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Cell proliferation, tumour growth and clinical outcome: gains and losses in intestinal cancer

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Cell proliferation is a key feature of the biology of normal intestinal mucosa and of malignant tumours. There have been numerous studies of proliferative indices in human intestinal cancer, using techniques such as tritiated thymidine (3H-TdR) labelling of tissue sections, stathmokinetics, and flow cytometry (FCM) derived S phase fraction (SPF) measurements. Mitoses can be counted in conventionally stained tissue sections, but they identify only a small fraction of the cycling population. Endogenous cell markers of proliferation such as proliferating cell nuclear antigen (PCNA) and Ki-67 have yet to prove their utility. With the exception of stathmokinetic studies, which measure the rate of accumulation of mitoses after metaphase arrest by agents such as colchicine *(1)*, and are very laborious, none of these techniques can be used to estimate *the rate of cell production* in clinical tumours.

The cell cycle concept facilitates the analysis of cell proliferation *(2)* (Fig. 1). 'Cell kinetics' is a general term used to encompass measurements which relate to the proliferating compartment *(3)*. These measures may be static indices, such as the labelling index (the proportion of cells labelled by the study antigen in the total study population) or the S phase fraction, or they may have a time component, such as the S phase duration (T_s), and the cell cycle time (T_c). During the S (DNA synthesis) phase the cell takes up extrinsic DNA base analogues, such as 3H-TdR or the halogenated pyrimidines bromo- and iododeoxyuridine (BrdUrd and IdUrd).

To visualise the entire proliferating compartment, S phase cells are best marked with such an extrinsic label. 3H-TdR has been a valuable tool for measurements of the *in vitro* labelling index in clinical studies *(4)*, but it has serious practical limitations. The halogenated pyrimidines have supplanted 3H-TdR in cell proliferation studies since monoclonal antibodies became available which recognised BrdUrd or IdUrd incorporated into DNA *(5)*. The same antibodies can be used for both histochemical and flow cytometric studies. BrdUrd and IdUrd are cytotoxic drugs which are free of acute toxicity when used as single, low-dose, intravenous boluses in patients with established malignancy *(6,7)*. *In vivo* labelling has advantages over *in vitro* techniques. Firstly, the label is distributed physiologically throughout the

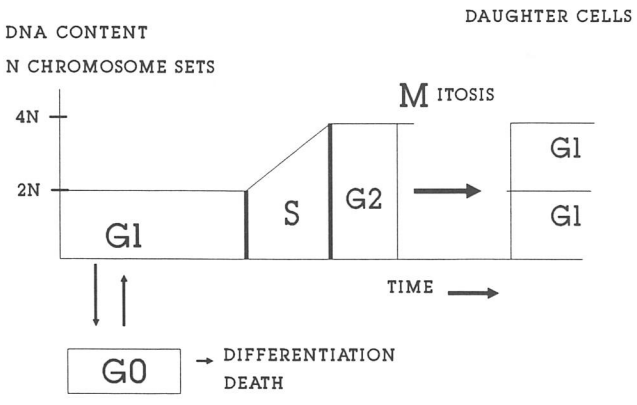

Figure 1. Diagram of the cell cycle designed to emphasise the increase in DNA content as the cell passes from the diploid to the tetraploid state through the S phase.

Based on a lecture for the Hunterian Professorship of The Royal College of Surgeons of England given to the British Association for Surgical Oncology at the Charing Cross and Westminster Medical School on 27 November 1992

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tumour mass. Secondly, pulse labelling with an interval between labelling and biopsy allows cohorts of labelled cells to be followed through their cycle. This introduces a time element to the analyses.

Single parameter FCM has been used to measure the DNA index and S phase fraction in many clinicopathological series of tumours *(8)*. The publication from Northwood in 1985 of a multiparameter FCM method for the measurement of cell kinetics was a considerable advance *(9)*. It allowed researchers to study single, low-dose BrdUrd or IdUrd pulse labelling of clinical tumours after *in vivo* drug administration, and to derive time-dependent cell kinetic data from single biopsies or surgical specimens. Multiparameter FCM and halogenated pyrimidine labelling provide the facility to measure simultaneously the DNA index, the labelling indices of the diploid and aneuploid populations, and the duration of the S phase (T_s) of the tumour cells in a single specimen. The details of the method have been described elsewhere *(7,9,10)* (Figs 2 and 3). The movement of labelled S phase cells through the DNA profile over a known time from pulse labelling to biopsy allows the S phase duration (T_s) to be estimated.

Tissue and tumour growth is a dynamic disequilibrium between cell production and cell loss. The proportion of cells capable of active proliferation in the total cell population is the growth fraction (GF). In most cell populations the GF is much less than 100%, because of the large number of quiescent, differentiated and dying cells, even within rapidly proliferating tumours. Together with the labelling index (LI) measured from the BrdUrd profile, the potential doubling time (T_{pot}) of the tumour can be calculated from the relationship $(T_{pot}) = (T_s/\mathrm{LI}) \times 0.8$. The T_{pot} is the time taken for cell numbers to double in a population where GF is less than unity and in the absence of cell loss.

The cell cycle concept also provides a framework for the study of proteins which act at specific points in the cycle to initiate, support, regulate, suppress or terminate proliferation. Multiparameter FCM allows the quantitative and cell-cycle-related study of these antigens and of nuclear proteins associated with normal and malignant cell proliferation.

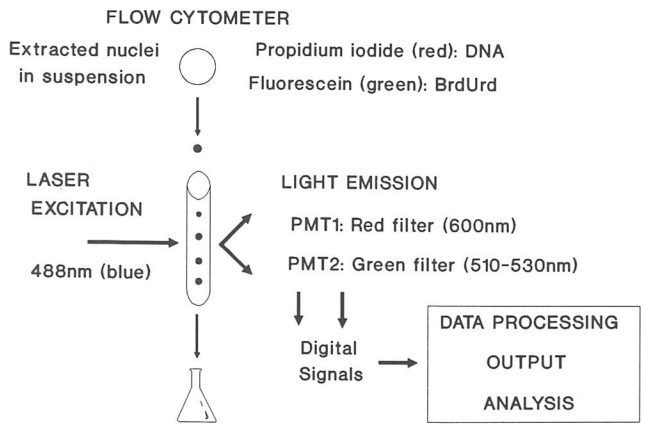

Figure 2. Diagram illustrating the method of multiparameter flow cytometric analysis of nuclei stained simultaneously for bromodeoxyuridine and DNA.

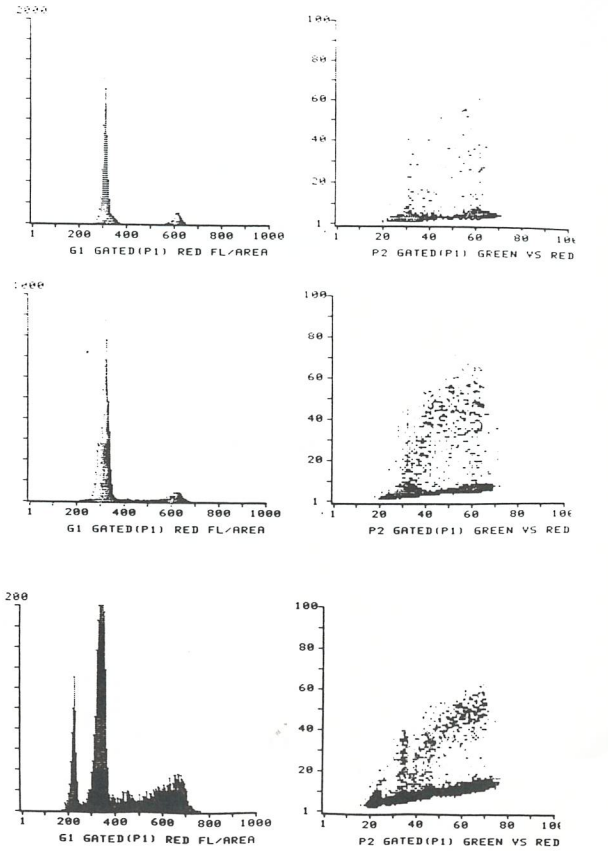

Figure 3. Illustrative pairs of bivariate (DNA *vs* BrdUrd) histograms. On the left are shown the DNA profiles, and on the right the cell cycle related (*x* axis) BrdUrd labelling (*y* axis). From top to bottom, they are 1. Diploid profile of normal colorectal mucosa. Note the relatively low BrdUrd labelling. 2. Diploid colonic tumour. Note the much higher BrdUrd labelling than is apparent in the mucosal profile. 3. Aneuploid rectal tumour.

Patients, methods and results

During this study, more than 100 patients with colorectal adenocarcinomas, 40 patients with gastric adenocarcinomas and nine with squamous oesophageal tumours kindly consented to receive an intravenous bolus of 250 mg bromodeoxyuridine between 2 and 16 h before conventional surgery or open biopsy in the case of inoperable tumours. Specimens of tumours, of intestinal mucosa, and of incidental polyps and adenomas were immediately preserved in ethanol (for flow cytometry) or formalin (for tissue sections). This material allowed the study of intestinal proliferation in a number of ways.

Firstly, the DNA index and BrdUrd labelling indices were measured, and the T_s and T_{pot} were calculated, in a large series of colorectal tumours. These findings have been published *(11)*. Intratumour heterogeneity for each parameter was assessed in up to six biopsies from 60 colorectal tumours, and considerable site-to-site variation in all indices was demonstrated. No correlations were

Table I. All labelling index data are corrected to take account of the differing times between injection and biopsy. In the case of aneuploid tumours, the T_{pot} was calculated using the labelling index of the aneuploid population. (*a*)–(*c*) denote paired data for tests of significance

Factor	*Median*	*Range*	*Significance*	*P =*
Histochemistry				
Mean LI data (hmn)				
LI% diploids ($n=25$)	17.9	4.0–31.8	NS	0.5
LI% aneuploids ($n=25$)	18.0	8.6–53.6		
All tumours LI_{hmn}%	17.9	4.0–53.3	(*a*)	>0.0001
Maximum LI data (hmx)				
LI% diploids ($n=25$)	30.9	6.8–48.7	NS	0.34
LI% aneuploids ($n=25$)	27.8	17.0–71.2		
All tumours LI_{hmx}%	30.5	6.8–71.2	(*a*)	
Flow cytometry				
Colorectal tumours *(10)*				
Total LI% ($n=100$)	9.0	0.7–22.2		0.009
Aneuploid LI% ($n=51$)	12.0	2.0–25.5		
T_s (hours) ($n=98$)	13.1	4.0–28.6		
$T_{pot\ fcm}$ days ($n=98$)	3.9	1.7–21.4		
Combined T_s and histochemical data				
$T_{pot\ fcm}$ days ($n=50$)	4.2	1.7–20.8	(*b*), (*c*)	
$T_{pot\ hmn}$ ($n=50$)	2.5	1.1–9.2	(*b*)	>0.0001
$T_{pot\ hmx}$ days ($n=50$)	1.4	0.6–5.4	(*c*)	>0.0001
Gastric tumours ($n=39$)				
Total LI%	4.9	0.9–18.5		
T_s (hours)	10.7	3.6–31.9		
T_{pot} (days)	5.2	0.8–39.4		
Squamous oesophageal carcinomas ($n=9$)				
Total LI%	5.3	1.4–17.4		
T_s (hours)	9.8	4.6–17.8		
T_{pot} (days)	4.3	2.7–17.9		

found with histological grade or Dukes' stage. The potential doubling time was consistently short (mean 5.9 days), indicating a rapid cell production rate. The FCM cell kinetic data for colorectal cancers has since been reproduced in other series *(12)*. The series of gastro-oesophageal tumour series is still being evaluated, and interim data are presented. Data are summarised in Table I.

Secondly, the proliferative architecture of colorectal tumours was studied in detail, and the BrdUrd labelling in histochemically stained sections was quantified. Studies were undertaken on formalin-fixed, wax-embedded tissue sections from 50 BrdUrd labelled tumours (Fig. 4*d*). There were 25 diploid and 25 aneuploid tumours. Counting was confined to the tumour cell compartment. Up to 200 tumour cells per region from ten different regions on each section were counted. The mean and maximal BrdUrd-derived tumour cell labelling indices (referred to as LI_{hmn} and LI_{hmx}, respectively) are reported, the latter being simply the highest region-on-region labelling index value in each section. The results are shown in Table I. Considerable local heterogeneity of the labelling index was seen within single tissue sections. There was minimal stromal labelling (the exception being within lymphoid follicles). Although the distribution of proliferating cells was often random, maximal proliferation was usually seen at the margins of the tumour. There were no correlations between kinetic data and the conventional indices of differentiation and tumour stage (Stradling and Rew, 1992, unpublished data).

Thirdly, proliferation was quantified in a large series of colorectal mucosal specimens. Mucosa is a valuable model for proliferation research. It is a highly structured tissue in which the distribution of proliferating cells is consistent. It is in a steady state, such that cell loss balances cell production. Each crypt displays in a microcosm all of the processes of cell production, maturation, differentiation and death. Computer-aided data analysis of the distribution of BrdUrd-labelled mucosal cells allowed a range of proliferative indices to be measured, including the crypt labelling index, the peak labelling position and the distribution of labelled cells. This data has also been published *(13)*. The technique gave sufficient discrimination to detect changes in the proliferative

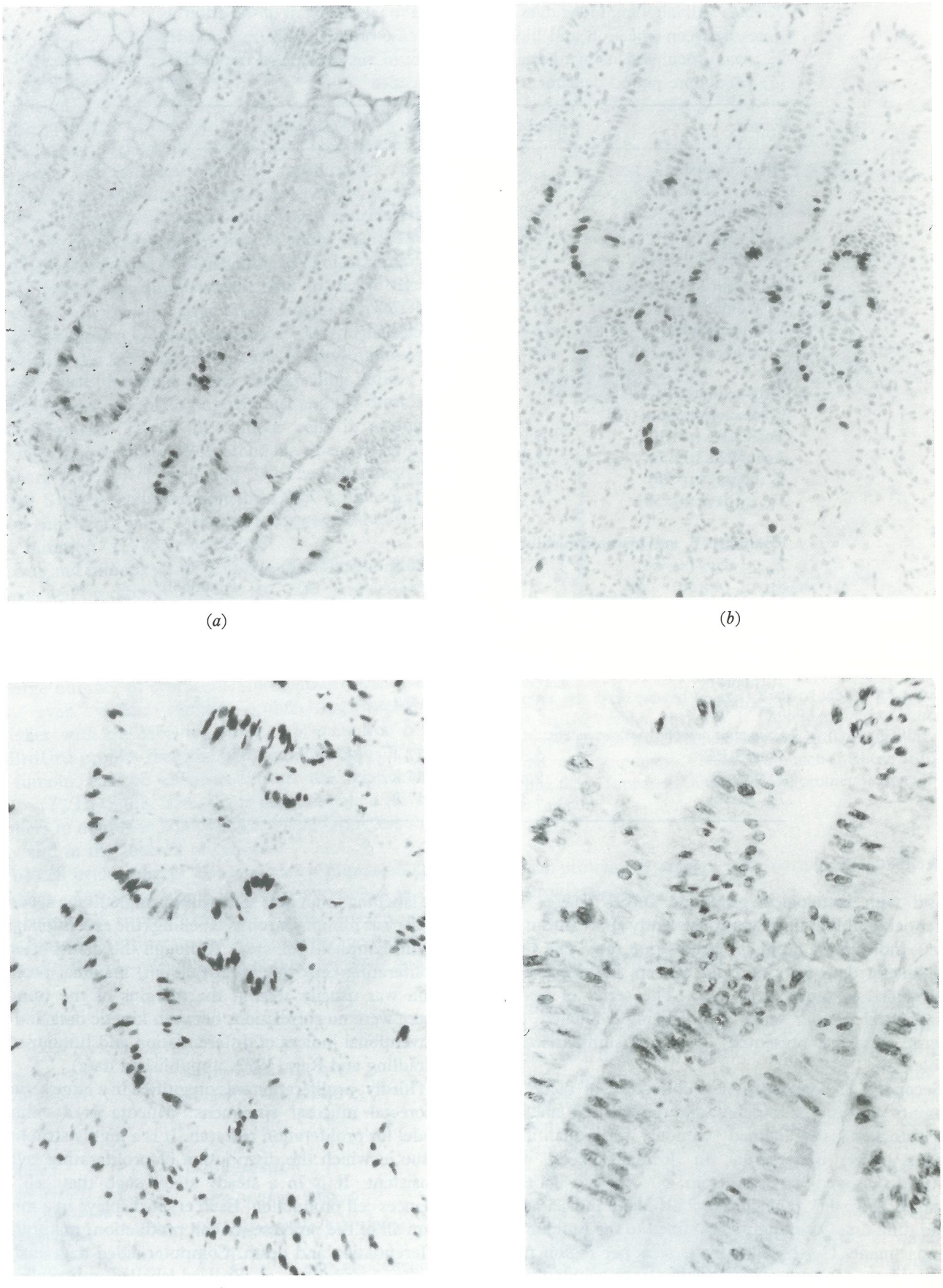

Figure 4. Composite black and white photomicrograph of four bromodeoxyuridine-labelled colorectal tissues, dark (peroxidase) stain identifying the S phase cells. (*a*), Normal colonic mucosa labelled with BrdUrd (×25). (*b*), Normal gastric mucosa. Note the complexity of the crypt anatomy compared with colorectal mucosa. (*c*), A villous adenoma (×25). (*d*), A rectal carcinoma (×40).

pattern in mucosa close to the primary tumour and in polyposis coli. There was no evidence for a field change in the pattern of proliferation in mucosa which might indicate a predisposition to intestinal cancer.

Studies were also undertaken on a smaller series of specimens of BrdUrd-labelled gastric body and antral mucosa (Fig. 4*b*) obtained from 25 patients with gastric carcinomas. Reproducible studies of gastric mucosal proliferation were much less easy to perform because of the anatomical variation and convolution of gastric mucosal crypts *(14)*.

Fourthly, qualitative observations were made on a small number of BrdUrd-labelled villous adenomas and microadenomas which were obtained from the bowel resection specimens. The proliferative architecture of these lesions is much more disorganised than in normal mucosa (Fig. 4*c*). In the polyposis coli microadenomas there was a dramatic inversion of the proliferative compartment at the junction with 'normal' mucosa.

Comparisons between flow cytometry and histochemistry data

The productivity of the FCM assay is only of use if the data produced is a true index of proliferation within the tissues. This was assessed by comparing directly the mean labelling indices obtained by FCM and histochemical counting in 50 tumours (Table I). Histochemical counts were consistently higher than FCM measurements because the observer deliberately excluded stromal cells. A limitation of multiparameter FCM is its inability to separate stromal from tumour nuclei in the G1 diploid population. Stromal cells exert a significant but variable dilutional influence on the flow cytometric tumour labelling index. This problem is overcome to some extent in aneuploid tumours, where analysis can be confined to the aneuploid, purely tumour cell population, which is more representative of the tumour cell population counted in stained sections.

S phase duration (T_s) data from FCM analysis allowed estimates of the potential doubling time (T_{pot}) of the tumour cell population to be made using either the FCM or the histochemical labelling indices. These calculations emphasise the comparative *rates* of cell proliferation. All calculations were standardised to compensate for variable time periods after pulse labelling between tumours. Highly significant differences were found to exist between each set of data ($P > 0.00001$). The median T_{pothmx} was only 1.6 days (Table I). The maximal labelling index may be a valuable measurement, because highly proliferative regions may have the greatest influence on clinical growth in tumour mass.

Correlations were studied between FCM cell kinetic data and clinical outcome for the series of patients with colorectal tumours, among whom there have been 54 documented deaths from all causes. At the time of analysis, the median follow-up was 42 months (range 28–51 months). The gastro-oesophageal tumour study is as yet incomplete and informative survival data are not yet available. Survival analysis was performed using the SAS computer program (SAS Institute, Cary, North Carolina, USA), using the Log-Rank and Wilcoxson tests. There was no correlation between survival and measured parameters. These were ploidy (diploid or aneuploid) ($\chi^2 = 0.28$, df = 1, $P = 0.59$), the flow cytometric total labelling index ($\chi^2 = 0.49$, df = 1, $P = 0.49$), S phase duration ($\chi^2 = 0.10$, df = 1, $P = 0.75$), potential doubling time ($\chi^2 = 0.86$, df = 1, $P = 0.35$) or mean G2 p62c-myc content ($\chi^2 = 2.55$, df = 1, $P = 0.11$) (see below), when each index was stratified by median values (Fig. 5). There was a highly significant difference in survival when tumours were stratified by Dukes' stages (A and B against C), ($\chi^2 = 21.1$, df = 1, $P = 0.0001$).

Nuclear oncoprotein studies

Flow cytometric assays also allow for the quantitative measurement of nuclear proteins which may have a role in the regulation of cell proliferation, such as the oncoprotein p62c-myc. Measurements can be made not only of the total numbers of labelled cells in the sample, but also of the absolute quantity of protein in each phase of the cell cycle in each cell within the biopsy. Protein epitopes, unlike BrdUrd and IdUrd, are often labile and require rigorous attention to the preparation and analysis conditions. Such assays are still very much under evaluation, as factors such as antibody concentration and even the choice of control antibody can have a major influence on the data obtained.

In a pilot study for such FCM assays, the expression of p62c-myc was measured in 87 colorectal carcinomas, in 18 gastric carcinomas, and in related mucosal samples from the BrdUrd labelled specimens described above *(15)*. Measurements were made in units of fluorescence in the G1 and G2 phases of the cell cycle. Mean ($\pm$ s.e.m.) levels of G2 p62c-myc in individual cells were significantly higher in intestinal mucosa (306 ± 24 units) than in tumours (188 ± 14 units). The nuclear p62c-myc content increased through the cell cycle with DNA content in both tumours and mucosa. This suggested a structural association between the protein and DNA. No differences in p62c-myc expression were found in tumours in relation to histological grade or to anatomical site, although considerable intratumour heterogeneity for p62c-myc was observed. There was an inverse relationship between G2 p62c-myc levels in tumours and mucosa and their *in vivo* BrdUrd labelling indices, and the protein was not shown to be a marker of tissue proliferation or of malignancy. It was concluded that deductions about the function and prognostic utility of such proteins in intestinal tumours should not be made unless the expression of the protein has also been studied in normal tissues.

Discussion

Neoplasia in intestinal tumours results from a complex and sequential series of genetic changes *(16)*. These

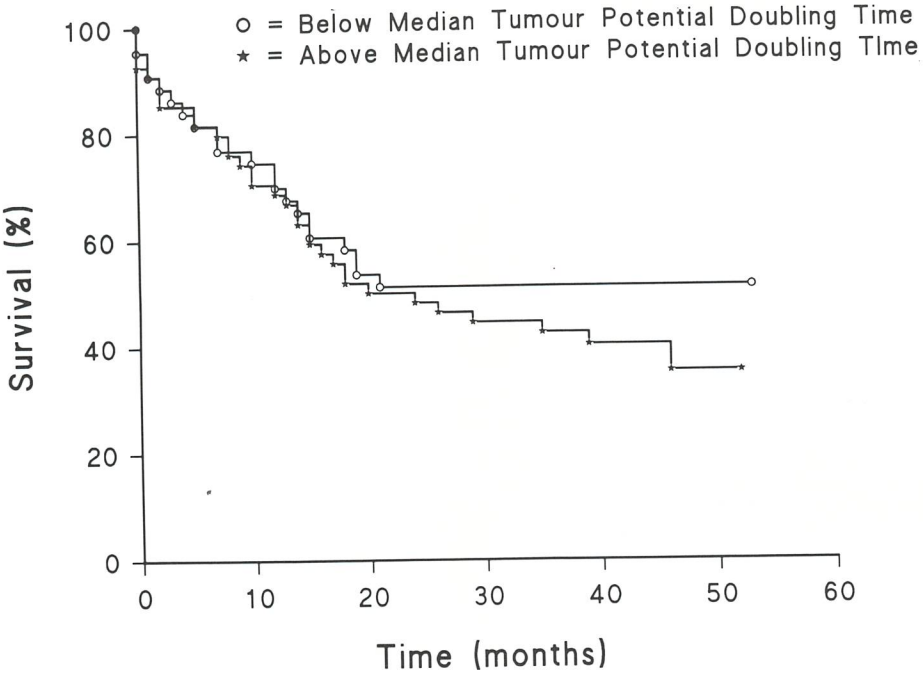

Figure 5. Survival curve using the median potential doubling time (3.9 days) to distinguish patients ($n = 97$) with rapidly from slowly proliferating colorectal tumours.

changes may be reflected in altered proliferation from normal colorectal mucosa, through polyps and adenomas to frank malignancy *(17)*. The true pattern and rate of growth of primary and metastatic intestinal tumours from single cells to advanced disease is not known. The volume doubling time (T_d) of intestinal tumours is difficult to measure, but it can sometimes be estimated indirectly by serial imaging, for example of chest or hepatic metastases.

In the series of interrelated studies reviewed in this essay, the new tool of multiparameter flow cytometry was evaluated in the measurement of the rates of cell production using bromodeoxyuridine as a proliferation marker in intestinal (and particularly colorectal) tumours and tissues. Data obtained was then related to clinical outcome and to BrdUrd-derived proliferation data from conventional histochemical studies. We have shown how primary intestinal tumours exhibit rapid proliferation when compared with tumours of the various classes which have been studied by flow cytometry and halogenated pyrimidine labelling (Fig. 6).

All methods of measuring tumour proliferation have practical limitations *(18)*, and multiparameter flow cytometry is no exception. The huge capacity for data generation must be offset against the problems of sample preparation and analysis, and the loss of tissue architecture with its information on the type and relationships of the cells under study. Quantitative histological studies of 3H-TdR or halogenated pyrimidine labelling, while subject to observer sampling and counting errors, and fatigue, and giving no indication of the *rate of turnover* of labelled cells, retain an important role in proliferation research. There is considerable synergy between histochemistry and flow cytometry.

Even if flow cytometry were remorselessly accurate and reproducible, and if it were possible to exclude stromal cells from every analysis, there are a number of reasons why accurate and detailed measurements of proliferation in intestinal tumours by flow cytometry might fail to predict clinical outcome. Firstly, intestinal tumours are often very heterogeneous in three dimensions, so small biopsies are unlikely to be representative of the entire tumour. Secondly, intestinal tumour growth results from a complex imbalance between cell proliferation and cell loss, in which the cell loss factor through exfoliation and necrosis is very high. Thirdly, proliferative data from a primary, resected colorectal tumour may not reflect the proliferative behaviour of the metastases

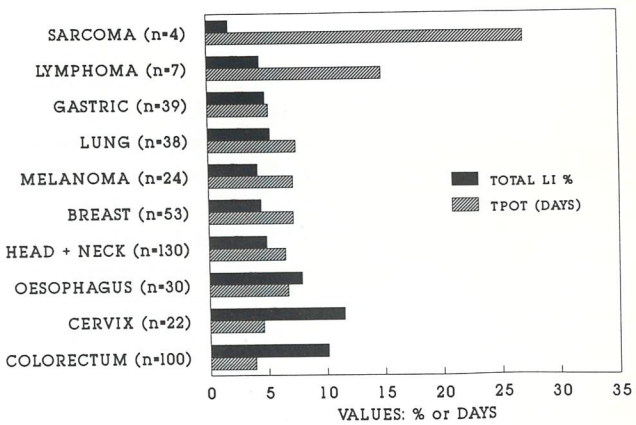

Figure 6. Histogram displaying flow cytometric data (total labelling index and T_{pot} for a range of tumours of varying pathology and site of origin, emphasising the rapidity of proliferation seen within colorectal tumours. (This data was compiled with Dr G D Wilson from a series of parallel clinical studies performed at the Gray Laboratory, Northwood).

which have a greater bearing on clinical outcome. Fourthly, tumour aggressiveness due to factors such as invasiveness, secretory processes and the propensity to metastasise is not described by measurements of the rate of tumour cell production. Fifthly, a measurement taken at any point in the life of the tumour may not be predictive either of the past or future growth characteristics of that tumour.

The problem posed by intratumour heterogeneity of labelling is compounded by the choice of method with which to measure the labelling index. The halogenated pyrimidines yield a range of indices which describe the spatial complexity and rates of cell proliferation within colorectal carcinomas. Paradoxically, this wealth of data confounds the use of halogenated pyrimidine-derived indices as simple prognostic markers. We are presented with a complex problem in deciding which of the many indices generated from one reliable S phase marker is most representative of the overall proliferation of the tumour. The LI_{max} and $T_{pot\ max}$ of the maximally proliferative clones may be appropriate because these regions are likely to have the greatest bearing on tumour behaviour, but we cannot be certain that the highly labelled foci that we observe would have continued to predominate over the more slowly growing regions. Unless a careful assessment of heterogeneity is made in multiple biopsies, the data is in any case unlikely to be a true reflection of proliferation in the whole tumour.

The importance of the cell loss factor

The cell loss factor and its contribution to the clinical volume growth of the tumour merits special consideration. For example, a highly proliferative tumour with a high cell loss factor may remain static in size or even regress, while a slowly proliferative tumour with no cell loss will continue to enlarge. Cell loss from tumours is caused by a number of processes. These include cell exfoliation into the bloodstream, lymphatics, body cavities or gut lumen, cell migration or metastasis; natural cell death (ageing and apoptosis); and tissue necrosis due to immunological response, tissue hypoxia or therapeutic action. Defective tumour cells may label in the S phase but then die. Cell loss from clinical tumours is not directly measurable by current technologies and can only be estimated indirectly in relatively few cases.

The cell loss factor is calculated from the ratio of the potential to the actual doubling time $[1-(T_{pot}/T_d)]$. Because the actual T_d of intestinal tumours and metastases often exceeds 100 days, demonstration of the rapid T_{pot} of colorectal tumours suggests that their cell loss factor may exceed 90%. This need not be surprising, as in a proliferating tissue in the steady state such as intestinal mucosa, cell production balances cell loss, and the cell loss factor is 100%. A relative reduction in cell loss, such as may be due to increased cell adhesiveness, may be as important as an increased rate of cell production in determining the rate of tumour volume growth.

The selective elimination of intestinal cells which have served their physiological purpose is an important process in the life of gut mucosa and tumours. Apoptosis or programmed cell death (PCD) is rapid and cell fragments are soon redistributed to other cells and macrophages. This process is not easily detected in tissue sections and no easily measurable quantitative indicator has yet been devised. The switches and controls of apoptosis may be as complex and important as those regulating cell proliferation. Cells with defective apoptosis mechanisms may survive unwanted, contributing to the growth of the tumour.

Future directions for proliferation research

The use of flow cytometry solely to study new or more refined prognostic indices in intestinal cancer would be inadequate exploitation of this remarkable technology. While therapeutic applications of proliferation data remain largely unexplored, practical lines of enquiry are emerging. For example, proliferation data may lead to less empirical adjuvant chemotherapy *(19)* and radiotherapy. The recognition of rapidly proliferating clones in radiosensitive tumours has led to the design of trials of continuous, hyperfractionated, accelerated radiotherapy (CHART) so as to enhance tumour kill by preventing proliferation during the course of treatment *(20)*. We do not yet know whether the CHART approach may be appropriate and effective in the treatment of selected rectal cancers.

The halogenated pyrimidines have proved to be potent markers of proliferating cells. Although they are yet to find useful clinical applications, they have taught us much about the proliferative biology of intestinal tumours. They remain a powerful research tool for detailed studies of the regulation of proliferation. They have served to emphasise how proliferation is only one side of the equation of tumour growth. The other side, that of cell loss, continues to be a problem of quantitative analysis. We have lost our innocence about the complexity of proliferation within malignant intestinal tumours, and the challenge that it poses to the development of better adjuvant therapies. No single or simple proliferation marker yet appears likely to be able to improve on the clinical or histological detection of metastases as an index of clinical prognosis, but the new technologies suggest a rewarding future for clinical research into the biological puzzles contained within intestinal tumours.

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