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S-PHASE FRACTION ANALYSIS IN DNA STATIC CYTOMETRY IN BREAST CANCER. COMPARISON WITH PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IMMUNOSTAINING

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ABSTRACT

The aim of our study was to evaluate human breast carcinomas for cell cycle data in cytologic material analyzed with DNA static cytometry (DNA SC) and to compare the results with the presence and distribution of PCNA immunostaining on tissue sections. The same cases were also investigated with a flow cytometric technique (DNA FC).
In three out of the six cases investigated, the static and flow cytometric analyses showed a histogram with a single G0-G1 peak containing the majority of the nuclei, accompanied by elements in the S and G2 compartments. As for the cell cycle data, good correspondence between the two techniques was observed, the closest being for the percentage of nuclei in S phase. The proportion of PCNA immunostained nuclei was high in all three cases. Those staining positively were subdivided into either intensely-stained nuclei with either a homogeneous or granular pattern, or lightly-stained nuclei with a granular pattern. The proportion of nuclei with the former appearance was similar to the percentage of S phase nuclei. In the remaining three cases, the static and flow cytometric analyses produced a histogram with two peaks, the first diploid and the second aneuploid, which were similar with both techniques. In these cases the cell cycle analysis for both peaks was performed only on the cytologic material using the two-cell-cycle option of the software. In the two cases where the two peaks were separated, information about the compartments was obtained and the average S-phase fraction was comparable to the proportion of intensely-stained nuclei with either homogeneous or granular pattern in the PCNA immunostaining. In case 5, the two peaks were closely positioned. Therefore, the cell cycle data were obtained only for the aneuploid population whose average S-phase fraction in DNA SC was lower than the percentage of intensely PCNA immunostained nuclei.
In conclusion, our study showed that cell cycle analysis is feasible in Feulgen-stained cytologic material in cases with either a single-cell population or with two distinct populations. The proportion of the most intensely-stained nuclei with the anti-PCNA antibody is generally comparable to that of the S phase nuclei.

Keywords: image analysis, static cytometry, flow cytometry, cell cycle analysis, PCNA.

INTRODUCTION

Cellular proliferation rate has been reported as one of the most important prognostic factors in cancer (Dawson et al., 1990, Fallenius et al., 1987, Kallioniemi et al., 1988, Lee et al., 1991). Recent reports

regarding this tumour capacity were carried out using flow cytometry, incorporation of radiolabelled material, such as tritiated thymidine, bromodeoxyuridine, and monoclonal antibodies for Ki-67 and proliferating cell nuclear antigen (PCNA). Also mitotic counts from tissue sections can be used (Baak et al., 1985, Collan et al., 1988, Haapasalo et al., 1989). The methods most commonly used are the thymidine labelling index and DNA flow cytometry (Meyer and Coplin, 1988, Silvestrini et al., 1985, Takahashi et al., 1991). Tritiated thymidine marks cells in the S-phase (replication of chromosomal DNA), and the S-phase cells are recognized by autoradiography. DNA FC can measure the nuclear DNA of individual cells and recognizes intermediate DNA content between that of G0-G1 and G2-M as a marker for S-phase cells. Presence of debris or sliced nuclei and artifactual cell-doublets can obscure the S-phase portion of the DNA histogram. DNA FC includes S-phase arrested cells in the S-phase count, whereas thymidine labelling does not (Schneller et al., 1987). These could be the reasons for the lack of a perfect correlation between the results of these two methods (Montironi et al., 1991). Apart from this, DNA flow cytometry is more widely used than the thymidine labelling index, because fresh, frozen and fixed material can be easily analyzed. However, there are cases or situations in which DNA FC is not feasible: the tumour nodule may be too small for both histologic study and DNA FC; tumour necrosis may be high; there may be too few tumour cells or they may be intermingled with normal cells; or lymphocytic stromal infiltration may conceal the neoplastic population in the DNA analysis. In these cases and situations DNA static (image) cytometry has been adopted, based on studies showing complementarity between DNA FC and DNA SC (Bauer et al., 1990, Cope et al., 1991). In particular, a previous paper from our group showed that the S-phase fraction of one-cell-cycle breast cancer can be evaluated in DNA SC with software commonly used for histogram analysis in flow cytometry (Montironi et al., 1991).

Proliferating cell nuclear antigen, also known or referred to as cyclin, is a recently-described 36 kD non-histone nuclear protein whose level of synthesis correlates directly with rates of cellular proliferation and DNA synthesis (Bravo and MacDonald-Bravo, 1987, Kurki et al., 1988, Morris and Mathews, 1989, Robbins et al., 1987, Takasaki et al., 1981, Takahashi et al., 1991). In fact, expression of PCNA is closely linked to the cell cycle, that is, elevated levels of PCNA appear in the nucleus during the late G1 phase immediately before the onset of DNA synthesis, peak during the S phase, and decline again during the G2 and M phases. Recent papers have shown that the immunological method of assessing this proliferating cell nuclear antigen has particular advantages over other techniques because the cellular and tissue architecture is maintained, the method is relatively simple and the results are rapidly obtained; neither *in vivo* nor *in vitro* labelling is required and the use of radioactivity is avoided (Coltrera and Gown, 1991, Woods et al., 1991).

The purposes of the present study were as follows: 1. to evaluate human breast carcinomas for the cell cycle in cytologic material using DNA SC; 2. to compare the results thereby derived with the presence and distribution of PCNA, visualised by an immunoperoxidase technique on formalin-fixed, paraffin-embedded tissue sections.

MATERIAL AND METHODS

The study was performed on six cases of infiltrating ductal carcinoma of the female breast which had been previously collected at the Institute of Pathological Anatomy and Histopathology of the Milan University School of Medicine, St Raffaele Hospital (Director: Prof. Angelo Cantaboni) for the purpose of evaluating the DNA index and the ploidy pattern. Fresh tissue was obtained from the surgical specimen at the time of frozen section. The tumour was divided into two parts. Imprints were obtained by touching the freshly-cut surface with glass slides. One part of the nodule was then fixed in 10% buffered neutral formalin for 12 to 18 h, dehydrated in alcohol (50%, 70%, 90% twice, 100% twice), cleared in xylene (twice), and embedded in paraffin for the histologic diagnosis and for the immunohistochemical evaluation with PCNA antibody, whereas the other part was used for DNA flow cytometry.

DNA static cytometry. DNA SC analysis was performed on imprints. The slides were air-dried for 30 min at room temperature, then fixed in 10% buffered neutral formalin for 30 min and washed in distilled water for 10 min. The imprints were then stained according to the standard Feulgen reaction procedure used in our Institute, ie, treated with 5 N HCl for 60 min and with the Feulgen stain solution for 60 min; then rinsed, washed, dehydrated, cleared in xylene and coverslipped (Montironi et al., 1991).

DNA SC analysis was performed with an IBAS Kontron image analyzer using the "DNA Measurement Software Package" (Release 2.0 - March 1991). Prior to measurements, the lamp was mechanically adjusted to obtain optimal light intensity and homogeneous illumination; measurements were started 15 min after switching on the system, including the lamp, ie, when the densitometric values had stabilized. Glare was corrected for by subtracting 10% of the 100% transmission level measured in a background area without visible particles. The integrated optical density of 1000 well-preserved and cytologically identified neoplastic cells was obtained by adopting a vertical stratified selection method at an objective magnification of 100 times (oil immersion; NA 1.32; filter 560 nm). Both the microscope image of each nucleus and the corresponding contoured image on the monitor were checked by the observer. The objects were accepted for analysis on the condition that the contoured one was satisfactory as evaluated by eye. This procedure allowed the interactive measurement of approximately 150 nuclei per hour.

The integrated optical density values (file format ASCII) were recorded in the IBAS host computer as a 256-channel histogram and analyzed using a software program written by Peter S Rabinovitch, commercially available as *MultiCycle* (Version 2.12; Phoenix Flow Systems, San Diego, CA). This is a highly sophisticated program, albeit fast and simple to use, employed in flow cytometers to calculate the cell cycle. The *MultiCycle* software for histogram analysis utilizes a curve-fitting system which ascribes a formula to the data. The parameters of the formula are adjusted iteratively until the best match between the formula and the data is obtained. The mathematical method of analysis used in this software, developed by Dean and Jett (1974), uses Gaussian distributions for the calculation of the G0-G1 and G2 curves, and a polynomial curve to calculate the S-phase. In a previous paper (Montironi et al., 1991), we gave some details of this software as applied to DNA SC data. In particular, a good correspondence in the cell cycle data between DNA FC and DNA SC was observed when the G2/G1 ratio was fixed and ranged from 1.95 to 2.00. The "Constraining the Ratio of G2/G1" option allows the position of the G2 peak to be restricted by constraining the ratio of the mean of the G0-G1 peak to the mean of the G2 peak. This ratio is usually near 2.0 or slightly less. This option is useful when the G2 peak is not distinct from the S-phase.

The following features were recorded: percentage of nuclei in G0-G1 (%G1), percentage of nuclei in S-phase (%S), percentage of nuclei in G2 (%G2), the G2/G1 ratio, and the coefficient of variation (standard deviation divided by the peak mean multiplied by 100) of the G0-G1 peak (CV G1). The DNA index was calculated as the ratio of the mean channel value of the G0-G1 peak of the tumour cell population to that of the G0-G1 peak of 100 small tumoral stromal lymphocytes (internal diploid reference). As shown previously (Montironi et al., 1991), before calculating the DNA index, the integrated optical density values of the lymphocytes had to be multiplied by a factor of 1.4 in order to compensate for the "proportionality error".

DNA flow cytometry. The analysis was performed on fresh tumour tissue. The specimen was mechanically and enzymatically dissociated and then stained with propidium iodine. Axillary lymph node tissue (without secondary deposits) was used as diploid reference. The nuclear suspensions were analyzed on a FACScan Becton Dickinson flow cytometer. A total count of at least 20,000 pulses was measured for the DNA distribution, displayed, and recorded as a 256-channel histogram. Cell cycle components (%G1, %S and %G2) as well as the G2/G1 ratio and CV G1 were evaluated, using the "DNA Cell-Cycle Analysis Software" (Ver C 5/87, Polynomial Model). The DNA index was calculated in a similar manner to the DNA SC data.

Immunohistochemical evaluation. Conventional histological sections (4 μm thick) were cut from formalin-fixed paraffin-embedded material. The sections were mounted on poly-lysine coated glass slides and air dried overnight at room temperature. The sections were dewaxed, taken through graded

alcohols and then immersed for 10 min in 25% phosphate-buffered saline in methanol, with 0.5% hydrogen peroxide to block endogenous peroxidase activity. The sections were then taken to water and immunostained with antibody to PCNA (PC10) using the ABC method (Dakopatts, UK), with primary incubation for 18 h at a dilution of 1:200. Diaminobenzidine-hydrogen peroxide was employed as a chromogen and a light haematoxylin counterstain was used.

Random fields were sampled with the aid of a random number table, and 1000 tumour nuclei were counted by one of our team (LD) using a x63 objective with an eye-piece graticule. The time needed to analyze each case was approximately 60 min. PCNA immunostaining was almost entirely confined to the nucleus and basically showed homogeneous, granular types of staining or a mixture of both. Nuclei with diffuse patterns appeared darker brown than the lighter granular and mixed patterns. The following features were recorded: percentage of stained or positive nuclei (%positive), percentage of darkly-stained nuclei with either homogeneous or granular pattern (%dark) and percentage of lightly-stained nuclei with granular pattern (%light). Mixed-pattern nuclei represented only a very small proportion of the stained nuclei and were grouped either with %dark or with %light, according to the prevalent pattern present in each nucleus. The intra-observer variation was 3%.

RESULTS

The six cases investigated were divided into two groups on the basis of the number of peaks present in DNA FC. One group contained three cases with one peak, that is, a single cell population (cases 1 to 3), whereas the other included the remaining cases which were characterized by two peaks, that is, two cell populations (cases 4 to 6).

As for the cases of the former group, the DNA FC analysis showed a histogram with a single G0-G1 peak containing the majority of nuclei, accompanied by elements in the G2-M compartment and in the S phase. All three cases showed a DNA index of 1.0 in both DNA FC and DNA SC. The DNA SC analysis based on the *Multicycle* software produced a histogram similar to those obtained in DNA FC, that is, a G0-G1 peak with a recognizable G2 compartment and an intermediate component corresponding to the S phase (Figure 1).

As for the cell cycle data, good correspondence between DNA FC and DNA SC was observed, the closest being for the %S values. The best agreement was present in case 3 (Table 1). The greatest discrepancy between DNA SC and DNA FC was observed in the G1 CVs whose values in static cytometry were more than double those in flow cytometry.

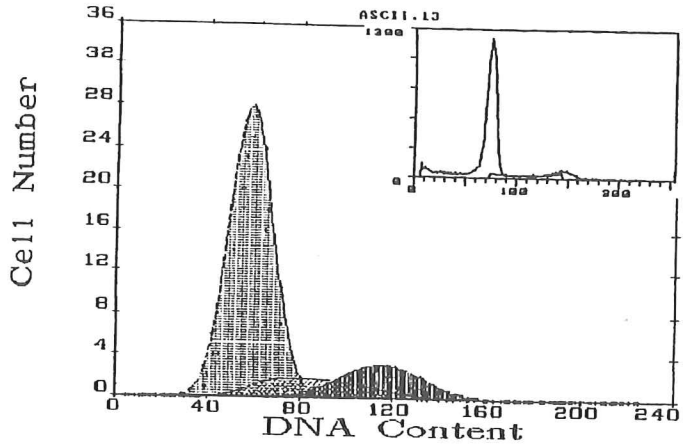

Figure 1.
Case 1: the DNA SC analysis based on the Multicycle software produced a histogram similar to those obtained in DNA FC (see inset), that is, a G0-G1 peak with a recognizable G2 compartment and an intermediate component corresponding to the S phase.

Table 1: Cell cycle data obtained in case 3 when the Multicycle software is applied to DNA SC values. The results are compared with those given in DNA FC

Cell cycle features	DNA SC*						DNA FC**
	1.95	1.96	1.97	1.98	1.99	2.00	
%G1	85.80%	85.70%	85.70%	85.70%	85.60%	85.60%	84.00%
%S	11.80%	12.00%	12.00%	12.20%	12.30%	12.40%	11.00%
%G2	2.40%	2.30%	2.30%	2.10%	2.10%	2.00%	5.00%
G1 CV	16.00	16.00	16.00	16.00	16.00	16.00	4.40

* Cell cycle changes according to the G2/G1 ratio fixed in the DNA SC data
** The G2/G1 ratio was 1.98

Table 2 shows the percentages of nuclei with different immunostaining patterns with PCNA. The proportion of stained nuclei was high in all three cases, the value being 62.00% in case 1, 53.00% in case 2 and 44.00% in case 3. The %dark/%light ratio was always below unity: 0.29 in case 1, 0.51 in case 2 and 0.51 in case 3. %dark values were the closest to the %S data, that is, %dark 14.00% vs DNA SC %S 12.00-15.40% (according to the G2/G1 ratios) and DNA FC %S 12.00% in case 1; %dark 18.00% vs DNA SC %S 21.50-22.60% and DNA FC %S 25.00% in case 2; %dark 15.00% vs DNA SC %S 11.80-12.40% and DNA FC %S 11.00% in case 3.

Table 2 : PCNA immunostaining data and %S values observed in DNA FC and DNA SC

features	case 1	case 2	case 3
PCNA immunostaining			
%dark	14.00%	18.00%	15.00%
%light	48.00%	35.00%	29.00%
%positive	62.00%	53.00%	44.00%
DNA FC %S	12.00%	25.00%	11.00%
DNA SC %S	12.00-15.40%	21.50-22.60%	11.80-12.40%

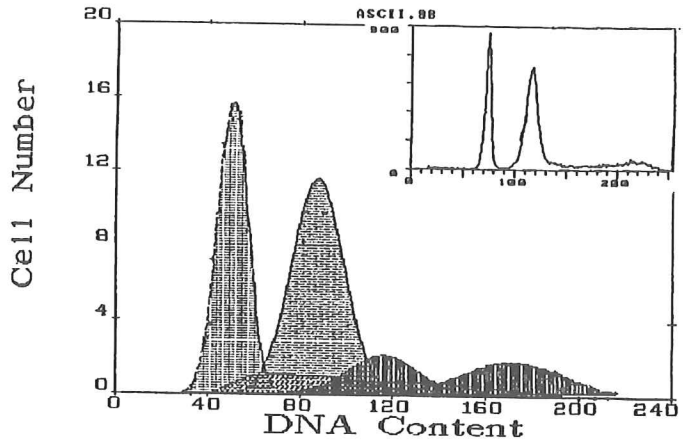

Figure 2.
Case 4: the pattern of the DNA SC histogram analyzed using the Multicycle software was almost identical to that obtained with the DNA FC (see inset), that is, two distinct peaks, the first diploid and the second aneuploid.

Three out of the six cases investigated were grouped together because the DNA FC analysis showed two distinct peaks, the first diploid and the second aneuploid. Unfortunately, the cell cycle had neither been evaluated in these three cases at the time of the original DNA FC analysis nor the values of the computer files stored for further analysis. However, the pattern of the DNA FC histogram was almost identical to that obtained with the DNA SC values analyzed using the *Multicycle* software. Since they deserve special attention, these three cases are briefly discussed later. For case 4 (Table 3 and Figure 2), the first peak was higher than the second which was considered aneuploid in both DNA FC and DNA SC. In DNA FC the ratio between the two peaks, that is, the DNA index, was 1.6. In DNA SC the mean G0-G1 channel value of the diploid cycle was 50.80, whereas that of the aneuploid population was 87.40, the difference being 36.60 and the ratio, or DNA index, 1.7. Cell cycle data for both peaks were obtained

Table 3: Cell cycle data obtained in the three cases with two peaks when the Multicycle software is applied to DNA SC values

Cell cycle features	case 4		case 5		case 6	
	D*	A*	D	A	D	A
%G1	75.20%	63.20%	100.00%	64.40%	52.60%	69.00%
%S	16.50%	23.30%	0.00%	19.00%	19.00%	20.50%
%G2	8.30%	13.50%	0.00%	16.60%	28.40%	10.50%
G1 CV	12.30	12.30	14.40	11.30	11.20	16.30
%total**	39.30%	60.70%	31.70%	68.30%	21.50%	78.50%

* D diploid; A aneuploid; ** %total indicates the proportion of the total number of nuclei measured present in each population

Table 4 : PCNA immunostaining data and %S values obtained from DNA SC analysis in the three cases with two peaks

features	case 4	case 5	case 6
PCNA immunostaining			
%dark	19.00%	21.00%	18.00%
%light	43.00%	45.00%	35.00%
%positive	62.00%	66.00%	53.00%
Average %S*	20.90%	13.00%	20.17%

* proportion of S phase nuclei to the total number of nuclei

using the *Multicycle* "two-cell-cycle" option. In the computer print out sheet the %S and %G2 values were preceded and followed by a question mark (?) because the values calculated were estimated by curve fitting in two populations which were closely positioned, therefore indicating a possible approximation in the calculation of the cell cycle compartments. 39.30% of the nuclei were allocated to the diploid population whose %G1, %S and %G2 were as follows: 75.20%, 16.50%, and 3.30% in %S, and 13.50% in %G2. The average %S, that is, the proportion in S phase compared to the total number of nuclei analyzed, was 20.90%. As for the PCNA, 62.00% of the nuclei were immunostained, %dark being 19.00% and %light 43.00%, with a ratio of 0.44. The %dark value was comparable to the average %S observed in DNA SC, that is, 20.90% (Table 4). For case 5 (Table3), in DNA FC the diploid peak was higher than the aneuploid whereas in DNA SC the second peak was higher than the first. In flow cytometry the DNA index was 1.6. In static cytometry the two peaks were closely positioned with some overlap, the latter feature being absent in DNA FC histogram. The mean G0-G1 channel value of the diploid cycle was 50.30, whereas that of the aneuploid population was 78.90, the difference being as small as 28.60 and the ratio (DNA index) 1.5. Therefore, when adopting the "two-cell-cycle" software option, 100% of nuclei of the diploid population, which accounted for 31.70% of the total population analyzed, were allocated to %G1 with 0.00% into %S and %G2, thus clearly underestimating the proportion of nuclei in the latter two compartments, whose elements were all included in the aneuploid population. The aneuploid, with 68.30% of the total population, was subdivided as follows: 64.40% into %G1, 19.00% into %S and 16.60% into %G2. The average %S observed in DNA SC was 13.00%. When considering the PCNA, 66.00% of the nuclei were immunostained. The %dark was 21.00% and %light 45.00%, respectively, with a ratio of 0.46. The former was much higher than the DNA SC %S (Table 4).

In case 6 (Table 3), the DNA FC analysis showed two very distinct peaks, the second being smaller and clearly broader than the diploid, and at a certain distance from it. Similarly, the histogram obtained in static cytometry showed the second peak broader, even though higher, than the first, the distance expressed as channel number being 86.2. The DNA index was 2.4 in DNA FC and 2.5 in DNA SC. The two populations were quite well-spaced, cell cycle analysis was feasible for both peaks. The diploid contained 21.50% of the total, with 52.60% in %G1, 19.00% in %S and 28.40% in %G2. The aneuploid population accounted for 78.50% of the total analyzed with 69.00% in %G1, 20.50% in %S and 10.50% in %G2. The average %S was 20.17%. When considering PCNA, 53.00% of the nuclei were immunostained. The %dark and %light were 18.00% and 35.00%, respectively, their ratio being 0.51. The former value was slightly lower than the average %S.

DISCUSSION

In a previous paper from our group (Montironi et al., 1991) it was shown that the S-phase fraction of one-cell-cycle breast cancer can be evaluated in DNA static (image) cytometry with software commonly used for histogram analysis in flow cytometry. In agreement with this study, the results of the present project show that cell cycle analysis can be performed even in cases with two populations on Feulgen-stained cytologic preparations.

To the best of our knowledge there have been no other attempts to investigate the feasibility of S-phase fraction evaluation in DNA histograms generated in static cytometry using flow cytometer software, this type of calculation being carried out routinely only in DNA FC. However, our methodological approach is very similar to that adopted by Felman et al. (1989) who assessed the reliability of DNA estimation on cytologic Feulgen-stained material from malignant lymphomas examined by image cytometry using a Quantimet 900 instrument. They applied Baisch's rectangular (planimetric) method (Baisch et al., 1975) and used manual calculations to evaluate the proportion of proliferating cells (S + G2-M) on 500 nuclei per case. Their study found a high coefficient of correlation between the values obtained in image cytometry and those in flow cytometry. In our study, some discrepancy in the %G1 and %G2 values exists between DNA SC and DNS FC. The probable reason is that the analysis in image cytometry was performed on tumour cells whereas in flow cytometry the cell suspension also contained lymphocytes, stromal cells and normal cells. This is in agreement with the recent study by Bauer et al. (1990), who made a prospective comparison of DNA quantitation by image and flow cytometry. They demonstrated that for some tumours, including breast cancers, there was a discrepancy in 10% of the cases because inflammatory and non-neoplastic cells present in the flow cytometry sample might have diluted the neoplastic population. On the other hand, in image analysis inflammatory cells are visually excluded and tumour cells are preferentially selected. Another reason for the mild discrepancy may be related to the fact that nuclear debris or fragments are generated together with nuclear clumping during tissue dissociation and then analyzed in flow cytometry (Bagwell et al., 1991, Kalionemi et al., 1988).

As for the CVs obtained in our DNA SC study, this can be seen as a serious problem, because large values preclude discrimination between two populations or their components (see case 5) with a similar DNA content. Wide CVs were observed by Lee et al. (1991) in some cases of near-diploid breast carcinomas whose imprints were analyzed with a CAS-200 image analyzer. However, these authors did not mention how the CV was calculated, and nor did Cope et al. (1991), whose study was partly conducted with an image analyzer similar to that used by Lee's group (1991). Cope et al. reported wide CV values in histologic sections of phaeochromocytomas which were similar to those obtained in our study. The same group found lower CVs when cytospin preparations were used. At this stage of our research we can only guess which reasons may be responsible for wide CVs and then try to avoid or solve them at a later stage. They could be related to sample preparation, including fixation, and staining. However, the present preparation and staining were planned according to the rules suggested in the literature (Dawson et al., 1990, Fallenius et al., 1987, Montironi et al., 1991).

In the recent medical literature there is a number of publications dealing with the proliferation assessment using anti-PCNA monoclonal antibodies (Bravo and MacDonald-Bravo, 1987, Kurki et al., 1988, Morris and Mathews, 1989, Robbins et al., 1987, Takasaki et al., 1981, Takahashi et al., 1991, Woods et al., 1991). In some of the papers the data are compared with the results of the DNA FC analyses performed in several types of human tumours, including breast carcinoma. Some of the studies have shown a linear correlation between the proportion of PCNA immunostained nuclei and the S-phase fraction, whereas others dealing with gastric cancer, breast cancer and with haemangiopericytomas have shown that the correlation is very poor because there appear to be more PCNA immunoreactive cells than expected. For instance, Garcia et al. (1989) observed that in breast carcinomas the anti-PCNA immunocytochemistry score was significantly higher than the DNA FC-determined proliferation index. They concluded that it remains to be explained why the percentages of positive tumour cells were much greater with the PCNA antibody method than with the percentage of S-phase cells calculated from flow cytometry histograms. In agreement with Garcia's data, Hall et al. (1990) reported that some doubts

remain as to the relationship between PCNA expression and cell proliferation in the context of at least some forms of neoplasia. Similarly, Dawson et al. (1990), in their study on the comparative assessment of proliferation and DNA content in breast carcinoma, observed that PCNA staining was greater than the S-phase fraction. Woods et al. (1991) assessed the PCNA immunostaining in primary gastrointestinal lymphomas. They observed high PCNA values which mildly correlated with the S + G2 + M phase fraction determined in flow cytometry. Our present study is in agreement with this type of observation. In fact, the proportion of PCNA-positive cells is almost double the number of S-phase nuclei in both static and flow cytometry.

In our study, in order to understand whether there was a way to improve agreement between the PCNA data and the S-phase values, we investigated the pattern of the PCNA staining and its degree of intensity, which were not identical in all the nuclei. Two main types of nuclei could be identified: one intensely-stained with either a homogeneous or granular pattern, and another more lightly-stained and granular. This is in agreement with the qualitative observation made by Celis and Celis (1985) according to which the variations in fluorescence staining pattern and intensity of the anti-PCNA antibody were cell cycle-dependent. In particular, they affirmed that cells in G1, G2 and mitosis exhibit weak staining with the antibody, while S-phase cells show variable patterns in terms of both intensity and distribution of the antigen. Early in S phase, PCNA is localized throughout the nucleoplasm with the exception of the nucleoli. A similar but stronger staining pattern is observed as cells progress throughout the S phase. At a later stage it is redistributed to reveal a punctated pattern with foci of staining throughout the nucleus. After the DNA synthesis has reached its maximum, the intensity of staining decreases. More recently Garcia et al. (1989) noticed some variation in the intensity of nuclear immunostaining in different positive cells. However, all immunostained nuclei, regardless of intensity, were scored as positive. When we first looked at the PCNA-stained sections, we had in mind the observation made by several authors according to which the number of positive cells were higher than the S-phase fraction. At the same time we were wondering whether only the most intensely-stained nuclei were comparable in number to the %S already estimated in static and flow cytometry. For this reason %dark and %light were counted separately. The results we obtained showed that %dark values were similar to those of the S-phase, the only discrepancy being observed in case 5. In this case, in fact, the two peaks were very close and the %S and %G2 associated with the first peak were not calculated by the computer. %S was therefore estimated only for the aneuploid population, whereas the %dark corresponded to the nuclei of both populations.

In conclusion, our study showed that cell cycle analysis is feasible in Feulgen-stained cytologic material in cases with either a single-cell population or with two distinct populations.The proportion of the most intensely-stained nuclei with the anti-PCNA antibody is comparable to that of the S phase nuclei, to which they might belong. Further studies are currently underway in our Institute to acquire additional information on the cell cycle related to PCNA through the use of quantitative microscopy.

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