Veterinary Research Communications, 22 (1998) 233–247

POTENTIAL TUMOUR DOUBLING TIME: DETERMINATION OF T_{pot} FOR VARIOUS CANINE AND FELINE TUMOURS

U. SCHWYN[1], N.E.A. CROMPTON[3], H. BLATTMANN[3], B. HAUSER[2], B. KLINK[1], A. PARVIS[1], D. RUSLANDER[1] AND B. KASER-HOTZ[1*]
[1]Veterinary Medicine Department, Radio-Oncology Programme and [2]Institute of Veterinary Pathology, University of Zürich, Winterthurerstr. 260, CH-8057 Zürich;
[3]Paul Scherrer Institute, CH-5232 Villigen, Switzerland
*Correspondence

ABSTRACT

Schwyn, U., Crompton, N.E.A., Blattmann, H., Hauser, B., Klink, B., Parvis, A., Ruslander, D. and Kaser-Hotz, B., 1998. Potential tumour doubling time: determination of T_{pot} for various canine and feline tumours. *Veterinary Research Communications*, **22(4)**, 233–247

Spontaneous tumours in dogs and cats are an excellent model for clinical human research, such as in developing proton conformation radiotherapy for humans. The kinetics of tumour cells can be used effectively to predict prognosis and response to therapy in patients with tumours. Knowledge of the kinetic parameters in these tumours is therefore important. In the present study the kinetic parameters evaluated included the labelling index (LI), relative movement (RM), mitotic index (MI), and potential doubling time (T_{pot}). These parameters were determined using *in vivo* labelling with bromodeoxyuridine, flow cytometry and histological preparation. Samples were obtained and evaluated from 72 dogs and 20 cats, presenting as patients in our clinic. Within the groups of epithelial and mesenchymal tumours from dogs and cats, the kinetic parameters LI, RM and MI were compared with T_{pot}. Significant correlations were observed for the comparison T_{pot} and LI. No correlation was found between T_{pot} and RM.

Keywords: bromodeoxyuridine, cat, dog, kinetics, potential doubling time, tumour

Abbreviations: BrdU, bromodeoxyuridine; HBSS, Hank's balanced salt solution; LI, labelling index; MI, mitotic index; RM, relative movement; T_{pot}, potential doubling time; T_S, duration of S phase

INTRODUCTION

Tumours have been described in small-animal medicine for many years and an increase in cancer cases has been observed over the past few years. They form an excellent model for clinical human tumours (Gillette, 1982). The WHO has provided staging criteria for a variety of animal tumours (Owen, 1980), and surgery, radiotherapy and chemotherapy are established cancer therapies in veterinary medicine today. Various imaging techniques, clinical work-up, histopathology and cytology are used during diagnosis, but it is difficult to assess the kinetic potential of a growing tumour at a particular time. Remvikos and colleagues (1991) described the importance of cell kinetics as a predictor in clinical tumour diagnostics. Knowing more precisely the speed at which a tumour mass is growing greatly enhances prognosis, and tumour therapy can be chosen on a more appropriate and individual patient basis. Potential

tumour doubling time (T_{pot}) is the theoretical time predicted for a tumour to double in cell number. T_{pot} is an important predictor in radiotherapy because, during standard treatments intended to kill the tumour cells, any radiotherapeutic procedure may be overridden by rapid growth of the surviving cells in those tumours with a short potential doubling time. This may also be true for some chemotherapeutic agents (Dewhirst *et al.*, 1995). In the method described in this paper, tumour cells are labelled *in vivo* and the T_{pot} of the tumour is determined from a single biopsy a few hours later.

Gratzner (1982) and Dolbeare and colleagues (1983) described a novel method of cell analysis utilizing a monoclonal antibody to bromodeoxyuridine (BrdU), which makes it possible to determine accurately the fraction of cells from a given population in a specified cell-cycle phase. BrdU is an analogue of uridine and is incorporated specifically into DNA in place of thymidine. Only those cells which are synthesizing DNA incorporate BrdU into their DNA, i.e. those cells in the S-phase of the cell cycle. The use of BrdU overcomes problems associated with injecting patients with radioactive substances and taking multiple samples. Begg and colleagues (1985) described a method for estimating the labelling index and the duration of the S-phase from measurements on a single tumour biopsy. These workers used flow cytometry to determine the fraction of cells from a given population in various phases of the cell cycle. T_{pot} can be calculated from a single sample of cells taken several hours after labelling with BrdU *in vivo*. When tumour cell kinetics are studied using this method, tumour proliferation rates can be monitored *in vivo* with a single injection of nontoxic and nonradioactive bromodeoxyuridine, requiring excision of only a single biopsy 4–8 h later, as shown by Begg (1989).

Numerous reports have been published of analyses of human tumours using this method (Mitchell *et al.*, 1983; Kinsella *et al.*, 1984; Dressler *et al.*, 1987; Wilson *et al.*, 1988a,b; Giordano *et al.*, 1991; Rew *et al.*, 1991, 1992; Shimomatsuya *et al.*, 1991; Popert *et al.*, 1993; Remvikos *et al.*, 1991; Cooke *et al.*, 1994), but little documentation about the various animal tumours is available. Carter and colleagues (1993) showed that the use of flow cytometry is as relevant in veterinary medicine as it is for human medicine. Flow cytometric analysis of BrdU-labelled cells has led to the description of various methods of determining T_{pot} from single veterinary samples. Vail (1993) examined lymphomas in dogs and discussed the potential benefit to the clinical oncologist of having quantitative kinetic assays for accurate prediction of the response to treatment and long-term survival. La Rue and colleagues (1994) obtained multiple samples from 30 dogs with osteosarcomas. Their study investigated the variation between tumours from different dogs and compared this with the variation between different samples from the same tumour for a number of kinetic parameters. The results of their study showed that there was more variation between tumours than within tumours for the kinetic growth parameters examined, such as T_{pot}.

The major goals of this report are to described the preparation of biopsies from different tumour types, to describe a simple, reliable protocol for producing single-cell suspensions, and to report on the spectrum of growth parameter values observed from a variety of canine and feline tumours. To our knowledge, only canine lymphomas and osteosarcomas have been studied to date, and no information is available on tumours from cats. The results of this predictive assay should be useful for cancer prognosis,

planning of therapy, and the incorporation of these measurements into clinical trials. Therefore, it is important to obtain good, reproducible results. T_{pot} values of various tumours from dogs and cats were compared with the labelling index (the fraction of cells which were synthesizing DNA at the time of BrdU administration), the relative movement (a measure of how far labelled cells progress through S-phase between BrdU labelling and biopsy excision), and the mitotic index (the fraction of cells observed to be in mitosis), all from the same tumours.

MATERIALS AND METHODS

The study involved an investigation of various tumours from 72 dogs and 20 cats, of both sexes, and of different breeds. The median age of the dogs was 9.5 years (range 0.7–16 years) and the median age of the cats was 13 years (range 1–16 years). The animals were presented to the Small Animal Clinic of the University of Zurich between January 1995 and May 1996. Owners were informed about the study and asked to monitor the animals for any subsequent side-effects. Patients mostly presented with a palpable mass at different body locations. Clinical diagnosis of neoplasia was based on histopathological examination. All tumour samples were examined microscopically at the Institute of Veterinary Pathology of the University of Zurich. The histological type of the tumour, its grade and mitotic index were determined. The mitotic index was determined using a standard method by counting the number of mitoses visible in several microscope fields of 0.17 mm^2 (magnification 400-fold). Samples of normal or inflammatory tissue were excluded from this analysis.

BrdU (5-bromo-2′-deoxyuridine), an analogue of thymidine, is incorporated into the DNA during the S-phase of the cell cycle (Cooke *et al.*, 1994). BrdU is not radioactive, nor is it toxic in the dose required for labelling the cells (Wilson *et al.*, 1988a; Giordano *et al.*, 1991; Cooke *et al.*, 1994). Infusions of twice the dose of BrdU can be tolerated for several weeks without myelosuppression (Mitchell *et al.*, 1983; Kinsella *et al.*, 1984). The BrdU used for intravenous injection in this study was prepared at the Paul Scherrer Institute, Villigen, Switzerland. BrdU (7.00336 mg/ml: Fluka Chemie AG, Buchs, Switzerland) was dissolved in pyrogen-free 0.9% NaCl, pH 6, and <1 mmol NaOH/L. BrdU solutions were tested for stability and sterility. After 6 months the stability of 500 mg BrdU dissolved in 72 ml sterile, pyrogen-free saline was still greater than 99%. The sterility test confirmed its suitability for use in veterinary medicine. All the dogs were given 500 mg BrdU and all the cats were given 100 mg BrdU, in single intravenous injections (Wilson *et al.*, 1988a; La Rue *et al.*, 1994). After an average post-injection incubation period of 5.78 ± 1.87 h, biopsies were obtained by excising the tumour. Half the biopsy was fixed in 50% ethanol and left overnight in a refrigerator. The other half was fixed in formalin for histological identification. Excess bromodeoxyuridine in the circulation is eliminated in a two-step process: first, BrdU is degraded to bromouracil, in a manner analogous to the degradation of thymidine; secondly, enzymatic dehalogenation takes place in the liver to form atomic bromine (Hughes *et al.*, 1964). The concentration of BrdU in the circulation falls to 30% of its original value after 24 h.

A single-cell suspension for flow cytometric evaluation was obtained from the biopsy which had been fixed in 50% ethanol at 4°C. First, the tumour sample was finely minced using two scalpels. During mincing, the tumour was submerged in approximately 1 ml of pepsin-HCl solution (0.4 mg pepsin (E. Merck, Darmstadt, Germany) per ml of 0.1 mol/L HCl; Schutte *et al.*, 1987; Wilson *et al.*, 1988a). Next, the minced tumour was transferred to a conical tube for digestion in 4 ml pepsin-HCl solution. HCl treatment promotes denaturation of DNA, and simultaneous treatment with pepsin decreases the subsequent amounts of debris and enhances the staining of nuclei (van Erp *et al.*, 1988). The tumour suspension was digested for 25 min at 37°C in a water bath, the tube occasionally being manually agitated. Incubation for more than 30 min causes overdigestion of the probe, unsatisfactory DNA profiles, and inaccurate determination of T_{pot} (Popert *et al.*, 1993). Afterwards, the supernatant was withdrawn with a syringe and filtered through a 40 μm mesh. The single-cell suspension so obtained was centrifuged (400*g*, 5 min), the supernatant was aspirated, and the pellet was resuspended in 3 ml Hanks' balanced salt solution (HBSS: Life Technologies, Basel, Switzerland). This suspension was also centrifuged (300*g*, 5 min), the supernatant was aspirated, and the pellet was resuspended in 500 μl 2 mol/L HCl, and incubated for 10 min at 37°C in a water bath, after which 3 ml HBSS was added. After further centrifugation (300*g*, 5 min), the supernatant was aspirated, and the pellet was resuspended in 3 ml HBSS. This wash step was repeated. After the next centrifugation (300*g*, 5 min), the supernatant was aspirated, and the pellet was resuspended in 3 ml HBSS with 0.5% Tween 20 (Serva Feinbiochemica, Heidelberg, Germany) and 0.5% bovine serum albumin (Readysysteme AG, Zurich, Switzerland). After another centrifugation (300*g*, 5 min), the supernatant was aspirated, and the pellet was again resuspended in 3 ml HBSS (White *et al.*, 1990). The cell density in this suspension was assessed using an electronic counter (Coulter counter). The suspension was centrifuged once more (300*g*, 5 min), the supernatant was aspirated, and the pellet was resuspended in 100 μl HBSS and 20 μl of anti-BrdU FITC-antibody (anti-BrdU-FITC with gelatine and 0.1% acid; Becton Dickinson, Basel, Switzerland). The cells were then incubated at room temperature for 25 min, the tube being occasionally manually agitated. Thereafter 3 ml of HBSS was added and the suspension was centrifuged (300*g*, 5 min), the supernatant was aspirated, and the pellet was resuspended in 1 ml HBSS and 20 μl propidium iodide (1 mg/ml). Propidium iodide is an intercalating dye which binds to double-stranded DNA (van Erp *et al.*, 1988). The pellet was mixed thoroughly and cells were left to stain for 5 min at 4°C, ready for flow cytometric analysis.

In the analysis, 20 000 cells were examined in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Procedures for flow cytometry have been described in detail previously (Begg *et al.*, 1985; Wilson *et al.*, 1985; Becton Dickinson, 1989). Briefly, four parameters were measured using an argon laser emitting light of 488 nm wavelength: forward-angle light scatter (cell volume), 90° side-angle light scatter (cell granularity), green fluorescence (BrdU content) and red fluorescence (DNA content). The cells were first identified on the basis of their size and granularity. Subsequently, bivariate density 'dot-plots' of DNA-area (the area of the DNA fluorescence signal) versus DNA-width (the width of the DNA fluorescence signal) permitted exclusion by

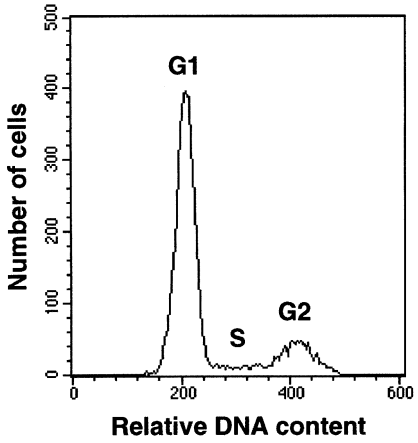

Figure 1. Flow cytometric DNA histogram of canine mammary carcinoma. The cell cycle phases are labelled: G1, gap 1 phase; S, DNA synthesis phase; G2, gap 2 phase. The units of DNA content are relative based on fluorescence signal size

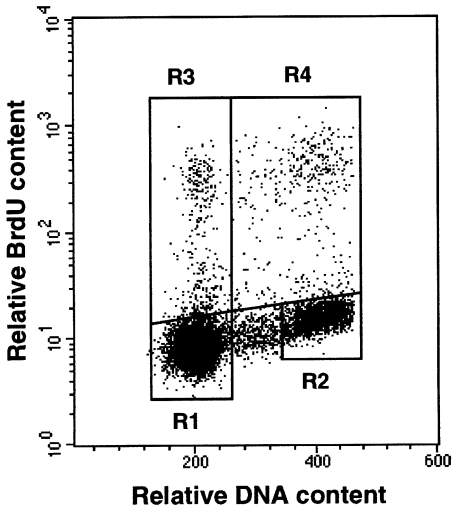

Figure 2. Bivariate density dot-plot of cellular DNA content versus bromodeoxyuridine (BrdU) labelling of a canine mammacarcinoma (see Figure 1). R1, G1-phase cells; R2, G2-phase cells; R3, labelled cells which have undergone cell division; R4, labelled cells which have not yet undergone cell division. The units of DNA content and BrdU content are relative based on fluorescence signal size

gating of abnormal signals and signals from unavoidable cell aggregates. A DNA-area histogram, and a DNA-area versus BrdU-incorporation bivariate density dot-plot were prepared from the data for the gated cells (see Figures 1 and 2).

The T_{pot} method involves calculations using flow cytometric data of BrdU incorporation (determined from the green fluorescence of the FITC-labelled antibodies) and total DNA content (determined from the red fluorescence of propidium iodide incorporated into the DNA) of the tumour cells. Four parameters must be measured: F_{G1}, F_{G2}, F_L and LI. F_{G1} is the average DNA fluorescence of cells in G_1-phase, F_{G2} that of cells in G_2-phase, and F_L that of cells which were BrdU-labelled but had not yet passed through mitosis (i.e. labelled, non-undivided), while LI is the fraction of cells labelled with BrdU. To determine F_{G1} and F_{G2}, G_1-phase cells were identified from the DNA histogram (Figure 1) and 'gated' and their average DNA content was determined automatically using Lysis II software (Becton Dickinson, San Jose, CA, USA). F_{G2} could then be determined as a function of the F_{G1} value simply multiplying by 1.9 (Vindeløv and Christensen, 1990). These values were often of assistance when separating undivided labelled cells from divided labelled cells on the dot-plots (Figure 2). F_L and LI values for calculating T_{pot} were obtained directly after 'gating'. This optimized analysis permitted a standard procedure to be applied to each specimen. To determine the average DNA fluorescence of labelled, undivided cells, the bivariate density dot-plot of DNA-area versus BrdU content was analysed. A gate was set around the labelled cells in order to determine labelling index. A gate was also set around the labelled cells which had not yet divided and had not yet entered the G_1-phase of the cell cycle. In the automated statistical subroutine of the Lysis II software, the average DNA content of F_L cells was automatically determined.

Using these data, relative movement (RM), duration of S-phase (T_S), and T_{pot} were determined. The methods of Begg and colleagues (1985) and White and colleagues (1990) were employed. RM was determined using: $RM = (F_L - F_{G1})/(F_{G2} - F_{G1})$. T_{pot} was calculated from: $T_{pot} = \lambda T_S/LI$. The calculation assumes that movement of labelled cells toward G_2 is linear with time. T_S can be derived from RM using $T_S = 0.5t/(RM - 0.5)$, where t is the time between intravenous administration of the BrdU and excision of the biopsy (Begg *et al.*, 1985). LI, the labelling index, is the fraction of cells which were synthesizing DNA at the time of BrdU administration and which therefore incorporated the label. λ is a correction factor which takes into account the nonlinear distribution of cells through the cell cycle; it can be estimated but not calculated exactly from data made available from a flow cytometric histogram (Terry *et al.*, 1991). A realistic estimate of λ is 0.8 and this is generally suitable for use with tumour biopsies (Steel, 1977; Wilson *et al.*, 1988b).

RESULTS

During these studies, the following observations were made regarding procedures which contributed to an optimal determination of T_{pot}. The size of the biopsies had to be at least 1–2 mm^3 in order for us to obtain sufficient cells for flow cytometry, because many cells were lost during the work-up procedure. The size of the biopsy also limited the speed of fixation by ethanol, which must penetrate into the centre of the sample, so the sample size should not be too large. Alanen and colleagues (1989) compared four standard fixation techniques for flow cytometric determination of T_{pot}: fresh, formalin-

fixed, ethanol-preserved and paraffin-embedded samples. Identical DNA indices and fairly constant S-phase values were obtained from 50% ethanol-preserved samples stored at 4°C for up to 5 months. In the present study, the samples were preserved in 50% ethanol and stored in a refrigerator at 4°C for at least 24 h. This method of preserving the biopsy permitted analysis of the samples even several days after the biopsies were taken. It was also possible to perform additional analyses at later dates, as long as sufficient material was still available. The pepsin-HCl incubation period for the diced sample, and the use of Hanks' balanced salt solution, were determined in a series of tests on different tumour types. During the course of these tests, three wash-steps were found to be necessary for obtaining suitable pH conditions for anti-BrdU-antibody binding following the HCl incubation. Both the duration and the temperature of antibody incubation were studied. Incubation at room temperature for 25 min gave optimum results.

BrdU was administered to 107 patients and biopsies were obtained. In 92 cases the T_{pot} value was measured successfully. In 9 of the 15 cases where no useful data could be obtained, histological examination revealed no tumour was present. In 2 other cases the biopsy was taken from a necrotic area of the tumour, and in the remaining 4 cases the biopsy sample was too small and too few labelled cells were available for analysis in the flow cytometer. Therefore, kinetic cell data were obtained from 85% of the samples. The majority of the biopsies were taken from dogs (73/92), while the rest (19/92) were taken from cats. These numbers represent approximately the ratio of dogs to cats presenting as tumour patients at our clinic. The majority of tumours were categorized as either epithelial or mesenchymal, because of the distinct ontogenetic origins of these two tumour types. Tumours such as insulinoma, mesothelioma, malpighioma and ameloblastoma, which were seldom seen, were listed separately. The potential doubling time (T_{pot}), labelling index (LI), relative movement (RM), and mitotic index (MI) were evaluated for all 92 tumours. Tables I and II show the histopathological classification and the kinetic values of the epithelial and mesenchymal tumours in the dogs and cats, respectively. In these tables, the median values and range are indicated for each category. Comparisons can be made both between and within the epithelial and mesenchymal tumour groupings from dogs and cats of T_{pot}, LI, and RM. Values of MI are displayed in Table III.

Regression analyses of $\log_e$ (LI) versus $\log_e$ (T_{pot}) for canine and feline mesenchymal and epithelial tumours are presented in Figures 3A to 3D. Figure 3A presents the regression line for $\log_e$ (LI) versus $\log_e$ (T_{pot}) of canine mesenchymal tumours. The same relationship for canine epithelial tumours is presented in Figure 3B. In Figure 3C, the regression curve of $\log_e$ (LI) versus $\log_e$ (T_{pot}) for feline mesenchymal tumours is shown, while in Figure 3D the same relationship is presented for feline epithelial tumours. In all four figures there is a significant slope and their data lie close to the regression curve. Figures 4A and 4B display the combined canine and feline mesenchymal and epithelial T_{pot} values in linear and logarithmic plots. Figure 4C presents the distribution of LI values, and Figure 4D the distribution of RM values. Whereas the RM values were normally distributed, both the T_{pot} and LI were log-normally distributed. The regression curve of RM versus $\log_e$ (T_{pot}) for all canine and feline mesenchymal and epithelial tumours is shown in Figure 5A. The correlation

TABLE I
Tumour kinetic parameters of all canine tumours studied

Tumour class	No.	Tumour type	Median potential doubling time in days (range)	Median labelling index in % (range)	Median relative movement (range)
Epithelial tumours	25	See text	9.5 (2.8–63.0)	2.6 (0.4–9.8)	0.87 (0.75–1.06)
Mesenchymal tumours	30	See text	19.3 (2.6–139.4)	2.4 (0.2–10.3)	0.88 (0.59–1.02)
Miscellaneous tumours	7	Mixed mammary cancer	15.6 (5.3–72.9)	1.4 (0.3–3.5)	0.86 (0.81–0.90)
Pigment cell tumours	4	Melanoma	14.9 (3.0–76.1)	2.8 (0.5–7.6)	0.91 (0.79–0.94)
Different tumours	4	Epulides	8.7 (8.5–10.1)	3.0 (2.7–3.5)	0.85 (0.78–0.85)
	1	Insulinoma	14.4	3.1	0.77
	1	Mesothelioma	4.2	5.4	0.95
	1	Malpighioma	8.1	2.3	0.96

TABLE II
Tumour kinetic parameters of all feline tumours studied

Tumour class	No.	Tumour type	Median potential doubling time in days (range)	Median labelling index in % (range)	Median relative movement (range)
Epithelial tumours	6	See text	5.0 (1.9–10.5)	3.9 (1.5–8.0)	0.91 (0.82–0.96)
Mesenchymal tumours	12	See text	5.9 (1.5–64.6)	3.7 (0.8–14.9)	0.87 (0.69–0.95)
Different tumours	1	Ameloblastoma	19.7	1.1	0.69

coefficient was close to zero and was not significant ($p = 0.25$). Figure 5B presents $\log_e$ (LI) versus $\log_e$ (T_{pot}) for the combined canine and feline mesenchymal and epithelial tumours. Figure 5C presents the regression curve of $\log_e$ (MI) versus $\log_e$ (T_{pot}) for the combined canine and feline mesenchymal and epithelial tumours. The modest correlation was highly significant ($p < 0.0001$). Figure 5D presents the regression curve of $\log_e$ (MI) versus $\log_e$ (LI) for combined canine and feline mesenchymal and epithelial tumours. The modest correlation was also highly significant ($p < 0.0001$).

TABLE III
Histological class, histological type, number of tumours and mitotic index of tumours in dogs and cats

Tumour class	Tumour type	No.	Median mitotic index (range)
Canine epithelial tumours	Anal sac adenocarcinoma	6	22.5 (11–72)
	Breast carinoma	4	27.5 (3–51)
	Squamous cell carcinoma	3	39 (9–58)
	Sweat gland carcinoma	3	6 (2–37)
	Adenocarcinoma of the lung	2	35.5 (17–54)
	Carcinoma of the pancreatic duct	1	44
	Adenocarcinoma of the cystic duct	1	20
	Hepatocellular carcinoma	1	0
	Signet-ring cell carcinoma	1	5
	Sebaceous adenoma	1	2
	Perianal adenoma	1	0
	Trichoblastoma	1	5
Canine mesenchymal tumours	Mast cell tumour	11	0 (0–11)
	Fibrosarcoma	6	5.5 (0–119)
	Haemangiosarcoma	5	25 (5–69)
	Lymphosarcoma	3	41 (34–60)
	Lipoma	2	0 (0–0)
	Spindle cell sarcoma	2	59 (48–64)
	Haemangiopericytoma	1	55
Different canine tumours	Mixed mammary cancer	7	5 (0–11)
	Melanoma	4	3.5 (0–94)
	Epulis	4	6.5 (0–11)
	Insulinoma	1	6
	Mesothelioma	1	32
	Malpighioma	1	59
Feline epithelial tumours	Squamous cell carcinoma	5	36 (10–107)
	Mammary cancer	1	62
Feline mesenchymal tumours	Fibrosarcoma	9	25 (0–76)
	Spindle cell sarcoma	1	84
	Haemangioma	1	0
	Intestinal mast cell tumour	1	49
Different feline tumour	Ameloblastoma	1	0

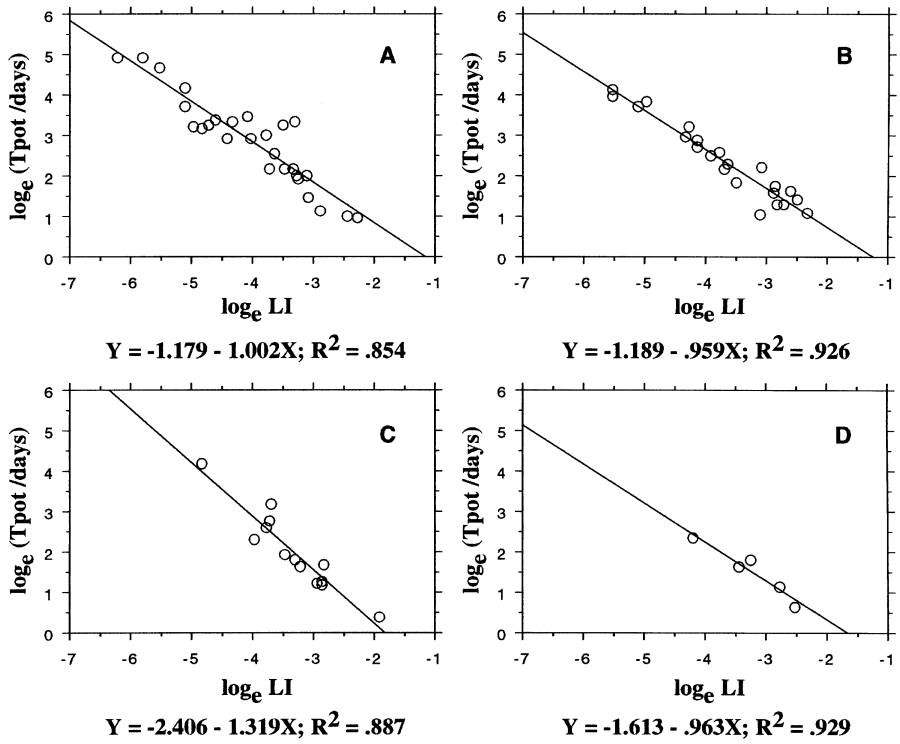

Figure 3. Regression analyses of $\log_e$ labelling index [log LI] versus $\log_e$ potential doubling time [$\log_e$ (T_{pot}/days)] for various small animal tumours. A, canine mesenchymal tumours; B, canine epithelial tumours; C, feline mesenchymal tumours; D, feline epithelial tumours. Labelling index is absolute, not a percentage. The formula for the regression curves and Pearson's correlation coefficients are given below the plots

DISCUSSION

Tumour-volume doubling time is an important clinical parameter but, unlike T_{pot}, it cannot be determined rapidly. T_{pot} is determined from the viable cells in a population; both quiescent and cycling cells. T_{pot} is more informative than tumour-cell cycle-time because it also takes into account the growth fraction, which is the proportion of cells that are actively dividing. However, at least three sources of potential difference between T_{pot} and tumour-volume doubling time exist. First, the contribution of cell loss through metastasis, exfoliation, cell death, etc., is not quantified by the T_{pot} method (Terry *et al.*, 1992). This can result in inappropriate T_{pot} values, especially for carcinomas, where cell loss contributes considerably to the tumour volume kinetics (Denekamp, 1970). Second, because T_{pot} measurements are based on the average of all

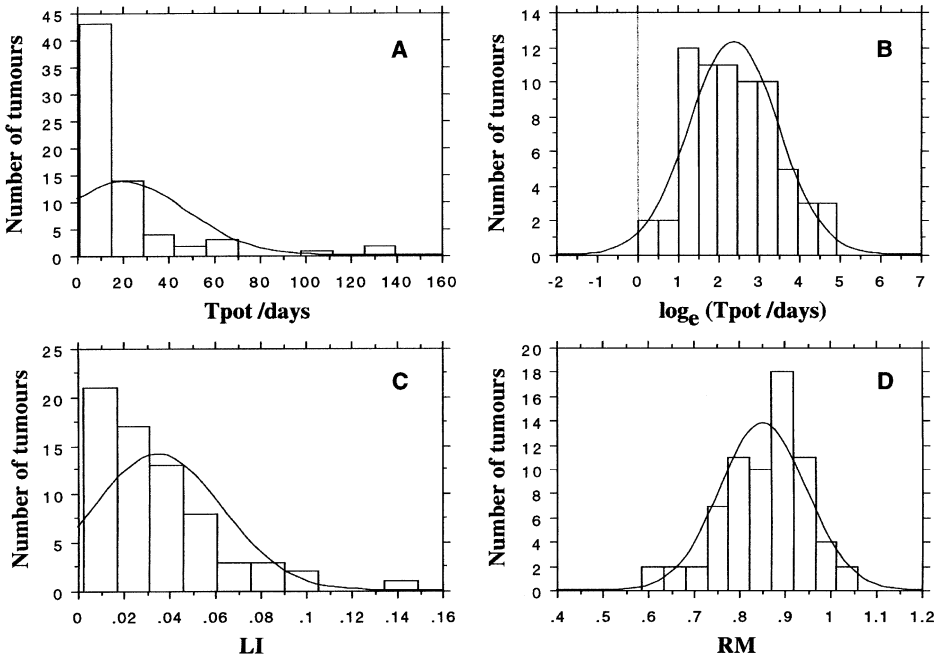

Figure 4. Histogram plots of various kinetic parameters from combined canine and feline mesenchymal and epithelial tumours. A, potential doubling time [T_{pot}/days]; B, $\log_e$ potential doubling time [$\log_e$ (T_{pot}/days)]; C, labelling index [LI]; D, relative movement [RM]. Labelling index is absolute, not a percentage. Relative movement is a dimensionless quantity. The continuous black line denotes the best fit of the data to a normal distribution

cells, this can be misleading when determined from tumours populated by only a small fraction of the tumour cells (clonogenic stem-cells). Third, heterogeneity in cell growth across the total tumour mass may contribute to biased T_{pot} values. Nevertheless, the potential doubling time expressed in days is an attractive prognostic parameter for the clinician. Furthermore, numerous authors have reported that there is less intratumour than intertumour variation, confirming that observed differences between T_{pot} values reflect actual differences in growth rate (Wilson *et al.*, 1988b; Giordano *et al.*, 1991; LaRue *et al.*, 1994). T_{pot} values have also been reported to be higher in metastases than in primary tumours (Holmgren *et al.*, 1995).

In the present study, all tumour types displayed a wide range of T_{pot} values, as previously reported by Fowler (1986). This emphasizes the need to determine the tumour kinetic parameters for each patient; no single tumour-characteristic value can be assumed. The T_{pot} values were log-normally distributed (see Figures 5A and 5B). This was also true of the LI and MI values. The tumours in the slow-growing 'tails' of

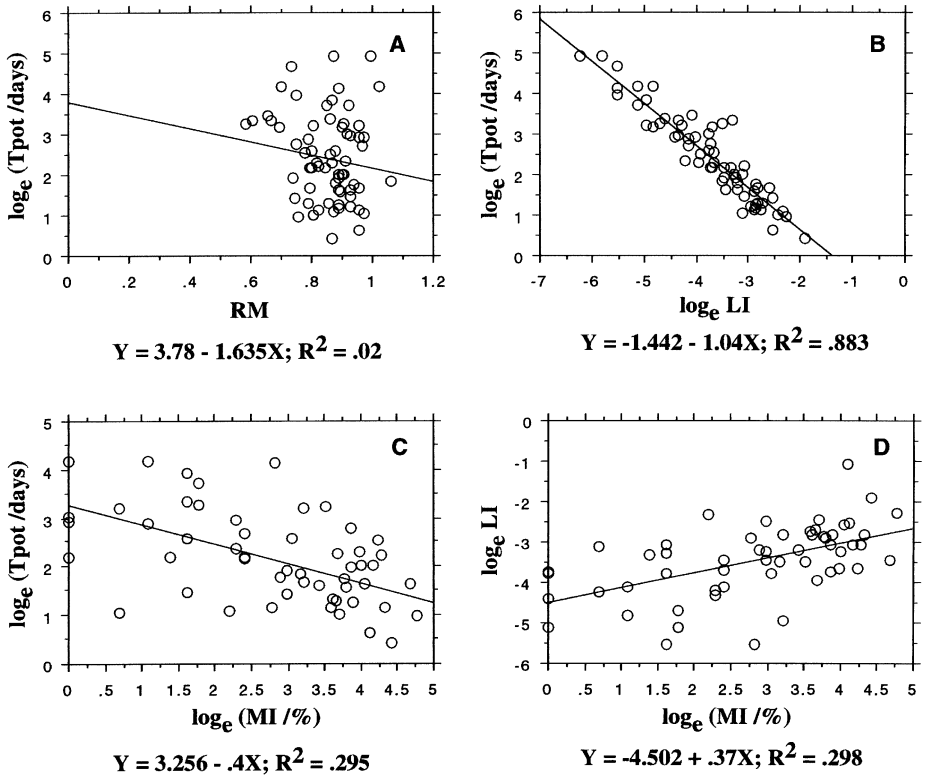

Figure 5. Regression analyses of various kinetic parameters from combined mesenchymal and epithelial tumours. A, relative movement [RM] versus $\log_e$ potential doubling time [$\log_e$ (T_{pot}/days)]; B, $\log_e$ labelling index [$\log_e$ LI] versus $\log_e$ potential doubling time [$\log_e$ (T_{pot}/days)]; C, $\log_e$ mitotic index [$\log_e$ (MI/%)] versus $\log_e$ potential doubling time [$\log_e$ (T_{pot}/days)]; D, $\log_e$ mitotic index [$\log_e$ (MI/%)] versus $\log_e$ labelling index [$\log_e$ LI]. Labelling index is absolute, not a percentage. Relative movement is a dimensionless quantity. The formula for the regression curves and Pearson's correlation coefficients are given below the plots

these distributions tend to represent the more radiotherapy-resistant tumours. Three experimental parameters contribute to T_{pot}: t, RM and LI. The average t value was 5.78 ± 1.87 h and was independent of tumour growth kinetics. The average RM was 0.85 ± 0.1, ranging from 0.59 to 1.06. T_{pot} is inversely proportional to 'RM – 0.5', which varied from 0.086 to 0.56, a 6.5-fold range. Compared to the range of LI values, the contribution of RM was negligible and no significant correlation with T_{pot} was observed (see Figure 5A). LI displayed a 75-fold range from 0.2% to 14.9% (average of combined data 3.5 ± 2.9%). The LI data were clearly skewed to small values: μ_3/

$\sigma = 1.4$ (see Figure 4C) and were log-normally distributed. The $\log_e$(LI) versus $\log_e$ (T_{pot}) regression plots for canine mesenchymal and epithelial tumours both displayed a distinct slope, with little scatter, indicating a high correlation (see Figures 3A and 3B). The same was true for feline mesenchymal and epithelial tumours (see Figures 3C and 3D). As LI is inversely proportional to T_{pot}, and because LI displayed a wide range of values, it was both expected and observed to be a strong predictor of T_{pot} (see Figure 5B). If T_{pot} is derived from LI data alone ($T_{pot} = 0.2365/\text{LI}$), the estimates of T_{pot} deviate from the experimental values by an average of only 28%.

The duration of the S-phase (T_S) was determined using t and RM values. The average T_S could also be derived from the slope of the $\log_e$ (LI) versus $\log_e$ (T_{pot}) regression curves, because the numerical value of the slope equals λT_S, and λ was fixed at 0.8 (Begg *et al.*, 1985). For canine mesenchymal tumours, T_S was 10.0 h; for canine epithelial tumours, 8.1 h; for feline mesenchymal tumours, 8.5 h; and for feline epithelial tumours, 5.4 h. This confirmed clinical observations indicating that epithelial tumours tend to grow more quickly than mesenchymal tumours. The T_S data also suggest that the S-phase in feline tumour cells is shorter than it is in canine tumour cells. Histological determination of MI and T_{pot} analysis were performed on the same biopsy sample by dividing the excised material into two small pieces. A modest but highly significant correlation between $\log_e$ (MI) and $\log_e$ (T_{pot}) and between $\log_e$ (MI) and $\log_e$ (LI) was observed (see Figures 5C and 5D). Significant correlations between these different tumour growth parameters were expected.

The method described in this study for evaluating tumour kinetic data using BrdU-labelled tumour cells is straightforward and reproducible. T_{pot} values were found to correlate well with the labelling index. In the future it may not be necessary to calculate T_{pot} using this method if adequate estimates based on labelling index can be made. It may even be possible to estimate labelling index noninvasively. This study demonstrated significant intertumour variation in the potential doubling time and re-emphasized the need for analysis of the data from individual patients. Determination of the kinetic growth of a tumour affords an additional prognostic step towards improved, patient-specific cancer therapy.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation: Grant 31-40482.94.

REFERENCES

Alanen, K.A., Klemi, P.J., Joensuu, H., Kujari, H. and Pekkala, E., 1989. Comparison of fresh, ethanol-preserved, and paraffin-embedded samples in DNA flow cytometry. *Cytometry*, **10**, 81–85

Becton Dickinson, 1989. Monoclonal antibodies detecting cell proliferation and activation. *Source Book Becton Dickinson Immunocytometry Systems, Mountain View, California, USA*, Section 2.80.1–3.80.2

Begg, A.C., 1989. Derivation of cell kinetic parameters from human tumours after labelling with bromodeoxyuridine or iododeoxyuridine. *British Journal of Radiology*, **19**, 113–119

Begg, A.C., McNally, N.J., Shrieve, D.C. and Kärcher, H., 1985. A method to measure the duration of the DNA synthesis and the potential doubling time from a single sample. *Cytometry*, **6**, 620–626

Carter, O.W., Robinson, J.P. and Narayanan, P.K., 1993. Flow cytometry: theory and clinical applications. *Proceedings of the American College of Veterinary Internal Medicine Forum*, **11**, 785–787

Cooke, L.D., Cooke, T.G., Forster, G., Jones, A.S. and Stell, P.M., 1994. Prospective evaluation of cell kinetics in head and neck squamous carcinoma: the relationship to tumour factors and survival. *British Journal of Cancer*, **69**, 717–720

Denekamp, J., 1970. The cellular proliferation kinetics of animal tumours. *Cancer Research*, **30**, 393–400

Dewhirst, M.W., LaRue, S.M. and Gerweck, L., 1995. Tumor physiology and cell kinetics. *Seminars in Veterinary Medicine and Surgery (Small Animal)*, **10**, 148–157

Dolbeare, F.A., Gratzner, H.G., Pallavicini, M.G. and Gray, J.W., 1983. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proceedings of the National Academy of Science of the USA*, **80**, 5573–5577

Dressler, L.G., Seamer, L., Owens, M.A., Clark, G.M. and McGuire, W.L., 1987. Evaluation of a modeling system for S-phase estimation in breast cancer by flow cytometry. *Cancer Research*, **47**, 5294–5302

Fowler, J.F., 1986. Potential for increasing the differential response between tumours and normal tissues: can proliferation rate be used? *International Journal of Radiation Oncology, Biology and Physiology*, **12**, 641–645

Gillette, E.L., 1982. Spontaneous canine neoplasms as models for therapeutic agents. In: I.J. Fidler and R.J. White (eds), *Design of Models for Testing Cancer Therapeutic Agents*, (Van Nostrand Reinhold), 185–192

Giordano, M., Riccardi, A., Danova, M., Brugnatelli, S. and Mazzini, G., 1991. Cell proliferation of human leukemia and solid tumors studied with *in vivo* bromodeoxyuridine and flow cytometry. *Cancer Detection and Prevention*, **15**, 391–396

Gratzner, H.G., 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science*, **218**, 474

Holmgren, L., O'Reilly, M.S. and Folkman, J., 1995. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Medicine*, **1**, 149–153

Hughes, W.L., Commerford, S.L., Gitlin, O., Krüger, R.C., Schultze, B., Shah, V. and Reilly, P., 1964. DNA metabolism *in vivo*: I. Cell proliferation and death as measured by incorporation and elimination of IUdR. *Federation Proceedings*, **23**, 640

Kinsella, T.J., Russo, A., Mitchell, J.B., Rowland, J., Jenkins, J., Schwade, J., Meyers, C.E., Colins, J.M., Speyer, J., Kornblith, P., Smith, B., Kufta, C. and Gladstein, E.A., 1984. A phase I study of intermittent bromodeoxyuridine with conventional fractionated irradiation. *International Journal of Radiation Biology and Physiology*, **10**, 69–76

LaRue, S.M., Fox, M.H., Withrow, S.J., Powers, B.E., Straw, R.C., Côté, I.M. and Gillette, E.L., 1994. Impact of heterogeneity in the predictive value of kinetic parameters in canine osteosarcoma. *Cancer Research*, **54**, 3916–3921

Mitchell, J.B., Kinsella, T.J., Russo, A., McPherson, S., Rowland, J., Kornblith, P. and Gladstein, E., 1983. Radiosensitization of hematopoietic precursor cells (CFUs) in glioblastoma patients receiving intermittent intravenous infusions of bromodeoxyuridine (BUdR). *International Journal of Radiation Oncology, Biology and Physiology*, **9**, 457–464

Owen, L.N., 1980. *TMN Classification of Tumours in Domestic Animals, ed. 1*. (World Health Organization, Geneva)

Popert, R.J.M., Joyce, D.J., Walmsley, B.H. and Coptcoat, M.J., 1993. Bromodeoxyuridine labelling of transitional cell carcinoma of the bladder – an index of recurrence? *British Journal of Urology*, **71**, 279–283

Remvikos, Y., Vielh, P., Padoy, E., Benyahia, B., Voillemot, N. and Magdelénat, H., 1991. Breast cancer proliferation measured on cytological samples: a study by flow cytometry of S-phase fractions and BrdU incorporation. *British Journal of Cancer*, **64**, 501–507

Rew, D.A., Wilson, G.D., Taylor, I. and Weaver, P.C., 1991. Proliferation characteristics of human colorectal carcinomas measured *in vivo*. *British Journal of Surgery*, **78**, 60–66

Rew, D.A., Campbell, I.D., Taylor, I. and Wilson, G.D., 1992. Proliferation indices of invasive breast carcinomas after *in vivo* 5-bromo-2′-deoxyuridine labelling: a flow cytometric study of 75 tumours. *British Journal of Surgery*, **79**, 335–339

Shimomatsuya, T., Tanigawa, N. and Muraoka, R., 1991. Proliferation activity of human tumors: assessment using bromodeoxyuridine and flow cytometry. *Journal of Cancer Research*, **82**, 357–362

Steel, G.G., 1977. *Growth Kinetics of Tumours: Cell Population Kinetics in Relation to the Growth and Treatment of Cancer*, (Clarendon Press, Oxford)

Schutte, B., Reynders, M.M.J., van Assche, C.L.M.V.J., Hupperets, P.S.G.J., Bosman, F.T. and Blijham, G.H., 1987. An improved method for the immunocytochemical detection of the bromodeoxyuridine labeled nuclei using flow cytometry. *Cytometry*, **8**, 372–376

Terry, N.H.A., White, R.A., Meistrich, M.L. and Calkins, D.P., 1991. Evaluation of flow cytometric methods for determining population potential doubling times using cultured cells. *Cytometry*, **12**, 234–241

Terry, N.H.A., White, R.A. and Meistrich, M.L., 1992. Cell kinetics: from tritiated thymidine to flow cytometry. *British Journal of Radiology*, **24**, 153–157

Vail, D.M., 1993. Analysis of tumor cell-cycle kinetics: a tool for predicting prognosis and treatment response. *Proceedings of the 11th ACVIM Forum*, 778–781

van Erp, P.E.J., Brons, P.P.T., Boezeman, J.B.M., de Jongh, G.J. and Bauer, F.W., 1988. A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation. *Cytometry*, **9**, 627–630

Vindeløv, L.L. and Christensen, I.J., 1990. A review of techniques and results obtained in one laboratory by an integrated system of methods designed for routine clinical flow cytometric DNA analysis. *Cytometry*, **11**, 753–770

White, R.A., Terry, N.H.A., Meistrich, M.L. and Calkins, D.P., 1990. Improved method for computing potential doubling time from flow cytometric data. *Cytometry*, **11** 314–317

Wilson, G.D., McNally, N.J., Dunphy, E., Karcher, H. and Pfagner, R., 1985. The labelling index of human and mouse tumors assessed by bromodeoxyuridine staining *in vitro* and *in vivo* and flow cytometry. *Cytometry*, **6**, 641–647

Wilson, G.D., McNally, N.J., Dische, S. and Bennett, M.H., 1988a. Cell proliferation in human tumours measured by *in-vivo* labelling with bromodeoxyuridine. *British Journal of Radiology*, **61**, 419–422

Wilson, G.D., McNally, N.J., Dische, S., Saunders, M.I., Des Rochers, C., Lewis, A.A. and Bennett, M.H., 1988b. Measurement of cell kinetics in human tumours *in vivo* using bromodeoxyuridine incorporation and flow cytometry. *British Journal of Cancer*, **58**, 423–431

(Accepted: 17 January 1998)