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A NEW METHOD FOR THE CYTOFLUORIMETRIC ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL USING THE J-AGGREGATE FORMING LIPOPHILIC CATION 5,5',6,6'-TETRACHLORO-1,1',3,3'-TETRAETHYLBENZIMIDAZOLCARBOCYANINE IODIDE (JC-1)

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A new method for the cytofluorimetric analysis of mitochondrial membrane potential in intact cells has been developed by using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), whose monomer emits at 527 nm after excitation at 490 nm. Depending on the membrane potential, JC-1 is able of forming J-aggregates that are associated with a large shift in emission (590 nm). The color of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized. In two human cell lines (K562 and U937), we have studied by flow cytometry the changes in membrane potential provoked by the K+ ionophor valinomycin, a drug known to affect mitochondrial membrane potential, while the K+/H+ ionophor nigericin, known to affect intracellular pH but not mitochondrial membrane potential, was used as control. The incubation with valinomycin for 10 min. at 37°C in a low K+ medium provoked a marked and dose-dependent reduction in JC-1 greenish orange fluorescence, while nigericin had no effect.

Flow cytometry (FCM) allows the characterization and analysis of several cell parameters and functions, such as lymphocyte phenotype, cell cycle, apoptosis, among others (1-6). A consistent advantage of FCM techniques is the possibility to analyze thousand of living cells in a few seconds. Fluorescent probes exist which allow the analysis of changes in mitochondrial membrane potential (rhodamine 123, Rh123) (7-13) or modifications of mitochondrial mass (nonyl acridine orange) (14, 15). However, the results these probe can put in evidence are expressed as decreases or increases in fluorescence intensity, so that variations of membrane potential are usually expressed as the shift of the mean peak channel of a single fluorescence. By using the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), we have developed a new FCM technique which is able to detect at a single cell level variations in mitochondrial membrane potential. This molecule, able to selectively entry into mitochondria (16), exists in a monomeric form emitting at 527 nm after excitation at 490 nm. However, depending on the membrane potential, JC-1 is able of forming J-aggregates that

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are associated with a large shift in emission (590nm) (17, 18). Thus, the color of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized (16). In order to establish whether such spectral properties could be applied in FCM, we have studied the effects on human cell lines of an ionophor such as valinomycin, known to reduce mitochondrial membrane potential. The potassium ionophor nigericin, usually used to equilibrate the pH inside and outside the cell, was used as control.

MATERIALS AND METHODS

K562 and U937 cell lines were cultured in complete medium, i.e. RPMI 1640 with 10% fetal calf serum (FCS), 1mg/mL penicillin-streptomycin, 2mM L-glutamine at 37°C in humidified atmosphere of 5% CO_2 in air, and collected during the log phase of growth. Such medium has a low K+ concentration. Valinomycin (Sigma, St. Louis, USA) was used at the concentrations of 100 nM - 10 μM; nigericin (Molecular Probes, Eugene, USA) was used at 0.1 - 10 μg/mL. Cells were incubated in complete medium with valinomycin or nigericin for 10 min. at 37°C and then stained with the mitochondrial probe. For this purpose, cell suspension was adjusted to a density of 0.5 $x10^6$ cells/ml and incubated in complete medium for 10 min. at room temperature in the dark with 10 μg/ml JC-1. JC-1 was from Molecular Probes, and was dissolved and stored according to the manufacturer's instruction. At the end of the incubation period the cells were washed twice in cold phosphate buffer saline (PBS), resuspended in a total volume of 400 μL and analyzed.

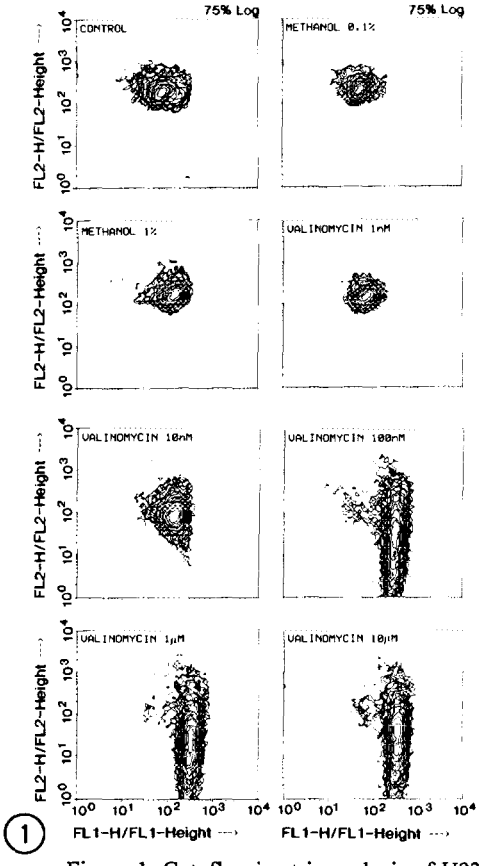
FCM was performed using a FACScan flow cytometer (Becton Dickinson, San Josè, USA) equipped with a single 488 nm argon laser. The filter in front of the FL1 photomultiplier transmits at 530 nm and has a bandwidth of 30 nm, the filter used in FL2 channel transmits at 585 and has a bandwidth of 42 nm. For the analysis of cells stained with JC-1, the photomultiplier (PMT) value of the detector in FL1 was set at 310 V, FL2 PMT at 259 V; FL1-FL2 compensation was 2.0%, FL2-FL1 compensation was 20.9%. A minimum of 10,000 cells per sample were analyzed. Data were acquired in list mode and analyzed with Lysys II software (Becton Dickinson).

RESULTS

Figures 1 shows the effects of the incubation with valinomycin on U937 cells stained with JC-1. Control cells have a high fluorescence emission both in FL1 and FL2 channels, while valinomycin-treated cells showed a slightly increased green emission but a significantly decreased orange emission. The Figure also demonstrates that such an effect is dose-dependent. Figure 2 shows that the same results can be obtained by using another cell line, i.e. K562. Figure 3 presents a separate analysis of the fluorescence emission in FL1 and in FL2. U937 cells treated with valinomycin showed a dose-dependent increase of the green fluorescence intensity (in FL1) and a concomitant decrease of the signal in FL2 (orange).

Figure 4 shows that the incubation with nigericin is not able to reduce mitochondrial membrane potential. Indeed, no modifications of JC-1 fluorescence emission were observed.

Figures 1-2 also indicate that the incubation with methanol (maximal dose 1% in complete medium), which was used to dissolve valinomycin, have no effects on the fluorescence emission of JC-1. The same results were obtained with lower doses of methanol



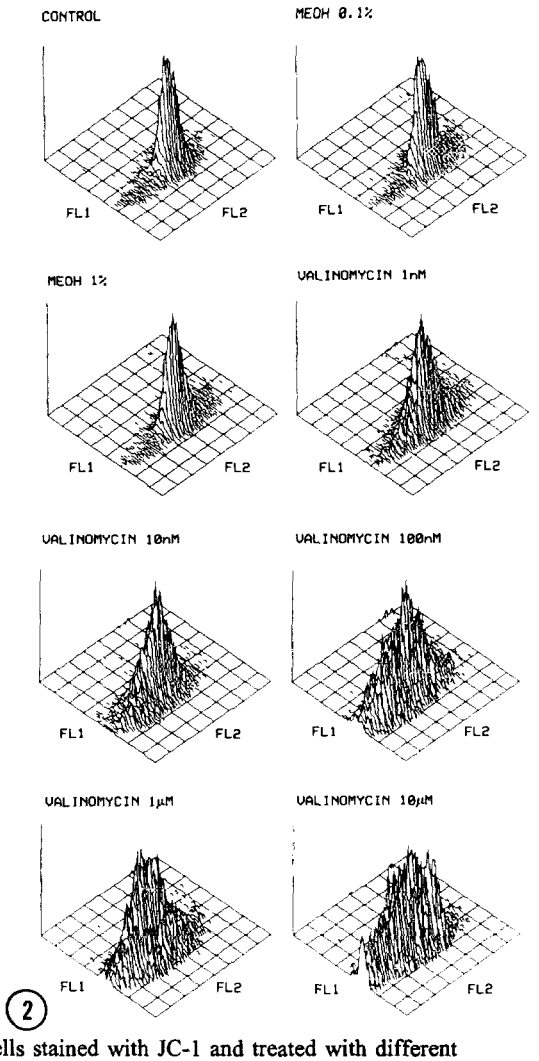


Figure 1. Cytofluorimetric analysis of U937 cells stained with JC-1 and treated with different doses of valinomycin, dissolved in methanol. In ascissa FL1 (green fluorescence, log. scale), in ordinate FL2 (orange fluorescence, log. scale). One experiment representative of 6 is shown.

Figure 2. Cytofluorimetric analysis of K562 cells stained with JC-1 and treated with different doses of valinomycin, dissolved in methanol (MEOH). FL1 indicates green fluorescence (log. scale), FL2 orange fluorescence (log. scale). Ordinate indicates the relative cell number. One experiment representative of 5 is shown.

or with dimethylformimide (DMF), used to dissolve nigericin (0.5% in complete medium) (data not shown).

Cell viability was measured in all samples both by FCM (propidium iodide exclusion method after the treatment with valinomycin or nigericin, or 7-amino actinomycin-D incorporation after staining with JC-1) and optical microscopy (tripan blue dye exclusion), and it was always >95% (data not shown). This was confirmed by the lack of modifications of the physical parameters of the cells, analyzed by FCM (forward and side angle scatters).

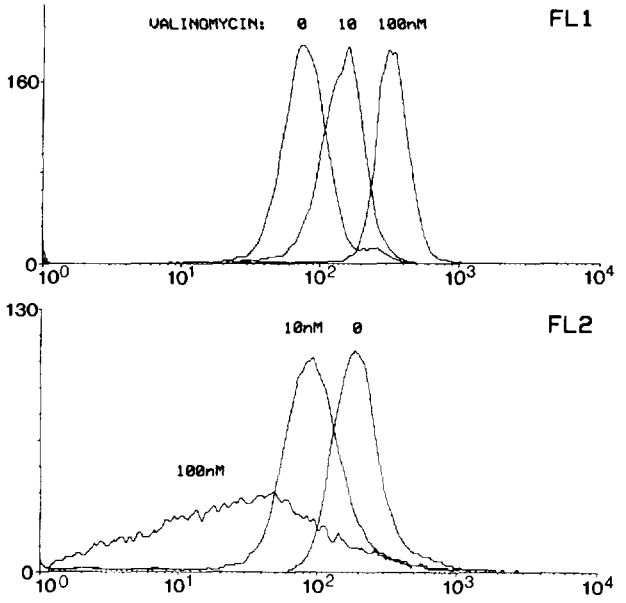


Figure 3. Separate analysis of JC-1 fluorescence emissions in U937 cells treated with different doses of valinomycin. Abscissa indicates fluorescence intensity, ordinate relative cell number.

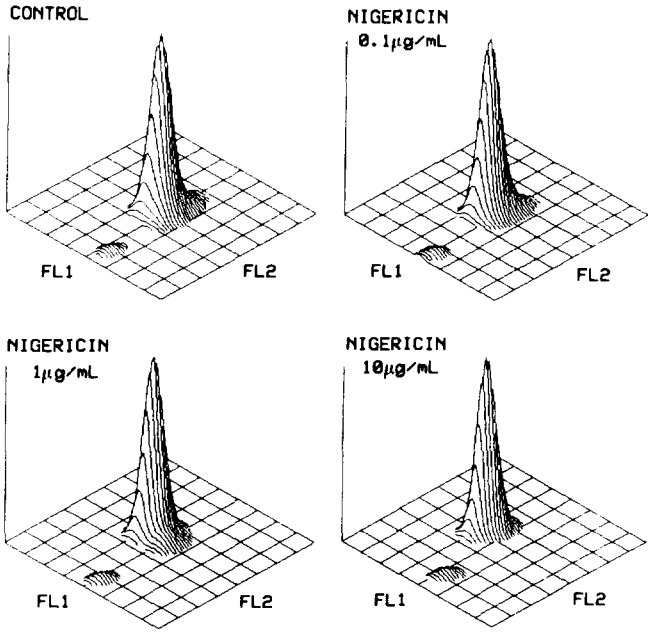


Figure 4. Nigericin has no effects on U937 cells stained with JC-1. FL1 indicates green fluorescence (log. scale), FL2 orange fluorescence (log. scale). Ordinate indicates the relative cell number. One experiment representative of 6 is shown.

DISCUSSION

In this paper we describe a new cytofluorimetric method for the rapid analysis of mitochondrial membrane potential variations. The dye used was 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), a lipophilic cation which exists in a monomeric form but is also able of forming multimers (19, 20). Such phenomenon has been observed within mitochondria, where it is dependent on their membrane potential (16). After excitation at 490 nm, the monomeric form emits at 527, while the J-aggregates are associated with a large shift in emission (590nm). Thus, the shift of the color of the dye (from green to orange) is an index of an increase of the polarization of mitochondrial membrane. As these wavelenghts are detectable by an argon laser equipped cytofluorimeter, we assessed whether a change in mitochondrial membrane potential could be evaluated, taking advantage from the fact that this instrument typically mount a filter for green fluorescence (FL1) and a filter for orange fluorescence (FL2).

Valinomycin, a classical K+ ionophor that dissipates the membrane potential but not the pH gradient, was used to reduce the orange fluorescence emission of JC-1. Although the drug was used in living cells instead of isolated mitochondria and in a low K+ medium, a significant and dose-dependent effect was observed both in U937 and in K562 cell lines, as shown in Figures 1 and 2. It is intesting to note that not only the emission in FL2 was affected by the drug, which was able to slightly increase the emission in FL1. In other words, cells treated with valinomycin and stained with JC-1 had a decreased orange fluorescence but also an increased green fluorescence. This can be due to the fact that the decrease of J-aggregate formation (giving an orange fluorescence) is logically mirrored by an increase of JC-1 monomers (green fluorescence).

As expected, nigericin, a drug which is not able to collapse the transmembrane potential, had no effect on JC-1 fluorescence emission.

The cytofluorimetric study of mitochondrial membrane potential in intact cells present several advantages. Indeed, it is possible to analyze at a single cell level potential variation, and this is of fundamental importance when researchers are coping with heterogeneous cell populations. Moreover, mitochondria can be studied in their physiological environment, i.e. within the cell. However, a disadvantage of such approach is that several substances able to interfere with mitochondrial activity, and which are widely used in studies on separated organelles, cannot be used as they are not able to pass plasmamembrane.

Mitochondrial membrane potential has been previously studied by FCM techniques by evaluating the changes of fluorescence of Rh123. However, Rh123 binding to mitochondria is difficult to calculate when the cell presents a certain mitochondrial heterogeneity due, for example, to a high number of mature or immature mitochondria, as in a continuously growing cell line. Moreover, it has been reported that in rat liver mitochondria different binding sites for Rh123 exist, i.e. sites which are freely accessible whatever the energy status of the mitochondria and sites which are hidden in the energized state and freely accessible in the deenergized form of the organelle (21). This has been attributed to different maturative states of the organelles (21). Thus, in a single cell the organelles can have different Rh123

mitochondrial binding sites with consequent different fluorescence emissions, and it is very difficult to ascertain whether or not mitochondria strongly bind Rh123 in an energy-dependent or -independent manner. The method we have described likely bypasses this problem, being both qualitative (considering the shift from green to orange JC-1 fluorescence emission) and quantitative (considering the pure fluorescence intensity, which can be detected in both FL1 and FL2 channels), and can be of great interest for the study of mitochondrial membrane potential variations in living cells.

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REFERENCES

1. Shapiro H.M. (1985) Practical Flow Cytometry, Alan R. Liss Ed., New York, USA.
2. Dolbeare, F., Gratzner, H., Pallavicini, M., and Gray, J. W. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5573-5577.
3. Darzynkiewicz, Z. and Crissman, H.A. Eds. (1990) Methods in Cell Biology, Vol. 33 - Flow Cytometry, Academic Press Inc., San Diego, USA.
4. Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991) J. Immunol. Methods 139, 271-279.
5. Cossarizza, A., Ortolani, C., Forti, E., Montagnani, G., Paganelli, R., Zannotti, M., Marini, M., Monti, D. and Franceschi, C. (1991) Blood 77, 1263-1270.
6. Barbieri, D., Troiano, L., Grassilli, E., Agnesini, C., Cristofalo, E.A., Monti, D., Capri, M., Cossarizza, A., Franceschi, C. (1992) Biochem. Biophys. Res. Commun. 187, 1256-1261.
7. Johnson, L.W., Walsh, M.L. and Chen, L.B. (1980) Proc. Natl. Acad. Sci. USA 77, 990-994.
8. Darzynkiewicz, Z., Staiano-Coico, L. and Melamed, M.R. (1981) Proc. Natl. Acad. Sci. USA 78, 6696-6698.
9. Goldstein, S. and Korczack, L.B. (1981) J. Cell Biol. 91, 392-398.
10. Petit, P.X., Diolex, P., Muller, P. and Brown, S.C. (1986) FEBS Lett. 196, 65-70.
11. O'Connor, J.E., Vargas, J.L., Kimler, B.F., Hernandez-Yago, J and Grisolia, S. (1988) Biochem. Biophys. Res. Commun. 151, 568-573.
12. Petit, P.X., O'Connor, J.E., Grunwald, D. and Brown, S.C. (1990) Eur. J. Biochem. 194, 389-397.
13. Pieri, C., Moroni, F. and Recchioni R. (1992) Cell. Immunol. 145, 210-217.
14. Maftah, A., Petit, J.M., Ratinaud, M.H. and Julien, R. (1989) Biochem. Biophys. Res. Commun. 164, 185-190.
15. Ratinaud, M.H., Leprat, P. and Julien, R. (1988) Cytometry 9, 206-212.
16. Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D. and Chen, L.B. (1991) Proc. Natl. Acad. Sci. 88, 3671-3675.
17. Hada, H., Honda, C. and Tanemura, H. (1977) Photogr. Sci. Eng. 21, 83-91.
18. Reers, M., Smith, T.W. and Chen, L.B. (1991) Biochemistry 30, 4480-4486.
19. Jelley, E.E. (1937) Nature 139, 631-632.
20. Sims, P.J., Waggoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315-3330.
21. Lopez-Mediavilla, C., Orfao, A., Gonzales, M. and Medina J.M. (1989) FEBS Lett. 254, 115-120.