FLOW CYTOMETRIC DETERMINATION OF PEROXIDASE ACTIVITY AND PEROXIDE FORMATION IN HEMATOPOETIC CELLS USING 2',7'-DICHLOROFLUORESCIN-DIACETATE

S.Burow, G.Valet

Mildred Scheel-Labor für Krebszellforschung
Max-Planck-Institut für Biochemie, D-8033 Martinsried

1. INTRODUCTION

The purpose of our study was to develop a standardized assay for flow cytometric determination of metabolic burst activity in hematopoetic cells.
2',7'-dichlorofluorescin-diacetate (DCFH-DA) was first used for the fluorimetric determination of peroxide concentration in solutions (1,2) and has more recently been introduced into flow cytometry (3). DCFH-DA diffuses through the cell membrane and is hydrolysed by cytosolic esterases to non-fluorescent polar 2',7'-dichlorofluorescin (DCFH). Intracellular oxidation of DCFH by means of peroxidases and intracellular generated H_2O_2 results in the formation of highly fluorescent 2',7'-dichlorofluorescein (DCF).

2. METHODS AND RESULTS

Model experiments with the non-fluorescent DCFH-DA cleavage product DCFH showed that DCFH in the absence of oxygen was quickly photooxidized to fluorescent DCF by UV-light, to a lower degree by daylight and even slowly autooxidized in the dark. Therefore the assays were protected from irradiation with light and prolonged storage of incubated assays was avoided. Besides photooxidation, DCFH is significantly converted to DCF by oxidation with H_2O_2. The reaction is markedly increased in the presence of peroxidase whereas catalase or superoxide-dismutase failed to show enhanced DCFH-oxidation. Intracellular DCFH mainly acts as an electron donor for peroxidases.

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The standardized four step assay for flow cytometric work with vital cells was 15min preincubation of 1000ul cells ($1x10^5$/ml) in HBS-buffer (0.15N NaOH with 5mM HEPES, pH 7.35) with 10ul dichlorofluorescin-diacetate (1mM in HBS with 10% DMF) at 37°C followed by:
->no further addition, in order to screen for spontaneous cellular H_2O_2-formation (fig.1A), or
->addition of 10ul H_2O_2 (100mM in HBS) to screen for intracellular peroxidase activity (fig.1B), or
->addition of 10ul phorbol-myristate-acetate (PMA, 15uM in HBS), for stimulation of metabolic burst activity and cellular H_2O_2-production (fig.1C), or
->addition of 10ul bacteria (E.coli, $3.2x10^9$/ml), for the generation of a physiological burst (fig.1D).
Propidium-iodide (PI, 60nM) was present in all assays in order to stain the DNA of dead cells.
The cell volume (hydrodynamically focused electrical measurement), the green DCF-fluorescence (500-530nm) or the red PI-fluorescence (550-700nm) of each cell were measured simultaneously with a FLUVO-METRICELL flow cytometer following excitation (400-500nm) with a HBO-100 mercury lamp.
Human peripheral blood cells and cells from rats' bone-marrow, spleen, peripheral blood and resident macrophages exhibited significant DCF-fluorescence after treatment with externally offered H_2O_2 indicating peroxidase activity.
Human granulocytes incubated with H_2O_2 or bacteria showed a single cell cluster of DCF-fluorescence (fig.1B,D) whereas stimulation with PMA, in contrast, always caused a bimodal distribution consisting of a high and a low activity granulocyte subpopulation (fig1.C). The percentage of active cells varied from 45-55% and was increased during disease (e.g.sepsis) to 70% and more. The two groups of neutrophil granulocytes were undistinguishable by morphological criteria.
Similarly two types of myeloblastic cells could be distinguished in rat bone-marrow upon stimulation with PMA whereas resident macrophages from peritoneum showed an unimodal increase in DCF-fluorescence. Granulocytes from peripheral rat blood and cells from rat spleen failed to be stimulated. Stimulation of cellular H_2O_2-formation by E.coli only was observed in resident rat macrophages and rat myeloblastic bone-marrow cells.

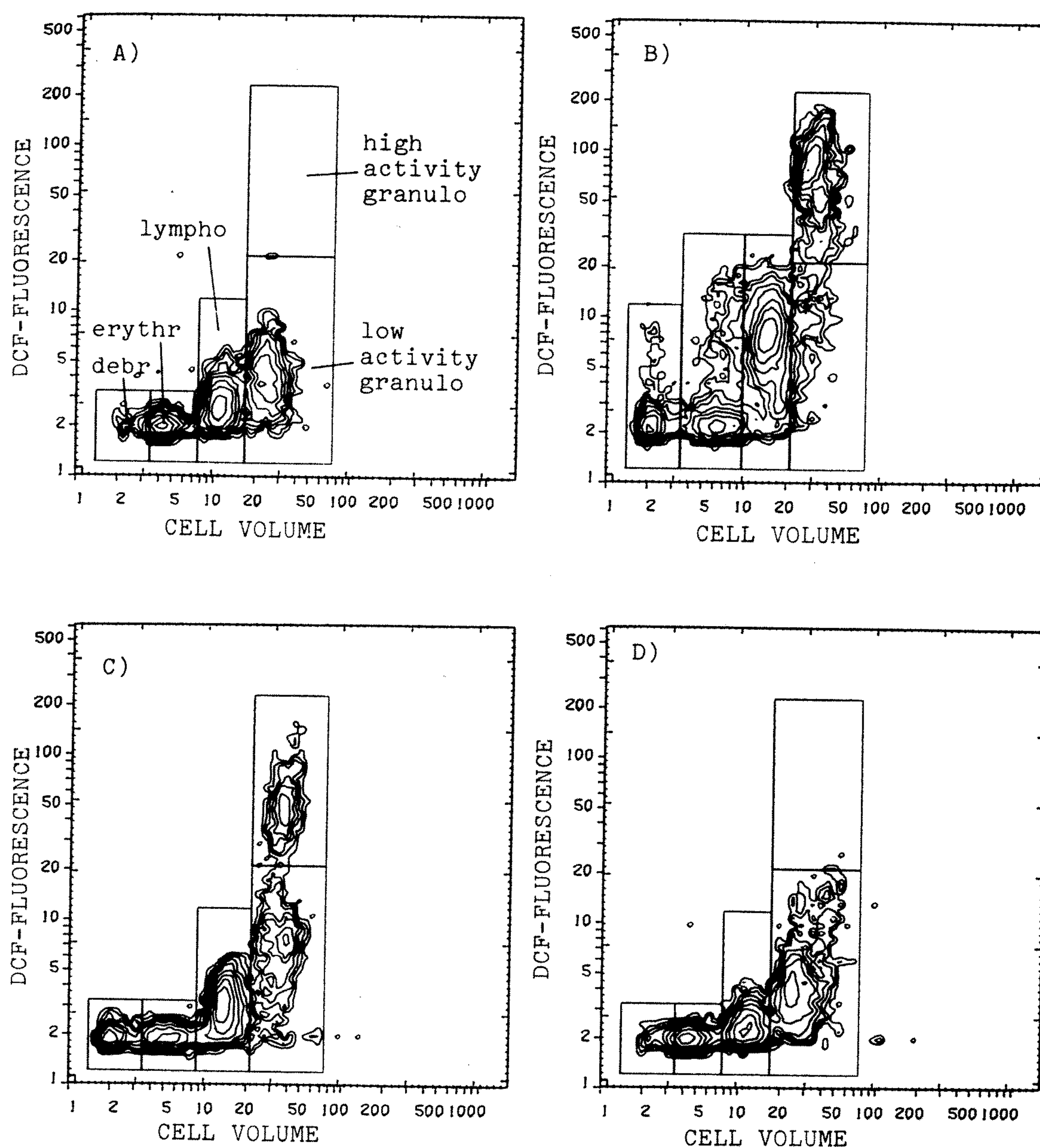

Fig. 1:DCF-stained human buffy-coat blood cells incubated with HBS alone (A), or together with 1mM H_2O_2 (B), or 150nM PMA (C) or $3.2x10^7$ E.coli bacteria/ml.

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G. Burger

Institut für Strahlenschutz, Neuherberg, FRG

J. S. Ploem

University of Leiden, The Netherlands

and

K. Goerttler

Deutches Krebsforschungszentrum, Heidelberg, FRG

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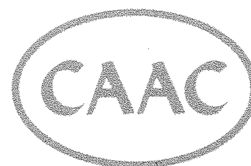

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