PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 229–233 (1980)

Osteosarcoma Associated Alkaline Phosphatase and *In Vivo* Growth and Development[1] (40853)

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Numerous different approaches have been taken to measure tumor cell kinetics. Many of the studies are directed at relating the susceptibility of the cells in cycle with the effectiveness of chemotherapy (1–5) or irradiation (6). Mathematical approaches not requiring cell cycle kinetic information to aid in the design of combination anticancer treatments are being developed as well (7). In the development of a relevant model, there are two major problems that must be overcome: (i) The growth of the tumor must be measured in a quantitative fashion *in vivo* in individual animals so that an accurate mathematical description of the growth can be derived; and (ii) The real cell cycle states of the tumor cell population at each stage of growth *in vivo* must be determined. If mathematical equations can be developed to describe the actual time related course of the growth of murine osteosarcoma (OS) *in vivo* and if the equations can be experimentally related to the real cell cycle kinetics taking place in the body, the model will be exceedingly useful for multiple modality treatment studies. Such equations can provide hypothetical information on the stage of growth of the tumor, i.e., whether maximum growth rate will be reached or the time when maximum tumor burden will take place in an individual, and also information on the distribution of the cells in the various states (G_0, G_1, S, G_2, and M) in the population at each stage of growth of the tumor. A murine OS has been used to investigate the first part of this problem. The purpose of the study has been to establish equations describing the natural growth of the murine osteosarcoma.

[1] This investigation was supported by Research Grants CA 16699 and CA 25965 from the National Cancer Institute, NIH.

Materials and methods. Determination of viable tumor cell numbers and total alkaline phosphatase (AP) levels in the circulation of tumor bearing mice was made in 6-week-old C_3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine) which were injected with 0.1 ml of 1 × 10^6 viable osteosarcoma cells subcutaneously. At different times during tumor growth (between the 13th and the 25th day), groups of three animals were sacrificed and the number of viable tumor cells per gram of tumor was measured for each mouse individually. Tumor was excised from the subcutaneous site of each animal. Any tumor nodules found in the lungs were carefully dissected free and combined with the subcutaneous tissue. The tumor mass was weighed, cut into small pieces, and dispersed with 10 ml 0.1% Pronase (EM Laboratories, Inc., Elmford, N.Y.) in tissue culture media (TC) 199 for 10 min at room temperature. The suspension was allowed to settle; the supernatant containing single cells was collected and centrifuged; then the cells were washed in TC 199 with 10% FCS. Pronase dispersion of the tumor cell mince was repeated twice more. The weight of undigested tumor and connective tissue was subtracted from that of the original tumor to give the weight of tumor cells released by enzyme digestion. The total number of viable tumor cells released was determined by cell counts and the representative number of tumor cells per gram tissue was established.

The total AP activity per mouse was made by measuring the activity in the plasma. Plasma (0.5 to 5.0 μl) from each animal was added to 1.0 ml *p*-nitrophenyl phosphate disodium salt (1.0 mg/ml) in substrate buffer (0.05 *M* Na_2CO_3 and 0.05 *M* $MgCl_2$; pH 9.8) and incubated at 37°C for 30 min. The reaction was stopped with the addition of 0.1 ml 1 *N* NaOH. The increase in

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absorbence with time is linear up to approximately 1 optical density unit. A control containing 5.0 μl plasma in 1.0 ml substrate buffer was run simultaneously. The samples were read at 400 nm in a Zeiss spectrophotometer. The total blood volume of the animal was calculated by multiplying the weight of the animal $\times$ 5.5 ml/100 g (8). Using the hematocrit value and the blood volume, the total circulating plasma AP per mouse in OD units was determined. The optical density units can be converted to the international units/l by multiplying with a factor 3.6×10^{-3}.

In a different experiment, five mice were injected sc with 1×10^6 OS tumor cells. The animals were monitored every 5 days for tumor growth by caliper measurements of the tumor's largest diameter and by determination of alkaline phosphatase levels in the plasma. Five control animals were also bled and tested individually to establish background levels of plasma AP per mouse. Background levels ranged between 34.3 and 68.5 optical density units in 106 individual test bleedings.

Results. The quantitative relationship between total number of viable tumor cells and total AP activity per mouse for 17 individual determinations (each point represents a single animal) is shown in Fig. 1. A linear relationship was observed between the number of viable tumor cells that could be recovered from the animal's body and the ln AP. A least square regression line through the experimental points provided the following equation $C = \alpha + \gamma \ln A$ where α = intercept, γ = slope, and A = total AP/mouse. With this equation, C, the tumor cell number could be estimated from AP determinations. The intercept was -483.5×10^6 (SE; 19.5×10^6; $P < 0.0001$) and the slope was 124.3×10^6 (SE; 3.71×10^6; $P < 0.0001$). The regression equation accounted for 98.7% of the total variation of the experiment.

In the second experiment, AP, a tumor-associated enzyme marker, was monitored in an attempt to estimate the *in vivo* tumor burden of C3H mice. Mice were injected sc with OS cells on Day 0. All animals were monitored for AP activity at intervals of 5 days for the first 35 days of the experiment. For each mouse, the AP values increased with the enlargement of sc tumor as measured by calipers (Tables I and II).

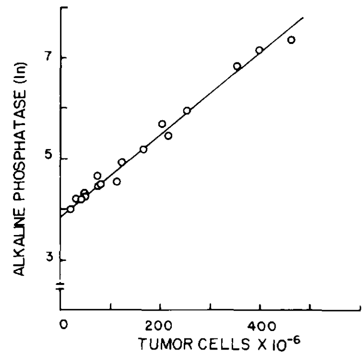

FIG. 1. Plot of the total alkaline phosphatase OD units/mouse versus the total number of viable tumor cells isolated from the animal. $C = -483.5 \times 10^6 + 124.3 \times 10^6 \ln A$.

Using the experimentally derived equation $C = -483 + 124 \ln A$, tumor cell numbers were estimated for each mouse (Table III). This average tumor cell number and the average AP level plotted against time showed an initial slow growth period which may be due to a lag period of growth of the transplanted neoplasm, a high turnover of AP, or the slow synthesis of AP as cells were divided more slowly. After 10 days, there was a period of relatively rapid growth of tumor up to 25 days, followed by a slowing down of tumor growth. The average tumor cell numbers, tumor size measurement by caliper, and AP levels increased with time giving a sigmoidal curve.

The equation relating viable tumor numbers and AP was derived

$$C = \alpha + \gamma \ln A \qquad [1]$$

Equation [1] suggests a plausible family of hypotheses in terms of the following bivariate system of differential equations.

$$\dot{C} = dC(t)/dt = \gamma_1 \phi(C,A,t), \qquad [2a]$$
$$\dot{A} = dA(t)/dt = \gamma_2 A \phi(C,A,t), \qquad [2b]$$

where γ_1 and γ_2 are proportionally constants, $\gamma_1/\gamma_2 = \gamma$, and $\phi(C,A,t)$ is a function of C, A, and t.

If the tumor growth in mice is assumed to follow the logistic growth law, i.e., $\phi(C,A,t)$

TABLE I. TIME-RELATED INCREASE OF ALKALINE PHOSPHATASE LEVELS IN INDIVIDUAL MICE WITH SUBCUTANEOUS IMPLANT OF OSTEOSARCOMA

Mouse No.	Units of alkaline phosphatase/mouse,[a] Days							
	5	10	15	20	25	30	35	43
1	63.8	49.6	144.0	438.6	926.0	1823.0	1577.0	1982.0
2	50.0	65.9	126.9	429.1	968.0	1039.0	[b]	
3	73.4	64.2	117.2	334.3	1116.0	1292.0	2035.8	2827.0
4	58.8	65.3	123.6	478.9	1430.7	1718.0	[b]	
5	65.1	67.9	136.0	372.4	1016.6	1147.0	1054.9	1490.0

[a] Mice were bled on the days reported and the plasma was assayed for the alkaline phosphatase activity as described under Materials and Methods.
[b] Mouse was dead prior to the assay date.

$= C(\beta\text{-}C)$ and $\gamma_1 = 4\delta/\beta^2$ in [2a] and [2b] then they become

$$dC(t)/dt = 4\delta C(\beta\text{-}C)/\beta^2, \quad [3a]$$

$$dA(t)/dt = \gamma_2 AC(\beta\text{-}C) = \gamma_2\beta 2AC/4\delta, \quad [3b]$$

where β = maximum size of tumor, and δ = maximum rate of tumor growth. The solution of [3a] and [3b] for the growth of tumor and alkaline phosphatase level is

$$C(t) = \beta/(1+\exp(-4\delta(t-\tau)/\beta)) \quad [4]$$

and

$$A(t) = A_0 \exp(k_1)/(1+\exp(-k_2t+k_3)), \quad [5]$$

where β = maximum size of tumor; δ = maximum rate of tumor growth; τ = time at which the tumor was half grown, and also the time at which the growth rate was at maximum; and A_0 = a constant. Equation 4 described the time-dependent increase of OS cells [$C(t)$]. Equation 5 described the time-dependent increase of AP [A(t)] in tumor-bearing animals. To define the tumor growth equations for the OS model, certain constants occurred in the growth curve and must be estimated. From these experiments, these were: $\hat{\beta}$, maximum tumor size, $=430.33 \times 10^6$; $\hat{\delta}$, maximum rate of tumor growth, $=31.229 \times 10^6$ cells/day; and, $\hat{\tau}$, time at which the tumor was at half its size and also the time at which the growth rate of the tumor is at its maximum, =18.3 days. For the time-dependent increase of AP, the background level of AP was given as $A(_0) = 56.37$ for these experiments. The essential constants to define the curve were calculated and were: $\hat{A}_0 = 56.37$; $k_1 = 3.33$, $k_2 = 0.326$, and $k_3 = 6.08$. The two equations define the entire course of growth of the neoplasm *in vivo*.

The experimental results for number of tumor cells versus time was compared with the calculated expected results using the above equations with the substituted constants (Fig. 2). Similarly, the time-dependent change of the experimentally determined AP activity was compared with the expected activity, using the experimentally derived constants (Fig. 3). As may be seen for both figures, the equations expressed

TABLE II. TIME-RELATED GROWTH OF TUMOR SIZE OF INDIVIDUAL MICE BEARING SUBCUTANEOUS IMPLANT OF OSTEOSARCOMA

Mouse No.	Tumor size (mm), Days						
	10	15	20	25	30	35	43
1	2.86	5.75	8.42	11.95	12.98	14.15	18.00
2	3.14	5.76	10.23	11.97	13.67	[a]	
3	3.03	5.55	9.02	12.03	12.82	13.50	17.78
4	4.11	8.03	9.22	11.21	14.32	[a]	
5	4.50	6.90	9.37	11.92	13.37	14.51	17.00

[a] Mouse was dead prior to the assay date.

TABLE III. TOTAL NUMBER OF TUMOR CELLS PER MOUSE POST-TRANSPLANTATION

Mouse No.	Tumor cells/mouse × 10^6,[a] Days						
	5	10	15	20	25	30	35
1	32		133	271	364	448	430
2	2	36	117	268	369	378	[b]
3	49	33	107	237	387	405	461
4	22	35	114	282	417	440	[b]
5	35	40	126	251	375	390	380

[a] Calculated with equation $C = \alpha + \gamma \ln A$, where C is the number of tumor cells × 10^6, α and γ are constants, and A is alkaline phosphatase units per mouse.
[b] Mouse was dead prior to the assay date.

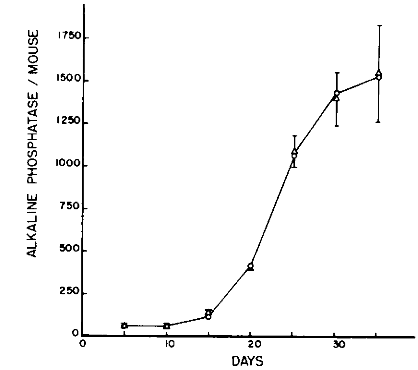

FIG. 3. Total alkaline phosphatase OD units/mouse versus days. Experimentally determined values/mouse (△) and calculated values using Eq. 5 (○).

the entire course of tumor growth and AP activity with time. The experimental points for both tumor cell numbers and AP showed good fit with $R^2 = 0.9972$ and $R^2 = 0.9991$, respectively.

Discussion. Computer simulation models of complex biological systems, are rapidly being recognized as valid and necessary tools in research (9–12). Until recently, utilization of the modeling capabilities of computers in cancer research has resulted in two relatively separate areas of development: (i) probabilistic models to simulate active cell growth based on discrete cell cycle phases and times (13–15); and (ii) continuous system models that describe cell growth in general or in terms of mathematical equations (1, 16–18).

Our investigations apply quantitative techniques to measure the growth of a murine OS tumor. The experimental measurement of tumor growth led to the formulation of equations which describe the entire natural growth of the tumor. The equations allow one to predict the AP or numbers of tumor cells for an animal with OS for any time interval based on a few AP measurements. While these relationships have been clearly established by these studies, the description by the equations of growth cannot reflect the events of a true biological model. In the biological model, all dividing cells follow a cyclic pattern which includes mitosis, G_1, S, G_2, and G_0 phases. Nevertheless, the mathematical definition of the growth of the tumor *in vivo* is the first step required for developing a kinetic model in which the actual numbers of tumor cells growing *in vivo* can be related to the numbers of cells in the different phases of the cell cycle.

In this study, individual mice given 1 × 10^6 tumor cells sc have been monitored for increased tumor size by caliper measurements and elevated AP levels with time. From AP measurements, the number of viable tumor cells in individual animals have been calculated for any instant of time, using an experimentally derived time independent equation, $C = \alpha + \gamma \ln A$.

Every measure of tumor growth applied (average tumor cell numbers, tumor size measurements by caliper and AP levels over time) has shown the growth curve as sigmoidal. The equations describing the growth of the murine OS have depended on assessing specific experimental growth constants. These have been defined as β,

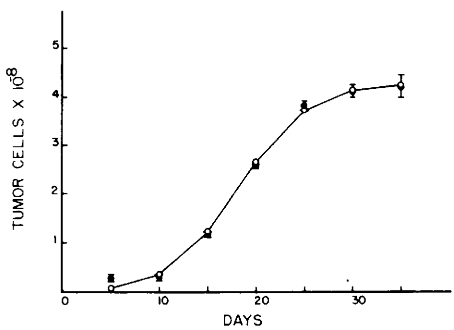

FIG. 2. Plot of total number of tumor cells/mouse × 10^{-8} versus days. Experimentally obtained values (●), tumor cell numbers obtained with the Eq. 4 (○).

maximum tumor size; δ, maximum rate of tumor growth; and τ, time at which the tumor is at half its size and also when the growth rate is maximum. While the equations have given an accurate measure of the increase in the number of cells *in vivo* with time, the cell cycle kinetics at each stage of the murine OS growth remains to be established by flow cytometry measurements (19, 20). The present equations however permit: (i) calculation for tumor cell numbers in individual animals at any instant of time based on experimental AP measurements and (ii) predictions to be made of the course of growth of the neoplasm.

The model gives the natural course of growth and is useful in predicting certain tumor growth parameters, AP or cell numbers, in a mouse with osteosarcoma. Potentially, with only two or three measurements of the AP levels in an individual mouse, irrespective of when or how many tumor cells were injected, we can locate the stage of growth of the tumor on the expected growth curve and its presumed course of growth. We can determine whether maximum growth rate has been reached or when it may be reached, and estimate the interval to maximum tumor burden and death. The predictive aspect of the OS growth model will be extremely useful if it can also be applied to the growth of metastasized lung tumors after the primary tumor has been removed. This area is currently being investigated.

Summary. Experimental methods and mathematical relationships were devised based on quantitative estimation of number of *in vivo* osteosarcoma cells and alkaline phosphatase levels from individual animals during the natural course of growth of the tumor implant. A least square regression plot of the experimental values gave the equation $C = \alpha + \gamma \ln A$ where α = intercept, γ = slope, A = total alkaline phosphatase/mouse, and C = the number of tumor cells/mouse. The average tumor cell number and the increase in alkaline phosphatase are time-related events and were expressed by two equations which then permitted estimation of the growth of the murine osteosarcoma before the event had taken place.

1. Simpson-Herren, L., and Lloyd, H. H., Cancer Chem. Rep. **54**, 143 (1970).
2. Skipper, H. E., Schabel, F. M., Mellett, L. B., Montgomery, J. A., Wilkoff, L. J., Lloyd, H. H., and Brockman, R. W., Cancer Chem. Rep. **54**, 431 (1970).
3. Hayes, F. A., and Mauer, H. M., J. Nat. Cancer Inst. **57**, 697 (1976).
4. Barlogie, B., Drewinko, B., Benjamin, R. S., and Freireich, E. J., Cancer Res. **36**, 2450 (1976).
5. Krishan, A., Pitman, S. W., Tattersall, M. H., Paik, K. D., Smith, D. C., and Frie, E., Cancer Res. **36**, 3813 (1976).
6. Steel, G. G., and Peckham, M. J., Int. J. Radiat. Oncol. Biol. Phys. **5**, 85 (1979).
7. Norton, L., and Simon, R. M., Clin. Res. **24**, 462A (1976).
8. Hiramoto, R. N., Ghanta, V. K., and Hamlin, N. M., J. Immunol. **111**, 1546 (1973).
9. Bell, G. I., J. Theor. Biol. **29**, 191 (1970).
10. Bell, G. I., J. Theor. Biol. **33**, 339 (1971).
11. Bell, G. I., J. Theor. Biol. **33**, 379 (1971).
12. Bischoff, K. B., Dedrick, R. L., Zaharko, D. S., and Longstreth, J. A., J. Pharm. Sci. **60**, 128 (1971).
13. Donaghey, C. E., *in* "Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells" (B. Drewinko *et al.*, eds.). Wilkins & Wilkins, New York (1977).
14. Schackney, S. E., Cancer Chem. Rep. **54**, 399 (1970).
15. Robinow, S. I., "Cold Spring Harbor Cell Proliferation 5," Book A, p. 528, Cold Spring Harbor Laboratory of Quantitative Biology, New York (1978).
16. Laird, A. K., Growth **29**, 249 (1965).
17. Hege, J. S., and Cole, L. J., J. Immunol. **97**, 34 (1966).
18. Sullivan, P. W., and Salmon, S., J. Clin. Invest. **51**, 697 (1972).
19. Darzynkiewicz, Z., Traganos, F., Andreef, M., Sharpless, T., and Melamed, M. R., J. Histochem. Cytochem. **27**, 478 (1979).
20. Steinkamp, J. A., Orlicky, D. A., and Crissman, H. A., J. Histochem. Cytochem. **27**, 273 (1979).

Received October 1, 1979. P.S.E.B.M. 1980, Vol. 164.