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Pan Cycle Expression of Proliferating Cell Nuclear Antigen in Human Colorectal Cancer and its Proliferative Correlations

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Proliferating cell nuclear antigen (PCNA) is a key cell cycle regulatory protein of known structure and function that also has an important role in DNA repair. Its use as a marker of proliferation can be assessed directly using a thymidine analogue in suitably labelled pathological material. Following optimisation studies, a quantitative and cell cycle phase–specific analysis of PCNA expression in fresh biopsies from 50 colorectal tumours (Series A) was undertaken using dual parameter flow cytometry (FCM). For comparison, quantitative histometric studies of PCNA expression were undertaken on 50 archival bromodeoxyuridine (BrdUrd) labelled colorectal tumours (Series B). In FCM assays, PCNA-specific fluorescence was displayed throughout the cell cycle in both cells and nuclei under all preparation conditions, but to a very variable extent. The mean PCNA labelling index ranged from 38.7% to 53.0% according to the method of cell extraction used. In the 27 diploid tumours in Series A, the median PCNA LI in GO/G1 was 71.5% (range 27.0–90.6%), in S it was 10.5% (3.3–29.5%), and in G2/M it was 17.4% (5.7–43.5%). In the histometric studies of Series B tumours, the mean [S.D.] PCNA labelling index (LI) was 38.8 [9.8]%, compared with the BrdUrd (histometric) LI of 21.1 [9.0]%. The BrdUrd LI measured by FCM was 12.4 [6.5]%. PCNA-PC10 is expressed throughout the cell cycle in human colorectal tumour biopsies, which is in keeping with the range of DNA repair, synthesis, and regulatory functions that it is now recognised to perform throughout the cell cycle. © 1995 Wiley-Liss, Inc.

Key terms: Flow cytometry, proliferating cell nuclear antigen, PCNA

'roliferating cell nuclear antigen (PCNA) is associated h DNA synthesis and repair. It is an auxiliary protein)NA polymerase delta and has been well characterised 5,11,12,18,26). FCM studies of cell systems have indied that PCNA exists in a detergent-extractable nuoplasmic form during G1, S, and G2, and a tightly ınd nuclear form associated with replisomes during S phase. In homogeneous cell lines grown exponenly in culture, the synthesis of PCNA initiates in G1, reases in late G1 and early S phase of the cell cycle, ıks in mid-S, and declines through G2/M (6,15,16,28). Multiparametric flow cytometry (FCM) is an estabıed research tool for the quantitative and cell cycle–ated study of tumour nucleoproteins, although at the ıt of loss of information on tissue architecture inherent ıistological studies. FCM studies of antigen expression solid tumours require careful attention to a range of hnical variables (23,27). Colorectal tumours are comx and asynchronous mixtures of stromal and maligıt cells of many lineages, unlike cells in culture. In this paper we report the measurement of PCNA expression through the cell cycle in a series of 50 freshly obtained primary colorectal tumours. We compare these results with data obtained by histometric and flow cytometric measurement of BrdUrd and PCNA labelling in an archival series of 50 primary, ethanol-fixed colorectal tumours labelled in vivo with BrdUrd (20).

MATERIALS AND METHODS

Series A: Flow Cytometry Studies of PCNA

In order to study PCNA by FCM under optimal conditions, colorectal adenocarcinomas (n = 50) were sampled immediately after excision. No tumour had been

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:reated with radio- or chemotherapy. This study of PCNA was divided into two parts. Optimisation studies were undertaken of tissue disaggregation using both mechanical and enzymatic methods, of cell and nuclear antigen preservation, of antibody titres, of controls, and of FCM profile analysis. The quantitative measurement of PCNA expression and of the S phase fraction in all 50 tumours was then undertaken.

Optimisation studies

Extraction of intact tumour cells. The tumour biopsies were minced in Hanks buffered saline solution (HBSS; Sigma, Poole, UK) for 10 min, then filtered through a 35 μm nylon mesh. For enzymatic disaggregation, the specimens were incubated in enzyme solution in HBSS without calcium or magnesium before filtration. Protease (Sigma, Poole), 0.5 mg/ml, and deoxyribonuclease, 0.005 mg/ml, extraction for 45 min at room temperature produced excessive digestion of tissues. Satisfactory cell suspensions, as assessed using a microscope haemocytometer, were obtained using 1.0 mg/ml collagenase type IV (Sigma) with deoxyribonuclease, 0.2 mg/ml, for 20 min at 37°C. The cells obtained were spun at 1,000 rpm for 5 min, washed in phosphate buffered saline (PBS), and spun again.

Extraction of tumour nuclei. Nuclei were extracted from these cell suspensions by adding 2 ml of a 25 mM sucrose, 0.33 mM calcium chloride and 0.25% Nonidet P-40 solution for 10 min on ice. This formulation had previously been standardised for cells in culture (28). The solution was then diluted with PBS and spun for 5 min at 2,000 rpm.

Fixation. Cells and nuclei were all fixed by resuspending in 200 μl of PBS to which 3 ml of absolute methanol at −20°C was added dropwise while vortexing. After 5 min the alcohol content was diluted to 70% with distilled water. Storage was then possible for 1 month or more without significant diminution of PCNA staining.

PC10 staining. The concentrations of cells or nuclei in all samples were adjusted using a haemocytometer to yield a total of 1×10^6 per vial, spun, and resuspended in 0.25 ml of PBS containing 0.25% bovine serum albumin and 0.5% Tween 20 (Sigma), and primary PC10-FITC conjugated antibody (Dako, Herts) at the selected dilution. This method was chosen as an alternative to a two (PC10 and second Ig-FITC) antibody binding process (28) to reduce the number of processing stages in the sample preparation. A control for nonspecific antibody binding was prepared for each sample using an FITC-conjugated mouse IgG2A (Dako) at the same equivalent antibody concentration as the PC10. The samples were incubated in the dark for 2 h at room temperature, washed in PBS, and resuspended in 2 ml of PBS containing 1 mg/ml ribonuclease and 20 μg/ml propidium iodide.

PC10 and control antibody dilution series. **PC10:** Two batches of PC10 were used in this study. A dilution series was performed for each batch to determine the optimum antibody concentration. Dilutions were selected that gave a PCNA labelling index at a point on the dilution curve most closely approximating to a plateau (19).

Control antibody: Non-specific fluorescence changes with the applied concentration of the mouse isotypic IgG selected as the non-PC10 control (28). So as to standardise this variable, the control IgG was always used at the equivalent dilution to that of PC10.

Flow cytometric analysis. All analyses were performed on a standard FACScan using a 15 mW argon laser at 488 nm with Lysys 2 and Cellfit software (Becton Dickinson, Oxford, UK). Green light (FITC) was collected in channel FL1 with a standard gain set at 555. Red light (PI) was collected in channel FL3 with a standard gain set at 300. Data were collected in list mode. Debris, doublets, and clumps were excluded using the automatic doublet discrimination function. PC10-stained and control cells/nuclei were always run at the same gains. Chick erythrocytes or human colorectal mucosa were used to define reference peaks for the diploid G0/G1 cells. The DNA index was calculated for each sample by using Lysys 2 to mark the G0/G1 diploid and aneuploid peak channels. Cellfit was used to calculate the S phase fractions.

Control gates. All paired control and PC10 specimens were run sequentially, and the resulting FL1-H vs. FL3-A profiles were displayed side by side for further analysis. For each sample, new gates were set around the control populations and transferred directly to the PC10 plots, as in Figures 1, 2, and 3.

There is considerable variation in the level of background fluorescence and of fluorescence attributable to the control antibody from one sample to the next (Fig. 4). Therefore, the gate settings were adjusted on the control profiles to a cutoff of 98% of fluorescent events, so as reproducibly to distinguish PCNA-PC10 positivity from control fluorescence. Ninety-eight percent gating is a compromise between incomplete and 100% gating out of control fluorescence. Some events with high fluorescence appear in most control profiles, and 100% control gating out would override virtually all PCNA-PC10 positive labelling. A small change in the control gate can have a major effect upon the PC10 measurement (Fig. 5).

Some cytograms showed variable upward distributions through the cell cycle (Fig. 1), while others remained virtually flat (Fig. 2). A variety of rectangular and trapezoid gate settings were assessed before adopting the rectangular model, which was a considerable aid to assay standardisation. The small percentage difference that such settings made to overall measurements was not found to be biologically significant. For example, in Figure 3a and 3b the difference between the rectangular model (21.2%) and the trapezoid model (19.9%) was 1.3% when each control was set at 98.0%.

The following quantitative PCNA parameters were measured in these tumours:

1. PCNA total labelling index, which is the proportion of labelled cells above the (98%) gate.
2. PCNA aneuploid labelling index, which is the pro-

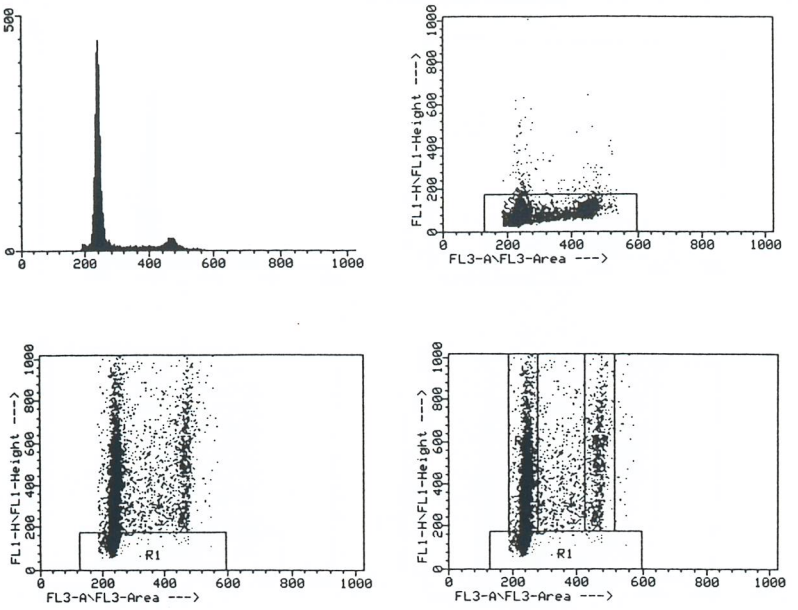


FIG. 1. Illustration of the setting (98%) of gates for a diploid tumour. **Upper left** is the DNA profile. **Upper right** is the 98% gate on the control profile. The FITC signal is on the FL1-H (Y) axis, and DNA content is on the FL3-A (X) axis. **Lower left** is the 98% gate transferred to the PC10 profile. **Lower right** are additional gates set around the PC10 "positive" cells in the G0/G1, S, and G2/M phases. The setting of these latter gates is subjective.

portion of labelled cells above the (98%) gate when gating on the aneuploid population alone.

3. Percentage of cells in G0/G1, S, and G2/M. These values were only calculated in the diploid tumours because of the inaccuracies introduced by overlapping populations in the aneuploid tumours.

4. The mid S phase PC10 staining ratio. To assess the specificity of PC10 expression in the S phase, the ratio of the mid-S phase PCNA staining within and without the 98% control gate was measured (Fig. 6).

5. The S phase fraction (SPF). The SPF was calculated using the models RFit, SOBR-3, and SOBR-8 within the Cellfit programme in each sample analysed for PCNA content.

Observer reproducibility. Gating of the PCNA positive cells into each cell cycle phase was performed subjectively. To assess the intraobserver reproducibility, five profiles from diploid tumours were reanalysed by one observer on consecutive days for the PCNA content of the G1, S, and G2/M phases. The ranges of the coefficients of variation of the measurements were G0/G1 (0.6–3.6%), S (5.6–13.5%), and G2/M (2.5–8.3%).

Series B: Histochemical Studies

A direct and quantitative comparison of PCNA and BrdUrd expression was undertaken using immunohistochemistry of serial tissue sections (Series B). Fifty blocks of formalin-fixed, wax-embedded specimens were selected at random from an archival collection of colorectal carcinomas that had been labelled in vivo with bromodeoxyuridine (20). Direct comparisons of PCNA and BrdUrd expression using flow cytometry were not possible because in preliminary studies the extraction of cells and nuclei from stored ethanol-fixed tissue blocks was found to destroy the PCNA-PC10 epitope. Tissue sections were cut at 4 μm, picked up onto poly-L-lysine–coated slides, and dried at 60°C for 30 min. Serial sections were stained for BrdUrd or PCNA-PC10 as follows.

Bromodeoxyuridine staining. After incubation in 5% H_2O_2 in methanol, sections were incubated in 0.1% trypsin (Sigma, Poole) in CaCl buffer (pH 7.8) at 37°C for 10 min. DNA was denatured by incubation in 1 N HCl at 60°C for 10 min. The anti-BRdUrd antibody (clone 20a, Dako, High Wycombe, Bucks) was then added (1 in 100) for 1 h at 20°C. Positivity was visualised by an indirect streptavidin biotin immunoperoxidase procedure (Dako "Duet" Reagents, Herts, UK) with diamino-benzidine tetrahydrochloride (Sigma, Poole) as a chromogen. Carrazi's haematoxylin was used for nuclear counterstaining.

PCNA staining with PC10 antibody. Sections were mounted on poly-L-lysine–coated slides and incubated for 1 h at 45°C to assist adherence. Endogenous peroxi-

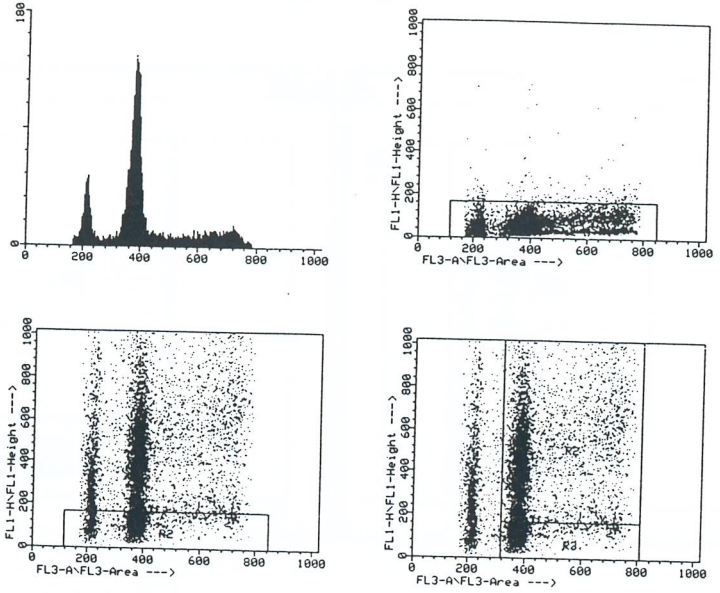


FIG. 2. Illustration of the (98%) gating for an aneuploid tumour. **Upper left** is the DNA profile. **Upper right** is the control-FITC profile set to exclude 98% events. **Lower left** is the 98% gate transferred to the PC10-FITC profile. **Lower right** is the gating set around the aneuploid population to yield the aneuploid labelling index. This gate includes some positive events from the G2/M phase of the diploid phase, overlapping the aneuploid peak.

dase activity was blocked in 1% H_2O_2 in methanol for 30 min. PC10 antibody (courtesy of Professor D.P. Lane, Dundee) was applied in dilutions of either 1/100, 1/200, or 1/400 for 1 h. After washing in Tris buffered saline, biotinylated anti-mouse IgG (Sigma Immunochemicals, Poole) was added for 30 min, and avidin-peroxidase conjugate was added for 45 min. Visualisation was achieved using diaminobenzidine for 10 min. Sections were counterstained with Harris haemotoxylin and mounted. Each section had a negative control by omission of PC10.

Quantitative counting. Tumour cells were counted by an observer using an eye graticule with a 10 × 10 grid. From each section, 10 representative areas were selected for counting. Each area contained between 100 and 200 cells. The mean PCNA or BrdUrd labelling index was thus derived from between 1,500 and 2,000 cells in each sample. In the case of the BrdUrd labelling data, a correction was applied to account for the variable extent of cell division that occurs in different tumours between BrdUrd injection and biopsy (up to 16 h). The labelling indices were corrected to T0 (the time of injection using correction factor derived from the BrdUrd/FCM analysis 3).

Clinical Correlates

Conventional assessment was undertaken by one senior pathologist in each case to report the Dukes staging and degree of differentiation. The site of the tumour was recorded.

RESULTS

Series A: Flow Cytometric Measurement of PCNA

Fifty patients with primary adenocarcinomas of the colon (n = 34) and rectum (n = 16) were studied. There were three Dukes A, 18 Dukes B, and 29 Dukes C tumours. Twenty-seven tumours were diploid and 23 were aneuploid. Series A was similar by conventional pathological criteria to the BrdUrd labelled series B tumours.

Optimisation studies. Five tumours were used for the optimisation studies.

PC10 Antibody dilution series. The optimum dilution was defined as the point at which PC10 labelling did not further increase with increasing concentration of primary antibody. In batch A of the PC10-FITC antibody, the optimum dilution was 1/25, and in batch B the optimum dilution was 1/10.

Gate standardisation. In cell suspensions with a high fluorescence attributable to non-specific IgG-FITC in the control sample, the change from 95% to 98% gating produced a large shift on the FITC fluorescence (FL1-H) axis. When transferred to the PCNA profile, this gate setting would make a large difference to the PCNA labelling index (Fig. 5). We thus standardised the control

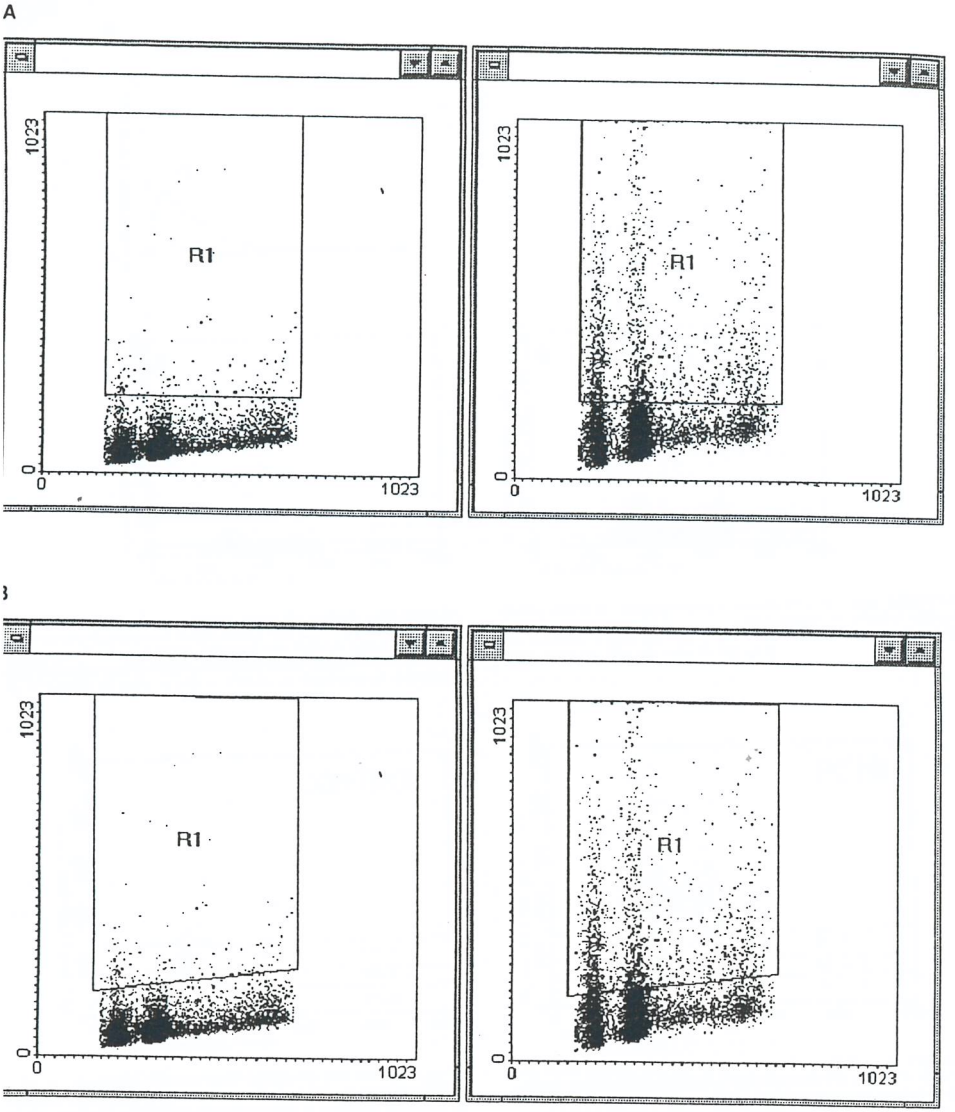
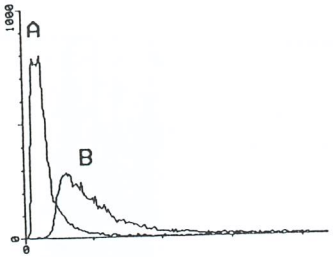
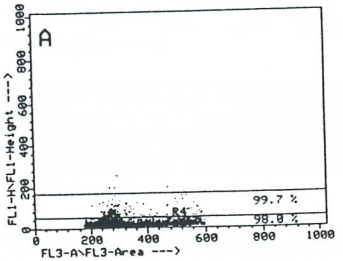


FIG. 3. A,B: The rectangular and trapezoid gates on the cytogram produce an absolute difference of 1.3% when the control gate is standardised at 98% (see text).

e setting as the 98% percentile for each tumour, her than at a prescribed channel setting.

Clinical series. Each tumour (n = 50) underwent ır different preparative procedures. These were mechanical extraction of cells (1) or nuclei (2), or enzymatic extraction of cells (3) or nuclei (4). Between one and four satisfactory PC10 profiles were obtained for each tumour. A profile was judged to be satisfactory if the





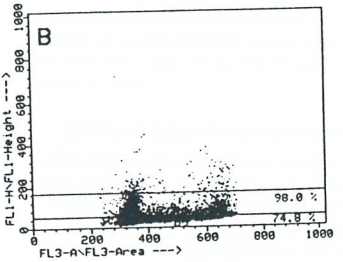
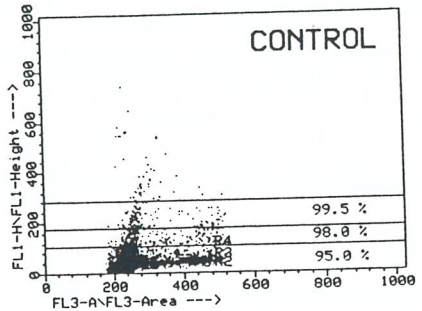


FIG. 4. Illustration of the problem posed by variable intensities in ntrol sample fluorescence in two diploid tumours. Tumour A has min- al non-specific fluorescence. Tumour B has high background fluores- nce. The upper profile directly compares these fluorescence profiles (y is = number of cells, x axis = FL1-H, or fluorescence intensity). Gating based on predetermined channels (FL1-H) would exclude very different proportions of background fluorescence in different tumours. For example, a gate set near channel 50 on the Y axis would exclude 98% of events in A but only 74.8% on B.



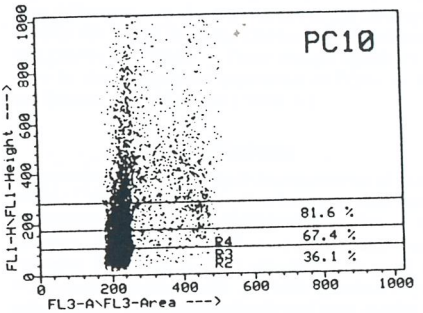


FIG. 5. Illustration of how a small variation in gate placement on the control profile (left) will have a major effect on the percentage of PCNA-PC10 "positive" cells within the gate (right) in a diploid colorectal tumour. A control with non-specific IgG (left) is paired with the correspond- ing PC10 profile. For example, a move from 95% to 99.5% on the control profile will move the positivity from 63.9% to 18.4%. One hundred percent gating of the control would require a gate at channel 800, which would yield less than 10% "positivity" on the PC10 profile.

DNA profile (FL3) was clearly defined, regardless of any difference in the FL1-H profile of the PC10-stained population when compared with the control. In six tumours, two regions were studied. In these cases, mean values for the PCNA data were used in subsequent analyses.

In 15 of 50 tumours, interpretable PCNA profiles were obtained by all four preparative procedures. In 17 tumours three procedures yielded data. In 11 tumours two procedures yielded data. In the remaining seven tumours only one procedure was successful. The quantitative

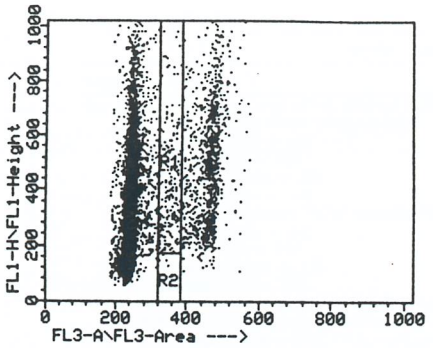


FIG. 6. This profile illustrates the method by which the mid S phase PCNA ratio is calculated in a diploid tumour. A narrow (50 channel width) gate or box is drawn in the mid S phase of the PCNA-PC10 positive sample, the 98% gate having previously been established from the control sample. The proportion of positive cells (R1) in the total sample (R1 + R2) yields the mid-S ratio. S phase cells unlabelled for PC10 will appear in R2.

PCNA-PC10 data obtained from these analyses are reported in Table 1.

Mechanical and collagenase extraction of cells yielded large numbers of clearly identifiable single cells from most tumours. Mechanical and collagenase based extractions yielded similar numbers of analysable samples (42 vs. 43 tumours), and there was no significant difference in the total PCNA labelling indices of the populations measured by either technique ($P = 0.92$). Mechanical extraction of nuclei yielded a marked increase in nuclear clumping or adhesiveness in the methanol solvent despite a variety of preemptive manoevres. These included vortexing, or the addition of Tween 20, EDTA or DNAase (Sigma, Poole UK). Collagenase extraction of nuclei was more effective than mechanical extraction (34 vs. 22 tumours), but there was no significant difference in the total PCNA labelling indices of the populations measured by either technique ($P = 0.62$).

Cell cycle analysis. PCNA expression was observed throughout the cell cycle in all specimens successfully studied. Quantitative analysis of the PCNA-PC10 content within the individual G1, S, and G2 phases was performed upon the cell extracts of the 27 diploid tumours. The median PCNA LI in the G0/G1 phase was 71.5%, with a range of 27.0–90.6%. The median PCNA LI in the S phase was 10.5%, with a range of 3.3–29.5%. The median PCNA LI in the G2/M phase was 17.4%, with a range of 5.7–43.5%. The overlapping of the various phases within aneuploid tumour profiles prevented such an analysis of aneuploid tumours.

Correlations with S phase fraction. The S phase fraction values calculated by the three different models Rfit, SOBR-3, and SOBR-8, and the total PCNA labelling index values were compared in profiles derived from analysis of 17 diploid tumours from which the cells were successfully extracted mechanically. In this group of tumours, all four SFP and PCNA data sets were available (Table 2). There was no correlation between the PCNA LI and SPF data points by any method, and the mean PCNA labelling index was significantly higher than the equivalent SPF value. There was a correlation of 0.46 between the S phase fraction, as determined using Cellfit, and the proportion of PCNA positive cells in the S phase, as determined by observer gate setting on the PCNA profile.

Mid-S phase PCNA ratio. The median mid-S phase PCNA ratio in diploid tumours ($n = 27$) was 62.7% (mean 64.9%, range 31.0–100%).

Clinical correlations (series A). There was no correlation detected between Dukes stage, differentiation, or site and the total PCNA-PC10 labelling indices determined by FCM.

Series B: Bromodeoxyuridine Labelled Tumours

There were 29 rectal and 21 colonic adenocarcinomas. These comprised two Dukes A, 24 Dukes B, and 24 Dukes C tumours. Twenty-five tumours were diploid and 25 were aneuploid. The quantitative BrdUrd and PCNA histochemical labelling index data and the corresponding BrdUrd labelling data obtained by flow cytometry (and previously reported) are presented in Table 1. Note that the FCM analyses included stromal cells.

There was a highly significant difference between BrdUrd and PCNA labelling ($P > 0.00001$) in the histometric study, with PCNA labelling being significantly higher than BrdUrd labelling in most tumours (Fig. 7). This difference was also seen between the flow cytometric data for PCNA labelling (Series A) and BrdUrd labelling (Series B) (Table 1). There was no significant difference in the histometric expression of PCNA or BrdUrd attributable to aneuploidy (Table 1).

DISCUSSION

Quantitative histochemical measurements of PCNA expression have been proposed to have practical value and prognostic potential in proliferation studies of clinical tumours, including those from the intestinal tract. For example, Mayer et al. (17) reported a range of PCNA-PC10 positivity from 3.0% to 54.6% (median 17.5%) in a series of 82 colorectal tumours and very similar values in 18 nodal metastases. Al-Sheneber et al. (1) reported a similar range of data in a smaller cohort of 40 colorectal tumours. Woods et al. (29) detected a correlation between the PC10 labelling index, histological grade, and survival in a series of 31 gastro intestinal lymphomas, while Jain et al. (14) reported that PCNA labelling of gastric carcinomas may have prognostic value.

The value of PCNA as a proliferation marker is under debate. PCNA is also expressed by cells undergoing DNA repair, so it may not be solely a proliferation or growth fraction marker (18). PCNA measurements using histochemical techniques are influenced by a number of preparative considerations, for example, the type and dura-

Table 1
Histometric and Flow Cytometric Data Obtained from Fresh Colorectal Tumour Biopsies and Archival BrdUrd Labelled Colorectal Tumours

Factor	Mean	S.D.	Range
Series A: Flow cytometry of PCNA labelled tumours			
1. Total population PCNA labelling (%)			
Mech. cells (n = 42)	53.0	26.0	5.8–99.7 (a)
Coll. cells (n = 43)	49.7	22.5	7.1–96.2 (a)
Mech. nuclei (n = 22)	47.1	23.5	9.6–99.6 (b)
Coll. nuclei (n = 34)	38.7	25.2	4.7–91.9 (b)
2. Aneuploid population PCNA labelling (%)			
Mech. cells (n = 22)	55.8	24.1	10.0–91.0 (c)
Coll. cells (n = 19)	66.6	18.7	23.8–99.2 (c)
Mech. nuclei (n = 13)	50.6	23.3	10.7–87.5 (d)
Coll nuclei (n = 14)	40.3	26.3	6.7–86.9 (d)
Series B: Histochemistry of BrdUrd labelled tumours			
BrdUrd tumour cell labelling indices (%)			
LI% diploids (n = 25)	20.7	8.0	4.4–35.4
LI% aneuploids (n = 25)	21.5	10.1	8.5–57.8
All tumours (n = 50)	21.1	9.0	4.4–57.8 (e) (f)
PCNA labelling indices (%)			
All tumours (n = 50)	38.8	9.8	16.9–63.9 (e)
Diploids (n = 25)	37.7	8.4	20.3–49.8
Aneuploids (n = 25)	39.9	11.1	16.9–63.9
Series B: Flow cytometry of BrdUrd labelled tumours			
BrdUrd Labelling Indices (%)			
All tumours (n = 50)	12.4	6.5	3.2–38.2 (f)
Aneuploid FCM LI% (n = 25)	14.7	7.4	5.2–38.2

Letters in parentheses [e.g., (a)] denote data sets that were compared for significance. (a–d): no significant differences; (e) $P > 0.00001$; (f) $P > 0.001$.
S.D. = standard deviation; Mech. = mechanically extracted; Coll = collagenase extracted.

Table 2
S Phase Fraction Data for 17 Diploid Tumour Cell Samples Following Mechanical Extraction, Calculated by the Three Algorithms Rfit, SOBR-3, and SOBR-8

Factor	Mean	SD	Range	Correlation
PCNA total LI%	53.4	27.4	5.8–94.1	
SPF% (RFit)	16.4	6.6	3.8–27.8	0.27
SPF% (SOBR-3)	11.5	5.4	4.3–24.2	−0.33
SPF% (SOBR-8)	7.8	3.3	3.8–15.3	−0.12

Correlations were made by linear regression analysis with the total PCNA LI in the same tumour sample.

tion of fixation (12,13,22), the concentration of applied antibody (19), and the actual antibody employed (25).

In contrast to microscope-based histometric studies, multiparameter flow cytometry allows quantitative and cell cycle phase–related analyses of nuclear antigen expression in tumour cells to be undertaken, although under the very different preparative conditions. PCNA expression has been evaluated in a range of cell lines using FCM (18,28), but solid tumours are a much more complex cell system than are cell lines grown in culture. The applications and technical limitations of monoparameter flow cytometry in the study of solid tumours have been well rehearsed (2,8,10,13,24,27). For example, the S phase analysis data demonstrated considerable variation on a case-by-case basis in the SPF calculations by the RFit, SOBR-3, and SOBR-8 methods, even though these were performed upon identical DNA profiles derived from fresh and optimally prepared tumour material. These

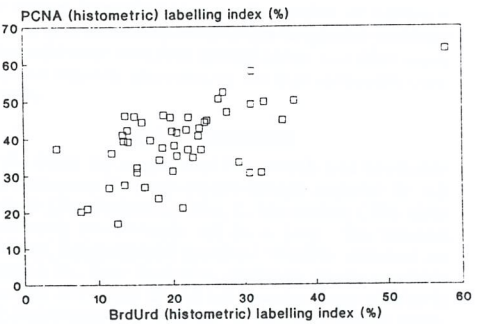


FIG. 7. This is a plot of the PCNA-PC10 and BrdUrd labelling indices calculated by microscope-based histometry in 50 colorectal tumours (Series B).

problems are compounded in multidimensional FCM analyses. Our study was thus designed to address a number of important technical problems in the flow cytometric analysis of PCNA in colorectal and other tumours. These include the following factors.

Firstly, the preservation of PCNA-PC10 epitope during the extraction of whole cells and nuclei from solid tumours is affected by the type and duration of fixation, and the method of disaggregation. Whole cells are more difficult to obtain, even from fresh biopsies of solid tumours, than are nuclear fragments. The extraction protocols were selected so as to preserve either whole cell or tightly bound nuclear PCNA. Mechanical mincing or grating procedures (7) appear to be adequate for extracting intact cells from colorectal tumour biopsies. Collagenase has previously been shown to produce high yields of viable whole cells from fresh tumours when compared with other enzymes (9). Mechanical or collagenase disaggregation made no difference to the results of the PCNA analysis.

Secondly, the epitope specificity, the batch of monoclonal antibody, and the choice of antibody-dye conjugate are important. Direct antibody-FITC conjugates may be preferable to indirect or multistage antibody marking in that they may simplify the preparation process. However, a two stage staining protocol using PC10 and an Ig-FITC conjugate (28) may decrease non-specific staining.

Thirdly, the evaluation of control antibodies and protocols is important. Autofluorescence is a variable feature of cell preparations, as is control antibody-associated fluorescence. We have shown that it is necessary to establish new control gates for each tumour analysed to prevent data from being heavily influenced by the inter-tumour variation in the baseline fluorescence. Nucleoprotein measurement must be undertaken with reference to a control specimen from which the PC10-FITC antibody has been omitted, because there is no discriminator on the continuum on the PCNA vs. DNA plot to distinguish labelled from unlabelled cells. Moreover, all cell and nuclear suspensions display a varying proportion of non-specific fluorescence attributable to both the isotypic control IgG binding and to intrinsic cell fluorescence. Some of this non-specific fluorescence can be intense, and a gate that was set on the control profile to exclude all non-specific fluorescence would also exclude most PCNA staining. The proportion and intensity of background staining also changes with the applied concentration of control antibody. There is no simple technical solution to the problem of control fluorescence subtraction, and in adopting our pragmatic solution we freely acknowledge the limitations of the technique, particularly in circumstances of low target protein positivity and high background fluorescence.

The flow cytometric measurement of PCNA expression in cell lines and haematogenous tumours has been reported reliably to yield an S phase fraction (16). In our series, all specimens studied appeared to stain satisfactorily for PCNA-PC10, but there was a wide variation in PC-10 labelling between tumours. Our FCM data indicate that PCNA labelling is substantial and that the protein is distributed through the cell cycle of colorectal tumour cells. This observation applies to both cells and nuclei. However, interpretation is handicapped by the inability of flow cytometry to distinguish easily diploid tumour cells from stromal cells, to identify those cells undergoing DNA repair, or to distinguish the transition between G0 and G1 cells. (This may be an important regulatory point for the initiation of PCNA production.) It is also interesting that the FCM assay only identifies around 65% PCNA positivity within the S phase (21). This may be an artefact of the technique, for example, due to S phase contamination with cell debris, or else the PC10 epitope may not be expressed in a significant proportion of proliferating cells.

In the parallel histochemical study of archival, formalin-fixed, BrdUrd-labelled colorectal tumours, we were able to compare directly the PCNA and BrdUrd labelling in serial sections (Table 1), and with flow cytometric data for this material (20). Histological BrdUrd labelling is significantly higher than corresponding FCM-derived labelling data because, unlike the human eye, FCM analyses cannot distinguish stromal from tumour cells (in the diploid compartment). PCNA labelling is seen consistently in a much higher percentage of tumour cells than is BrdUrd. In contrast, FCM appears to detect a much higher percentage of PCNA positivity than does histochemistry. However, direct comparisons of PCNA expression in the two series may be handicapped by differences in preservation and preparation.

In conclusion, PCNA analysis using FCM is a valuable model for the development of multiparametric FCM assays of nucleoproteins in solid tumours. Further studies of the expression of PCNA and of other nucleoproteins should pay careful attention to the evaluation and description of all conditions under which all analyses were undertaken, as even apparently minor variables can have a major influence on the data obtained in flow cytometric studies. Interlaboratory standardisation of multiparametric flow cytometry assays poses far greater problems than is the case with DNA analysis alone, and these issues must be urgently addressed by the flow cytometry community.

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