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FLOW CYTOMETRIC MEASUREMENT OF RNA SYNTHESIS BASED ON BROMOURIDINE LABELLING AND COMBINED WITH MEASUREMENT OF DNA CONTENT OR CELL SURFACE ANTIGEN

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RNA synthesis can be analysed in nuclei or cells labelled with 5-bromouridine (BrUrd) and stained using cross-reacting anti-bromodeoxyuridine (BrdUrd) antibody. Flow cytometric dual parameter analysis of BrUrd incorporation and DNA content in nuclear suspensions of human blood lymphocytes showed that RNA synthesis increased within the first 24 hours of phytohemagglutinin (PHA) stimulation, reaching a maximum at 48 hours, when cells had entered the cell cycle. Using a new method for flow cytometric dual parameter analysis of BrUrd incorporation and a cell surface antigen, spontaneous RNA synthesis in HL-60 and K-562 cells was measured simultaneous with CD13 expression.

Measurement of RNA synthesis may be of interest in a variety of studies on activation and inhibition of cell functions. Thus, increased RNA-synthesis may be expected as a result of stimulating quiescent cells with various growth factors (1). We have recently described a method for flow cytometric measurement of RNA synthesis, using BrUrd labelling and cross-reacting anti-BrdUrd antibodies (2). Intense and specific staining of nuclear BrUrd incorporated into RNA was obtained with the ABDM antibody of Partec and B-44 antibody of Becton Dickinson. Here, we show a new application of the previously described method for combined analysis of BrUrd and DNA content (2). Furthermore, we describe a new method for combined analysis of BrUrd and a cell surface antigen:

Example 1 (BrUrd/DNA method). Demonstration of flow cytometric, combined measurement of RNA synthesis and recruitment into cell cycle, as induced by PHA stimulation of human blood lymphocytes (3).

Example 2 (BrUrd/surface antigen method). Demonstration of flow cytometric, combined measurement of spontaneous RNA synthesis and expression of the myeloid differentiation marker CD13 in HL-60 and K-562 cells.

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Material and Methods

Cells. Human lymphocytes were obtained as the mononuclear cell fraction of peripheral blood from healthy male donors by standard Ficoll/hypaque density gradient centrifugation and transferred to culture medium (1×10^6 cells/ml in RPMI 1640 with 10% fetal calf serum, penicillin 100 U/ml and streptomycin 0.1 mg/ml). Cells were then stimulated with PHA (phytohemagglutinin M, Difco) at a concentration of 0.1 mg/ml and grown in the dark at 37°C in a 5% CO_2 humidified atmosphere. HL-60 and K-562 human leukemia cell lines were obtained from American Type Culture Collection (ATCC). Another HL-60 cell line (a DNA hypotetraploid subclone) was kindly supplied by Dr. B. T. Mortensen, Dept. of Hematology L, Rigshospitalet, Copenhagen. HL-60 and K-562 cultures were passaged twice a week at a seeding density of 10^5 cells/ml in medium as above, to maintain exponential growth.

BrUrd-labelling. One hour before harvest, BrUrd (Aldrich 03824HP) was added to part of the cultures to a final concentration of 1 mM. Cells harvested for BrUrd/DNA analysis (method 1) were washed once with cold PBS (Ca- and Mg-free Dulbecco's phosphate buffered saline, pH 7.2), that means diluted with PBS and centrifuged at 200 G for 10 min, followed by partial aspiration of supernatant, leaving approx. 150–200 μl fluid. The washed cells were lysed and fixed according to the method of Landberg & Roos (4): Samples of approximately 1.5×10^6 washed cells were prepared into nuclei by addition of 0.5 ml 5°C lysing buffer (PBS with 0.5% Triton X-100, 1% bovine serum albumin (BSA) and 0.2 μg/ml EDTA), immediately whirlimixed, and slowly agitated for 15 min on icebath. Samples were then fixed with 3 ml −10°C methanol, mixed by turning the tubes upside down, and slowly agitated for 15 min on icebath. Fixed samples were either stained immediately or stored at −20°C for later staining. Cells harvested for BrUrd/surface antigen analysis (method 2) were washed once in cold PBS with 0.1% BSA.

Staining of BrUrd/DNA (method 1). Samples of approx. 3×10^5 fixed nuclei of BrUrd labelled and unlabelled lymphocytes or HL-60 cells were stained at 0–5°C, as previously described (2): After a wash in cold PBS, 50 μl monoclonal anti-BrdUrd antibody (ABDM; Partec, Münster, Germany), diluted 1 : 10 in PBS with 0.1% v/v Nonidet P40 (BDH Chemicals), was added. The samples were agitated for further 60 min and then washed. 50 μl polyclonal FITC-conjugated rabbit anti-mouse antibody (DAKO F-313, $F[ab']_2$ fragment), diluted 10 × in PBS containing 5% normal rabbit serum (DAKO X-902), was added, and the samples were agitated for 60 min and washed. Finally, 50 μl of propidium iodide 50 μg/ml (Sigma P-4170) in PBS was added, and the samples agitated for at least 15 min before flow cytometry. With regard to RNA specificity controls (see (2)).

Staining of BrUrd/surface antigen (method 2). To samples of approx. 2×10^5 cells was added 100 μl R-phycoerythrin-conjugated monoclonal antibody, diluted in PBS with 0.1% BSA, for 30 min at room temperature in the dark. For staining of HL-60 and/or K-562 cells, we used an anti-CD13 antibody (Coulter Clone, MY7-RD1, IgG_1, diluted 30 ×) and as isotype control an irrelevant mouse antibody (DAKO, X-928, IgG_1, diluted 1 : 30). Excessive amounts of antibody were removed by washing once in cold PBS. For fixation and permeabilization, similar to the method of Carayon & Bord (5), 1 ml of PBS with 1% paraformaldehyde and 0.05% Nonidet P40 was added at room temperature, the cells were mixed by turning the tubes upside down, and the samples were slowly agitated for 15 min in the dark. Fixed samples could be kept in the refrigerator for several days until further staining. Then paraformaldehyde was removed by washing in cold PBS with 1% glycine. To verify the RNA specificity of the BrUrd signal, some samples were treated at this point with 0.5 ml RNase (Sigma R-4875, 1 mg/ml in PBS) for 30 min at 37°C. All samples had a second wash in cold PBS, and then received 100 μl of FITC-conjugated anti-BrdUrd antibody (Becton Dickinson 7583, diluted 25 × in cold PBS with 0.1% BSA and 0.5% Nonidet P40). After incubation in the dark at room temperature for 45 min, the samples were washed in PBS with 0.1% BSA. Washed samples were resuspended with 200 μl PBS containing 0.1% BSA and 0.1% Nonidet P40.

Flow cytometry. Samples were analysed with a Becton Dickinson FACS IV cell sorter, upgraded with a Consort 30 data system for list mode acquisition of forward and orthogonal light scatter and two colours of fluorescence, and using argon laser excitation at 488 nm, 400 mW. For BrUrd/DNA analysis, log FITC fluorescence was collected at 510–540 nm and linear propidium iodide fluorescence at > 620 nm, and counting of 10^4 nuclei was triggered by the PI fluorescence. For BrUrd/surface marker analysis, log FITC fluorescence was collected at 510–540 nm and log phycoerythrin fluorescence at 565–580 nm, and counting of 10^4 cells was triggered by the forward scatter signal. Sample flow rate was approx. 0.5 μl/sec, and a 70 μm nozzle was used. Sheath fluid was destilled water. The instrument was calibrated using propidium iodide stained, unfixed chicken erythrocyte nuclei. Electronic compensation of FITC/phycoerythrin emission overlap was adjusted with Calibrite beads (Becton Dickinson). Cell debris was eliminated from the dual fluorescence data by initial gating in forward and orthogonal side scatter.

Results and Discussion

In exponentially growing cultures of HL-60 cells pulse-labelled with 1 mM BrUrd for 1 h, a spontaneous RNA synthesis could be demonstrated in almost all cells (> 95%), according to flow cytometric analysis of nuclear BrUrd (method 1, Fig. 1). The peak-to-peak ratio between

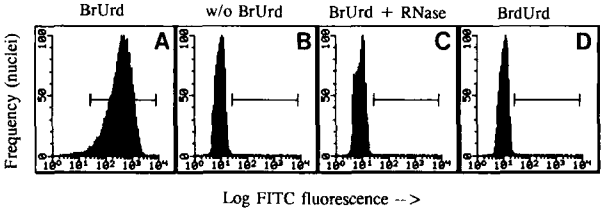

Fig. 1. Flow cytometric measurement of RNA synthesis in HL-60 cells, based on pulse-labelling with bromouridine (BrUrd). Incorporated BrUrd was immunochemically measured as the log FITC-fluorescence of fixed nuclei stained with cross-reacting anti-BrdUrd antibody (Partec) (method 1). Markers indicate histogram regions of BrUrd positive nuclei. A) Cells were incubated with 1 mM BrUrd for 1 h (98% positive nuclei). B) Cells were incubated without BrUrd (0.3% positive nuclei). C) Cells were incubated with BrUrd, but fixed nuclei were treated with RNase before staining with anti-BrdUrd antibody (0.3% positive nuclei); D) Cells were labelled with 10 μM BrdUrd instead of BrUrd (0.4% positive nuclei).

Table

*Bromouridine-labelling of PHA-stimulated peripheral blood mononuclear cells**

PHA (h)	Individual No. 1	Individual No. 2	Pooled data
0	3.2 ± 2.9	0.4 ± 0.8	1.7 ± 2.6
1	2.4 ± 0.4	2.1 ± 3.2	2.2 ± 2.1
24	11.8 ± 6.0	11.7 ± 8.6	11.8 ± 6.5
48	30.8 ± 2.8	34.1 ± 14.9	33.3 ± 11.2
72	27.5 ± 9.3	25.5 ± 14.5	26.1 ± 12.0

* Percentage of BrUrd-positive nuclei, using thresholds as indicated in Fig. 2. Mean value ± SD from 2–4 culture experiments.

the FITC fluorescence intensities of BrUrd labelled and unlabelled control samples was in the order of 10–50. It was indicated that the FITC signal was specific for RNA, by the fact that treatment with RNase before staining reduced the signal to the level of BrUrd unlabelled nuclei. Also, deliberately BrdUrd labelled nuclei stained negatively. Similar results have been obtained using the K-562 erythroleukemia cell line, human breast cancer and bladder cancer cell lines, and mouse fibroblast cell lines.

Dual parameter analysis of FITC (BrUrd) and propidium iodide (DNA) fluorescence in samples of BrUrd pulse-labelled, unstimulated human blood lymphocytes revealed a small subpopulation of BrUrd positive nuclei, indicating spontaneous RNA synthesis (method 1, Table, Fig. 2). The samples harvested after 24 h of stimulation with PHA showed increased BrUrd incorporation, with respect to the fraction of positive nuclei as well as FITC fluorescence intensity, thus indicating an early response in RNA synthesis to stimulation by PHA. Stimulation for 48 h elicits a maximum response in RNA synthesis along with the entrance of approximately half of the cells into cell cycle.

Using a new staining method, cellular BrUrd incorporation could be related to a phenotypic subpopulation (method 2, Fig. 3). Cells were stained with a phycoerythrin-conjugated antibody against the specific cell surface molecule, in this example CD13 on HL-60 cells, and then fixed and stained with FITC-conjugated cross-reacting anti-BrdUrd antibody. The green and red fluorescence signals representing cellular BrUrd and CD13 respectively were bright and specifically measured. As with method 1, treatment with RNase before staining of BrUrd reduced the FITC-fluorescence to the level of BrUrd unlabelled cells. The CD13 fluorescence was well separated from the background fluorescence of phycoerythrin-conjugated isotype control antibody. As a model of a heterogeneous cell system, we stained a mixture of BrUrd labelled and unlabelled cells from the myeloid cell line HL-60 (high CD13 expression) and the erythroid cell line K-562 (low CD13 expression). In the analysis of the mixture, clusters representing all four combinations could be identified (Fig. 4). The small increase in the background of FITC and phycoerythrin fluorescence in the cell mixture, compared to its components, is likely to arise from unintended BrUrd

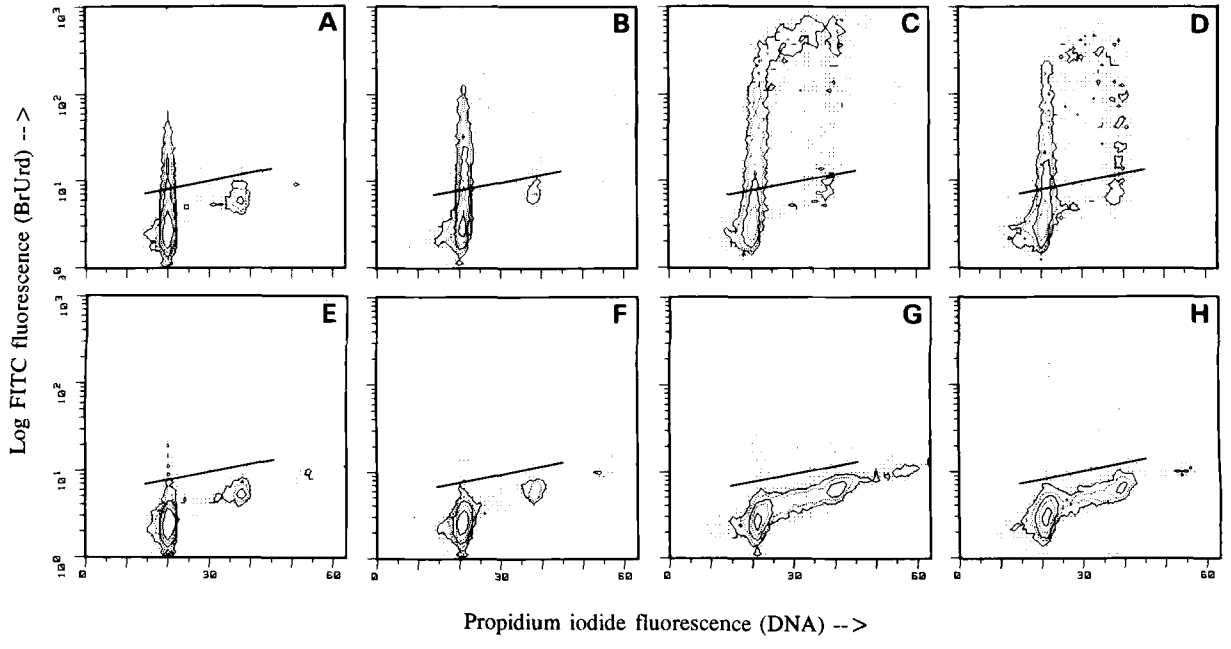

Fig. 2. According to method 1: combined analysis of BrUrd (log FITC fluorescence) and DNA content (propidium iodide fluorescence) of human blood lymphocytes. Cells were incubated with 1 mM BrUrd (A–D) or without (E–H) for 1 h before harvest. The lymphocytes were harvested before (A and E) and after stimulation with PHA for 1 h (B and F), 48 h (C and G), and 72 h (D and H). The discrimination of BrUrd positive nuclei is illustrated by the bars.

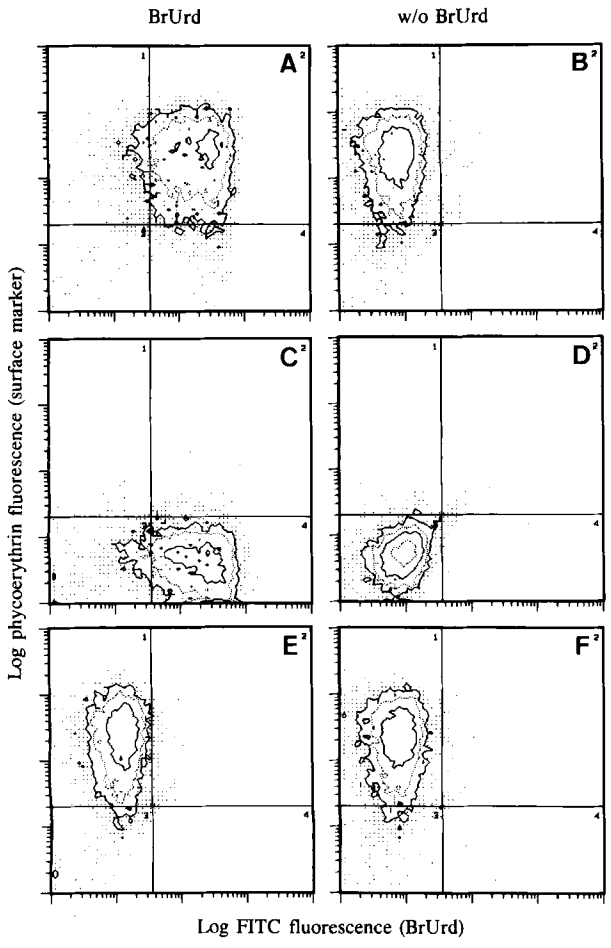

Fig. 3. According to method 2. Combined analysis of BrUrd incorporation (log FITC fluorescence, 4 decades) and CD13 (log phycoerythrin fluorescence, 4 decades) of HL-60 cells (DNA hypotetraploid subclone). Cells were incubated with 1 mM BrUrd (A, C and E) or without (B, D and F) for 1 h. Before fixation, cells were stained either with phycoerythrin conjugated CD13 antibody (A, B, E and F) or an isotype control antibody (C and D). All cell samples were stained with cross-reacting FITC-conjugated anti-BrdUrd antibody (Becton Dickinson). To test the RNA specificity of the FITC signal, cells were treated with RNase prior to staining with anti-BrdUrd antibody (E and F).

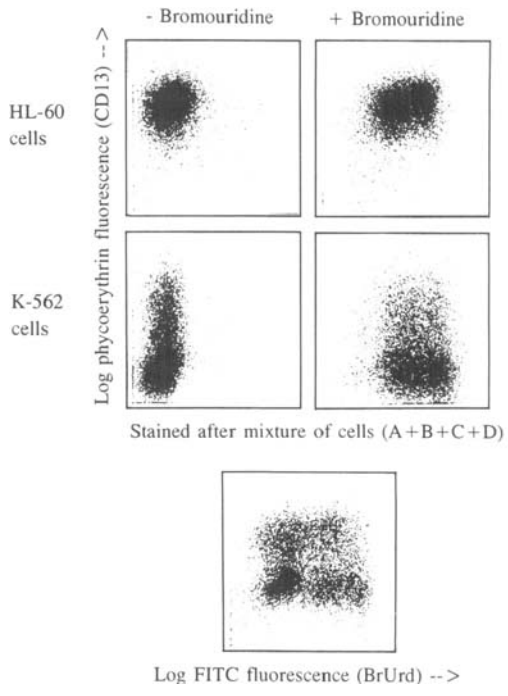

Fig. 4. According to method 2. Combined analysis of BrUrd incorporation (log FITC fluorescence, 3 decades) and CD13 (log phycoerythrin fluorescence, 3 decades) of HL-60 and/or K-562 cells. Cells were incubated with 1 mM BrUrd (B and D) or without (A and C) for 1 h. Before staining and fixation, cells from A, B, C and D were mixed (E). All cell samples were stained with phycoerythrin conjugated CD13 antibody and with a cross-reacting FITC-conjugated anti-BrdUrd antibody (Becton Dickinson).

labelling and possibly exchange of soluble CD13, going on during the short period the cells were mixed together and before washing and fixation were completed. This background could possibly have been avoided if the medium had been removed by washing the cell samples before their mixture. In preliminary experiments, phenotypic discrimination together with measurements of RNA synthesis were also obtained with the surface markers CD4 and CD25 on PHA stimulated lymphocytes (data not shown). After 2 days of PHA treatment we found BrUrd incorporation in both CD4 positive and CD4 negative cells, but all BrUrd incorporating cells were CD25 positive.

We conclude that RNA synthesis can be studied by flow cytometry, according to the incorporation and allocation of BrUrd to the nucleus and the entire cell respectively, and furthermore that the measurement of RNA synthesis can be correlated to cell cycle distribution and differentiation phenotype as well. Further studies are needed to evaluate the potential significance of this method in analysis of leukemia and lymphoma.

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