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The hemolytic component of cancer anemia: effects of osmotic and metabolic stress on the erythrocytes of rats bearing multifocal inoculations of the Walker 256 tumor

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Abstract

Cancer anemia is classified as an anemia of chronic diseases, although it is sometimes the first symptom of cancer. Cancer anemia includes a hemolytic component, important in the terminal stage when even transfused cells are rapidly destroyed. The presence of a chronic component and the terminal complications of the illness limit studies of the hemolytic component. A multifocal model of tumor growth was used here to simulate the terminal metastatic dissemination stage (several simultaneous inoculations of Walker 256 cells). The hemolytic component of anemia began 3-4 days after inoculation in 100% of the rats and progressed rapidly thereafter: Hb levels dropped from 14.9 ± 0.02 to 8.7 ± 0.06 from days 7 to 11 (~5 times the physiologically normal rate in rats) in the absence of bleeding. The development of anemia was correlated (r^2 = 0.86) with the development of other systemic effects such as anorexia. There was a significant decrease in the osmotic fragility of circulating erythrocytes: the NaCl concentration causing 50% lysis was reduced from 4.52 ± 0.06 to 4.10 ± 0.01 ($P<0.01$) on day 7, indicating a reduction in erythrocyte volume. However, with mild metabolic stress (4-h incubation at 37°C), the erythrocytes showed a greater increase in osmotic fragility than the controls, suggesting marked alteration of erythrocyte homeostasis. These effects may be due to primary plasma membrane alterations (transport and/or permeability) and/or may be secondary to metabolic changes. This multifocal model is adequate for studying the hemolytic component of cancer anemia since it is rapid, highly reproducible and causes minimal animal suffering.

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Key words
- Hemolytic component
- Cancer anemia
- Osmotic fragility
- Systemic effects
- Walker 256 tumor

Introduction

Cancer anemia is usually classified as an anemia of chronic diseases (ACD), but is sometimes the first symptom to appear and therefore not necessarily a chronic manifestation of cancer (1-3). Cancer anemia includes a hemolytic component which markedly increases in the final stage of the illness, when even transfused red blood cells (RBC) are rapidly destroyed (4,5).

The mechanisms underlying the hemolytic

component are not well understood, and their study is difficult because of the simultaneous presence of a chronic component, and because of ethical difficulties, treatments and other complications, particularly in the terminal stages.

In experimental studies such as those previously performed with the Walker 256 tumor inoculated at a single subcutaneous (*sc*) site, the development of anemia showed the general characteristics of ACD (5,6). However, if instead of the usual unifocal tumor growth model, the studies are initiated using multifocal simultaneous tumor inoculations (simulating the metastatic dissemination of the final stage), the systemic effects of cancer, including anemia, appear rapidly and synchronously in all animals (7,8). The development of anemia under these conditions is similar to that found in terminal cancer patients, in which the rate of RBC destruction is very high (4,9). This multifocal model using the Walker 256 tumor therefore seems to be particularly suited for the study of the hemolytic component of cancer anemia.

In the present study, we examined the time-course of the changes occurring in hemoglobin levels (Hb) and RBC osmotic fragility (OF) and correlated them with other systemic effects of cancer such as anorexia. The OF was studied in fresh whole blood samples and in the same RBC after metabolic stress (*in vitro* incubation at 37°C).

Material and Methods

Tumor and animals

The Walker 256 "A" tumor line was kindly provided by Dr. Maria C. Cintra Gomes, Department of Physiology, IB/UNICAMP. The line originally came from the National Cancer Institute Bank, Cambridge, MA, USA. The tumor is currently kept in the laboratory under liquid N_2 and is maintained through intraperitoneal or *sc* passages in rats.

Thirty-five 9-week-old male Wistar rats were used. The animals were housed at controlled temperature (21°C), on a 12-h light-dark cycle and fed a commercial rat diet (Labina/Purina, Campinas, SP, Brazil). The rats were randomly divided into experimental (tumor) and pair-fed control groups of 20 and 15 animals, respectively. The tumor bearers were allowed free access to food, while the pair-fed controls were allowed to eat only the amount ingested the day before by the tumor bearers. Both had free access to water. Tumor-bearing rats received four dorsal *sc* inoculations of 5×10^6 tumor cells each in 0.25 ml of Ringer-lactate spaced at least 1 cm apart. Healthy tumor cells with 98% viability (assessed by Trypan blue) were obtained from the ascitic fluid of a donor rat. Control rats received four identical inoculations of vehicle only.

The general UKCCR guidelines for animal welfare were followed (10). One of the authors was responsible for the daily clinical evaluation of the animals. Somatic and visceral pain was explored by palpation and gentle compression of the limbs and axillary, inguinal, tumoral, peritoneal and thoracic regions. No animal needed to be sacrificed before the end of the experiment.

Experimental design

Before tumor inoculation, the rats were anesthetized with ether and a 0.7-ml blood sample was collected from the suborbital plexus into 0.05 ml heparin (5000 IU/ml). The tumor bearers were sacrificed on days 4, 7 and 11 (5, 5 and 10 rats, respectively) after tumor inoculation, and the pair-fed controls on days 5, 8 and 12 (5, 5 and 5 rats, respectively). At sacrifice, the thorax was opened under deep ether anesthesia and a large (~6 ml) blood sample was collected into 0.3 ml of heparin, via direct heart puncture with an 18 G needle. All blood samples were used for hemograms and OF determinations.

Hemogram

Hemograms were performed using a Celldyn 1600 apparatus and the results were corrected for the relative volume of heparin present in each blood sample.

Osmotic fragility and RBC incubation tests

Red blood cell OF was determined as described by Dacie et al. (11). Packed RBC obtained from tumor bearers and controls (0.2 ml, followed by a 5-min centrifugation at 978 *g*) at sacrifice were incubated for 4 h at 37°C after resuspension in different media: a) RL (1.0 ml of Ringer-lactate), b) RL + G (1.0 ml of Ringer-lactate + 2 mg of glucose), c) PLa (1.0 ml of autologous plasma) and d) PLa + G (1.0 ml of autologous plasma + 1 mg of glucose). After incubation, the OF was again determined. To facilitate data analysis the median corpuscular fragility (MCF) was often used. MCF has been defined as the NaCl concentration (g/l) causing 50% of RBC lysis (11).

Autopsy

Autopsies were performed on all tumor-bearing rats. The tumors were dissected and weighed, and special attention was paid to the eventual presence of metastases, the invasion of important tissues and bleeding.

Statistical analysis

The results are reported as means ± SEM. The statistical significance of the changes in fresh blood was determined by the Student paired *t*-test. The significance of the metabolic stress studies was tested by ANOVA and by the paired Student *t*-test (12).

Results

Tumor development

Tumors grew at all inoculated sites (4/rat) and were palpable within 3 days. Their mean weights were 2.8 ± 0.2 g and 10.3 ± 0.6 g on days 7 and 11, respectively.

Development of anemia

Figure 1 shows the time-course of the changes in Hb and food intake during the experiment. The Hb levels decreased rapidly ($P<0.001$) in tumor bearers, dropping from 14.9 ± 0.2 to 8.7 ± 0.6 g/dl in the last 4 days (1.55 g dl^{-1} day^{-1}, about 5 times the normal rate of RBC destruction in rats). The decrease in Hb levels was correlated with the decrease in food intake: on days 4, 7 and 11 Hb levels decreased 2.3 ± 2.1, 16.7 ± 1.1 and 48.3 ± 3.3%, while the respective average food intake decreased 27, 34 and 72% (r^2 = 0.86, $P<0.001$).

Osmotic fragility changes in fresh RBC

Figure 2 shows the OF curves of fresh RBC from tumor bearers and pair-fed controls, 4, 7 and 11 days after tumor inoculation. A significant shift to the left was observed in all tumor bearers (Figure 2A, B and C) which, expressed in MCF, corresponded to -0.25 ± 0.04, -0.42 ± 0.06 and -0.36 ± 0.07 g/l, respectively ($P<0.01$ in all instances). In the control rats there was a slight shift to the left on day 4 but no changes on days 7 and 11

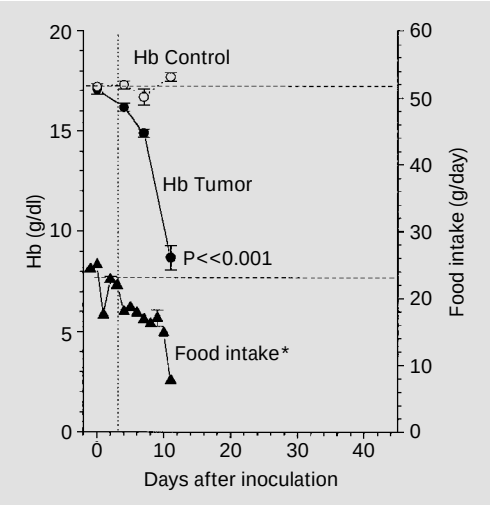

Figure 1 - Changes in the hemoglobin levels and daily food intake of rats with multifocal inoculations of the Walker 256 tumor and of their pair-fed controls. The pair-fed control data were shifted backwards one day in order to cancel the methodological delay in the protocol (see Material and Methods). *Tumor-bearing and pair-fed control animals.

(Figure 2D, E and F).

Consistent with the changes in OF the hemogram showed a decrease in median RBC corpuscular volume, expressed as fl, with values of 53.2 (range 53-54), 51.6 (range 51-52) and 50.0 (range 49-52) for tumor bearers on days 4, 7 and 11 after tumor inoculation, respectively ($r^2 = 0.77$, $P<0.001$). In the control group, the values were 51.6 (range 51-53), 51.8 (range 50-53) and 50.2 (range 49-52), respectively ($r^2 = 0.21$, not significant).

Another change observed on day 11 was the decrease in the slope of the RBC OF curves (Figure 2C). The maximal derivative value, which corresponds to the maximal percent variation in RBC lysis, induced by a change of 0.5 g/l in NaCl concentration dropped from 51.7 ± 1.8% on day zero to 43.7 ± 2.1% per 0.5 g/l on day 11 (-8.0 ±

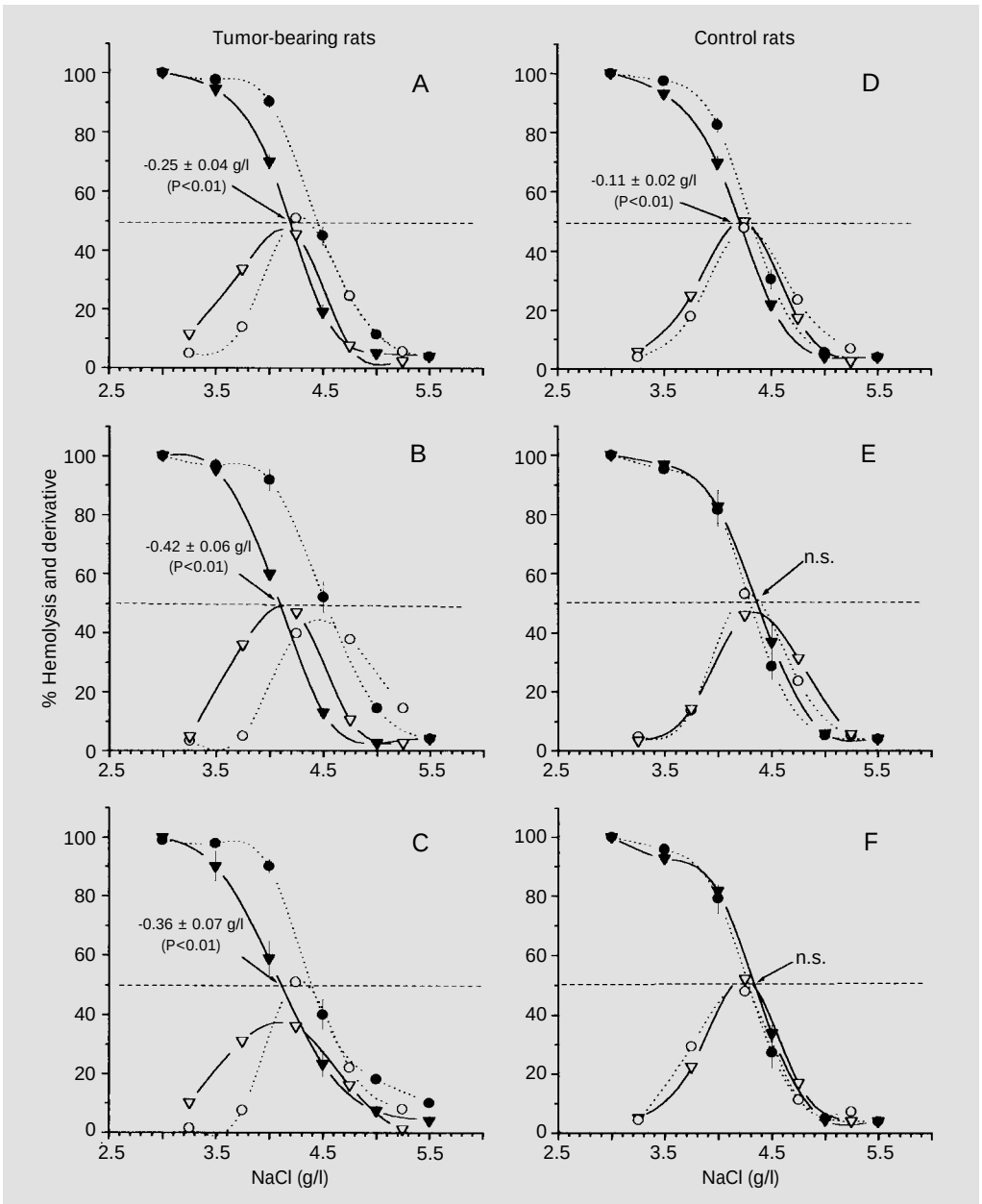

Figure 2 - Osmotic fragility curves for fresh RBC from rats multifocally inoculated with Walker 256 tumor (left) and for the pair-fed controls (right) sacrificed on days 4 (A and D), 7 (B and E) and 11 (C and F) after inoculation. Filled symbols: % hemolysis. Open symbols: derivative values (difference between two consecutive points on the hemolysis curve). Broken lines and circles: mean values on day zero. Solid lines and inverted triangles: mean values on the day of sacrifice. n.s.: Nonsignificant.

2.1% per 0.5 g/l, P<0.01). Under the same conditions the changes in the control group were not significant, i.e., 52.0 ± 2.4 to 48.0 ± 1.0% per 0.5 g/l (-4.0 ± 2.2% per 0.5 g/l).

Osmotic fragility after *in vitro* incubation

The sensitivity of RBC from tumor-bearing rats and their pair-fed controls to the metabolic stress induced by incubation at 37ºC for 4 h was tested using Ringer-lactate buffer as incubation medium and autologous plasma with or without glucose addition. This treatment caused only a small increase in the MCF of RBC from the pair-fed controls, whereas RBC from tumor bearers were more sensitive to the stress, particularly 11 days after tumor inoculation when the increase in MCF was marked and the difference, compared with the controls, was significant for all incubation media tested (Figure 3).

Autopsy

There were no signs of bleeding or macroscopic visceral metastases. Only some enlarged regional lymph nodes were observed, particularly in the retroperitoneum and around the thymus, but in no instance were neighboring tissues invaded.

Discussion

All rats rapidly and synchronously developed anemia and anorexia, the systemic effects characteristic of the final stage of cancer, after multifocal tumor inoculation. The effects began 3-4 days after inoculation and progressed rapidly thereafter, and would have resulted in death 8-9 days later (7,8) if the rats had not been sacrificed at fixed times according to the experimental protocol. The effects developed in the absence of complications such as bleeding, invasions or visible metastases, as confirmed at autopsy. The early onset, rapid course and magnitude of the decrease in Hb levels in the tumor bearers indicated an acute form of anemia which, in the absence of bleeding, could only be explained by the rapid destruction of RBC. Based on the rate of decrease in Hb levels, the RBC destruction in the final four days was about five times the normal physiological rate for this phenomenon.

The rapid progress of the anemia seen with multifocal tumor inoculation is quite different from the progress observed after unifocal inoculation. Figure 4 shows unpublished data (Vieira-Matos AN and Rettori O) from our laboratory similar to those reported by others using the unifocal Walker 256 model (5,13). The average results obtained under these conditions suggest that this cancer causes moderate chronic anemia and mild temporary anorexia. These conclusions can be partially explained by the fact that in this model the periodic transverse averaging

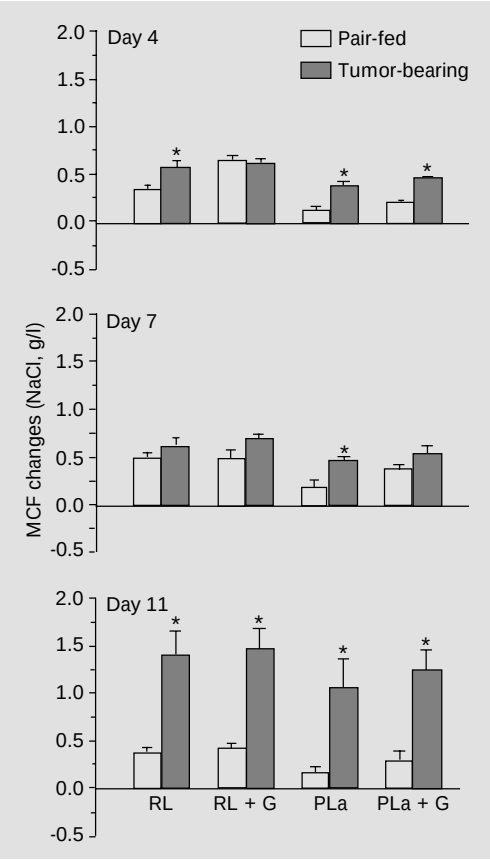

Figure 3 - Effect of a 4-h incubation at 37ºC on the osmotic fragility of RBC from tumor-bearing and pair-fed control rats in different incubation media: RL: Ringer-lactate; RL + G: Ringer-lactate + glucose (2 mg/ml); PLa: autologous plasma; PLa + G: autologous plasma + glucose (1 mg/ml). MCF: Median corpuscular fragility = NaCl concentration (g/l) causing 50% of RBC lysis. *P<0.05 (paired Student *t*-test).

of data mixes values from groups of animals at very different stages of the illness with data from animals with still undetectable homeostatic alterations. In addition, the exclusion of animals that died at the end of the experiment, together with the survival of some resistant animals generates an artifact that suggests an apparent improvement in the illness condition. Guaitani et al. (13) recognized these influences while studying the anorexia induced by the Walker 256 tumor and suggested a different treatment of the data, in which the independent variable was changed. Instead of time, this author proposed the use of "the percentage of the survived time". Using this approach, at the time of death (100% of survived time) for example, all animals in the group were totally anorectic. A further improvement in the handling of this problem was described by Rettori et al. (7). The multifocal tumor model avoids all of these problems by synchronizing the onset and progress of the systemic effects of the final stage of cancer among the individuals of the experimental group (7,8). Under the latter conditions, the hemolytic component of cancer anemia is of particular importance as described for humans by Hyman and Harvey in the early 50s (4). These authors demonstrated what is generally recognized in oncology, namely, that in terminal cancer patients even the half-life of transfused RBC is markedly reduced, so that frequent transfusions are of little help in maintaining reasonable Hb levels.

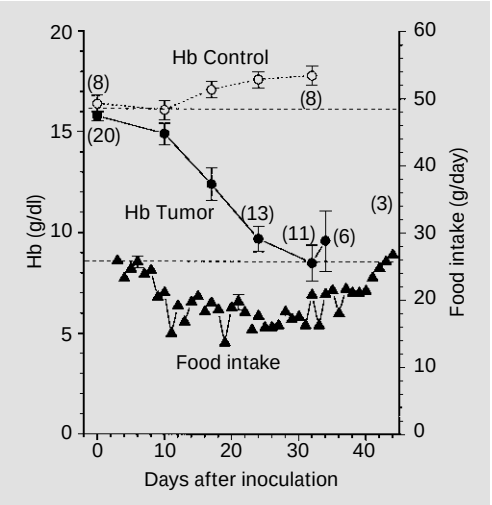

Figure 4 - Changes in hemoglobin levels and daily food intake of rats bearing unifocal inoculations of the Walker 256 tumor (Vieira-Matos AN and Rettori O, unpublished results similar to those reported by others, see text). (n): Number of individuals in the group or remaining in it.

The increased importance of the hemolytic component of the anemia of terminal cancer patients is probably associated with metastatic dissemination. In this regard, the multifocal inoculation model simulates the terminal stage of cancer, with the advantage of dissociating the hemolytic component of anemia from the chronic complications that mask it.

Changes in red blood cell osmotic fragility induced by the Walker 256 tumor

The OF of fresh RBC reflects their ability to take up water without lysis and is determined by their volume-surface area ratio (11).

In the present study, the development of acute anemia was accompanied by a decrease in the OF of RBC (a shift to the left in the OF curves), indicating a reduction in the volume of circulating erythrocytes. Indeed, the blood count showed that the mean corpuscular volume of RBC from tumor-bearing rats was significantly reduced compared with control RBC. On the other hand, the higher increases in MCF after a 4-h incubation at 37°C when compared with pair-fed controls indicated that the RBC of tumor bearers had an increased propensity to swell during incubation, thus decreasing the resistance to metabolic stress. This effect was cumulative since it became marked only on day 11 and was absent or slight on days 4 and 7. The complexity of the factors associated with the changes in OF induced by this tumor was further suggested by the reduction in the slope of the OF curve on day 11.

Mechanisms involved in RBC alterations

The above results shed little light on the molecular basis of the observed phenomenon. Hereditary alteration in RBC glycolytic

enzymes, such as pyruvate kinase deficiency, is an example of how a primary alteration in RBC metabolism is able to induce anemia associated with a decrease in the volume (decreased OF) and resistance to metabolic stress (marked OF increase after incubation) of fresh RBC. On the other hand, hereditary and acquired membrane defects are also known to decrease the RBC volume-surface area ratio, and hence decrease the OF (11). In contrast, the induction of cell volume changes leads to important cellular metabolic alterations (14) such that circulating molecules of tumor and/or host origin, cytokines or other effector molecules (15) acting on RBC metabolism and/or its plasma membranes may be involved in inducing the observed phenomenon. This hypothesis is supported by observations that the tumor-induced OF effects seen in the present study may be reproduced under special conditions by incubating normal RBC with plasma from tumor anemic animals (Vieira-Matos AN and Rettori O, unpublished results). A reduction in RBC OF in anemic tumor-bearing mice has been reported, and may be caused by structural alterations in the RBC (16). We have also observed a marked decrease in RBC OF associated with anemia in rats bearing DMBA-induced mammary cancer (Cavalcanti TC and Rettori O, unpublished results).

Relevance of the present observations

The high correlation ($r^2 = 0.86$) between the development of anemia and other well-known systemic effects such as anorexia induced by cancer suggests the possibility of a common molecular basis for the serious homeostatic alterations occurring in the final stage of cancer. Recent studies have proposed an imbalance between catabolic and anabolic hormones and cytokines of tumor and/or host origin as the molecular basis for cancer cachexia (15). A main role has been suggested for the following cytokines: IL-α, IL-ß, IL-6, IL-8, TNF-α, INF-α and γ, but conclusive data are often lacking (17,18), and because the circulating levels usually do not correlate well with cachexia, an alternative model of abnormal generalized high local production of cytokines through positive feedback systems has been proposed (17).

The possibility should be explored that the study of the alterations in the RBC plasma membrane and/or metabolism could help to identify these molecules and to explain the mechanism of cancer anemia, cachexia and the so-called multiple organ failure syndrome, frequently referred to as the cause of death in cancer patients.

The multifocal tumor inoculation model

The explanation as to why several small tumors (little more than 100 mg each on days 3-4) were more effective in inducing the systemic effects of cancer than a single large tumor (sometimes 40 g or more) is probably related to the "cell kinetics" of tumor growth. Studies in this field suggest that the sum of proliferative tumor cells (PTC) in several small tumors is larger and increases faster than that of a single, big tumor, in which most cells are non-proliferative (NPTC). Recent work has demonstrated that in tumors of up to 100 mg (days 3-4 in our model) about 90% of the mass would be PTC and would grow fast (exponentially), but they would rapidly reach an almost steady state of about 1-2 g (19). One explanation for this, consistent with the current concepts of tumor cell proliferation (20), would be that, due to the rapidly increasing irrigation deficit, about half of the new cells formed after each replication would stay in G_1 or go to G_0, with many of the latter progressing to apoptosis or necrosis. Thus tumors larger than 1-2 g would grow mainly at the expense of NPTC, i.e., cells that are not effective in inducing the systemic effects of cancer. In the final stage, when metastatic dissemina-

tion occurs, the multiple small foci of neoplastic growth, each bearing high relative populations of PTC, would produce a high enough "total" rate of PTC growth to induce the serious homeostatic alterations that culminate in host death.

In addition to the already mentioned advantages of the multifocal model (synchronism among individuals, rapidity, reproducibility and absence of complications), this model also involves minimal animal suffering.

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