[CANCER RESEARCH 29, 2334–2338, December 1969]

The Improvement of the Animal Tumor Model[1]

Daniel E. Bergsagel and Chan H. Park

Department of Medicine, University of Toronto, and the Ontario Cancer Institute, Toronto, Ontario, Canada

Summary

It is clear that the tumor model, which has been used to explain tumor growth and to guide the design of new treatment schedules, needs to be improved. The study of tumor models requires the use of direct assays so that the validity of the hypothesis suggested by the model can be tested. The assays which have been used to study tumor stem cells are reviewed, and some preliminary results of attempts to study these cells in cell culture are presented. The existence of special classes of tumor stem cells, such as nonproliferating cells, and tumor stem cells which have an increased capacity to reproduce themselves and a reduced tendency to differentiate are postulated. The use of physical methods to study tumor cells is suggested as possible methods for detecting and studying special classes of tumor stem cells.

I have been very concerned about the title of this talk, for a review of the tumor models used to explain the kinetics of tumor growth and to guide chemotherapy has revealed that our knowledge of this subject is incomplete. Important new discoveries must be made before the working models now in use can be improved. In the absence of this new knowledge, I have decided to discuss the methods which have been used to study the effects of treatment on tumors and to indicate the important need for improved assay technics.

Some of the methods used to assess the effects of treatment on tumors have been listed in Table 1. Tumor size changes have been useful for detecting the antitumor activity of chemical agents, but this measurement provides inaccurate or misleading information for the comparison of the effectiveness of different agents or dosage schedules (9, 14). Within the last two decades it has become clear that tumors contain heterogeneous populations of cells (2, 5, 8). The most important class of cells are the tumor stem cells which have the extensive proliferative capacity required for tumor regeneration. This class of cell has also been referred to as *competent*, *viable*, and *malignant*. The important properties of tumor stem cells are that they have the ability to reproduce themselves and the proliferative capacity to form colonies and to regenerate the tumor. The transplantation of a single, viable, tumor stem cell results in the growth of a tumor which leads to the death of the animal (4, 13, 16).

Table 1

1. Tumor size
2. Tumor stem cells
a. Limiting dilution
b. Median survival time
c. Colony formation
(i) Spleen
(ii) Lung
(iii) Cell culture
3. Required: Assays for special classes of tumor stem cells such as the following hypothetical types:
a. Nonproliferating tumor stem cells
b. Tumor stem cells with a reduced tendency to differentiate
c. Others.

Tumor assay methods.

Tumor Stem Cell Assays

Several methods for assaying tumor stem cells are listed in Table 1.

The limiting dilution assay, introduced by Hewitt (5, 6), was the first quantitative method for measuring tumor stem cells. This technic is also the most sensitive of the experimental assays for these cells. In this assay dilutions of tumor cell suspensions are transplanted, and the frequencies of tumor induction in the recipients are determined. If the data fit a Poisson distribution, the application of the Poisson distribution law would imply that the dilution with 37% failures would contain an average of one tumor stem cell in the volume administered to each recipient. That is, with the injection of a set volume of this dilution into 100 animals, one would expect that 37 animals would receive no tumor stem cells (failures) and 63 would receive one or more.

The determination of the numbers of tumor stem cells present in single cell suspensions of various tumors by the limiting dilution assay, shown in Table 2, has shown that some tumors are very heterogeneous. In the rapidly growing mouse leukemias and lymphomas, from 33 to 50% of the cells are tumor stem cells with the proliferative capacity to kill the host. Tumors with slower growth rates are more heterogeneous, for only one in 100 to one in 10,000 of the cells from these tumors have the proliferative capacity of tumor stem cells. The figures shown in Table 2 have been determined in different laboratories by investigators who have used

[1] This work was supported by a grant from the Ontario Cancer Treatment and Research Foundation.

Table 2

Mouse tumor (route of injection)	No. of tumor cells required to induce tumors in 63% of assay mice	Reference
A. Rapid growth rate		
1. L1210 (i.p.)	2	13
2. CBA lymphocytic leukemia (i.p.)	3	7
3. Leukemia P388 (i.p.)	3	12
B. Slower growth rate		
1. Osteosarcoma C22LR (s.c.)	400–3000	19
2. Shinogi Ca 115 (s.c.)	approx. 1000	*a*
3. Ca 755 (i.c.)	approx. 5000	9
4. Adj. PC-5 myeloma (i.v.)	1000–4000	2

Limiting dilution assays for tumor stem cells of mouse tumors. i.c., intracerebral.

[a] J. W. Meakin, 1968, personal communication.

different technics. For example, tumor cells have been injected into multiple subcutaneous sites, intracerebrally, intraperitoneally, or intravenously, and positive results have been read as the development of a palpable tumor or the death of the animal. Some investigators (6, 12, 19; J. W. Meakin, 1968, personal communication) have been careful to relate the frequency of tumor induction to the number of tumor cells judged to be viable by morphologic criteria, while others (2, 9) have related the frequency of tumor takes to the total number of nucleated cells in the cell suspension. Bergsagel and Valeriote (2) found that approximately 63% of the animals injected with 10^4 nucleated cells prepared from the spleens of animals bearing advanced Adj. PC-5 myeloma died with active tumor. The cells of this plasma cell tumor are all larger than 12 μ, and from 10 to 40% of the cells in the suspensions used for these experiments were identified as tumor cells in the hemocytometer chamber. Thus, the frequency of stem cells in the population of Adj. PC-5 tumor cells recognized by morphologic criteria is in the range of one in 1,000 to one in 4,000.

Technical differences in these limiting dilution assays make direct comparisons of the results hazardous. The most important factors which will influence the frequency of stem cells detected in a tumor cell suspension are the methods used to prepare the cell suspension and the criteria used to identify "viable" tumor cells in the injected dilutions. However, the results shown in Table 2 were obtained by investigators who prepared tumor cell suspensions under conditions that were designed to keep tumor cells viable. The tumors were minced under aseptic conditions, the cells were suspended in a protein-containing medium and kept cold throughout the *in vitro* manipulations, and similar criteria were used to identify morphologically intact tumor cells (i.e., large cells which exclude vital dyes such as trypan blue and eosin). It is possible that improved methods of handling tumor cells may increase the proportion of tumor stem cells detected in the cell suspensions of slowly growing tumors, but it is unlikely that the frequency can be increased to that found in the rapidly growing leukemias and lymphomas.

It would appear that only a fraction of the tumor cells, judged to be viable on the basis of their ability to exclude vital dyes, have the extensive proliferative capacity required for the formation of a tumor. This is strong evidence for the view that tumors contain at least two types of cells, i.e., tumor stem cells, and a second type of tumor cell which has a limited proliferative potential. The tumor stem cell fraction in slowly growing tumors is smaller than in tumors with a rapid growth rate.

The determination of the median survival time of mice following therapy, or following the transplantation of cell dilutions containing unknown numbers of tumor stem cells, has been used extensively by investigators at the Southern Research Institute (9, 16). This measurement will give an order-of-magnitude estimate of the surviving fraction of tumor stem cells if the tumor is one which causes death when the number of tumor cells have multiplied to a fixed lethal number. Some tumors do not meet this necessary growth condition. Hewitt (6) was unable to demonstrate a good correlation between the mean tumor stem cell dose and the survival time of mice injected intraperitoneally with a transplantable CBA lymphocytic leukemia. After treating spontaneous AKR mouse lymphoma with cytotoxic agents, Dr. Bruce has found that tumor stem cells regenerate rapidly until they reach the pretreatment level and then remain stable for prolonged periods; death occurs at variable times after the number of tumor stem cells reaches a stable value (W. R. Bruce, personal communication). In tumors with these growth characteristics, the median survival time of mice cannot be used to assay the surviving fraction of tumor stem cells.

Assays for tumor cells with the proliferative capacity to form transplantable tumor colonies are more direct assays for tumor stem cells. Many tumors form colonies in the spleen following intravenous inoculation (2, 3, 21), and this assay has provided much useful information. Other tumors may form lung colonies. Williams and Till (20) demonstrated a linear relationship between the number of polyoma-transformed rat embryo cells injected into the tail veins of weanling rats and the numbers of lung surface colonies at 16–17 days. Hill and Bush (7) have also used this assay for the tumor stem cells of a transplanted mouse sarcoma.

Some tumor stem cells will form colonies in cell culture when they are grown under special conditions. The colony shown in Fig. 1 is formed by Adj. PC-5 mouse myeloma cells grown in semisolid agar over a feeder layer of mouse kidney tubules for 28 days with the daily addition of fresh medium. This colony contains approximately 10^4 cells. Metacentric marker chromosomes characteristic of the tumor are found in the metaphases of the cells in these colonies, and the transplantation of colonies such as this back into BALB/c mice initiates the growth of a plasma cell tumor which produces the same myeloma protein as the original tumor (C. Park and D. E. Bergsagel, unpublished data). The relationship obtained between the number of tumor colonies formed per plate and the number of tumor cells planted, shown in Chart 1, is not quite linear. If the cultural conditions can be improved to the point where a linear relationship is obtained, this technic will form a useful method for assaying the stem cells of this mouse plasma cell tumor. At the Ontario Cancer Institute, Drs. Meakin and Kumar (J. W. Meakin and R. S. Kumar, personal

communication) have also had some success in getting the Shinogi carcinoma 115 to form tumor colonies in cell culture. These cell culture methods have the promise of being useful in the study of human tumors.

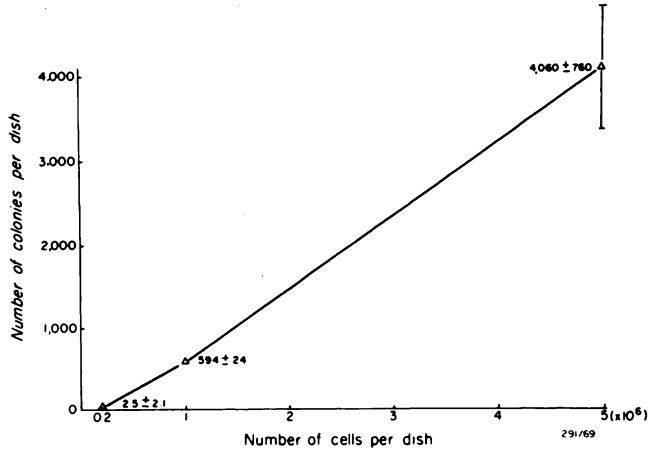

Chart 1. This is a plot of the number of Adj. PC-5 tumor colonies per Petri dish containing more than 50 cells on Day 11 *versus* the total number of cells planted per dish. The figures shown for each point represent the mean and standard deviation of the numbers of tumor colonies found in four dishes.

Special Classes of Tumor Stem Cells

The development of assays for tumor stem cells and their use in evaluating the effectiveness of chemotherapy has permitted investigators to design rational treatment regimens for eliminating tumor stem cells from advanced animal tumors (9, 13, 17, 18). If the use of these assays leads to the development of cures for all animal and human tumors there will be no need to improve the animal tumor model. It has been the failure of effective chemotherapeutic agents to cure some animal tumors which has led investigators to postulate that there may be a population of nonproliferating tumor stem cells; these form the fraction which is resistant to our best agents. Compartment B of Skipper's model (15) was created for these cells. However, it must be recognized that this special class of tumor stem cell is still hypothetical, for the evidence which suggests its existence is indirect and open to several interpretations. It is also possible that there may be a special class of tumor stem cell which has a great potential for reproducing itself and a small probability of differentiating and losing some of this proliferative potential. A special population of hemopoietic stem cells with these properties has been found in mice (R. G. Worton, E. A. McCulloch, and J. E. Till. Physical Separation of Hemopoietic Stem Cells Differing in Their Capacity for Self-renewal. Submitted to J. Exptl. Med.) It is difficult to visualize direct assays for these hypothetical special classes of tumor stem cells. However, it may be possible to separate different classes of tumor stem cells on the basis of their physical properties. Some of the physical methods for the characterization of cell populations are listed in Table 3. The development of methods for separating different classes of tumor stem cells which will permit us to assay them and to study their properties should lead to an improvement in the animal tumor model.

Table 3

Technic	Physical principle	Cell property determining selection
Density gradient centrifugation (10)	Sedimentation equilibrium	Cell density
"Staput" sedimentation (11)	Sedimentation velocity	Cell density X radius^2
Counter current separation (1)	Partition between selected pairs of media	Cell membrane properties
Pulse-height analysis[a]	Multichannel analyzer coupled with Coulter counter	Cell volume

Physical methods for the characterization of cell populations.
[a] R. G. Miller, personal communication.

REFERENCES

1. Albertsson, P. A., and Baird, G. D. Counter-Current Distribution of Cells. Exptl. Cell Res., *28:* 296–322, 1962.
2. Bergsagel, D. E., and Valeriote, F. A. Growth Characteristics of a Mouse Plasma Cell Tumor. Cancer Res., *28:* 2187–2196, 1968.
3. Bruce, W. R., and Van der Gaag, H. A Quantitative Assay for the Number of Murine Lymphoma Cells Capable of Proliferation *in Vivo.* Nature, *199:* 79–80, 1963.
4. Furth, J., and Kahn, M. C. The Transmission of Leukemia of Mice with a Single Cell. Am. J. Cancer, *31:* 276–282, 1937.
5. Hewitt, H. B. Studies of the Quantitative Transplantation of Mouse Sarcoma. Brit. J. Cancer, *7:* 367–392, 1953.
6. Hewitt, H. B. Studies of the Dissemination and Quantitative Transplantation of a Lymphocytic Leukemia of CBA Mice. Brit. J. Cancer, *12:* 378–401, 1958.
7. Hill, R. P., and Bush, R. S. A Lung Colony Assay for Determining the Cellular Radiosensitivity of a Solid Tumour. Internatl. J. Radiation Biol., in press.
8. Lasnitzki, I. The Behavior of Ascites Tumour Cells in Vivo and in Tissue Culture. J. Pathol. Bacteriol., *64:* 252–253, 1952.
9. Laster, W. R., Mayo, J. G., Simpson-Herren, L., Griswold, D. P., Jr., Lloyd, H. H., Schabel, F. M., Jr., and Skipper, H. E. Success and Failure in the Treatment of Solid Tumors. II. Kinetic Parameters and "Cell Cure" of Moderately Advanced Carcinoma 755. Cancer Chemotherapy Rept., in press.
10. Leif, R. C., and Vinograd, J. The Distribution of Buoyant Density of Human Erythrocytes in Bovine Albumin Solutions. Proc. Natl. Acad. Sci. U. S., *51:* 520–528, 1964.
11. Peterson, E. A., and Evans, W. H. Separation of Bone Marrow Cells by Sedimentation at Unit Gravity. Nature, *214:* 824–825, 1967.
12. Porter, E. H., and Berry, R. J. The Efficient Design of Transplantable Tumor Assays. Brit. J. Cancer, *17:* 583–595, 1963.
13. Schabel, F. M., Jr. *In Vivo* Leukemic Cell Kinetics and "Curability" in Experimental Systems. *In:* The Proliferation and Spread of Neoplastic Cells, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, pp. 379–408. Baltimore: The Williams & Wilkins Co., 1968.
14. Skipper, H. E. Kinetic Considerations Associated with Therapy of Solid Tumors. *In:* The Proliferation and Spread of Neoplastic Cells,

The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, pp. 213–233. Baltimore: The Williams & Wilkins Co., 1968.

15. Skipper, H. E. Improvement of the Model Systems. Cancer Res., *29:* 2329–2333, 1969.
16. Skipper, H. E., Schabel, F. M., Jr., and Wilcox, W. S. Experimental Evaluation of Potential Anticancer Agents. XIII. On the Criteria and Kinetics Associated with "Curability of Experimental Leukemia." Cancer Chemotherapy Rept. *35:* 1–111, 1964.
17. Skipper, H. E., Schabel, F. M., Jr., and Wilcox, W. S. Experimental Evaluation of Potential Anticancer Agents. XXI. Scheduling of Arabinosylcytosine to Take Advantage of Its S-Phase Specificity against Leukemia Cells. Cancer Chemotherapy Rept. *51:* 125–165, 1967.
18. Valeriote, F. A., Bruce, W. R., and Meeker, B. E. Synergistic Action of Cyclophosphamide and 1,3-Bis(2-chloroethyl)-1-nitrosourea on a Transplanted murine lymphoma J. Natl. Cancer Inst., *40:* 935–944, 1968.
19. Van Putten, L. M. Tumor Reoxygenation during Fractionated Radiotherapy; Studies with a Transplantable Mouse Osteosarcoma. Europ. J. Cancer, *4:* 173–182, 1968.
20. Williams, J. F., and Till, J. E. Formation of Lung Colonies by Polyoma-transformed Rat Embryo Cells. J. Natl. Cancer Inst., *37:* 177–184, 1966.
21. Wodinsky, I., Swinarski, J., and Kensler, C. J. Spleen Colony Studies of Leukemia L1210. I. Growth Kinetics of Lymphocytic L1210 Cells *in Vivo* as Determined by Spleen Colony Assay. Cancer Chemotherapy Rept., *51:* 415–421, 1967.

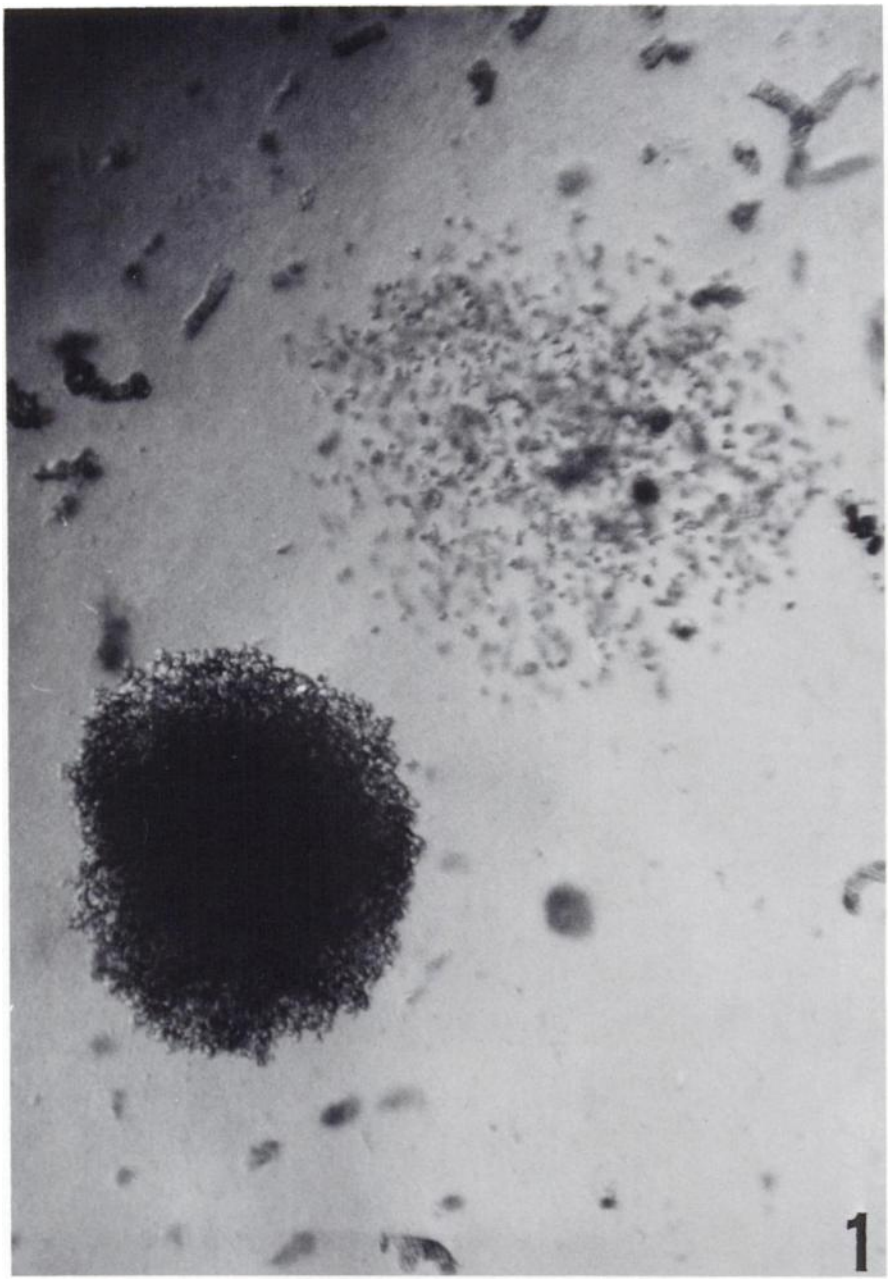

Fig 1. Adj. PC-5 plasma cell tumor colony in semisolid agar. A single cell suspension of a BALB/c mouse spleen infiltrated with Adj. PC-5 tumor cells was prepared, and 10^6 cells were planted in semisolid agar over a solid agar feeder layer containing mouse kidney tubules. The cell culture was fed by the daily addition of fresh medium. This photograph was taken on Day 28. The tumor cells grow over one another to form a colony which is several cells thick. At this stage the tumor colonies contain at least 10^4 cells. The diffuse colony formed by a single layer of cells is composed of normal cells which resemble macrophages.