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FROM THE ABTEILUNG FÜR STRAHLENBIOLOGIE AM ZENTRUM FÜR STRAHLENMEDIZIN AND THE FACHKLINIK HORNHEIDE, UNIVERSITÄT MÜNSTER, D-4400 MÜNSTER, AND THE INSTITUT FÜR ZOOLOGIE, TECHNISCHE HOCHSCHULE DARMSTADT, D-6100 DARMSTADT, WEST GERMANY.

CYCLING S-PHASE CELLS IN ANIMAL AND SPONTANEOUS TUMOURS

I. Comparison of the BrdUrd and 3H-thymidine techniques and flow cytometry for the estimation of S-phase frequency

E.-M. LANGER, H. R. RÖTTGERS, M. G. SCHLIERMANN, E.-M. MEIER, H.G. MILTENBURGER, J. SCHUMANN and W. GÖHDE

Abstract

Evaluation of the proliferative activities of cell populations has mainly been restricted to the use of autoradiography and flow cytometric measurements. The introduction of a new BrdUrd specific antibody makes it possible to determine exactly the DNA synthesizing cells. The BrdUrd technique is safe with respect to handling and the results are obtained within five hours. The suitability of the BrdUrd labelling procedure has been studied in different cell lines and compared with 3H-thymidine autoradiography and flow cytometry.

In experimental and clinical oncology the proliferative activity of cells is of great interest. A new BrdUrd specific antibody (7) enables us to measure the frequencies of DNA synthesizing cells in cell cultures, experimental tumour cell lines, and spontaneous human tumours. We have started to apply this technique to human tumours, and it seems possible to estimate S-phase frequencies before and during therapy even in the daily clinical routine.

In contrast to 3H-thymidine (3) the BrdUrd technique allows early estimation (within 4–5 hours after biopsy) of the proliferative activity of an individual tumour, which can be taken into account for therapy planning. In the present investigation, estimations of S-phase frequencies in various cell lines by flow cytometry, 3H-thymidine microautoradiography, and the BrdUrd technique are compared. Results in irradiated experimental and spontaneous human tumours will be reported in subsequent publications (4, 6).

Material and Methods

Cells and growth conditions. Chinese hamster ovary (CHO) cells were grown in 5 ml Petri dishes in MEM with 10% fetal calf serum, to a density of 1×10^5 cells/ml. At intervals of 24 h samples were taken from the culture.

Bone marrow cells were washed out from mice femora.

Ehrlich-ascites tumour (EAT) cells (2×10^6 cells in the logarithmic phase of growth) were inoculated into female NMRI mice. At intervals of two days the mice were killed and the EAT cells were harvested.

Flow cytometry. Cells were fixed in 70% ethanol immediately after harvesting and stained according to GÖHDE et coll. (1). The DNA per cell frequency distributions (DNA histograms) were measured using an instrument from Partec (Switzerland). The frequencies of cells in the different cell cycle phases were estimated by a method proposed by MEIER (5).

3H-thymidine labelling of DNA. Cell suspensions were incubated in MEM with 10% fetal calf serum

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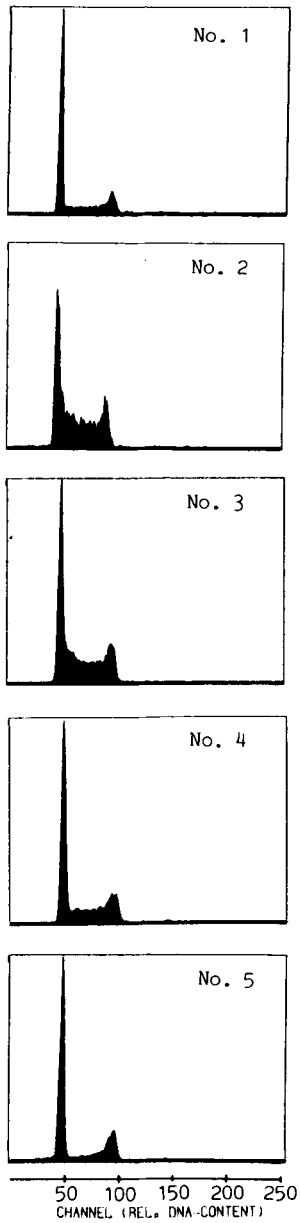


Fig. 1. DNA histograms of Chinese hamster ovary cells and S-phase percentages obtained by BrdUrd and 3H-thymidine labelling and flow cytometry analysis representing different stages of a cell culture after a growth time of 0–96 h.

Histogram No.	Growth time (h)	S-phase percentages obtained by: BrdUrd	3H-TDN	FCM
1	0	30.4	24.8	30.2
2	24	57.8	61.7	66.8
3	48	55.1	58.0	57.3
4	72	40.2	36.9	38.9
5	96	8.7	7.9	26.4

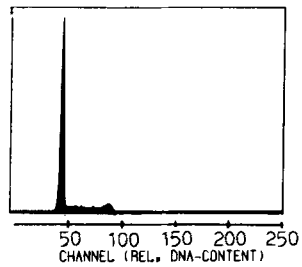


Fig. 2. DNA histogram of murine bone marrow cells and S-phase percentages obtained by BrdUrd labelling (21.6%), 3H-thymidine labelling (22.6%) and flow cytometry analysis (21.2%).

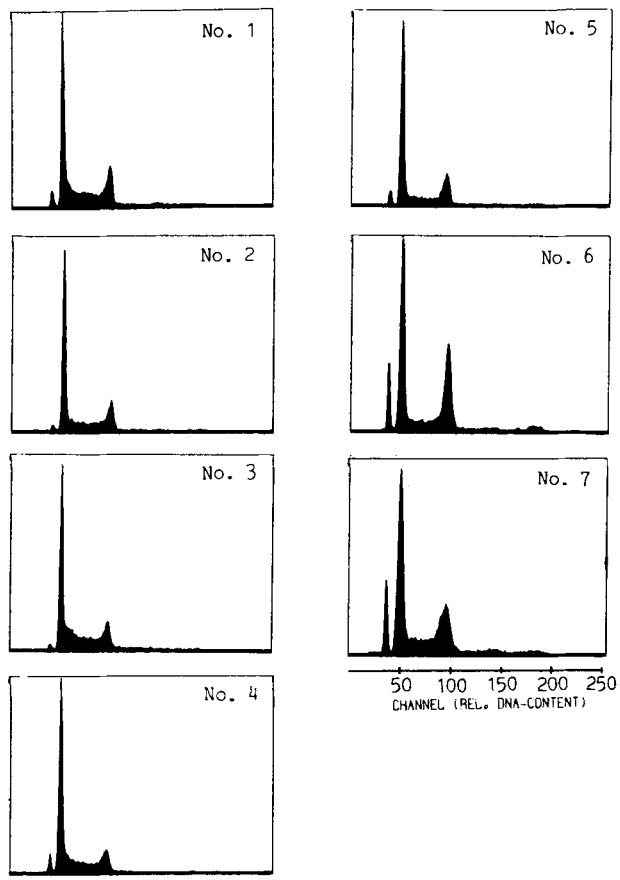
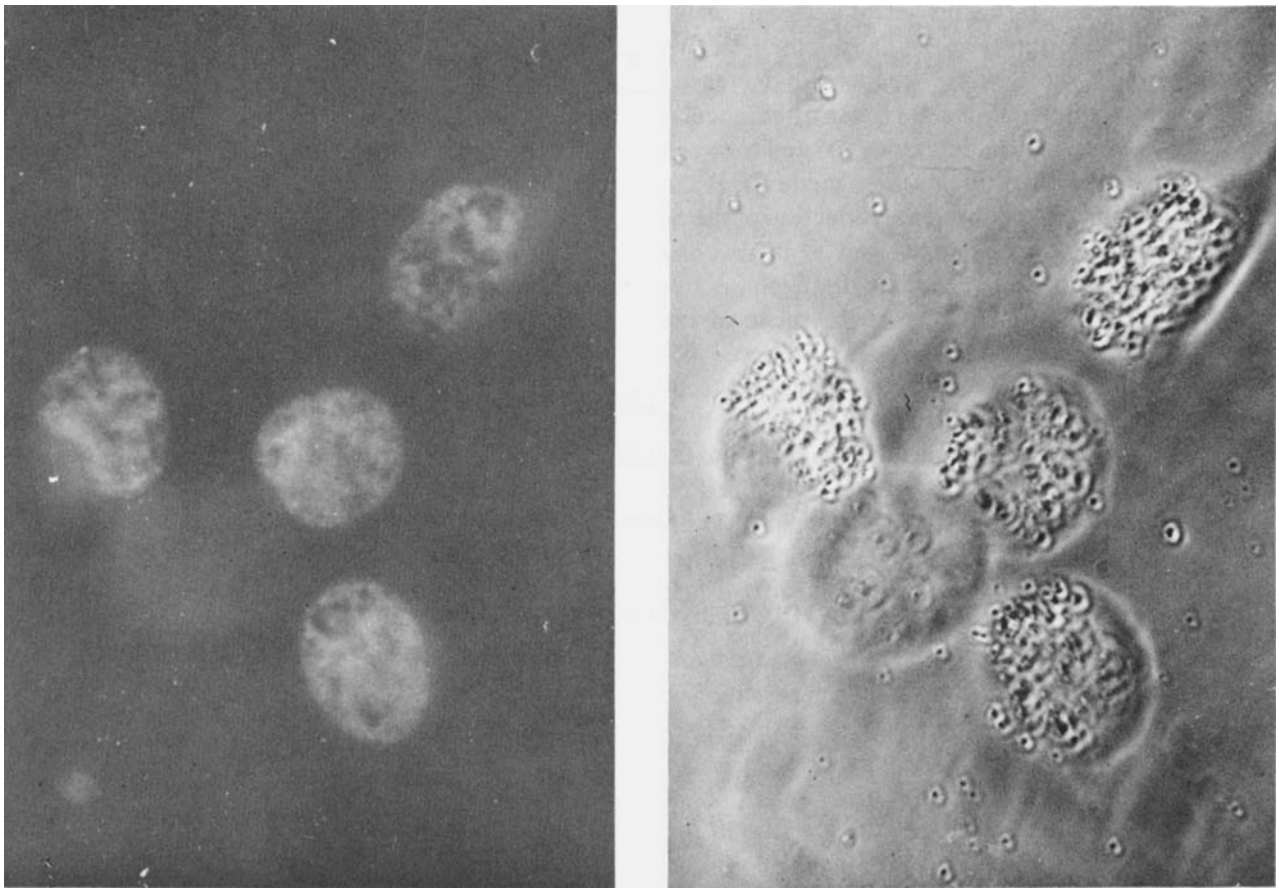


Fig. 3. DNA histograms of Ehrlich-ascites tumour cells and S-phase percentages obtained by BrdUrd and 3H-thymidine labelling and by flow cytometry analysis for tumour growth times of from 2 to 14 days.

Histogram No.	Growth time (h)	S-phase percentages obtained by: BrdUrd	3H-TDN	FCM
1	2	54.5	51.0	53.5
2	4	36.9	35.3	39.8
3	6	34.6	34.9	47.8
4	8	25.4	30.5	39.1
5	10	21.1	23.4	26.0
6	12	13.2	12.8	25.3
7	14	10.5	10.4	28.0



a b

Fig. 4. Double cell labelling of CHO cells with BrdUrd and 3H-thymidine. The S-phase CHO cells stimulated by ultraviolet light only produce a green fluorescence because of the BrdUrd-FITC labelling (a). Silver grains caused by 3H-thymidine labelling become visible in phase contrast light (b). A fifth cell, not labelled, shows neither BrdUrd-FITC fluorescence nor silver grains.

containing tritiated thymidine (37 kBq/ml, spec. activity 925 GBq/mmol) for 15 min at 37°C and fixed in 70% ethanol. The fixed cells were mounted on slides and covered with photographic emulsion (Ilford, K 5). After exposure for two days at 4°C in the dark, the slides were developed (Kodak, D 9). For each analysis 1 000 cells were counted, and the labelling index was calculated.

BrdUrd monoclonal antibody labelling. The growing cells were transferred to MEM supplemented by 10% fetal calf serum containing equimolar amounts of BrdUrd and deoxycytidine (final concentration 15 μm). After an incubation time of 30 min the cells were fixed in 70% ethanol. For antibody labelling the cells were removed from the ethanol and resuspended in 1.5 n HCl for 20 min at room temperature, washed twice in phosphate-buffered saline (PBS) and incubated in PBS containing 0.5% Twen 20, 0.5% bovine serum albumin (BSA) and a 1:500 dilution of monoclonal anti-BrdUrd (Partec, Switzerland) for one hour at 37°C. After this treatment the cells were washed once in PBS containing 0.5% Tween 20, 0.5% BSA, 1% neutral goat serum and 1% fluorescein labelled goat anti-mouse IgG. After 10 min the S-phase cells show a bright fluorescence when stimulated. Five hundred cells/slide were counted and the labelling index was calculated. For detailed description of the BrdUrd labelling technique, see SCHLIERMANN (7).

CHO samples were labelled both with BrdUrd and tritiated thymidine, incubated with the antibodies, and covered with photographic emulsion.

Results

Simultaneous microautoradiography, flow cytometry and BrdUrd labelling were applied for the determination of the S-phase frequencies of all three cell lines.

The CHO cells in the logarithmic phase of growth

showed an S-phase frequency of 30 per cent, whatever method was used (Fig. 1). During the first two days of growth the S-phase frequencies increased because of 'stimulation' of cell growth after inoculation. Up to this time no difference existed between the estimates obtained by the three methods. If the medium remained unchanged the reduction of the S-phase frequencies began at the end of the second day. Four days after inoculation the S-phase frequency reached its minimum. At the onset of the stationary phase a discrepancy was observed between the estimates obtained by the different techniques. The 3H-thymidine and the BrdUrd labelling methods showed a continuous decrease in the S-phase frequency whereas the estimates obtained by flow cytometry remained at a much higher level. After 96 h, flow cytometry gave a four times higher S-phase frequency estimate than the BrdUrd and 3H-thymidine methods.

When S-phase frequencies were analysed in murine bone marrow (a cell system representing a steady state situation) the values obtained by different techniques were almost identical (Fig. 2).

EAT cells grown for two days in female NMRI mice had an S-phase frequency as high as 50 per cent, because of 'stimulation' after inoculation (Fig. 3). Two and four days later a decrease in the S-phase frequency was registered by all three methods. With increasing time after inoculation an increasing discrepancy in the S-phase frequency obtained by the different methods was observed. At the end of the survival time of the animals, fourteen days after inoculation, flow cytometry gave three times higher values than 3H-thymidine and BrdUrd labelling methods.

Fig. 4 shows CHO cells after double labelling with 3H-thymidine and BrdUrd. The cells containing silver grains also show the antibody FITC-fluorescence.

Discussion

The DNA content per cell measured by flow cytometry cannot discriminate between actually synthsizing cells and those having an S-phase equivalent DNA content but remaining in proliferative abeyance. 3H-thymidine and BrdUrd labelling give information on the frequency of cycling S-phase cells and thereby on the proliferative activity and progress in the cell populations.

Up to now, the evaluation of DNA synthesizing cells has mainly been based on autoradiography. Although this technique delivers satisfactory results it suffers from the disadvantage of technical complications connected with the use of radioactive substances. Furthermore, its practical use is limited by the exposure time (minimum 2 days).

The BrdUrd antibody technique (2, 7) delivers labelling indices which exactly agree with those obtained by autoradiography after 3H-thymidine labelling.

Double cell labelling with tritiated thymidine and BrdUrd, furthermore, confirms that the BrdUrd antibody technique is a method equivalent to autoradiography. The BrdUrd labelling technique offers the advantage of simple and safe handling and a comparatively short processing time; the results are available within 4 to 5 hours. The BrdUrd antibody labelling technique may develop into a powerful and rapid method for investigations on cell proliferation for research and clinical application as well.

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