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Optimized fluorescent probe combinations for evaluation of proliferation and necrosis in anthracycline-treated leukaemic cell lines

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Abstract. Proliferation and multidrug resistance status are key predictors of therapeutic outcome in acute myeloid leukaemia (AML). Anthracyclines such as daunorubicin (DNR) are typically used to treat AML and can induce drug resistance. The goal of the studies described here was to select a combination of fluorescent probes that could be used in combination with flow cytometry to monitor cell proliferation vs. cell death/necrosis as a function of anthracycline uptake. Propidium iodide (PI), the most commonly used marker of membrane integrity, cannot be used to evaluate necrosis in DNR-containing cells because of spectral overlap. A membrane integrity probe compatible with the use of a dye dilution method using PKH67 to study cell proliferation was also selected. The results show that DAPI and Cascade Blue (CB), like PI, were able to detect necrotic cells when no DNR was present, although CB gave less resolution between viable and necrotic cells than PI or DAPI. In the presence of DNR, DAPI cannot be used owing to the fluorescence quenching by DNR. However, it was found that a combination of DNR, CB, and PKH67 allows simultaneous identification of chemoresistant cells, based on reduced DNR accumulation, necrotic cells based on CB incorporation, and proliferating cells based on partitioning of PKH67 fluorescence between daughter cells. It was also found that unless a marker of necrosis is used in combination with the dye dilution assay, a moderate decrease of fluorescence as a result of necrosis may be incorrectly interpreted as proliferation.

INTRODUCTION

Proliferation and multidrug resistance status are key predictors of therapeutic outcome in acute myeloid leukaemia (List 1996). The aim of this study was to be able to detect proliferation of chemoresistant leukaemic cells cultured with daunorubicin (DNR). Classical methods for proliferation assessment, such as tritiated thymidine (^{3}H-TdR) or

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bromodeoxyuridine (BrdUrd) incorporation, are correlated with treatment outcome (Lacombe *et al.* 1994), but they are time consuming and difficult to standardize and require cell fixation (Dolbeare 1996). As an alternative, a dye dilution method has been developed using PKH67 to monitor proliferation of drug sensitive and resistant cell lines. PKH67 is one of a family of lipophilic, fluorescent membrane intercalating dyes (Horan *et al.* 1990). PKH dyes contain two long alkyl chains, which allow a strong anchorage in the lipid bilayer. When labelled cells divide, the resulting daughter cells receive half the label, reducing the fluorescence intensity to one-half that of parent cells. As a consequence, the proliferation of labelled cells leads to proportionate decreases in fluorescence (Yamamura *et al.* 1995).

Typical multidrug resistance (MDR) can be induced by anthracycline drugs (Foon & Gale 1992) such as DNR. Chemoresistant cells expressing molecules such as glycoprotein Gp170 (PGP) and the multidrug resistance-related protein (MRP) (Loe, Deeley & Cole 1996) can be distinguished by flow cytometry from chemosensitive cells on the basis of their reduced DNR content (Van Acker, Van Hove & Boogaerts 1993), monitored as emission of an orange red fluorescence when excited at 488 nm (Krishan & Ganapathi 1980). In order to use PKH molecules in chemoresistant models, it has been previously demonstrated that these molecules were not extruded by PGP or MRP (Boutonnat *et al.* 1998). The main drawback in using DNR is that the emission spectrum overlaps the spectrum of propidium iodide (PI), the most widely used fluorescent dye for evaluating membrane integrity.

Therefore, DAPI (4′,6-diamino-2-phenylindol) (Trotta *et al.* 1996) and cascade blue (CB) (Haugland *et al.* 1991) were evaluated as alternatives to propidium iodide (PI) for detecting necrotic cells. Two kinds of cell death were used: (i) direct necrosis by hyperthermia, and (ii) both necrosis and apoptosis induced by DNR and by vincristine (VCR) resulting in membrane integrity loss. VCR does not fluoresce and this allows comparison of the differents probes used to detect the loss of membrane integrity, although DNR emits a red fluorescence which overlaps PI fluorescence. After UV light excitation, DAPI emits a blue fluorescence. This molecule is classified as a semipermeant dye and can be considered as a marker of membrane integrity if the delay between dye contact and uptake measurement is controlled. CB is the amine-reactive derivative of sulphonated pyrene (Haugland *et al.* 1991). It can be excited by a UV laser and emits a blue fluorescence that can be collected by the same optical filter set used for DAPI. As compared to the aminocoumarin derivatives such as 7-amino-4-methylcoumarin-3-acetic acid (AMCA), cascade blue displays less spectral overlap with fluorescein, an important advantage for multicolour applications (Lowy 1995). As reported here, this novel application of cascade blue to monitor necrosis has allowed the development of a three colour method for studying, in a single tube, necrosis and proliferation, and the accumulation of anthracyclines, without unacceptable spectral overlap.

MATERIALS AND METHODS

Reagents

PKH67 and Diluent C were supplied by Phanos Technologies (Beverly Hills, CA, USA) or obtained from Sigma Aldrich (St Quentin Fallavier, France). Ethidium bromide, acridine orange, DNR and VCR were obtained from Sigma Aldrich. Cell growth medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and cellulose syringe filters (0.45 mm) were purchased from Gibco BRL (Cergy Pontoise, France). PI, CB, DAPI were provided by Molecular Probes (Interchim, France).

Cell lines

The human myeloid leukaemic K562 cell line, derived from a chronic myeloid leukaemia (Lozzio & Lozzio 1975), and its adriamycin resistant subline, K562/Adr, were generously provided by Prof J.P. Marie (Hotel Dieu, Paris, France). Parental and resistant K562 cultures are designated in the text as K562S and K562R, respectively. All cell lines were routinely cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 ng/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Exponentially growing cells were used for all experiments. Each cell line was tested to ensure freedom from mycoplasma contamination prior to use. Chemoresistance was maintained by continuous exposure to adriamycin at 10^{-6} M for the K562R. Resistant cells were grown in adriamycin free medium for three weeks to avoid interference from adriamycin fluorescence.

Methods

Comparative analysis of DAPI, CB and PI as markers of membrane integrity

To induce DNR-independent necrosis so that the different probes could be directly compared without interference from DNR, three tubes containing 10^6 K562S cells were incubated at 50°C for 10 min. Control and heated cells were then incubated with PI (final concentration: 50 μg/ml) or DAPI (final concentration: 0.1 μg/ml) or CB (final concentration: 1 or 10 μg/ml; in order to evaluate the best concentration) for 30 min at 20°C.

For analysis of necrosis induced by VCR, 3×10^6 K562S cells were incubated in complete medium with VCR at 150 nM for 24 h at 37°C. Cells were distributed in three tubes and, respectively, incubated with PI (final concentration: 50 μg/ml) or DAPI (final concentration: 0.1 μg/ml) or CB (final concentration: 1 μg/ml) for 30 min at 20°C.

For analysis of necrosis induced by DNR, 2×10^6 K562S cells were incubated in complete medium with DNR at 10^{-6} M for 24 h at 37°C. Cells were distributed in two tubes and incubated with CB (1 μg/ml) or DAPI (0.1 μg/ml) for 30 min at 20°C. Control cells (cells untreated by drugs) were incubated with the same dyes.

Assessment of proliferation, necrosis and chemoresistance status using three colour analysis

A mixture of 40% K562S cells (4×10^6 cells) and 60% K562R cells (6×10^6 cells) was labelled with PKH67 as follows. Cell suspensions were added to a polypropylene tube, centrifuged and carefully aspirated to maximize removal of PBS but minimize cell loss. The 2× staining solution of PKH67 was prepared in polypropylene tubes by diluting 20 μl 1 mM ethanolic dye solution in the Diluent C (both supplied with the kit) immediately prior to cell staining. Staining was initiated by adding a 2× concentrated cell suspension prepared by resuspending the cell pellet in 1 ml Diluent C to 2× concentrated dye solution. Staining was stopped after 3 min by adding an equal volume (2 ml) of FBS over a period of one minute and subsequently an equal volume (4 ml) of complete medium containing 10% FBS. Cells were then centrifuged and washed three times with 10 ml complete medium. All the steps were performed at room temperature. PKH67 stained cells were cultured with 10^{-7} M DNR for two days and analysed each day by flow cytometry.

Before each analysis, CB was added (final concentration: 1 μg/ml) for 30 min at 20°C. An aliquot of PKH67 labelled cells was fixed in 2% paraformaldehyde on day 0 and used as a reference control for further analysis.

Flow cytometric analysis

Cell fluorescence was detected using a FACStar^{Plus} flow cytometer (Becton Dickinson, Le Pont de Claix, France) equipped with a 5-W UV Laser (Coherent, Orsay, France) and a 100 mW

488 nm laser (Ion Laser Technology, Salt Lake City, UT, USA). PKH67 fluorescence was collected on the FL3 channel equipped with a filter at 530 nm ± 36 nm, DAPI and CB fluorescence were collected on the FL1 channel equipped with a filter at 424 nm ± 24 nm, PI fluorescence was collected on the FL4 channel equipped with a filter at 575 nm ± 26 nm. For each PKH67 acquisition, calibrated beads (Polyscience #425844, Cat. 18604) were placed in a fixed channel number each day by adjusting the photomultiplier voltage to obtain day to day interanalysis reproducibility. Thresholds levels and regions which combine fluorescence and FSC parameters were applied as selection criteria to select positive cells

This three colour combination required electronic compensation: FL3 − FL4 = 25% and FL4 − FL3 = 40%. All the data were acquired using Cell Quest software (Becton Dickinson) and analysed using MacCyt software (MacCyt, Grenoble, France).

RESULTS

Comparative analysis of DAPI, CB and PI as markers of membrane integrity, in the absence of DNR

The heat treatment induced 11% necrosis in the K562S cells, as detected by DAPI (Figure 1a) with a mean of fluorescence for the necrotic population around 400 a.u. Using PI (Figure 1b), 12% of K562S cells were detected as necrotic with a mean fluorescence value around 510 a.u. With CB (Figure 1c,d) at 1 and 10 μg/ml, respectively, the same fraction of necrotic cells was obtained but less separation was observed between viable and necrotic cell populations. Increasing the concentration of CB to 10 μg/ml led to a 10-fold increase of the mean fluorescence value of dead and viable cells but no improvement in resolution. Each experiment was carried out in triplicate and the maximum difference among triplicates and the different probes was less than 2% for the fraction of necrotic cells and less than 12 a.u. for the mean fluorescence intensity of the various membrane integrity markers. For each probe the repeatability between different experiments was never more than 6%.

Comparative analysis of DAPI, CB and PI as markers of membrane integrity, in the presence of DNR or VCR

Incubation of K562S cells with 10^{-7} M DNR for 24 h at 37°C induced modifications in size and granularity parameters as shown in region A of Figure 2a. Most cells with altered light scatter properties also displayed altered membrane permeability, as determined by a back-gate on the CB-positive populations in Figure 2c (data not shown). DAPI or CB were used in order to avoid spectral interference from DNR. Using DAPI, no fluorescence was detected (Figure 2b). Using CB, Figure 2c shows 12% of necrotic cells with a mean fluorescence value approximately 33 a.u. To determine whether the failure to observe DAPI fluorescence was a result of interference from DNR, cells were incubated with 150 nM VCR at 37°C for 24 h (Figure 2e,f). Under these conditions, similar results were obtained for both PI and DAPI: 19% necrotic with PI vs. 17% with DAPI. For each probe the repeatability between different experiments was never more than 6% whereas the variability between triplicates and the differents probes was less than 3%.

Three colour analysis using PKH67, DNR, and CB

The first analysis was carried out 3 h after PKH67 labelling and DNR incubation (day 0, Figure 3). The admixed K562S + K562R cell population is homogenous by light scatter (Figure 3a). Biparametric analysis of PKH67 and DNR fluorescence (Figure 3b) revealed 61% (PKH67+ DNR−) cells, 39% (PKH67+ DNR+) cells, in good agreement with the

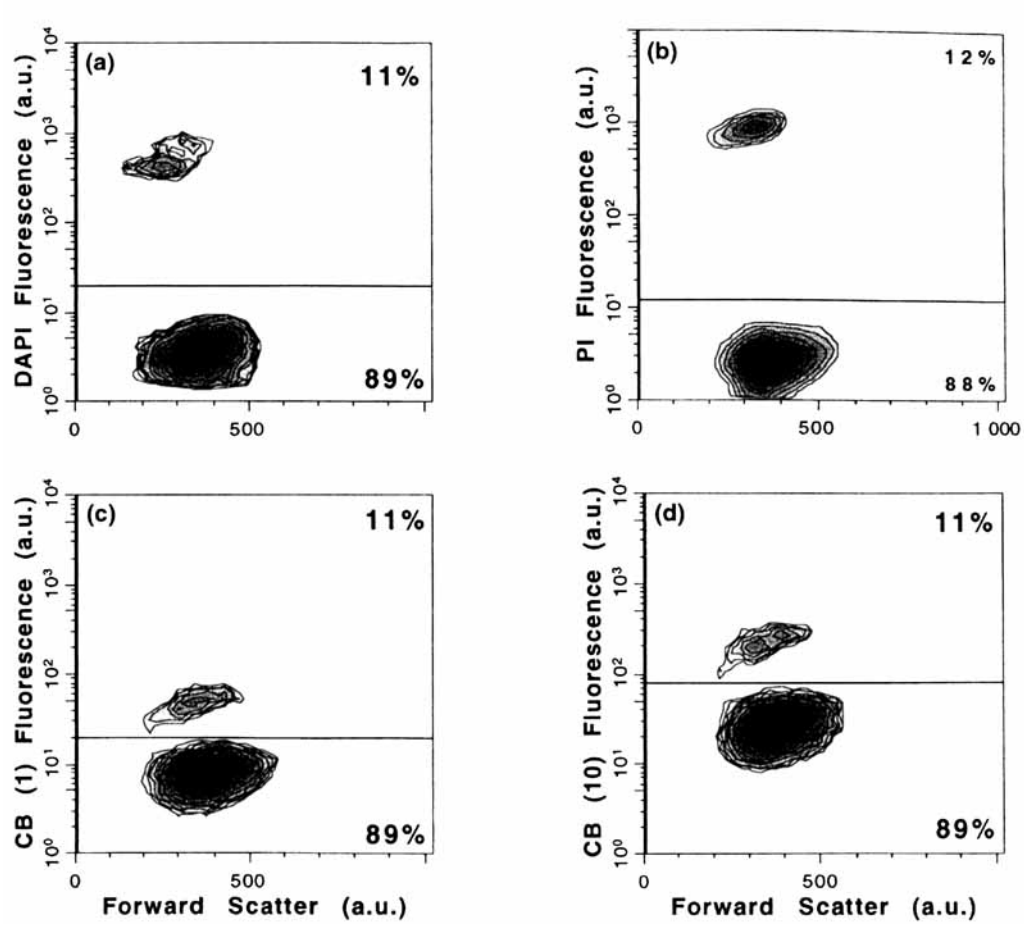


Figure 1. Comparative analysis of DAPI, CB and PI as markers of membrane integrity, in the case of heat induced necrosis. (a) Necrotic cells detection using DAPI (11%); (b) necrotic cells detection using PI (12%); (c) necrotic cells detection using 1 μg/ml CB (11%); and (d) necrotic cells detection using 10 μg/ml CB (11%)

expected 40% of K562S cells in the mixture. Also as expected after this relatively brief exposure, no necrotic (CB+) cells were observed. Biparametric analysis of DNR and CB fluorescence (Figure 3d), showed 41% (DNR+ , CB−) cells, 60% (DNR− CB−) cells, again in good agreement with the predicted percentages of K562S and K562R cells in the mixture, respectively.

By day 1, a decrease of PKH67 fluorescence was observed for DNR-treated cells (mean fluorescence value of PKH67 at day 0 = 1300 a.u., mean fluorescence value of PKH67 at day 1 = 711 a.u.). A decrease was also observed in the percentage of cells which had incorporated DNR (40% at day 0 and 32% at day 1). Figure 3f reveals an increase in the fraction of necrotic cells (4% CB+).

It was expected by this analysis to find an increase of cell granularity corresponding to necrotic cells, some necrotic cells detected by CB and DNR-positive or negative cells according to their chemoresistance. Also it was expected to find a decrease of PKH67 corresponding to the K562R which would have proliferated.

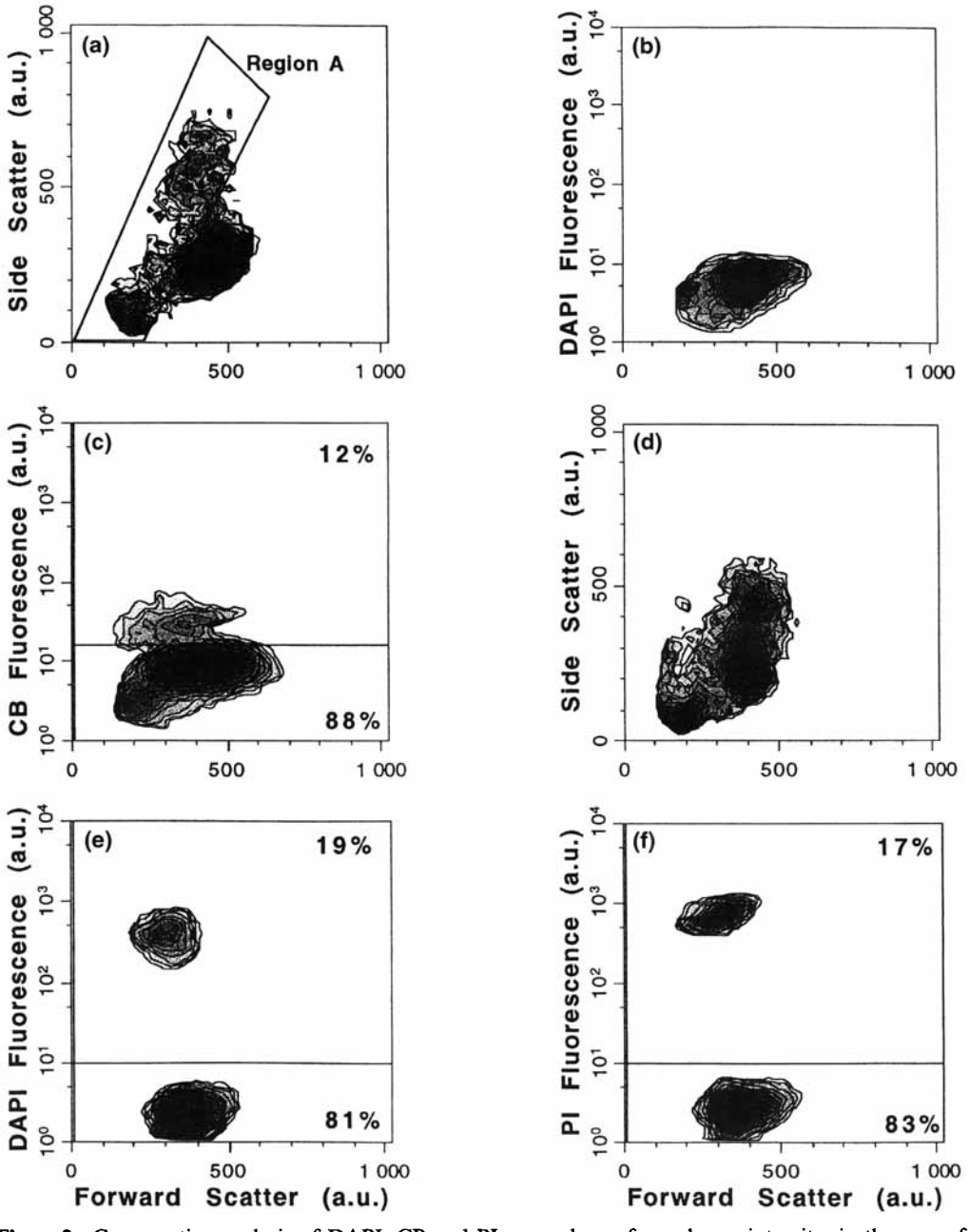


Figure 2. Comparative analysis of DAPI, CB and PI as markers of membrane integrity, in the case of by DNR (a–c) or VCR (d–f) induced necrosis. (a) Biparametric analysis of side and forward scatter of cells incubated with DNR 10^{-6}M for 24 h. Region A were cells with an increased cellular granularity and a decreased cell size. (b) Corresponds to the fluorescence of cells incubated with DAPI. No necrotic cells were detected. (c) Detection of necrotic cells using CB (12%). (d) Biparametric analysis of side and the forward scatter of cells incubated with vincristine 150 nM for 24 h. This dot shows an increased cellular granularity and decreased cell size for a fraction of the population. (e) Detection of necrotic cells using DAPI (19%). Panel (f) shows the PI fluorescence by forward scatter and 17% of the cells have an increased fluorescence (620 a.u.) and were considered as positive.

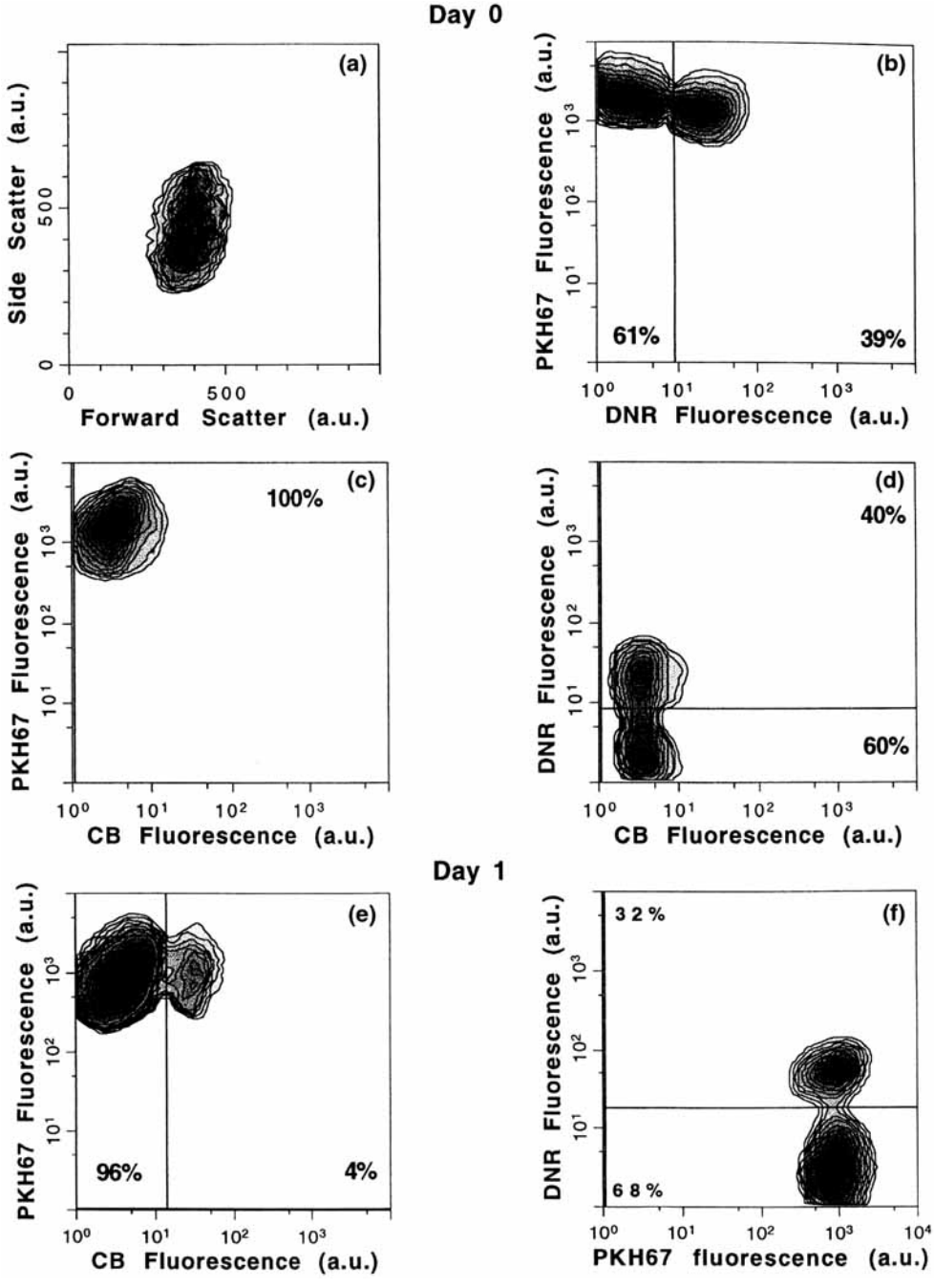
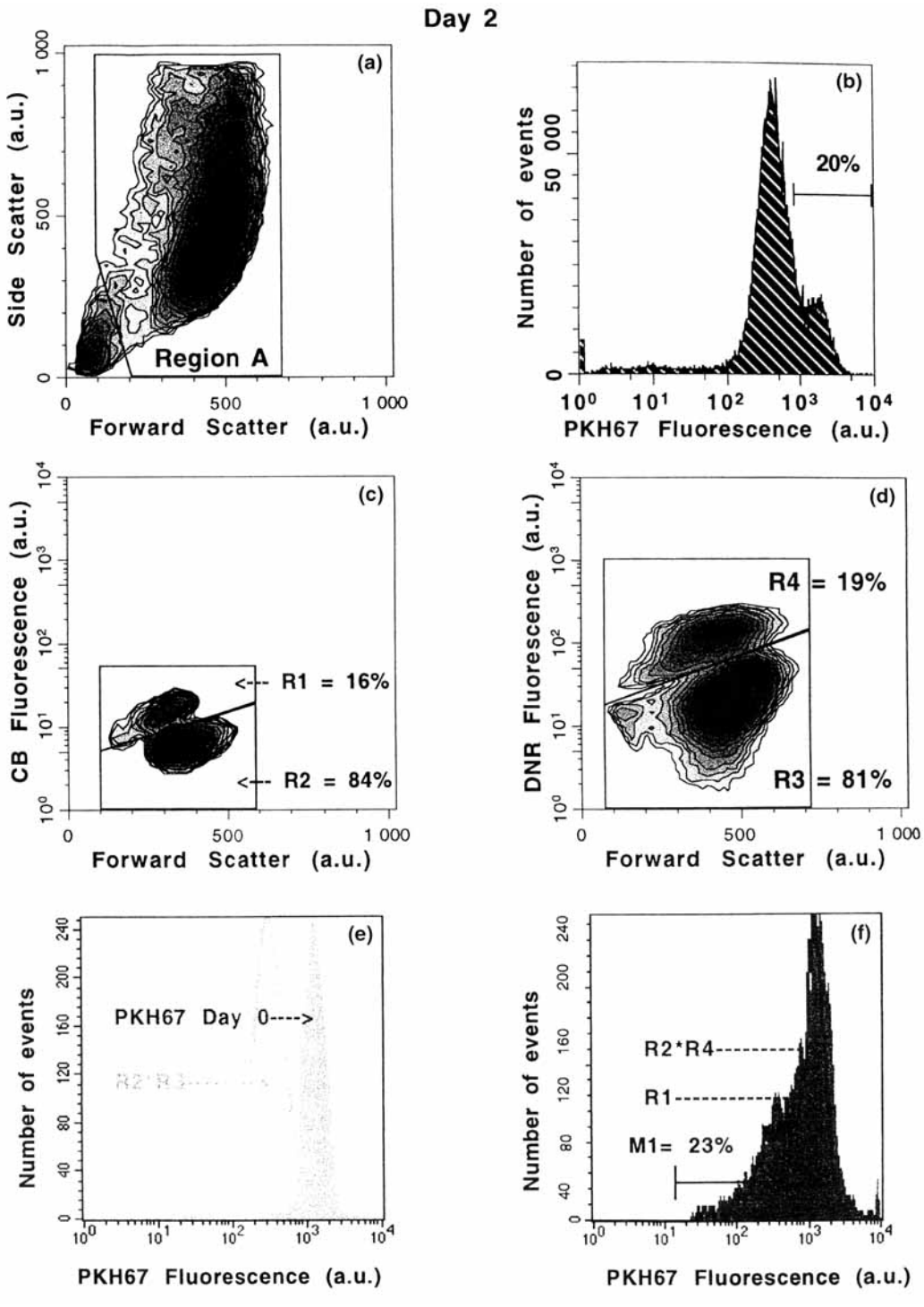


Figure 3. Three colour analysis of PKH67, DNR and CB By day 0: (a) Homogenous distribution of cell granularity and cell size of the (K562S/K562R) mixture; (b) 61% of cells were (PKH67+ and DNR), 39% of cells were (PKH67+ and DNR+); (c) no necrosis was detected; and (d) 40% of cells were positive for DNR; all cells were negative for CB. By day 1: (e) 32% of cells display a high level of fluorescence associated to DNR; and (f) 4% of cells were detected as necrotic based on the CB fluorescence.


Day 2
(a)
Side Scatter (a.u.)
Forward Scatter (a.u.)
Region A
(b)
Number of events
PKH67 Fluorescence (a.u.)
20%
(c)
CB Fluorescence (a.u.)
Forward Scatter (a.u.)
<--- R1 = 16%
<--- R2 = 84%
(d)
DNR Fluorescence (a.u.)
Forward Scatter (a.u.)
R4 = 19%
R3 = 81%
(e)
Number of events
PKH67 Day 0---->
PKH67 Fluorescence (a.u.)
(f)
Number of events
R2*R4
R1
M1= 23%
PKH67 Fluorescence (a.u.)

By day 2, it was found that:

1 an increased light scatter heterogeneity is seen (Figure 4a). The PKH67 fluorescence distribution of cells in region A displayed a biphasic profile, where 20% of cells had preserved the initial intensity of fluorescence and where 80% of cells had decreased their fluorescence (Figure 4b).
2 Figure 4c (CB fluorescence/FSC) showed 16% of necrotic cells. Two regions were drawn: one including cells which have incorporated CB (R1) and another one around viable cells (R2). The measurement of the forward scatter parameter was necessary to distinguish these two populations because the fluorescence of the largest viable cells was equivalent to the fluorescence of necrotic cells with a low size. Figure 4d shows that 19% of cells are maintained at a high level of DNR (R4 = 19%).
3 In order to determinate the proliferative population, PKH67 fluorescencewas evaluated using the region combinations described above. Figure 4e shows in bold grey, PKH67-associated fluorescence at day 0; the grey line representing the histogram extracted from the combination of region R2 (viable cells) and R3 (cells negative for DNR fluorescence). The mean fluorescence values were 1300 a.u. and 350 a.u. at day 0 and day 2, respectively.
4 Figure 4f shows the histogram (black line) extracted from a combination of R2 (viable cells) and R4 (cells with high intracellular levels of DNR) and the histogram (bold grey) extracted only from R1, corresponding to necrotic cells. The histogram (with black contours) indicates that the majority of these cells did not proliferate and surprisingly 23% (marker M1) of the cells underwent proliferation. The histogram (bold grey) extracted only from R1, corresponding to necrotic cells overlaps the histogram extracted from region (R2 × R4). This latter fact pointed out that necrotic cells have lost PKH67 fluorescence probably by cell membrane disruption.

DISCUSSION

This paper describes the choice of membrane integrity markers when studying proliferation and necrosis of chemosensitive and chemoresistant cells cultured with DNR. In the case of heat induced necrosis (i.e. when interference from DNR is not a problem), DAPI, CB and PI were able to detect the same percentage of cells that have lost membrane integrity. However, DAPI and PI allows a better discrimination between viable cells and dead cells (mean intensity of fluorescence of dead cells was about 10-times higher with DAPI and PI than with CB). In these experimental conditions, DAPI can be considered as a good alternative to PI.

In the case of DNR-treated cells, necrosis can be detected by FSC and SSC parameters (Darzynkiewicz *et al.* 1997) but no cells were fluorescent with DAPI. The absence of

Figure 4. (opposite) By day 2: (a) FSC and SSC parameters identify a more heterogeneous cell population. (b) PKH67 fluorescence extracted from region A (dot 1). Twenty per cent of cells had maintained their initial fluorescence intensity and 80% of cells had decreased their fluorescence. (c) Two populations were identified: necrotic cells which have incorporated CB (R1) and viable cells (R2). (d) Nineteen per cent of cells maintained a high level of DNR (R4). (e) The histogram (bold grey PKH67) associated fluorescence at day 0, the grey line representing the histogram extracted from the combination of region R2 (viable cells) and R3 (cells negative for DNR fluorescence). The mean fluorescence values were 1300 a.u. and 350 a.u. at day 0 and day 2, respectively. (f) The histogram (black line) extracted from region (R2*R4), the histogram (bold grey) extracted only from R1, corresponding to necrotic cells. The latter overlaps the histogram extracted from region (R2*R4).

fluorescence emission is probably a result of an energy transfer phenomenon between DAPI and DNR. This hypothesis is consistent with the following facts: (i) DNR is an intercalating agent which binds to 5′A/TCG and 5′A/TGC; (ii) DAPI binds preferentially AT clusters (Trotta *et al.* 1996) (there is a spatial proximity); and (iii) the emission spectrum of DAPI overlaps with the excitation spectrum of DNR. In addition, it is worth noting that DNR is already partially quenched when bound to DNA (Frezard & Garnier-Suillerot 1991). In order to test the energy transfer hypothesis, the detection of necrotic cells by DAPI and PI were assessed when necrosis was induced by VCR (a cytostatic agent which does not emit fluorescence after 488 nm excitation). The fact that DAPI and PI give equivalent results in this case confirms that DAPI is a marker of membrane integrity and allows detection of necrotic cells, and also argues for the energy transfer phenomenon in the case of co-localization with DNR.

CB acetyl azide is a polar tracer and an impermeant dye (Haugland *et al.* 1991) which can bind cellular amines. In the case of dead cells it was supposed that fluorescence will be increased secondary to CB binding to intracellular amines, allowing differentiation between viable and necrotic cells. The present results have demonstrated that CB can be used for evaluating necrosis in the presence of DNR despite a lower sensitivity compared to DAPI and PI. The fluorescence of viable cells incubated with CB is slightly increased compared to the autofluorescence level secondary to CB binding extracellular amines. However, in the case of cells having lost their membrane integrity, cells have increased blue fluorescence compared to viable cells.

CB was therefore used, in association with PKH67, to evaluate the proliferation and necrosis on a mixture with 40% of K562S and 60% of K562R cells treated with DNR. This triple staining permits the study of proliferation through the decrease of PKH67 fluorescence in viable cells having accumulated greater or lesser amounts of DNR. In this case studied, DNR at the concentration of 10^{-7} M has not immediately completely blocked K562S cell proliferation and has induced necrosis. Interestingly, it was found that the PKH67 fluorescence distribution of necrotic (CB+) cells was superimposable on the PKH67 fluorescence distribution of DNR+ viable cells, and that both distributions had a high proportion (77%) of high intensity cells and a small fraction (23%) of cells with reduced PKH67 intensity. Without using a marker of membrane integrity (here CB), the observed decrease in PKH67 fluorescence intensity seen for the CB+ cells in R1 could be mistaken for cell proliferation. These results confirm the necessity of having a marker of membrane integrity, when measuring proliferation with PKH67 or any other dye dilution technique. For detection of CB-labelled cells attention must be paid to the FSC parameter, in order to avoid inadequate detection of positive cells.

To the authors' knowledge, CB has been used for the labelling of secondary antibodies (Lowy 1995) but not as a marker of membrane integrity. The mean fluorescence value of dead cells stained by CB being lower compared to the fluorescence of viable cells can be a limiting factor. Perhaps other dyes could be used which do not have this drawback. This hypothesis remains to be demonstrated.

CONCLUSION

Daunorubicin, the cytostatic agent most commonly used to detect chemoresistant cells, has the disadvantage of having an orange–red fluorescence which cannot be eliminated using electronic compensation. The DNR fluorescence overlaps the PI fluorescence and the quenched DAPI fluorescence. In this paper, CB has been demonstrated as a marker of

membrane integrity. Unlike DAPI, CB exhibits no energy transfer phenomenon with DNR. The use of CB with PKH67 and DNR allows triple labelling to study proliferation, necrosis and chemoresistance. This rapid method is potentially applicable to routine studies of proliferation and necrosis on leukaemic cells.

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