CHAPTER 51

Laser Cytometry of Human Tissues and Tumors: Proliferation and Therapeutic Applications

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I. Introduction

Treatment strategies for cancers are often inadequate. Surgery, chemotherapy, and radiotherapy all have serious limitations. The clinician thus seeks help from science and technology to achieve better clinical outcomes. The modern tools of cytometry have given us a whole new range of investigative capabilities at the cell and tissue level. Nevertheless, the trained human eye and the brain of the cytologist and histopathologist remain the fastest, most efficient, and most versatile image processor. The conventionally stained histological section contains an immense amount of information about tissue architecture, constitution, cell size, and characteristics. Histology remains the gold standard of clinical diagnosis and prognosis, because of its facility to describe the architecture and the class of tumor, for example, an adenocarcinoma; the grade of the tumor, or its degree of differentiation, usually based on the semiquantitative scale of well, moderately, or poorly differentiated features; and the presence of tumor in metastases. Visual assessment can be enhanced, where appropriate, by techniques such as histochemical staining or automated image analysis. Most descriptive information about tumors is still derived from visualization, either from inspection of the lesion, its local invasion, and its metastases *in situ* or from microscopy to describe the cell and tissue architecture.

Cytometric technologies must thus provide information not otherwise available to the trained observer across a range of applications in clinical oncology. These include the attainment of a precise clinical diagnosis, prognostication, research in the basic sciences, and therapeutic research. We may broadly classify human tumors for cytometric purposes as those presented in suspension, such as in blood or effusions; in homogeneous solid form such as lymphomas, carcinoids, sarcomas, poorly differentiated carcinomas, and melanomas; or in heterogeneous solid form allowing classification on tissue morphology alone, as are most carcinomas. A useful cytometric measurement of outcome must thus complement rather than substitute for tissue morphological criteria. Cytometers can help further classify tumors in those situations where morphology is homogeneous and insufficiently discriminatory. In the case of reticuloendothelial tumors such as lymphomas, cytometric immunophenotyping allows rapid, precise classification of lymphomas and the optimization of treatment (see Chapter 46 of this volume).

Tumors in suspension such as leukemias are relatively easy to study using cytometric instruments. Fresh and archival samples of solid, nonhematological tumors pose considerable problems for cytometric assays. These problems include tissue disaggregation and cell extraction, tumor heterogeneity, and biomarker preservation. Solid tumors must be disaggregated by mechanical or enzymatic techniques. These can introduce cell and epitope damage, and they may yield an unrepresentative sample. All these factors have a significant bearing on the interpretation of cytometric data.

Tumor heterogeneity poses critical and unresolved problems in cytometric data evaluation. Tissue and tumor extracts invariably comprise a complex admixture of

cells of many different tumor, stromal, and inflammatory lineages. At the cell, subcellular, and organelle level, there are many similarities between cells and nuclei from tumors and normal tissues. Cell size, granularity, and scatter characteristics are generally heterogeneous, thus ruling out simple classifications based on the standard morphologies and parameters that help characterize hematological lineages. Intratumor heterogeneity of morphology, form, and expression of many biomarkers can be considerable, both within a planar microscope field of tissue section and from site to site within a tumor. This renders measurements based on one or a few samples per tumor unreliable. They may not be representative either of the entire tumor or of the most biologically aggressive regions within it (Rew, 1996). Heterogeneity also varies with time during the growth of each tumor, as for example, heterogeneity of DNA content (Shackney and Shankey, 1995; Shackney *et al.,* 1995).

II. Ploidy and Proliferation in Surgical Oncology

A. Prognostic Studies

Tumor behavior is unpredictable. The analysis of tumor samples for prognostic purposes seeks to detect predictors of future biological behavior, including studies that measure the rate of tumor growth (Steel, 1977). Many biomarkers and tumor characteristics have been studied by cytometric assays in terms of outcome measures, commonly time to local recurrence or death of advanced disease. However, it is of little use to the patient or to the clinician to be able to predict the precise hour of death using the best tools of cytometric science if survival or quality of life cannot be improved.

The best index of the biological aggressiveness of a tumor remains evidence of its spread to regional or distant lymph nodes, liver, or other metastatic site. No measurement of any marker on the primary tumor yet equates as an independent prognostic indicator. Nevertheless, tumors do offer clues as to their future behavior in their morphological appearance, with poorly differentiated tumors tending toward greater aggression. Clinical cytometric studies of prognosis fit two general categories: retrospective and prospective.

B. Retrospective Studies

Retrospective studies are usually conducted on archival series of clinical pathology samples. They offer the considerable advantage of established clinical follow-up of patients, often over many years, and they allow the study of rare and infrequent tumor classes. However, the cytometric study of archival samples can be flawed. The investigator has no control over the sampling and representative nature of the tissue block from the original tumor. Such series are rarely able to address the problem of intratumor heterogeneity, and randomly archived samples may well not be representative of the entire tumor.

C. Prospective Studies

Prospective studies are planned in advance of tissue collection. They have the advantage of allowing the investigator to collect fresh material in optimal conditions and to select specific areas of tumor for study. They have the major disadvantage of duration and unpredictability of specimen availability, the dependence on close liaison with surgeons and oncologists, and the need for collocation of clinical and research facilities.

This chapter offers examples of how laser cytometry has helped our understanding of clinical tumor behavior with examples from the literature and from our own studies. We also consider the constraints to the use of cytometric technologies in cancer medicine, and how laser scanning cytometry in particular promises to advance our knowledge, as with our studies of fluorochromatic, cytotoxic drug uptake in tumors.

III. Cytometric Studies of Proliferation

A. Ploidy Measurements as Prognostic Markers

The cell cycle model (Howard and Pelc, 1951) underpins much cytometric research. Cell cycle related DNA content (ploidy) and the derived S-phase fraction (SPF) have been intensively studied in all classes of clinical tumors, both in prospective and archival clinicopathological series, with respect to prognostic outcome. These studies were aided by techniques for the extraction of cell nuclei from wax-embedded, formalin-fixed archival tissue blocks (Frankfurt *et al.,* 1984, 1986; Hedley, 1989).

Ploidy studies have highlighted the frequency of aneuploidy in clinical tumor series. Aneuploidy is a feature of some 75% of tumors. We are still uncertain whether the gross chromosomal disorder of aneuploidy is a cause or a consequence of malignant change (Rew, 1994). Conventional cytometry fails to detect subtle changes in DNA such as translocations and mutations that may cause cancer. In global terms, ploidy measurements have not proved to be useful independent prognostic indicators in clinical practice, when compared to histological assessment of tumor grade and stage, even in the widely studied model of breast tumors.

B. S-Phase Fraction Measurements as Prognostic Markers

The SPF indicates the proportion of cells or nuclei in the S-phase compartment at the time of measurement. Measurement of the SPF by flow cytometry (FCM) poses a number of problems. Within aneupoid populations, it is difficult to distinguish the S-phase cells of the diploid population within the overlapping aneuploid populations. The interpretation of ploidy and SPF data is further complicated by methodological problems in many series, including different

analytical protocols, methods of sample preparation, and instruments (Wheeless *et al.,* 1991). We must thus treat with caution data which purport to demonstrate a correlation between the SPF and clinical behavior of tumors. The SPF is often presented as a surrogate measure of proliferation. However, even where measured accurately using sophisticated deconvolution models, the SPF gives no measure of time, nor of the rate of transition of the cells through the cell cycle. Thus, a tumor with a large S-phase fraction of slowly transiting cells may be much less proliferative than a tumor with a small S-phase fraction of rapidly transiting cells.

C. Cytometry of Other Clinical Tumor Markers

The labeling index (LI) is a generic way to quantify biomarker expression in cell populations. It is the proportion of cells expressing the biomarker above a given threshold in the total population under study. The LI describes a snapshot of the number of labeled cells in a tissue or tumor sample at one time point. It gives no indication of the rate of turnover or transit of the labeled cells in the population.

Cytometry allows quantitation of many cell surface, cytoplasmic, and nuclear proteins and epitopes, for which monoclonal antibodies and fluorescent tags offer accurate identification (Watson, 1992). Such markers include those that are closely associated with key regulatory processes such as proliferation, apoptosis, cell signaling, and oncoprotein function. Many proliferative biomarkers are known to act in the cell cycle, and their expression changes between quiescent and cycling cells, and with the phase of the cell cycle (Quinn and Wright, 1990). Examples include Ki-67, PS1, and the cyclin-dependent kinases. Multiparameter assays that plot DNA content against the expression of a chosen protein marker allow study of its cell cycle related expression, as for example, c-Myc (Rew *et al.,* 1991b) proliferating cell nuclear antigen (PCNA) (Hall *et al.,* 1990; Sawtell *et al.,* 1995), p53 (Rew *et al.,* 1996), and the cyclins (Darzynkiewicz *et al.,* 1996).

IV. The Halogenated Pyrimidines in Cell Proliferation Research

Proliferating cells may also be identified by an exogenous label, usually a thymidine analog. The nonradioactive halogenated pyrimidine (HP) thymidine analogs bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd) are robust and reliably incorporated into living cells during the S phase. They can be detected by a range of monoclonal antibodies developed in the early 1980s (Gratzner, 1982; Gonchoroff *et al.,* 1985; Gray, 1985; Vanderlaan and Thomas, 1985; Sasaki *et al.,* 1986). These can in turn be identified by fluorochromatically or histochemically labeled probes suitable for cytometric detection and analysis. BrdUrd was

originally developed and used as a tumor radiosensitizer in the 1950s, to be given as an adjunct to radiotherapy by intravenous injection in doses of 1 g per day for up to 40 days (Kinsella *et al.,* 1984). Iododeoxyuridine has similar properties to BrdUrd and is used as an alternative for *in vivo* kinetic studies. It is cross-reactive to some anti BrdUrd monoclonal antibodies. The HP analogs can be used in one of three ways to obtain data on the proliferating compartment (Waldman *et al.,* 1988): (1) by *in vitro* incubation of freshly obtained tumor samples (Miwa *et al.,* 1989), (2) by *in vivo* infusion over a period of hours to "saturate" the replicating fraction of cells (these studies have been reviewed in detail by Dolbeare, 1995a,b, 1996), and (3) by *in vivo,* intravenous pulse labeling preoperatively to obtain dynamic indices, of which we have particular experience as reviewed in this chapter.

The incorporation of HPs into DNA raises concerns about mutagenicity. In experimental cell systems, ultraviolet light increases the damage to DNA containing BrdUrd in tumor cells in culture and inhibits cell differentiation (Bar[redacted] *et al.,* 1978; Kaufman, 1986; Wright, 1986; Raffel *et al.,* 1988). Findings from these models must be interpreted with caution, as the unusual experimental conditions of the animal or cell model may not translate to the human body. For example, a DNA strand break assay may not take into account damage reversal by the DNA repair enzymes that regulate the integrity of chromosomes. HPs continue to be used in high doses as radiosensitizers, such as for brain tumors, and for the delivery of therapeutic doses of radioiodine to liver metastases (Speth *et al.,* 1989). Photosensitization has been reported with high doses (Fine and Breathnach, 1986). No untoward acute or long-term effects from low dose, single shot labeling have come to light in studies of more than 2500 cancer patients to date.

A. *In Vitro* Studies of Halogenated Pyrimidine Labeling

In vitro cytometric studies of human tumor biopsies by incubation with HPs allow derivation of an S-phase LI (Dolbeare *et al.,* 1983, 1985; Lloveras *et al.,* 1994), but they provide no measurement of the S-phase transit time. Incubation of freshly harvested and viable tissue and tumor cells can be performed in the laboratory without the need for *in vivo* injection or clinical consent. It has been used by many groups over a wide range of tumor and tissue types, as reviewed in detail by Dolbeare (1995a,b). The comparability between the tritiated thymidine and the nonisotopic analogs *in vitro* has been established in studies of human breast tumors (Meyer *et al.,* 1993; Maas *et al.,* 1996) and human colorectal and cervical tumors (Wilson *et al.,* 1985).

B. *In Vivo* Halogenated Pyrimidine Labeling Indices

In vivo labeling offers clear advantages over *in vitro* techniques. When given intravenously (iv) or intraperitoneally (ip) in animal models, the HP label is delivered physiologically to the tumor mass. Over short infusion periods immedi-

ately prior to, or at the time of biopsy, the labeled fraction will consist almost entirely of cells in the S phase, and a few that have passed into G_2/M.

The administration of a HP by continuous iv infusion over several hours progressively saturates cycling cells with label, such that, as more and more cells enter and pass through and out of the S phase, the labeled fraction approaches the growth fraction. *In vivo* HP labeling index (S-phase fraction) data have been reported on various classes of tumor (Wilson *et al.,* 1985; Raza *et al.,* 1985), including intracranial gliomas (Hoshino *et al.,* 1985, 1989), bronchial tumors (Tinnemans *et al.,* 1995), meningiomas (Langford *et al.,* 1996), transitional cell carcinoma of the bladder (Popert *et al.,* 1993), renal carcinoma (Larsson *et al.,* 1994), squamous cell carcinomas (SCC) of head and neck (Kotelnikov *et al.,* 1995a,b), and breast tumors (Sasaki *et al.,* 1987; Goodson *et al.,* 1993; Christov *et al.,* 1994).

C. Time-Dependent Parameters of Tumor Proliferation

Time is a key component of biological processes. Tumors grow, regress, stabilize, or evolve with time. Using HPs, proliferation measurements with a time component can be made in clinical tumors. These "dynamic" indices include the S-phase duration (Ts), the cell cycle time (Tc), and the potential doubling time (Tpot). The data from such clinical measurements are reviewed at greater length here.

Continuous prebiopsy infusion denies the option of time-dependent data provided by pulse labeling. A pulse label of an HP and multiparameter flow cytometry can be used to derive time-dependent data from a single biopsy of a tumor following *in vivo* administration by the intraperitoneal or intravenous route (Begg *et al.,* 1985, 1988; Begg, 1989; Terry *et al.,* 1991). HP markers are robust within tumor biopsies, surviving degradation, enzymatic extraction, and acid denaturation during analysis, and surviving long-term storage in ethanol- and in formalin-fixed tissues. It is a remarkable and fortunate observation that these markers survive metabolism, sequestration, and dilution, enter the tumor mass, pass into proliferating cells, and incorporate into S-phase DNA within an hour of intravenous injection.

The technique is illustrated by a study of BrdUrd incorporation after bolus intraperitoneal injection into the human HT29 colorectal tumor grown in a mouse model in Fig. 1. In this model, the G_0/G_1 phase of the principal tumor cell population is centered on channel 40 on the *X* axis. Within 2 hr, labeled S-phase cells are clearly identified in the G_2M phase. By 5 hr, many labeled cells have appeared in G_0/G_1 of the daughter cell cycle. By 14 hr, the majority of labeled cells have reached this phase. By 29 hr, daughter cells are streaming through the S phase once again. The cell cycle duration is measured by plotting the proportion of labeled cells in the mid S phase against time (Fig. 2). Such serial biopsy is rarely practical in the clinical setting, where single biopsies must usually suffice.

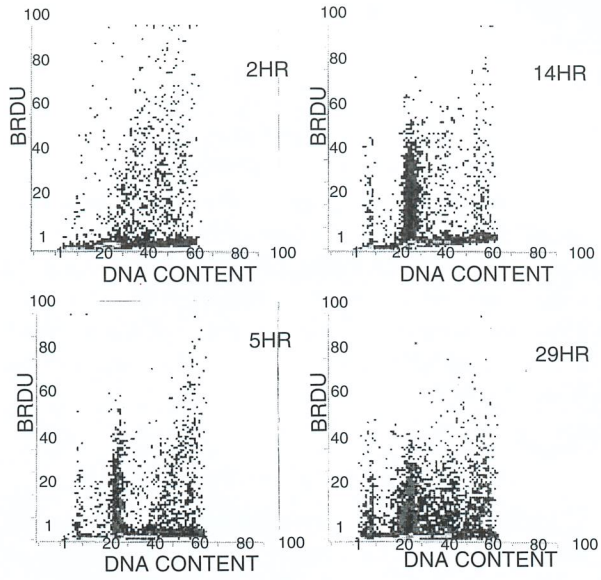


Fig. 1 A study of BrdUrd incorporation after bolus intraperitoneal injection into the human HT29 colorectal tumor grown in a mouse model. The G_0/G_1 phase of the principal tumor cell population is centered on channel 40 on the *X* axis. BrdUrd fluorescence is represented on the *Y* axis. By 2 hr, labeled S-phase cells are seen in the G_2/M phase. By 5 hr, many labeled cells have appeared in G_0/G_1 of the daughter cell cycle. By 14 hr, the majority of labeled cells have reached that phase. By 29 hr, many of the daughter cells have reentered the S phase.

D. Correlation of Proliferation Data and Biomarker Expression

The *in vivo* labeling of clinical tumors with a robust S-phase label provides a unique opportunity for correlative studies of biomarker expression in proliferative cells. They provide a framework for the study of proteins that act at specific points in the cycle to initiate, regulate, suppress, or terminate DNA replication, such as the oncoproteins p62-c-Myc and p53 (Rew *et al.,* 1991b, 1996), or proteins associated with apoptosis, such as bc12 (Wilson *et al.,* 1996).

V. Clinical Studies of Cell Production Rates with Thymidine Analogs

Studies of cell production rates *in vivo* using tritiated thymidine and the fraction of labeled mitoses (FLM) long predated the halogenated pyrimidines (Tubiana

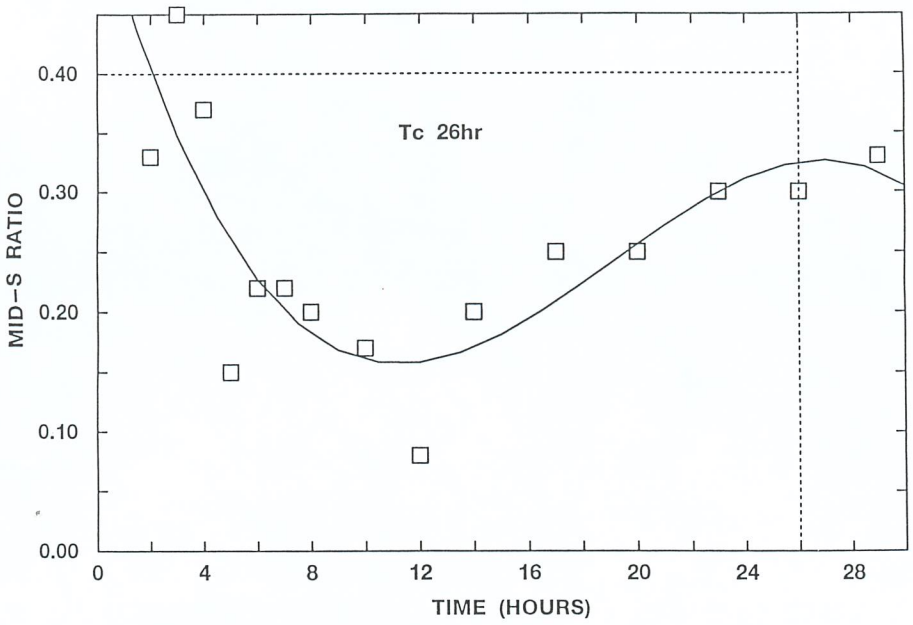


Fig. 2 The method of estimation of the cell cycle duration in the model described in Fig. 1 by plotting the proportion of labeled cells in the mid S phase against time. The cell cycle length in this model is of the order of 26 hr.

and Courdi, 1989). However, the HP/flow cytometry method represented a quantum leap in clinical utility and speed in analysis (Rew and Wilson, 1991). Studies reporting time-dependent parameters derived from the HP/FCM method fall into four general categories: Those descriptive studies that report proliferative data, including heterogeneity studies; those that correlate such data with clinical outcome; those that correlate such data with response to therapy; and those that combine data from immunohistochemical (IHC) measurements of labeling indices with flow cytometric measures of S-phase duration in related samples. These combined studies allow estimates of proliferation rates in selected areas of heterogeneous tissues and tumors, or in tissues such as epithelia where proliferative cells display geographic organization.

In early clinical studies, BrdUrd was shown to be a reliable marker when given intravenously in a subclinical bolus dose of 100–250 mg a few hours before surgical biopsy (Fig. 3). Wilson *et al.* (1988) reported the *in vivo* measurement of the LI, Ts, and Tpot of 26 evaluable human tumors of various lineages obtained by local biopsy. Riccardi *et al.* (1988) reported the cell kinetics of 46 acute leukemias, 27 gastric carcinomas, and 16 gliomas.

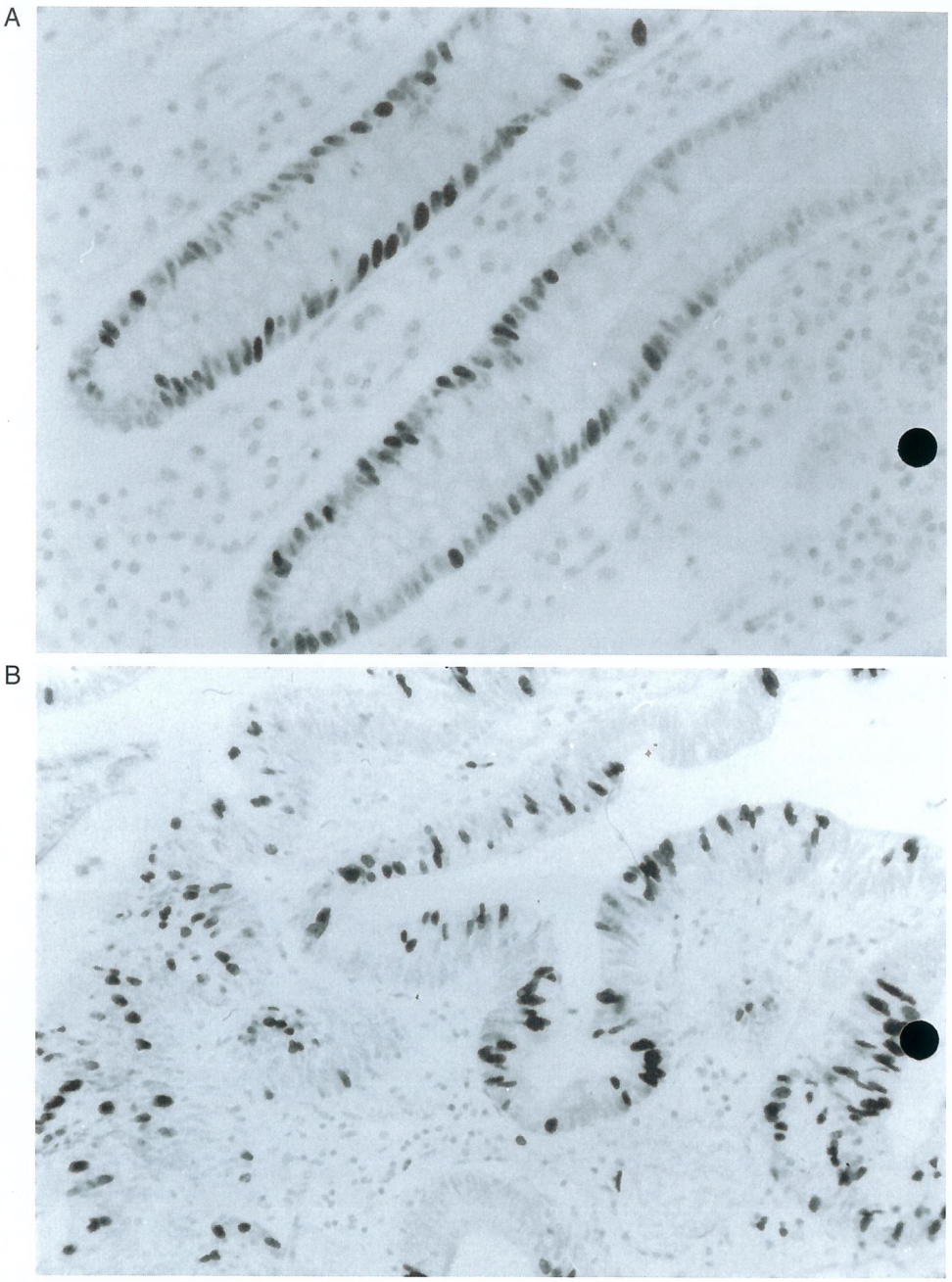
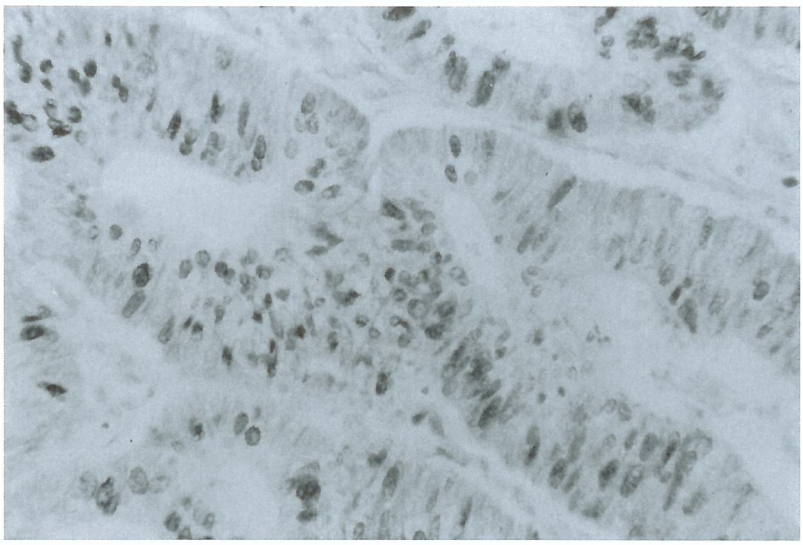


Fig. 3 Series of photomicrographs of colorectal tissues obtained at surgery, where the BrdUrd pulse-labeled proliferating cells are detected immunohistochemically (see text) with peroxidase counterstain. (A) Highly ordered distribution of labeled cells in the base of normal mucosal crypts. (B) Villous adenoma of the rectum. (C) Well-differentiated invasive adenocarcinoma of the colon. Microadenoma in mucosa from a patient with familial polyposis coli. (D) Note the migration of the poliferating compartment towards the luminal face.

C



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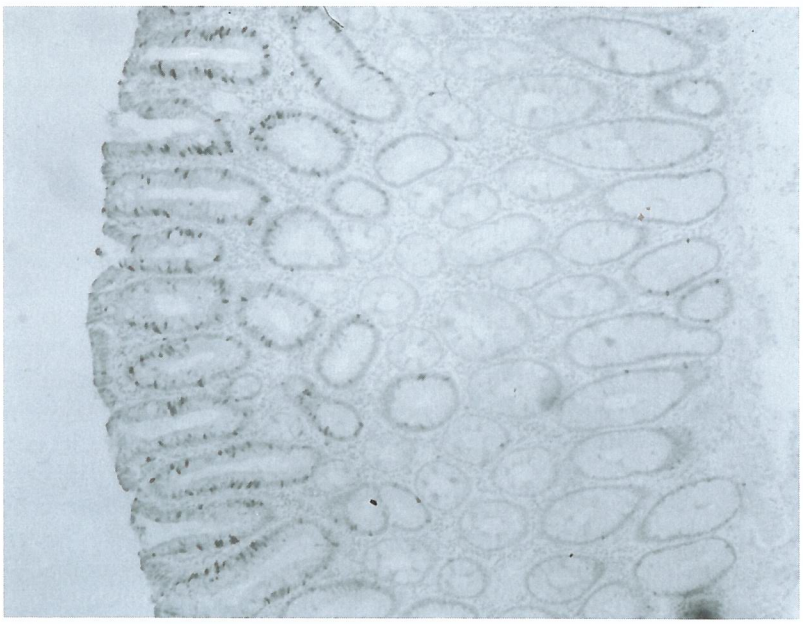

Fig. 3 (*continued*).

A. Descriptive Clinical Studies of Dynamic Tumor Proliferation

1. Squamous Carcinomas

a. 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 challenge that they pose, their radioresponsiveness, and their accessibility to biopsy and to direct observation of clinical growth rates.

Forster *et al.* (1992) reported cell kinetics in a series of 82 HN-SCC tumors. Cooke *et al.* (1994) reported interim survival data for this series. They found no significant correlation between LI, Ts, and Tpot and tumor stage, node status, or tumor site. Bennett *et al.* (1992) assessed tumor proliferation of HN-SCC by both IHC and flow cytometric (FCM) analysis. Using FCM data alone, 46% of the tumors 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. Jones *et al.* (1994) reported data from 75 patients with HN-SCCs. Nylander *et al.* (1994) studied 31 such tumors by both FCM and IHC following IdUrd labeling. The utility of prognostic correlations were limited by small numbers and incomplete follow-up. Benazzo *et al.* (1995) studied 46 head and neck cancers. BrdUrd LI and TS significantly correlated with histological differentiation grading: Grade III tumors showed higher LI values and shorter TS values than Grade I and Grade II tumors. Similar study results are reported by Kotelnikov *et al.* (1995a,b) and Wilson *et al.* (1995). These data are recorded in Table I. Figure 4A illustrates the typical proliferation patterns of two diploid tumors labeled *in vivo* with BrdUrd, a basal cell carcinoma and a squamous cell carcinoma of the pinna (Rew, 1991).

b. Squamous Esophageal Tumors

The Northwood (England) group has reported the proliferation parameters in the biopsies of 30 esophageal squamous tumors using BrdUrd pulse labeling (Wilson, 1991; Rew *et al.*, 1991a) (Table II). Haustermans *et al.* (1994, 1995, 1997) reported a similar series of 31 patients. IdUrd was injected 6 to 10 hr before surgery, and five biopsies per tumor were taken ($n = 305$). Tumor-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 tumors. Haustermans *et al.* (1994) also reported the heterogeneity of proliferation in these tumors. The coefficient of variation (cv) of intratumor Tpot measurements from up to five biopsies from each of 30 tumors ranged from 7.0 to 39%, or up to 5 days. In a sample of 30 biopsies from a single squamous tumor to assess intratumor heterogeneity, the mean Tpot was 4.2 ± 0.9 [standard deviation (SD)] days, with a range of 2.0–6.4 days.

c. Squamous Carcinomas of the Uterine Cervix

Cervical tumors are also significant for their radioresponsiveness and thus have been a focus for several labeling studies. Bolger *et al.* (1993) assessed 120

Table I
Tumors of the Head, Neck, and Lung

Reference	No.	Median LI, %	Median Ts, hr	Median Tpot (range), days	Comments
Head and Neck					
Bennett *et al.* (1992)	123	6.8	9.9	5.7	BrdUrd
Forster *et al.* (1992)	105	7.0 (1.3–21.9)	14.0 (7.0–106)	5.9 (1.3–67.5)	BrdUrd
Cooke *et al.* (1994)					
Jones *et al.* (1994)	75	8.9 (1.6–25.0)	14.8	Check	BrdUrd
Nylander *et al.* (1994)	31 FCM	13.6 (3.6–26.4)	16.1 (3.9–32.4)	4.6 (1.3–12.2)	IdUrd
	IHC	9.1 (1.6–35.0)		5.4 (1.1–36.2)	
Wilson *et al.* (1995)	165	5.0 diploid	9.9	1.8 diploid	
		9.3 aneuploid	5.4–21.9	3.2 aneuploid	
Benazzo *et al.* (1995)	46/52	7.9 (2–18)	11.6 (6–28.5)	5.7 (2–30)	BrdUrd
Kotelnikov *et al.* (1995)	12	>20	12.1 (5.1–21.5)	43.2 hr (18.8–84.5 hr)	IdUrd + BrdUrd histochemistry
Bourhis *et al.* (1996)	70	6.3–7.7	8.3–9.3	4.6–5.6	BrdUrd
Corvo *et al.* (1996)	82	8.0 (1.5–28.0)	10.0 (6.0–14.0)	5.0 (2.0–20.0)	BrdUrd
CNS tumors					
Astrocytoma, Shibuya *et al.* (1993)	100		9.2 (6.0–13.7)	1 to 60 days	
Meningioma, Riccardi *et al.* (1988)	22	2.1 (0.9–3.9)	16.7	63.2	BrdUrd
Malignant, Struikmans *et al.* (1997)	71	0.03	4.5	5.4	BrdUrd
Benign, Struikmans *et al.* (1997)	52	0.01	4.7	20.9	BrdUrd
CNS mets, Struikmans *et al.* (1997)	14	0.03	3.9	3.9	BrdUrd

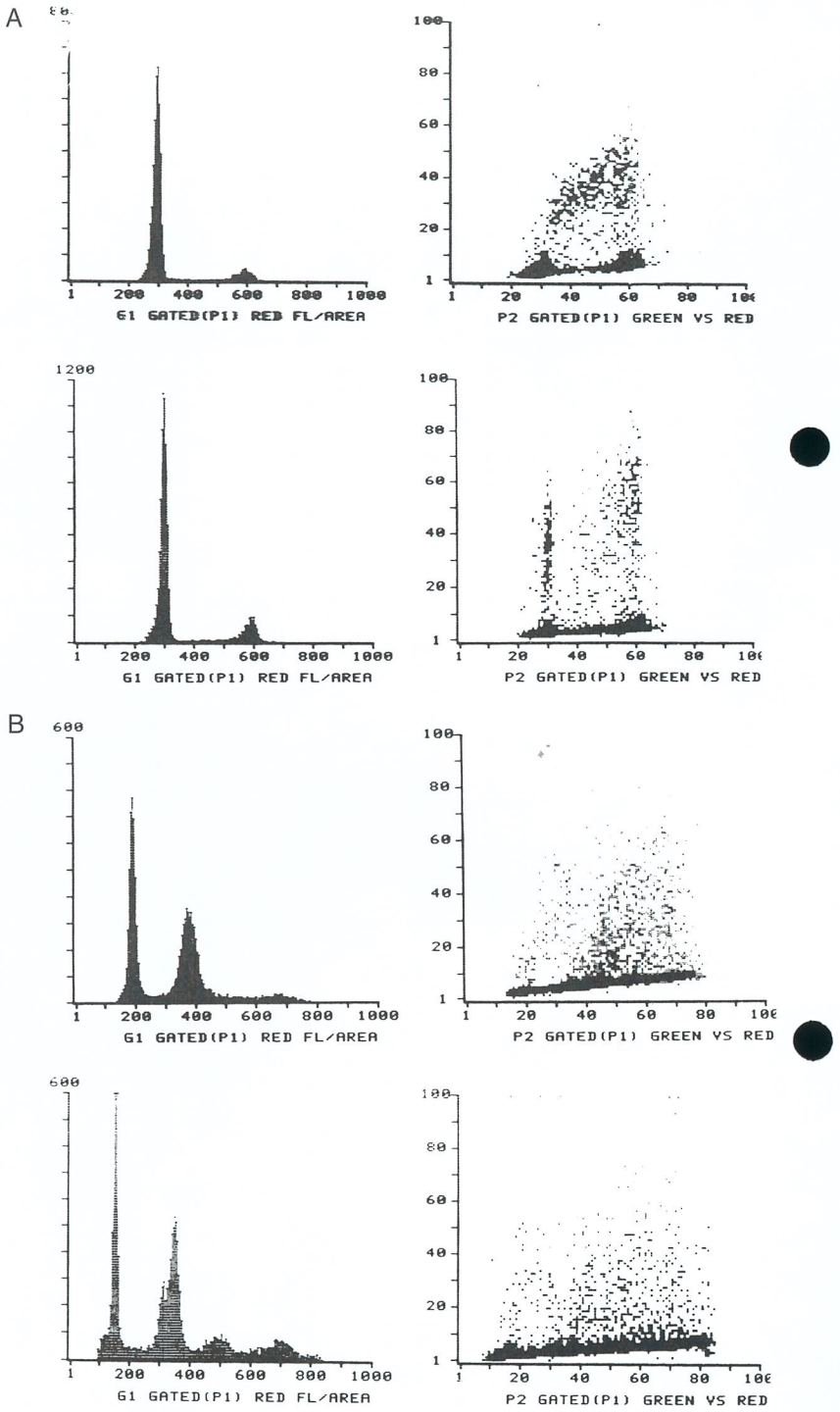
A
G1 GATED(P1) RED FL/AREA
P2 GATED(P1) GREEN VS RED
G1 GATED(P1) RED FL/AREA
P2 GATED(P1) GREEN VS RED
B
G1 GATED(P1) RED FL/AREA
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Fig. 4

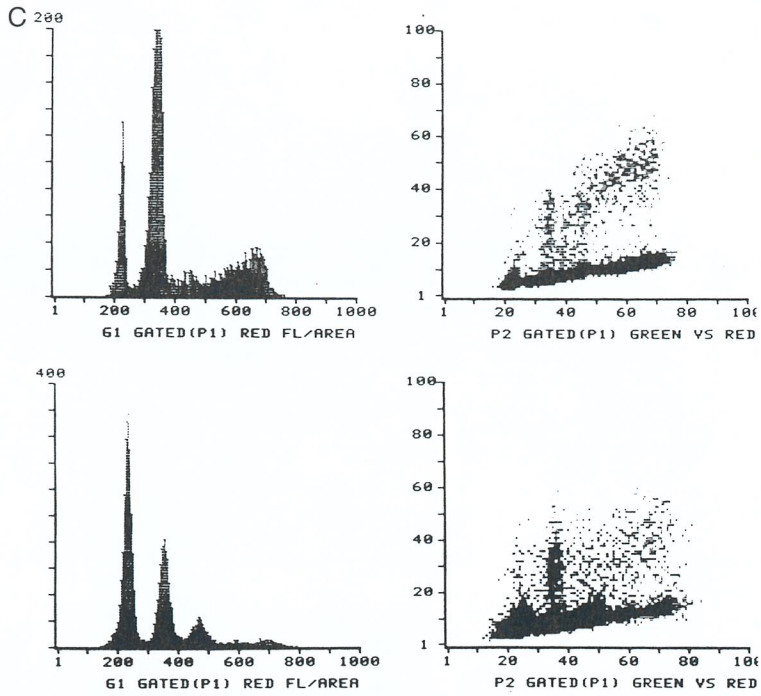

Fig. 4 Paired histograms and dot plots displaying the DNA profiles and corresponding multivariate BrdUrd (*Y* axis) versus DNA (*X* axis) analyses of a series of tumors pulse labeled with 250 mg BrdUrd *in vivo* (see text). All analyses were undertaken on an Orthocytofluorograph cytometer at the GRAY Laboratory, Northwood, England. (A) Two diploid tumors. The upper profile is from a giant basal cell carcinoma of the scalp, and the lower from a squamous cell carcinoma of the pinna. (B) Typical gastric adenocarcinoma (upper profile) and its liver metastasis (lower profile). (C) Two typical aneuploid colorectal tumors.

cervical tumors. In 89% both static and temporal kinetic parameters could be measured. The analysis of multiple biopsies from each tumor revealed marked intratumor heterogeneity (Table III). There was an elevation in the LI, but no difference in Ts, between tumor and non-neoplastic cervical tissue. There was a significant elevation of the LI, in proliferating cells from advanced stage and large size tumors. In a further study, this group reported (Bolger *et al.* (1993) no correlation between these parameters and a response to conventional radiotherapy was found other than an association between a high BrdUrd labeling index and pelvic tumor recurrence. Tsang *et al.* (1995) measured the pretreatment Tpot values in carcinomas of the uterine cervix in 46 patients. For 25 patients where Tpot measurements were performed at two separate laboratories, systematic laboratory differences were detected in the data.

Table II
Gastrointestinal Tract Tumors

Reference	No.	Median or mean LI (ranges), %	Median Ts (range), hr	Mean or median Tpot (range), days	Comments
Upper intestinal tumors					
Esophageal squamous, Haustermans *et al.* (1994)	31	19.0 ± 7.0 (SD)	17.2 ± 8.0	4.4 (2.0–6.4)	IdUrd
Esophageal squamous, Rew (1993)	9	5.3 (1.4–17.4)	9.8 (4.6–17.8)	4.3 (2.7–17.9)	BrdUrd
Esophageal squamous, Wilson (1991)	50	7.8 (0.4–27.5)	12.4 (6.9–28.6)	5.2 (1.6–56.8)	BrdUrd
Gastric adenocarcinoma, Rew (1993)	39	4.9 (0.9–18.5)	10.7 (3.6–31.9)	5.2 (0.8–39.4)	BrdUrd
Gastric adenocarcinoma, Haustermans (1995); Haustermans *et al.,* 1995)	32	16.0 ± 9.0 (SD)	15.8 ± 6.7	5.6 (2.1–9.5)	IdUrd
Gastric adenocarcinoma, Riccardi *et al.* (1988)	22	9.9 (5.7–14.0)	15.2 (13.4–22.7)	9.8 (6.8–13.5)	BrdUrd
Colorectal					
Camplejohn (1982)	19	—	—	192 hr	Stathmokinetics
Bergstrom and Stenling (1990)	?	13 (2.9–27.0)	16 (7.0–35.0)	3.6 (2.5–14.0)	IdUrd
Rectal, Terry *et al.* (1995)	101	21.0 (13.5–27.0)	20.0	3.3 (2.4–5.6)	BrdUrd 6 hr before biopsy
Rew *et al.* (1991a)	100	9.0 (0.7–22.2)	13.1 (4.0–28.6)	3.9 (1.75–21.4)	BrdUrd
Michel *et al.* (1997)	19	5.3–15.4	9.7–16.6	4.5 (1.2–21)	BrdUrd
Wilson *et al.* (1993a,b)	125	12.7 (0.6–33.8)	14.1 (5.2–48.3)	4.5 (0.9–53.8)	IudUrd

Table III
Female Reproductive Tract Tumors

Reference	No.	Median LI (range), %	Median Ts (range), hr	Median Tpot (range), days	Comments
Breast					
Rew *et al.* (1992)	75	4.2 (0.6–15.4)	8.7 (2.7–22.2)	8.2 (1.8–47.5)	BrdUrd
Stanton *et al.* (1996)	84	3.2	12.0	12.5	BrdUrd
Gynecology					
Ovarian, Erba *et al.* (1994)	55	6.1–7.2	14.7	12.5	BrdUrd
Cervix, Bolger *et al.* (1996)	120	9.8 (6.7–14.5) interquartile	12.8 (11.1–14.9) interquartile	4.0 (3.1–6.3) interquartile	BrdUrd
Cervix (squamous), Tsang *et al.* (1995)	39			5.5	BrdUrd
Cervix (adenocarcinoma), Tsang *et al.* (1995)	7			6.6	BrdUrd
Ovarian metastases, Rew (1991)	2	5.1, 5.4	12.5, 13.9	3.5, 6.5	

2. Central Nervous System Tumor

Most studies of HP labeling *in vivo* on brain tumors have measured labeling indices alone (see Dolbeare, 1995a). For example, Shibuya *et al.* (1993) studied 100 brain tumors double labeled with BrdUrd and IdUrd. Struikmans *et al.* (1997) reported on 71 malignant and 52 benign brain tumors and 14 cerebral metastases. Labeling indices were low, and there was little difference between data for benign and malignant tumors. Studies of meningiomas (Riccardi *et al.,* 1988) and astrocytomas (Danova *et al.,* 1991) have also been reported.

3. Adenocarcinomas

a. Gastric Adenocarcinomas

Gastric adenocarcinomas are very heterogeneous in their clinical growth behavior, from the flat, infiltrative to the markedly exophytic lesions. The contribution of cell production rates to their biological aggressiveness is uncertain. Static labeling index measurements of gastric tumors have been studied, particularly by Japanese groups (Miwa *et al.,* 1993; Ohyama *et al.,* 1990). A number of series of time-dependent indices of gastric adenocarcinomas are reported from Europe. We have reported data on 39 such tumors (Rew 1991, 1993). Haustermans *et al.* (1997) reported a series of 32 tumors labeled with IdUrd. Intratumor, intertumor, 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 intratumor Tpot measurements from up to five biopsies from each of 30 tumors ranged from 16.0 to 51% (Table I). Figure 4b illustrates the proliferation patterns of a typical gastric adenocarcinoma and its liver metastasis following *in vivo* BrdUrd labeling (Rew, 1991).

b. Colorectal Adenocarcinomas

Colorectal proliferation studies are of particular value for the facility to correlate proliferative data and labeling patterns with form and function across the mucosa–adenoma–tumor sequence. They also provide valuable insights into the gross proliferative biology of this common surgical disease.

Cell proliferation studies on these tumors predate the HP assay. Camplejohn (1982) published estimates of colorectal tumor proliferation rates using laborious stathmokinetic measurements of tritiated thymidine labeling. The efficacy of BrdUrd labeling of colorectal tumors *in vivo* was established by Risio *et al.* (1988) and by Khan *et al.* (1988), who published labeling studies of small series.

Three large studies have subsequently provided dynamic data on proliferation rates in primary colonic and rectal cancers using HP labeling *in vivo.* Rew *et al.* (1991a) found no correlation between any kinetic parameters and the Dukes stage or histological classification of 100 colorectal tumors labeled *in vivo* with BrdUrd. A series of tumors labeled with IdUrd (Wilson *et al.,* 1993a,b) yielded

similar data. Terry *et al.* (1995) reported the cell kinetics of 101 rectal cancers. A smaller series of 19 tumors was reported by Michel *et al.* (1997).

These series also demonstrated considerable intratumoral heterogeneity of proliferative parameters according to site of biopsy, and between aneuploid and diploid tumors and populations. A cross study of samples between these institutions demonstrated considerable reliability and reproducibility of the measurements, irrespective of institution, choice of HP, or cytometer used. Figure 4c illustrates the proliferation patterns of two typical aneuploid colorectal tumors following *in vivo* BrdUrd labeling from the Northwood series (Rew *et al.,* 1991a).

c. Breast Carcinomas

Breast cancers are an important clinical challenge and a valuable model for proliferation studies. Primary and recurrent lesions are accessible to biopsy, and volume growth is more easily estimated in the absence of exfoliation or necrosis as significant causes of cell loss, as for example, in tumors treated by tamoxifen or other chemotherapy alone.

Two series report the *in vivo* labeling of human breast tumors with BrdUrd. In a series of 69 patients with invasive breast carcinoma (Rew *et al.,* 1992), there were no significant differences in the total LI, Ts, or Tpot when patients were stratified according to lymph node status, tumor size, tumor grade, or menopausal status. Histological counting of labeling indices in this series of breast tumors yielded data comparable to flow cytometric analysis (Ashton-Key *et al.,* 1993). Stanton *et al.* (1996; Stanton, 1996) reported a similar series of 84 cases from Glasgow. LIs were significantly higher in aneuploid tumors and in tumors not expressing estrogen receptors, but they were not correlated with tumor size, nodal status, or expression of c-erbB2 (Table III).

d. Ovarian Carcinomas

Erba *et al.* (1994) 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 tumor stage, histological grading, residual tumor size, or DNA ploidy (Table III).

4. Other Tumors

a. Malignant Melanoma

Primary malignant melanoma lesions are difficult to study because of their small size and heterogeneous schirrhous admixture of skin and tumor cells, and because of the limitation of losing key pathological information on depth and planes during experimental biopsy. Laing *et al.* (1992) have reported one large series of 83 primary and metastatic malignant melanomas. Thin, good prognostic primary lesions (<1.5 mm thickness) displayed significantly slower proliferation than did the thicker lesions.

b. Hematological Malignancy

Hematological tumors are of interest for the ease with which multiple analyses can be performed on blood samples. Hematological stem cells are among the most actively proliferating subsets in the body (Raza *et al.,* 1992). Giordano *et al.* (1993) reported the kinetics of acute nonlymphoblastic leukemia (ANLL). Sixty-five patients with untreated ANLL and 15 patients with solid tumors and normal bone marrow (BM) received 250 mg/m^2 BrdUrd. Raza *et al.* (1997) studied bone marrow of 68 patients with myelodysplastic syndromes (MDS) who received sequential infusions of IdUrd and/or BrdUrd (Table IV).

c. Lung Carcinomas

Wilson (1993) reported a series of 38 lung tumors yielding 88 samples (Table IV). Tinnemans *et al.* (1993) studied bronchoscopy specimens of 27 lung cancers and 11 benign biopsies after *in vivo* labeling with 50 mg/m^2 BrdUrd. They obtained cytokinetic data from seven samples of small cell lung cancer (SCLC) and 20 samples of non-small cell lung cancer (NSCLC). No significant differences were observed between the mean values of the cytokinetic parameters of SCLC and NSCLC.

d. Urological and Other Tumors

Other tumor types, including tumors of the urological tract carcinoma of the bladder (Rew *et al.,* 1991c), lymphomas, and sarcomas, have been studied in smaller numbers (Table V).

B. Descriptive Studies of Cell Production Rates in Nonmalignant Epithelium

The *in vivo* labeling of human tumors also provides opportunities for the qualitative and quantitative histological study of patterns of proliferation in histological samples of tumors and adjacent normal tissue. A number of such studies have been reported. They provide valuable descriptive and quantitative information on the proliferation of epithelia in particular (Potten *et al.,* 1992a,b). Labeled cells can be clearly distiguished by conventional immunohistochemical techniques. Figure 3 illustrates this point with a series of photomicrographs of BrdUrd pulse-labeled colorectal tissues obtained from surgical resection specimens (see Rew *et al.,* 1991a). In Fig. 3a, the distribution of labeled cells in the base of normal mucosal crypts is highly ordered. In Fig. 3b, the cells in the epithelium of this villous adenoma of the rectum, a premalignant lesion, have lost their spatial discipline but remain confined to the epithelial plane. In Fig. 3c, a well-differentiated invasive adenocarcinoma of the colon, the proportion of proliferating tumor cells is much higher, associated with a greater degree of spatial indiscipline. Figure 3d shows how HP labeling can highlight disordered proliferation controls in disease states. It illustrates a microadenoma in mucosa from a patient with familial polyposis coli and a concurrent carcinoma. The

Table IV
Other Tumors

Reference	No.	Median LI (range), %	Median Ts (range), hr	Median Tpot (range), days	Comments
Melanoma					
Laing *et al.* (1992)					
Primary tumors	24	4.2 (1.3–13.6)	10.7 (6.3–20.5)	7.2 (3.5–41.3)	BrdUrd
Metastases	61	5.4 (0.6–16.8)	11.6 (6.1–26.2)	7.2 (2.3–139.0)	
Hematology					
Normal bone marrow Giordano *et al.* (1993)	15	11.8 ± 3.1	10.1 ± 2.0	3.5 ± 5.6	BrdUrd
Myelodysplastic syndrome Raza *et al.* (1997)	68	28.4	11.8	40.8 hr cell cycle time	Double labeling
MDS + acute myeloid leukemia Raza *et al.* (1992)	10	27.7 (22–37.5)	15.5 (11.4–24.6)	58.3 hr cell cycle time	BrdUrd
Acute non-lymphocytic leukemia Giordano *et al.* (1993)	54	6.1 ± 2.9	16.2 ± 6.1	18.1 ± 18.9	BrdUrd
ANL leukemia Riccardi *et al.* (1988)	42	6.2 (0.9–11.7)	12.1 (6.9–27.8)	8.5 (2.8–16.7)	BrdUrd
Lung					
Wilson (1993) Tumors	38	8.0 (0.7–28.2)	15.1 (5.5–37.8)	7.3 (1.4–132.0)	
Samples	88				
Normal lung	9	2.7 (1.2–7.8)	11.1 (4.2–41.1)	17.3 (6.5–32.0)	BrdUrd
Diploid tumors	14	5.0 (1.1–11.9)	6.9 (3.6–13.5)	9.6 (1.9–28)	
Aneuploid tumors	13	14.7 (3.8–33.6)	14.0 (5.7–29.4)	6.7 (1.5–20.7)	
Tinnemans *et al.* (1993)					

Table V
Unpublished Data and Rare Tumors[a]

Tumor type	No.	Mean LI (range), %	Mean Ts (range), hr	Mean Tpot (range), days	Comments
Lymphomas	2	5.4 (0.9–13.4)	11.0 (8.3–16.0)	15.8 (2.5–23.2)	
Hodgkin's nodes	3	2.6 (0.9–4.6)	10.4 (9.4–11.0)	17.7 (5.5–39.6)	
Basal cell carcinoma	1	5.4	10.7	6.6	
Sarcoma	2	1.5, 2.7	7.1, 22.0	8.8, 48.8	
Liposarcoma	1	0.6	2.4	13.5	
Osteosarcoma	1	0.8	8.8	36.7	
Urological					
TCC bladder, Rew *et al.* (1991c)	19	2.5 (0.5–10.0)	6.2 (3.5–9.7)	17.1 (3.6–40.0)	BrdUrd
Renal adenocarcinoma, Rew *et al.* (1991c)	2	1.9, 6.6	7.1, 11.9	4.5, 18.0	BrdUrd
Prostate carcinoma, Rew *et al.* (1991c)	1	1.9	9.0	7.7	

[a] G.D. Wilson and D.A. Rew, unpublished data, 1988–1991.

distribution of proliferating cells is completely inverted as compared with normal mucosa.

The rate of cell proliferation in normal tissues is of particular interest in comparison with tumor proliferation rates, where samples are obtained in the course of labeling studies on cancer cases. The ordered architecture of epithelium does not allow direct measurement of labeling indices derived from tissue homogenates using the automated techniques of laser cytometry. The counting of labeled cells must be in a structured fashion, and it must be correlated directly with the tissue architecture (Potten *et al.,* 1992a,b) (Table VI).

1. Skin and Mucosa

Van Erp *et al.* (1996) studied the cell cycle kinetics of normal skin epidermal cells in 14 lymphoma patients with IdUrd using serial biopsies. Esophageal squamous mucosa also has a well-defined proliferative zone at the base of the epidermis. The proliferation rates in labeled cells from normal esophageal mucosa from esophageal tumor resection specimens has been reported (Rew, 1991; Haustermans, 1995). 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 analyzed. Patel *et al.* (1993) 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.* (1992a) conducted a systematic quantitative analysis of the distribution of BrdUrd-labeled cells in human colorectal mucosa, taken from sites throughout the colorectum. A range of proliferative indices were measured, including the crypt labeling index, the peak labeling position, and the detailed distribution of labeled cells, along with the Ts median value of 8.6 hr. The cell cycle time was calculated to be 30 hr. The entire crypt turnover time was thus calculated to be 82 hr. These data were very consistent with earlier calculations made using tritiated thymidine in much smaller series and with animal model studies.

2. Proliferation in Transition Tissues

Colorectal adenomas are of interest for their malignant potential and for the intermediate level of proliferative disorder that they display between mucosa and invasive tumors. In a small study, incidental adenomas analyzed from colorectal tumor resection specimens, including cases of familial polyposis coli, displayed patterns of disorder and values for labeling indices intermediate between mucosal crypts and tumors (Rew, 1993). Ts values were similar to those in mucosal and tumor cells (Table VI).

Table VI
Normal and Nonmalignant Tissues

Reference	No.	Mean LI (range), %	Ts	Tpot	Comments
Skin					
Van Erp *et al.* (1996) Gastrointestinal Epithelium		3.5	9.7 ± 0.6 hr	28.4 hr cell cycle time	
Squamous esophageal mucosa, Rew (1991)	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 esophageal mucosa, Haustermans (1995)	53	5.2 ± 2.1 (SD)	9.6 ± 2.8 (SD)	8.4 ± 3.8 (SD)	IdUrd
Gastric mucosa, Patel *et al.* (1993)	27	2.7 (1.1–4.7)	9.6 (3.8–19.7)	15.6 (3.4–59.7)	FCM data on tissue disaggregates
Colorectal mucosa, Rew (1991) (flow cytometry analysis)	157	2.0 (0.6–8.4)	10.7 (3.1–37.5)	24.8 (2.9–101)	FCM data on tissue disaggregates
Colorectal mucosa, Potten *et al.* (1992a,b) (histometric analysis)	147	Varies with position in crypt	As above	Cell cycle time 30 hr	Same series as Rew, 1991; histochemistry and manual counting
Villous adenoma, Rew *et al.* (1991a)	6	5.3 (2.3–9.1)	8.6 (4.8–12.8)	6.1 (3.6–10.6)	BrdUrd
Metaplastic polyp, Rew *et al.* (1991a) (colorectal mucosa)	10	4.9 (0.6–20.1)	10.6 (5.2–19.6)	6.3 (2.5–53.6)	BrdUrd