

Comparison of proliferating cell nuclear antigen (PCNA) staining and BrdUrd-labelling index under different proliferative conditions *in vitro* by flow cytometry

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Abstract. PC10 is a monoclonal antibody against proliferating cell nuclear antigen (PCNA). The staining pattern in immunocytochemistry depends on fixation and detergent extraction treatment. The aim of this study was to validate the flow cytometric PCNA assay against Bromodeoxyuridine-labelling index (BrdUrd-LI) under different proliferative conditions *in vitro*. Expression of PCNA in methanol fixed cells with, and without, prior detergent extraction with EDTA/Triton was compared to BrdUrd-labelling index in NIH-3T3 fibroblasts and human Caski tumour cells in exponential phase and under confluent conditions. Serum stimulation and serum starvation conditions were studied. The results for BrdUrd-LI and PCNA-index after extraction showed good correlation for 3T3 fibroblasts and for Caski cells, with some differences for serum withdrawn Caski cells. There was no correlation between the number of cells that were positive for PCNA without extraction and BrdUrd-LI. Spheroid cells with G₁-DNA-content showed an almost synchronous recruitment and progression through the cell cycle after trypsinisation and replating. Tightly bound PCNA paralleled this synchronicity whereas total PCNA did not change significantly. The results demonstrate that immunochemical detection of non-extractable PCNA-index gives similar results as compared with BrdUrd-labelling index under different proliferative conditions *in vitro* for different monolayer cell lines, whereas without extraction PCNA does not correlate with BrdUrd-LI in these fast growing cell lines due to its long half-life. PCNA expression parallels the progression through the cell cycle in V79 spheroids, a primitive model of tumour growth.

Several markers (S phase fraction, Ki-67, different proto-oncogenes) are presently being evaluated as tools for determining the proliferative capacity of tumours (Yu, Woods & Levison 1992).

S phase fraction can be assessed by [³H]-thymidine labelling or by DNA flow cytometry. However, DNA histograms are difficult to evaluate for tumours with cell populations of different ploidy. Dolbeare *et al.* (1983) published a non-radioactive method to determine S phase fraction using Bromodeoxyuridine (BrdUrd). Begg *et al.* (1985) described a method for calculation of

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potential doubling time (T_{pot}) from a single sample. In an ongoing phase III study, Begg *et al.* (1992) showed that the BrdUrd-assay might help to choose between conventional or accelerated hyperfractionated radiotherapy in head and neck tumours. Potential doubling time and S phase labelling index (LI) provide strongly related information in this study. Though the BrdUrd-LI currently is the gold-standard for S phase detection, a cell inherent S phase marker would be desirable.

Ki-67 is a monoclonal antibody that apparently determines growth fraction and is of good prognostic value in lymphoma and leukaemia. Its value in solid tumours has not finally been determined (Brown & Gatter 1990). The cDNA-sequence of the gene that codes for the Ki-67 antigen has been completely sequenced and the definition of the antigen in molecular terms has recently been reviewed (Schlüter *et al.* 1993, Duchrow, Gerdes & Schlüter 1994).

PCNA was initially described as a corresponding antigen to an autoantibody in sera of patients with Lupus Erythematosus (Miyachi, Fritzler & Tan 1978) and is identical with the nuclear protein 'Cyclin' (Mathews *et al.* 1984). It is a non-histone protein with a molecular weight of 36 kD which functions as an auxiliary protein of DNA-polymerase delta (Prelich *et al.* 1987). PCNA is expressed beginning in late G_1 , with a two to three-fold increase in S and a decrease at the S/ G_2 transition and during $G_2 + M$. Two intranuclear populations exist (Bravo & McDonald-Bravo 1984, Galand & Degraef 1989, Humbert *et al.* 1992). A tightly bound nucleolar form is only detectable from late G_1 to $G_2 + M$. A second diffuse nucleoplasmic form, which can be extracted by an EDTA/Triton-containing lysing buffer prior to fixation with methanol (Landberg & Roos 1991), is detectable during the whole cell cycle and is probably PCNA that has left the replisomes, and is acting as a reserve pool. It is degraded with a half-life of 20 h (Bravo & McDonald-Bravo 1987).

PCNA is involved in DNA-repair and behaves like synthesis-associated PCNA in methanol or paraformaldehyde-Triton treatment (Shivji, Kenny & Wood 1992, Stivala *et al.* 1993, Toschi & Bravo 1988, Vriza *et al.* 1992). After serum stimulation of quiescent cells, PCNA gene expression was detected by Northern blotting after 10 h, with a maximum after 16–18 h (Bravo & McDonald-Bravo 1984). PCNA is expressed after *c-myc* and *c-fos* following serum stimulation (Vriza *et al.* 1992) and is necessary for the G_1/S transition that can be inhibited by antisense nucleotides (Jakulski *et al.* 1988). It seems to be one of the final events that lead to DNA replication (Bravo & McDonald-Bravo 1984).

Results of immunohistochemical studies using PCNA on normal and tumour tissue have been conflicting. Depending on the method of fixation and the antibody that was applied, either the S phase fraction or the proliferation fraction were detected (Sasaki, Kurose & Ishida 1993). Thus, studying PCNA *in vitro* under different proliferative conditions may help to interpret *in vivo* data.

For exponentially growing cell lines *in vitro*, a good correlation between BrdUrd-LI and the tightly bound PCNA-index was shown (Landberg & Roos 1991, Sasaki *et al.* 1993).

We report on flow-cytometric detection of proliferating cell nuclear antigen (PCNA) in comparison with BrdUrd as an S phase marker using different rodent and human monolayer cell lines under different proliferative conditions *in vitro*, with and without detergent extraction prior to methanol fixation. Additionally, expression of PCNA in partially synchronized V79 spheroids was studied.

MATERIALS AND METHODS

Cell lines and endpoints

Caski human cervix carcinoma cells and NIH-3T3 mouse fibroblasts were grown as monolayer cultures in 20 ml culture flasks with RPMI 1640 medium, supplemented with 1% L-glutamine and

10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO₂. For serum starvation and stimulation experiments medium was first changed to RPMI 1640 containing 1% L-glutamin and a residual concentration of 1% FCS to prevent cell-detaching for both exponentially growing cells and cells that were confluent for 6 days. Cells were then trypsinized (EDTA 0.2%; 2 × 3 min at 37°C, Trypsin 0.2%; 5 min at 37°C, followed by blocking with 10% FCS-containing medium) and replated using medium supplemented with 10% FCS. Cells were trypsinized and harvested as described above, 24 h after serum withdrawal as well as 0, 4, 6, 11, 15 and 19 h after serum induction (exponentially growing 3T3 cells were more closely monitored after serum withdrawal (e.g. see Figure 4a)). They were then fixed, stained and analyzed as described below. V79 cells were grown as spheroids in spinner bottles with DMEM (1% L-glutamin, 10% FCS). After reaching a median diameter of approximately 300 μm, spheroids were disaggregated (Trypsin 0.2%, 1 ml; EDTA 0.2%, 1 ml, 10 min at 37°C) and replated as single cells. Aliquots of these cells were harvested 0, 2 (not shown), 4, 5, 6, 8, 10, 12, 14 and 24 h after plating.

BrdUrd-labelling

Cells (5×10^6) were incubated with BrdUrd (15 μmol/l) for 45 min. Immediately afterwards they were trypsinized. No BrdUrd labelling was performed on V79 spheroids.

Fixation and extraction

After trypsination cells were resuspended in 600 μl PBS and the suspension was then divided into three equal portions. For BrdUrd-detection, cells were fixed in 3 ml 70% ethanol at 4°C for 30 min. For detection of total PCNA cells were fixed in 3 ml of absolute methanol at -20°C for 10 min, then kept at 4°C for 30 min. For detection of DNA-bound PCNA, soluble PCNA was extracted by incubating the cells in 500 μl EDTA 0.2% containing Triton X-100 (2.5 μl/ml) and BSA (5 μl/ml) for 15 min on ice, according to a protocol published by Landberg & Roos (1991). Then the samples were fixed with methanol as described above.

Immunochemical staining

BrdUrd

For BrdUrd detection a modification of the method originally described by Dolbeare *et al.* (1983) was used. DNA denaturation was performed with 2N HCl in a waterbath at 37°C for 30 min. Between all following steps cells were washed in 3 ml PBS and taken up in a 24 G syringe several times.

The staining protocol is a standard two-step procedure. One millilitre of PBS containing BSA (10 μl/ml) and Triton X-100 (50 μl/ml) was used for further permeabilization and non-specific blocking for 15 min at room temperature. The primary antibody (anti BrdUrd, B44, Becton Dickinson, San Jose, CA, USA) and, after washing, the FITC conjugated goat-anti-mouse IgG (Becton Dickinson) as a secondary antibody were used, each at a dilution of 1:10 in 100 μl PBS containing BSA (2 μl/ml) for 30 min at room temperature. DNA-staining was achieved with 1.4 ml propidium iodide (20 μg/ml) together with RNAase (30 μg/ml) to remove double-strand RNA (15 min in the dark, room temperature). Samples were then stored at 4°C until analysis. For negative controls an irrelevant isotypic antibody was used as the primary antibody, or no primary antibody at all was used, for both extracted and methanol fixed nuclei.

PCNA

No DNA denaturation was performed after fixation. Monoclonal anti-PCNA (PC10, Dianova, Hamburg, Germany) was used as primary antibody at a dilution of 1:10. Otherwise, processing was performed as described above.

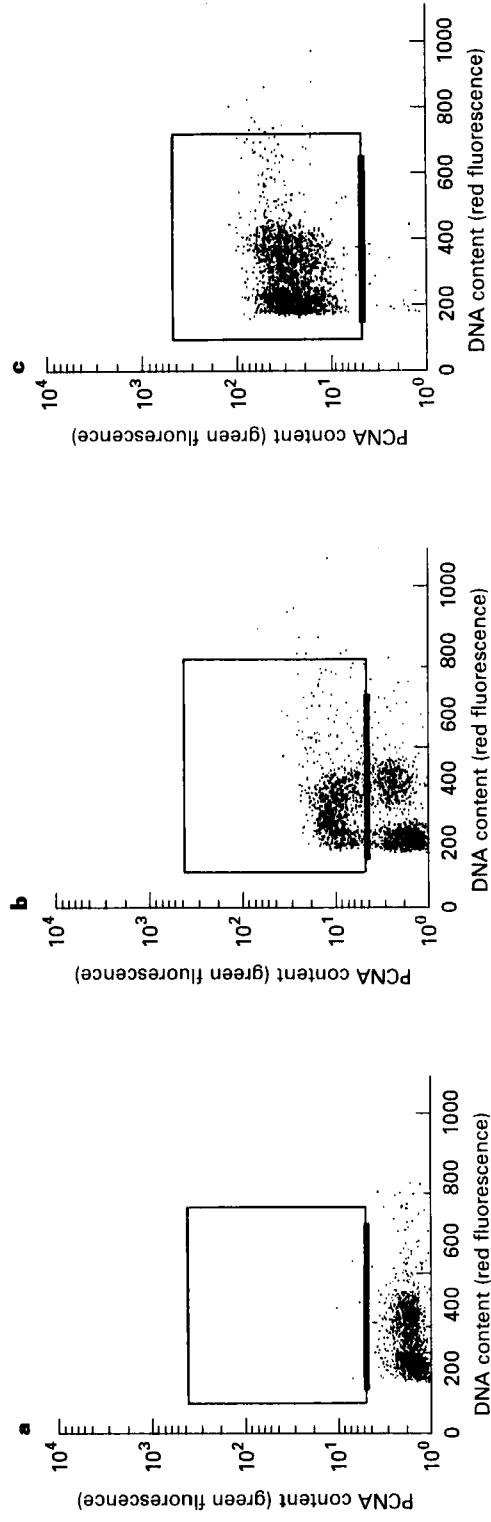


Figure 1. DNA/anti-PCNA plots with set gates for exponentially growing 3T3 fibroblasts: **a** negative controls (irrelevant primary antibody) **b** methanol fixation after extraction with lytic buffer, **c** methanol fixation.

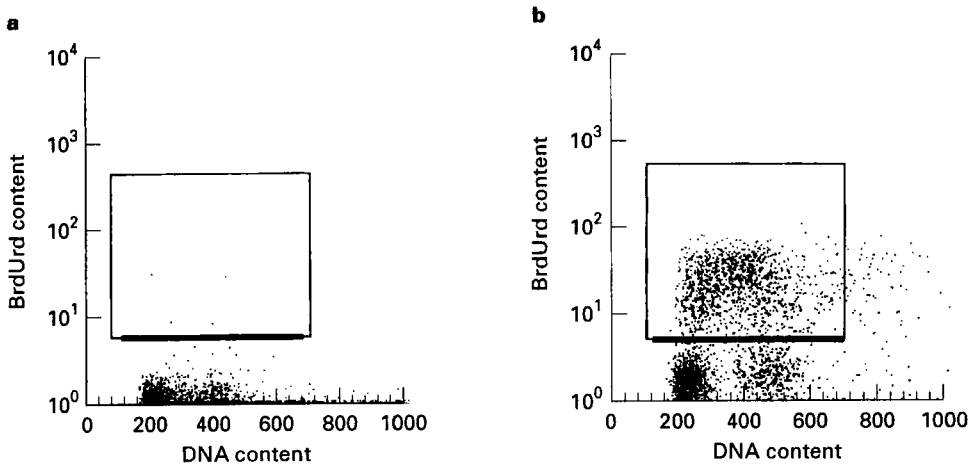


Figure 2. DNA/anti-BrdUrd plots with set gates for exponentially growing 3T3 fibroblasts: **a** negative controls (irrelevant primary antibody), **b** ethanol fixation.

Flow cytometry

Analysis was performed with a FACScan flow cytometer (Becton Dickinson). Amplification had to be set differently for BrdUrd and PCNA-detection because of the influence of DNA denaturation but was identical for the detection of total and tightly bound PCNA. Negative controls for both extracted and non-extracted cells were used to determine instrument settings for comparable measurements. At least 10 000 events were acquired for each data point. No live gating was performed since few doublets were measured. Remaining doublets were excluded by individual window settings for each measurement. Lower window levels for positive cells were set as shown in Figure 1 so that a maximum of 2% false positive cells were accepted for negative controls. Experiments were repeated. The results are plotted as the mean of at least two experiments. The difference between actual values and the mean was always less than 15%.

RESULTS

Fixation and extraction

Figure 1 shows biparametric plots of DNA content v. anti-PCNA-FITC fluorescence in negative controls (non-extracted, methanol fixed), detergent extracted and non-extracted exponentially growing 3T3 cells after fixation with methanol. Staining after extraction gave good S phase specificity and the cut-off between positive and negative cells could be easily placed. Figure 2 shows negative controls and anti-BrdUrd-staining for the same cells after ethanol fixation, which was superior to methanol fixation in this respect (not shown).

3T3 fibroblasts

The index for tightly bound PCNA after detergent extraction showed excellent correlation with BrdUrd-LI under all conditions examined for 3T3 mouse fibroblasts. After serum induction, tightly bound PCNA and BrdUrd-LI increased at the same time and this was paralleled by an increase of cells with an S phase DNA-content in the DNA profile (Figure 3). For both exponential and plateau-phase the number of cells expressing tightly bound PCNA paralleled the fraction of BrdUrd-positive cells after serum withdrawal and after serum induction within less

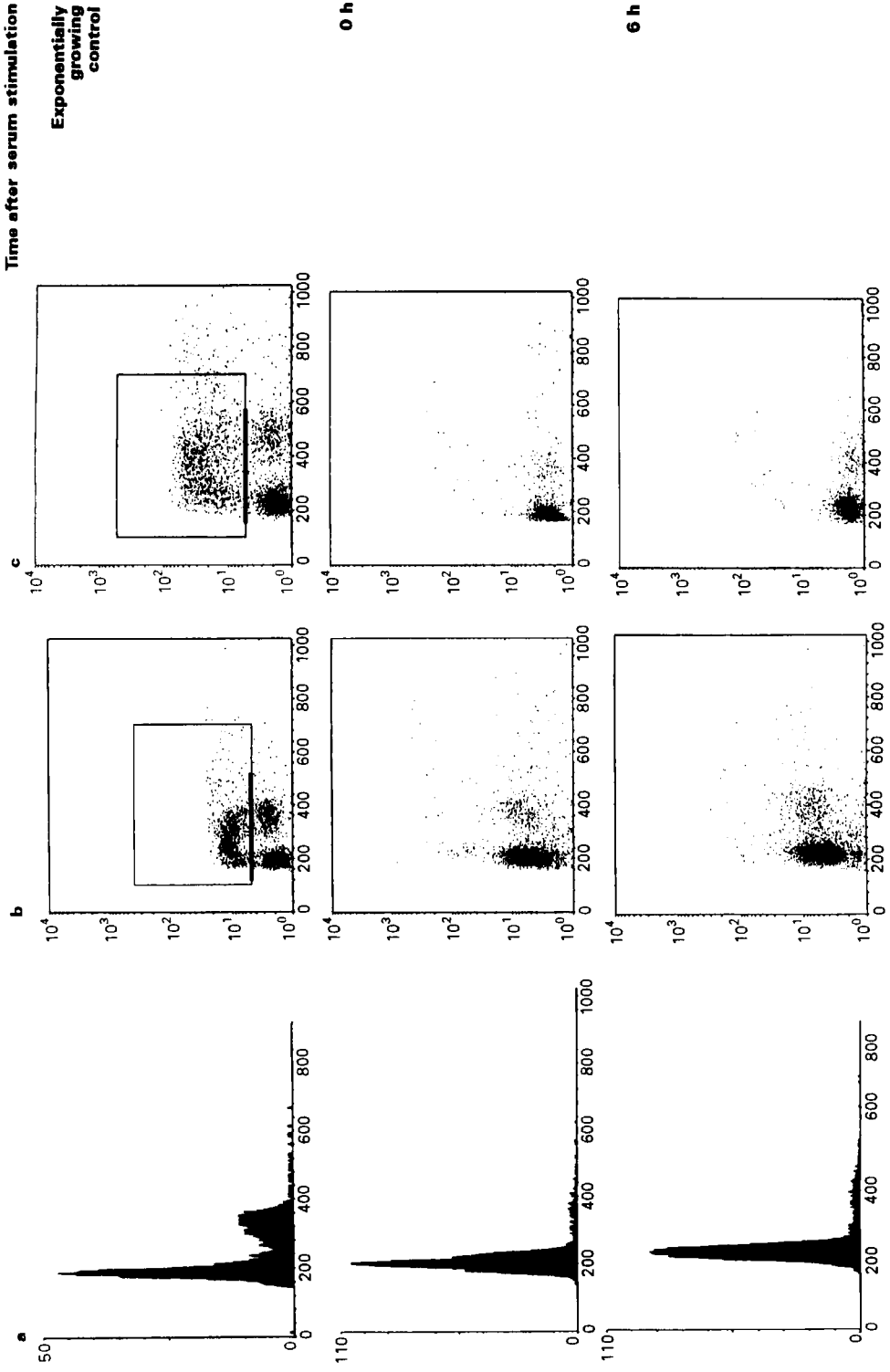


Figure 3. a DNA-histograms, b DNA/anti PCNA (tightly bound) plots and c biparametric DNA/anti-BrdUrd plots for exponentially growing 3T3 fibroblasts at different times after serum induction.

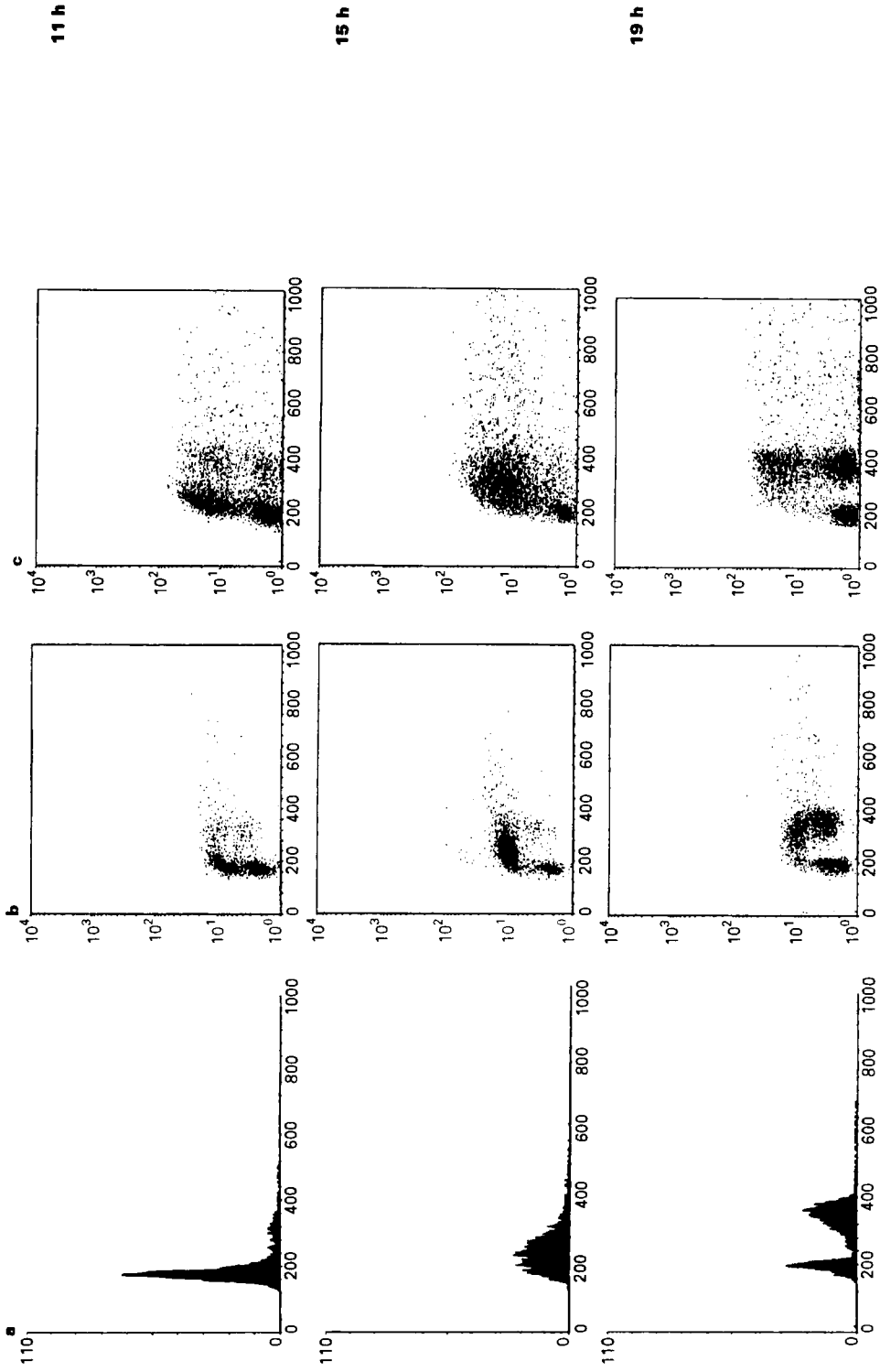


Figure 3.—continued.

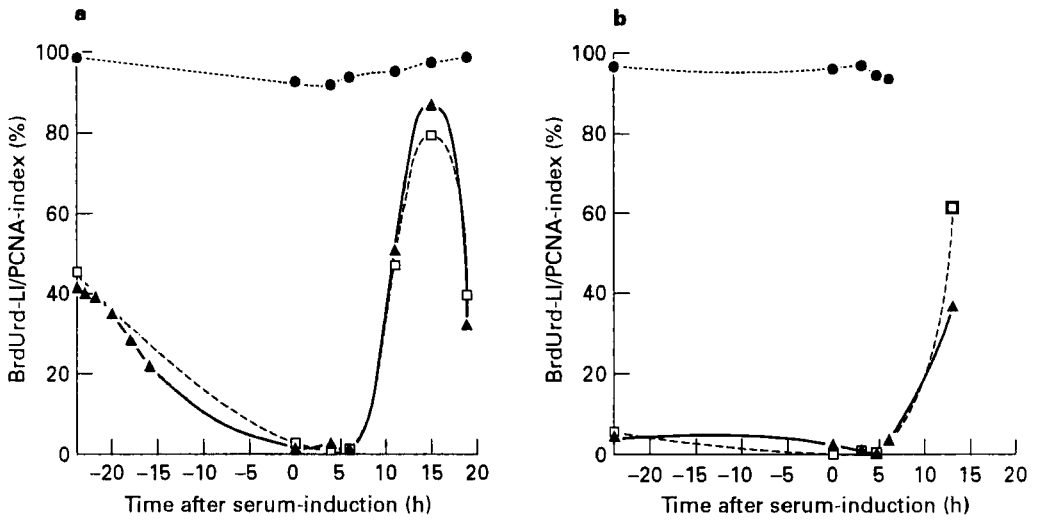


Figure 4. a BrdUrd-LI, total PCNA-index and tightly bound PCNA-index (all as %) after serum withdrawal (-24 h) and serum induction (0 h) for exponentially growing 3T3 fibroblasts. b BrdUrd-LI, total PCNA-index and tightly bound PCNA-index after serum withdrawal (-24 h) and serum induction (0 h) for 3T3 fibroblasts in plateau-phase (6 days of confluency). ▲, tightly bound PCNA index; □, BrdUrd-LI; ●, total PCNA index.

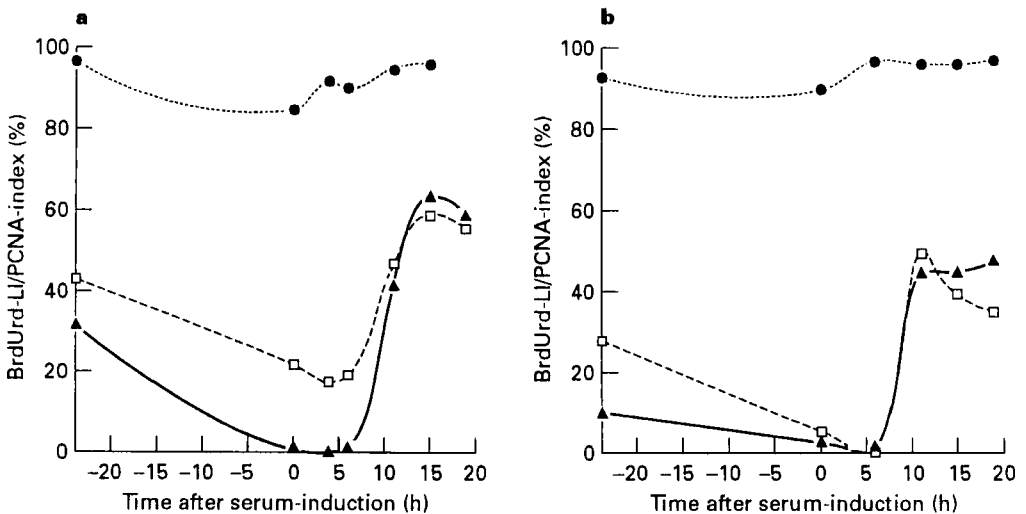


Figure 5. a BrdUrd-LI, total PCNA-index and tightly bound PCNA-index after serum withdrawal (-24 h) and serum induction (0 h) for exponentially growing human Caski cells. b BrdUrd-LI, total PCNA-index and tightly bound PCNA-index after serum withdrawal (-24 h) and serum induction (0 h) for human Caski cells in plateau-phase (6 days of confluency). ▲, tightly bound PCNA index; □, BrdUrd-LI; ●, total PCNA index.

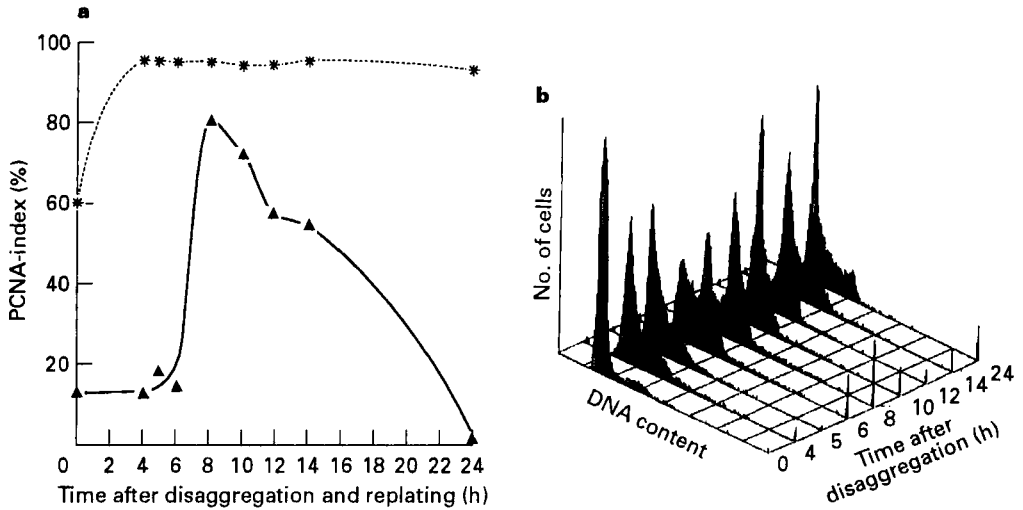


Figure 6. **a** PCNA-index at different times after disaggregation of V79 spheroids and replating as monolayers. \blacktriangle , tightly bound PCNA index; * total PCNA index. **b** DNA profiles at different times after disaggregation of V79-spheroids and replating as monolayers.

than 3% variation, except for the data at 10 h (Figure 4b), 15 and 19 h (Figure 4a) where a variation of 8 to 23% was seen (Figure 4a,b). Total PCNA was close to 100% positive cells in all experiments (Figure 4a,b).

Caski cells

For Caski cells there was also a correlation between BrdUrd-LI and tightly bound PCNA index. While, however, after serum induction the fractions of positive cells were similar (Figure 5a,b), after serum withdrawal of exponentially growing cells up to 20% BrdUrd-positive cells could be detected while almost no cells were positive for tightly bound PCNA (Figure 5a). Similarly, almost 30% BrdUrd-positive cells were observed with only 10% positivity for tightly bound PCNA for confluent cells prior to serum withdrawal. Yet, mean anti-BrdUrd fluorescence decreased parallel to the decrease in tightly bound PCNA (not shown).

V79 spheroids

The appearance of total PCNA and tightly bound PCNA paralleled the appearance of S phase cells in the DNA histogram after 4–6 h (Figure 6). Tightly bound PCNA declined as the S phase fraction declined when the partially synchronized cells progressed through the cell cycle. This was confirmed by determination of the maximum mitotic index (not shown). Total PCNA remained elevated.

DISCUSSION

In immunohistochemistry, using different anti-PCNA antibodies and different fixation protocols, either granular or diffuse nuclear staining was reported, resembling either the S phase fraction or values similar to the Ki-67-index (Bravo & McDonald-Bravo 1984, Bravo & McDonald-Bravo 1987, Landberg & Roos 1991, Shrestha *et al.* 1992, Sasaki *et al.* 1993). Its prognostic value has been discussed with some controversy (Sullivan & Mortimer 1993). Among others, one reason

may be that the above mentioned antibodies detect different epitopes which differ in stability to fixation (Roos *et al.* 1993). Studying PCNA *in vitro* under different proliferative conditions may help to interpret this *in vivo* data.

Using flow cytometry instead of immunohistochemistry has the advantage of overcoming topical heterogeneity of PCNA-expression in biopsies. However, this may also be a disadvantage since information about the spatial distribution of proliferation is lost. Moreover, identification of PCNA-positive *v.* negative cells is more reliable with flow cytometry than with immunohistochemistry, since placement of the cut-off between negative and positive cells is easier than in histochemistry because antibody dilution influences the intensity of PCNA staining (McCormick *et al.* 1993).

Since the BrdUrd assay is currently the standard method for detection of S phase cells in cell cultures or tumours, any possible alternative method has to be compared to this standard. The resulting good S phase specificity and DNA profiles in our study with the antibody of the clone PC10 confirm the results of Landberg and Wilson (Landberg & Roos 1991, Wilson *et al.* 1992).

As the functional association of PCNA with DNA-replication suggests, in our studies the tightly bound PCNA-index showed excellent correlation with BrdUrd-LI for 3T3 mouse fibroblasts, except for the data points late after serum induction. However, these data points are in the steep parts of the curves and, thus, little variation in timing between induction and measurement may cause large variations in PCNA-expression.

For human Caski cells there was also good correlation, although the results were not identical, since after serum starvation in exponentially growing cells up to 20% BrdUrd-positive cells could be detected, while almost no cells were positive for tightly-bound PCNA. For confluent cells prior to serum withdrawal there was also up to 25% BrdUrd-positive cells with only 10% positive cells for tightly bound PCNA. However, mean anti-BrdUrd-fluorescence decreased, indicating a reduced rate of DNA-synthesis.

The observed phenomenon suggests some remaining DNA-synthesis that is either independent of PCNA-expression under *in vitro* conditions of restrained growth (serum starvation and confluency) or which only requires small levels of PCNA whose fluorescence level is below our detection threshold (cut-off). This discrepancy is somewhat contradictory to the theory that malignant and benign cells that are adjacent to tumours show enhanced PCNA protein expression even when not cycling because of paracrine deregulation of protein transcription (Hall *et al.* 1990). However, studies which have suggested this mechanism have lacked a marker such as BrdUrd as a standard. Other authors did not find such a phenomenon (Bleiberg, Morret & Galand 1993). More recently, true growth stimulation (possibly paracrine) of cells was considered more likely (Scott *et al.* 1991). Yet, the possibility of deregulation should not totally be dismissed since there is evidence (Ottavio *et al.* 1990) of increased PCNA-expression at the mRNA-level in fully quiescent cells after destruction of intron 4 in the PCNA-gene. Another possible, but unlikely explanation, is that tightly bound PCNA might be more easily extractable in serum depleted Caski cells than in 3T3-cells. Increased PCNA protein expression without DNA-synthesis, on the other hand, was also observed (Sasaki *et al.* 1993) *in vitro*, indicating that there is not always an exact coordination between cell proliferation and PCNA-expression.

The results with spheroids as a model for a 3-D-cell system with oxygen, proliferation and nutrient-gradients with most cells supposed to be in the G₀ phase are in agreement with the results obtained from monolayer cells. The increase and decrease of tightly bound PCNA paralleled the appearance and disappearance of cells with S phase DNA-content after disaggregation.

The results of our flow cytometric studies in all three cell lines confirm previous data obtained by immunoblotting techniques concerning the expression of PCNA after serum induction (Bravo & McDonald-Bravo 1987). Being close to 100% for all the performed experiments, even after

24 h serum withdrawal, the total PCNA index does not reflect changes in proliferative status in these rapidly cycling cells, mainly due to the long half life of PCNA. However, the proliferative status of more slowly proliferating human tumours does not change as quickly as in cultured cells. Thus, the total PCNA content in human tumours might be correlated to tightly bound PCNA. The feasibility of flow cytometric PCNA detection in human tumours has been reported (Danova *et al.* 1988, Teague & El-Naggar 1994, Inada *et al.* 1993). Correlation of total PCNA-content to tightly bound PCNA is suggested by the good rank correlation between the PCNA-index without extraction and the BrdUrd-LI in three rat tumour sublines and the variations in non-extracted PCNA-index between 5 and 70% in human head and neck tumours (Lohr *et al.* 1993, Wenz *et al.* 1994). However, a reason for this might be that, after disaggregation, some soluble PCNA is extracted and, even without detergent lysis, preferably tightly bound PCNA is measured in solid tumours. However, both theories provide a rationale for using PCNA as an operational marker of proliferation in human tumours.

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