European Journal of Surgical Oncology 2000; **26**: 405–417
doi:10.1053/ejso.1999.0907, available online at http://www.idealibrary.com on IDEAL®

EDUCATIONAL SECTION

Cell production rates in human tissues and tumours and their significance. Part II: clinical data

D. A. Rew* and G. D. Wilson*†

Southampton University Hospitals and The Gray Laboratory, Northwood, Middlesex HA6 2JR, UK

This paper reviews the available data for cell production rates of human tissues and tumours, measured *in vivo* using halogenated pyrimidine labelling and laser cytometry. The technique has now been widely evaluated, and we draw general inferences from the proliferative data over a broad range of tumour and tissue types.

Estimates of the S-phase duration, the time taken for DNA synthesis in cycling cells, are consistent over a narrow range with a median value of around 10 hours, notwithstanding the constraints of the experimental and statistical technique, in normal tissues and tumours. This suggests that Ts values may be a species-specific constant. The more easily measured labelled S-phase fraction, or labelling index, shows much greater intra and intertumour variation within any one tumour class. It may thus be a surrogate for time dependent measurements to a first order approximation.

The cell production rate, described by the potential doubling time (Tpot), is remarkably rapid in most tumours, a median value of the order of 5 days, and much faster than clinical volume doubling times for most lesions. The rapid cell production rates in normal tissues and tumours highlight the importance of cell loss in the growth and modelling of biological structures.

Cell production rate measurements do not adequately describe the biological aggressiveness of tumours. They may be used to refine adjuvant strategies for radiotherapy and chemotherapy in experimental research. Dynamic halogenated pyrimidine labelling has provided unique and valuable insights into the living biology of human tissues and tumours.

Key words: **cell proliferation; flow cytometry; prognosis; clinical outcome; bromodeoxyuridine; iododeoxyuridine; cancer.**

Introduction

In the first of these two articles, we reviewed the techniques of measurement of time dependent proliferation parameters in human tissue and tumour samples.[1] The data is derived principally from labelling *in vivo* by intravenous pulse injection with a halogenated pyrimidine (HP), bromo- or iododeoxyuridine (BrdUrd, IudUrd). The principal indices are: the labelling index (LI), which is the proportion of labelled cells in any designated tumour cell population. The LI is a 'static' parameter which lacks a time element. Those possessing a time element ('dynamic' indices) include the S-phase duration (Ts, measured in hours), which is the time taken for cells to pass through the DNA synthesis phase of the cell cycle; and the potential doubling time (Tpot, days), which is the time which the tumour cells would take to double in number if there were no cell loss. We here review the data on time dependent indices of human tumour and tissue proliferation which has been acquired in the past decade, and its correlations with prognosis and therapy. A much larger body of data is available which relates to the measurement of labelling indices alone using halogenated pyrimidine labelling, often from *in vitro* rather than *in vivo* incubation of tumour samples with the labelling agent. Such data has been reviewed in detail by Dolbeare,[2,3] and we have generally omitted reference to such studies, except where the data illustrates important points.

Studies on time dependent parameters fall in four general categories, viz:

(a) those descriptive studies which report proliferative data, including heterogeneity studies: this category includes those studies which combine data from immunohistochemical (IHC) measurements of labelling indices with flow cytometric (FCM) measures of S-phase duration in related samples. These studies allow estimates of

Correspondence to: D. A. Rew MA MChir FRCS, The Dept. of Surgery, The Royal Hants Cancer Centre, Southampton University Hospitals, Brinton's Terrace, Southampton SO14 0YG, UK.

0748–7983/00/040405 + 13 $35.00/0

proliferation rates in selected areas of heterogenous tissues and tumours.

(b) those studies which correlate proliferation data with measures of clinical outcome, such as time to death or to recurrence, or with surrogate measures of outcome, such as prognostic factors. The *in vivo* labelling of tumours with these robust S-phase labels provides a unique opportunity for correlative studies of biomarker expression in proliferative cells. They provide a framework for the study of proteins which act at specific points in the cycle to initiate, regulate, suppress or terminate DNA replication, including oncoproteins such as c-myc and p53.

(c) those clinical studies which correlate proliferative data with evidence of response to radiotherapy.

(d) descriptive studies of normal tissue proliferation. The *in vivo* labelling of human tumours also provides opportunities for the qualitative and quantitative study of patterns of proliferation in normal tissues such as epithelia where proliferative cells display geographic organization.

Descriptive studies and correlations

The following section describes the published proliferation data for the various general categories of tumour studied, along with correlations with clinical stage and grading, clinical outcome, and with other biomarkers and prognostic factors. In general terms, the series are selected and subject to research bias, such as in the obtaining of informed consent, or the omission of small tumours where access to samples for analysis may be limited, such as for small breast carcinomas. Correlations with other prognostic markers are generally based upon data derived from IHC or FCM counts of tissue samples.

Squamous carcinomas of the head and neck

Squamous cell carcinomas of the head and neck (HN-SCC) have been of particular interest in clinical research, because of the therapeutic opportunity which they present through their relative radioresponsiveness; and their accessibility to biopsy and to direct observation of clinical growth rates. A number of descriptive series of dynamic proliferation data from HN-SCC tumours have been published, data for which are given in Table 1. Forster *et al.* reported from Glasgow a series of 82 tumours in 1991.[4] Jones *et al.*[5] reported data from 75 patients with HN-SCCs. Nylander *et al.*[6] studied 31 such tumours by both FCM and IHC following IudUrd labelling. Follow-up data was incomplete. Bennett *et al.*[7] assessed tumour proliferation of HN-SCC by both IHC and flow cytometric (FCM) analysis. Using FCM data alone, 46% of the tumours exhibited a Tpot of less than 5 days. When the Ts from the FCM data was combined with the average IHC LI, 84% of Tpot values were less than 5 days and with the maximum LI in any one tissue section, 99% were less than 5 days. Marchal *et al.*[8] reported descriptive data using both FCM and IHC quantitation of 37 nasopharyngeal carcinomas using BrdUrd. FCM and IHC counts of the LI did not correlate. Hoyer *et al.*[9] reported a study of 99 patients with HN-SCC given IudUrd. Eighty-seven tumours were quantified by FCM and 45 also by IHC. Benazzo *et al.*[10] studied 46 head and neck cancers. BrdUrd LI and TS significantly correlated with histological differentiation grading: Grade III tumours showed higher LI values and shorter TS values than Grade I and Grade II tumours. Similar study results are reported by Kotelnikov *et al.*[11,12] This group found no correlation between the LI and expression of the cell cycle associated protein, cyclin D1, or the apoptotic fraction, when measured by IHC.[13] Cooke *et al.* reported interim survival data for 105 patients from the Glasgow series.[14] They found that there was no significant correlation between LI, Ts and Tpot and tumour stage, node status or tumour site, or with survival. Their data are also recorded in Table 1.

Central nervous system (CNS) tumours

A number of studies of cerebral tumour proliferation have been published. Most studies of HP labelling *in vivo* on brain tumours have measured labelling indices alone.[3] For example, Kakinuma *et al.* have recently reported BrdUrd labelling studies in a series of 182 meningiomas in relation to clinical progression, and recurrence after surgery. Proliferation indices were inversely correlated with tumour volume growth rate calculated from serial imaging.[15]

Studies of dynamic indices in CNS tumours have included a small series of meningiomas by Riccardi *et al.*[16] and astrocytomas by Danova *et al.*[17] Shibuya *et al.*[18] studied 100 brain tumours double labelled with BrdUrd and IudUrd. Struikmans *et al.*[19] reported data for 71 malignant and 52 benign brain tumours and 14 cerebral metastases. Labelling indices were low and there was little difference between data for benign and malignant tumours. Clinical variables including age, and duration of first symptoms and local progression free survival were significantly associated with proliferation data.[20] Data are presented in Table 1.

Squamous oesophageal tumours

Limited data is available on cell proliferation in oesophageal tumours. The Northwood, UK group reported the proliferation parameters in the biopsies of 30 oesophageal squamous tumours using BrdUrd pulse labelling.[21,22] Haustermans *et al.*[23–25] reported a similar series of 31 patients. IudUrd was injected 6–10 hours before surgery, and five biopsies per tumour were taken ($n = 305$). Tumour stage, pathological node status and sex significantly influenced the disease free survival (DFS). When DFS was studied as a function of Tpot, no significant difference was found between fast and slow proliferating tumours. Haustermans *et al.*[24] also reported the heterogeneity of proliferation in these tumours. The coefficient of variation (cv) of intratumour measurements of LI values from up to five biopsies from each of 30 tumours ranged from 7.0 to 39%, or Tpot values by up to 5 days. In a sample of 30 biopsies from a single squamous tumour to assess intratumour heterogeneity, the mean Tpot was 4.2 [SD 0.9] days, with a stage of 2.0–6.4 days. Further data are given in Table 1.

Table 1. Tumours of the head, neck and lung

Reference	No.	Median LI %	Median Ts (h)	Median Tpot days	Comment
Head and neck					
Bennett (1992)[7]	123	6.8	9.9	5.7	BrdUrd
Forster (1992)[4]	105	7.0 (1.3–21.9)	14.0 (7.0–106)	5.9 (1.3–67.5)	BrdUrd 200 mg
Cooke (1994)[14]					
Jones (1994)[5]	75	8.9 (1.6–25.0)	14.8	Check	BrdUrd mg
Nylander (1994)[6]	31 FCM	13.6 (3.6–26.4)	16.1 (3.9–32.4)	4.6 (1.3–12.2)	IudUrd 100 mg
	IHC	9.1 (1.6–35.0)		5.4 (1.1–36.2)	
Wilson (1995)[67]	165	5.0 Diploid	9.9	1.8 Dp	
		9.3 Aneuploid	5.4–21.9	3.2 An	
Benazzo (1995)[10]	46/52	7.9 (2–18)	11.6 (6–28.5)	5.7 (2–30)	BrdUrd 250 mg
Kotelnikov (1995)[11,12]	12	>20 %	12.1 (5.1–21.5)	43.2 hrs (18.8–84.5 hrs)	IudUrd + BrdUrd Histochemistry
Bourhis (1996)[74]	70	6.3–7.7	8.3–9.3	4.6–5.6	BrdUrd 200 mg
Corvo (1996)[77]	82	8.0 (1.5–28.0)	10.0 (6.0–14.0)	5.0 (2.0–20.0)	BrdUrd 250 mg
Marchal (1997)[8]	37	3.8	10.8	10.9	BrdUrd
Hoyer (1998)[9]	99	12.9 (3.1–46)		4.1 (0.6–19.5)	IudUrd
CNS tumours					
Astrocytoma: Shibuya (1993)[18]	100		9.2 (6.0–13.7)	1 to 60 days	
Meningioma: Ricccardi (1988)[16]	22	2.1 (0.9–3.9)	16.7	63.2	BrdUrd
Malignant: Struikmans (1997)[19]	71	0.03	4.5	5.4	BrdUrd *in vivo*
Benign: Struikmans (1997)[19]	52	0.01	4.7	20.9	BrdUrd *in vivo*
CNS mets: Struikmans (1997)[19]	14	0.03	3.9	3.9	BrdUrd *in vivo*

LI: labelling index; Ts: S-phase duration; Tpot: potential doubling time.

Adenocarcinomas

Gastric adenocarcinomas

Static LI measurements of gastric tumours have been extensively studied by Japanese groups.[26,27] A number of series of time dependent indices of gastric adenocarcinomas are reported from Europe. Riccardi *et al.* published the first series in 1988.[16] Rew *et al.* collected data on 39 gastric adenocarcinomas labelled with BrdUrd and resected surgically.[22,28] Haustermans *et al.*[24] reported a series of 32 tumours labelled with IudUrd. Intratumour, intertumour and interlaboratory heterogeneity of proliferation indices have also been reported by this group. In a sample of 30 biopsies from a single adenocarcinoma, the mean Tpot was 5.2 [1.7] days, with a range of 2.1–9.5 days. The cv of intratumour Tpot measurements from up to five biopsies from each of 30 tumours ranged from 16.0 to 51%. Data are listed in Table 1. The correlation of cell production rates with biological aggressiveness is unknown, as insufficient data is available from which to draw valid conclusions.

Colorectal adenocarcinomas

Colorectal proliferation studies are of particular interest for the facility to correlate proliferative data and labelling patterns with form and function to correlate proliferative data and labelling patterns with form and function across the mucosa–adenoma–tumour sequence. They also provide valuable insights into the gross proliferative biology of this common surgical disease. Cell proliferation studies on these tumours predate the HP assay. Camplejohn published estimates of colorectal tumour proliferation rates using laborious stathmokinetic measurements of tritiated thymidine labelling in 1973.[29] The efficacy of HP labelling of colorectal tumours *in vivo* was established by Risio *et al.*,[30] and by Khan *et al.*,[31] who published labelling studies of small series of colorectal tumours labelled with BrdUrd in 1988.

There is now substantial dynamic data on proliferation rates in primary colonic and rectal cancers derived from HP labelling *in vivo.* Data are presented in Table 2. Rew *et al.*[32] found no correlation between any kinetic parameters and the Dukes' stage or histological classification of 100 colorectal tumours labelled *in vivo* with BrdUrd. Wilson *et al.* studied a similar series of tumours labelled with IudUrd,[33] which yielded similar data. Terry *et al.* reported the cell kinetics of 101 rectal cancers.[34] Palmqvist *et al.* reported a further series of 109 tumours[35] labelled with IudUrd, and quantified both by FCM and IHC. There was significantly higher proliferative activity in the superficial and paraluminal regions of these tumours.[36] Heterogeneity was studied in up to eight samples per tumour. A smaller series of 19 tumours was reported by Michel *et al.*[37] These series also demonstrated considerable intratumoral heterogeneity of proliferative parameters according to site of biopsy, and between aneuploid and diploid tumours and populations.[22,36,38,39] A cross study of samples between laboratories by Wilson *et al.*[38] demonstrated reliability and reproducibility of the measurements, irrespective of institution, choice of HP or type of equipment used.

The limited data on correlations between dynamic proliferation indices and survival is equivocal. Rew *et al.* found no correlation between proliferative indices and interim survival data in a cohort of patients followed to 3 years after surgery.[28] Interestingly, in the Swedish series[35,36] longer survival times were associated with more rapidly proliferating tumours. There is also some data on

Table 2. Gastrointestinal tract tumours

Reference	No.	Median or mean LI % range	Median Ts (h) range	Mean/median Tpot (days) (range)	Comment
Upper intestinal tumours					
Oesophageal squamous					
Hausterman (1994)[23]	31	19.0 (7.0) (SD)	17.2 (2.0–6.4)	4.4 (2.0–6.4)	IudUrd 200 mg
Rew (1993)[22]	9	5.3 (1.4–17.4)	9.8 (4.6–17.8)	4.3 (2.7–17.9)	BrdUrd
Wilson (1991)[21]	50	7.8 (0.4–27.5)	12.4 (6.9–28.6)	5.2 (1.6–56.8)	BrdUrd
Gastric adenocarcinoma					
Rew (1993)[28]	39	4.9 (0.9–18.5)	10.7 (3.6–31.9)	5.2 (0.8–39.4)	BrdUrd
Haustermans (1995)[25]	32	16.0 (9.0) (SD)	15.8 (6.7)	5.6 (2.1–9.5)	IudUrd 200 mg
Riccardi (1988)[16]	22	9.9 (5.7–14.0)	15.2 (13.4–22.7)	9.8 (6.8–13.5)	BrdUrd
Colorectal					
Camplejohn (1973)[29]	19	—	—	192 hours	Stathmokinetics
Bergstrom (1990)[39]	33	13 (2.9–27.0)	16 (7.0–35.0)	3.6 (2.5–14.0)	IudUrd
Rew (1991)[32]	100	9.0 (0.7–22.2)	13.1 (4.0–28.6)	3.9 (1.75–21.4)	BrdUrd
Michel (1997)[37]	19	5.3–15.4	9.7–16.6	4.5 (1.2–21 –	BrdUrd
Wilson (1993)[33,39]	125	12.7 (0.6–33.8)	14.1 (5.2–48.3)	4.5 (0.9–53.8)	IudUrd
Rectal					
Terry (1995)[34]	101	21.0 (13.5–27.0)	20.0	3.3 (2.4–5.6)	BrdUrd 200 mg 6 hrs before biopsy

LI: labelling index; Ts: S-phase duration; Tpot: potential doubling time.

correlations between proliferative indices and the expression of other biomarkers, including the oncoproteins p62-cmyc[40] and p53.[41]

Tumours of the female breast and reproductive system

Breast carcinomas

Breast cancers are an important clinical problem and a valuable model for proliferation studies. Primary and recurrent lesions are accessible to biopsy and volume change in response to therapy is more easily estimated, as for example, in tumours treated by tamoxifen or other chemotherapy alone. Static proliferative indices have been extensively studied, particularly using 3H TdR labelling, and more recently using BrdUrd *in vivo*. For example, Goodson *et al.* (1998) studied 145 cases by the latter technique.[42] Both the BrdUrd and the Ki67 labelling indices correlated positively with tumour size, node positivity and tumour grade, but not with age or oestrogen receptor status. In 30 breast cancers from this series, BrdUrd LIs were correlated with concurrent regional node metastases. The tumour LI ranged from 0.1% to 34% (median, 10.3%) and axillary lymph node metastases labelling index ranged from 0.1% to 27.7% (median, 10.0%). There was strong correlation between the values for primary tumour LIs and their metastases.

Two series report dynamic data derived from the *in vivo* labelling of human breast tumours with BrdUrd. In a series of 69 patients with invasive breast carcinoma,[43] there were no significant differences in the total LI, Ts or Tpot when patients were stratified according to lymph node status, tumour size, tumour grade or menopausal status. Quantitative histological counting of labelling indices in this series of breast tumours yielded data comparable to flow cytometric analysis.[44] Stanton *et al.*[45] reported a similar series of 84 cases from Glasgow. LIs were significantly higher in aneuploid tumours, and in tumours not expressing oestrogen receptors, but were not correlated with tumour size, nodal status, or expression of c-erbB2 (Table 3). Robertson *et al.*[46] reported that in a study of 53 BrdUrd labelled tumours from this series, there were correlations between the LI and Tpot and epidermal growth factor receptor (EGFR) expression. There are as yet no informative data on correlations between proliferative indices and survival in breast cancer patients.

Ovarian and other gynaecological tumours

There is limited data on ovarian tumours. Erba *et al.*[47] reported the kinetic parameters of human ovarian adenocarcinoma *in vivo* using BrdUrd incorporation in 55 untreated patients. LI and TS were not correlated with clinical tumour stage, histological grading, residual tumour size or DNA ploidy (Table 3). No informative data on survival correlates are available.

Squamous carcinomas of the cervix

Cervical tumours are also significant for their radio-responsiveness, and thus have been a focus for several labelling studies. Bolger *et al.*[48] assessed 120 cervical tumours. In 89% both static and temporal kinetic parameters could be measured. The analysis of multiple biopsies from each tumour revealed marked intratumour heterogeneity (Table 2). There was an elevation in the LI, but no difference in Ts, between tumour and non-neoplastic cervical tissue. There was a significant elevation of the LI, in proliferating cells from advanced stage and large size tumours. In a further report on outcomes in this series,[49] there was no

Table 3. Female breast and reproductive tract tumours

Reference	No.	Median LI % range	Median Ts (h) range	Median Tpot (days) range	Comment
Breast					
Rew (1991)[42,43]	75	4.2 (0.6–15.4)	8.7 (2.7–22.2)	8.2 (1.8–47.5)	BrdUrd 250 mg
Stanton (1996)[45,46]	84	3.2	12.0	12.5	BrdUrd 200 mg
Gynaecology					
Ovarian					
Erba (1994)[47]	55	6.1–7.2	14.7	12.5	BrdUrd 250 mg
Cervix					
Bolger (1996)[48,49]	120	9.8 (6.7–14.5)	12.8 (11.1–14.9)	4.0 (3.1–6.3)	BrdUrd 200 mg
(Squamous)		Interquartile	Interquartile	Interquartile	
Tsang (1995)[50]	39			5.5	BrdUrd 200 mg
(Adenocarc)					
Tsang (1995)[50]	7			6.6	BrdUrd 200 mg
Ovarian metastases					
Rew (1991)[22]	2	5.1, 5.4	12.5, 13.9	3.5, 6.5	BrdUrd 250 mg

LI: labelling index; Ts: S-phase duration; Tpot: potential doubling time.

correlation between these parameters and response to conventional radiotherapy, other than for an association between a high BrdUrd labelling index and pelvic tumour recurrence. Tsang *et al.*[50] measured the pre-treatment Tpot values in 46 cervical tumours. Proliferative data and duration of treatment did not correlate significantly with disease free survival. For 25 patients where Tpot measurements were performed at two separate laboratories, systematic differences were detected in the data.

Other tumours

Malignant melanoma

Primary malignant melanomas are difficult to study because of their small size, heterogenous and schirrhous admixture of skin and tumour cells, and because of the practical limitation of losing key pathological information on depth and planes during experimental biopsy. Laing *et al.*[51] have reported one large series of 83 primary and metastatic malignant melanomas. Thin, good prognostic primary lesions (<1.5 mm thickness) displayed significantly lower proliferation indices than did the thicker lesions. No informative survival correlates are otherwise available.

Haematological malignancy

Haematological tumours offer the opportunity of multiple and serial analyses which can be performed upon blood samples. Haematological stem cells are among the most actively proliferating subsets in the body. Raza *et al.*[52,53,54] studied acute leukaemias, and the bone marrow of 68 patients with myelodysplastic syndromes (MDS), who received sequential infusions of iodo- and/or BrdUrd. Giordano *et al.*[55] reported the kinetics of acute non-lymphoblastic leukaemia (ANLL). Sixty-five patients with untreated ANLL and 15 patients with solid tumours and normal bone marrow (BM) received 250 mg/m^2 BrdUrd. Data are presented in Table 4. No informative correlations with survival are otherwise available.

Lung carcinomas

No large series of lung tumour proliferative data have been published. Wilson *et al.*[57] reported a series of 38 lung tumours yielding 88 samples (Table 4). Tinnemans *et al.*[58] studied bronchoscopy specimens of 27 lung cancers and 11 benign biopsies after *in vivo* labelling with 50 mg/m^2 BrdUrd. They obtained cytokinetic data from seven samples of small cell lung cancer (SCLC) and 20 of non-small cell lung cancer (NSCLC). No significant differences were observed between the mean values of the cytokinetic parameters of SCLC and NSCLC. No informative correlations with survival are available.

Urological tumours

A number of studies have reported static proliferative indices in urological tumours using BrdUrd labelling and IHC. Nemoto *et al.* reported such studies of transitional cell carcinomas of the bladder (TCCB), and prostatic adenocarcinomas.[58–60] In a series of 103 patients with Grade II superficial TCCBs labelled *in vitro* with BrdUrd, Tachibana *et al.*[61] reported that a BrdUrd LI greater than 5.3%, and the presence of aneuploidy, were strong predictors of future recurrence. Ota *et al.*[62] studied 11 patients with recurrent TCCBs by BrdUrd labelling. Higher LIs correlated with more rapid tumour growth rates. Larsson *et al.*[63] studied proliferation in 136 samples from renal carcinomas labelled with IudUrd by four different FCM and IHC methods to measure the S-phase fraction, IudUrd and PCNA (proliferating cell nuclear antigen, a marker of growth fraction) LIs. They found that each technique yielded comparable information on proliferative activity. In an earlier study of 200 samples from the 33 renal tumours in this series, they found no correlation between LI and tumour size.

Table 4. Other tumours

Reference	No.	Median LI	Median Ts (h)	Median Tpot (days)	Comment
Melanoma					
Primary tumours	24	4.2 (1.3–13.6)	10.7 (6.3–20.5)	7.2 (3.5–41.3)	BrdUrd 250 mg
Metastases					
Laing (1993)[51]	61	5.4 (0.6–16.8)	11.6 (6.1–26.2)	7.2 (2.3–139.0)	
Haematology					
Normal bone marrow					
Giordano (1993)[55]	15	11.8 (±3.1)	10.1 (±2.0)	3.5 (±5.6)	BrdUrd 250 mg/m^2
Myelodysplastic syndrome					
Raza (1997)[54]	68	28.4	11.8	40.8 hours cell cycle time	Double labelling
MDS + Acute Myeloid					
Leukemia Raza (1992)[53]	10	27.7 (22–37.5)	15.5 (11.4–24.6)	58.3 hours cell cycle time	BrdUrd
Acute non-lymphocytic leukemia Giordano (1993)[52]	54	6.1 (±2.9)	16.2 (±6.1)	18.1 (±18.9)	BrdUrd 250 mg/m^2
ANL leukemia					
Riccardi (1988)[16]	42	6.2 (0.9–11.7)	12.1 (6.9–27.8)	8.5 (2.8–16.7)	BrdUrd 250 mg/m^2
Lung					
Wilson (1993)[56]	38	8.0 (0.7–28.2)	15.1 (5.5–37.8)	7.3 (1.4–132.0)	
Normal lung	9	2.7 (1.2–7.8)	11.1 (4.2–41.1)	17.3 (6.5–32.0)	BrdUrd 50 mg/m^2
Diploid tumours	14	5.0 (1.1–11.9)	6.9 (3.6–13.5)	9.6 (1.9–28)	
Aneuploid tumours	13	14.7 (3.8–33.6)	14.0 (5.7–29.4)	6.7 (1.5–20.7)	
Tinnemans (1993)[57]					

LI: labelling index; Ts: S-phase duration; Tpot: potential doubling time.

There is little data on cell proliferation dynamics in tumours of the urological tract. TCCBs have been studied in small numbers by Rew *et al.*[64] Numbers were insufficient to draw correlations between proliferation rates with clinical behaviour. In a follow-up to this series, Popert *et al.*[65] noted that the BrdUrd label was reliably detected by IHC in all resected tumours. There was a significantly higher mean LI (8.4% *vs*. 3.4%) in those tumours which recurred during the follow-up period of 30 months. IHC thus tended to identify a much larger value for the BrdUrd LI than when it is measured by FCM.

Radiotherapy is an important treatment modality for prostatic adenocarcinomas. Haustermans *et al.*[66] studied seven patients with such lesions by *in vivo* IurdUrd administration. LIs were less than 2.5%, yielding long estimates for Tpots, and suggesting that hyperfractionated therapy may not be of value for these tumours.

Other tumour classes

We have also studied proliferative indices in small numbers of other tumours, including lymphomas and sarcomas.[22] The data are recorded in Table 5.

Correlations with therapy

The rate of cell proliferation in individual tumours, as indicated by the Tpot, may be hypothesized to influence the tumour response to chemotherapy or radiotherapy. In the absence of cell loss and other factors influencing biological aggressiveness (such as invasiveness, metastatic potential), rapidly proliferating tumours might be expected to repopulate more rapidly during treatment, and thus be relatively resistant to treatment. They may mandate more frequent courses of fractionated therapy to seek out and destroy the proliferating cells. The option of frequent fractionation schedules is more attractive for radiotherapy than chemotherapy, because side effects are not quite so critical and unmanageable with radiotherapy, but the principles may also apply to chemotherapy regimes.

Radiotherapy and squamous cell carcinomas of the head and neck

Most studies correlating proliferation data with therapeutic response have been performed upon squamous carcinomas of the head and neck, for which radiotherapy is often the primary treatment modality. The hypothesized presence of rapidly proliferating clones in radiosensitive tumours suggested the design of trials of continuous, hyperfractionated, accelerated radiotherapy (CHART). This is intended to enhance tumour kill by preventing proliferation during the course of treatment. Studies have focused upon head and neck cancer in particular.[67–69] In a series reported from Northwood, data were complete for 90 patients.[70] No kinetic parameter predicted the outcome of patients treated by CHART. When the Tpot was calculated using a combination of histology LI and FCM TS, diploid tumours showed more rapid median proliferation rates (Tpot 1.8 days) than aneuploid tumous (3.2 days). A study of the expression of bcl2, a protein associated with apoptosis, indicated a positive correlation with survival in this series.[71]

Bourhis *et al.*[72] studied the predictive value of pre-treatment Tpot and LI, in 70 patients with head and neck squamous cell carcinoma treated with conventional

Table 5. (Unpublished) data and rare tumours (GDW and DAR data)

Reference	No	Mean LI (range)	Mean Ts (range)	Mean Tpot (range)	Comment
Lymphomas[22]	2	5.4 (0.9–13.4)	11.0 (8.3–16.0)	15.8 (2.5–23.2)	BrdUrd *in vivo*
Hodgkins nodes[22]	3	2.6 (0.9–4.6)	10.4 (9.4–11.0)	17.7 (5.5–39.6)	As above
Basal cell carcinoma[22]	1	5.4	10.7	6.6	As above
Sarcoma[22]	2	1.5, 2.7	7.1, 22.0	8.8, 48.8	As above
Liposarcoma[22]	1	0.6	2.4	13.5	As above
Osteosarcoma[22]	1	0.8	8.8	36.7	As above
Urological					
TCCB Popert (1993)[65]	19	3.1 (ta tumours) 8.6 (t1 tumours) 16.4 (t2/3 tumours)			BrdUrd *in vivo* IHC stain Manual counts
TCCB Rew (1991)[64]	19	2.5 (0.5–10.0)	6.2 (3.5–9.7)	17.1 (3.6–40.0)	BrdUrd *in vivo*
Renal Ca Larsson (1994)[63]	33	1.06 ± 0.59			IudUrd *in vivo*
Renal Ca Rew (1991)[64]	2	1.9, 6.6	7.1, 11.9	4.5, 18.0	BrdUrd *in vivo*
Prostate Hausterman (1997)[66]	7	0.6–2.4	9.5–13.2	16–61	IudUrd *in vivo*
Prostate Ca Rew (1991)[64]	1	1.9	9.0	7.7	BrdUrd *in vivo*

LI: labelling index; Ts: S-phase duration; Tpot: potential doubling time.

radiotherapy (70 Gy in 7 weeks). No relationship was found between the Tpot or LI and the tumour stage, nodal status, histological grade, and the site of the primary. The mean Tpot of the tumours that relapsed locally was 5.3 days, compared to 6.1 days for those who did not relapse locally (not significant, NS). The TS, LI, DNA index, and Tpot were not associated with local relapse, or disease free survival (DFS). Corvo *et al.*[73,74] reported a further such series, correlating Tpot positively with longer term survival in 82 patients.

Interim reports have been published from the EORTC (European Organisation for Research into the Treatment of Cancer) phase III trial comparing conventional fractionation (70–72 Gy in 7–8 weeks, 1.8–2.0 Gy/fraction) to a split-course accelerated treatment (72 Gy in 5 weeks, 1.6 Gy/fration, 3 fractions per day with a 12–14 day split after 8 days). An analysis of 60 cases demonstrates that pre-treatment Tpot data could not discriminate patients with improved (>4 days) or impaired (<4 days) survival, even though the clinical data has shown benefit for the accelerated schedule.[75,76] A similar finding has emerged from the Paris study[77] with longer follow-up.

A study from Genoa[78] of 69 patients with a median follow-up of 47 months treated by conventional fractionation and a boost schedule suggested that a Tpot >5 days could predict ($P = 0.04$) improved (68%) local control at 3 years compared to 13% in fast tumours (<5 days). This study supports the Tpot as a predictor of outcome. Overall the 3 year local control rate was 54% in patients with a Tpot of >5 days and 25% in those with a Tpot <5 days ($P = 0.004$). In a report from Sweden[79] in which 89 patients were treated by conventional radiation with a median follow-up of 30 months, nodal involvement and Tpot were correlated with local control. Hoyer *et al.*[9] found no correlation between proliferation data generated by IudUrd labelling and indices of loco-regional radiotherapy control in their series of 99 tumours.

Thus, the ability of Tpot measurements to consistently predict the outcome in conventional radiation treatment remains questionable. Studies of their predictive value in accelerated fractionation schedules have failed to reveal any significance of this measure of proliferation in either concomitant boost, split course accelerated treatment or in the CHART schedule.[80,81] An analysis of correlations between kinetic data and the outcome of treatment of 476 patients in 11 centres by at least 6 weeks conventional radiotherapy by Begg *et al.*[82] demonstrated that the labelling index was the only significant variable in a univariate analysis for no control. Multivariate analysis showed no significant correlations, although comparisons were hampered by inter-centre variation in measurements.

The results of these studies have thus not been consistent and no consensus has yet emerged as to whether Tpot will be accepted as a useful clinical predictive test. There have been several drawbacks to the published studies, in the lack of randomization, small patient numbers, short follow-up and lack of formal quality control of the Tpot measurements.

Cell production rates in normal epithelium

The study of the rates of cell proliferation in normal tissues provides insights into the biology of the normal cell. It allows comparison with tumour proliferation rates, thus suggesting the optimization of antiproliferative treatment strategies to minimize side effects to normal tissues. The ordered architecture of epithelium does not allow direct measurement of labelling indices derived from tissue homogenates using the automated techniques of laser cytometry. The counting of labelled cells must be undertaken in a structured fashion, and correlated directly with the tissue architecture.[83]

Van Erp *et al.*[84] studied the cell cycle kinetics of normal skin epidermal cells in 14 lymphoma patients with IudUrd using serial biopsies. Oesophageal squamous mucosa also has a well defined proliferative zone at the base of the epidermis. The proliferation rates in labelled cells from normal oesophageal mucosa from oesophageal tumour resection specimens has been reported by Rew[22] and

Table 6. Normal and non-malignant tissues

Reference	No.	LI	Ts	Tpot	Comment
SKIN					
Van Erp (1993)[84]		3.5 %	9.7 ± 0.6 hrs	28.4 hrs cell cycle time	
Renal cortex					
Larsson (1994)[63]	33	0.08 ± 0.09			IudUrd *in vivo*
GI epithelium					
Squamous oesophageal mucosa					
Rew (1991)[22]	7	3.7 (0.4–8.4)	12.3 (6.5–18.2)	25.3 (4.3–67.6)	FCM data on tissue disaggregates
Squamous oesophageal mucosa					
Haustermans (1995)[25]	53	5.2 (2.1 SD)	9.6 (2.8 SD)	8.4 (3.8 SD)	IudUrd 200 mg
Gastric mucosa					FCM data on tissue
Patel (1993)[85]	27	2.7 (1.1–4.7)	9.6 (3.8–19.7)	15.6 (3.4–59.7)	disaggregates
Colorectal mucosa					
Rew (1991)[22]	157	2.0 (0.6–8.4)	10.7 (3.1–37.5)	24.8 (2.9–101)	FCM data on tissue disaggregates
Flow cytometry analysis					
Colorectal mucosa					
Potten (1992)[86,87]	147	Varies with position in crypt	As in box above	Cell cycle time 30 hours	Same series as above Histochemistry and manual counting
Histometric analysis					
Villous adenoma					
Rew (1991)[22]	6	5.3 (2.3–9.1)	8.6 (4.8–12.8)	6.1 (3.6–10.6)	BrdUrd 250 mg
Metaplastic polyp					
Rew (1991)[22] (colorectal mucosa)	10	4.9 (0.6–20.1)	10.6 (5.2–19.6)	6.3 (2.5–53.6)	BrdUrd 250 mg

LI: labelling index; Ts: S-phase duration; Tpot: potential doubling time.

Haustermans *et al.*[23] Small numbers have been studied and there is no information on the relationship between mucosal proliferation rates and squamous malignant change. The complex, convoluted architecture of gastric and duodenal mucosa has also been analysed. Patel *et al.*[85] used Ts data from tissue homogenates, in conjunction with standard histochemical counting, to estimate the cell turnover time of gastric mucosa, at between 11.5 and 28.1 days, according to anatomical site.

Colorectal mucosa is a structured tissue in which the proliferating cells are linearly ordered in the depths of each columnar mucosal crypt. Potten *et al.* conducted a systematic quantitative analysis of the distribution of BrdUrd labelled cells in human colorectal mucosa, taken from sites throughout the colorectum.[86,87] A range of proliferative indices were measured, including the crypt labelling index, the peak labelling position and the detailed distribution of labelled cells, along with the Ts median value of 8.6 hours. The cell cycle time was calculated to be 30 hours. The entire crypt turnover time was thus calculated to be 82 hours. These data were very consistent with earlier calculations made using tritiated thymidine in much smaller series and with animal model studies. Data are presented in Table 6.

Proliferation in transition tissues

Colorectal adenomas are of interest for their malignant potential and for the intermediate level of proliferative disorder which they display between mucosa and invasive tumours. In a small study, incidental adenomas analysed from coloretal tumour resection specimens, including cases of familial polyposis coli, displayed patterns of disorder and values for labelling indices intermediate between mucosal crypts and tumours.[28] Ts values were similar to those in mucosal and tumour cells (Table 6).[22,87]

Discussion

An overview of proliferation data in human tissues and tumours

The halogenated pyrimidines have proved to be a potent tool for measuring tumour cell production rates when given intravenously as a small pulse labelling dose, and when labelled cell populations are analysed by laser flow or scanning cytometry.[88] The technique has been evaluated by a number of groups using different equipment and analytical techniques, and results have been found to show high concordance from one centre to another. Validation of the type and distribution of labelled cells in tumour and tissue samples using immunohistochemistry give added confidence that these are meaningful measurements and not artefactual. The versatility of the technique has also transformed the volume and analysis of cell proliferation data using the time parameter, when compared with the limitations of stathmokinetics and tritiated thymidine studies.

The labelling index

The generation of quantitative data conceals considerable methodological problems, whatever measurement technique is used. In the case of immunohistochemistry, stains such

as peroxidase or the avidin–biotin complex can be very variable in their affinity for tissues, such that even single tissue sections may be very unevenly labelled, regardless of true heterogeneity of labelling of the halogenated pyrimidine marker. The proportion of proliferating cells (the labelling index) also varies considerably from region to region within a tumour, even on one tissue section. The proportion of S-phase cells in any one local area of a tumour may range from less than 1% to more than 50%. Tumours are architecturally complex, with admixture of stroma and tumour cells, and the observer's eye is naturally drawn to the more heavily stained areas. True objectivity is thus very difficult to achieve, given the numerical and time constraints of manual counting. Conversely, the observer can focus on areas of tumour and ignore labelled stromal tissues.

Flow cytometry presents different problems. It allows a much more rapid quantitation of large numbers of cells, and is free of observer bias, although there remains a selection bias in the sample presented for analysis. We have seen how there may be marked variation in proliferation, for example, between the margin and the centre of a tumour, which may introduce a large systematic bias into any correlations of a measured LI with clinical outcome or with other biomarkers.

Flow cytometric analysis is indiscriminate, and the technology cannot readily discriminate tumour from stromal nuclei in diploid populations during analysis. Thus, tumour labelling indices will be diluted to a variable and indeterminate degree by stromal contamination, which may be a very high fraction in the case of breast tumours, for example. Nuclei within aneuploid populations can be generally assumed to be derived from tumours. It is thus advisable to study samples both by IHC or at least by conventional histology, and FCM, to estimate the proportion of stroma in the sample. A further problem with flow cytometric analysis is that the cut off between labelled and unlabelled nuclei is subjective (see Part I), and thus subject to random or systematic error.

The S-phase duration

The S-phase duration (Ts) shows much less variation within and between tumours, and between tumours and normal tissues. Many studies reveal that Ts in human cells appears to be relatively consistent with most values falling between 10–20 hours across a wide range of tissues and tumours, notwithstanding the complex and heterogenous nature of tumours. Given the profound conservation of key biological processes in nature, it seems very likely that the time taken to duplicate DNA, the duration of S-phase, is a species specific constant for a given number of chromosomes and volume of DNA. The variation observed in the Ts measurement may thus be due to experimental artefact. Indeed, the cell cycle is such an evolutionarily conserved and powerfully regulated process that it might be surprising if the duration of DNA synthesis were significantly different in tumour cells, with the exception of markedly aneuploid cells. These may be expected to have a longer Ts in proportion to the additional DNA to be replicated.

Measurement of the Ts becomes less reliable at low fractions of HP labelling, because of the method by which the Ts is estimated from the absolute numbers of labelled cells in each phase of the cell cycle. This may give rise to inappropriately long Tpot calculations, and underestimations of the true rate of cell production.

Is the S-phase a species specific constant?

It seems highly likely that the S-phase duration is a species specific constant in cells with normal DNA content, because cell cyle control is such a highly conserved and critical process to the continuity of life. It also seems likely that cell cycle control is conserved in tumour cells, as they would not be able to replicate if it were significantly disrupted. If DNA duplication proceeds at a constant rate, then one variable in the Ts duration might be the absolute quantity of abnormal DNA in the tumour cells, reflected in the DNA index, or aneuploidy. However, we found no significant difference in the Ts of cohorts of diploid and aneuploid colorectal tumour samples to support this hypothesis.[32] Much of the variation in measured values of the Ts in clinical samples may thus be due to the inevitable experimental and biological variation inherent in this assay, which is dependent on successful delivery of label to the tumour, and its transport and incorporation into tumour DNA in competition with endogenous thymidine with a few hours of intravenous administration.

The potential doubling time and tumour volume growth rates

The Tpot is a derivative calculation of the ratio Ts/LI. There are a number of interesting aspects to the Tpot data. One feature of the data across the many series of tumours is the similarity rather than the differences in calculated cell production rates between classes of tumour of very different histology and behaviour. Another is the high rate of cell production in many tumours, with Tpots of the order of 5 days. The observed volume doubling time of tumours in clinical practice is commonly of the order of 100 days or more. This provides strong corroborative evidence that the differencee between the calculated and the observed doubling times is due to a high cell loss factor. This might be expected to be due principally to exfoliation and necrosis, as often observed in those highly exfoliative tumours in the gastrointestinal and urogenital tract. However, the indication of high cell loss factors in non-luminal tumours, such as of the breast, suggests that apoptosis may be playing a very major role in cell loss. This is to be expected where large numbers of abnormal cells are produced in these proliferative neoplasms. These observations indicate that cell loss is a dominant rather than a marginal factor in tumour growth. Apoptosis is most likely to account for a large proportion of cell loss.

The static labelling index may be a useful surrogate measure of dynamic proliferation. If the Ts is relatively constant from one cell population to another, then the observed experimental variation Tpot is largely attributable to site to site and intertumour variation in the LI. If we assume the Ts to be a species constant and take the median value as representative, then we may use the LI as an index of

proliferation rate to a reasonable first order approximation. This would simplify the approach to proliferation measurements, as static, *in vitro* and histochemical measurements of laboratory practice then would be the results of dynamic *in vivo* studies. It would also allow us to draw upon the large body of data derived from tritiated thymidine and halogenated pyrimidine labelling of tumours *in vivo* and *in vitro*.

Proliferation data and clinical outcome

It is commonly assumed that a higher rate of tumour cell production increases the biological aggressiveness of tumours. Within the many HP labelling studies reported, it is possible to find correlations between proliferative characteristics and surrogate histological or clinical markers of outcome, such as tumour grade, size or node status. As there is no absolute measure of 'speed' in tumour growth, it is common practice to define it by the median Tpot value in any one tumour series, the longer Tpots representing the more slowly growing lesions. In those series where survival has been recorded, there has generally been poor correlation between proliferation parameters and time to death. The reasons for the lack of correlation between proliferation indices and biological aggressiveness have been discussed in Part I.[1]

Correlations with other biomarkers

The incorporation of BrdUrd or IudUrd in DNA provides a robust marker of proliferation which is highly resistant to degradation under severe extraction and preparation conditions. This provides a valuable phase specific marker of proliferating cells for the detailed study of the expression of regulatory proteins and oncoproteins, for example. Immunohistochemistry offers limited options in this regard, because it is qualitative and because double labelling of single cells is not practical. FCM allows parallel or simultaneous quantitation in cell nuclei of DNA, a HP and one or more biomarkers, and thus a range of detailed investigational possibilities which have not been fully explored. This facility is extended by the visualization capabilities of laser scanning cytometry.[88]

Static, histochemistry derived quantitative studies of biomarker expression in tumour biology, whether or not in association with proliferation markers, are often of limited utility and biological significance. They fail to address the problem of heterogeneity and intracellular turnover. Just as with proliferation parameters, a count of labelled cells in a tissue section will fail to identify, or to quantify, the intracellular content or the transient expression of key regulatory molecules, or processes such as mitosis or apoptosis.

Cell production rates in normal tissues

The concept of the Tpot is also applicable to normal tissues, where at maturity, cell production is in a steady state balance with cell loss. Where normal tissue samples, such as gastrointestinal mucosa, have been studied, cell proliferation rates are similar to those of the tumours. This strengthens the theory that tumour growth may be due not so much to an acceleration of cell production rates, as to a reduction in cell loss rates. One proviso to generalizations about normal tissue proliferation rates is that much of the available data on normal tissue samples has been obtained from incidental biopsies from surgical tumour resection cases. This raises the question as to whether the proliferation patterns in the normal tissues may have been modified, for example through exposure to tumour growth factors. This possibility, though unlikely to produce significant anomalies in proliferative patterns, cannot be ruled out from the available data.

Cell proliferation and experimental therapy

Cell proliferation research may help improve adjuvant chemotherapy[89,90] and radiotherapy[91–93] in a number of ways. HP labelling is now a standard laboratory technique for measuring normal and perturbed cell proliferation in experimental models. For example, it allows the point of action of drugs in the cell cycle to be inferred from the cell cycle profiles of experimental cell populations. The block to the cell cycle by individual agents causes proliferating cells to 'pile up' in specific phases of the cycle.[3] The technique also allows the assessment of the proliferative response of tissues to damage, such as induced by hypoxia, in tumour cell cultures and animal models.[94]

Cell production rate measurements are yet to find useful clinical applications, either in prognostication or in guiding therapeutic strategy. Nevertheless, they have taught us much about the proliferative biology of human tumours. They remain a powerful research tool for laboratory studies of the regulation of proliferation. They have served to emphasize how cell production 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 now recognize the complexity of proliferative behaviour within and between tumours and normal tissues, 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 may help elucidate patterns of behaviour within solid tumours. Halogenated pyrimidine labelling is now a well established technique for laboratory experiments and in clinical research. Studies into anti-proliferative therapeutic strategies continue. The complex biology of cancer challenges the practical applications of this valuable research tool. Nevertheless, tumour proliferation research has come a long way in a decade.

Acknowledgements

We thank the many surgeons, oncologists and patients who have supported our own studies and collaborations in this field of clinical research, including Mr P. C. Weaver, Mr M. R. Thompson and Dr V. Svoboda at Portsmouth, and Professor I. Taylor and Mr C. D. Johnson at Southampton between 1988 and 1991. Elements of our work have been supported in turn by the Cancer Research Campaign,

Wessex Cancer Trust and the NHS Research and Development Executive in Trent Region.

References

1. Rew DA, Wilson GD. Cell proliferation rates in human tissues and tumours: Part I. *Eur J Surg Oncol* 2000; **26**:
2. Dolbeare F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics [Review]. *Histochem J* 1995; **27**: 339–69.
3. Dolbeare F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part II: Oncology, chemotherapy and carcinogenesis. *Histochem J* 1995; **27**: 923–64.
4. Forster G, Cooke TG, Cooke LD, Stanton PD, Bowie G, Stell PM. Tumour growth rates in squamous carcinoma of the head and neck measured by *in vivo* bromodeoxyuridine. *Br J Cancer* 1992; **65**: 698–702.
5. Jones AS, Roland NJ, Caslin AW, Cooke TG, Cooke LD, Forster G. A comparison of cellular proliferation markers in squamous cell carcinoma of the head and neck. *J Laryngol Otol* 1994; **108**: 859–64.
6. Nylander K, Anneroth G, Gustafsson H, Roos G, Stenling R, Zackrisson B. Cell kinetics of head and neck squamous cell carcinomas. Prognostic implications. *Acta Oncol* 1994; **33**: 23–8.
7. Bennett MH, Wilson GD, Dische S, Saunders MI, Martindale CA, Robinson BM, O'Halloran AE, Leslie MD, Laing JH. Tumour proliferation assessed by combined histological and flow cytometric analysis: implications for therapy in squamous cell carcinoma in the head and neck. *Br J Cancer* 1992; **65**: 870–8.
8. Marchal S, Marchal C, Parache RM, Benjaafar N, Odda M, Leclerc A, Merlin J-L, El Gueddarri B, Bey P. Combined flow cytometry and immunohistochemistry analyses for the assessment of nasopharyngeal carcinoma cell kinetics *in vivo* by bromodeoxyuridine infusion. *Cytometry* 1997; **29**: 165–72.
9. Hoyer M, Jorgensen K, Bundegaard T, Johansen LV, Bentzen SM, Overgaard M, Hansen O, Sogaard H, Overgaard J. Lack of predictive value of potential doubling time and iodeoxyuridine labelling index in radiotherapy of squamous cell carcinoma of the head and neck. *Radiotherapy and Oncology* 1998; **46**: 147–55.
10. Benazzo M, Mevio E, Occhini A, Franchini G, Danova M. Proliferative characteristics of head and neck tumours. *In vivo* evaluation by BrdUrd incorporation and flow cytometry Orl. *J Oto-Rhino-Laryngol* 1995; **57**: 39–43.
11. Kotelnikov VM, Coon JS, Taylor S, Hutchinson J, Panje W, Caldarelli DD, LaFollette S, Preisler HD. *In vivo* labelling with halogenated pyrimidines of squamous cell carcinomas and adjacent non-involved mucosa of head and neck region. *Cell Proliferation* 1995; **28**: 497–509.
12. Kotelnikov VM, Coon JS, Haleem A, Taylor S, Hutchinson J, Panje W, Caldarelli DD, Griem K, Preisler HD. Cell kinetics of head and neck cancers. *Clin Cancer Res* 1995; **1**: 527–37.
13. Kotelnikov VM, Coon JS, Mundle S, Kelanic S, LaFollette S, Taylor IVS, Hutchinson J, Panje W, Caldarelli DD, Preisler HD. Cyclin D1 expression in squamous cell carcinomas of the head and neck and in oral mucosa in relation to proliferation and apoptosis. *Clinical Cancer Res* 1997; **3**: 95–101.
14. Cooke LD, Cooke TG, Forster G, Jones AS, Stell PM. Prospective evaluation of cell kinetics in head and neck squamous carcinoma: the relationship to tumour factors and survival. *Br J Cancer* 1994; **69**: 717–20.
15. Kakinuma K, Tanaka R, Onda K, Takahashi H. Proliferative potential of recurrent intracranial meningiomas as evaluated by labelling indices of BrdUrd and Ki-67, and tumour doubling time. *Acta Neurochirurgica* 1998; **140**: 26–32.
16. Riccardi A, Danova M, Wilson GD, Ucci G, Dormer P, Mazzini G, Brugnatelli S, Girino M, McNally N, Ascari E. Cell kinetics in human malignancies studied with *in vivo* administration of bromodeoxyuridine and flow cytometry. *Cancer Res* 1988; **48**: 6238–45.
17. Danova M, Gaetani P, Lombardi D, Giordano M, Riccardi A, Mazzini G. Prognostic value of DNA ploidy and proliferative activity in human malignant gliomas. *Med Sci Res* 1991; **19**: 613–5.
18. Shibuya M, Ito S, Davis RL, Wilson C, Hoshino T. A new method for analyzing the cell kinetics of human brain tumors by double labeling with bromodeoxyuridine *in situ* and with iododeoxyuridine *in vitro*. *Cancer* 1993; **71**: 3109–13.
19. Scruikmans H, Rutgers DH, Jansen GH, Tulleken CAF, Van der Tweel I, Batterman JJ. S phase fraction, bromodeoxyuridine labelling index, duration of S phase, potential doubling time and DNA index in benign and malignant brain tumours. *Radiation Oncol Investigations* 1997; **5**: 170–9.
20. Struikmans H, Rutgers DH, Jansen GH, Tulleken CAF, Van der Tweel I, Batterman JJ. Prognostic relevance of cell proliferation markers and DNA ploidy in gliomas. *Acta Neurochirurgica* 1998; **140**: 140–7.
21. Wilson GD. Assessment of human tumour proliferation using bromodeoxyuridine—current status. *Acta Oncologica* 1991; **30**: 903–10.
22. Rew DA. The *in vivo* proliferation kinetics of human solid tumors. MChir Thesis, University of Cambridge, UK, 1991.
23. Haustermans K, Vanuytsel L, Geboes K, Lerut T, Van Thillo J, Leysen J, Coosemans W, van der Schueren E. *In vivo* cell kinetic measurements in human oesophageal cancer: what can be learned from multiple biopsies? *Eur J Cancer* 1994; **30A**: 187–91.
24. Haustermans K, Fowler F, Geboes K, Lerut T, van der Schueren E. Do cell kinetics have prognostic and/or predictive value in oesophageal cancer treated by surgery? *Eur J Surg Oncol* 1997; **23**: 293–7.
25. Haustermans K. *In vivo* growth kinetic measurements in human oesophageal cancer. PhD Thesis, University of Leiden, Holland, 1995.
26. Miwa H, Wada R, Abe H, Ohkura R, Yang SW, Watanabe H, Ogihara T, Hamada T, Sato N. Diagnosis of gastric adenoma versus early gastric cancer by bromodeoxyuridine immunohistochemistry from gastric biopsy specimen. *J Gastroenterol Hepatol* 1993; **8**: 133–7.
27. Ohyama S, Yonemura Y, Miyazaki I. Prognostic value of S phase fraction and DNA ploidy studied with *in vivo* administration of bromodeoxyuridine on human gastric cancers. *Cancer* 1990; **65**: 116–21.
28. Rew DA. Cell proliferation, tumor growth and clinical outcome: gains and losses in intestinal cancer. *Ann R Coll Surg Engl* 1993; **75**: 397–404.
29. Camplejohn RS. Cell kinetics of colorectal tumours. *Recent Results Cancer Res* 1982; **83**: 21–30.
30. Risio M, Coverlizza S, Ferrari A, Candelaresi GL, Rossini FP. Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel. *Gastroenterology* 1988; **94**: 899–906.
31. Khan S, Raza A, Petrelli N, Mittleman A. *In vivo* determinations of labelling index of metastatic colorectal carcinoma and normal colonic mucosa using intravenous infusions of bromodeoxyuridine. *J Surg Oncol* 1988; **39**: 114–8.
32. Rew DA, Wilson GD, Taylor I, Weaver PC. Proliferation characteristics of 100 colorectal tumours measured *in vivo*. *Br J Surg* 1991; **78**: 60–6.
33. Wilson MS, West CM, Wilson GD, Roberts SA, James RD, Schofield PF. An assessment of the reliability and reproducibility of measurement of potential doubling times in human colorectal cancers. *Br J Cancer* 1993; **67**: 754–9.
34. Terry NH, Meistrich ML, Roubein LD, Lynch PM, Dubrow RA, Rich TA. Cellular kinetics in rectal cancer. *Br J Cancer* 1995; **72**: 435–41.
35. Palmqvist R, Oberg A, Bergstrom C, Rutegard JN, Zackrisson B, Stenling R. Systematic heterogeneity and prognostic significance of cell proliferation in colorectal cancer. *Br J Cancer* 1998; **77**: 917–25.
36. Bergstrom C, Palmqvist R, Denekamp J, Oberg A, Tavelin B, Stenling R. Factors influencing the estimates of proliferative labelling indices in rectal cancer. *Radiother Oncol* 1998; **46**: 169–77.
37. Michel P, Hermet J, Paresy M, Menard JF, Laquerriere A,

Scotte M, *et al.* Comparison between endoscopic and surgical sampling for the measurement of potential doubling time in colorectal cancer. *Cytometry* 1997; **29**: 273–8.
38. Wilson MS, West CM, Wilson GD, Roberts SA, James RD, Schofield PF. Intratumoral heterogeneity of tumor potential doubling times in colorectal cancers. *Br J Cancer* 1993; **68**: 501–6.
39. Bergstrom C, Stenling R. Proliferative heterogeneity in colorectal cancer, studied using iododeoxyuridine. Abstract to European Study Group for cell proliferation. ESPOO XVII, Finland Sept 1990.
40. Rew DA, Taylor I, Watson JV, Wilson GD. The c-myc protein product is a marker of DNA synthesis but not of malignancy in gastrointestinal tissues and tumours. *Br J Surg* 1991; **78**: 1080–3.
41. Rew DA, Karkera R, Stradling R, Mullee M, Julious S, Wilson GD. The flow cytometric analysis of total p53 protein content and proliferation indices in colorectal cancer, in relation to clinical outcome. *Eur J Surg Oncol* 1996; **22** 5: 508–15.
42. Goodson III WH, Moore II DH, Ljung B-M, Chew K, Florendo C, Mayall B, Smith HS, Waldman FM. The functional relationship between *in vivo* BrdUrd labelling index and Ki-67 proliferation index in human breast cancer. *Breast Cancer Research and Treatment* 1998; **49**: 155–64.
43. Rew DA, Campbell I, Taylor I, Wilson GD. The *in vivo* proliferation kinetics of invasive carcinoma of the breast. *Br J Surg* 1992; **79**: 335–9.
44. Ashton-Key M, Coddington R, Rew DA, Campbell ID, Taylor I. Quantitative indices of proliferation using bromodeoxyuridine: A study of sixty invasive breast carcinomas. *The Breast* 1993; **2**: 42–7.
45. Stanton PD, Cooke TG, Forster G, Smith D, Going JJ. Cell kinetics *in vivo* of human breast cancer. *Br J Surg* 1996; **83**: 98–102.
46. Robertson KW, Reeves JR, Smith G, Keith WN, Ozanne BW, Cooke TG, Stanton PD. Quantitative estimation of epidermal growth factor receptor and c-erbB2 in human breast cancer. *Cancer Res* 1996; **56**: 3823–30.
47. Erba E, Giordano M, Danova M, Mazzini G, Ubezio P, Torri V, Mangioni C, Landoni F, Bolis P, Tenti P, *et al.* Cell kinetics of human ovarian cancer with *in vivo* administration of bromodeoxyuridine. *Annals Oncol* 1994; **5**: 627–34.
48. Bolger BS, Cooke TG, Symonds RP, MacLean AB, Stanton PD. Measurement of cell kinetics in cervical tumors using bromodeoxyuridine. *Br J Cancer* 1993; **68**: 166–71.
49. Bolger BS, Cooke TG, Symonds RP, MacLean AB, Stanton PD. Prediction of radio-therapy response of cervical tumors through measurement of proliferation rate. *Br J Cancer* 1996; **74**: 1223–6.
50. Tsang RW, Fyles AW, Kirkbride P, Levin W, Manchul LA, Milosevic MF, Rawlings GA, Banerjee D, Pintilie M, Wilson GD. Proliferation measurements with flow cytometry Tpot in cancer of the uterine cervix: correlation between two laboratories and preliminary clinical results. *Int J Radiat Oncol Biol Phys* 1995; **32**: 1319–29.
51. Laing JHE, Rew DA, Wilson GD. Cell kinetics of human solid tumours. *Br J Radiol* 1992; Suppl 24: 163–7.
52. Raza A, Ucar K, Preisler HD. Double labelling and *in vitro* versus *in vivo* incorporation of BrdUrd in patients with acute nonlymphocytic leukemia. *Cytometry* 1985; **6**, 6: 633–41.
53. Raza A, Yousuf N, Bokhari SA, *et al. In situ* cell cycle kinetics in bone marrow biopsies following sequential infusions of IUdR/BrdUrd. *Leuk Res* 1992; **16**: 299–306.
54. Raza A, Alvi S, Broady-Robinson L, Showel M, Cartlidge J, Mundle SD, Gregory SA, *et al.* Cell cycle kinetic studies in 68 patients with myelodysplastic syndromes following intravenous iodo- and/or bromodeoxyuridine. *Experimental Hematology* 1997; **25**: 530–5.
55. Giordano M, Danova M, Mazzini G, Gobbi P, Riccardi A. Cell kinetics with *in vivo* bromodeoxyridine assay, proliferating cell nuclear antigen expression, and flow cytometric analysis. Prognostic significance in acute non-lymphoblastic leukemia. *Cancer* 1993; **71**: 2739–45.
56. Wilson GD. HP Beck-Bornholdt (ed). *Medical Radiology, Current Topics in Clinical Radiobiology of Tumours.* Berlin: Springer Verlag, 1993.
57. Tinnemans MM, Schutte B, Lenders MH, Ten Velde GP, Ramaekers FC, Blijham GH. Cytokinetic analysis of lung cancer by *in vivo* bromodeoxyuridine labelling. *Br J Cancer* 1993; **67**: 1217–22.
58. Nemoto R, Hattori K, Sasaki A, Miyanaga N, Koiso K, Harada M. Estimations of the S phase fraction *in situ* in transitional cell carcinoma of the renal pelvis and ureter with bromodeoxyuridine labeling. *Br J Urol* 1989; **64**: 339–44.
59. Nemoto R, Uchida K, Hattori K, Shimazui T, Nishijima Y, Saito S, Koiso K, Harada M. S phase fraction of human bladder tumor measured *in situ* with bromodeoxyuridine labeling. *J Urol* 1988; **139**: 286–9.
60. Nemoto R, Uchida K, Hattori K, Shimazui T, Koiso K, Harada M. Immunochemical demonstration of S phase cells by anti-bromodeoxyuridine monoclonal antibody in human prostate adenocarcinoma. *J Urol* 1989; **141**: 337–40.
61. Tachibana M, Miyakawa A, Nakamura K, Baba S, Murai M, Tazaki H. Role of proliferative activity estimated by BrdUrd labeling index in determining predictive factors of recurrence in superficial intermediately malignant bladder tumors. *J Urol* 1996; **156**: 63–9.
62. Ota T, Akaza H, Hattori K, Uchida K. The relationship between the estimated tumour growth speed and indicees of BrdUrd incorporation and PCNA expression in superficial bladder cancer. *Japn J Urol* 1997; **88**: 868–73.
63. Larsson P, Roos G, Stenling R, Wilson GD, Ljungberg B. Cell proliferation in renal cell carcinoma—a comparative study of cell kinetic methods. *Urol Research* 1996; **24**: 281–5.
64. Rew DA, Thomas DJ, Coptcoat MJ, Wilson GD. *In vivo* measurement of urothelial tumor kinetics. *Br J Urol* 1991; **68**: 44–8.
65. Popert RJ, Joyce AD, Thomas DJ, Walmsley BH, Coptcoat MJ. BrdUrd labelling of transitional cell carcinoma of the bladder: an index of recurrence? *Br J Urol* 1993; **71**: 279–83.
66. Haustermans KMG, Hofland I, Van Poppel H, Oven R, Van de Voorde W, Begg AC, Fowler JF. Cell kinetic measurements in prostate cancer. *Int J Radiat Biol Phys* 1997; **37**: 1067–70.
67. Wilson GD, Dische S, Saunders MI. Studies with bromodeoxyuridine in head and neck cancer and accelerated radiotherapy. *Radiother Oncol* 1995; **36**: 189–97.
68. Withers HR, Taylor JMG, Maciejewski B. The hazard of accelerated tumor clonogen repopultation during radiotherapy. *Acta Oncol* 1988; **27**: 131–46.
69. Dische S, Saunders MI. Continuous, hyperfractionated, accelerated radiotherapy (CHART). *Br J Cancer* 1989; **59**: 325–6.
70. Saunders MI, Dische S, Grosch E, Fermont DC, Ashford R, Maher J, Makepeace AR. Experience with CHART. *Int J Radiat Oncol Biol Phys* 1991; **21**: 871–8.
71. Wilson GD, Grover R, Richman PI, Daley FM, Saunders MI, Dische S. BCL2 expression correlates with favourable outcome in head and neck cancer treated by accelerated radiotherapy. *Anticancer Res* 1996; **16**: 2403–8.
72. Bourhis J, Wilson G, Wibault P, Chavaudra N, Janot F, Luboinski B, Eschwage F, Malaise EP. *In vivo* measurement of the potential doubling time by flow cytometry in oropharyngeal cancer treated by conventional radiotherapy. *Int J Radiat Oncol Biol Phys* 1993; **26**: 793–9.
73. Corvo R, Giaretti W, Sanguineti G, Geido E, Orecchia R, Barra S, Margarino G, Bacigalupo A, Vitale V. Potential doubling time in head and neck tumors treated by primary radiotherapy: preliminary evidence for a prognostic significance in local control. *Int J Radiat Oncol Biol Phys* 1993; **27**: 1165–72.
74. Corvo R, Giaretti W, Sanguineti G, Geido E, Orecchia R, Guenzi M, Margarino G, Bacigalupo A, Garaventa G, Barbieri M, Vitale V. *In vivo* cell kinetics in head and neck squamous cell carcinomas predicts local control and helps guide radiotherapy regimen. *J Clin Oncol* 1995; **13**: 1843–50.
75. Begg AC, Hofland I, Moonen L, Bartelink H, Schraub S, Bontempts P, Le Fir R, Van den Bogaert W, Caspers R, van Glabbeke M, Horiot J-C. The predictive value of cell kinetic measurements in a European trial of accelerated fractionation

in advanced head and neck tumors: an interim report. *Int J Radiat Oncol Biol Phys* 1990; **19**: 1449–53.
76. Begg AC, Hofland I, Van Glabbeke M, Bartelink H, Horiot J-C. Predictive value of potential doubling time for radiotherapy of hand and neck tumour patients: results from the EORTC cooperative trial 22851. *Seminars in Radiat Oncol* 1992; **2**: 22–5.
77. Bourhis J, Dendale R, Hill C, Bosq J, Janot F, Attal P, Fortin A, Marandas P, Schwaab G, Wibault P, Malaise EP, Bobin S, Luboinski B, Eschwege F, Wilson G. Potential doubling time and clinical outcome in head and neck squamous cell carcinomas treated with 70 GY in 7 weeks. *Int J Radiat Oncol Biol Phys* 1996; **35**: 471–6.
78. Antognoni P, Bignardi M, Cazzaniga LF, Poli AM, Richetti A, Bossi A, Rampello G, Barbera F, Soatti C, Bardelli D, Giordano M, Danova M. Accelerated radiation therapy for locally advanced squamous cell carcinomas of the oral cavity and oropharynx selected according to tumor cell kinetics—a phase II multicenter study. *Int J Radiat Oncol Biol Phys* 1996; **36**: 1137–45.
79. Zackrisson B, Gustafsson H, Stenling R, Flygare P, Wilson GD. Predictive value of potential doubling time in head and neck cancer patients treated by conventional radiotherapy. *Int J Radiat Oncol Biol Phys* 1997; **38**: 677–83.
80. Corvo R, Giaretti W, Geido E, Sanguineti G, Orecchia R, Scala M, Garaventa G, Mora E, Vitale V. Cell kinetics and tumor regression during radiotherapy in head and neck squamous cell carcinomas. *Intl J Cancer* 1996; **68**: 151–5.
81. Horiot JC, Bontemps P, van den Bogaert W, Le Fur R, van den Weijngaert D, Bolla M, Bernier J, Lusinchi A, Stuschke M, Lopez-Torrecilla J, Begg AC, Pierart M, Collette L. Accelerated fractionation (AF) compared to conventional fractionation (CF) improves loco-regional control in the radiotherapy of advanced head and neck cancers: results of the EORTC 22851 randomized trial. *Radiother Oncol* 1997; **44**: 111–21.
82. Begg A, Haustermans K, Wilson GD. The value of pretreatment cell kinetic parameters as predictors for radiotherapy outcome in head and neck cancer: a multicentre analysis. *Radiother Oncol* 1999; **50**: 13–23.
83. Dolbeare F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part III. Proliferation in normal, injured and diseased tissue, growth factors, differentiation, DNA replication sites and *in situ* hybridization. [Review]. *Histochemical J* 1996; **28**: 531–75.
84. Van Erp PE, Boezeman JB, Brons PP. Cell cycle kinetics in normal human skin by *in vivo* administration of iododeoxyuridine and application of a differentiation marker—implications for cell cycle kinetics in psoriatic skin. *Analytical Cellular Pathology* 1996; **11**: 43–54.
85. Patel S, Rew DA, Taylor I, Potten CS, Roberts S. Studies of proliferation in human gastric mucosa following *in vivo* bromodeoxyuridine labeling. *Gut* 1993; **34**, 7: 893–6.
86. Potten CS, Kellett M, Roberts S, Rew DA, Wilson GD. Measurement of *in vivo* proliferation in human colorectal mucosa using bromodeoxyuridine. *Gut* 1992; **33**: 71–8.
87. Potten CS, Kellett M, Roberts S, Rew DA. Proliferation in human gastrointestinal epithelium using bromodeoxyuridine *in vivo*: data for different sites, proximity to a tumor, and polyposis coli. *Gut* 1992; **33**: 524–9.
88. Rew DA, Reeve L, Wilson GD. A comparison of flow and laser scanning cytometry for the assay of cell proliferation in human solid tumours. *Cytometry* 1998; **33**: 355–61.
89. Tannock I. Experimental chemotherapy and concepts related to the cell cycle. *Int J Radiat Biol* 1986; **49**: 335–55.
90. Van Putten LM. *Cell Kinetics, a guide for chemotherapy? Controversies in Cancer*, Masson Publishing 1979; pp 117–9.
91. Denekamp J. Cell kinetics and radiation biology. *Int J Radiat Biol* 1986; **49**, 2: 357–80.
92. Kummermehr J, Trott K-R. Rate of repopulation in a slow and fast growing mouse tumour. In: Karcher KH *et al.* (eds). *Progress in Radio-oncology II*, New York: Raven Press 1982, pp 299–308.
93. Terry NHA. Predictive assays for radiotherapy: the role of tumour proliferation (Tpot) measurements. *Onkologie* 1996; **19**: 322–7.
94. Webster L, Hodgkiss RJ, Wilson GD. Cell cycle distribution of hypoxia and progression of hypoxic tumour cells *in vivo. Br J Cancer* 1998; **77**: 227–34.