7. Fidler IJ. Origin and biology of cancer metastasis. *Cytometry* 1989, **10**, 673–680.
8. Daidone MG, Silvestrini R, Valentinis B, *et al.* Proliferative activity of primary breast cancer and of synchronous lymph node metastases evaluated by [^{3}H]-thymidine labelling index. *Cell Tissue Kinet* 1990, **23**, 401–408.
9. Meyer JS, Lee JY. Relationships of S-phase fraction of breast carcinoma in relapse to duration of remission, estrogen receptor content, therapeutic responsiveness, and duration of survival. *Cancer Res* 1980, **40**, 1890–1896.
10. Meyer JS, McDivitt RW. Reliability and stability of the thymidine labeling index of breast carcinoma. *Lab Invest* 1986, **54**, 160–164.
11. Allegra JC, Barlock A, Huff KK, Lippman ME. Changes in multiple or sequential estrogen receptor determinations in breast cancer. *Cancer* 1980, **45**, 792–794.
12. Holdaway IM, Bowditch JV. Variation in receptor status between primary and metastatic breast cancer. *Cancer* 1983, **52**, 479–485.
13. Hahnel R, Twaddle E. The relationship between estrogen receptors in primary and secondary breast carcinomas and in sequential primary breast carcinomas. *Breast Cancer Res Treat* 1985, **5**, 155–163.
14. Silvestrini R, Daidone MG, Valagussa P, Di Fronzo G, Mezzanotte G, Bonadonna G. Cell kinetics as a prognostic indicator in node-negative breast cancer. *Eur J Cancer Clin Oncol* 1989, **25**, 1165–1171.
15. Silvestrini R, Daidone MG, Valagussa P, *et al.* ^{3}H-Thymidine labeling index as a prognostic indicator in node-positive breast cancer. *J Clin Oncol* 1990, **8**, 1321–1326.
16. Silvestrini R, Daidone MG, Valagussa P, Salvadori B, Rovini D, Bonadonna G. Cell kinetics as a prognostic marker in locally advanced breast cancer. *Cancer Treat Rep* 1987, **71**, 375–379.
17. Tubiana M, Pejovic MH, Koscielny S, Chavaudra N, Malaise E. Growth rate, kinetics of tumor cell proliferation and long-term outcome in human breast cancer. *Int J Cancer* 1989, **44**, 17–22.
18. Courdi A, Hery M, Dahan E, *et al.* Factors affecting relapse in node-negative breast cancer. A multivariate analysis including the labeling index. *Eur J Cancer Clin Oncol* 1989, **25**, 351–356.
19. Meyer JS, Province M. Proliferative index of breast carcinoma by thymidine labeling: prognostic power independent of stage, estrogen and progesterone receptors. *Breast Cancer Res Treat* 1988, **12**, 191–199.
20. McGuire LW, Tandon AK, Allred DC, Chamness GC, Clark GM. How to use prognostic factors in axillary node-negative breast cancer patients. *J Natl Cancer Inst* 1990, **82**, 1006–1015.
21. EORTC Breast Cancer Cooperative Group. Standards for the assessment of estrogen receptors in human breast cancer. *Eur J Cancer* 1973, **9**, 379–381.
22. Scatchard G. The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949, **51**, 600–672.
23. Gentili C, Sanfilippo O, Silvestrini R. Cell proliferation and its relationship to clinical features and relapse in breast cancer. *Cancer* 1981, **48**, 974–979.
24. Kamby C, Rose C, Iversen H, Holm NV, Andersen KW, Thorpe SM. Pattern of metastases in human breast carcinoma in relation to estrogen receptor status. *Anticancer Res* 1986, **6**, 107–112.
25. Bertuzzi A, Daidone MG, Di Fronzo G, Silvestrini R. Relationship among estrogen receptors, proliferative activity and menopausal status in breast cancer. *Breast Cancer Res Treat* 1981, **1**, 253–262.
26. Meyer JS, Prey MU, Babcock DS. Breast carcinoma cell kinetics, morphology, stage, and host characteristics. *Lab Invest* 1986, **54**, 41–51.

Eur J Cancer, Vol. 28A, No. 12, pp. 2010–2017, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00
Pergamon Press Ltd

Flow Cytometric Characterisation of Proliferating Cell Nuclear Antigen using the Monoclonal Antibody PC10

George D. Wilson, Richard S. Camplejohn, Christine A. Martindale, Adrian Brock, David P. Lane and Diana M. Barnes

Anti-PCNA antibodies have aroused considerable interest recently as potential immunohistochemical markers of proliferation for use on clinical samples. PC10 is a monoclonal antibody which has been shown to recognise its epitope on formalin-fixed, paraffin-embedded, archival material. However, whilst PC10 gives the expected labelling pattern for growth fraction in normal tissues and some tumours, discrepant results have been obtained, for example, in carcinoma of the breast. By means of flow cytometry, we have attempted to characterise the different staining patterns that can be obtained with PC10. Intact fixed cells from proliferative mammalian cultures show 100% labelling, consistent with a growth fraction estimate. In contrast, detergent-extracted nuclei show S-phase specific staining. Nuclei extracted by treatment of fixed cells with pepsin show a different staining pattern again, with many G1 cells weakly stained and staining intensity increasing through S-phase into G2. The results demonstrate that multiparametric flow cytometry can define the cell populations which label with proliferation-related antibodies, such as PC10, under a variety of experimental conditions.
Eur J Cancer, Vol. 28A, No. 12, pp. 2010–2017, 1992.

INTRODUCTION

IDENTIFICATION AND characterisation of the cellular proteins involved in the control of cell proliferation is essential for understanding the mechanisms of growth regulation in both normal and neoplastic tissue. Knowledge of proliferation in human tumours can provide valuable information which may be used prognostically or diagnostically to select appropriate treatments or treatment scheduling, and biologically it may give insight into tumour progression and, in particular, metastatic potential.

Traditionally, proliferation in human tumours, has been assessed using *in vitro* incorporation of tritiated thymidine

([^{3}H]TdR) and autoradiography. This technique has been used in a wide variety of tumours giving information on labelling index (LI) and clinical outcome (see [1] for review). The introduction of flow cytometry (FCM) allowed the measurement of S-phase fraction, analogous to the LI but without the need to incorporate a DNA precursor [2]. More recently, the two techniques have been combined using bromodeoxyuridine (BrdUrd) incorporation, and simultaneous measurement of its uptake and total DNA content by FCM [3, 4]. This approach yields more information by generating both the LI and the duration of S-phase (T_S), thus making it possible to compute potential doubling time (T_{pot}). However, this technique requires the administration of a DNA precursor to the patient and a biopsy specimen to be removed several hours later.

There is a need for a non-invasive marker of proliferation that can also be used on conventionally fixed histological material. The monoclonal antibody Ki67 has been widely used as a measure of growth fraction [5], although its antigen remains to be identified. Recently, attention has been focused on other cell cycle-related proteins (see [6] for review) such as DNA polymerase α, ribonucleotide reductase and, in particular, proliferating cell nuclear antigen (PCNA).

Synthesis of PCNA occurs in late G1, presumably after the restriction point, and throughout S-phase [7], although the protein is detectable throughout the proliferating cell cycle due to its relatively long half life (20 h). Levels of PCNA are negligible in long-term quiescent cells [8]. PCNA has been identified as an auxiliary factor to DNA polymerase δ at the replication fork [9] and exists in at least two forms, one of which is tightly bound in the replisome or at sites of DNA repair and a second which shows diffuse distribution throughout the nucleoplasm [8]. The distribution of the two forms of PCNA around the cell cycle has been studied using indirect immunofluorescence of cells, 2-D gel electrophoresis, and by FCM [11, 12].

The latter technique offers the most direct approach to study cell cycle distribution of PCNA, but the studies, so far, have produced conflicting results. Using the autoantibody derived from patients with systemic lupus erythematosus, only S-phase staining results on methanol-fixed cells [7]. However, studies using the monoclonal antibodies 19F4 and 19A2 failed to show S-phase specific staining after methanol fixation. Instead, paraformaldehyde, with or without lysolecithin, followed by methanol fixation, was required to obtain S-phase-specific staining [12].

Recently, a new series of monoclonal antibodies against PCNA have been described [13]. These were raised against recombinant PCNA protein cloned in a series of bacterial expression vectors using the cDNA for rat PCNA. Of the 11 antibodies produced, PC10 was selected for histological studies as it had the highest affinity for PCNA. Several studies have now been reported using the PC10 antibody in histological sections of normal and neoplastic tissues. Although these studies have shown the 'expected' distribution of proliferating cells in normal tissues and some tumours, there have also been unexpected results in other tumour types, notably breast [14].

Using FCM, the present study was undertaken to characterise PC10 staining following a series of alcohol and aldehyde fixation procedures and, after enucleation, using detergents or pepsin. The aim was to produce staining techniques useful for experimental studies with PCNA, but also techniques that could be applied to human tumours and which give insight into the nature of PC10 binding using immunohistochemistry.

METHODS AND MATERIALS

Tissue culture

V79 379A Chinese hamster fibroblast cells were taken from stocks kept at both centres; those taken from stocks kept at the Gray Laboratory are designated V79*. Three of the cell lines used, the HeLa human cervix, MV Chinese hamster ovary fibroblasts, a clone of CHO cells, and V79 lines were grown both as monolayers and in suspension. HeLa cells were grown in RPMI 1640 medium with 20 mmol/l HEPES (Gibco) and 10% fetal calf serum (FCS) (Gibco). MV and V79 monolayers were grown in Eagles minimal essential medium (MEM) with Hanks salts and 25 mmol/l HEPES (Gibco) supplemented with 10% FCS and non-essential amino acids. Suspension cultures of these cells were grown in MEM modified for suspension culture with 20 mmol/l HEPES (ICN Flow Laboratories) and supplemented with 7.5% FCS and non-essential amino acids. ZR75 human breast tumour cells were grown as monolayers in Dulbeccos modified Eagles Medium (Gibco) with 10% FCS and 10^{-8} mol/l oestradiol. V79* cells were grown in MEM modified for suspension culture (ICN Flow Laboratories) with 2.5 mg/ml Fungizone (Gibco) and 7.5% FCS. EJ, a human bladder carcinoma cell line, was grown in alpha-modified MEM with 2.5 mg/ml Fungizone and 10% FCS. Glutamine 20 mmol/l, penicillin 125 μg/ml and streptomycin 125 μg/ml were added to all culture media and all cells were maintained in exponential growth at 37°C. Monolayers to which no HEPES had been added were maintained in an environment of 5% CO_2. Cells were harvested during exponential growth. Monolayers were trypsinised in a solution of 0.25% trypsin (Difco Ltd., Detroit, Michigan), 100 mmol/l NaCl, and 10 mmol/l sodium citrate at 37°C for varying lengths of time. After washing in PBS, they were enucleated or fixed as described below. Suspension cultures were harvested by centrifugation at 300 *g* for 5 min.

Nuclei extraction

Nuclei were extracted using two methods. Firstly, 2×10^6 unfixed cells were washed twice in ice-cold PBS. They were resuspended in 2 ml of 0.25 mmol/l sucrose (Sigma Chemical Co.), 0.33 mmol/l calcium chloride (Sigma Chemical Co.) and 0.25% Nonidet P-40 (Sigma Chemical Co.) for 10–12 min on ice. The suspension was then diluted with PBS and centrifuged for 5 min at 700 *g*. The resulting nuclei pellet was either fixed as below or stained immediately. A non-detergent enucleation procedure was achieved by incubation of 2×10^6 ethanol fixed cells with 0.4 mg/ml pepsin (Sigma Chemical Co.) in 0.1 mol/l HCl for 10 min at 37°C. This was followed by two washes in PBS.

Fixation procedures

For fixation in ethanol, cells or nuclei were resuspended in 200 μl PBS to which 5 mls of ice-cold 70% ethanol was added. For methanol fixation, cells or nuclei were resuspended in 200 μl PBS to which 2 mls of absolute methanol at −20°C was added.

Correspondence to G.D. Wilson.
G.D. Wilson and C.A. Martindale are at the CRC Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR; R.S. Camplejohn and A. Brock are at the Richard Dimbleby Department of Cancer Research, St Thomas' Hospital, London SE1 7EH; D.P. Lane is at the Cell Transformation Research Group, Cancer Research Campaign Laboratories, Department of Biochemistry, The University, Dundee DD1 4HN; and D.M. Barnes is at the ICRF Clinical Oncology Unit, Guy's Hospital, London SE1 9RT, U.K.
Revised 1 Apr. 1992; accepted 26 May 1992.

After 5 min, the solution was diluted to 70% with distilled water. Paraformaldehyde fixation required suspension of cells or nuclei in 1 ml PBS containing 1% paraformaldehyde (BDH, Poole) for 2 min. The sample was then centrifuged and treated as for the methanol fixation. However, 10 min postfixation and permeation was allowed before dilution. A variation of this method of fixation was to resuspend the sample in 1 ml PBS containing 1% paraformaldehyde and 500 μg/ml lysolecithin (Sigma Chemical Co.) for 5 min. The sample was then centrifuged and treated with methanol as for the paraformaldehyde-fixed sample. All samples were stored at 4°C after fixation at a concentration of 1×10^6/ml.

PC10 staining

All samples were washed once in PBS and 1×10^6 cells or nuclei were resuspended in 0.25 ml of PBS containing 0.5% normal goat serum (NGS) (Sigma Chemical Co), 0.5% polyoxyethylene sorbitan monolaurate (Tween 20) (Sigma Chemical Co) and 1:50 dilution of the PC10 antibody. In each case, a control sample containing no antibody was prepared. There was no difference in background staining levels between using an irrelevant isotypic antibody or simply using the PBS/Tween-20 solution. The samples were incubated for 1 h at room temperature. After washing with PBS, the samples were resuspended in PBS/NGS/Tween solution containing a 1:25 dilution of a goat anti-mouse IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugate (Sigma Chemical Co.) for 30 min at room temperature. The samples were again washed in PBS and finally resuspended in 2 ml of PBS containing 1 mg/ml ribonuclease (Sigma Chemical Co.) and 10 μg/ml propidium iodide (Sigma Chemical Co.).

BrdUrd incorporation and staining

1×10^6 cells were incubated with 20 μmol/l BrdUrd for 20 min. After washing with PBS, they were trypsinised or collected by centrifugation and fixed in 9 mls of 70% ethanol after resuspension in 1 ml of PBS. After overnight fixation, cells were washed in PBS then incubated in 2 mol/l HCl for 25 min. The acid was neutralised by a further two washes of PBS. The cells were resuspended in 0.5 ml PBS/NGS/Tween solution containing a 1:20 dilution of rat anti-BrdUrd monoclonal antibody (Dr M. Ormerod, Institute of Cancer Research, Sutton, Surrey) for 1 h at room temperature. The cells were then washed and resuspended in a further 0.5 ml PBS/NGS/Tween solution containing a 1:20 dilution of a goat anti-rat IgG (whole molecule) FITC conjugate (Sigma Chemical Co.) for a further 30 min. The cells were then washed and resuspended in 2 ml PBS containing 10 μg/ml propidium iodide.

Flow cytometric analysis

The samples were analysed at both laboratories using Becton Dickinson FacSCAN flow cytometers equipped with 15 mW argon-ion lasers emitting an excitation beam of 488 nm. Green fluorescence from FITC was collected by fluorescence detector 1 after filtration using a 530 nm band pass filter. Red fluorescence from propidium iodide was collected by detector 2 after filtration using a 585 nm band pass filter. All data were collected in list mode and doublets and debris were excluded by gating on the area and width signals from the detector. At least 2000 events were collected for each sample after doublet discrimination. The data presented were collected at different FL1 PMT settings in order to accommodate the different fluorescence intensities between whole cells and nuclei. To directly compare these two situations, the PMT was calibrated using a control population and the data expressed at a single FL1 PMT voltage of 670, the voltage at which nuclei were analysed.

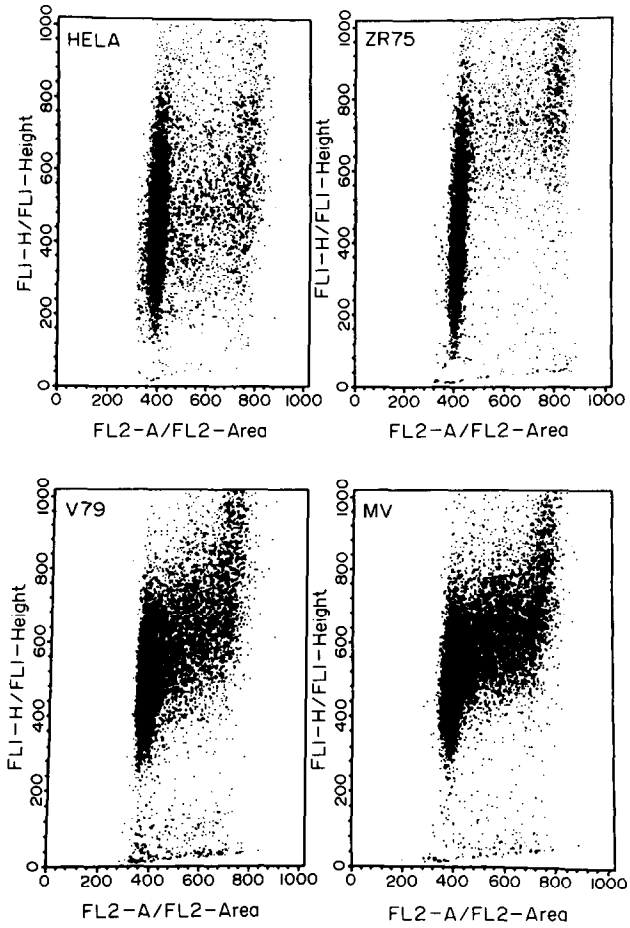

Fig. 1. Bivariate cytograms of DNA content vs. PC10 distribution in methanol-fixed intact cells from different cell lines.

RESULTS

Intact cells

Figure 1 shows examples of bivariate cytograms of DNA content vs. PCNA content for intact methanol fixed cells. The shapes of the profiles were similar for all the cell lines tested. All cells showed positive staining with PC10. In all cases, levels of PCNA vary within the G1 population, the cells which progress into S-phase emanating from populations of G1 cells with the higher levels of PCNA. PCNA content increased linearly through S-phase before starting to plateau in G2/M. Figure 2 shows the effect of different methods of fixation on cell cycle-dependent variation in PCNA staining. The curves represent variation in mean fluorescence through the cell cycle assessed by setting regions in each phase of the cell cycle according to DNA content. There was little difference in the shape of the PCNA cell cycle distribution obtained after various methods of fixation. However, cells fixed in paraformaldehyde had levels of fluorescence which were a factor of 20 times higher than those determined for the other three methods of fixation. The control samples, fixed in paraformaldehyde, also showed very high levels of fluorescence which were comparable to those of the positively stained samples for the other methods of fixation. Cells fixed in methanol and paraformaldehyde with lysolecithin

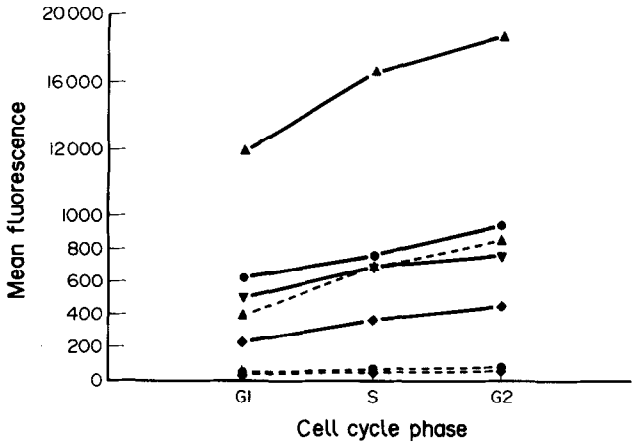

Fig. 2. Fluorescence intensity of PC10 staining as a function of cell cycle phase in intact V79 cells after different modes of fixation. The fluorescence values are means for each population. The controls are indicated by the dashed lines. The symbols represent (▲) paraformaldehyde, (●) methanol, (▼) paraformaldehyde and lysolecithin, and (◆) ethanol.

had very similar levels of fluorescence, with the ethanol fixed cells having the lowest levels. Mean fluorescence values for each cell cycle phase are shown in Table 1 for methanol fixed cells. In the three cell lines for which both suspension cultures and monolayers were studied, the cells grown as monolayers had higher levels of PCNA throughout the cell cycle. Overall, however, there was little difference in levels of fluorescence between cell lines, with the exception that HeLa cells grown as monolayers exhibited twice as much PC10 fluorescence as the other cell lines and that EJ cells exhibited low levels of PC10 fluorescence.

Nonidet extracted nuclei

Examples of bivariate cytograms of DNA content vs. PCNA content for nuclei extracted using Nonidet prior to methanol fixation are shown in Fig. 3. In contrast with those obtained for whole cells, the profiles show S-phase specific staining with the majority of cells in G1 or G2/M being visible as separate populations with very low levels of PCNA. There is a steep increase in levels of fluorescence as the cells move through late G1 and early S-phase, with levels peaking in mid-S and declining as cells move through late S-phase and into G2/M. Figure 4 shows the effect of different methods of fixation. The S-phase specific staining was retained with all four methods of fixation, with ethanol producing the most poorly defined profile. Fixation in paraformaldehyde and lysolecithin or paraformaldehyde alone produced very similar levels of fluorescence. However, fixation in aldehyde yielded levels of mid-S fluorescence which were

Table 1. Cell cycle-dependent expression of PCNA in whole, methanol-fixed cells

Cell line	G1	S	G2
HeLa S	1200	1369	1590
HeLa M	2161	2400	2383
V79 S	1369	1690	2167
V79 M	1435	1805	2394
MV S	1047	1288	1559
MV M	1164	1532	1793
ZR75	1171	1805	1889
EJ	555	755	755

Units represent relative fluorescence after subtraction of control values. S or M refers to the cell line grown as a suspension or monolayer culture.

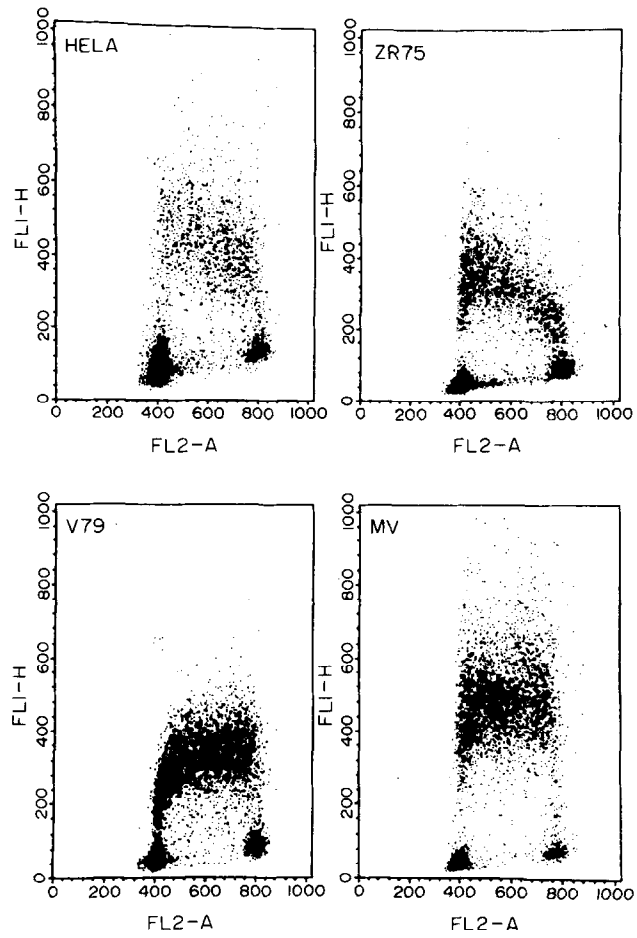

Fig. 3. Bivariate cytograms of DNA content vs. PC10 distribution in methanol-fixed detergent-extracted cells from different cell lines.

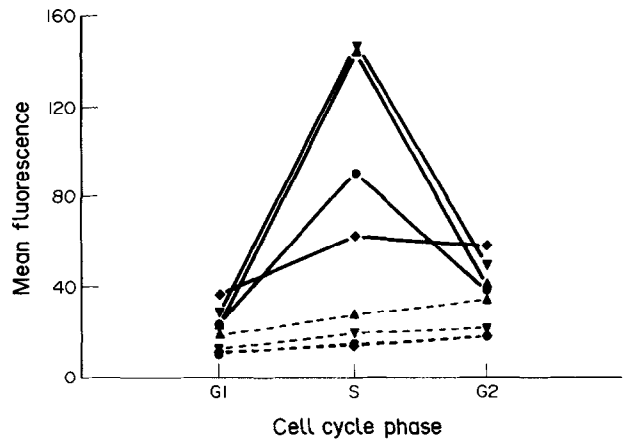

Fig. 4. Fluorescence intensity of PC10 staining as a function of cell cycle phase for detergent-extracted V79 cells with different modes of post-fixation. The controls are indicated by the dashed lines. The symbols represent (▲) paraformaldehyde, (●) methanol, (▼) paraformaldehyde and lysolecithin, and (◆) ethanol.

Table 2. Cell cycle-dependent expression of PCNA in NP40 extracted methanol fixed nuclei

Cell line	G1	S	G2
HeLa	46	449	72
HeLa M	34	410	54
V79 S	27	309	42
V79 M	21	232	29
MV S	17	501	30
MV M	19	533	35
ZR75	18	425	32
EJ	5	155	17

Units represent relative fluorescence after subtraction of control values. S or M refers to the cell line grown as a suspension or monolayer culture.

twice those determined for the alcohol-fixed nuclei. Mean fluorescence in each phase of the cycle is shown in Table 2. In contrast to the results for whole cells, levels of fluorescence from cells grown as monolayers are lower than those grown in suspension in two out of the three cell lines examined. Levels of fluorescence in G1 and G2 are similar to those of control samples, with S-phase nuclei having levels of fluorescence ranging from six to 30-fold higher than those in G1 and G2.

Nonidet nuclei/whole cells

Table 3 shows the levels of mid-S phase fluorescence, defined as a narrow window of 50 channels around the mid-S DNA content, in Nonidet extracted nuclei as a percentage of levels in a similar region from whole cells. Values range from 11 to 40%; with all three cell lines grown as suspension cultures showing retention of a higher percentage of PCNA following Nonidet extraction than those grown as monolayers.

Pepsin extracted nuclei

Figure 5 shows examples of bivariate cytograms of DNA content vs. PCNA content for nuclei extracted from ethanol-fixed cells using pepsin. It was not possible to use methanol-fixed cells, as in the study of whole cells and Nonidet extracted nuclei, because the DNA profiles obtained from nuclei extracted using pepsin from methanol-fixed cells were very poor. Some of the cell lines studied were very sensitive to the action of pepsin, particularly EJ line, which produced only debris at the

Table 3. Percentage of PCNA retained in mid-S after Nonidet extraction of nuclei

Cell line	Nuclei/cells Mid-S ratio (%)
HeLa S	33
HeLa M	17
V79 S	18
V79 M	13
MV S	40
MV M	33
ZR75	24
EJ	21

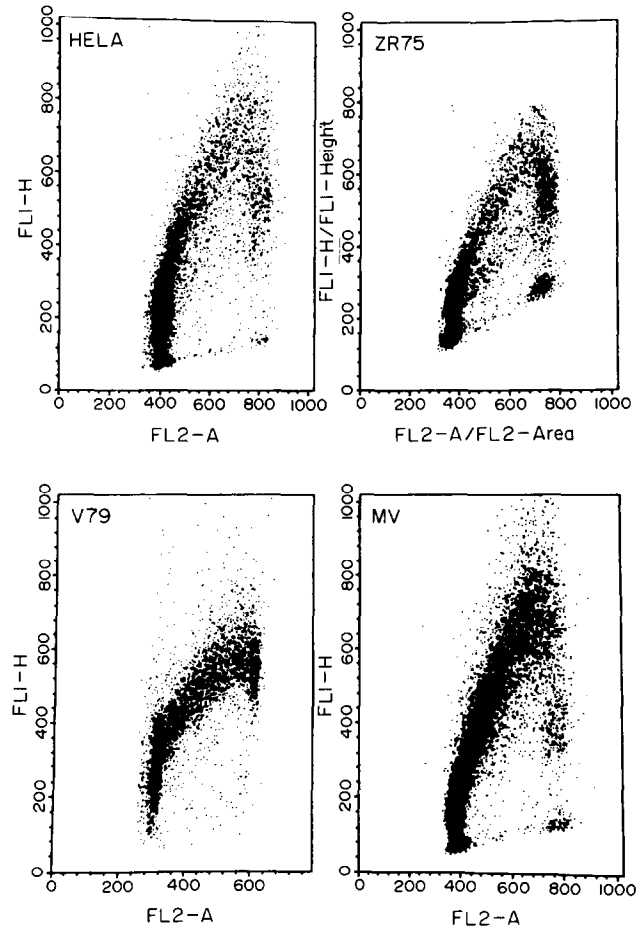

Fig. 5. Bivariate cytograms of DNA content vs. PC10 distribution in ethanol-fixed, pepsin-treated cells from different cell lines.

concentration of pepsin used in this study. Similar profiles to those obtained from other cell lines could be achieved using reduced pepsin concentrations. There was much more variation in the shape of the profiles than had been seen for the whole cells and Nonidet-extracted nuclei. However, most profiles showed a wide variation in PCNA levels in G1, followed by a linear increase through S-phase, peaking in late S-phase and decreasing as the cells moved into G2/M. Table 4 shows a wider variation in mean fluorescence levels between cell lines than those found for Nonidet-extracted nuclei. However, as for

Table 4. Cell cycle-dependent expression of PCNA in pepsin extracted nuclei

Cell line	G1	S	G2
HeLa S	96	271	280
HeLa M	50	124	132
V79 S	33	92	85
V79 M	28	77	82
MV S	23	62	67
MV M	22	70	63
ZR75	19	70	64
EJ	99	207	223

Units represent relative fluorescence after subtraction of control values. S or M refers to the cell line grown as a suspension or monolayer culture.

Nonidet-extracted nuclei, levels of fluorescence are higher in cells from suspension cultures than in those from monolayers for the HeLa and V79 cells, but not the MV line.

PCNA/BrdUrd LI

Figure 6 shows a plot of the labelling index obtained from PCNA profiles of Nonidet-extracted methanol-fixed nuclei vs. the labelling index obtained from BrdUrd profiles. The PC10 index was defined as the percentage of positively stained cells compared with controls without the monoclonal antibody. A regression line has been fitted through the points and the 95% confidence limits calculated. The correlation coefficient was 0.82 ($P < 0.0008$).

DISCUSSION

A major impetus to the study of cell proliferation has been the development of monoclonal antibodies against nuclear proteins. Potentially, the pathologist can perform relatively simple immunohistochemical staining procedures to obtain information concerning proliferative characteristics of neoplastic disease which may facilitate more effective management of patients. A fundamental pre-requisite for any clinically orientated proliferation measurement is a knowledge of the identity and cell cycle distribution and regulation of the particular protein. Some monoclonals, such as Ki-67, have found their way into clinical use without identification of the antigen being recognised, although there is no doubt about its association with cell proliferation [5].

PCNA has been the subject of a good deal of recent study, at the molecular level, because of its role in DNA replication. Although much has still to be learned in terms of its regulation, its identity and function are well established. PCNA represents an ideal candidate marker for clinical application. However, confusion exists in the application of PCNA detection to human cancer, due to the heterogeneity of staining patterns that have been reported using different monoclonal or auto antibodies and fixation procedures.

We have attempted to study the influence of sample processing procedures on the cell cycle distribution of PCNA in a variety of mammalian cell lines of differing proliferative characteristics. We have used FCM to achieve our aims with the belief that this

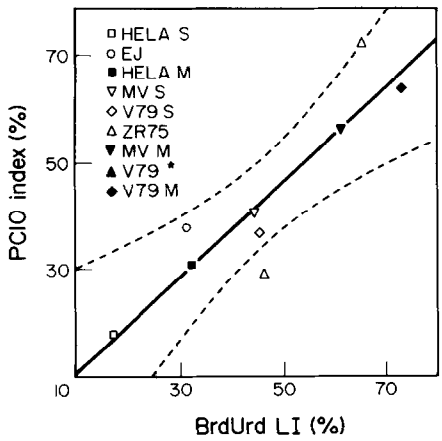

Fig. 6. Comparison of BrdUrd LI and PC10 index in different cell lines. The PC10 index was derived from detergent-extracted, methanol-fixed cells. S or M refers to the cell lines grown as a suspension or monolayer culture. The line fitted to the data is the regression analysis with 95% confidence limits.

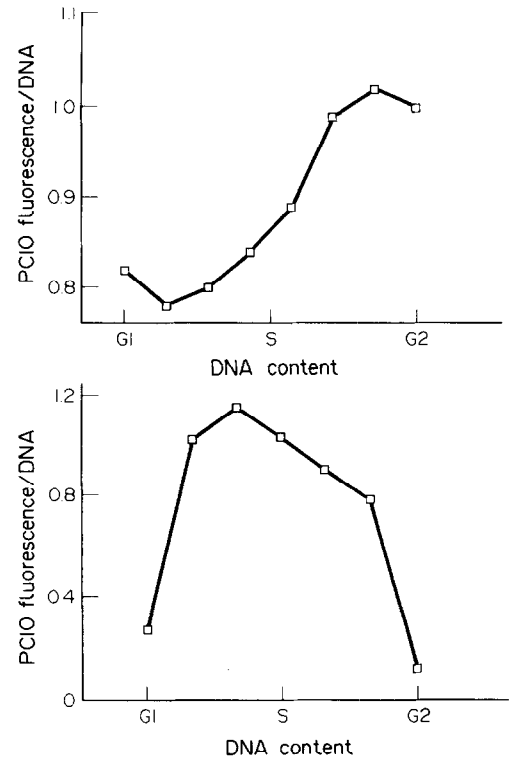

Fig. 7. Content of PCNA as a function of cell cycle position. Multiple regions were set throughout the cell cycle and the fluorescence intensity of PC10 staining was expressed as a function of DNA content after subtraction of control PC10 fluorescence values. (a) Shows intact methanol-fixed V79 cells. (b) Shows detergent-extracted, methanol-fixed V79 cells.

may give insight into the information that can be generated from immunohistochemical studies.

PC10 appears to be the best monoclonal antibody for routine application to histological material, as the epitope it binds to is relatively resistant to conventional fixation procedures [13, 14]. Our studies clearly demonstrate that PC10 recognition of PCNA can be manipulated profoundly by extraction, but less so by fixation. Alcohol or aldehyde-fixed intact cells all showed PC10 positivity consistent with a growth fraction type measurement. Changes in the cell cycle distribution of PCNA (Fig. 7a) mirrored those which have been observed with total or nuclear protein by flow cytometry [15], i.e. a sharp increase through G1 (G1A → G1B) followed by an almost linear increase through S-phase plateauing in late S and G2 at a level which is twice that seen in early G1 cells. There was little to choose between the fixatives, with one exception; an enormous increase in fluorescence signal (20-fold greater than other methods) was observed with paraformaldehyde (see Fig. 2), due apparently to autofluorescence. This effect was abolished in intact cells by the addition of lysolecithin and was not observed in paraformaldehyde-fixed nuclei extracted with Nonidet (Fig. 4) or pepsin (Fig. 5). Qualitatively, methanol fixation produced the best staining profiles in terms of PC10 fluorescence intensity and integrity of the DNA profile.

Extraction of cells with Nonidet-P40 followed by fixation, produced staining profiles consistent with S-phase-specific staining. These profiles were not obtained in the absence of postfixation, but could be achieved in unfixed extracted nuclei if they were incubated in 0.1 mol/l HCl for 20 min prior to staining (results not shown). Again, there was very little to choose

between the fixatives postextraction. Qualitatively, methanol produced the best profiles for the same reasons as discussed for intact cells. Compared with intact cells, Fig. 7b shows a completely different pattern of PCNA expression as a function of cell cycle distribution. G1 and G2 cells are essentially negative, with strong staining of S-phase cells.

In order to estimate the ratio of tightly bound, detergent non-extractable PCNA to total PCNA, the methanol-fixed profiles were analysed by setting a narrow region in mid-S phase of both intact and extracted cells. After subtraction of control values, the ratios were calculated (Table 3). There was some variation in the ratio of chromatin-associated to total PCNA between the cell lines (11–40%), but the average value of 24% for all the cell lines is in good agreement with the few previous estimates of 20–35% which are available [8, 11]. These results confirm that most of the PCNA present in cells is loosely bound and easily extractable.

The correlation between PC10 index and BrdUrd LI in detergent-extracted nuclei (Fig. 6) suggests that, under the appropriate staining conditions, PCNA can be used as an S-phase marker. However, results from the present study highlight the dangers of applying histochemical markers of proliferative activity in ignorance of what the staining patterns actually mean. We have shown that at least three distinct patterns can be achieved on the same type of cell with the same monoclonal antibody, in this case by staining whole cells and detergent or pepsin-extracted nuclei. Whether true S-phase staining can be achieved by manipulation of immunohistochemical procedures remains to be established. Previous reports of immunohistochemical staining have produced confusing results. Robbins *et al.* [16] were amongst the first to use the auto antibodies on tissue sections and obtained PCNA indices which were consistent with S-phase detection, despite the intensity of the labelling being weak. Unfortunately, no measurement was made of LI or SPF (S-phase fraction) in this study. Several groups have reported results using either the 19A2 or 19F4 monoclonal antibodies. In formalin-fixed material, the PCNA positivity rate resembles the Ki67 index [17]; this would then be a putative growth fraction estimate. When tissue sections were fixed in 1% paraformaldehyde followed by methanol, Van Dierendonck *et al.* [17] found a close correlation between positivity and BrdUrd LI. However, Coltera and Gown [18], using similar fixation, reported PCNA indices which were intermediate between a BrdUrd LI and the Ki67 index. Galand and Defraef [19] have reported a close correlation between PCNA staining and tritiated thymidine incorporation in methanol-fixed histological material. These results are rather surprising in the light of our FCM studies in which no S-phase specificity was seen using any of the fixatives on intact cells.

The results generated using the pepsin enucleation procedure are difficult to explain. On the cell lines, the pattern of labelling seen after pepsin enucleation was different to that seen with both intact cells and detergent-extracted nuclei. When cells were stained intact, virtually 100% showed positive staining; in detergent-extracted cells, only S-phase cells were positive; in pepsin-treated cells, a small proportion of G1 cells (20%) are negative; with the remainder of G1, all of S-phase and G2 + M are positive. There is a linear increase in PCNA staining intensity through S-phase and G2, although in some cell lines there is a slight reduction in G2 (Fig. 5). Interestingly, the level of fluorescence is less in pepsin-extracted cells than after Nonidet extraction (Table 4). This may indicate that pepsin treatment causes a partial digestion of both forms of PCNA. One other possible explanation for the pepsin profiles is that, under these conditions, a nucleolar form of PCNA is being detected. This would be consistent with the Sf and Sg distribution described by Celis and Celis [20] in which nucleolar localisation was seen in mid to late-S. These results do indicate that enucleation alone is not sufficient to account for S-phase specificity.

The confusion that exists between different reports in the literature may arise, not only due to the different cells and fixation procedures studied, but also to the different antigenic determinants of the antibodies used. PC10 is similar to the auto-antibodies in that it detects a folded form of PCNA [13], whereas 19A2 and 19F4 were raised from SDS-denatured PCNA [21] and are unable to immunoprecipitate native PCNA.

We have shown that caution should be exercised in interpreting staining patterns obtained with anti-PCNA antibodies, as these patterns can be profoundly influenced by tissue processing and staining conditions. However, our results on cell lines demonstrate that S-phase specific staining is achievable and it may also be possible to obtain a growth fraction estimate. This latter possibility requires confirmation in experimental solid tumours, particularly as a recent study suggested that PCNA may over-estimate the growth fraction compared with Ki67 and pulse-labelled mitosis analysis in a human tumour xenograft [22]. Providing that PCNA staining on tissue sections can be shown to be consistent for a given tissue, and that it measures some proliferation-related parameter, it may have a role as a simple operational marker of proliferative activity in clinical material.

1. Tubiana M, Courdi A. Cell proliferation kinetics in human solid tumors: Relation to probability of metastatic dissemination and long-term survival. *Radiotherapy and Oncology* 1989, **15**, 1–18.
2. Barlogie B, Raber NM, Schumann J, *et al.* Flow Cytometry in clinical cancer research. *Cancer Res* 1983, **43**, 3982–3997.
3. Wilson GD, McNally NJ, Dische S, *et al.* Measurement of cell kinetics of human tumours *in vivo* using bromodeoxyuridine incorporation and flow cytometry. *Br J Cancer* 1988, **58**, 423–431.
4. Begg AC, McNally NJ, Shrieve DC, Karcher H. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* 1985, **6**, 620–626.
5. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation associated with human nuclear antigen defined by the monoclonal antibody Ki67. *J Immunol* 1984, **133**, 1710–1715.
6. Hall PA, Woods AL. Immunohistochemical markers of cellular proliferation: achievements, problems and prospects. *Cell Tissue Kinet* 1990, **23**, 505–522.
7. Bravo R, MacDonald-Bravo H. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *Embo J* 1985, **4**, 655–661.
8. Bravo R, MacDonald-Bravo H. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: Association with DNA replication sites. *J Cell Biol* 1987, **105**, 1549–1554.
9. Lee SH, Eki T, Hurwitz J. Synthesis of DNA containing the Simian Virus 40 origin of replication by the combined action of DNA polermerase α and δ. *Proc Natl Acad Sci USA* 1989, **86**, 7361–7365.
10. Liu YC, Bambara R. Gene expression of PCNA/Cyclin in adult tissues and the R323OAC mammary tumor of rat. *Biochem Biophys Res Comm* 1989, **161**, 873–882.
11. Morris GF, Matthews MB. Regulation of proliferating cell nuclear antigen during the cell cycle. *J Biol Chem* 1989, **264**, 13856–13864.
12. Kurki P, Ogata K, Tan EM. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/Cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. *J Immunol Methods* 1988, **109**, 49–59.
13. Waseem NH, Lane DP. Monoclonal antibody analysis of PCNA structural conservation and detection of nucleolar form. *J Cell Sci* 1990, **96**, 121–129.
14. Hall PA, Levison DA, Woods AL, *et al.* Proliferating cell nuclear

antigen (PCNA) immunolocalisation in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol* 1990, **164**, 285–294.
15. Roti Roti JL, Higashikubo R, Blair OC, Uygur N. Cell-cycle position and nuclear protein content. *Cytometry* 1982, **3**, 91–96.
16. Robbins BA, De la Vega D, Ogata K, Tan EM, Nakamura M. Immunohistochemical detection of proliferating cell nuclear antigen in solid human malignancies. *Arch Pathol Lab Med* 1987, **111**, 841–845.
17. Van Dierendonck JH, Wijsman JH, Keijzer R, Van de Velde CJH, Cornelisse CJ. Cell-cycle related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies. *Am J Pathol* 1991, **138**, 1165–1172.
18. Coltera MD, Gown AM. PCNA/Cyclin expression and BrdU uptake define different subpopulations in different cell lines. *J Histochem Cytochem* 1991, **39**, 23–30.
19. Galand P, Defraef C. Cyclin/PCNA immunostaining as an alternative to ^{3}H-Thymidine pulse labelling for marking S-phase cells in paraffin sections from animal and human tissues. *Cell Tissue Kinetics* 1989, **22**, 383–392.
20. Celis JE, Celis A. Cell-cycle dependent variations in the distribution of the nuclear protein cyclin in cultured cells. *Proc Soc Natl Acad Sci USA* 1985, **82**, 3262–3266.
21. Ogata K, Ogata Y, Celis JE, Nakamura RM, Tan EM. Monoclonal antibodies to a nuclear protein (Cyclin/PCNA) associated with DNA replication. *Exp Cell Res* 1987, **168**, 475–486.
22. Scott RJ, Hall PA, Haldane JS, *et al.* A comparison of immunohistochemical markers of cell proliferation with experimentally derived growth fraction. *J Pathol* 1991, **165**, 173–178.

Eur J Cancer, Vol. 28A, No. 12, pp. 2017–2024, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Biochemical Modification of the Toxicity and the Anti-tumour Effect of 5-Fluorouracil and cis-Platinum by WR-2721 in mice

Clasina L. van der Wilt, Jan A.M. van Laar, Fruzsina Gyergyay, Kees Smid and Godefridus J. Peters

WR-2721 (ethiofos) was tested on Balb/c mice for its chemoprotective capacity against 5-fluorouracil (5FU) monotherapy. In this combination WR-2721 was not active, but WR-2721 pretreatment allowed an elevation of the cisplatin (CDDP) dose in 5FU/CDDP combination therapy in these mice. Thrombocytopenia caused by the 5FU/CDDP (100 and 7 mg/kg, respectively) therapy was prevented by WR-2721 (200 mg/kg) and a partial protection against leukopenia was observed in C57Bl/6 mice. Various WR-2721/CDDP/5FU combinations were tested on two murine colon tumour models. The best antiproliferative effect against Colon 26 (in Balb/c mice) and the lowest toxicity were found with 5FU (100 mg/kg) and CDDP (5.5 mg/kg) delivered together 30 min after WR-2721 (200 mg/kg). The increased efficacy of WR-2721/CDDP/5FU both in Colon 26 and Colon 38 (in C57Bl/6 mice) compared to single 5FU or 5FU/CDDP treatment at the same dose could not be explained by enhanced inhibition of thymidylate synthase (TS), the 5FU target enzyme. The protection by WR-2721 against toxicity of CDDP/5FU might enable the use of high doses of CDDP in this combination.
Eur J Cancer, Vol. 28A, No. 12, pp. 2017–2024, 1992.

INTRODUCTION

s-2(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOTIC ACID (WR-2721, ethiofos), has initially been developed and used as a radioprotector [1, 2]. Lately it has been investigated for its chemoprotectivity against toxic side-effects of cis-platinum (CDDP) and other agents [3, 4]. WR-2721 has to be activated to its metabolite WR-1065 by dephosphorylation catalysed by alkaline phosphatase (AP), a plasma membrane enzyme and as such responsible for membrane passage and hydrolysis of WR-2721 [5, 6]. WR-1065 is the active derivative, which protects DNA against several chemotherapeutic agents [6, 7]. A high uptake of WR-1065, after administration of labelled WR-2721 has been shown in kidney, liver, bone marrow, heart and spleen; low uptake occurs in the central nervous system, muscle and tumour [8–11]. Selective protection of WR-2721 in non-tumour tissue against cytotoxic effects can be explained by difference in absorbtion due to bad vascularisation and low AP activity in the tumour [12, 13]. In the clinic WR-2721 has been demonstrated to be protective against nephro-, neuro- and ototoxicity induced by CDDP without loss of anti-tumour activity [14].

5-Fluorouracil (5FU) is widely used for the treatment of solid tumours, such as advanced colorectal cancer, breast cancer and squamous cell carcinoma of the head and neck. 5FU has to be metabolised and converted to the nucleotide level to become active [15]. The metabolite 5-fluoro-2′ deoxyuridine-5′-monophosphate (FdUMP) inhibits thymidylate synthase (TS), leading to a cessation of DNA synthesis. In the presence of 5,10-

Correspondence to G.J. Peters.
C.L. van der Wilt, J.A.M. van Laar, K. Smid and G.J. Peters are at the Department of Oncology, Free University Hospital, PO Box 7057, 1007 MB Amsterdam, The Netherlands, and F. Gyergyay is at the National Institute of Oncology, Budapest, Hungary.
Revised 13 May 1992; accepted 23 June 1992.