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Growth Characteristics of a Mouse Plasma Cell Tumor[1]

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SUMMARY

A quantitative spleen-colony assay was developed and used to evaluate the growth characteristics of the Adj. PC-5 colony-forming units (CFU). There was a positive correlation between the number of colonies per spleen and the number of tumor cells injected, which is compatible with the assumption that each colony arises from one malignant cell. At 21 days, each colony contains $8.4 \pm 2.1 \times 10^7$ cells, but less than 4.4% of these are tumor stem cells with the proliferative capacity to form a colony.

The growth rate of tumor CFU was estimated in the spleen (CFU T_D = 20 ± 4 hours) and femoral marrow (CFU T_D = 29 ± 5 hours). A survival T_D of 36 ± 2 hours was estimated from the survival of mice injected with graded numbers of tumor cells as an indication of the growth rate of the total tumor.

The proliferative capacity of the tumor CFU was completely suppressed during a 10-hour exposure to vinblastine *in vivo*. This result suggests that the majority of tumor CFU have a T_G of less than 10 hours. All of the tumor CFU appear to be in cell cycle, for no detectable tumor CFU survived a 10-hour exposure to vinblastine, and 70% of these cells were killed by a 20-minute exposure to tritiated thymidine *in vitro*.

Based on these findings, a model is proposed for the growth kinetics of this tumor. The model states that a small fraction of the tumor population is composed of cells in a short generation cycle with unlimited proliferative capacity (i.e., tumor stem cells). The remaining tumor cells lack the proliferative capacity to form a colony. This model for the Adj. PC-5 tumor is compared with that of a transplantable AKR lymphoma, and the chemotherapeutic implications are discussed.

INTRODUCTION

The kinetics of tumor cell proliferation have an important influence on the sensitivity of a tumor to many chemotherapeutic agents. Quantitative studies of the killing of hematopoietic and tumor stem cells by chemotherapeutic agents have shown that the selective killing of tumor cells by many agents depends, to a great extent, on their proliferative state (4). Populations of proliferating cells, such as are present in a transplanted AKR lymphoma and in regenerating marrow, are much more sensitive to the toxic effects of many chemotherapeutic agents than are resting, nonproliferating cells in the G_0 state (13), such as the hemopoietic stem cells of untreated mice (2, 6, 23). The transplanted tumors used in these investigations have been of the rapidly growing, undifferentiated type, in which the majority of tumor cells were proliferating and the cell generation time was short (5, 7, 24).

In order to obtain a more general view of the mechanism of the action of chemotherapeutic agents, it is important to evaluate the effects of these agents on a wide spectrum of tumors and to relate the effectiveness of the agents to the growth characteristics of the tumor. Thus, if the conclusions drawn from the studies outlined above are generally applicable, one would predict that agents which are selectively toxic for rapidly proliferating cells would be less effective in the treatment of a tumor which grows slowly because the tumor stem cells have a long generation time, or if a significant proportion of these cells are in G_0. On the other hand, a tumor growing slowly because the growth fraction (15) is small and all of the tumor stem cells are in rapid cell cycle should be very sensitive to agents that are selectively toxic for proliferating cells.

As a prelude to the investigation of the effects of chemotherapeutic agents on slowly growing tumors, we have studied the growth characteristics of the mouse plasma cell tumor Adj. PC-5 (20). This tumor grows relatively slowly and differentiates to produce a specific myeloma protein (19).

In this paper we describe a quantitative assay for the stem cells of the Adj. PC-5 plasma cell tumor. This assay is similar to that used for the transplanted AKR lymphoma (9) and depends on the fact that colonies of tumor cells are formed in the spleens of BALB/c mice following the injection of appropriate numbers of tumor cells intravenously. The tumor cells which lodge and grow in the spleen and possess the extensive proliferative capacity required for the formation of macroscopic colonies are referred to as colony-forming units (CFU). The growth characteristics of the mouse plasma cell tumor, Adj. PC-5, were determined using this assay. The tumor was found to grow slowly because a small proportion of the tumor cells are stem cells with extensive proliferative capacity. The tumor stem cells appear to have a short generation time, and none were detected to be resting in the G_0 state.

MATERIALS AND METHODS

Mice. Six- to eight-week-old, female, BALB/c mice from the Jackson Laboratory, Bar Harbor, Maine, were used for these studies. They were kept in groups of five to ten to a cage and allowed Rockland Mouse Diet (Tekland Inc., Monmouth, Ill.) and water as desired.

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Transplanted Cell Line. The Adj. PC-5 plasma cell tumor used in these studies was induced by incomplete Freund's adjuvant and heat-killed staphlococci and isolated by Dr. Michael Potter from a BALB/c mouse (20). Dr. Potter had carried this tumor line in the ascitic form for 97 transplant generations when we received it. We transplanted these ascitic tumor cells intravenously into isologous recipients and found that some of these animals died with marked hepatosplenomegaly and plasma cell infiltration throughout the marrow, spleen, liver, and other tissues. This tumor line was then passaged at weekly intervals by the intravenous injection of 10^7 cells harvested from the spleens of animals given similar injections 17 to 21 days earlier. Cells from the 2nd to 31st intravenous transplant generations were used for the following investigations.

Preparation of Cell Suspensions. Cell suspensions were prepared from the spleens and femoral marrows of mice previously injected with tumor cells. The spleens were minced finely with scissors, washed through a fine wire mesh screen, and suspended in CMRL-1066 (18). The femora were cleaned, their ends cut off, and, with an appropriate syringe and needle, the marrow cavity was repeatedly aspirated with CMRL-1066. Samples of pooled cell suspensions from five to ten animals were diluted, and 500 to 2000 cells were counted in a hemocytometer. The initial cell suspensions were diluted in CMRL-1066 to achieve the concentration required for the subsequent injections. All suspensions were kept at ice-water temperature before use. The number of tumor cells in the spleen and femoral marrow cell suspensions was not determined by differential counts. Throughout this paper the number of "tumor cells" injected refers to the number of hemocytometer-counted cells in a spleen or femoral marrow cell suspension obtained from a tumor-bearing mouse. These suspensions contained large, polypoid tumor cells, but normal spleen and marrow cells are also included in the hemocytometer counts. At 16 days after the intravenous injection of 10^7 tumor cells, the spleens contained, on the average, 7×10^8 cells. The number of cells per femur remained relatively constant at between 1 and 4×10^7 cells following the intravenous injection of 10^7 tumor cells, but the proportion of large, polyploid plasma cells increased.

Histology and Hematology. Histologic examinations of the organs studied were made after fixing in Bouin's fixative and 70% ethanol and staining with hematoxylin and eosin. Blood smears and organ imprints were stained with Wright's stain.

Incorporation of Tritiated Thymidine *in Vitro*. Thymidine-methyl-^{3}H (TdR-^{3}H) in sterile water (specific activity 17,100 mc/mmole, 1.0 mc/ml) was obtained from the Radiochemical Centre, Amersham, England. Tritiated water (^3H_2O) at 1 c/ml was obtained from the New England Nuclear Corporation, Boston, Mass. These solutions were made isotonic by the addition of an appropriate amount of NaCl. The desired amount of TdR-^{3}H of ^3H_2O was pipetted into a 50-ml centrifuge tube. To this was added 4×10^7 tumor cells from a spleen suspension prepared with CMRL-1066 lacking thymidine. The initial concentration of tumor cells was adjusted so that the total volume in each tube was 2.0 ml. Two controls were used. As a control against loss of CFU through incubation alone, 4×10^7 tumor cells were incubated in 2.0 ml CMRL-1066 lacking thymidine. To evaluate any effect due to the irradiation of cells by radioisotope in the medium, a second control was used in which 600 μc ^3H_2O were added to 4×10^7 tumor cells in CMRL-1066 lacking thymidine. Incorporation of TdR-^{3}H was achieved by incubating the cell suspensions in a water bath at 37°C for 20 minutes with frequent, gentle agitation of the centrifuge tubes by hand. The controls were incubated in the same manner. The incorporation of the isotope was then stopped by the addition of 18.0 ml of cold (4°C) CMRL-1066 containing 10 mg/liter of unlabeled thymidine. The suspensions were then assayed for CFU by the intravenous injection of 0.5 to 2×10^5 cells in 0.5 ml into assay animals.

Vinblastine. Vinblastine sulphate (obtained from Lilly Pharmaceutical Co., Toronto) was dissolved in 0.15 M phosphate-buffered saline. The required dose (0.5 mg) was injected intraperitoneally as a single dose.

RESULTS

Spleen Colony Assay for Tumor Colony-forming Units

The spleen colony assay has proven to be a useful tool for studying the growth characteristics of hemopoietic stem cells (2, 3, 6, 7, 23), the tumor stem cells of the transplanted AKR lymphoma (4, 5, 7, 9, 14) and L1210 leukemia (26) in mice. Hemopoietic spleen colonies have been shown to arise from single hemopoietic stem cells with extensive proliferative capacity (3), and quantitative assays were developed to measure the survival of hemopoietic and malignant stem cells following treatment with chemotherapeutic agents (4).

Experiments were first designed to see if such spleen colonies could be obtained with cells from the plasma cell tumor. We found that colonies composed of large, polyploid plasma cells were formed in the spleens of recipient mice following the injection of cells from the tumor. This finding indicated that it was possible to develop a quantitative assay for these tumor cells.

In the following experiments, we describe the assay developed for the Adj. PC-5 plasma cell tumor and its validity for the quantitative assay of tumor stem cells with extensive proliferative capacity. A suspension of cells was prepared from the femoral marrow of tumor-bearing mice, and graded numbers of these cells were injected intravenously into isologous recipient mice. When fewer than 3×10^5 tumor cells were injected, discrete, macroscopic nodules were found in the spleens of animals killed 20 to 22 days later. The appearances of the colonies following fixation of the spleens in Bouin's solution are shown in Fig. 1. The colonies became confluent when larger numbers of cells were injected. Microscopically, the discrete nodules appeared as colonies of large, immature plasma cells with prominent nucleoli.

For this assay procedure to be quantitative, however, it is necessary that the number of colonies per spleen be directly related to the number of tumor cells injected into the recipient mice. To test if this was the case, a cell suspension was first prepared from the femoral marrow of tumor-bearing mice. The concentration of cells in this initial suspension was determined by counting in a hemocytometer, and graded numbers of these

cells were injected intravenously into groups of 15 recipient mice. Four separate experiments were done. The average number of colonies per spleen obtained from these mice is shown in Chart 1. The number of colonies per spleen was found to increase linearly with the number of cells injected over the range of 2.5×10^4 to 3.0×10^5 cells. A straight line was fitted through these data by the least squares method, and the 95% confidence interval on the ordinate was found to include zero. This linearity is compatible with the assumption that each of the colonies arises from one malignant cell (11). The injection of approximately 3×10^4 cells resulted in the formation of a mean of one colony per spleen.

These results indicate that this procedure can be used to determine the number of CFU in a cell suspension. The above assay procedure can be applied to determine the effects of chemotherapeutic agents on this tumor cell population. The results of such a study will be reported in subsequent publications. In this paper we have used the spleen colony assay as a tool for the investigation of the growth characteristics of this tumor.

Fraction of Tumor Colony-forming Cells Lodging in the Spleen

The CFU content of a cell suspension is a measure of the number of cells with the proliferative capacity required for the formation of a colony, but it does not determine the absolute number of these cells in a cell suspension, for only a fraction of the injected cells lodge in the spleen. The following experiment was done to determine the fraction of tumor stem cells present in the spleen 24 hours after injection. A group of 10 recipient mice was injected intravenously with 1.8×10^7 tumor cells/mouse. These mice were killed 24 hours later, and the spleens were removed, pooled, and minced. The cells were washed through a fine wire mesh with 10 ml of CMRL 1066 and were counted by hemocytometer. A group of 30 assay mice were then injected intravenously with 2×10^7 recipient spleen cells/assay mouse. When these mice were killed 21 days later, a total of 3 colonies were found in the 30 assay spleens. The figures used to estimate the fraction value of 0.002 are shown in Table 1.

Table 1

CFU injected (1 CFU/3 × 10^4 tumor cells)/ recipient mouse	6.0×10^2
Total cells recovered/recipient spleen (average)	2.26×10^8
Number of recipient spleen cells injected into each assay mouse	2.0×10^7
Colonies/assay spleen (mean ± S.E.)	0.1 ± 0.06
CFU/recipient spleen	1.13
CFU per recipient spleen/CFU injected (fraction)	0.002

Fraction of colony-forming units (CFU) recovered at 24 hours from the spleens of mice injected intravenously with 1.8×10^7 tumor cells.

An attempt was also made to determine the fraction value at 2 hours, but no CFU were detected in the spleen cell suspensions of recipient mice killed 2 hours after the injection of 2×10^7 tumor cells.

The Composition of Tumor Spleen Colonies at 21 Days

Ten well-separated colonies were dissected from the spleens of mice injected 21 days earlier with 3×10^3 to 3×10^4 tumor cells intravenously. These colonies were individually minced and washed through a fine wire mesh into 10 ml of

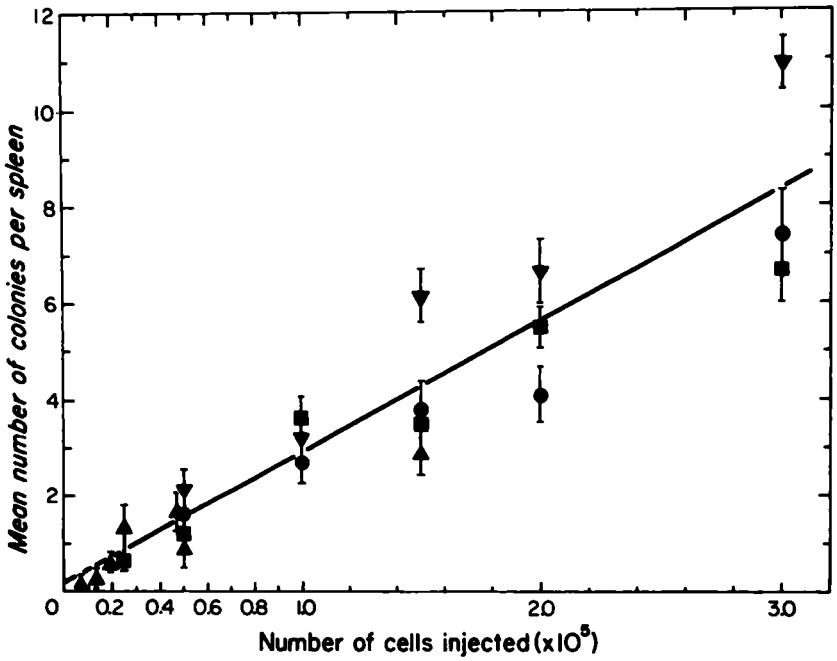

Chart 1. The mean number of colonies per spleen is shown as a function of the number of cells injected. Confidence intervals represent one standard error of the mean.

CMRL 1066. The numbers of cells in these suspensions were determined by hemocytometer counting, and the concentration of CFU was determined. The procedure and the results of this experiment are shown in Table 2.

Table 2

Colony	Cells/colony ($\times 10^7$)	CFU/colony ($\times 10^3$)	Tumor stem cells/colony ($\times 10^6$)
1	7.6	<69.0	
2	4.8	4.8	2.40
3	6.4	12.8	6.40
4	10.0	<10.0	
5	10.0	<10.0	
6	10.0	1.7	0.83
7	8.0	<1.6	
8	12.0	8.0	4.00
9	6.4	11.5	5.80
10	8.4	3.4	1.68
Mean ± S.D.	8.4 ± 2.1	7.02 ± 4.11	3.52 ± 2.07

The composition of tumor spleen colonies at 21 days. CFU, colony-forming units. Groups of 10 mice were injected intravenously with 3×10^3 and 3×10^4 tumor cells. These mice were killed 21 days later and 10-well demarcated colonies were dissected from the spleens. These colonies were individually minced and washed through a fine wire mesh into 10 ml of CMRL 1066. Hemocytometer counts were done on this cell suspension. The total number of cells per colony are shown in the second column. From 1 to 5×10^3 cells from each colony were then injected into 10 assay mice for the determination of the number of CFU in the cell suspension. The number of CFU/colony, shown in the third column, was estimated from the average colonies/assay spleen and the total cells/colony. No spleen colonies were found in the assay spleens for Colonies 1, 4, 5, and 7; the CFU/colony in these spleens were less than the figures shown, which represent what the values would have been if one spleen colony had been detected in each group of assay spleens. The total number of tumor stem cells/colony, shown in the fourth column, was estimated by multiplying the CFU/colony by one/f (f = the fraction of tumor stem cells which locate and grow in the spleen = 0.002).

These colonies contained a mean total of $8.4 \pm 2.1 \times 10^7$ cells and $7.02 \pm 4.11 \times 10^3$ tumor CFU. If the fraction value of 0.002 is used, a mean total of $3.52 \pm 2.07 \times 10^6$ tumor stem cells per colony is estimated, representative of only 4.4% of the total number of cells in the colony.

Growth of Adj. PC-5 in the Spleens and Femurs of Tumor-bearing Mice

The spleen colony assay was then used to determine the growth rate of tumor CFU in the spleens and femurs of tumor-bearing mice. Mice were injected with 2×10^7 cells (approximately 1.5×10^3 tumor CFU) obtained from spleen suspensions of tumor-bearing mice. Groups of 5 to 10 recipient mice were then killed from 2 hours to 17 days later. Cell suspensions were prepared from the spleens and femurs of each group and assayed for their content of tumor CFU. The number of CFU in each organ increased with time so that it was necessary to inject fewer cells into the assay mice at longer periods of time.

The data for the growth of tumor CFU in the spleen from a typical experiment are shown in Table 3, and the results for both spleen and femoral marrow are shown graphically in Chart 2*a* and 2*b* respectively. The results of two separate experiments are shown. With the cell numbers used, no tumor CFU were detected in the spleen or femoral marrow cell suspensions prepared at 2 hours, or in the femoral suspension prepared at 2 days after transplantation. Tumor CFU were detected in the spleen on the second day and in the femoral marrow on the fourth day after transplantation. The number of tumor CFU in these organs then increased exponentially. The increase in tumor CFU in both of these organs was fitted to an exponential curve by the least squares method.

Table 3

Time after injection	Cells/recipient spleen	Number of cells injected into assay mice	Mean number of colonies/"assay" spleen	CFU/recipient spleen (± S.E.)
2 hours	2.1×10^8	5×10^7	0	<0.5
2 days	2.2×10^8	5×10^7	0.1	0.5 (± 0.5)
4 days	2.6×10^8	2×10^7	0.1	1.5 (± 1.5)
8 days	3.4×10^8	4×10^6	1.5	$1.3 (\pm 0.3) \times 10^2$
12 days	6.1×10^8	4×10^5	6.0	$1.0 (\pm 0.1) \times 10^4$
16 days	6.7×10^8	2×10^5	9.3	$3.1 (\pm 0.4) \times 10^4$

Number of tumor colony-forming units (CFU) recoverable from the spleen 2 hours to 16 days after the intravenous injection of 2×10^7 cells (1.2×10^3 tumor CFU).

During the course of these experiments, the cellularity of the spleens of the recipient animals increased greatly (see Table 3); the mean number of cells recovered per recipient spleen increased from 2×10^8 at two hours to 7×10^8 at 16 days. Between the second and sixteenth days, the doubling time of the tumor CFU in the spleen was found to be 20 ± 4 hours. The mean number of cells recovered per recipient femur did not change significantly during the course of the experiment, being 3×10^7 at two hours and 1×10^7 on the sixteenth day. The doubling time for the tumor CFU in the femoral marrow was found to be 29 ± 5 hours. The error terms shown here were obtained by calculating the 95% confidence limits of the slopes of these curves.

The difference in the doubling time for tumor CFU in the spleen and femoral marrow may reflect solely the anatomical difference between the organs. The spleen is an expansible organ, whereas the femur is relatively constrained. This is shown by the changes in the cellularity of these organs during the growth of the tumor cell population. While the spleen expands in volume to accommodate the increased number of tumor cells, it seems most likely that in the case of the femoral marrow, a fraction of the tumor cell population must continually migrate from this organ, and possibly some of these cells migrate to the spleen. Because of this, we have assumed that the true doubling time for the tumor CFU is somewhere between 20 and 29 hours and have chosen, arbitrarily, a value of 25 hours.

Survival of Mice following the Intravenous Injection of Graded Numbers of Tumor Cells

The analysis of the median survival times of mice injected with graded numbers of tumor cells provides a rapid method

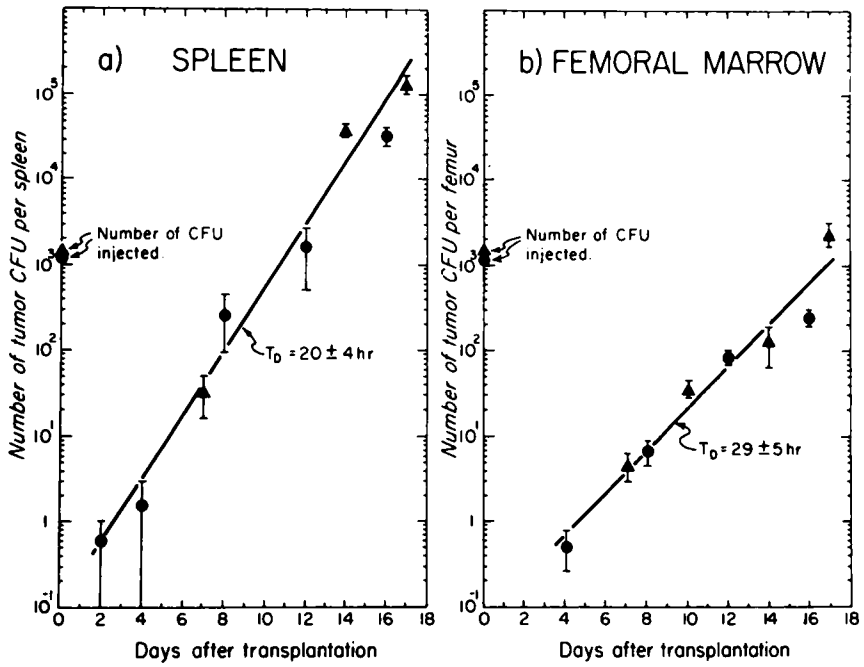

Chart 2. The number of tumor colony-forming units (CFU) in (*a*) the spleen and (*b*) the femoral marrow of tumor-bearing mice as a function of time after the administration of 2×10^7 tumor cells (containing about 1.5×10^3 tumor CFU). The results of two experiments for each organ are shown. Confidence intervals shown represent one standard error of the mean. The error term on the doubling time is obtained from the 95% confidence limits on the slopes of these curves.

for determining the *in vivo* doubling time (survival T_D) of a tumor cell population. This method has been used previously for mouse lymphomas and leukemias (5, 14, 20, 26). Unfortunately there are many assumptions implicit in this method, such as: (*a*) no lag phase in the growth of the tumor cells; (*b*) an exponential growth in the number of tumor cells in the population throughout the growth phase; and (*c*) the recipient animals die when the number of tumor cells reaches some critical value. However, the T_D obtained from such a determination does give a rough estimation of the growth rate, and we have used this method to provide additional data for the relatively long T_D for tumor CFU found by the spleen colony assay.

To determine the survival T_D of Adj. PC-5 cells in recipient mice, graded numbers of tumor cells, obtained from the spleens of tumor-bearing mice, were injected intravenously into groups of animals, and the time to death of these recipients was measured. The groups were given either 10^3, 10^4, 10^5, 10^6, or 10^7 cells. The survival of the animals was followed for 90 days. The survival data of several experiments have been pooled and are shown in Chart 3. The median day of death of the animals in each group is plotted as a function of the cell number injected. Of the animals given 10^5, 10^4, 10^3 cells, 96, 58, and 15% of the respective groups died. The median survival time of only those animals that died is represented in Chart 3. The data were fitted to a straight line by the least squares method. This survival curve was characterized by a 36-hour decrease in survival for each doubling of the number of cells injected. Dead animals were examined grossly, and preparations of spleen, liver, and femoral marrow

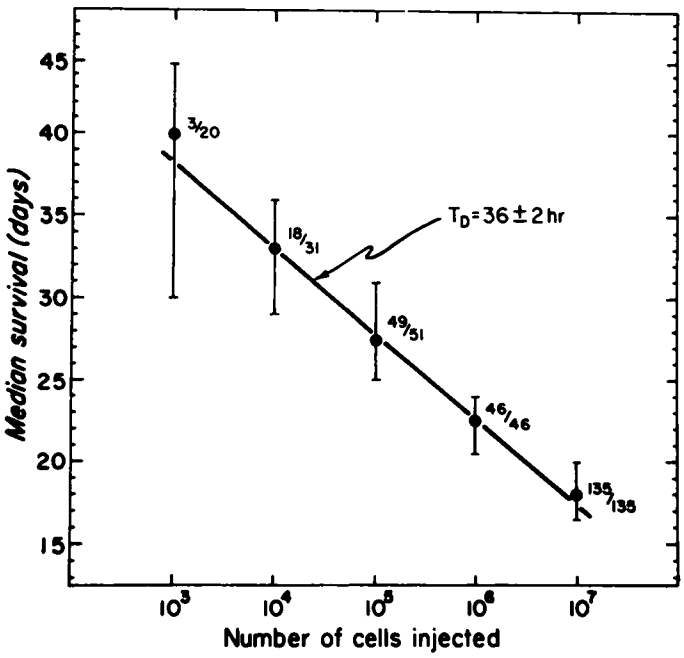

Chart 3. Median survival time of mice dying following the injection of graded doses of tumor cells. For mice injected with 10^4 to 10^7 cells, the confidence intervals shown represent limits of the second and third quartile (25% to 75% survival). The range of the first and last day of death is shown for mice injected with 10^3 cells. The fractions shown are the ratio of the total number of animals dead to the total number injected.

were made for microscopic examination. All animals that died had gross hepatosplenomegaly caused by the proliferation of large plasma cells. Hematomas frequently formed in the spleen, and occasionally these ruptured intraperitoneally. Plasma cell infiltration was widespread, and occasionally hind limb paralysis was noted shortly before death. The results again show that the tumor cell population which we are investigating has a relatively long doubling time.

Sensitivity of Tumor CFU to Vinblastine

The growth rate of the plasma cell tumor CFU in the spleen and femur was found to be relatively slow, with a doubling time (T_D) in the order of 25 hours. This, of course, does not mean that the generation time (T_G) of these cells is also long, for the loss of proliferative capacity by the tumor cells through cellular death or differentiation would result in the T_D being much larger than the T_G. We therefore determined the sensitivity of the tumor CFU to varying periods of exposure to the drug vinblastine.

Vinblastine has been shown to kill cells during the mitotic phase (and possibly late G2) of the cell cycle (10). This observation suggested that this drug might be used for the estimation T_G, for the surviving fraction of cells capable of forming colonies exposed to effective concentrations of vinblastine should fall to zero in the time required for a complete cycle. The time required for the surviving fraction of L-cells, *in vitro* (10) and AKR lymphoma cells *in vivo* (24) to fall to zero provided T_G estimates which were compatible with the times anticipated for these cells. Valeriote *et al.* (24) found that the intraperitoneal injection of 0.05 mg of vinblastine/mouse was sufficient to maintain the drug concentration above the critical level required to destroy the proliferative capacity of AKR lymphoma cells throughout the generation cycle. The reduction in the surviving fraction of lymphoma CFU was independent of dose in the range 0.05 to 0.5 mg of vinblastine/mouse.

Mice were injected intravenously with 10^7 cells from the spleens of tumor-bearing mice, and, 16 days later, 0.5 mg of vinblastine/mouse was injected intraperitoneally. The number of tumor CFU/femur was determined in groups of untreated animals and in treated groups killed from one to ten hours after the administration of vinblastine. Cells were obtained from the femurs of groups of five animals, pooled, counted, and diluted appropriately for the determination of the CFU content. The surviving fractions of tumor CFU/femur at one to ten hours after vinblastine administration are shown for three separate experiments in Chart 4. The average tumor CFU content of the femurs of untreated animals was 167 and decreased rapidly to about 2 CFU/femur at nine hours. No tumor CFU could be detected in the femoral marrow at 10 hours following vinblastine administration. The total cell count of the femurs taken 1 to 10 hours after treatment with vinblastine did not change significantly.

Thus, 98 percent of the tumor CFU are killed during a nine-hour exposure to vinblastine. This result suggests that the majority of tumor CFU have a T_G of less than nine hours. Also, since the proliferative capacity of the tumor CFU is completely suppressed within 10 hours, it appears that the majority of these cells are in cell cycle and few, if any, are in a resting (G_0) state. The concave shape of the curve is quite different from the slightly convex survival curve for AKR lymphoma CFU under the same experimental conditions (24). The shape of these survival curves will be considered in the discussion.

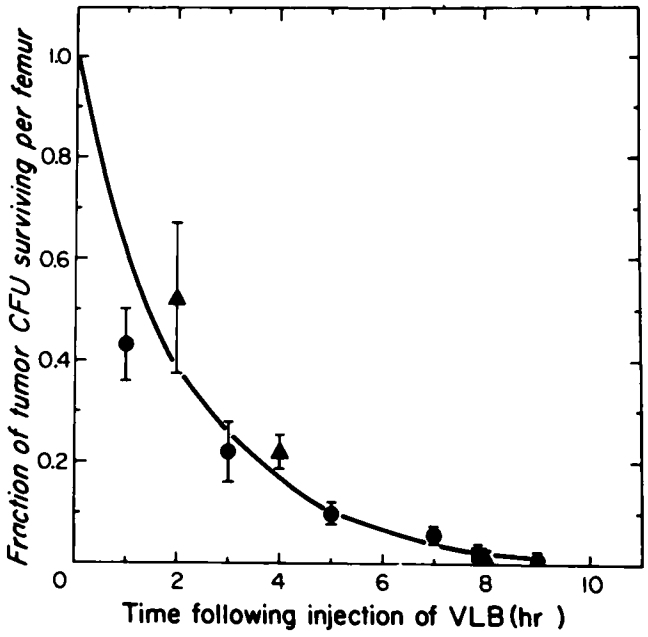

Chart 4. The fraction of tumor colony-forming units (CFU) surviving in the femoral marrow as a function of time following the administration of 0.5 mg VLB/mouse. Confidence intervals shown represent one standard error of the mean. VLB, vinblastine.

Determination of the Proportion of Tumor CFU in the S-Phase of the Cell Cycle

The preceding results suggested that most of the tumor CFU were in cell cycle. If this were so, one would expect to find a large fraction of these cells in the S-phase of the cell cycle, since this phase usually occupies a large part of the cell generation time (1). Therefore, the proportion of tumor CFU in the S-phase was determined quantitatively to confirm the finding that the majority of tumor CFU are in cell cycle. The method used for this investigation was the TdR-^{3}H suicide technic (2, 25).

The proportion of tumor CFU in the S-phase of the cell cycle was determined by incubating suspensions of these cells for 20 minutes in CMRL-1066 lacking thymidine, but containing from 0 to 600 μc TdR-^{3}H/ml, and measuring the surviving fraction of tumor CFU. The intranuclear radiation from the TdR-^{3}H incorporated into DNA of cells during the S-phase results in a loss of their proliferative capacity (25). The cells that form colonies are those which did not incorporate TdR-^{3}H, that is, were not in the S-phase during the 20-minute exposure. It is known that the survival of resting cells, such as normal hemopoietic stem cells, is unaffected by exposure to TdR-^{3}H (2). To rule out the effect of ^{3}H activity external to the cell, a control cell suspension was incubated in the presence of 600 μc ^3H_2O/ml.

The results of this experiment are shown in Table 4. With TdR-^{3}H activity of 100 μc/ml or greater, the fractional survival of tumor CFU was reduced to a constant value of about 0.30. Thus, approximately 30 percent of the tumor CFU did not incorporate sufficient TdR-^{3}H to cause a loss of their colony-forming ability, and similarly, at least 70 percent of these cells were in S-phase during the 20-minute incubation period *in vitro*. Thus, the majority of the tumor CFU are in the cell cycle.

DISCUSSION

The growth rates of spontaneous and transplanted tumors and tumor cells cultured *in vitro* have been related to the kinetics of cell proliferation as revealed by studies with TdR-^{3}H (12, 15, 16, 22).

Mendlesohn (15, 16) found that spontaneous breast carcinomas of C3H mice were composed of proliferating and nonproliferating populations of tumor cells. He devised a method for estimating the growth fraction (proliferating cells/total cells) of a tumor. Steel *et al.* (22) studied two transplanted tumors with volume-doubling times which differed by a factor just over 8. The mean cell cycle times of the proliferating cells in the tumors were estimated to be in the ratio of 2.6:1. The difference in the growth rates of the two tumors appeared to be largely due to differences in the growth fractions. The proportion of proliferating cells was about 95% in the faster growing tumor and 30% in the slower one. Similar observations have been reported by Frindel *et al.* (12), who studied the kinetics of cell proliferation of a transplanted fibrosarcoma growing *in vivo* and *in vitro* during an early phase when growth is rapid and again in a later phase when the growth rate has slowed considerably. The duration of the cell cycle did not change as the rate of growth slowed, but there was a marked decrease in the growth fraction. The slowing of the growth rate was explained mainly by the decrease in the growth fraction and by increasing cell death. This evidence suggests that the size of the growth fraction is an important factor in regulating the growth rate of a tumor. However, studies using TdR-^{3}H to label proliferating cells are difficult to interpret, for they suffer from the limitations discussed by Mendlesohn (17). The two limitations which are of major concern to the present study are: (*a*) TdR-^{3}H labels all proliferating cells and does not distinguish between tumor stem cells with unlimited proliferative capacity, which are capable of forming a colony and perpetuating a tumor, and cells at a later stage of development (? differentiated cells), which may not have the proliferative capacity required to form a colony or perpetuate the tumor. (*b*) Studies with TdR-^{3}H give no information about the nonproliferating population of tumor cells. This nonproliferating population may contain sterile cells, which have lost irreversibly the ability to proliferate, but a group of resting G_0 cells capable of regaining the ability to proliferate may be hidden in the fraction of cells that are not labeled by TdR-^{3}H.

For these reasons we have developed an assay for the Adj. PC-5 tumor cells which lodge in the spleens of isologous mice and possess sufficient proliferative capacity to form macroscopic colonies. The injection of approximately 3×10^4 cells resulted in the formation of a mean of one colony per spleen, which is compatible with the view that only a relatively small proportion of Adj. PC-5 tumor cells possessed proliferative capacity sufficient to form a tumor. However, this proportion needs to be corrected for the fact that appreciable numbers of tumor stem cells failed to reach the spleen and therefore were unable to form colonies even though they may have possessed the potential to do so. An attempt was made to determine the fraction of tumor stem cells which lodge in the spleen. No CFU were found in recipient spleens two hours after the injection of 2×10^7 tumor cells, and 24 hours after the injection of 1.8×10^7 tumor cells, an average of only about 1 CFU per recipient spleen was recovered. The results shown in Table 1, obtained with the 31st intravenous transplant generation, indicate a low value for a fraction of 0.002 or less. The growth curve shown in Chart 2*a* was done with cells from the 3rd and 9th intravenous transplant generations. The extrapolation of this growth curve to zero time yields an even lower estimate of fraction. These results show that the proportion of tumor stem cells could be greater by several orders of magnitude than the proportion of tumor CFU. A more reliable estimate of the proportion of tumor stem cells may be provided by the finding (Chart 3) that only a small fraction of animals given 10^3 Adj. PC-5 tumor cells died within ninety days. This result indicates that the proportion of cells capable of regenerating the tumor is of the order of 1 cell in 10^3.

The growth characteristics of the plasma cell tumor also support the view that only a small proportion of the tumor cells possess a large proliferative capacity. The growth of the tumor cells was studied in three different ways. The experiments using vinblastine (Chart 4) yielded an estimate of 9 hours for the maximum generation time (T_G) for tumor CFU. The growth curves for tumor CFU in spleen and marrow (Chart 2) yielded estimates of 20 and 29 hours for the doubling time of tumor colony-forming units (CFU T_D). The change in survival time of mice injected with graded numbers of tumor cells (Chart 3) yielded an estimate of 36 hours for the doubling time of the total tumor cell population responsible for the death of the animal (survival T_D). Thus, the doubling time for the total tumor cell population is greater than the doubling time of the tumor CFU, which in turn is greater than the generation time of tumor CFU. These are the results which would be expected if many tumor stem cells lose their extensive proliferative capacity through differentiation and/or cell death. The difference between the CFU T_D and the survival T_D is the result which would be expected if death and removal of a fraction of tumor cells from the animal occurs during the growth of the tumor, since the assay for CFU estimates the growth rate of the small proportion of tumor cells with unlimited proliferative capacity, while the survival T_D estimates the growth rate of the total tumor cell population in the animal. The fact that the Adj. PC-5 tumor produces a specific, homogeneous myeloma protein (19) which has not changed during more than 100 transplant generations indicates that these tumor cells retain the ability to differentiate along a predetermined, heritable pathway. It is possible that the differentiation entailed in the production of this protein is associated with a reduction in the proliferative potential of these cells. Thus, all the results presented in this

paper are compatible with the view that the Adj. PC-5 plasma cell tumor appears to grow slowly because it has a small proliferative pool of stem cells capable of regenerating a tumor.

If the mean T_G of the tumor CFU were uniform and the cells were distributed evenly throughout the cell cycle, one would expect that the killing of these cells by vinblastine as they enter mitosis (10, 24) would cause the surviving fraction to decrease at a constant rate, reaching zero in a period equal to the longest T_G. In our system we found that the tumor CFU were rapidly killed initially and more slowly killed as the time of exposure to vinblastine increased. This change in the rate of loss of CFU resulted in a concave survival curve (Chart 4). A survival curve of this shape could result from a number of factors, such as a skewed distribution of generation times or a concentration of the CFU in a particular phase of the cell cycle at the time the experiment was performed. For example, Conners *et al.* (T. A. Conners, J. Strackey, and M. E. Whisson, Changes in Chromosome Number and Sensitivity to Alkylating Agents Occurring in a Plasma Cell Tumor during Conversion to the Ascitic Form, personal communication) have recently found that an unexpectedly large fraction (37%) of tumor cells in an Adj. PC-5 tumor carried by subcutaneous transplantation are in the G_2 phase. Although the explanation for the rapid loss of tumor CFU exposed to vinblastine is unknown, the failure to detect tumor CFU after a ten-hour exposure to vinblastine indicates that none of the tumor CFU were protected from the action of the drug by being in a resting (G_o) state. The demonstration that 70% of the tumor CFU are in the S-phase of the cell cycle (Table 4) provides confirmatory evidence that the majority of these cells are in cell cycle and that few, if any, are in G_o state.

It is of interest to compare the growth characteristics of the rapidly growing, transplantable, AKR lymphoma studied by Bruce and his colleagues (4, 5, 7, 9, 23, 24) with those of the more slowly growing Adj. PC-5 plasma cell tumor (Table 5). For the AKR lymphoma, the survival T_D is 12 hours, the CFU T_D in the femoral marrow is 11.5 hours, and the T_G, as measured with vinblastine, is 10 hours. These data indicate that the tumor doubles in size in approximately the time required for one cell generation cycle. The tumor grows rapidly. Shortly before death each femur contains about 10^5 lymphoma CFU, and one spleen colony is formed for each 100 femoral marrow cells injected.

If the tumor stem cell compartment of the Adj. PC-5 plasma cell tumor is appreciably smaller than that of the AKR lymphoma, it should be possible to cure animals with advanced disease with agents which are selectively toxic for proliferating cells. Cyclophosphamide can be administered to animals bearing AKR lymphoma in doses which reduce the fraction of surviving tumor CFU by 10^{-7} (8), but since animals with advanced disease have more than 10^7 tumor stem cells, some of the lymphoma stem cells survive, proliferate, and kill the mouse. If the Adj. PC-5 tumor stem cells are equally sensitive to the action of cyclophosphamide, one would predict that it should be possible to cure animals with advanced disease which contain fewer than 10^7 tumor stem cells. This prediction assumes that a large proportion of Adj. PC-5 tumor cells are differentiated cells with a limited life span and limited proliferative potential, and that dedifferentiation of differentiated tumor cells to tumor stem cells does not occur.

Table 4

μc/ml	Number of cells injected per animal	Mean number of colonies/spleen	Mean number of CFU per 10^6 nucleated cells (± S. E.)	Fractional survival
TdR–^{3}H				
0	5×10^4	3.4	67 (± 8.3)	1.0
100	1×10^5	1.7	17.3 (± 3.0)	0.25
200	2×10^5	5.2	26.0 (± 2.5)	0.38
400	2×10^5	3.9	19.7 (± 2.4)	0.29
600	2×10^5	4.1	20.3 (± 2.6)	0.30
^3H_2O				
600	1×10^5	7.3	72 (± 6.9)	1.08

The effect of incubating adj. PC–5 tumor cells for 20 minutes *in vitro* with tritiated thymidine (TdR–^{3}H) or ^3H_2O on their colony-forming ability. CFU, colony-forming units.

Table 5

	AKR lymphoma	Adj. PC -5
T_D survival (hours)	12	36 ± 2
T_D CFU, femur (hours)	11.5 ± 0.5	29 ± 5
T_G CFU (hours)	10.0	9.0
CFU/femur (day before death)	10^5	10^3
Frequency of tumor CFU in femoral marrow cell suspensions	$1:10^2$	$1:3 \times 10^4$
Evidence of tumor cell differentiation	none	Myeloma protein

Growth characteristics of a transplantable AKR lymphoma and the Adj. PC-5 plasma cell tumor. CFU, colony-forming units.

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REFERENCES

1. Baserga, R. The Relationship of the Cell Cycle to Tumor Growth and the Control of Cell Division: A Review. Cancer Res., *25:* 581–595, 1965.
2. Becker, A. J., McCulloch, E. A., Siminovitch, L., and Till, J. E. The Effect of Differing Demands for Blood Cell Production on DNA Synthesis by Hemopoietic Colony-forming Cells of Mice. Blood, *26:* 296–308, 1965.
3. Becker, A. J., McCulloch, E. A. and Till, J. E. Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells. Nature, *197:* 452–454, 1963.
4. Bruce, W. R. The Action of Chemotherapeutic Agents at the Cellular Level and the Effects of These Agents on Hematopoietic

and Lymphomatous Tissue. Proc. Seventh Canadian Cancer Research Conference. Toronto: Pergamon Press, pp. 53–64, 1967.
5. Bruce, W. R., and Meeker, B.E. Dissemination and Growth of Transplanted Isologous Murine Lymphoma Cells. J. Natl. Cancer Inst. *32:* 1145–1159, 1964.
6. Bruce, W. R., and Meeker, B. E. Comparison of the Sensitivity of Hematopoietic Colony-forming Cells in Different Proliferative States to 5-fluorouracil. J. Natl. Cancer Inst., *38:* 233–245, 1967.
7. Bruce, W. R., Meeker, B. E., and Valeriote, F. A. Comparison of the Sensitivity of Normal Hematopoietic and Transplanted Lymphoma Colony-forming Cells to Chemotherapeutic Agents Administered *in vivo*. J. Natl. Cancer Inst., *37:* 233–245, 1966.
8. Bruce, W. R., Valeriote, F. A. and Meeker, B. E. Survival of Mice Bearing a Transplanted Syngeneic Lymphoma following Treatment with Cyclophosphamide, 5-fluorouracil and 1, 3-bis (2-chloroethyl)-1-nitrosourea. J. Natl. Cancer Inst., *39:* 257–266, 1967.
9. Bruce, W. R., and Van Der Gaag, H. A Quantitative Assay for the Number of Murine Lymphoma Cells Capable of Proliferating *in vivo*. Nature, *199:* 79–80, 1963.
10. Bruchovsky, N., Owen, A., Becker, A. J., and Till, J.E. Effects of Vinblastine on the Proliferative Capacity of L-cells and Their Progress through the Division Cycle. Cancer Res., *25:* 1232–1237, 1965.
11. Dulbecco, R., and Vogt, M. Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., *99:* 167–182, 1954.
12. Frindel, E., Malaise, E. P., Alpen, E., and Tubiana, M. Kinetics of Cell Proliferation of an Experimental Tumor. Cancer Res., *27:* 1122–1131, 1967.
13. Lajtha, L. G., Gilbert, C. W., Porteous, D. D., and Alexanian, R. Kinetics of a Bone-marrow Stem-cell Population. Ann. New York Acad. Sci. *113:* 742–752, 1964.
14. Maruyama, Y., and Brown, B. W., Jr. The Growth of Murine Lymphomatous Tumor-cells as Determined by Host Survival Time. Int. J. Radiol. Biol., *8:* 59–73, 1964.
15. Mendelsohn, M. L. Autoradiographic Analysis of Cell Proliferation in Spontaneous Breast Cancer of C3H mouse. III. The Growth Fraction. J. Natl. Cancer Inst. *28:* 1015–1029, 1962.
16. Mendelsohn, M. L. The Kinetics of Tumor Cell Proliferation. *In:* Cellular Radiation Biology. Baltimore: Williams and Wilkins, pp. 498–513. 1965.
17. Mendelsohn, M. L., and Dethlefson, L. A. Tumor Growth and Cellular Kinetics. 21st Annual Symposium on Fundamental Cancer Research, The University of Texas M.D. Anderson Hospital and Tumor Institute, 1967. (in press)
18. Parker, R. C. Methods of Tissue Culture. Third Ed. New York: Hoeber, p. 77, 1961.
19. Potter, M., Appella, E., and Geisser, S. Variations in the Heavy Polypeptide Chain Structure of Gamma Myeloma Immunoglobulines from an Inbred Strain of Mice and a Hypothesis as to their Origin. J. Mol. Biol., *14:* 361–372, 1965.
20. Potter, M., and Robertson, C. L. Development of Plasma-cell Neoplasms in BALB/c Mice after Intraperitoneal Injection of Paraffin-oil Adjuvant, Heat-killed Staphylococcus Mixtures. J. Natl. Cancer Inst., *25:* 847–861, 1960.
21. 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.
22. Steel, G. G., Adams, K., and Barrett, J. C. Analysis of the Cell Population Kinetics of Transplanted Tumors of Widely Differing Growth Rate. Brit. J. Cancer, *20:* 784–800, 1966.
23. Valeriote, F. A., and Bruce, W. R. Comparison of the Sensitivity of Hematopoietic Colony-forming Cells in Different Proliferative States to Vinblastine. J. Natl. Cancer Inst., *38:* 393–399, 1967.
24. Valeriote, F. A., Bruce, W. R., and Meeker, B. E. A Model for the Action of Vinblastine *in vivo*. Biophys. J., *6:* 145–152, 1966.
25. Whitmore, G. F., and Gulyas, S. Synchronization of Mammalian Cells with Tritiated Thymidine. Science, *151:* 691–694, 1966.
26. Wodinsky, J., Swiniarski, 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. *S1:* 415–421, 1967.

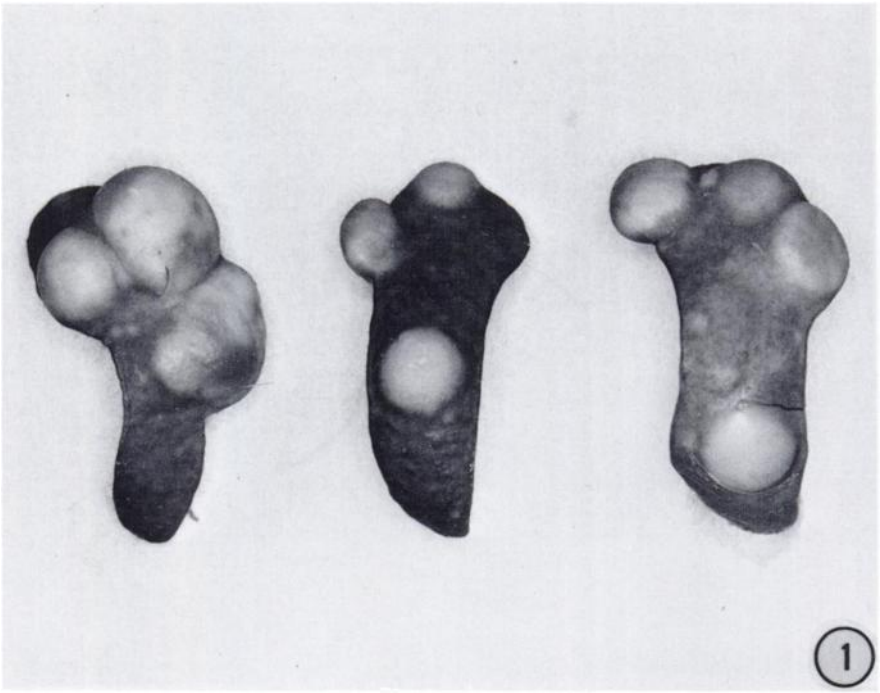

Fig. 1. Gross appearance of colonies in the spleens of mice receiving 10^5 cells of an isologous spleen cell suspension containing Adj. PC-5 tumor cells. The spleens were removed at 22 days following the intravenous injection of tumor cells.