THE FLOW-CYTOMETRIC DETERMINATION OF INTRACELLULAR CALCIUM IN VITAL CELLS WITH THE INDO-1 DYE

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1. INTRODUCTION

Calcium plays an important role for normal cell growth and cell function. Calcium concentrations are usually low in the cytoplasm (nM to µM range) but high in the mitochondria and in the cell nucleus (0.1 to 1mM) (1). Most of the calcium is protein bound. Approximately 10% of the calcium is present in free form. Calcium can be determined by fluorescent dyes which is of interest for flow cytometry.

The INDO1-AM (INDO-1 acetoxy-methyl) ester (2) penetrates the cell membrane, and enriches in vital cells after liberation of free INDO-1 by intracellular esterases. The colour of emitted fluorescence light of the INDO-1 dye shifts to shorter wavelengths on calcium binding. This shift is detectable by a simultaneous fluorescence measurement at two different wave lengths similarly as for intracellular pH-measurements (3). The ratio of both fluorescence intensities can be measured at different calcium concentrations in the presence of a Ca^{2+}-ionophore (4) to obtain a saturation curve (5) for intracellular calcium. This curve can be used as a calcium calibration curve when the amount of intracellular INDO-1 is known.

2. DETAILED PRESENTATION

$1x10^6$ Ehrlich ascites cells or rat bone marrow cells in 10mM HEPES buffered saline pH 7.35 (HBS) were incubated for 15min at 22°C with 20µg/ml INDO1-AM ester to stain vital cells and simultaneously with 40µg/ml of propidium iodide to stain the DNA of dead cells. Cellular fluorescence was excited by a HBO100 high pressure mercury arc lamp between 300 and 400nm. The blue (F1: 418 to 440nm) and the green

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plus red fluorescence (F2: 500 to 700nm) were simultaneously measured with the electrical volume of each cell in a FLUVO-METRICELL II flow cytometer (HEKA-Elektronik, D-6734 Lambrecht/Pfalz, FRG) in list-mode operation. The cell volume versus blue fluorescence histogram (fig.1a) and the cell volume versus the F1/F2 ratio histogram were calculated from all measured cells and plotted (fig.1b).

The intracellular enrichment of INDO-1 was different for different cell types. It was, therefore, important to determine the amount of intracellular INDO-1 i.e the task was to calibrate the fluorescence scale of fig.1a. This was accomplished in the following way: Human lymphocytes were isolated over Ficoll from peripheral blood and $1x10^7$ cells were loaded with INDO1-AM as indicated above. The cells were then pelleted through a cushion of water insoluble dibutylphthalate (2min, 12000xg) to separate intracellular from extracellular INDO-1. The supernatant was removed and the cell pellet was lysed with 50µl of distilled water followed by dilution with 2ml HBS, recentrifugation and removal of the supernatant which contained all the INDO-1 liberated from the 2µl of lymphocyte sediment ($1x10^7$ lymphocytes x $2x10^2 \mu m^3$). The INDO-1 concentration in the supernatant was 3.1µM as determined fluorimetrically from an INDO-1 calibration curve. The intracellular INDO-1 concentration in lymphocytes was, therefore, 3.1mM which corresponded to a 150 fold enrichment over the 20µM INDO1-AM ester during the initial incubation in the HBS-buffer. One lymphocyte contained $0.62x10^{-15}$Mol of INDO-1 or $3.72x10^8$ INDO-1 molecules/cell The cellular fluorescence of single lymphocytes corresponded to 10 logarithmic fluorescence units on the fluorescence scale of fig.1a. Based on this calibration, the granulocytes contained $1.24x10^{-15}$Mol INDO-1 (2.85mM) and Ehrlich ascites cells $3.1x10^{-15}$Mol INDO-1 (0.95mM). A mean overall intracellular calcium concentration for Ehrlich-ascites cells of 70µM was obtained from the measured F1/F2 ratio (fig.1b), the amount of intracellular INDO-1 (fig.1a) and the cellular calcium saturation curve (5).

The cell volume versus F1/F2 ratio histogram (fig.1b) shows that the tumor cells contain slightly more calcium than the granulocytes. The F1/F2 ratio of the tumor cells decreased when the cell membranes were permeabilized by 100µM of the non fluorescent Ca^{2+}-ionophore A23187Br (4) in the presence of 1mM EDTA. The decrease corresponded to an extractable calcium concentration of 10µM. The F1/F2 ratios increased for both cells in the presence of 1mM external calcium and the Ca^{2+}-ionophore. The results suggest that only cytoplasmic Ca^{2+} is accessible under these conditions. No changes of intracellular calcium concentrations were

observed when 1mM EDTA or 1mM calcium were present in the suspending buffer without Ca^{2+}-ionophore.

The flow-cytometric INDO-1 method for intracellular calcium determination has been quite useful e.g. to measure the influx of calcium into granulocytes which are stimulated by bacteria, or to monitor elevated Ca^{2+}-levels in tumor cells which are resistant against the cytostatic drug doxorubicine.

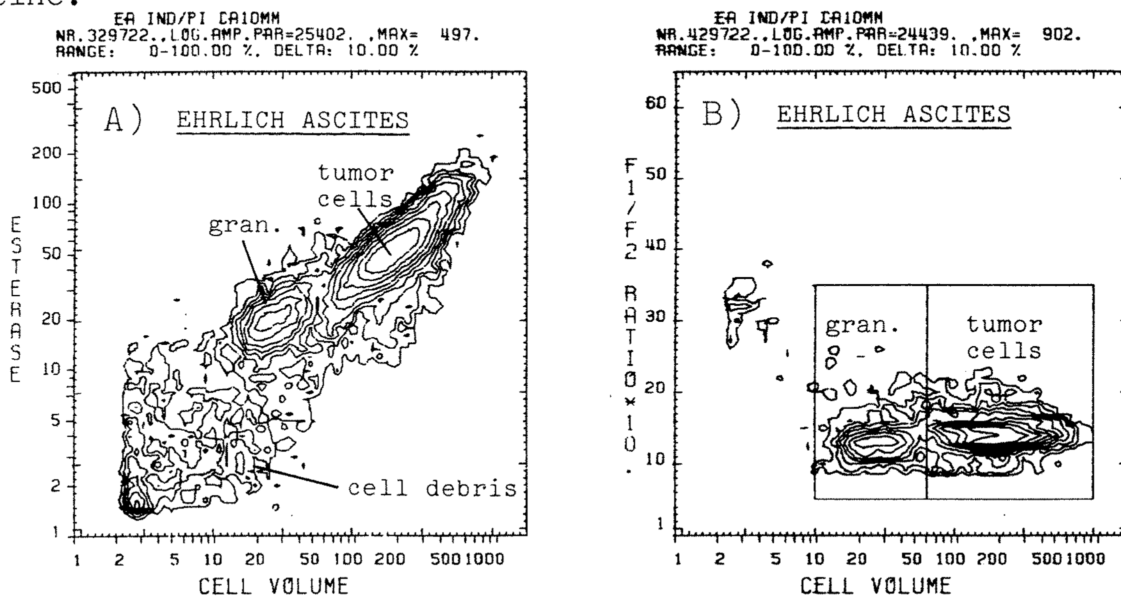

Fig.1a Cell volume versus esterase activity of the vital cells of a mouse Ehrlich ascites after 15min incubation with 20ug/ml INDO1-AM ester and 40ug/ml propidium iodide. The dead cells were gated out by computer evaluation of the list-mode data. The large tumor cells are well separated from the smaller granulocytes and the cell debris. The graph is standardized to the maximum logarithmic channel content (902 cells). Contour lines are plotted from the maximum in linear 10% intervals downwards. A total of 25400 cells and particles was measured. 1 logarithmic volume unit corresponds to $21.7\mu m^3$ and 1 logarithmic fluorescence unit to 6.2×10^{-17}Mol INDO-1 per cell.

Fig.1b Cell volume versus F1/F2 ratio display of the measurement of fig.1a. The F1/F2 ratio is a measure of the intracellular calcium concentration. Tumor cells have a slightly higher calcium concentration than granulocytes.

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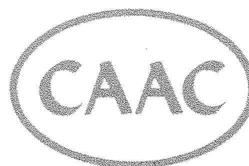

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