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Cell Kinetics of Human Brain Tumors: *In Vivo* Study with Bromodeoxyuridine and Flow Cytometry

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Abstract—*Bromodeoxyuridine (BUDR) is a thymidine analog which is incorporated into the DNA of proliferating cells. Since the dose of BUDR needed to label cells is not toxic, cell labelling can be accomplished* in vivo, *by infusing the substance in patients. A monoclonal antibody against BUDR is then used to identify BUDR-labelled cells. The same cell population can also be stained for DNA content with propidium iodide (PI). Using bivariate flow cytometry (FCM) for measurements, both the percentage of BUDR-labelled cells and their total DNA content can be evaluated. This technique allows one to obtain the labelling index (LI) and the DNA synthesis time (TS). The potential doubling time (T_{pot}) and the fractional turnover rate (FTR) can be mathematically derived, so that a complete picture of tumor growth can be obtained. Our aim was to ascertain whether this method is clinically applicable and whether the kinetic values obtained are reliable.*

We studied 22 *patients with benign and malignant brain tumors, and observed no immediate toxicity from BUDR administration. The BUDRLI obtained ranged from* 0.9% *to* 3.9% (*median:* 2.0%) *in meningiomas and from* 3.8% *to* 7.6% (*median:* 6.3%) *in malignant gliomas* ($P<0.01$). *The fraction of S-phase cells determined with the BUDR FCM technique was statistically similar to that found by single DNA flow cytometric analysis performed on duplicate samples of both benign and malignant brain tumors.*

The TS obtained in malignant gliomas ranged from 10.5 *to* 227 *h* (*median:* 12.8). *The calculated T_{pot} ranged from* 7.6 *to* 26.8 *days* (*median:* 11.6), *and the calculated FTR ranged from* 3.7 *to* 13.1 *cells/*100 *cells/day* (*median:* 8.8).

These data suggest that in vivo *BUDR infusion coupled with FCM can be performed in clinical settings, and it is reliable and can easily be used for kinetic studies in clinical trials aimed at evaluating the prognostic relevance of proliferative parameters and in planning tumor treatment.*

INTRODUCTION

THE BIOLOGICAL properties of human brain tumors have been only partially elucidated by histological [1, 2], ultrastructural [3, 4] and tissue culture data [5, 6]. Cell kinetic studies might increase our understanding in this field [7–9] but, until quite recently, the only method available for measuring cell kinetic parameters was to use radiolabelled precursors of DNA, in particular tritiated thymidine ([^{3}H]TdR) and autoradiography [10]. This method has several disadvantages that have limited its applicability and popularity, namely the radiation hazard of tritium to normal tissues and to the environment and the long processing time required for autoradiography [11–13]. The development of automated flow cytometry (FCM) has made it possible to obtain a measurement similar to the [^{3}H]TdR labelling index (LI) by quantitating the intensity of DNA

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fluorescence in individual tumor cells [14]. FCM analysis provides a rapid method for determining the number of cells in DNA synthesis and it has been employed in various studies on human brain tumors [15–19]. Unfortunately, the lack of sophisticated computer programs which enable one to obtain the proliferative activity when the tumor shows more than one population (aneuploid clones) has limited routine use of single DNA FCM for this purpose. The development of a monoclonal antibody (MoAb) against bromodeoxyuridine (BUDR), a thymidine analog that is incorporated into DNA during the S-phase of the cell cycle, has greatly facilitated the analysis of tumor cell kinetics [20–22]. Cell labelling with BUDR can be accomplished not only *in vitro* but also *in vivo*, because BUDR is neither radioactive nor myelotoxic at the doses needed for this type of study and it is also employed as an anticancer drug [23–30]. The anti-BUDR MoAb can be detected on slides by immunocytochemical methods or by immunofluorescence.

In this study we coupled *in vivo* BUDR administration and bivariate FCM (to detect BUDR and DNA content simultaneously), in order to study the proliferative characteristics of 22 human brain tumors. The flow cytometric BUDR LI was rapidly obtained on a large number of cells (even in aneuploid tumors) and in some cases (malignant gliomas) it was also possible to estimate the S-phase duration (DNA synthesis time, TS) using only one tissue sample and then calculate other important cytokinetic parameters, such as the potential doubling time (T_{pot}) and the fractional turnover rate (FTR).

MATERIAL AND METHODS

Patients

This study was performed on surgical tumor samples from 22 patients with intracranial neoplasms (Tables 1 and 2) who underwent surgery in the neurosurgical division of the Department of Surgery of the University of Pavia from November 1986 to April 1987.

In vivo *BUDR administration*

Permission to administer BUDR was given by the Ethical Committee at the Department of Internal Medicine of the University of Pavia, and informed consent was obtained from each patient. Patients received *in vivo* BUDR infusion before tumor specimens were obtained for diagnostic (including cytologic and histologic inspection) and/or therapeutic (curative or palliative surgery) purposes. Patients were given a 15–20 min infusion of BUDR, 250 mg/m^2 in 100 ml sodium chloride. Tumors were sampled 1 or 4–6 h later.

Sample processing

Single cell suspensions were obtained by a purely mechanical method. After removal of the blood and electrocoagulated portions each sample was carefully minced with a sharp blade and syringed through decreasing gauge needles. Cell suspensions were washed twice in PBS, filtered through a 35 μm-pore nylon filter, resuspended in PBS and fixed in 70% ethanol at a concentration not exceeding 10^6 cells/ml.

BUDR and DNA staining

The method for BUDR and DNA staining has been described in detail elsewhere. Briefly, cell suspensions were incubated with 2 N HCl for 30 min at 37°C, to denature the double stranded DNA (this allows the anti-BUDR MoAb to react with BUDR in the DNA chain) and later with 1 ml of PBS containing 0.5% Tween-20 and 0.5% normal goat serum (NGS) for 15 min at 37°C. Cells were then washed in PBS and incubated again in 0.5 ml of PBS containing 10 μl of the anti-BRDU MoAb (Becton Dickinson, Lab Impex Ltd, Twickenham, Middx, U.K.), for 30 min at room temperature with occasional mixing. They were then washed twice and incubated with PBS containing Tween-20 and NGS for 15 min, and later with the second antibody (10 μl of goat anti-mouse IgG FITC conjugate, Sigma Chemicals), in 0.5 ml of PBS/Tween-20/NGS for 1 h. After two washings in PBS, the cells were resuspended for 15 min in 4 ml PBS containing 10 μg/ml propidium iodide (PI) which stains DNA.

A portion of each cell suspension was also stained for single DNA only with PI before treatent with HCl, utilizing a method described in detail elsewhere [19].

Flow cytometry

Bivariate distributions of BUDR labelling (green) vs. DNA content (red) were measured utilizing an Ortho System 50-M cytofluorograph (Ortho Instruments, Westwood, MA) [31]. All data were collected in the list mode and analyzed using an Ortho 2150 computer. Debris, cell doublets, triplets, etc. were excluded from the analysis using an appropriate gated analysis on the cytogram of the distributions of DNA values vs. cell area. For each specimen, 20,000–50,000 cells were analyzed. Non-specific staining, as evaluated on cells stained for DNA and treated with the second antibody without previous incubation with the anti-BUDR monoclonal antibody, was subtracted from the measurements with the computer.

A separate single DNA measurement was performed for each tumor, and a sample of normal human brain obtained by biopsy during surgery for head

Table 1. *Clinical data, nuclear ploidy and S-phase values, expressed as bromodeoxyuridine labelling index of* 16 *patients with meningioma*

Patient No.	Sex/age	Type	Location	DNA I	BUDR LI (%)
1 G.P.	M/62	Fibroblastic	Parietal convexity	1.00	1.3
2 R.C.	M/54	Meningomatous	Sphenoid ring	1.00	1.1
3 M.O.	M/42	Fibroblastic	Parietal convexity	1.00	0.9
4 S.L.	F/44	Psammomatous	Parietal convexity	1.00	0.9
5 S.P.	M/54	Anaplastic	Frontal convexity	1.00	3.9
6 B.L.	F/48	Meningomatous	Parietal convexity	1.00	1.7
7 F.G.	F/48	Psammomatous	Frontal convexity	1.00	3.0
8 D.G.	M/63	Fibroblastic	Parietal convexity	1.00	2.0
9 R.L.	F/58	Fibroblastic	Parietal convexity	1.00	3.1
10 G.A.	M/50	Psammomatous	Sphenoid ring	1.00	3.0
11 D.A.	M/52	Meningomatous	Falx	1.00	1.6
12 D.M.	M/60	Meningomatous	Parietal convexity	1.00	2.6
13 P.G.	F/54	Fibroblastic	Falx	1.00	3.0
14 L.M.	M/64	Fibroblastic	Frontal convexity	1.00	3.1
15 S.M.	F/53	Psammomatous	Tentorial	1.00	2.1
16 P.F.	M/60	Meningomatous	Parietal convexity	1.00	1.5

DNA I = DNA index; BUDR LI = bromodeoxyuridine labelling index.

Table 2. *Clinical data, nuclear ploidy and cell kinetic parameters in six patients with malignant glioma*

Patient No.	Sex/age	Type	Location	DNA I	BUDR LI (%)	TS	T_{pot}	FTR
1 S.V.	M/59	Anaplastic astrocytoma	Occipital	1.00/1.4	7.1(7.7*)	16.7	9.8	10.2
2 A.G.	M/65	Glioblastoma	Parietal	1.00	3.9	20.0	26.8	3.7
3 M.M.	F/58	Anaplastic astrocytoma	Temporal	1.00/1.5	5.6(5.4*)	11.8	8.7	11.2
4 V.M.	F/54	Anaplastic astrocytoma	Occipital	1.00/1.4	7.6(7.8*)	13.9	7.7	13.1
5 F.M.	F/57	Glioblastoma	Parietal	1.00/1.3	7.0(7.9*)	22.7	13.5	7.4
6 F.B.	M/60	Glioblastoma	Parietal	1.00	3.8	10.5	13.4	7.4

DNA I = DNA index; BUDR LI = bromodeoxyuridine labelling index; *BUDR LI of the aneuploid population; TS: DNA synthesis time, T_{pot} potential doubling time, FTR fractional turnover rate.

injuries was used as a diploid standard in each determination [19].

BUDR LI evaluation

The BUDR LI was determined on either 1 h or 4–6 h samples. In the 1 h samples, BUDR-labelled, green-fluorescing cells are distributed in the S-phase, with a peak distribution in the middle of the S-phase. The window for LI determination comprises all these green fluorescing cells, and the LI is their percentage figure over the whole population. In the 4–6 h samples (which are also used to determine the TS), a number of cells labelled with BUDR at the time of BUDR infusion have already moved toward G2 and some, after mitosis, have recycled in that they are found labelled in G1 (see below). In these cases, the LI value, obtained as in the 1 h samples, must be corrected by adding one half the percentage of these G1 BUDR-labelled cells. This procedure is biologically and mathematically correct (Giordano *et al.*, unpublished data).

In some tumors containing both diploid and aneuploid cells that have sufficiently distinct modal DNA content, an estimate of both the diploid and aneuploid population LI can be obtained.

BUDR TS evaluation

The method we used to determine the DNA synthesis time was developed by Begg *et al.* in 1985 [33]. It involves a simple calculation using flow cytometric data about cell BUDR incorporation and DNA content. Two assumptions are made: (a) that at the time of pulse labelling (1 h following BUDR infusion) the peak distribution of S-phase cells (green fluorescence) is approximately in the middle of the interval between the $2n$–$4n$ peaks (red fluorescence); (b) that the movement of cells through the S phase is constant. The procedure is illustrated in Fig. 1.

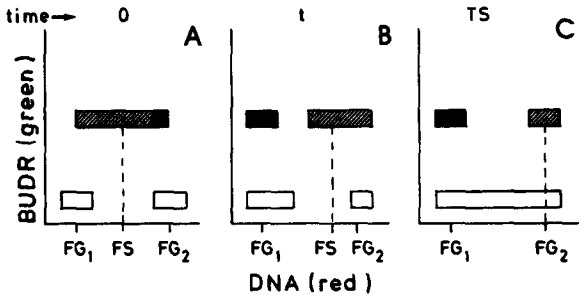

Fig. 1. Method for the evaluation of DNA synthesis time (TS) [33]. Assumptions are that at time 0 (A), the mean DNA content(s) (red fluorescence) of bromodeoxyuridine (BUDR)-labelled S-phase cells (green fluorescence) is about in the middle (0.5) of the interval between the G1 and G2 peaks, and that the movement of cells through the S-phase is constant. At the time of tumor sampling (t) (B), S-phase cells have progressed towards G2 and some have already recycled. At a time corresponding to TS (C), all S-phase cells have reached G2. To calculate TS, the position of the S-phase cells at the time of tumor sampling (B) is measured from their mean red fluorescence. The relative movement (RM) of S-phase cells, with respect to the time 0 position, is then calculated as follows: RM = FS−FG1/FG2−FG1, where F is the mean red fluorescence of the corresponding phase of the cell cycle. The TS is then calculated with the formula: TS = 0.5 × t/RM−0.5, where t is the sampling time.

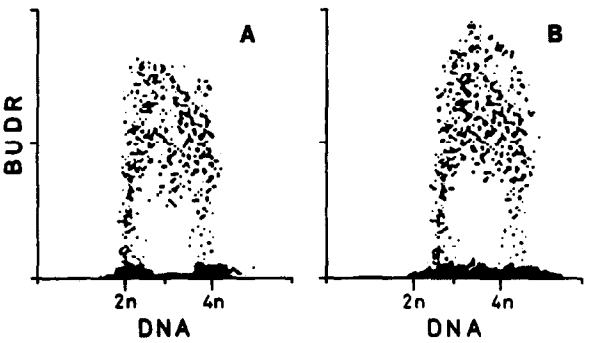

Fig. 2. A: Bivariate distribution of BUDR and DNA values from one patient with a meningioma of the brain. Measurements were performed 1 h following BUDR infusion. Almost all cells with a DNA content typical of the S-phase (2n–4n) have incorporated BUDR. B: Bivariate distribution of BUDR and DNA values from one patient having glioblastoma. Measurements were performed 1 h following BUDR infusion. A great number of cells have DNA content typical of S-phase without incorporating BUDR (U-cells). They do not enter the window for BUDR LI evaluation.

Calculated cell kinetic parameters

Once LI and TS were experimentally obtained, two additional kinetic parameters were calculated: the rate at which the tumor duplicates (potential doubling time, T_{pot}) and the rate of cell production per unit of time (fractional turnover rate, FTR). For these calculations it was assumed that tumor populations represent cell renewal systems in steady state.

The T_{pot} calculated by the formula:

$$T_{pot}\ (\text{days}) = (\text{TS}/\text{LI})/24.$$

The reciprocal gives the FTR:

$$\text{FTR}\ (\text{cells}/100\ \text{cells}/\text{day}) = \text{LI}/\text{TS}) \times 24.$$

Ploidy evaluation

Cell suspensions utilized for single DNA study were analyzed either alone or after mixing with an aliquot of normal brain cells. At first the normal brain cells and the tumor population under examination were measured separately. The normal brain was employed to calibrate the instrumentation and also served as an external diploid standard. The tumor population was then measured under the same experimental conditions. The presence of aneuploidy was determined by evaluating the PI DNA index (DNA 1 = modal channel of the G0/G1 peak of the studied population/modal channel of the G0/G1 peak of the normal brain, used as a diploid standard) [19].

Theoretically, the DNA 1 of a diploid tumor population is 1.00. Minor deviations (of the order about 10% or less) from this value were found for the G0/G1 peak of a number of tumors, having either unimodal or bimodal DNA distribution. In these cases, measurements were repeated by mixing the tumor cells with the normal diploid standard, i.e. the normal brain served as an internal standard. Under these conditions, diploidy was assessed when mixed normal brain and tumor cells gave a single G0/G1 peak, with CV identical to those determined for normal or tumor cells measured alone. Aneuploidy was assessed when mixed normal brain and tumor cells gave two separate peaks.

RESULTS

The results are summarized in Figs 2–5 and in Tables 1 and 2.

No patient experienced toxicity or adverse reactions from BUDR infusion.

Representative cytograms

Figures 2–5 show examples of the staining profiles obtained several hours after administration of BUDR to patients with benign and malignant brain tumors, respectively. Figure 2A shows the bivariate distribution of BUDR and DNA values from a patient with a meningioma removed 1 h after injection of BUDR. The percentage of BUDR-labelled cells represents the BUDR LI. In this patient, almost all cells with a DNA content typical of the S-phase (2*n*–4*n*) show BUDR incorporation, i.e. enter the window for the BUDR LI determination.

Figure 2B shows the same staining profile obtained 1 h after BUDR administration to a patient with a glioblastoma. In this patient a number of cells having 2*n*–4*n* DNA content do not incorporate BUDR. Thus, they are not considered in the BUDR LI evaluation.

Figure 3 shows the DNA profile on the left and the bivariate distributions of BUDR and DNA

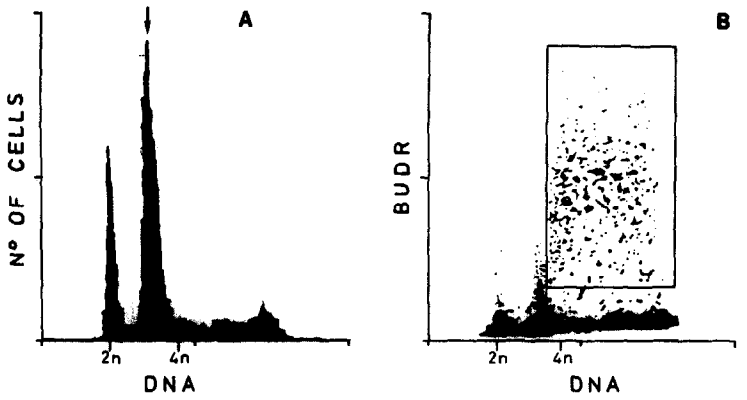

Fig. 3. DNA profile (left panel) and bivariate distribution of BUDR and DNA values (right panel) from a patient with a glioblastoma having both diploid and aneuploid (arrow) DNA distribution. Measurements were performed 1 hr *following BUDR infusion. The BUDR LI can be calculated for all S-phase cells. In tumors such as this, where the modal DNA content of the two populations is well separated, an estimate of the LI for the population with the greater modal DNA content can also be accomplished by excluding the population with the lower DNA content from the measurements.*

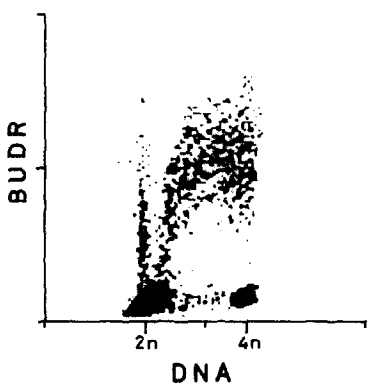

Fig. 4. Bivariate distribution of BUDR and DNA values (right panel) from a patient with a glioblastoma. Measurements were performed 3 h *following BUDR infusion for both the LI and TS determinations. With respect to the cytogram in Fig.* 1, *BUDR-labelled cells have moved through the S-phase (their distribution is shifted to the right) and some of them have already recycled (they have a diploid DNA content). Calculation of TS was accomplished according to the procedure described in Fig.* 2.

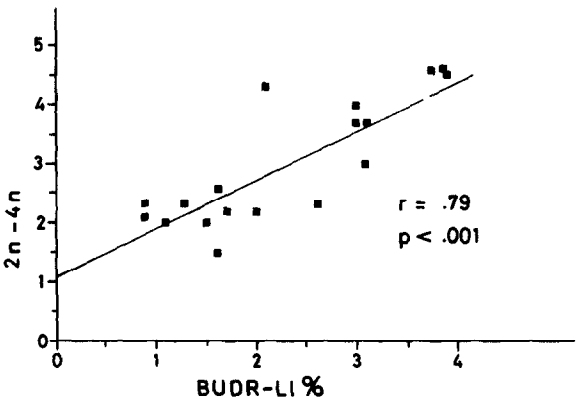

Fig. 5. Correlation between the S-phase values obtained on duplicate samples in 22 *patients with brain tumors, using* in vivo *bromodeoxyuridine (BUDR LI %) administration coupled with flow cytometry (FCM) and single DNA FCM* (2n–4n).

values on the right, from a patient with a malignant glioma having both diploid and aneuploid tumor cells. The BUDR LI was calculated for both cell populations.

Figure 4 shows the bivariate distribution BUDR/DNA from a patient with a glioblastoma. Measurements were performed 6 h following BUDR infusion. One can see that BUDR-labelled cells have moved through the S-phase (their distribution is shifted to the right), and some of them have already recycled (they have a diploid DNA content). Calculation of TS was accomplished according to the procedure described in Fig. 1.

Labelling index

The median BUDR LI in meningiomas was 2.9% (range 0.9–3.9%) (Table 1) and in malignant gliomas it was 6.3% (range 3.8–7.6%) (Table 2). The difference was statistically significant ($P < 0.01$).

Figure 5 shows that a good correlation exists between the S-phase values obtained on duplicate samples in our patients, using the *in vivo* BUDR FCM technique and single DNA FCM ($P < 0.01$).

DNA synthesis time

Only the cytograms obtained from the six malignant brain tumors were also utilized for TS determination. Reasons for the failure in determining the TS in meningiomas were the presence of few viable cells in the biopsy, the lack of sufficient cells for FCM (due to the nature of the biopsy) and the occasionally very low LI, which made reliable measurement of the relative movement of this small cohort of S-phase cells in the 4–6 h samples, very difficult.

The TS obtained in malignant gliomas ranged from 10.5 to 22.7 h (median: 12.8). The calculated T_{pot} ranged from 7.6 to 26.8 days (median: 11.6)

and the calculated FTR ranged from 3.7 to 13.1 cells/100 cells/day (median: 8.8) (Table 2).

Ploidy

All 16 meningiomas had unimodal DNA distribution, with diploid G0/G1 modal DNA content. The DNA 1 of the G0–G1 peaks ranged, in fact, from 0.94 to 1.12, when compared with the normal brain cells used as an external diploid standard; and we were unable to find two G0/G1 peaks when normal brain was mixed with the tumor population as an internal standard (Table 1).

Four out of six malignant tumors were aneuploid, showing two evident peaks. The first one was always diploid and the median value of the DNA 1 of the second (aneuploid) peak was 1.4 (range 1.3–1.6) (Table 2).

DISCUSSION

Since 1970 several cytokinetic analytical studies of human brain tumors have been reported [7–9, 12]. However, the techniques applied in these studies have several drawbacks. For example, the necessity of using radioactive tracers restricted the *in vivo* measurement of the labelling index of human tumors to only a few cases and led to the development of *in vitro* techniques for labelling biopsy material [11, 12]. In addition, when other cell kinetic parameters were needed, for example in the labelled mitoses curve technique, the necessity of repeated tissue sampling and the time-consuming nature of cytoautoradiography (including sample preparation and scoring) had to be taken into account.

With the development of FCM rapid analysis of the DNA distribution of cell populations has become feasible [14]. With this technique it has been possible to evaluate the S-phase size of a tumor population by determining the percentage of cells with DNA content intermediate between the diploid ($2n$) and the tetraploid ($4n$) values ($2n$–$4n$ %). Single DNA FCM has not become entirely clinically feasible because of two major problems. First, the accuracy of the $2n$–$4n$ cell % in estimating the S-phase has been hampered by the presence of $2n$–$4n$ cells which do not incorporate a DNA precursor such as [^{3}H]TdR. These S-phase-arrested cells (frequently found in tumors) are not considered as proliferating by single DNA FCM, while they actually are not. Second, the proliferative activity cannot be evaluated by DNA FCM when an aneuploid population is present because all the mathematical computer programs presently available, tend to overestimate the S-phase size [15–19]. Single DNA FCM is also inadequate to obtain other temporal cell kinetic parameters besides LI.

The recently introduced method using *in vivo* BUDR administration and anti-BUDR MoAb to study tumour cell kinetics has created new possibilities in this field of research [20–22]. Like TdR, BUDR is incorporated into the DNA of proliferating cells, but it is not radioactive and has been used as a radiosensitizing agent for more than 10 years without causing serious side-effects [23–30]. The dose of BUDR (250 mg/m^2) given intravenously to each patient in this study was far below the therapeutic dose and had no appreciable side-effects.

Nuclei containing BUDR can be identified by an anti-BUDR MoAb, which, in turn, can be detected with a peroxidase target or a fluorescein isothiocyanate (FITC) conjugated second antibody. This allows evaluation of the size of the S-phase as the BUDR LI *in vitro* as well as *in vivo* and within a few hours after tissue sampling. Several studies employing immunocytochemical methods for detecting *in vivo* BUDR-labelled cells on slides [34–39] have demonstrated that the BUDR LI and [^{3}H]TdR LI are very close [31].

In this study we utilized the *in vivo* BUDR technique combined with FCM to carry out simultaneous measurements of the total DNA and BUDR content [31, 32, 40] of cells from human brain tumors. This method is rapid and, from a theoretical point of view, the BUDR LI (the proportion of cells that have incorporated BUDR after a pulse label) measured with FCM is statistically more precise than both the [^{3}H]TdR and BUDR LI determined from slide inspection, in that a much greater number of cells is evaluated. The problem of cytoautoradiographic background, which is a difficulty in [^{3}H]TdR LI evaluation, is lessened because non-specific staining is negligible in the BUDR preparations used for FCM and it can also be subtracted using a computer. With respect to the other methods for evaluating the S-phase, e.g. the measurement of the $2n$–$4n$ cell % with single DNA FCM, the BUDR FCM method avoids counting the S-phase-arrested cells as proliferating; and, in tumors having both diploid and aneuploid cell lines this method allows one to obtain information on the LI of both populations.

In addition, simultaneous measurements of *in vivo* incorporated BUDR and DNA content by bivariate FCM have the potential to give a considerable amount of kinetic information. For instance, in addition to the LI, it may be possible to measure the S-phase duration if the sample is taken a few hours after injecting BUDR. From the experimentally obtained LI and TS and assuming a steady stage proliferation model, other cytokinetic parameters like the T_{pot} and the FTR can be mathematically derived.

We have demonstrated in this study the feasibility of measuring cell kinetics in human brain tumors by using *in vivo* BUDR administration and FCM.

This technique allows one to obtain relevant cytokinetic information within a day of biopsy without undue stress to the patient. This could be important both in benign and in malignant brain tumors. For instance, because the growth potential and rate of recurrence are of major prognostic importance in treating patients with meningiomas, the determination of the BUDR LI can be an extremely helpful procedure. In malignant gliomas, it may be expected that patients whose tumors have a relatively high T_{pot} should have their treatment whether it be drugs or radiation, with brief intervals between administrations, in order to reduce tumor cell repopulation. Hence the technique we described could allow researchers in evaluating the prognostic meaning of proliferative activity and physicians to plan therapy based on the cytokinetic characteristics of individual tumors.

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