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Cell Kinetics in Human Malignancies Studied with *in Vivo* Administration of Bromodeoxyuridine and Flow Cytometry¹

Alberto Riccardi,² Marco Danova, George Wilson, Giovanni Ucci, Peter Dörmer, Giuliano Mazzini, Silvia Brugnattelli, Margherita Girino, Nicholas J. McNally, and Edoardo Ascari

Istituto di Clinica Medica II, Dipartimento di Medicina Interna e Terapia Medica, Istituto di Ricovero e Cura a Carattere Scientifico Policlinico, San Matteo [A. R., M. D., G. U., S. B., M. G., E. A.], and Centro di Studio per l'Istochimica del Consiglio Nazionale delle Ricerche, Dipartimento di Biologia Animale [G. M.], Università di Pavia, 27100 Pavia, Italy; Cancer Research Campaign, Gray Laboratory, Mount Vernon Hospital, Northwood HA6 2RN, England [G. W., N. J. M.]; and Institut für Experimentelle Hämatologie der Gesellschaft für Strahlen- und Umweltforschung, D-8000, München, West Germany [P. D.]

ABSTRACT

Bromodeoxyuridine (BrdUrd) is a pyrimidine analogue which is incorporated into the DNA of proliferating cells. When *in vivo* BrdUrd infusion is coupled with bivariate flow cytometry to measure cell BrdUrd incorporation and DNA content, both the percentage of DNA-synthesizing cells [BrdUrd-labeling index (LI)] and the DNA synthesis time (T_S) can be determined on the same tissue sample. From experimentally determined LI and T_S , the potential doubling time of the population and its cell production rate are calculated. To ascertain whether the BrdUrd infusion method is clinically feasible and if data are reliable, we studied patients with leukemia, refractory anemia, multiple myeloma, and brain and gastric tumors. The BrdUrd incorporation data were compared with those determined on duplicate samples with the techniques conventionally used for LI and T_S values, *i.e.*, ³H- and ¹⁴C-labeled thymidine autoradiography, respectively. The complete BrdUrd procedure takes 6–9 h, and no immediate toxicity from BrdUrd administration has been observed. In an 8-month period, 154 patients were studied. Successful LI and T_S determinations were obtained in 78.9 and 59.7% of cases, respectively, more often in hematological than in solid tumors. The values for LI and T_S assessed with the BrdUrd technique were very close to those found with ³H- and ¹⁴C-labeled thymidine autoradiography ($r = 0.88$, $P < 0.005$, and $r = 0.89$; $P < 0.005$, respectively). The potential doubling time and production rate were accordingly similar. These data indicate that *in vivo* BrdUrd infusion coupled with flow cytometry measurements can be performed in clinical settings and that this method is reliable. It could be used for kinetic studies in clinical trials aimed at evaluating the prognostic relevance of proliferative parameters and for planning radio- and/or chemotherapy.

INTRODUCTION

A number of clinical trials (1–7) have suggested that proliferative characteristics are of prognostic relevance in tumors. However, controversies still exist on this topic, and extensive investigations are made difficult by the fact that accurate kinetic data are not easy to obtain in clinical settings. The method most commonly used to measure proliferative activity is evaluation of the S-phase fraction of the tumor population (8). This is done with autoradiography, determining the percentage of cells incorporating [³H]dThd³-labeling index, or with FCM, determining the percentage of cells with DNA content intermediate between the diploid (2n) and the tetraploid (4n) values

(2n–4n cell%). However, the S-phase fraction gives only a limited insight on cell proliferation, for which the time parameters of the cell cycle are also necessary (9). More complex techniques yield a wider experimental panel of kinetic parameters, including the duration of the cell cycle phases, but are exceedingly difficult to apply in humans. For example, the method of the labeled mitoses curve involves the *in vivo* administration of radioisotopes and multiple tumor sampling, in addition to time-consuming autoradiographic procedures. This topic has been extensively reviewed by Steel (9).

It is theoretically possible to obtain a complete kinetic picture easily by administering *in vivo* BrdUrd and by performing measurements with bivariate FCM (10). BrdUrd is a nonradioactive pyrimidine analogue which is taken up by DNA-synthesizing nuclei, and BrdUrd-incorporating cells are detected with an anti-BrdUrd monoclonal antibody (11). After staining the same cell population with propidium iodide (which stoichiometrically intercalates into DNA), the distribution of BrdUrd-labeled cells and their total DNA content are simultaneously measured with FCM (12). From these measurements, both the LI and the DNA T_S are determined on a single tumor sample (13). Other cell kinetic parameters such as the tumor doubling time and production rate are then mathematically derived.

In a previous study (14), we presented some preliminary results with *in vivo* BrdUrd infusion coupled with FCM measurements. We report here the results obtained on a much larger cohort of patients, and we compare the BrdUrd kinetic data with those determined on duplicate samples with the techniques conventionally used for studying cell kinetics (15), *i.e.*, tritium- (for LI) and carbonium-labeled (for T_S) thymidine autoradiography (16, 17).

MATERIALS AND METHODS

From September 1986, to August 1987, 154 patients with neoplastic diseases received *in vivo* BrdUrd infusion before cell specimens were obtained for diagnostic (including cytological and histological examination) and/or therapeutic (curative or palliative surgery) purposes (Table 1).

Patients with gastric and brain cancers and refractory anemia with blast excess (18) had newly diagnosed disease. Patients with AL were either untreated or relapsed following successful treatment. Patients with MM had very advanced disease, with clinical and hematological features (the BM plasma cell infiltrate was >91%) of the acute terminal phase (19).

Permission to administer BrdUrd 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. Additional procedures performed on duplicate cell samples from a part of the patients were *in vitro* [³H]dThd and [¹⁴C]dThd (16, 17, 20) incorporation studies.

BrdUrd Incorporation Study

***In Vivo* BrdUrd Administration.** Patients were given a 15- to 20-min infusion of BrdUrd, 500 mg in 100 ml sodium chloride solution,

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² To whom requests for reprints should be addressed, at Istituto di Clinica Medica II, Policlinico San Matteo, 27100 Pavia, Italy.

³ The abbreviations used are: dThd, thymidine; FCM, flow cytometry; BrdUrd, bromodeoxyuridine; LI, labeling index; T_S , DNA synthesis time; AL, acute leukemia; MM, multiple myeloma; BM, bone marrow; PBS, phosphate-buffered saline; MoAb, monoclonal antibody; NGS, normal goat serum; T_{pot} , potential doubling time; PR, production rate.

prepared by the Department of Pharmacology, Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo. Tumors were sampled 1 h or 4–6 h after completion of the BrdUrd infusion.

Sample Processing. In patients with hematological malignancies, 2-ml BM samples were obtained by sternal aspiration. Two patients with leukemia were studied three times, once at presentation and twice after disease remissions (29 and 181 days and 33 and 231 days after presentation, respectively). BM aspirate was also obtained in 10 solid tumor patients having normal BM cytology and histology. They are referred to as normal BM in Table 1. Cells were layered (in a 1:1 ratio) on Ficoll-Hypaque and collected after centrifugation ($6.0 \times g$ for 30 min).

In all patients with brain tumors and in 17 of the 35 patients with gastric cancers, a 2- to 4-mm-diameter tissue sample (with no gross signs of tissue necrosis) was obtained during surgery. In 6 patients with gastric cancer two samples from different areas of the tumor were obtained. In the remaining 12 patients with gastric cancer, 2–5 much smaller endoscopic biopsies were pooled for the study. These tissue samples were processed to obtain single cell suspensions for FCM by using disaggregation methods which differed according to the macroscopic appearance of the tumor sample. For soft and fragile samples (as those from most brain tumors), purely mechanical devices were used. After removal of blood and electrocoagulated portions, samples were carefully minced with a sharp blade and syringed through decreasing (from 19 to 25) gauge needles. Both these procedures were carried out in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} , since the absence of these divalent cations prevents fragile tissues from sticking or clumping together. Special care was also taken to avoid both excessive shearing and formation of air bubbles, which are a cause of cell damage. This disaggregation procedure was continued until only the connective tissue remained, and it took an average of 20–30 min. For harder tumors (as often gastric tumors are, especially when scirrhous), a mechanical technique was coupled with enzymatic digestion. These samples were first washed in PBS and cut into 1- to 2-mm³ fragments, and then incubated at 37°C in Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution containing 0.2% collagenase, type I (Worthington Biochemicals Co.; with respect to collagenase IV, collagenase I has lower tryptic activity and produces much "cleaner" cell suspensions, *i.e.*, cell suspensions lacking cellular debris), with constant agitation (using either a rotating wheel apparatus, to which 25-ml universal bottles containing the tissue and the enzyme solution are attached, or a shaking water bath or a magnetic stirrer in a conical flask). Avoiding Ca^{2+} in the medium does not lower collagenase dissociation (notwithstanding a widespread belief to the contrary) and has the added advantage of preventing clumps (see above). The median collagenase incubation time was 30 (range, 15–60) min. This incubation time was chosen empirically for individual tumors by trying to dissociate the sample with pipeting at 15-min intervals to see if it has started to break up. After incubation the tumor pieces were dissociated as described above.

Cell suspensions from both hematological and solid tumors were washed twice in PBS, filtered through a 35- μm -pore nylon filter and resuspended in PBS. If the suspension was "dirty" following filtration (*i.e.*, it contained debris), it was either spun slowly ($0.6 \times g$ for 10 min) or spun through a higher density medium (such as bovine serum albumin, sucrose, or Ficoll) to remove debris. However, this was not usually necessary. After counting in a Bürker's chamber, cells were ultimately fixed in 70% ethanol at a concentration not exceeding 1×10^6 cells/ml.

BrdUrd and DNA Staining. The method used to detect BrdUrd-labeled cells has been described in detail elsewhere (10, 14). Briefly, cell suspensions were first incubated with 2 N HCl (for 30 min at 37°C) to denature the double strand DNA. This allows the anti-BrdUrd MoAb to react with BrdUrd in the DNA chain (11, 21). About 1×10^6 of these cells were later suspended in 1 ml of PBS containing 0.5% Tween 20 and 0.5% NGS for 15 min at 37°C, washed in PBS, and then incubated in 0.5 ml of PBS containing Tween 20 and NGS and 10 μl of the anti-BrdUrd MoAb (Becton Dickinson, Lab Impex, Ltd., Twickenham, Middlesex, United Kingdom) for 30 min at room temperature with occasional mixing. Following two washings in PBS, the cells were resuspended in 1 ml of PBS containing Tween 20 and NGS for 15 min, and later with the second antibody (10 μl of goat anti-mouse IgG

fluorescein isothiocyanate conjugate; Sigma Chemical Co.), in 0.5 ml of PBS/Tween 20/NGS for 30 min. After two further washings in PBS, the cells were resuspended in 4 ml PBS containing 10 $\mu\text{g}/\text{ml}$ propidium iodide (that stoichiometrically stains DNA) for 15 min.

Flow Cytometry. Bivariate distributions of BrdUrd labeling (green) versus DNA content (red) were measured by utilizing an Ortho System 50-M Cytofluorograph (Ortho Instruments, Westwood, MA) (10) and the data, collected in the list mode, were analyzed with an Ortho 2150 computer. An appropriate window (which was chosen from the cytogram of the red fluorescence peak versus cell area signal) was used to eliminate debris, cell doublets, triplets, etc., from the analysis. For each specimen, $20\text{--}50 \times 10^3$ cells were analyzed. Nonspecific staining, as evaluated on cells stained for DNA and treated with the secondary antibody without previous incubation with the anti-BrdUrd MoAb, was negligible. In any event, it was subtracted from the measurements by using the computer.

DNA histograms were constructed with the same instrumentation. As diploid reference standard, normal tissues were used according to the type of tumor under study; for example, normal gastric mucosa was used when a gastric carcinoma was to be measured. The presence of aneuploidy was estimated by evaluating the DNA index, *i.e.*, the ratio between the modal channel of the $\text{G}_0\text{--}\text{G}_1$ peak of the tumor population and the modal channel of the $\text{G}_0\text{--}\text{G}_1$ peak of the reference standard. In cases with unimodal DNA distribution, the percentages of cells with $2n$ ($\text{G}_0\text{--}\text{G}_1$ phase), $4n$ (G_2 - and mitotic phases) and $2n\text{--}4n$ (S phase) DNA content were determined as previously described (20).

Evaluation of BrdUrd-LI. The BrdUrd-LI was determined on either 1-h or 4- to 6-h samples. In the 1-h samples BrdUrd-labeled, green-fluorescing cells belonging to the S phase have a peak DNA content distribution in the middle of the $2n\text{--}4n$ interval. The window for the LI determination comprises all these cells, and the LI is their percentage figure over the whole population, as measured with a larger window (22). In the 4- to 6-h samples (which are used to determine both the LI and the T_s ; see below) the S-phase cells labeled with BrdUrd at the time of BrdUrd infusion have moved toward G_2 , so that their peak DNA content distribution is shifted toward $4n$. Furthermore, some of them have recycled to $\text{G}_0\text{--}\text{G}_1$ following mitosis, so that they are found in the cytogram as labeled cells with $2n$ DNA content. In these cases the LI value is the percentage, over the whole population, of the S-phase cells plus one-half the percentage of the $2n$ BrdUrd-labeled cells.

Evaluation of BrdUrd- T_s . The BrdUrd- T_s was assessed on cell samples taken 4–6 h after completion of BrdUrd infusion. The whole procedure for T_s calculation has been described by Begg *et al.* (13) and is illustrated in Fig. 1. Basically, it involves assuming that at the time of BrdUrd infusion the mean DNA content of BrdUrd-labeled, S-phase cells is in the middle of the interval between the $2n$ ($\text{G}_1\text{--}\text{G}_0$) and $4n$ (G_2) peaks and that the rate of cell progression through the S phase is constant. At the time of tumor sampling, *i.e.*, 4–6 h later, the position of the BrdUrd-labeled S-phase cells (as measured from their mean DNA content) allows one to determine the rate at which they have progressed through the S phase. All S-phase cells are expected to have reached G_2 at a time corresponding to T_s , which can be calculated from this progression rate.

³H]dThd and [¹⁴C]dThd Incorporation Studies

A part of the same cell sample (from normal, AL, and MM BM) used for the BrdUrd-LI and BrdUrd- T_s determination was also used to evaluate the LI (48 patients) and the T_s (13 patients) with [³H]dThd and [¹⁴C]dThd autoradiography, respectively. Autoradiography was not performed on solid tumor cells because the laborious disaggregation technique these tumors require may alter the metabolism of these cells, and hence their radioactive precursors' incorporation.

The [³H]dThd-LI was determined by a standard method (20). At least 1000 cells were scored for each determination.

The technique of quantitative [¹⁴C]dThd autoradiography for evaluating the T_s is based on the concept that, under standardized experimental conditions, the *in vitro* uptake of [¹⁴C]dThd by the cell nucleus is proportional to the rate of DNA synthesis, from which the T_s is calculated as the time needed to duplicate the diploid dThd content of the nucleus at this rate of dThd incorporation. This method has been

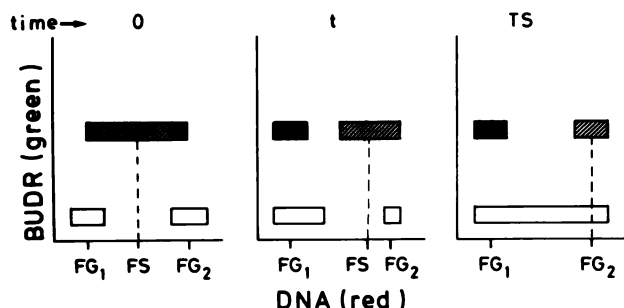


Fig. 1. Method for the evaluation of DNA T_s . Assumptions are that at time 0, the mean DNA content (red fluorescence) of BrdUrd (BUDR)-labeled S-phase cells (green fluorescence) (shaded plus black area) is in the middle of the interval between the G_1 and G_2 peaks (their position, i.e., their relative movement (RM1) with respect to the interval between G_1 and G_2 , is 0.5 ($RM1 = F_s - FG_1 / FG_2 - FG_1$), where F is the mean red fluorescence of the corresponding phase of the cell cycle) and that the rate of progression of cells through the S phase is constant. At the time of tumor sampling (t), S-phase cells have progressed toward G_2 , and some (black area) have already recycled. The new position of the S-phase cells is measured from their mean DNA content (red fluorescence), and their relative movement (RM2) at this time is calculated according to the above formula. All S-phase cells are expected to have reached G_2 at a time corresponding to T_s . The T_s is hence calculated with the formula:

$$T_s = \frac{0.5}{RM2 - 0.5} \times t$$

For example, assuming that RM2 is 0.75 at $t = 6$ h, S-phase cells would reach 1.0 at 12 h, and this is an estimate of T_s .

thoroughly described elsewhere (16, 17). Briefly, heparinized BM is filtered to avoid cell clumps and incubated with 5-fluorodeoxyuridine (in order to prevent the utilization of endogenous dThd) for 6 min at 37°C. The [14 C]dThd is then added and the incubation is prolonged for 7 min more. Cells are then smeared onto slides, which undergo an autoradiographic procedure. The radioactivity incorporated into every single cell nucleus is quantitated photometrically (with a Leitz MPV2 microscope photometer equipped for incident light bright field measurements) by comparing the blackening present over the nucleus of a labeled S-phase cell with the blackening produced by a standard source of known radioactivity ([14 C]polymethyl methacrylate) attached to the same slide. The rate of dThd incorporation in the labeled nuclei is derived from these data, since the incubation time, the concentration, and the specific activity of the [14 C]dThd used are known. The T_s is calculated as the time needed by the cell to duplicate the dThd content of the diploid nucleus (23) at the measured rate of dThd incorporation. At least 200 labeled cells were randomly measured for each T_s determination.

The harmonic mean (16) of the T_s of the entire population was used for successive calculations. It minimizes the bias of sampling, due to the different probabilities of becoming labeled that cells with different dThd incorporation rates have. Furthermore, the single T_s values could not represent the real duration of DNA synthesis for a given cell, since small variations in the rate of dThd uptake occur during the S phase. In cases where tumor cells were aneuploid by DNA flow cytometry, the harmonic mean of the T_s was corrected by taking into account the DNA index, which is known from DNA histograms (24).

Calculated Cell Kinetic Parameters

Once the LI and T_s were experimentally obtained (from BrdUrd or from [3 H]dThd and [14 C]dThd incorporation data), two additional kinetic parameters were calculated, namely the T_{pot} and the cell PR (9, 16, 17).

For these calculations, a steady state condition (9) was arbitrarily assumed for the studied populations. The T_{pot} is hence calculated by the formula:

$$T_{pot} \text{ (days)} = \frac{T_s \times 100}{LI \times 24}$$

The reciprocal is the PR:

$$PR \text{ (cells/100 cells/day)} = \frac{LI}{T_s} \times 24$$

RESULTS

The results are summarized in Tables 1 and 2 and in Figs. 2–8.

None of the 154 patients who received BrdUrd infusion experienced immediate toxicity or adverse reactions.

Representative Cytograms. Fig. 2 shows typical bivariate distributions of BrdUrd and DNA values from the BM of two previously untreated patients, from whom samples were taken for BrdUrd-LI evaluation 1 h after BrdUrd infusion. In Fig. 2A almost all cells (from a patient with lymphoblastic AL) having a DNA content ($2n$ – $4n$) typical of the S phase show BrdUrd incorporation, i.e., enter the window for the BrdUrd-LI determination. In Fig. 2B a number of cells (from a patient with MM in acute terminal phase, with plasma cells representing the 98% of BM cells) having $2n$ – $4n$ DNA content do not incorporate BrdUrd. Thus, they are not considered in the BrdUrd-LI evaluation.

Fig. 3 shows a similar cytogram from a patient with gastric cancer who had both diploid and hyperdiploid cells. In this case, BrdUrd-LI could be calculated for the whole tumor (13%) and for the hyperdiploid population (17.5%).

Fig. 4 shows a cytogram obtained in a patient with nonlymphoblastic AL for the T_s determination 6 h following BrdUrd infusion. With respect to that in the cytograms of Fig. 2, the position of the BrdUrd-labeled cells is shifted to the right. This means that in the interval between BrdUrd infusion and tumor sampling they have moved through the S phase. Some of them have already recycled and now show a diploid DNA content. Fig. 5 shows a similar cytogram obtained in a brain glioblastoma. Calculation of the T_s from cytograms of this type is accomplished according to the procedure described in Fig. 1.

BrdUrd-LI and BrdUrd- T_s Determination. To obtain the LI and T_s values, the complete BrdUrd procedure takes 8–9 h from the start of the BrdUrd infusion and 2–3 h from the tumor sampling.

The LI was successfully determined in 123 of 154 cases (79.8%) who received BrdUrd infusion, using either 1-h or (usually) 4- to 6-h tissue samples. The LI could be assessed less often on solid (54 of 78 cases = 69.2%) than on hematological tumor (69 of 76 cases = 90.7%) samples. Failure was in fact more frequent in solid than in hematological neoplasias. Reasons for the failure were, in decreasing order, the lack of sufficient cells for FCM (due to insufficient material obtained: this occurred in 7 of 12 gastric cancers that underwent endoscopic biopsy), the presence of few viable cells in the biopsy (due to histologically proven necrosis), and the difficulty of obtaining clean single-cell suspensions (this happened in two brain tumors).

The T_s could be derived in 92 of 154 (59.7%) patients who underwent BrdUrd infusion, all of whom also had successful LI measurement. The causes that hampered the LI evaluation also hampered that of the T_s . In addition, the T_s could not be determined in cases (mostly brain meningiomas) with a very low (less than 2%) LI, due to the difficulty in reliably measuring the relative movement (Fig. 1) of this small cohort of S-phase cells in the 4- to 6-h samples (Fig. 6).

Comparison of BrdUrd-LI and BrdUrd- T_s with Autoradiographic LI and T_s . Fig. 7A shows the close linear correlation

Table 1 Median values and range of kinetic data obtained in human malignancies with *in vivo* administration of bromodeoxyuridine. To calculate T_{pot} and PR from the experimentally determined LI and T_s , a steady state model for cell proliferation was assumed.

Diagnosis	No. of patients studied	LI determination		Other kinetic parameters' determination			
		No. of patients	LI	No. of patients	T_s (h)	T_{pot} (days)	PR (cells/100 cells/day)
Normal BM ^a	10	10	15.4 (12.4–25.4) ^b	10	13.7 (11.8–21.4)	3.6 (3.3–4.0)	27.3 (23.2–28.5)
Gastric tumors	35	22	9.9 (5.7–14.0)	17	15.2 (13.4–22.7)	9.8 (6.8–13.5)	10.1 (7.2–14.4)
Brain tumors Meningiomas	31	22	2.1 (0.9–3.9)	1	16.7	63.2	1.44
Gliomas	12	10	6.3 (2–7.6)	10	15.3 (10–22.7)	13.4 (4.6–63.2)	6.3 (3.6–11.2)
RAEBt	10	9	9.5 (4.3–15.5)	8	9.7 (6.5–15.0)	6.1 (2.3–14.5)	25.9 (6.7–41.2)
AL	46	42	6.2 (0.9–11.7)	40	12.1 (6.9–27.8)	8.5 (2.8–16.7)	14.4 (3.6–30)
Multiple myeloma, terminal phase	10	8	11.7 (10.4–24.5)	6	10.4 (7.5–25.0)	2.9 (0.6–4.2)	28.3 (14.6–55.2)

^a Normal BM, morphologically normal bone marrow from patients with gastric and brain tumors; RAEBt, refractory anemia with blast excess in transformation.

^b Numbers in parentheses, range.

Table 2 Gastric cancer: kinetic values measured on two samples (a and b) obtained from different areas of the same tumor

Case	Sample	LI	T_s (h)	T_{pot} (days)	PR (cells/100 cells/day)
1	a	8.0	23.4	2.9	8.2
	b	7.5	24.4	3.2	7.3
2	a	13.4	13.8	1.0	23.3
	b	14.0	15.9	1.1	21.1
3	a	10.5	16.8	1.6	15.0
	b	12.8	15.3	1.2	20.1
4	a	9.8	15.2	1.5	15.4
	b	10.1	14.2	1.4	17.0
5	a	7.0	22.7	3.2	7.4
	b	7.8	22.2	2.8	7.6
6	a	13.1	18.2	1.3	17.2
	b	14.0	18.0	1.2	18.6

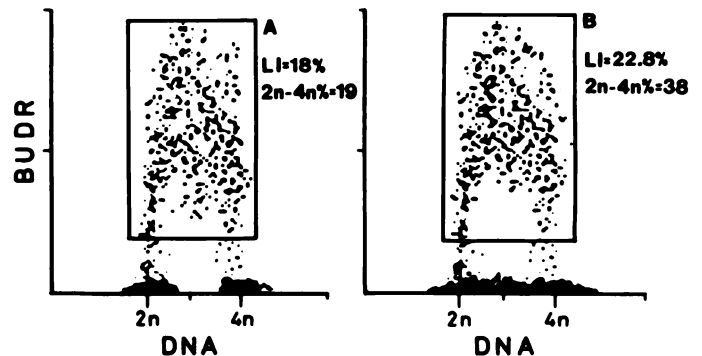


Fig. 2. Bivariate distribution of BrdUrd (*BUDR*) incorporation and DNA values from two patients, one with acute lymphoblastic leukemia (*A*) and the other with multiple myeloma in acute terminal phase (*B*), with plasma cells representing the 98% of the bone marrow cells. Measurements were performed 1 h following BrdUrd infusion. In (*A*) all the cells with a DNA content intermediate between the diploid ($2n$) and the tetraploid ($4n$) value ($2n-4n$), i.e., typical of the S phase, have incorporated BrdUrd, so that the $2n-4n$ cell percentage ($2n-4n\%$) and the BrdUrd-LI ($LI\%$) are similar. In (*B*) a number of $2n-4n$ cells did not incorporate BrdUrd, so that the LI is lower than the $2n-4n\%$.

between the LI values obtained on duplicate samples from the BM of 48 patients (having hematological tumors or normal BM; Table 1) using *in vivo* BrdUrd administration and *in vitro* [3H]dThd autoradiography.

Fig. 7*B* shows that a correlation also exists between the T_s values obtained on duplicate samples in 13 patients using *in vivo* BrdUrd administration and *in vitro* quantitative [^{14}C]dThd autoradiography. BrdUrd values were systematically a bit higher than the [^{14}C]dThd values. As detailed elsewhere (16), this is due to the fact that [^{14}C]dThd- T_s determination is performed by calculating a "flux" parameter, while BrdUrd- T_s is a "compartment" parameter. Flux values are slightly smaller than compartment values, and the longer the T_s values become, the less this makes a difference.

As expected, the T_{pot} and PR values determined from BrdUrd infusion and from autoradiographic data were also linearly correlated ($r = 0.91$, $P < 0.05$; and $r = 0.83$, $T < 0.05$, respectively).

Cumulative Kinetic Data in Human Tumors. Table 1 summarizes kinetic data obtained with the BrdUrd technique. The highest LI and PR values and the shortest T_{pot} were found in the morphologically normal BM and in the BM plasma cell populations of patients with MM in acute terminal phase. Brain meningiomas had the lowest proliferative activity. Intermediate values for LI, PR, and T_{pot} were found in AL, refractory anemia with blast excess, gastric cancer, and brain gliomas.

Two patients with AL had three sequential BrdUrd studies, one at presentation and two during cytostatic-induced remission of the disease. This was possible because there was no residual BrdUrd labeling in the blood 4 days following each BrdUrd infusion. The LI and the PR were low at presentation (Fig. 8), when all BM cells were leukemic blasts, and rose at remission, when BM cytology was normal.

Table 2 shows that two tumor samples obtained from differ-

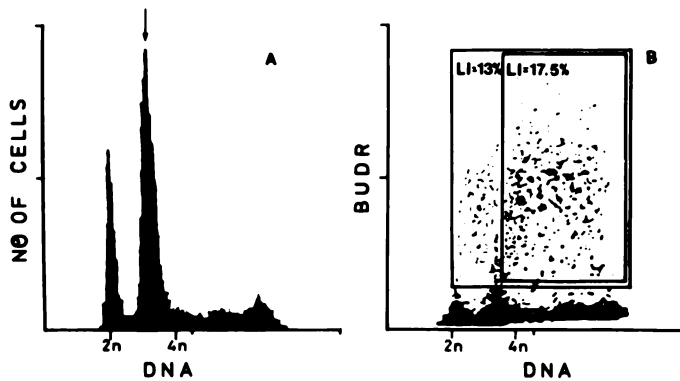


Fig. 3. DNA profile (A) and bivariate distribution of BrdUrd (BUDR) and DNA (B) from a patient with gastric cancer having both diploid (2n) and hyperdiploid (arrow) cells. Measurements were performed 1 h following BrdUrd infusion. In tumors such as this (where the modal DNA contents of the two populations are well separated), both the LI of the total tumor population (13%) and that of the population with the greater modal DNA content (17.5%) can be determined (by including or excluding the population with the lower DNA content from measurements).

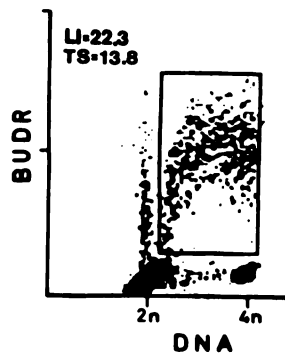


Fig. 4. Bivariate distribution of BrdUrd (BUDR) and DNA values from a patient with nonlymphoblastic acute leukemia. Measurements for both LI (%) and T_s (TS) (h) determinations were performed 6 h following BrdUrd infusion. With respect to the cytograms in Fig. 2, BrdUrd-labeled cells have moved through the S phase (their mean distribution is shifted to the right) and some of them have already recycled (showing a diploid DNA content). Calculation of T_s is accomplished according to the procedure described in Fig. 1.

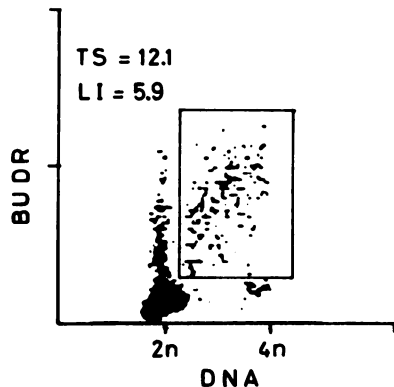


Fig. 5. Bivariate distribution of BrdUrd (BUDR) and DNA values from a patient with brain glioblastoma (explanations as in Fig. 4).

ent areas of 6 gastric cancers did not give very different LI and T_s values, except in case 3.

In 20 patients (16 of whom had solid tumors), both diploid and highly hyperdiploid cells coexisted in the same tumor sample, and the two DNA peaks were sufficiently separated to allow determination of the BrdUrd-LI both for the overall and for the aneuploid cell population (as in Fig. 3). Median LI was 7.6% (range, 0.9–21.7) for the overall and 10.2% (range, 2.4–24.5) for the hyperdiploid population ($P < 0.05$). The hyperdip-

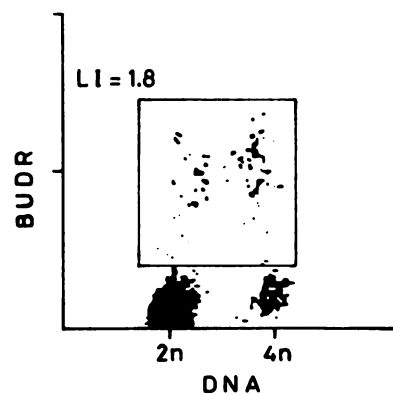


Fig. 6. Bivariate distribution of BrdUrd (BUDR) and DNA values from a patient with brain meningioma. As in Figs. 4 and 5, measurements were performed 6 h following BrdUrd infusion. However, in this case the T_s could not be calculated due to the difficulty in reliably measuring the relative movement (Fig. 1) of the small (less than 2%) cohort of S-phase cells.

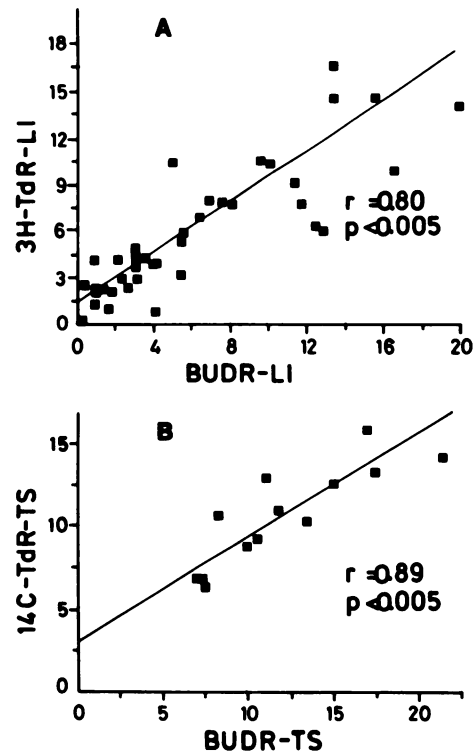


Fig. 7. A, correlation between the LI values obtained from *in vivo* BrdUrd administration (BUDR-LI) and *in vitro* [3 H]dThd autoradiography (3H-TdR-LI) on duplicate samples. B, correlation between the DNA T_s obtained from *in vivo* BrdUrd administration (BUDR-TS) and *in vitro* quantitative [14 C]dThd autoradiography (14C-TdR-TS) on duplicate samples.

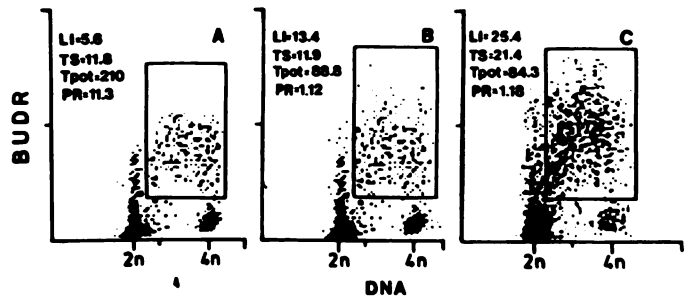


Fig. 8. Sequential determinations of kinetic parameters in a patient with acute nonlymphoblastic acute leukemia who achieved remission of the disease. A, at presentation; B, 29 days later, at early remission; and C, 6 months later, during stable remission.

loid population LI was lower ($P < 0.05$) than the overall population LI in 1 case, and greater in 12 cases. In 7 cases the two values were similar.

DISCUSSION

Our data show that administering BrdUrd *in vivo* and using FCM for measurements allows one to obtain reliable experimental measures for the LI and T_s in humans, while using only one tumor sample. Other kinetic parameters depicting tumor growth were calculated. The *in vivo* BrdUrd technique was feasible in a clinical setting and we were able to study a large number of patients in a short time.

The use of BrdUrd to label S-phase cells relies on the fact that BrdUrd is incorporated, like [^3H]dThd, into proliferating cells (25), both *in vitro* and *in vivo* (10). The BrdUrd-incorporating cells are identified with an anti-BrdUrd MoAb (11), by using immunofluorescence or immunohistochemistry to detect the reaction. The percentage of BrdUrd-labeled cells, *i.e.*, the BrdUrd-LI, is a reliable estimate of the size of the S phase. As occurred in this investigation, the BrdUrd-LI and the traditional [^3H]dThd-LI have been found to be very close, both in experimental models (10, 26) and in humans (27). Statistically, the BrdUrd-LI values measured with FCM, as in this study, are more accurate than both [^3H]dThd-LI and BrdUrd-LI values determined on slides, since a much greater number of cells is evaluated with FCM than by visual counting. The problem of background, which is a difficulty in the [^3H]dThd-LI evaluation (9), is also lessened, in that nonspecific staining is low in BrdUrd preparations used for FCM and can also be subtracted with the computer. The other traditional method for evaluating S phase is to measure the 2n–4n cell percentage with conventional DNA FCM (8). With regard to this method, the BrdUrd technique has the advantage that it avoids counting as proliferating those cells which have 2n–4n DNA content but do not actually synthesize DNA, because they are not labeled with BrdUrd (Fig. 2B). These cells are also documented by determining the Feulgen-DNA content on slide preparations from tumor cells previously exposed to [^3H]dThd *in vitro* (28). They lead to an overestimation of the S phase with DNA FCM as compared to the [^3H]dThd autoradiographic determination (8, 29).

In this study, we used the *in vivo* administration of BrdUrd to label S-phase cells, coupled with two-parameter FCM for rapid measurement of BrdUrd-labeled cell progression through the S phase in the 4–6 h following BrdUrd infusion. With respect to the *in vitro* BrdUrd method, this allows experimental measurement of T_s on the same sample used for the LI determination (Figs. 4 and 5), assuming that cell progression through the S phase is constant (13). The T_s depicts the rate at which proliferating (*i.e.*, BrdUrd-labeled) cells move through the S phase, a parameter that is exceedingly difficult to determine in humans, especially in solid tumors (9). *In vivo* BrdUrd infusion was used to label leukemia cells, which 1–2 h later were also exposed to [^3H]dThd *in vitro* (30). This allows the T_s to be obtained with a simplified “double-labeling technique” (9), but time-consuming autoradiography and visual counting are not avoided with this procedure.

The values for T_s drawn from the FCM-BrdUrd incorporation analysis must be viewed as accurate. In this research they were strictly related to the values drawn on duplicate samples from quantitative [^{14}C]dThd autoradiography (Fig. 7B), which is another accepted method for calculating T_s (15). For solid

tumors and AL these values were also similar to those obtained with other autoradiographic techniques (9, 31).

From the experimentally obtained LI and T_s , a number of temporal kinetic parameters can be calculated (9, 16, 17). We have selected only the T_{pot} , *i.e.*, the time at which the whole tumor duplicates, and the PR, *i.e.*, its rate of cell production, as the most meaningful in giving an overall picture of tumor growth. The T_{pot} and PR values calculated from the LI and T_s give rates of tumor growth which are more rapid than those experimentally derived from direct measurements of tumor masses, due to cell loss (9). Cell loss could therefore be derived in cases where both measurements can be obtained, as shown in solid tumors (9) and MM (32).

It must be noticed that in deriving T_s from FCM cytograms (Fig. 1) we arbitrarily used Begg's algorithm (13), and in calculating T_{pot} and PR from LI and T_s we assumed a steady state model for cell proliferation. Other algorithms for T_s (33) and other more complex models for cell proliferation (9) have been described and could have easily been adopted. Deciding which mathematics would be theoretically more appropriate in interpreting experimental FCM data was, however, beyond the scope of this research, whose aim was to obtain experimental data from BrdUrd-DNA cytograms.

Our study indicates that the *in vivo* BrdUrd procedure is feasible in clinical settings. Immediate adverse reactions following BrdUrd infusion were neither observed in this study nor reported. Long-term BrdUrd toxicity is not likely, since BrdUrd is neither radioactive nor myelotoxic at the dosage used (32). At larger doses it is actually used as radiosensitizer (34, 35). The *in vivo* BrdUrd method requires only one tumor sample, which is needed anyway for diagnostic or therapeutic purposes, and takes 8–9 h from the start of BrdUrd infusion and only 2–3 h from the tumor sampling. These times are distinctly less than those necessary for [^3H]dThd and especially for [^{14}C]dThd autoradiography, which take several days or weeks to furnish LI and T_s values, respectively.

With FCM-BrdUrd incorporation analysis, we were able to study 154 patients in an 8-month period; LI values were obtained in 79.8% of cases and T_s , T_{pot} , and PR values were obtained in 59.7% of cases. As expected, kinetic values could be determined more often on hematological than on solid tumors. Failure in calculating both LI and T_s was due to not obtaining suitable FCM samples, and this happened almost exclusively in solid tumors. Reasons for this failure were the insufficient cell harvesting from biopsied gastric cancers and, much more infrequently, the presence of histologically proven necrosis in some brain and gastric tumor samples, and the difficulty in obtaining a clean (*i.e.*, without cellular debris) cell suspension. With an adequate surgical sample and by using the cell dissociation technique we have described, this latter occurrence was absolutely uncommon in brain and gastric cancers we studied. Failure in obtaining the T_s in cases who had successful LI determination was that in tumors with very low LI (almost exclusively in brain meningiomas) it was not possible to measure reliably the relative movement of the small cohort of S-phase cells in the 4- to 6-h samples.

Although no attempt was made in performing statistical analysis, the measurements for LI obtained on BM populations appear to be reliable. According to the literature on [^3H]dThd-LI levels, the BrdUrd-LI values were greater in morphologically normal BM than in active AL and refractory anemia with blast excess in transformation (2, 4, 19). They rose, as expected (4), in two patients with AL who achieved remission of the disease (Fig. 6). As was found in our cases, very low S-phase values

have been reported in meningiomas (36–38). There is no sense in discussing the accuracy of the LI values in gastric cancer and gliomas (and of the T_s values in all pathologies), since the literature data on these topics are only occasional (9).

For solid neoplasias a long-discussed possibility is that kinetic parameters are different in different areas of the tumor, so that the proliferative characteristics obtained could be partly determined by the site of tumor sampling (*i.e.*, they may not be representative of whole tumor kinetics). FCM-*in vivo* BrdUrd incorporation analysis (possibly coupled with direct visual inspection of BrdUrd incorporation on tissue sections) (39) seems an adequate method for ascertaining this point. For example, in this study 6 patients with gastric tumors had LI and T_s measured on two different tumor samples, and the intratumoral kinetic variation was quite low.

With respect to the methods of determining the LI and T_s on slides (using either [^3H]dThd or BrdUrd for LI, and [^{14}C]dThd for T_s), a limitation of the BrdUrd labeling and FCM technique is that the values obtained are mean values for the whole sample population, since cell morphology cannot be taken into consideration. Hence, the kinetic parameters depict the proliferative activity of the tumor only if essentially all the cells in the sample are neoplastic as, for example, in overt AL, advanced myeloma, and most solid tumors. In cases in which the sample population is a mixture of neoplastic and residual normal cells (for example, in hematological diseases with only partial bone marrow involvement, such as MM or refractory anemia with blast excess), the LI and T_s values obtained and the parameters calculated are, accordingly, mean values for this mixture. To overcome this limitation, a marker for the neoplastic cells could be used, for example, a B-cell marker in myeloma (40). Based on this marker, neoplastic and normal cells could be sorted and then measured separately for BrdUrd incorporation and DNA content. Another possibility would be to use instrumentation capable of measuring at least three parameters simultaneously (41); that is, the tumor population marker, BrdUrd incorporation, and DNA content.

Besides the theoretical interest in knowing the pattern of human tumor growth, two clinical advantages could be offered by the *in vivo* BrdUrd technique. First, the ease with which kinetic parameters are obtained in large numbers of patients could substantiate, or challenge, the clinical relevance of pre-treatment tumor proliferative characteristics in prospective series. Second, BrdUrd results are time scale suitable for clinicians to use in planning treatments in individual cases. For example, in radiotherapy a gain in local disease control might be obtained in tumors having doubling times less than 5 days, if the 6- to 7-week treatment is drastically shortened by giving 2–3 fractions per day (42).

We conclude that cell kinetics can be reliably studied in large numbers of human tumors with homogenous cell populations by combining *in vivo* BrdUrd administration and FCM measurements. Possible clinical advantages involve the evaluation of the prognostic meaning of proliferative activity and aid in planning antitumor treatments.

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