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A comparison of proliferation markers (BrdUrd, Ki-67, PCNA) determined at each cell position in the crypts of normal human colonic mucosa

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SUMMARY

Samples of microscopically normal human sigmoid colon fixed in 70% ethanol from 15 patients who had received bromodeoxyuridine (BrdUrd) prior to surgery have been reanalysed using a combination of proliferation markers. The specimens have been immunostained for proliferating cell nuclear antigen (PCNA) and after microwave treatment, they have been stained for BrdUrd and Ki-67. The 15 patients selected comprised 5 patients whose mucosa previously gave high BrdUrd labelling indices in the crypt, 5 that gave median values for BrdUrd labelling and 5 that gave low values for bromodeoxyuridine labelling on a previous analysis using tissue fixed in 70% ethanol and formal saline and using a different antibody (Potten *et al.*, 1992). The relative levels of labelling at each cell position in the crypts has been compared using the 3 proliferation markers with the data being compared with the BrdUrd labelling as a standard labelling for S phase cells. One objective was to see whether all three proliferation markers discriminated equally well between the three groups of patient samples. The data show that the distinction between high, medium and low values seen with BrdUrd labelling was retained when Ki-67 immunostaining was analysed. PCNA immunostaining resulted in high levels of labelling and the different levels of labelling seen with BrdUrd and Ki-67 were largely lost.

INTRODUCTION

The measurement of proliferation in samples of tumour and normal human tissue particularly tissue associated with the gastrointestinal tract, has in the past been limited. The problems are: the availability of appropriate material, questions concerning in vitro incubation procedures, the use of radioisotopes in humans and the lack of markers that do not require the incorporation of molecules into the DNA. Over the last decade, advances have been made in the use of antibodies

resistant PCNA co-localises with BrdUrd (McCormick and Hall, 1992) ie it becomes an S-phase marker. PCNA is also expressed in areas of normal tissue adjacent to tumours (Hall *et al.*, 1990) it can be induced by growth factors (Baserga, 1991) and is associated with chromatin at all phases of the cell cycle after ultra violet exposure (McCormick and Hall, 1992) ie it might be associated with DNA repair activity.

Clearly PCNA can be present in many cells and its selective removal by various fixation and processing steps or detergent treatment results in the visualisation of different concentration or binding fractions. It can thus variably stain all proliferating cells or at the other extreme just S-phase cells. Using a small study in rats with Carnoy's fixation we demonstrated conditions where PCNA labelled all the proliferative cells in the crypt, ie, the growth fraction.

MATERIALS AND METHODS

Patients

The full details of the patients used are presented elsewhere (Potten *et al.*, 1992c). Briefly, 147 samples were obtained from 75 patients (ages 45-86 years) who consented to receive a single intravenous dose of 250 mg 5-bromo-2'-deoxyuridine administered 2.4-16h before surgery. The samples were taken from ostensibly normal tissue more than 5 cm from surgically resected tumour. Strips of mucosa from the sigmoid colon were dissected and stored in 70% ethanol at -20°C.

Tissue and fixation

The ethanol fixed material was processed to wax by standard methods using a Vacuum Infiltration Processor (Bayer UK Ltd). 5 samples (patients) were selected on the basis of previous BrdUrd labelling as having high labelling levels, 5 as having median labelling and 5 as having low labelling levels for analysis here. The earlier BrdUrd studies made use of a post-ethanol fixation in formal saline and the slides were not subjected to microwave treatment. Since archival material was being used and the availability of *in vivo* labelling in tumour is scarce other fixation protocols could not be studied.

Antibodies

PCNA immunochemistry was performed using a monoclonal murine anti-PCNA antibody, PC10 developed by Waseem and Lane (1990) and now commercially available from Dako UK Ltd. Polyclonal rabbit and anti-human Ki-67 raised against synthetic Ki-67 polypeptide (Dako UK Ltd) was used to stain Ki-67 antigen. A mouse monoclonal anti-BrdUrd obtained from Dako UK Ltd was used to stain BrdUrd . All other sera and immunoreagents were from Dako UK Ltd. Dilutions were made in TBS (tris-HCI 0.05 M pH 7.6 NaCl 0.15 M).

Immunochemistry

3µm sections were cut onto silane coated slides and dried overnight at 37°C, 36 sections were cut serially from each specimen, and then 12 sections were non-serially selected for each of the 3 immuno-staining groups. The microwave time was selected by experimentation for Ki-67 and BrdUrd. Microwaving for PCNA immunostaining made no detectable difference and so was not used further. Although not originally planned, the use of ethanol as a fixative allowed all three markers to be used on semi-serial sections and hence permitted a good comparison.

Microwave method

The slides were dewaxed and rehydrated then placed in two plastic racks (R A Lamb, London UK), using every other space, and immersed in 1 litre of 0.01 M citrate buffer pH 6.0 in a microwaveable plastic bowl and microwaved for 25 minutes on high power in a Matsui model M180TC oven. Afterwards the slides were left in the hot solution for 15 minutes prior to cooling in running tap water then soaking in TBS.

The section were stained for PCNA, Ki-67 and BrdUrd as follows.

PCNA

Slides were dewaxed in xylene, rehydrated through graded ethanol and soaked in TBS then incubated for 10 minutes in 10% normal rabbit serum prior to incubation with PC 10 antibody diluted 1/100 or as a control in TBS and incubated overnight at 4°C. After washing in TBS, sections were incubated at room temperature in 1/400 biotinylated rabbit and anti-mouse $f(ab')_2$

that recognise various cell cycle related proteins, eg Ki-67, PCNA. However, the practical application of these has suffered because of the necessity in some cases to use frozen tissue sections and the highly variable results attributable to differences in fixation, tissue processing and immunohistochemical methods. Recent developments in microwave technology for antigen retrieval and the standardisation of fixation, handling and processing stages have suggested that these techniques now should have wide application.

There have been relatively few studies using DNA precursors in vitro to label the S phase fraction of cells. We have recently reported the BrdUrd labelling of S phase cells in a large series of ethanol and formalin fixed samples of human mucosa which had been labelled with BrdUrd intravenously prior to surgery (Potten *et al.*, 1992 a, b and c). These studies defined in detail the distribution of BrdUrd labelled cells in the human crypt and showed that peak levels of labelling (S phase cells) were observed at around the 15th-16th cell position from the bottom of the crypt in both the rectum and colon. The absolute levels of labelling differed slightly in these two regions with peak values of around 22% labelling in the rectum and peak values of nearly 30% in the colon; the differences probably reflecting differences in the fraction of proliferating cells (growth fraction) to differentiated cells (goblet cells and mature columnar cells) and in cell cycle characteristics. The studies also showed that there was very considerable variability from individual to individual, an observation also reported elsewhere (Balzi *et al.*, 1993) part of which may have been due to variation in preparative protocols. Some patients gave inherently low readings for the BrdUrd labelling while others gave high readings or intermediate ones.

This present study has been made to compare the usefulness and application of various other cell proliferation markers and techniques. Here, we have reanalysed 15 of the original 75 specimens selecting 5 that gave the high labelling index, 5 that gave the low labelling index and 5 around the median level of labelling. This was done in order to check the validity of the previous BrdUrd readings, but also to provide samples that might have different proliferative characteristics to test other proliferative markers. Tissue was cut from the original specimens, which were stored in ethanol, processed to wax and new sections have been cut. We have immunostained semi-serial sections for BrdUrd, Ki-67 and PCNA and compared the results. It was found to be necessary to microwave the sections used for BrdUrd and Ki-67 immunostaining but not PCNA immunostaining.

Antibodies to BrdUrd recognised BrdUrd in a single stranded DNA (produced by partial denaturation of double stranded DNA) (Gratzner, 1982). BrdUrd is incorporated during the S phase of the cell cycle, ie DNA synthesis, less efficiently than its normal counterpart thymidine. However, because of its specificity for DNA synthesis it is a reliable marker for S phase cells. The antigen recognised by Ki-67 antibodies is claimed by some to assess the growth fraction of normal tissues, since it is expressed throughout the cell cycle (Gerdes *et al.*, 1984). The function of Ki-67 antigen, a protein doublet of 345 and 395 kDa, is not known but it may be a structural protein that maintains the high order structure of DNA during mitosis (Sawhney and Hall, 1992). Ki-67 antigen can be recognised by antibodies in paraffin sections after microwave treatment. (Cattoretti *et al.*, 1992). Proliferating cell nuclear antigen (PCNA) is a 36 kD auxiliary protein to DNA polymerase delta (Bravo *et al.*, 1987). Monoclonal antibodies have been made to genetically engineered PCNA (Waseem and Lane, 1980). One of these, PC10, has been shown to demonstrate proliferating cells in conventionally processes tissue (Hall *et al.*, 1990). PCNA has a relatively long half-life and detectable levels of PCNA persist after cells exit from M-phase. Contrary views have been expressed on the correlation between Ki-67 and PCNA staining (Leonardi *et al.*, 1992, Rosa *et al.*, 1992). However two recent studies (Cher *et al.*, 1995 and Sallinen *et al.*, 1994) suggest that Ki-67 is the preferred marker of cellular proliferation for pathological purposes. This is probably explained by the complexity of the immunostaining variables of PCNA (Hall *et al.*, 1990) and the complexity of the relationship between antigen levels and the cell cycle (McCormick and Hall, 1992). PCNA levels rarely become undetectable in formaldehyde fixed cells (Bravo and McDonald-Bravo, 1987) and detergent

fragments for 30 minutes, washed in TBS and finally incubated in streptavidin ABC-HRP for 30 minutes followed by washing in TBS. The stain was developed in 0.03% H_2O_2 0.5 mg/ml 3.3, diaminodenzidine 4-HC1 (Sigma) in TBS for 7 minutes, washed in water and counterstained in stabilised haematoxylin activity 1 (Life Sciences International, Ltd, UK) prior to dehydration and coverslipping.

Ki-67

The microwaved slides were incubated in 10% normal swine serum for 10 minutes followed by rabbit anti Ki-67 diluted 1/50 for 16 hours at 4°C. The following day the slides were washed in TBS and incubated at room temperature in 1/400 biotinylated swine anti-rabbit $f(ab')_2$ fragments for 30 minutes washed and treated as above for PCNA.

BrdUrd

The microwaved sections were denatured in 1M HCl for 15 minutes at 37°C, washed in water then TBS. The sections were incubated in 10% normal rabbit serum for 10 minutes followed by mouse anti-BrdUrd diluted 1/30 for 16 hrs at 4°C. The slides were washed in TBS and incubated in biotinylated rabbit anti-mouse $f(ab')_2$ fragments as above and then treated as described for PCNA.

Contiuous tritiated thymidine labelling in outbred Wistar rats

Two pairs of rats (150 g body weight) were injected with tritiated thymidine (^{3}HTdR) (1μCi/g or 37 kBq/g). The first pair received one injection and were killed 40 minutes later. The second pair received 3 injections 6 h apart with sacrifice 40 minutes later, ie 12 h 40 min after the first injection. Ileal samples were fixed in Carnoy's fixative and sections were prepared for autoradiography after staining for PCNA. The frequency of single and double labelled cells was recorded on a cell positional basis. This experiment was used to check the comparability of PCNA staining with repeated ^{3}HTdR labelling which labels all the proliferating cells, ie the growth fraction. It was performed in rats since it could not be undertaken in humans.

Scoring

The scoring of all samples made use of the crypt cell positional scoring techniques and statistical methods described elsewhere (Potten *et al.*, 1982; Chwalinski *et al.*, 1988; Potten *et al.*, 1990). Briefly, the labelling characteristics of at least 50 half crypt sections selected on the basis of their longitudinal profiles were recorded on a cell position by cell position basis starting at the base of the crypt. In a few of the human samples only about 10 crypt sections were scored because of orientation difficulties and the size of the piece of tissue. For each of the rats 100 half crypt sections were scored. Cell positional scoring in this way permits a comparison of proliferation characteristics of cells in relation to their hierarchical or cell lineage status (Potten *et al.*, 1982). Cells at or near the crypt base are likely to be primitive cells or stem cells, those in the mid-crypt region rapidly dividing transit cells and the upper regions of the crypt contain cells decycling associated with a differentiated function.

RESULTS

Continuous labelling in rats

The results reported in Figure 1 show that the levels of ^{3}HTdR labelling in the rat increase with increasing number of injections of ^{3}HTdR reaching levels of about 95% in the mid crypt region after the third injection ie, they rise from about 55% after one injection to about 95% after the third. At this time, the levels of labelling correspond reasonably well with the PCNA labelling suggesting that on the whole the latter does measure the growth fraction when Carnoy's fixation is used. There are slight differences in the upper half of the crypt between the PCNA labelling in rats receiving one or three injections of ^{3}HTdR.

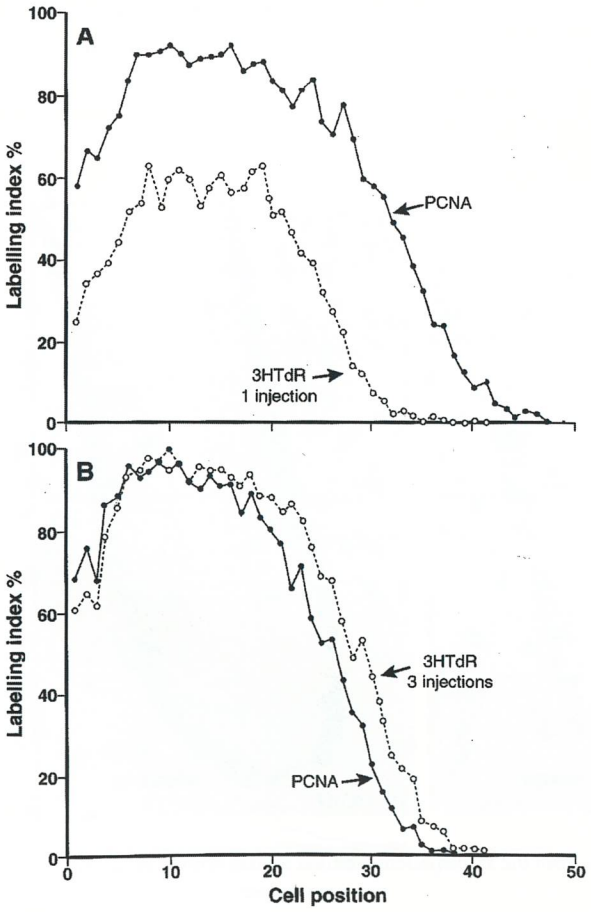
Human tissues

Figure 2 shows the typical patterns of immunostaining seen with BrdUrd, Ki-67 and PCNA for the human colon.

BrdUrd labelling

Figure 3 shows that microwave treatment of the sections results in labelling index frequency plots that have the same general shape as seen previously (Potten *et al.*, 1992, band c) with peak values at cell positions 10-20. However, the microwaving results in 5-10% more labelled cells at the peak and in a shift to the right in the distribution of about 10 cell positions. The absolute position of the higher labelled cell is, however, about the same at cell position 75-85. The group representing low BrdUrd labelling when analysed previously showed a shift to the right after microwaving in the present reassessment, but no increase in labelling levels at the position of the peak.

For the 3 groups, the previously high, medium and low labelling were still showing these same trends after microwaving.



Ki-67 immuno-staining

The results for the 3 BrdUrd groups when analysed for Ki-67 are shown in Figure 4. The 3 groups clearly retain a differential staining intensity, however, the frequency of Ki-67 stained cells is generally about 25-30% higher in all groups but the position of the labelled cells in the crypt ie, the shape of the frequency plot is very similar. Ki-67 is thus labelling more cells than BrdUrd ie, more than just the S phase cells, but as will be shown below Ki-67 labels fewer cells than PCNA.

PCNA immuno-staining

PCNA immunolocalisation is seen at levels of 70-80% at the base of the crypt (Figure 5) and the labelling declines slightly up to about 55th cell position where the labelling index is 50-60%. Thereafter, it declines more sharply to reach zero at about cell position 90 only slightly higher than for Ki-67 or BrdUrd (about cell position 70-80). There is little difference in labelling with PC10 between the high, median and low BrdUrd labelling groups unlike the situation seen after Ki-67 labelling.

PCNA may be detecting nearly all actual and potential (Go) proliferating cells, ie the growth fraction. The unlabelled cells after PCNA staining probably represent the fully functional, non-proliferative, differentiated cells such as mature goblet and endocrine cells.

Figure 6 summarises the data for the three proliferation markers. As can be seen from the difference plots (Fig. 6B) up to 25-30% more cells are labelled in the lower to mid crypt region by Ki-67, but the overall shape of the curve is simi-

Fig. 1 - Labelling index values against cell position in the crypt, with the bottom of the crypt to the left of the diagram. Frequency plots are shown for ^{3}HTdR (open circles) and PCNA (solid circles) labelling for 100 crypts scored from each of 2 rats at each time. Data have been smoothed over 3 cell positions.

1A shows the tritiated thymidine labelling 40 mins after a single injection in comparison to the PCNA labelling index for the same sections from 2 rats.

1B shows the tritiated thymidine labelling curve 40 mins after the third injection of tritiated thymidine each being spaced 6 h apart compared with the PCNA labelling for these two rats. The 2 curves are much more similar and both approach 100% in the middle regions of the crypt.

lar to that for BrdUrd suggesting that Ki-67 detects more than just S phase cells, but that the extra Ki-67 labelled cells are in the same relative proportions as the length of S (Ts) to the duration of the cell cycle (Tc) ie, Ki-67 is labelling S-phase plus a constant fraction of the cell cycle. The PC10 antibody to PCNA detects about 45% more cells at the crypt base, 10-15% more cells at cell position 20 and up to 35% more cells at about the 60th cell position. If PCNA immunostaining is labelling most of the proliferative cells (the growth fraction) ie, detecting cells at all (or most) stages of the cell cycle and any cells in G_0 then the difference plot (Fig. 6C) suggests that the cell cycle duration differs significantly with cell position - an observation measured and reported for murine small intestine (Potten, 1986). The rat data suggest that PCNA is indeed detecting something close to the total proliferative population (growth fraction). There is less difference in shape between the PCNA and repeated ^{3}HTdR labelling data in the rat suggesting that their may be less variation in cell cycle duration with cell position in the crypts of the rat.

DISCUSSION

Technical considerations

In the original experiments (Potten *et al.*, 1992c) the tissue was doubled fixed, first in ethanol and then in buffered formalin, this made the tissue very variable in staining intensity by routine immunohistochemical methods. When this tissue was microwaved in an attempt to give more consistent results, then virtually all cells in the specimen immunostained positively for PCNA. The double fixation probably fixed the majority of the PCNA in the tissue by a combination of coagulation and cross-linking, and then the microwaving lowered the immunological detection threshold (McKee *et al.*l, 1993). Ki-67 immunostaining did not work on this double fixed tissue. The use of ethanol fixation alone gave a more consistent staining pattern for PCNA, with or without microwave treatment and allowed staining for BrdUrd and Ki-67 if the sections were microwaved. This is surprising as most uses of microwaves for immunostaining have been directed against formalin fixed tissue. However, this could

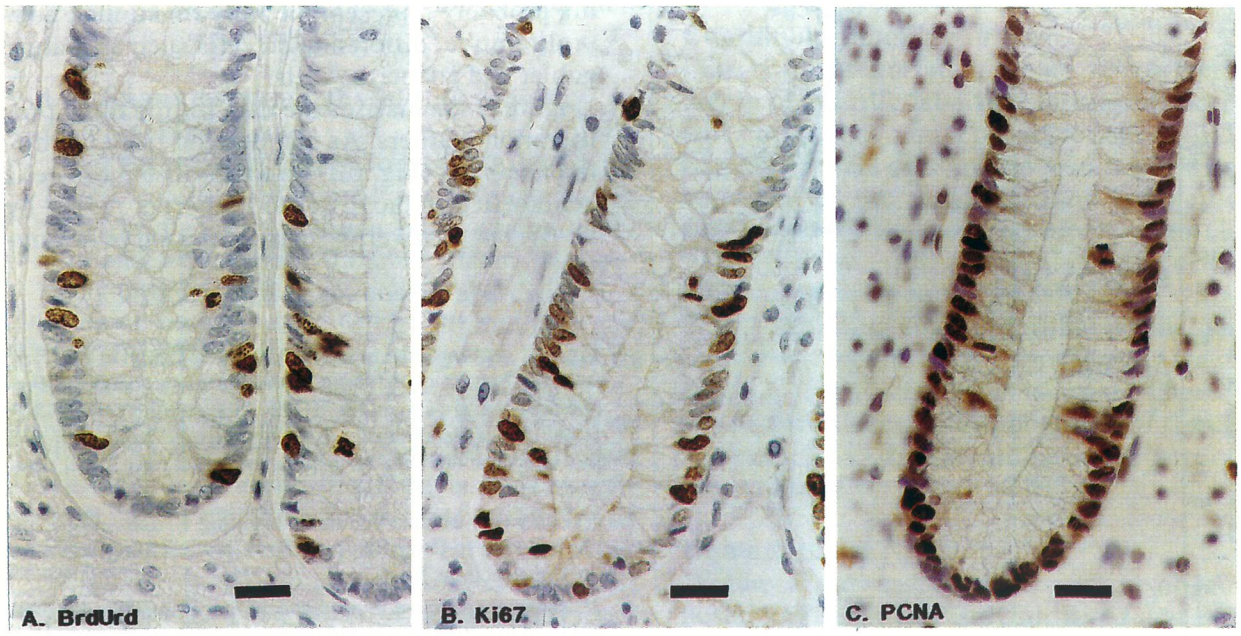
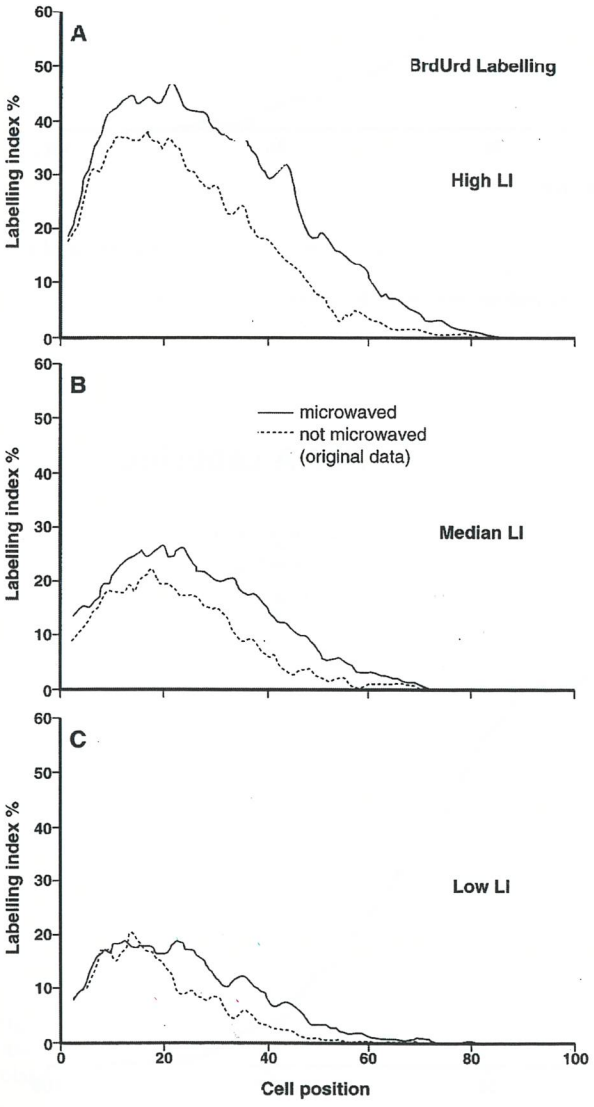


Fig. 2 - Photomicrographs showing the pattern of labelling in sections from the same patient labelled with bromodeoxyuridine (A), Ki-67 (B) and PCNA (C). All X500. Bar represents 20μm.

be because the tissue was stored in alcohol for several months.

Exactly what is being demonstrated by PCNA staining is confused and depends on many factors (Hall *et al.*, 1990) including the type and duration of fixation, the processing time, the concentration and type of the primary antibody and exactly what is being counted as a labelled cell (ie, visual detection threshold). Other factors include microwaving and increasing the sensitivity of the staining method, these tend to reverse the negative aspects of the factors mentioned above.



The caveat that applies to using different antibodies to PCNA probably also applies to the type of cells used in the study, for example cells *in vitro*, in frozen sections or in paraffin sections. Even when the cells are fixed in the same fixative they may well behave differently because they have variable amounts of damage caused by cut membranes, freeze-thawing or different processing reagents. Whilst this applies to any antigen stained in cultured cells or frozen or paraffin section it is particularly relevant for PCNA because of its dual location and different forms.

Biological implications

BrdUrd and ^{3}HTdR delivered as pulse labels reliably detect the S phase fraction of a tissue. Several studies have shown that they label the same cohort of cells (eg Chwalinski *et al.*, 1988). These techniques can be regarded as the "gold standards" for cell proliferation studies. Thresholds for detection of ^{3}HTdR labelled cells in autoradiographs can be precisely set but this is more difficult with immunohistochemical reactions. However, in practice this does not seem to be a major problem.

Differences in the S phase fraction (labelling index) with BrdUrd or ^{3}HTdR such as seen here with the three groups of human samples most likely reflects differences in cell cycle duration (ratio of S phase to the total cycle length) and/or (a) differences in the growth fraction (proportion of proliferative cells to non-proliferative differentiated cells) or (b) differences in the proportion of quiescent (Go) cells.

The pattern of BrdUrd labelling in the human colon (Fig. 3) is similar in shape to that seen in the rat for ^{3}HTdR (Fig. 1) and in the mouse (Potten *et al.*, 1992a) with low levels of label at the crypt base and a peak of labelling in a broad band in the middle of the crypt. The lower labelling at the crypt base in the mouse has been attributed to the presence or differentiated non-pro-

Fig. 3 - A comparison between the bromodeoxyuridine labelling with (solid line) and without (dashed line) microwaving action on the sections. Panel A for the 5 patients with a high labelling index; Panel B for the 5 patients with the median labelling index; and Panel C for the 5 patients with the low labelling index. In all 3 cases, the microwaving results in slightly higher readings.

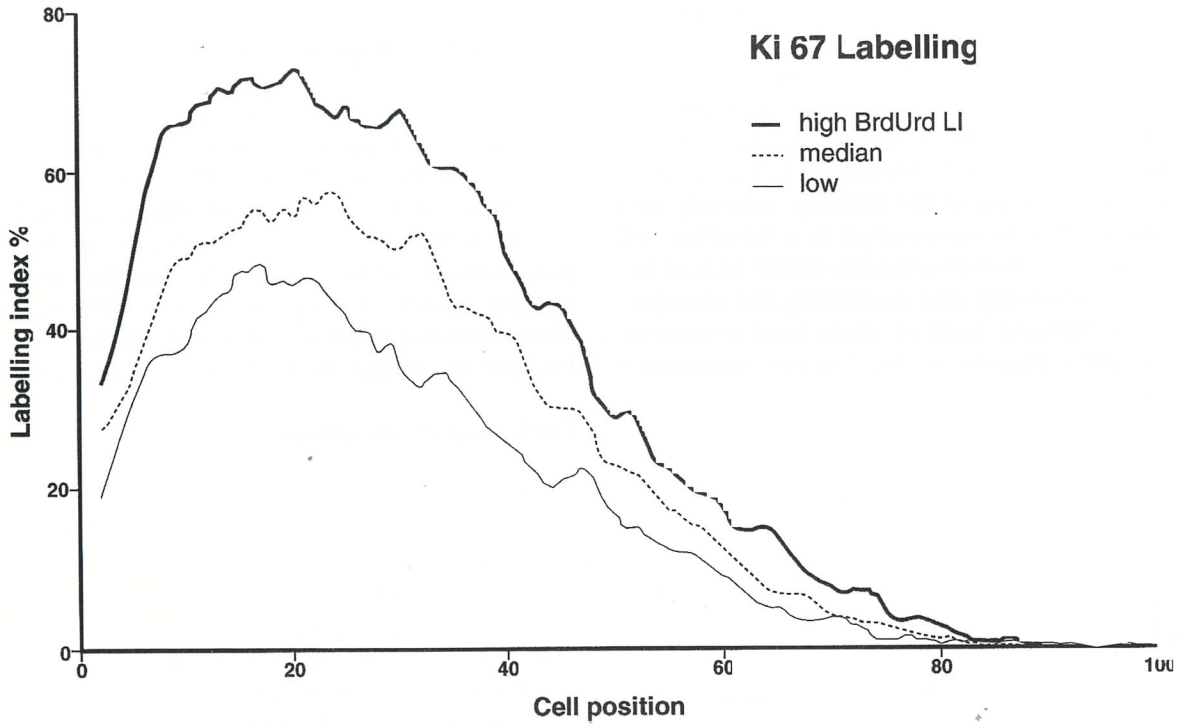
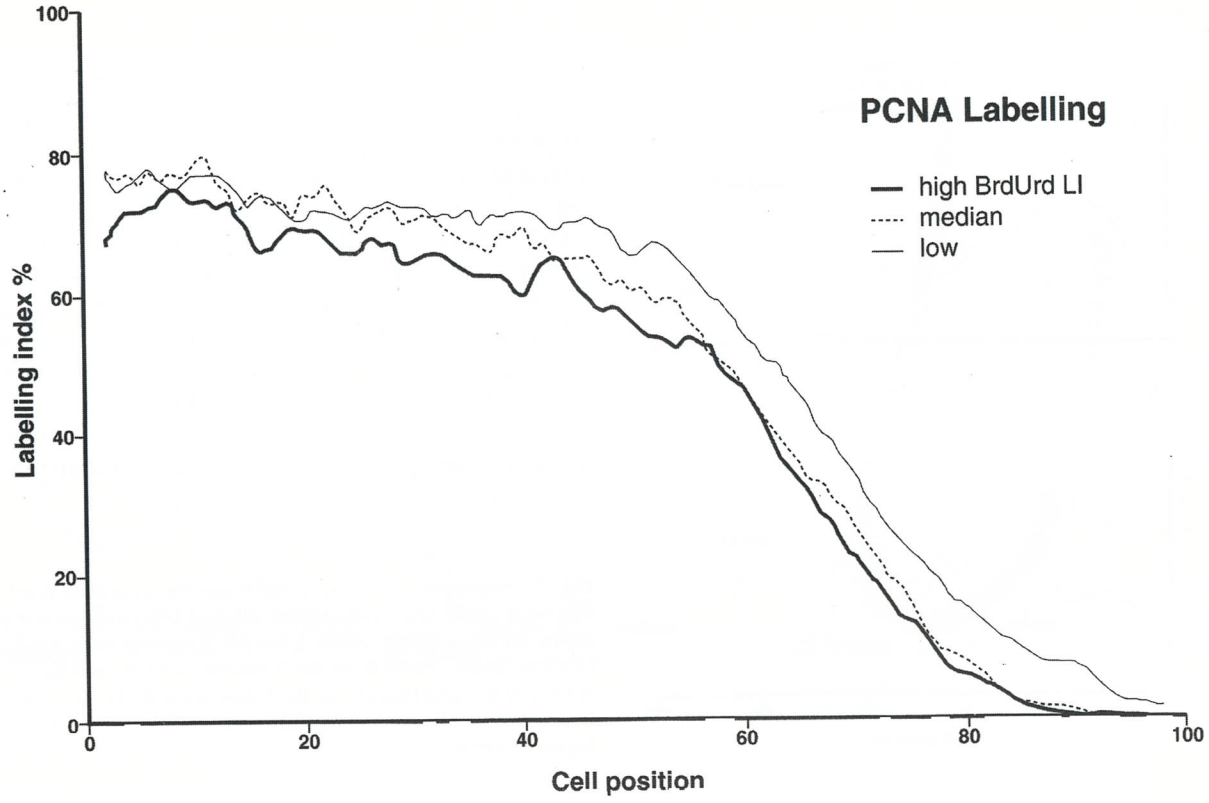


Fig. 4 - A comparison of the Ki-67 labelling in the 5 patients that had high bromodeoxyuridine labelling, median and low labelling. For the Ki-67 labelling, the same relationship is seen between the 3BrdUrd groups. In all cases, the curves are somewhat higher in terms of labelling index than the corresponding curves for bromodeoxyuridine, but show the same general shape.



liferative cells and proliferating cells with a longer cell cycle (Potten, 1986). The main difference between human and rodent, other than turnover, is in the size (length) of the crypt. When this is taken into account (Potten *et al.*, 1992a) the shapes of the curves are very similar. The amplitude (height) of the peak tends to be lower in humans, suggesting a longer cell cycle or lower growth fraction in the mid-crypt.

Ki-67 labels significantly more cells than BrdUrd (Figs 4 and 6) and this clearly labels more than just the S phase fraction. The difficulty is in knowing exactly how much more. Does it label all cells in G_2 and G_1 and thus essentially all the cycling cells? The results would suggest that Ki-67 is detecting S phase cells plus a constant fraction of the rest of the cycle, perhaps all the cell cycle except G_0.

Early studies with human PCNA auto-antibodies (Bravo and McDonald-Bravo, 1987b) demonstrated the existence of two populations of PCNA, one at sites of DNA replication seen after methanol fixation. The other nucleoplasmic form seen together with the first type after formaldehyde fixation. A similar situation was seen with PC10 antibody, where granular staining was seen after fixation with organic solvents and granular plus diffuse staining after fixation with formalin (Hall *et al.*, 1990).

Different PCNA antibodies recognising different epitopes of the molecule have different properties because the epitopes are affected in different ways by fixation and processing (McCormick and Hall, 1992). Conclusion drawn from immunostaining PCNA with one antibody cannot be applied in general to all PCNA antibodies. For example PCNA detected by the 19A2 antibody after methanol fixation is closely associated with S-phase (Galand and Degraet, 1989). However this was not seen with PC10 except under special conditions for flow cytometry (Beppu *et al.*, 1994).

When tumour tissue sections were extracted with detergent prior to fixation in methanol, the resulting PC10 stained cells were found to be equivalent to S-phase cells by double labelling with BrdUrd (Sasaki *et al.*, 1994). This was also found in lymphocytes when PCNA staining using PC10 was compared with ^{3}HTdR pulse labelling (Galand *et al.*, 1995). McCormick *et al.* (1993) found that PC10 at a dilution of less than 1 in a 100 stained 100% of the growth fraction of formalin fixed paraffin embedded xenografts compared to 42% stained for Ki-67 with the MIB-1 antibody (Cattoretti *et al.*, 1992). A much smaller proportion of cells stained with PC10 in the granular pattern. The PCNA staining reported here after fixation with ethanol obviously represents S-phase cells plus cells containing nucleoplasmic PCNA.

The situation with PCNA is the most complex, difficult to interpret and because of previously mentioned factors the most difficult of the antibodies to use. A difference is seen with PCNA when the shape of the cell position versus labelling index curve is compared between rodent and man. In the rat the PCNA curve falls at the bottom of the crypt reflecting the fall also seen with ^{3}HTdR (Fig. 1). In man it is much flatter at all cell positions (Fig. 5 and 6). This may be a reflection of (a) an absence of G_0 cells in the rodent and/or (b) the occurrence of differentiated functional cells (Paneth cells) in rodent ileum that are absent in human colon. PCNA used in the way shown here may be a useful detector of the total proliferative compartment (cycling and quiescent G_0 cells). In the rodent, labelling reaches 90% in the mid crypt suggesting that the growth fraction here is close to unity. In human colon the growth fraction as detected by PCNA is about 75% for the lower and mid-crypt region. Here the non-growth fraction is assumed to be comprised mainly of functional differentiated cells (such as goblet cells) which can occur at many cell positions in the crypt.

In the rat experiment (Fig. 1) the repeatedly ^{3}HTdR labelled samples gave slightly higher labelling than the PCNA in the upper regions of the crypt. This is undoubtedly due to the fact that cells are moving up the crypt dur-

Fig. 5 - The mean labelling index for PCNA for the high bromodeoxyuridine labelling, median and low labelling groups. In this case, all 3 curves are much more similar, the curves have a different shape to the Ki-67 and bromodeoxyuridine, particularly in relation to the lower cell positions.

ing the course of the ^{3}HTdR administration.

The slight differences between the two PCNA labelled samples in the rat (Figs 1A and B) may be due to (a) sampling problems associated with the small group size (b) circadian variations, since the group injected three times were sacrificed at a different time of day or (c) variations in crypt length due to either of the two preceding processes. The latter could be accomodated by a crypt normalisation adjustment.

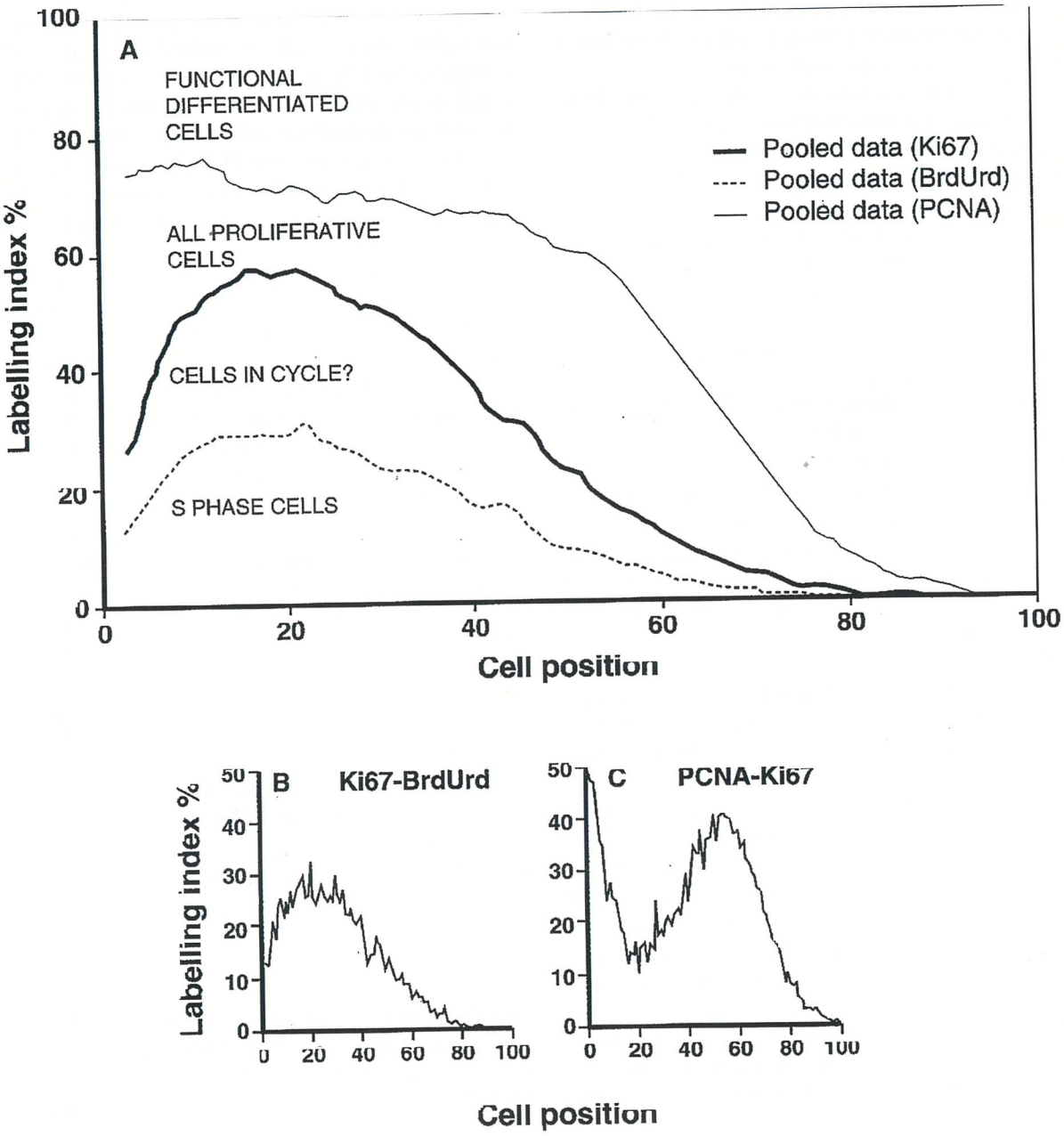


Fig. 6 - The overall mean of all 3 bromodeoxyuridine labelling groups for bromodeoxyuridine, Ki-67 and PCNA with the inserts 6B, showing the difference between the Ki-67 and the bromodeoxyuridine curve, ie, the cells that are exclusively detected by Ki-67, after the S phase cells have been subtracted. Panel 6C shows the difference between the PCNA and Ki-67 labelling, ie, the cells that are exclusively detected by PCNA. The possible populations of cells marked by the three techniques are indicated in Panel A (for more detail see text).

In the human, the PCNA labelling index is at its maximum level at low cell positions. The long half-life of PCNA compared with Ki-67 may generate PCNA staining higher up the crypt. Because of this long half-life the cut-off point between a positively stained cell and a PCNA negative cell may also be harder to determine because of a more gradual tailing off of the PCNA antigen concentration.

CONCLUSION

BrdUrd is the most reliable and robust immunohistochemical proliferation marker because it identifies only S-phase cells. PCNA when fixation, processing and immunostaining are optimised labels the greatest proportion of crypt cells probably the entire proliferative compartment including any G_0 cells. Ki-67 appears to label a proportion of cells intermediate between the two, probably the S-phase cells plus some constant fraction of G_0 and/or G_1 but perhaps excluding the portion of G_1 associated with major variation in cell cycle duration and excluding any cells in quiescence or G_0.

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