Cell Prolif. 2005, **38**, 301–311

Comparison of apoptosis and mortality measurements in peripheral blood mononuclear cells (PBMCs) using multiple methods

S. Glisic-Milosavljevic*,†, J. Waukau*, S. Jana*, P. Jailwala*, J. Rovensky* and S. Ghosh*

**Max McGee National Research Center for Juvenile Diabetes and Human Molecular and Genetics Center, Medical College and Children's Hospital of Wisconsin, WI, USA and †'VINCA' Institute of Nuclear Sciences, Belgrade, Serbia and Montenegro*

Received 29 *June 2005; revision accepted* 22 *August* 2005

Abstract. Death through apoptosis is the main process by which aged cells that have lost their function are eliminated. Apoptotic cells are usually detected microscopically by changes in their morphology. However, determination of early apoptotic events is important for *in vitro* (and *ex vivo*) studies. The main objective of the present study is to find the most sensitive method for apoptosis detection in human peripheral blood mononuclear cells (PBMCs) by comparing six different methods following five different means of immunological stimulation at 3 and 5 days. Each of six apoptosis quantification methods, except the trypan blue exclusion test, is a combination of two stains, one for the specific detection of apoptotic cells and the other for the unspecific detection of dead cells. Values for apoptosis and mortality were compared with a reference method. The choice of apoptosis detection method is more important following 3 days of stimulation than after 5 days of stimulation ($P = 2 \times 10^{-6}$ versus $P = 1 \times 10^{-2}$). In contrast, we find mortality measurements following the different means of stimulation highly significant at both 3 and 5 days ($F_{2.28} = 7.9$, $P = 1.4 \times 10^{-6}$ at 3 days and $F_{2.28} = 8.5$, $P = 4.5 \times 10^{-7}$ at 5 days). Variation as a result of the combination of specific PBMC stimulation and the method used to detect apoptosis is reduced considerably with time ($F_{1.58} + 3.7$, $P + 3 \times 10^{-7}$ at 3 days to $F = (1.58) = 0.97$, $P = 0.5$ at 5 days). Based on Tukey's test, YO-PRO-1 is the most sensitive stain for apoptosis and, when combined with 7-AAD, provides an accurate measure of apoptosis and mortality. In conclusion, we propose YO-PRO-1/7-AAD as a new combination and low-cost alternative for the sensitive detection of early apoptosis.

INTRODUCTION

Proliferation and death regulate the size of cell populations and apoptosis plays a key role in this balance. As a result of this tightly regulated, active physiological process, apoptotic cells undergo engulfment by phagocytic cells before leakage of their intracellular contents, thus averting an

Correspondence: Soumitra Ghosh MD PhD, Max McGee National Research Center for Juvenile Diabetes, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA. Tel.: (414) 456 4901; Fax: (414) 456 6663; E-mail: sghosh@mail.mcw.edu

inflammatory immune response. Apoptosis plays multiple roles in the immunological system that have an impact on homeostasis. It is crucial in the deletion of autoreactive T cells in thymus (central tolerance) and also it regulates the size of the T-cell clone pool during proliferation, after receiving appropriate stimuli [for example, activation-induced cell death (AICD), activated T-cell autonomous death (ACAD)]. Of great interest, apoptosis in responder T cells is the primary mechanism for suppression induced by T regulatory cells (Grossman *et al.* 2004; Gondek *et al.* 2005). Furthermore, apoptosis is a non-inflammatory way to eliminate cells at the end of their life.

There are recognizable changes in cell morphology and biochemistry that can be used to measure apoptotic rates (Vermes *et al.* 2000). When a cell receives a signal to begin apoptosis, the cell size decreases, although membrane integrity remains intact. With the progression of apoptosis, caspases become activated. Depending on a starting signal, different initiator caspases (caspase-2, -8, -9, -10) begin further cascade reactions (Thornberry & Lazebnik 1998). These cascades result in activation of executioner caspases (Green & Reed 1998; Green 2003; Abraham & Shaham 2004) that are responsible for the remaining changes during the apoptotic process. Several organelles play a key role in apoptosis, activating or releasing enzymes involved in the mechanism (Bursch 2001; O'Brien & Bolton 1995). With advanced apoptosis, organelles lose membrane integrity, leading to diffusion of ions. As a consequence, the pH of the cytosol in the apoptotic cell is changed, altering the chemical balance and enzymatic reactions within the cell. As a last step, the cell disintegrates into apoptotic bodies that can be easily taken up by macrophages in the process of phagocytosis.

Given such a multifaceted pathway, many different techniques have been developed for apoptosis identification and quantification (Darzynkiewicz *et al.* 2001). One feature is that the method(s) chosen should be focused on morphological and biochemical characteristics of the process, making it possible to distinguish apoptosis from other mechanisms of active cell death (e.g. cellular suicide), where lysosomal, autophagic and proteasomal mechanisms of cell destruction may dominate (Bursch 2001).

Available techniques for the determination of apoptosis differ in their sensitivity, reliability, cost and the phase of the apoptosis process they capture. Therefore, it is important to compare assays in order to choose the most appropriate method(s) for a particular cell type, experimental condition and design.

The objective of the study described here was to compare six methods for measuring apoptosis under different stimulation regimes. One of the methods is a new stain combination we have devised, YO-PRO-1/7-AAD. Hence, we provide a choice for investigators in their decision for appropriate apoptosis detection methods. To the best of our knowledge, there has been no other recent publication that carefully compares commonly used methods for detecting apoptosis in healthy human PBMCs. Each apoptosis quantification method (except the trypan blue exclusion test) is a combination of two stains, one for detection of apoptotic cells and the other for detection of dead cells, which are analysed simultaneously. Because dyes detecting apoptotic cells can detect dead cells as well, in order to generate specific information (percentage of apoptotic cells versus mortality percentage), we simultaneously stained treated cells with a viability stain as well. Cells that were neither apoptotic nor dead were viable. Therefore, each of the tested methods gave us additional data for estimating viability.

MATERIALS AND METHODS

Amongst the methods that we tested, some are commonly used by researchers, for example AnnexinV/PI and AnnexinV/7-AAD. One of the methods we employed is intracellular staining of the active form of caspase-3, the key molecule in the apoptotic process. Intracellular staining

of caspase-3 was combined with ethidium monoazide (EMA) in order to detect cells that were dead before fixation. Other methods capture changes in membrane permeability, making it possible for the staining process to occur. For example, trypan blue stains the cytoplasm and nucleus evenly, while other molecules just stain the DNA (YO-PRO-1, 7-AAD, EMA and PI). Although most of the molecules used in this study bind to DNA, there are differences between them in their affinity and binding preference: some intercalate in dsDNA (EMA), while others bind not only to dsDNA but also to ssDNA and RNA (PI, YO-PRO-1). Finally, some prefer GC-rich regions in exposed nucleic acids (7-AAD).

Preparation and culture of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat of anonymous healthy donors by centrifugation on Ficoll-Hypaque (Amersham Pharmacia, Uppsala, Sweden) density gradient, washed and re-suspended in RPMI-1640 culture medium (Biowhittaker, Walkersville, MD, USA). The medium was supplemented with 10% pooled human serum type AB (Atlanta Biologicals, Lawrenceville, GA, USA), 50 μg/ml streptomycin and 50 U/ml penicillin (Sigma, St Louis, MO, USA), 10 mM HEPES (Invitrogen, Carlsbad, CA, USA) and 1 mM L-glutamine (Invitrogen, Carlsbad, CA, USA). Cell numbers were determined by the trypan blue exclusion test. PBMCs were treated by non-specific stimulation (mouse $IgG_{1,\kappa}$ 10 ng/ml, BD Biosciences, San Diego, CA, USA) for 48 h, rested for 48 h and re-stimulated with different stimulation for 72 (3 days) and 120 h (5 days) in a 37 °C incubator with 5% CO_2 and saturated humidity in air at a cell density of 10^6 cells/ml. Initially, we observed that non-specific stimulation caused no extra apoptotic cell death (data not shown). Secondary stimulation was performed in one of the following ways: (i) 10 ng/ml anti-CD3 (Clone UCHT1, Ancell Immunology Research Products, Bayport, MN, USA); (ii) 40 U/ml recombinant human IL-2 (BD Biosciences); (iii) 10 ng/ml of anti-CD3 and 40 μ/ml of rhIL-2; (iv) 10 ng of both anti-CD3 and anti-CD28 (Clone 28.2, BD Biosciences); (v) 10 ng/ml phorbol myristate acetate (PMA, Sigma) with 500 ng/ml ionomycin (Fisher, Pittsburgh, PA, USA). Proliferation of PBMCs was determined by adding [^{3}H]-thymidine for the last 16 h in culture.

Methods for measurement of proliferation and apoptosis

Proliferation was measured after 16 h incubation with 2.5 μCi/well [^{3}H]-thymidine and counts per minute (cpm) were determined using NXT TOPO Count (Perkin Elmer, Wellesley, MA, USA). Apoptosis in cell cultures was assessed by the trypan blue exclusion test using a haemacytometer and light microscope, and by five flow cytometric methods: (i) fluorescein isothiocyanate (FITC)-AnnexinV/PI (BD Biosciences); (ii) FITC-AnnexinV and 7-AAD (BD Biosciences); (iii) YO-PRO-1 (Molecular Probes, Eugene, OR, USA) and PI; (iv) YO-PRO-1 and 7-AAD; (v) FITC-anti-activeCas-3 mAb (BD Biosciences) and EMA (Molecular Probes) combinations. Mortality of cells in the cultures was also measured.

Trypan blue exclusion method

PBMCs were seeded in six-well plates at a density of 10^6/ml. After stimulation for 72 and 120 h, respectively, 40 μl of culture were taken twice for counting with the trypan blue exclusion method. Trypan blue distinguishes apoptotic cells by revealing their rough membranes, different shapes and sizes, and their nuclear condensation. Evenly blue cells were considered as dead when using trypan blue.

AnnexinV-FITC/PI combination

A minimum of 3×10^5 cells were washed and were re-suspended in 50 μl annexin-binding buffer; 5 μl of annexinV-FITC was added to the culture. After 20-min incubation at room temperature

in the dark, 200 μl of binding buffer was added. Five minutes before acquisition, PI was added in a final concentration of 50 μg/ml. Cells stained with annexinV-FITC only were considered apoptotic. Cells stained by PI and by both PI and annexinV-FITC were considered dead.

AnnexinV-FITC/7-AAD combination

A minimum of 3×10^5 cells were washed, re-suspended in annexin-binding buffer and 5 μl of annexinV-FITC were added to the culture. After 20-min incubation at room temperature in the dark, 200 μl of binding buffer were added. Ten minutes before acquisition, 0.25 μg of 7-AAD was added. Together, these two molecules can distinguish early apoptotic cells from dead and live cells. Cells stained with annexinV-FITC only were considered apoptotic. Cells stained by 7-AAD and by both annexinV-FITC and 7-AAD were considered dead.

YO-PRO-1/PI combination

A minimum of 10^5 cells were washed and re-suspended in 0.5 ml of phosphate-buffered saline (PBS). YO-PRO-1 was added at a final concentration of 1 μM. Cells were stained for 20 min in the dark at room temperature and, 5 min before acquisition, PI was added in a final concentration of 50 μg/ml. Together, these two molecules distinguish early apoptotic cells from dead and live cells. Cells stained with YO-PRO-1 only were considered apoptotic. Cells stained by PI and by both PI and YO-PRO-1 were considered to be dead.

YO-PRO-1/7-AAD combination

A minimum of 10^5 cells were washed and re-suspended in 0.5 ml of PBS. YO-PRO-1 was added to a final concentration of 1 μM. Staining lasted 20 min at room temperature in the dark. Ten minutes before acquisition, 0.25 μg of 7-AAD was added. Together, these two stains can distinguish early and late apoptotic cells from dead and live cells. Cells stained with YO-PRO-1 only were considered apoptotic. Cells stained by 7-AAD and by both YO-PRO-1 and 7-AAD were considered to be dead.

FITC-aaCas-3/EMA combination

Ethidium monoazide (EMA) is a viability stain which intercalates into nucleic acids of dead cells with high efficiency. After covalent binding to DNA it needs to be photoactivated to yield fluorescently labelled nucleic acids. A mixed population of live and dead cells labelled with this reagent retains its staining pattern after aldehyde-based fixation. EMA fluorescence identifies cells that were dead before fixation (Green 2003). In the present study, we combined EMA with intracellular staining of the active form of caspase-3.

A minimum of 5×10^5 cells were washed and re-suspended in 100 μL of PBS. Ten microlitres of 50 μg/ml EMA was added and cells were incubated at 4 °C in the dark for 15 min followed by exposure to light for 10 min (for photo-activation of EMA). Cells were next washed with PBS and fixed in 100 μL of Cytofix/CytoPerm buffer (BD Biosciences) for 15 min at 4 °C. Cells were then washed in 1 ml of Perm buffer and stained with 10 μl of FITC-anti-active Cas-3 mAb in 50 μl of Perm buffer at room temperature in the dark for 30 min. After washing in Perm buffer, cells were further analysed in PBS on LSRII. Cells stained with FITC-aaCas-3 only were considered apoptotic. Cells stained by EMA and by both FITC-aaCas-3 and EMA were considered to be dead.

FACS analysis

All five flow cytometric methods were analysed on LSRII (BD Biosciences, Franklin Lakes, NJ, USA). PBMCs treated with 1 μM; camptothecin (CPT) for 6 h were used for compensation and as positive control for methods measuring apoptosis in live cultures. Non-treated freshly

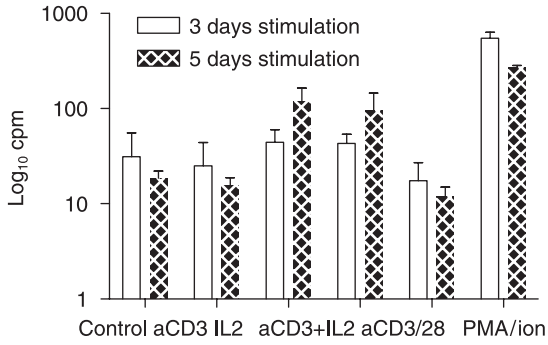

Figure 1. Proliferation of stimulated cell populations after 3 and 5 days in culture.

isolated PBMCs were used as a negative control. For intracellular staining, CPT-treated PBMCs were fixed, kept at −20 °C in ethanol and used for caspase-3 staining when needed. Figure 2 shows positive control apoptosis measured with all five flow cytometric methods. Analysis was performed using BD FACSDiva software (BD Biosciences).

Statistics

Results for five different PBMC treatments obtained with tested methods were compared by a two-way ANOVA test. *P*-values = 0.05 were considered as significant (Analyse-it). A post-hoc Tukey's test was also conducted to show which method contributes most to the significant results.

RESULTS

With all applied means of stimulation a notable proliferative response was detected (cpm in the range 5000–326 000). Among tested means of stimulation, the PMA/ionomycin combination caused the highest level of proliferation by far, at both 3 days and 5 days in culture (Fig. 1).

Measurement of the apoptotic index

PBMCs under the stimulation conditions described above were analysed for apoptosis by six different methods. Apoptosis is presented as an apoptotic index calculated as the number of apoptotic cells divided by the total cell number. The apoptotic index in the FACS-based methods represents cells stained with the apoptosis stain only (Q1 quadrant, Fig. 2). Figure 2 shows apoptosis results of six detection methods recorded with a positive control (PBMCs treated for 6 h with CPT); freshly isolated PBMCs were used as a negative control.

Applied stimulation resulted in a significant difference in apoptotic indices (two-way ANOVA $F_{2.28} = 21.9$, $P = 3 \times 10^{-16}$ at 3 days and $F_{2.28} = 13.2$, $P = 1 \times 10^{-10}$ at 5 days). When all methods of creating apoptosis were taken together, there was a large drop in the level of significance by two-way ANOVA, from $F_{2.27} = 7.8$, $P = 2 \times 10^{-6}$ for 3 days to $F_{2.27} = 3.2$, $P = 10^{-2}$ for 5 days. This corroborates the greater degree of concordance seen between apoptotic detection methods after 5 days in culture than after 3 days. In parallel, differences are reduced considerably with time for the type of stimulation/apoptosis detection method combination, known as the interaction term in the two-way ANOVA, which dropped from $F_{1.58} = 3.7$, $P = 3 \times 10^{-7}$ at 3 days to $F_{1.58} = 0.97$, $P = 0.5$, at 5 days.

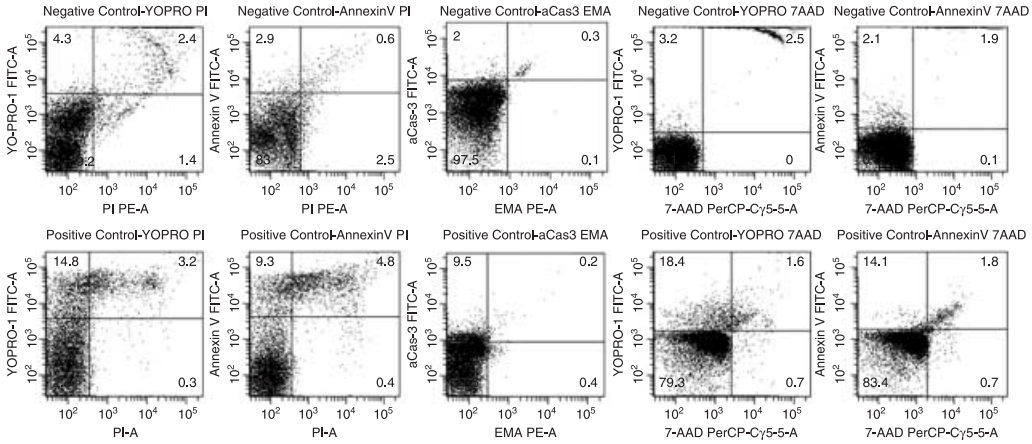

Figure 2. Apoptotic indices of positive and negative controls measured by five flow cytometric methods used in this study. Freshly isolated PBMCs were used as a negative control. The positive control was generated by incubation of PBMCs with CPT for 6 h.

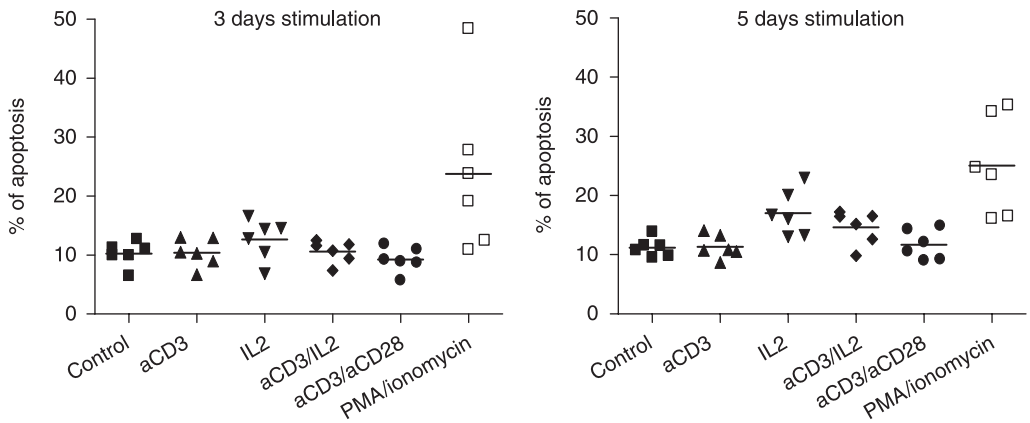

Figure 3. PMA/ionomycin stimulation caused the highest level of apoptosis. Cells were stimulated by different immunological regimes that caused varying levels of apoptosis, measured by all six methods. At both time points (3 and 5 days), the highest mean apoptosis was caused by PMA/ionomycin stimulation.

The presence of the active form of caspase-3, as a key executioner molecule, is a sign of ongoing and irreversible apoptosis. Intracellular staining of caspase-3 is considered as a reliable method for capturing the apoptotic process. Therefore, we used this method as a reference.

As PMA/ionomycin stimulation yielded the highest apoptotic index (Fig. 3), a reasonable assumption is that the apoptosis quantification method which gives the highest apoptotic index following PMA/ionomycin stimulation is the most sensitive. Figure 4 shows that the method resulting in the highest apoptotic index after 3 days of PMA/ionomycin stimulation is YO-PRO-1/7-AAD. This is the only method that gave a significantly higher apoptotic index when compared with aCas-3/EMA staining following PMA/ionomycin stimulation for 3 days ($P < 0.01$ by Tukey's test). None of the other tested methods provided statistically significant differences in apoptotic indices compared with aCas3/EMA after 5 days in culture.

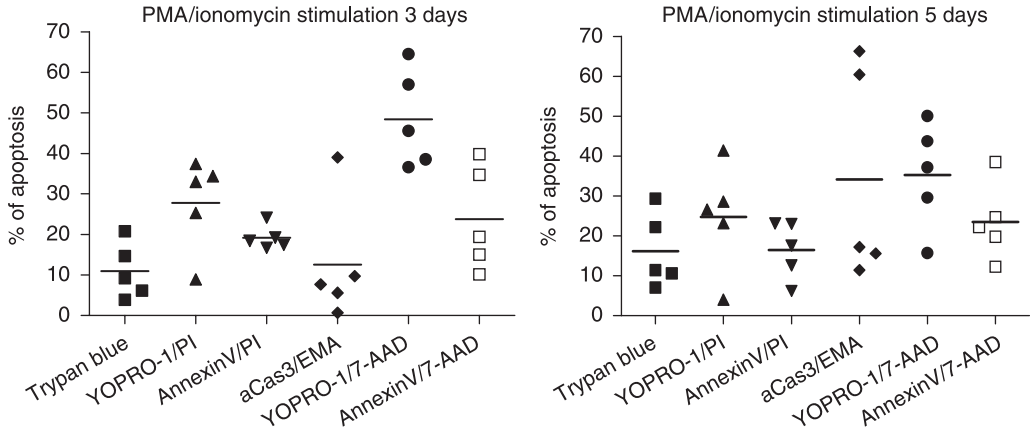

Figure 4. YOPRO-1/7-AAD detects the highest level of apoptosis after 3 days in culture. When compared with other methods employed, YOPRO-1/7-AAD detected the highest apoptosis after 3 days; after 5 days apoptotic levels were not significantly different.

Measurement of mortality index

Every apoptosis detection method we tested, except the trypan blue exclusion test, included a dye for staining of dead cells. Mortality was expressed as a mortality index, which was calculated as the number of dead cells divided by total number of cells. The trypan blue method has been used as a reference. In FACS-based methods, mortality was counted as percentage for cells positive with either apoptosis-plus-death stains or with a death stain only (Q2 + Q4 quadrants, Fig. 2).

The different stimulation methods used caused a significant difference in mortality indices ($F_{2.28} = 36.2$, $P = 7 \times 10^{-24}$ at 3 days and $F_{2.28} = 51.4$, $P = 2 \times 10^{-30}$ at 5 days). The two-way ANOVA test showed that the difference between mortality detection methods varies widely at 3 days, $F_{2.27} = 7.9$, $P = 1.4 \times 10^{-6}$ and also at 5 days, $F_{2.28} = 8.5$, $P = 4.4 \times 10^{-7}$. Furthermore, there was no added influence from the type of stimulation/mortality method interaction at 5 days $F_{1.58} = 0.23$, $P = 0.99$ which is nevertheless apparent at 3 days $F_{1.58} = 2.5$, $P = 3.8 \times 10^{-4}$. Figure 5 shows mortality indices after applied immunological stimulation at the two time points. As with the apoptosis results, PMA/ionomycin stimulation caused the highest mortality. When compared with trypan blue exclusion and under PMA/ionomycin stimulation, only YO-PRO-1/PI and aCas3/EMA gave significantly higher values ($P < 0.05$) at 3 days, but there were no statistically significant differences between methods at 5 days (Fig. 6).

DISCUSSION

The objective of the present study was to discover the most sensitive method for detection of the apoptotic process, following immunological stimulation of human PBMC by five different methods. There are numerous methods for evaluating the apoptotic index that might capture different steps of the overall event. Methods tested here are commonly in use amongst investigators. We investigated the measurement of apoptosis by multiple assays and we provide here a new stain combination, YO-PRO-1/7AAD, that is more sensitive in detecting apoptosis, while simultaneously providing consistent mortality measurements. We tested (i) methods for assessment of

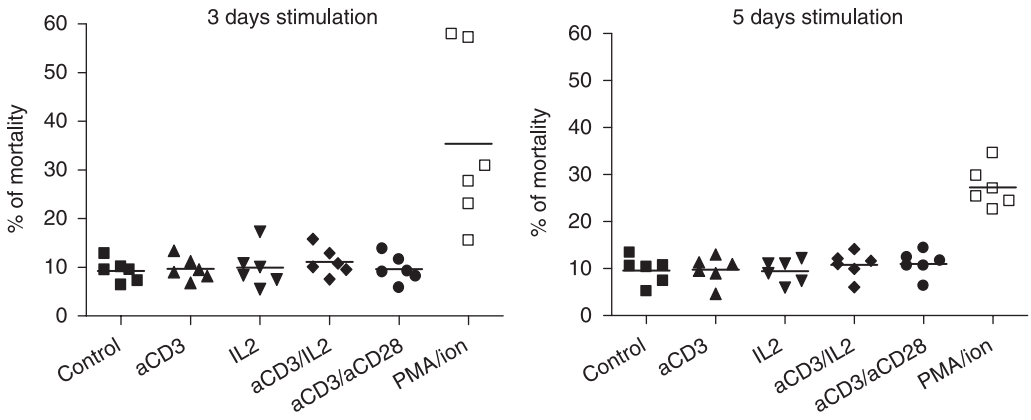

Figure 5. PMA/ionomycin stimulation caused the highest percentages of mortality. Cells were stimulated with different immunological stimulations that resulted in the mortality indices. At both time points (3 and 5 days), the highest mortality was caused by PMA/ionomycin.

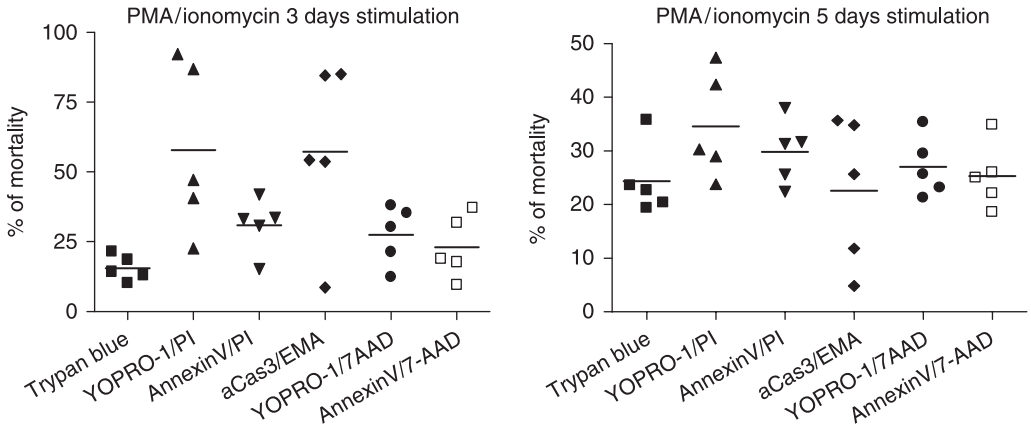

Figure 6. 7-AAD is the most reliable stain of mortality. When compared with other tested methods, only 7-AAD detected cell mortality as reliably as trypan blue exclusion; after 5 days similar mortality levels were detected by all tested methods not employing PI.

membrane integrity breakdown (trypan blue exclusion method, 7-AAD, PI, EMA, YO-PRO-1), (ii) translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (AnnexinV) and (iii) intracellular staining of the active form of the key executioner molecule in the apoptotic cascade (caspase-3).

We chose to study four nucleic acid stains that can label DNA for the detection of apoptosis for two reasons. First, some of them are commonly used amongst researchers, such as PI (with FITC-AnnexinV) and, second, because of their properties. These include:

1 the capability of entering a cell at an early stage of apoptosis (YO-PRO-1);
2 selective binding to DNA only (7-AAD) following entry into the cell;
3 the capability of covalent binding to DNA which makes the fixation of cells (and therefore intracellular staining of protein) possible (EMA).

All these differences depend on the chemical properties and molecular weight of each of molecule. Therefore, although all of them bind to nucleic acids, there are substantial differences in the dynamics of this.

To find the best apoptotic stain, we stimulated PBMCs using several methods. Stimulation with PMA/ionomycin resulted in the highest apoptotic index. Surprisingly, we made the important observation that the choice of method for detecting apoptosis was critical at 3 days ($P = 2 \times 10^{-6}$) but not critical, although still significant, at 5 days ($P = 1 \times 10^{-2}$). This marked drop in significance level was not seen in similar analyses with mortality measurement, which stayed significant throughout. Furthermore, at 3 days, there are additional interaction effects depending on the type of stimulation and the type of detection method combination, which are not apparent at 5 days. These interactions from the ANOVA results show the same trend for apoptosis and mortality (data not shown).

Our post-ANOVA analysis confirms that only YO-PRO-1/7-AAD gave a higher apoptotic index compared with the measurement of the active form of caspase-3 following PMA/ionomycin stimulation for 3 days, but after 5 days the differences disappeared. The higher apoptotic index at 3 days indicates that YO-PRO-1/7-AAD is more sensitive at an earlier stage in the apoptosis pathway. Possible explanations for the higher values of apoptosis recorded at 3 days by the YO-PRO-1/7-AAD combination compared with other methods lie in the mechanisms of staining, the affinity stains have for binding to nucleic acid and DNA sequence preference. Biochemical and photochemical comparison of these molecules show that:

1 YO-PRO-1 is a small molecule (MW 629.32);
2 it intercalates into dsDNA with high fluorescent quantum yield and into ssDNA and RNA with lower fluorescent quantum yield without sequence preference;
3 it has narrow emission band width (491 up to 509 nm) that allows multicolour application (easy to compensate).

7-AAD is:

1 a bigger molecule (MW 1270.45);
2 binds to DNA with sequence preference (GC regions of DNA only);
3 finally, the emission fluorescence is detected above 650 nm.

PI is:

1 a relatively small molecule (MW 668.40);
2 it intercalates every four to five nucleotides into dsDNA, ssDNA and RNA without sequence preference and with high affinity;
3 emission fluorescence is detected from 550 nm up to 670 nm, which is a broad emission spectrum suggesting that proper compensation is essential.

Ethidium monoazide (EMA) is:

1 a small molecule (MW 420.31);
2 after entering into cell in dark, needs light for photolysis to bind to nucleic acids without sequence preference;
3 after binding to nucleic acid, emission is narrowed to 600 ± 10 nm.

Additionally, YO-PRO-1/7-AAD is a low-cost method with similar cost per sample as YO-PRO-1/PI (Idziorek *et al.* 1995; Guillerm *et al.* 1998; Wronski *et al.* 2002). Furthermore, the YO-PRO-1/7-AAD combination is also able to detect mortality better than most other stains. There are several studies that successfully used 7-AAD only as a stain for apoptosis determination (Lecoeur & Gougeon 1996; Donner *et al.* 1999; Prieto *et al.* 2000). However, the combination of 7-AAD with the apoptotic stains DiOC6 (Herault *et al.* 2002) and AnnexinV (Hasper *et al.* 2000) distinguished apoptotic cells from live and dead cells in a more precise manner. These cited studies, along with supported data in this study, gave credence to the claim that 7-AAD is a good

stain for the detection of mortality. We note that specific experimental conditions can have a great impact on apoptosis detection outcome. This important observation has been made by other authors also (O'Brien & Bolton 1995; King *et al.* 2000; Schuurhuis *et al.* 2001). The present work avoids common pitfalls in analysis of apoptosis by flow cytometric methods (Darzynkiewicz *et al.* 1997).

We present YO-PRO-1/7-AAD as a reliable, sensitive and easy-to-perform, low cost FACS-based stain combination for apoptosis determination. Even more, YO-PRO-1/7-AAD is capable of detecting apoptotic cells after both short (3 days) and longer culture time (5 days) in addition to giving sensitive quantification of mortality and viability.

Therefore, we claim that YO-PRO-1/7-AAD is not only the most sensitive method for detecting apoptosis but that it also detects earlier changes in apoptosis captured at 3 days when compared with 5 days.

ACKNOWLEDGEMENTS

The authors would like to thank to Dr Shigemi Matsuyama for helpful advice. This study was supported by General Clinical Research Centre Grant MO1-RR00058 from the National Institutes of Health. None of the authors has connections with any of the companies that produce the tested drugs.

REFERENCES

Abraham MC, Shaham S (2004) Death without caspases, caspases without death. *Trends Cell Biol*. **14**, 184.

Bursch W (2001) The autophagosomal–lysosomal compartment in programmed cell death. *Cell Death Differ*. **8**, 569.

Darzynkiewicz Z, Bedner E, Smolewski P (2001) Flow cytometry in analysis of cell cycle and apoptosis. *Semin. Hematol*. **38**, 179.

Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F (1997) Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* **27**, 1.

Donner KJ, Becker KM, Hissong BD, Ahmed SA (1999) Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry* **35**, 80.

Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol*. **174**, 1783.

Green DR (2003) Overview: apoptotic signaling pathways in the immune system. *Immunol. Rev*. **193**, 5.

Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* **281**, 1309.

Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21**, 589.

Guillerm C, Robert-Hebmann V, Hibner U, Hirn M, Devaux C (1998) An anti-CD4 (CDR3-loop) monoclonal antibody inhibits human immunodeficiency virus type 1 envelope glycoprotein-induced apoptosis. *Virology* **248**, 254.

Hasper HJ, Weghorst RM, Richel DJ, Meerwaldt JH, Olthuis FM, Schenkeveld CE (2000) A new four-color flow cytometric assay to detect apoptosis in lymphocyte subsets of cultured peripheral blood cells. *Cytometry* **40**, 167.

Herault O, Domenech J, Georget MT, Clement N, Colombat P, Binet C (2002) All-trans retinoic acid prevents apoptosis of human marrow CD34+ cells deprived of haematopoietic growth factors. *Br. J. Haematol*. **118**, 289.

Idziorek T, Estaquier J, De Bels F, Ameisen JC (1995) YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J. Immunol. Meth*. **185**, 249.

King MA, Radicchi-Mastroianni MA, Wells JV (2000) There is substantial nuclear and cellular disintegration before detectable phosphatidylserine exposure during the camptothecin-induced apoptosis of HL-60 cells. *Cytometry* **40**, 10.

Lecoeur H, Gougeon ML (1996) Comparative analysis of flow cytometric methods for apoptosis quantitation in murine thymocytes and human peripheral lymphocytes from controls and HIV-infected persons. Evidence for interference by granulocytes and erythrocytes. *J. Immunol. Meth*. **198**, 87.

O'Brien MC, Bolton WE (1995) Comparison of cell viability probes compatible with fixation and permeabilization for combined surface and intracellular staining in flow cytometry. *Cytometry* **19**, 243.

Prieto A, Reyes E, Diaz D, Hernandez-Fuentes MP, Monserrat J, Perucha E, Munoz L, Vangioni R, de La Hera A, Orfao A, Alvarez-Mon M (2000) A new method for the simultaneous analysis of growth and death of immunophenotypically defined cells in culture. *Cytometry* **39**, 56.

Schuurhuis GJ, Muijen MM, Oberink JW, de Boer F, Ossenkoppele GJ, Broxterman HJ (2001) Large populations of non-clonogenic early apoptotic CD34-positive cells are present in frozen–thawed peripheral blood stem cell transplants. *Bone Marrow Transplant*. **27**, 487.

Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. *Science* **281**, 1312.

Vermes I, Haanen C, Reutelingsperger C (2000) Flow cytometry of apoptotic cell death. *J. Immunol. Meth*. **243**, 167.

Wronski R, Golob N, Grygar E, Windisch M (2002) Two-color, fluorescence-based microplate assay for apoptosis detection. *Biotechniques* **32**, 666.