C. Cell Production Rates and Therapy

Most clinical research on the correlations between therapy and cell production rates has been conducted during radiotherapy for squamous cell carcinomas of the head and neck. The rate of cell proliferation in individual tumors, as indicated by the local Tpot, may influence the tumor response to fractionated radiotherapy. The presence of rapidly proliferating clones in radiosensitive tumors suggested the design of trials of continuous, hyperfractionated, accelerated radiotherapy (CHART). This is intended to enhance tumor kill by preventing proliferation during the course of treatment. Studies have focused on head and neck cancer in particular (Withers *et al.,* 1988; Dische and Saunders, 1989; Saunders *et al.,* 1991). In a series reported from Northwood, data were complete for 90 patients (Wilson *et al.,* 1995). 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 tumors showed more rapid median proliferation rates (Tpot 1.8 days) than aneuploid tumors (3.2 days).

Bourhis *et al.* (1993) studied the predictive value of pretreatment Tpot and LI in 70 patients with head and neck squamous cell carcinoma treated with conventional radiotherapy. No relationship was found between the Tpot or LI and the tumor stage, nodal status, histological grade, and the site of the primary. The mean Tpot of the tumors that relapsed locally was 5.3 days, compared to 6.1 days for those who did not relapse locally (not significant). The TS, LI, DNA index, and Tpot were not associated with local relapse, nor with disease-free survival (DFS). Corvo *et al.* (1993, 1995) reported a further such series of 82 patients (data shown in Table I).

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/fraction, three fractions per day with a 12–14 day split after 8 days). An analysis of 60 cases demonstrates that pretreatment Tpot data could not discriminate patients with improved (>4 days) or impaired (<4 days) survival even though the clinical data have shown benefit for the accelerated schedule (Begg *et al.,* 1990, 1992). A similar finding has emerged from studies from Paris (Bourhis *et al.,* 1996) in which 70 patients with oropharyngeal tumors treated by 70 Gy in 7 weeks were evaluated.

A study from Genoa (Antognoni *et al.,* 1996) 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 tumors (<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 (Zackrisson *et al.,* 1997) 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.

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 (Corvo *et al.,* 1996; Horiot *et al.,* 1997).

An analysis by Begg *et al.* (1998) of correlations between kinetic data and the outcome of treatment of 476 patients in 11 centers by at least 6 weeks of conventional radiotherapy demonstrated that the labeling index was the only significant variable in a univariate analysis for no control. Multivariate analysis showed no significant correlations, although comparisons were hampered by intercenter 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.

D. An Overview of Proliferation Data in Human Tissues and Tumors

The halogenated pyrimidines have proved to be potent tools for measuring tumor cell production rates when analyzed by laser flow or scanning cytometry (Rew *et al.,* 1998). The technique has been evaluated by a number of groups using different equipments and analytical techniques, and results have been found to show high concordance from one center to another. There are nevertheless a number of factors that confound the interpretation of proliferation data derived from human tumor biopsies.

The reliability of analyses is a key factor in the interpretation of proliferation measurements (Wilson, 1993). There is considerable technical, observer, and institutional variability in the data, contributing to the spread of data within and between tumors (Wheeless *et al.,* 1991; Haustermans *et al.,* 1995). Concern has been expressed by one group about the stoichiometry of antibody binding after DNA denaturation (Gilliland *et al.,* 1997; Williamson *et al.,* 1994).

Heterogeneity of architecture and cell content confounds the interpretation of data in solid tumors (Rew, 1996). Site to site variation for proliferation parameters is highly significant in tumors. The labeling index can vary considerably from region to region within a tumor. In any one local area of a tumor it may range from less than 1% to more than 50%, and it may vary according to the technique selected for measurement of the HP LI. For example, histochemical measures of the labeling index derived by manual counting are usually higher than equivalent FCM-derived indices (Bennett *et al.,* 1992; Ashton-Key *et al.,* 1993).

We are also presented with a complex problem in deciding which of the many indices generated by a HP label is most representative of the proliferation of the tumor. For example, the LI(max) and Tpot(max) of the maximally prolifera-

tive clones may have the greatest relevance to tumor cell behavior. In the study illustrated in Fig. 5, we have calculated the Tpot by the method of Begg for 60 colorectal tumors from our series using the same Ts values but with labeling index data derived in one of three ways: by flow cytometry, by using average counts from histochemically labeled sections, and by counting the maximally proliferative zones on the tissue sections. The series displayed upward of twofold variation in proliferation rates depending on the LI counting method chosen.

1. The S-Phase Duration

The S-phase duration (Ts) generally shows much less variation than the LI within and between tumors and between tumors and normal tissues. Many studies reveal that Ts in human cells appears to be relatively consistent, with most values falling between 10 and 20 hr across a wide range of tissues and tumors. Given the profound conservation of key biological processes in nature, it seems probable 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

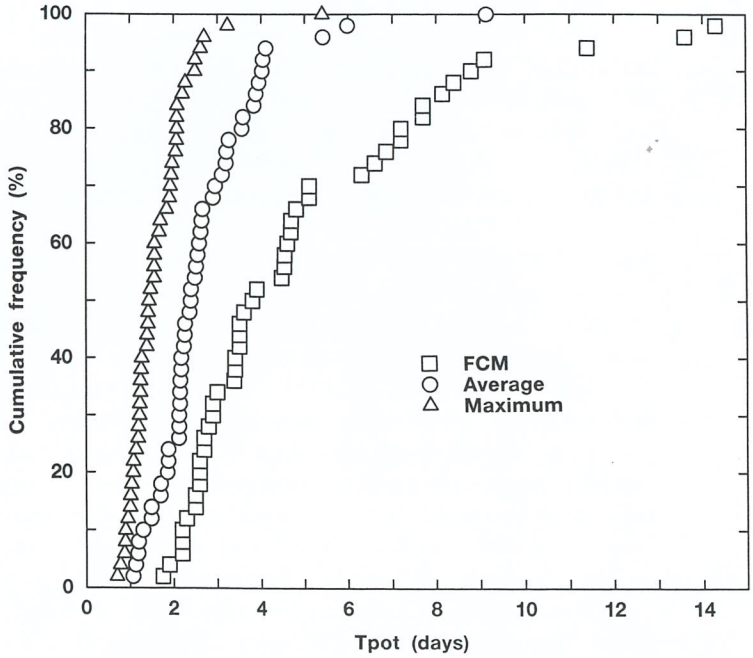


Fig. 5 The problem of heterogeneity of potential doubling time calculations according to the method of estimation of the labeling index in a series of 60 human colorectal tumors: FCM, flow cytometric method; Average, average counts from histochemically labeled sections; Maximum, the counts from maximally proliferative zones on the tissue sections (see text).

variation observed in the Ts measurement may thus be due to experimental artifact. Indeed, DNA duplication is such an evolutionarily conserved and powerfully regulated process that it might be surprising if the duration of DNA synthesis were significantly different between normal cells and viable tumor cells of similar lineages and DNA content.

Measurement of the Ts becomes less reliable at low fractions of HP labeling, because of the method by which the Ts is estimated from the absolute numbers of labeled cells in each phase of the cell cycle. This may give rise to prolonged estimates of the Tpot.

It thus seems likely that the S-phase duration is a species-specific constant in cells with normal DNA content. 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 tumor cells, reflected in the DNA index, or aneuploidy. We found no significant difference in the Ts of cohorts of diploid and aneuploid colorectal tumor samples to support this hypothesis (Rew *et al.,* 1991a), although such differences may be concealed within experimental error.

2. The Potential Doubling Time and the Cell Loss Factor

There are a number of interesting aspects to the Tpot data presented. One feature of the data across the many series of tumors reported is the similarity rather than the differences in calculated cell production rates between classes of tumors of very different histology and behavior. Another is the high rate of cell production in many tumors, with Tpots of the order of 5 days. The observed volume doubling time of tumors in clinical practice is commonly of the order of 100 days or more. This provides strong corroborative evidence that the difference 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 is often observed in tumors in the GI and uropenital tract.

The cell loss factor has a major bearing on the clinical volume growth of a tumor. Tissue and tumor growth is a dynamic disequilibrium between cell production and cell loss. Proliferation measurements do not take into account tumor and tissue cell loss during growth. There is a disparity between the actual volume growth of tumors, as measured directly or by serial radiology, and the potential doubling time. Cell loss from tumors is caused by processes that include exfoliation, cell migration or metastasis, necrosis, and apoptosis. Thus, a highly proliferative tumor with a high cell loss factor may remain static in size or even regress, whereas a slowly proliferative tumor with no cell loss will continue to enlarge.

The indication of high cell loss factors in nonluminal tumors 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 tumor growth. Apoptosis is most likely to account for a large proportion of cell loss.

3. The Labeling Index as a Surrogate Measure of Dynamic Proliferation

If the Ts is relatively constant from one cell population to another, then the observed experimental variation Tpot is thus largely attributable to site to site and intertumor 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 proliferative markers would be considerably simpler to obtain in service laboratory practice than would be the results of dynamic *in vivo* studies.

4. 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 tumors. This strengthens the theory that tumor 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 clinical tissue samples has been obtained from surgical tumor 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 tumor growth factors.

5. Proliferation Data, Biological Aggressiveness, and Clinical Outcome

It is commonly assumed that a higher rate of tumor cell production increases the biological aggressiveness of tumors. There is no absolute measure of "speed" in tumor growth, and it is thus accepted practice to stratify data sets by the median Tpot value in any one tumor series. In those series where survival has been recorded, there has generally been little or no correlation between proliferation parameters and time to death.

Proliferation measures must be regarded as an inadequate measure of tumor growth and of biological aggressiveness. They do not measure cell loss, and they are unable to predict invasive and metastatic potential. There may be differences in proliferative behavior between primary and metastatic lesions, such that assumptions cannot be made about the overall behavior of the tumor mass from measurements on the primary tumor alone. Proliferative measurements are usually made on the primary tumor, and these may have little relevance to the proliferative biology of metastatic or invasive clones.

Proliferative biology may also change with time. Tumor growth fractions and proliferation rates may change with growth and time. A snapshot biopsy measurement taken at any point in the life of the tumor may not be predictive either of the past behavior or of future growth rates of that tumor.

6. Cell Proliferation and Experimental Therapy

Cell proliferation research may help improve adjuvant chemotherapy (Tannock, 1986; Van Putten, 1979) and radiotherapy (Denekamp, 1986; Kummermehr and Trott, 1982; Terry, 1996) in a number of ways. HP labeling 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 (Dolbeare, 1995b). The technique also allows the assessment of the proliferative response of tissues to damage, such as induced by hypoxia, in tumor cell cultures and animal models (Webster *et al.,* 1998).

E. Concluding Comments on Cell Proliferation Studies

In vivo proliferation data provide a sound, evidence-based framework for modeling the growth of tumors and tissues, for studying chemotherapy and radiotherapy in the laboratory, for understanding the importance of cell loss and apoptosis in tumor progression, and for providing new hypotheses for clinical trials of treatment of those many tumors whose behavior defies surgical excision.

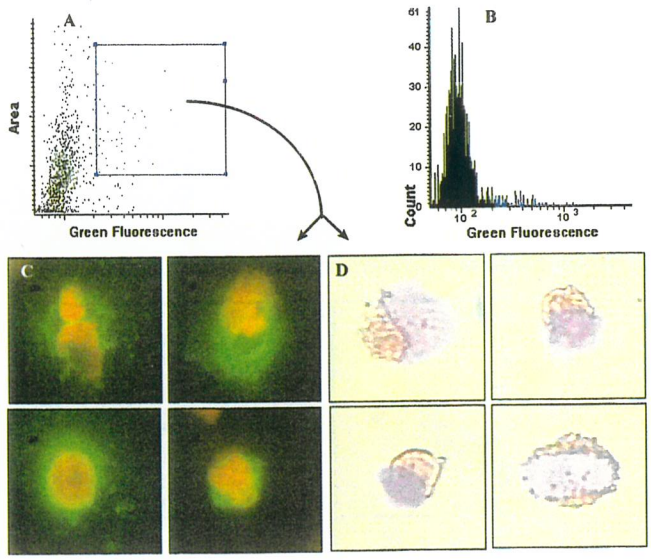
Cell production rate measurements are yet to find useful clinical applications, either in confident prognostication or in guiding therapeutic strategy. Nevertheless, they have taught us much about the proliferative biology of human tumors (Rew and Wilson, 2000a,b). They have served to emphasize how cell production is only one side of the equation of tumor growth. The other side, cell loss, continues to be a problem of quantitative analysis. We now recognize the complexity of proliferative behavior within and between tumors and normal tissues, along with 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.

VI. Further Applications of Cytometry in Clinical Oncology

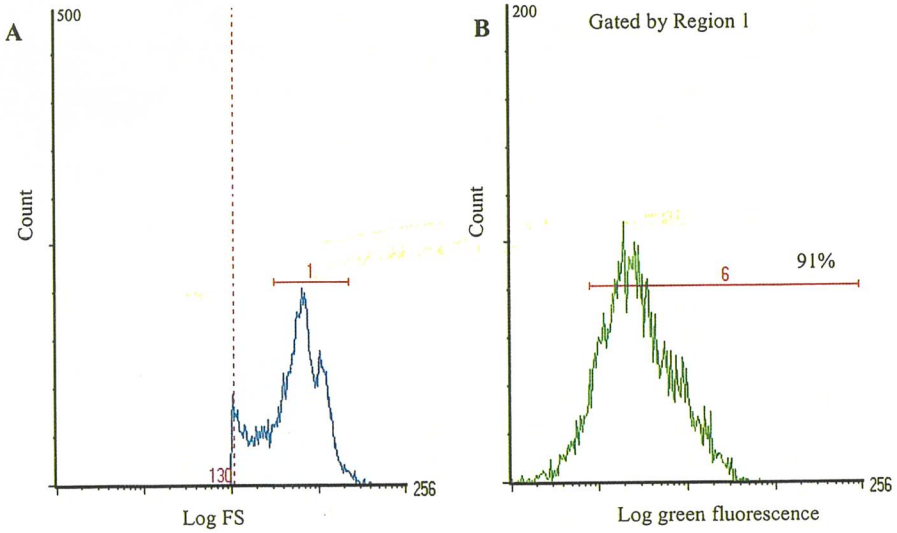
A. Laser Scanning Cytometry in Clinical Studies

The inability of flow cytometry to correlate fluorimetric data with cell morphology on a precise, cell by cell basis renders it unsuitable for the study of many aspects of solid tumor biology. It prevents the confident discrimination of cell types in complex populations of cells with very variable morphology, scatter characteristics, lineage, and viability.

Laser scanning cytometry (Kamentsky and Kamentsky, 1991) creates a direct link between cell morphology, laser excitation, and fluorochromatic quantitation. The fixing of the cell and tissue in space and time beneath the familiar microscope



Chapter 51, Fig. 6 The capabilities of laser scanning cytometry in clinical sample analysis. A Cytospin of human asthmatic sputum has been stained with monoclonal anti-human major basic protein specific antibody (BMK13) and secondary Oregon Green conjugated goat anti-mouse antibody and analyzed on the Compucyte LSC. Plotted are peak green fluorescence signal against cell size of cells captured with propidium iodide nuclear counterstain (A, B). Examples of relocation and still video image capture of cells within the region of interest under epifluorescence with the IB excitation cube, wide band (C) and after chromatic restaining of the same slide under bright-field microscopy with chromotrope 2R (D) (with thanks to Dr. G. Woltmann, Leicester, England).



Chapter 53, Fig. 6 Flow cytometric analysis of *B. anthracis* spores incubated in polysaccharide media for 30 min at 37°C. A specific FITC-conjugated anti-cell wall polysaccharide MAb stained emerging vegetative *B. anthracis* cells (region 6) (viable cells) but did not stain encapsulated *B. anthracis* cells, dormant *B. anthracis* spores, or other *Bacillus* species. Forward light scatter was used to identify vegetative cell population in the sample (region 1).

objective of a laser scanning cytometer vastly increases the scope of analysis, interpretation, and experimentation when compared with the flow cytometer (Rew *et al.,* 1999). It allows the corroboration of descriptive morphology with qualitative and quantitative measures of light scatter and fluorescence, and their spatial distribution within and around the cell. It allows study of dynamic patterns of transport of fluorochromes, of subcellular concentration of dyes, and of normal and abnormal cell physiology. The visualization capabilities of epifluorescence, bright-field microscopy, and image processing are further enhanced by direct linkage to an image processing system (Woltmann *et al.,* 1998).

Laser scanning cytometry replicates the range of assays on clinical material available in flow cytometry, including DNA ploidy (Martin-Reay *et al.,* 1994; Luther and Kamentsky, 1996; Sasaki *et al.,* 1996; Chapter 31 of this volume), immunophenotyping (Clatch *et al.,* 1998; Clatch and Forman, 1998; Chapter 46 of this volume), and cell proliferation (Rew *et al.,* 1998). It also offers unique capabilities in terms of sample presentation on a microscope slide, accommodating fine needle aspirates, smears, imprints, viable cells in irrigated chambers, cells grown to confluence *in situ* (Yang *et al.,* 1998), and complex samples such as asthmatic sputum (Woltmann *et al.,* 1999) (Fig. 6). It may also be used with limited resolution for subcellular analyses such as fluorescence *in situ* hybridization (Kamentsky *et al.,* 1997) and genotoxicity testing using the mouse micronucleus assay (Rew and Styles, 1998). Cell and tissue culture techniques on a microscope chamber slide also provide for direct quantitative assay of processes such as cell signaling, growth factor response, molecular translocation, and incorporation of viral vectors in contiguous cell samples (Musco *et al.,* 1998). The laser scanning cytometer can also analyze conventional tissue sections to a limited degree, using discriminators such as nuclear staining. Measurement is still constrained by problems with cell overlap and sectioning and by boundary discrimination, but this may improve with better software algorithms and new staining techniques.

B. Cytometric Assays in Cancer Therapy

The LSC offers considerable advantages in the cytometric analysis of complex cell populations from solid tumors, whose variable light scatter characteristics or DNA content do not allow simple classification of cell subtypes. Specific cell types or assay results can be validated by direct inspection, whereas rare event analysis and cell sorting on the slide can be undertaken in the course of normal routines.

Research using laser cytometry has opened up many new vistas on the cancer cell and its behavior. However, none of the applications in solid tumor biology have become a standard part of the clinicopathological assessment. Most cancer treatments carry a significant morbidity, and the selection of prognostic groups also helps to optimize treatments and to avoid unnecessary treatment for some categories of patients. In cancer biology, the greatest immediate value would

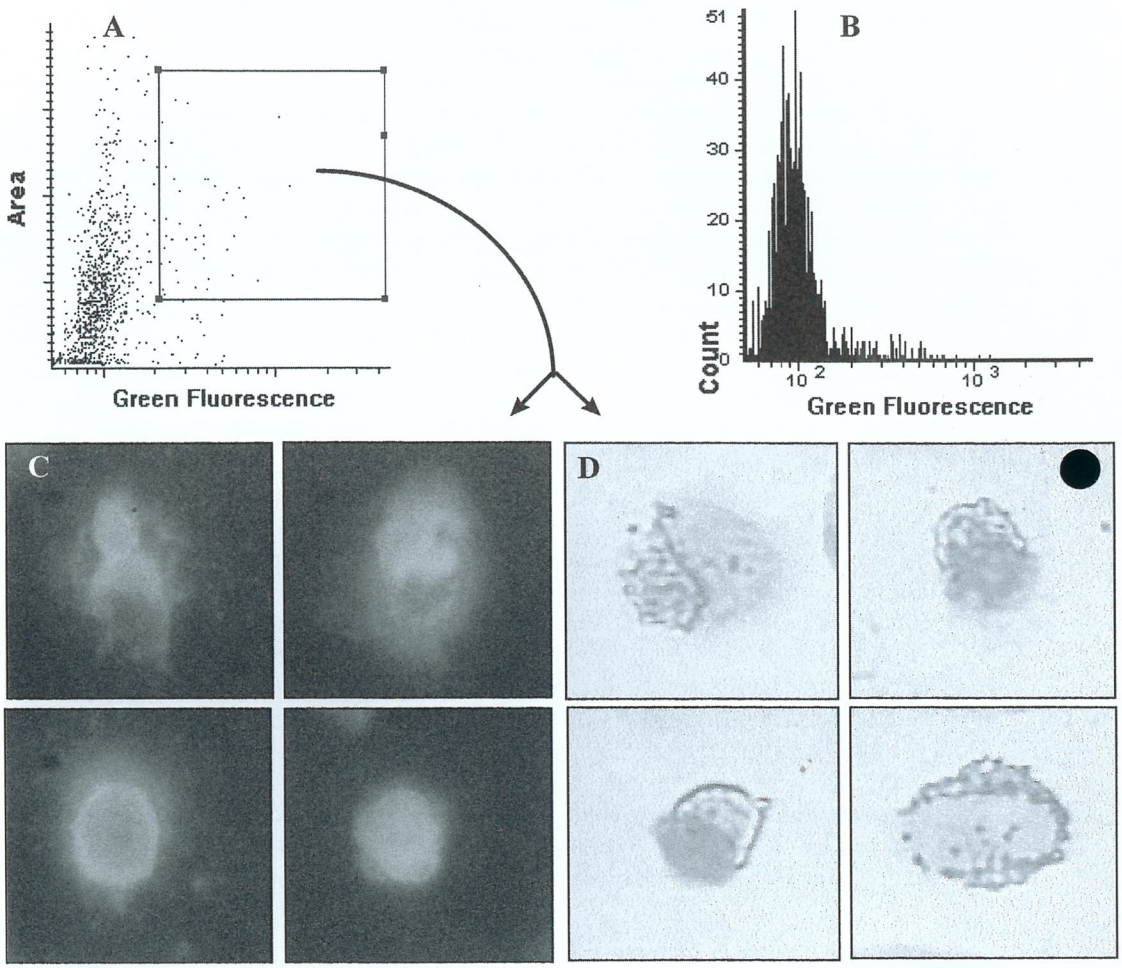


Fig. 6 The capabilities of laser scanning cytometry in clinical sample analysis. A Cytospin of human asthmatic sputum has been stained with monoclonal anti-human major basic protein specific antibody (BMK13) (Bradsure Biologicals, Loughborough, England) and secondary Oregon Green conjugated goat anti-mouse antibody (Molecular Probes, Europe, BV) and analyzed on the Cytocyte LSC. Plotted are peak green fluorescence signal against cell size of cells captured with propidium iodide nuclear counterstain (A, B). Examples of relocation and still video image capture of cells within the region of interest under epifluorescence (C) and after chromatic restaining of the same slide under bright-field microscopy with chromotrope 2R (D) (courtesy of Dr. G. Woltmann, Leicester, England) (Woltmann *et al.,* 1999).

derive from techniques that allow us to make better use of existing adjuvant therapies on a case by case basis. Cytometric techniques which thus aided the selection or rejection of specific cancer therapy might identify mechanisms for the circumvention of drug resistance, the specific targeting of individual drugs to tumors, or the optimum use of radiotherapy fractionation and radiosensitizers.

C. Cytometric Assays of Fluorescent Cytotoxic Drugs

The decision to use adjuvant chemotherapy for primary, metastatic, or recurrent tumors, and the selection of particular cytotoxic agents thereafter, is much influenced by empiricism and individual practice in clinical oncology. This may be depriving many patients with cancers of real benefits from individually targeted adjuvant chemotherapy. The absence of simple clinical assays of cytotoxic drug uptake by complex populations of viable, nonviable, drug-sensitive, and drug-resistant tumor cells has been a serious handicap.

The anthracycline/anthraquinone families of cytotoxic agents include Adriamycin, daunorubicin, mitozantrone, epirubicin, and idarubicin. These agents have a number of potent cytotoxic actions, which include intercalation of DNA and interference with the topoisomerase proteins and other DNA repair enzymes. These compounds possess intrinsic fluorescence; they absorb light around 470 nm and emit around 560 nm (Krishan and Ganapathi, 1980; Krishan *et al.,* 1987; Krishan and Sauerteig, 1992). They are expelled from the cell by the p170 glycoprotein pump, which confers multidrug resistance (MDR), and this mechanism may help limit their cytotoxicity (Herweijer *et al.,* 1989; Gheuens *et al.,* 1991; Smith *et al.,* 1992; Carpentier *et al.,* 1992; Tiberghien and Loor, 1996; Homolya *et al.,* 1996; Haugland and Larison, 1996; Landon, 1997). Flow cytometric studies of anthracycline uptake into tumor cells in suspension from hematological tumors, effusions, and ascites have been reported elsewhere in this book. It is also possible to demonstrate fluorochromatic drug uptake into solid tumor disaggregates, as with tumor cells in suspension in blood or effusions, using FCM. However, efforts to assay these drugs are confounded by heterogeneity of cell size, origin, and viability.

These drugs are potent anticancer agents, but their systemic toxicity has restricted their clinical use. Laser cytometry may offer a variety of techniques to circumvent systemic toxicity. The capabilities of the LSC in cell verification by direct visualization suggested a feasible strategy for quantitation of drug uptake and comparative studies of fluorochromatic drug uptake into tumor cells, as validated in our early studies (Reeve, 2000). One way may be to study means by which their target cell toxicity may be promoted at lower systemic doses through the use of MDR blocking agents. Another may be to identify those tumors that fail to concentrate the agents, and to spare these patients from inappropriate therapy. A third way may be to identify from a panel of agents the drug most reliably concentrated in tumor cells from surgical biopsies or fine needle aspiration. Indeed, proof of specific drug uptake would be a considerable advance over the current empiricism inherent in single agent, multiple agent, and sequential adjuvant therapy. Fine needle aspirates and endoscopic biopsies would be well suited to the monitoring of accessible tumors for changes in drug resistance during a course of therapy. Better assays may encourage the earlier use of these agents as prophylaxis against minimal residual disease after surgery over a range of tumor types.

The assay of drug uptake is alone insufficient to indicate cell killing. For example, fluorescent drug may be metabolized, sequestered, or otherwise ren-

dered inert within target cells. Thus, additional assays of drug efficacy must be developed. These might produce evidence of induced cell death (Furuya *et al.,* 1997) or disruption of other vital functions, such as evidenced by the accumulation of cell cycle phase specific markers or mitotic disruption.

More speculatively, cytometric assays may encourage drug designers to develop "detectability," by intrinsic fluorescence or monoclonal immunogenicity, in other anticancer agents. For example, fluorescence techniques have also been used to detect cis-platinum–rhodamin 123 conjugates (Assaraf *et al.,* 1989), and methotrexate (Teicher *et al.,* 1986) in cell models. A more difficult step will be to translate laboratory studies into practical randomized controlled clinical trials, so as to evaluate current unselective, empirical chemotherapy with more selective strategies.

VII. Conclusions

The complex morphology and behavior of human cells, tissues, and tumors challenges the designers of cytometric instrumentation. The range of clinical applications of flow cytometry is well defined, and the inherent constraints of the technology limit its utility in the clinical laboratory. A cytometer must above all provide useful information not otherwise readily obtainable by other means. The new generation of instruments open new horizons in clinical diagnosis and therapeutics, and we may expect further advances and insights in the next few years. Early in the twentieth century, the pathologist James Ewing (1919) hoped that "The twentieth century . . . may, thereby prove to be the era of successful therapeutics and prophylactics." His speculation was not fully vindicated for the solid tumors that preoccupy surgeons and oncologists. Nevertheless, technologies that Ewing could not know, and at which we must still marvel, offer us much excitement and optimism in the new century.

Acknowledgments

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