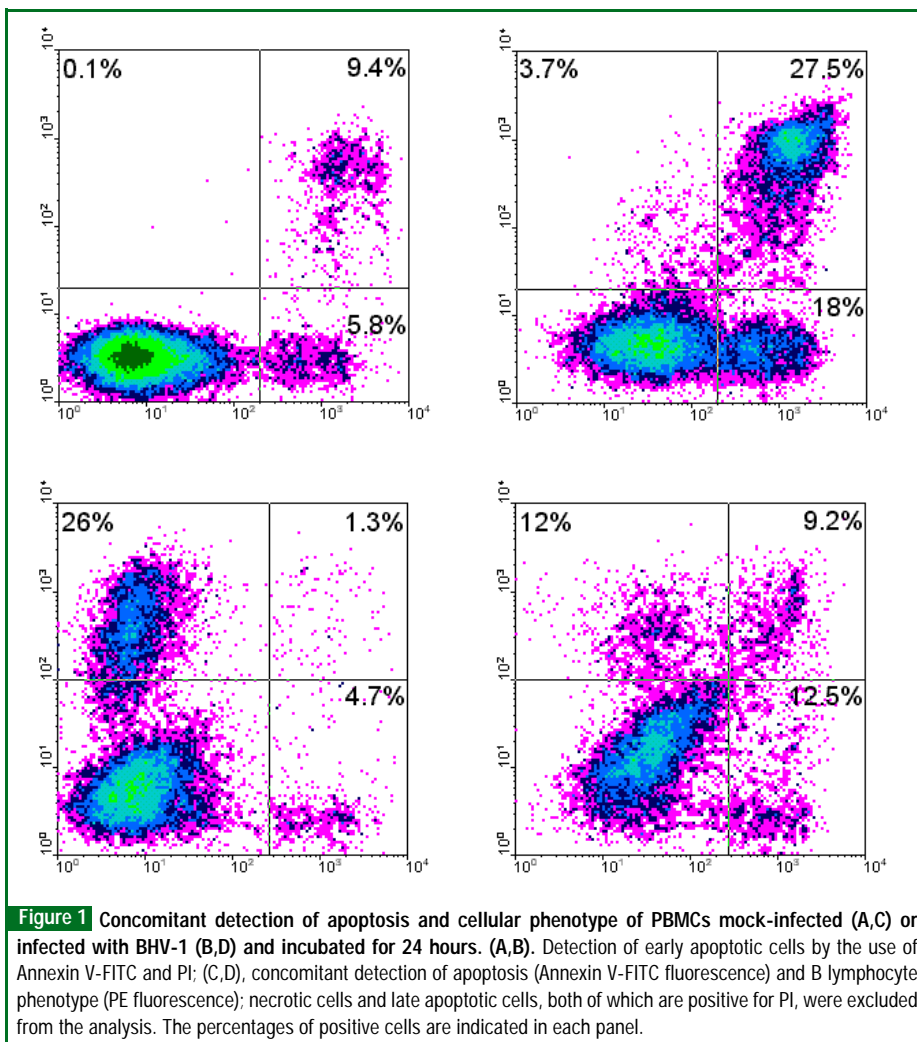


Figure 1 Concomitant detection of apoptosis and cellular phenotype of PBMCs mock-infected (A,C) or infected with BHV-1 (B,D) and incubated for 24 hours. (A,B), Detection of early apoptotic cells by the use of Annexin V-FITC and PI; (C,D), concomitant detection of apoptosis (Annexin V-FITC fluorescence) and B lymphocyte phenotype (PE fluorescence); necrotic cells and late apoptotic cells, both of which are positive for PI, were excluded from the analysis. The percentages of positive cells are indicated in each panel.

lated PBMCs (5). We used this experimental model to investigate whether concomitant detection of apoptosis and cellular phenotype characterization are two compatible procedures. After 24 hours of incubation, cells were harvested and processed such that the cellular phenotype could be characterized by indirect immunofluorescence using a PE-conjugated secondary antibody. The cells were then further treated so that early apoptotic cells could be detected by the use of Annexin V-FITC and PI. As shown in Figure 1B, BHV-1 increased the percentage of early apoptotic cells, which are defined as positive for Annexin V-FITC (Green fluorescence) and negative for PI (red fluorescence). By excluding PI-positive cells (necrotic and late apoptotic cells) from the analysis, we were able to determine the percentage of early apoptotic B lymphocytes. Indeed, concomitant detection of Annexin V-FITC (green fluo-

rescence) and PE-conjugated secondary antibody (orange fluorescence) indicated, that 9.2% of B lymphocytes in BHV-1-infected PBMC cultures were apoptotic as compared to 1.3% in mock-infected cultures (Figure 1C and D).

These results provide evidence that concomitant detection of apoptosis, using Annexin V-FITC, and cellular phenotype characterization are two compatible procedures. The method described here can be extended to all membrane-associated antigens (including cell cycle-associated proteins, viral glycoproteins, or activation markers). Furthermore, since this procedure is entirely realized without any fixation or cellular permeabilization, it allows the execution of additional investigations, including cell sorting by flow cytometry and characterization of discrete cellular events occurring in early apoptotic cells.

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Product	Cat. No.	Pack Size
Annexin-V-FLUOS	1 828 681	250 tests
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