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18. The relationship between the PCB concentration in whole bodies and molts was not consistent for the three collections. In November 1974 PCB concentrations in molts were relatively high. Molts represent about 8 percent of the euphausiid's dry weight; therefore, it can be calculated that approximately 18 percent of the euphausiid's PCB body burden was associated with the outer surface of the animals collected at that time. On the other hand, the fraction associated with molts collected in January 1975 was only about 5 percent. The fluctuation in the relationship between the concentrations in whole animals and molts could be explained if the predominant route of accumulation of PCB's in the exoskeleton is by surface adsorption from the surrounding water. This mode of uptake is thought to play an important role in the accumulation of organochlorine compounds by euphausiids and other small aquatic invertebrates [see J. L. Cox, *Fish. Bull.* **69**, 627 (1971); R. F. Addison, in *Effects of Pollutants on Aquatic Organisms*, A. P. M. Lockwood, Ed. (Cambridge Univ. Press, Cambridge, England, 1976), p. 127]. If this is the case, then significant short-term changes in PCB concentrations in the water would be more closely reflected by PCB concentrations in the molts than those in whole bodies which include tissues not in direct contact with the water. On the other hand, whole animal PCB concentrations should be more representative of long-term integrated PCB concentrations in the water mass or in the food, or both.
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Primary Bioassay of Human Tumor Stem Cells

Abstract. *A simple method has been developed to support human tumor stem cell colony growth in soft agar. The technique appears suitable for culture of a variety of neoplasms of differing histopathology. Tumor stem cell colonies arising from different types of cancer have differing growth characteristics and colony morphology. This bioassay should be suitable for clinical studies of effects of anticancer drugs or irradiation on human tumor stem cells.*

Tumor stem cells are the cell renewal source of a neoplasm and also serve as the seeds of metastatic spread of cancer. Studies of transplantable tumors in animals indicate that tumor stem cell colony-forming assays (in vivo or in vitro) can be used to study the biological properties of these cells and to delineate differences in individual sensitivity to a variety of chemotherapeutic agents (*1*, *2*). For example, the development of an in vitro colony-forming assay for stem cells from transplantable mouse myeloma (*3*) (a plasma cell neoplasm) permitted detailed analysis of the effects of anticancer drugs in vitro, and the assays are predictive of therapeutic responses even in animals with advanced mouse myeloma (*1*).

The ability to grow colonies from primary tumor cell explants in semisolid culture media has even greater potential application. Unfortunately, primary explantation of human tumors for colony formation in vitro has met with little success. One major problem has been the creation of an environment that gives tumor cells a selective advantage over normal cells. Several investigators have had occasional success in obtaining colony growth in soft agar with pediatric solid tumors (*4*). Most recently, the effect of drugs on human stem cell colonies has been studied with the use of xenografts established in nude mice and then culturing cells from these grafts in agar in diffusion chambers intraperitoneally implanted in mice that had been irradiated (*5*). However, such multiple-step systems have not been clinically practical. A standard colony-forming assay for human tumor stem cells could be used to determine the sensitivity of tumor cells from individual patients to drugs, irradiation, and other therapeutic modalities and would permit the demonstration of resistant clones of cells in previously treated patients. The development of such a colony-forming assay seems especially important in view of evidence (*6*) indicating that the only valid measure of drug efficacy in killing an established culture of human lymphoma cells was the inhibition of their colony-forming capability.

Using the studies of mouse myeloma stem cells as a model, we developed an in vitro assay that permitted colony formation by human myeloma stem cells in soft agar and appeared to preclude growth of normal hematopoietic precursors. Using bone marrows or malignant effusions as the source of metastatic tumor cells, we subsequently proceeded to other tumor types.

A feeder layer that was analogous to those used for murine systems was required for support of human tumor colony growth (*7*, *8*). While several feeder layers proved useful for our studies on humans, the preferred one consisted of a "conditioned medium" prepared from the adherent spleen cells of BALB/c mice that had been primed with 0.2 ml of mineral oil injected intraperitoneally 4 weeks previously. The adherent cells were obtained as follows. The spleens were teased with needles to form a single-cell suspension, and 5×10^6 cells were placed in a 60-mm Falcon petri dish for 2 hours to permit the cells to adhere. The dishes were then rinsed three times in cold phosphate-buffered saline. (All tissue culture media, buffers, and serums were obtained from Grand Island Biological Co.) Cells were incubated for 3 days at 37°C in RPMI 1640 medium with 15 percent heat-inactivated fetal calf serum (*7*). The conditioned medium was decanted and centrifuged at 400*g* for 15 minutes, and the supernatant was then passed through a 0.45-μm Nalgene filter and stored at −20°C.

Bone marrow cells were collected aseptically (in preservative-free heparin) from consenting normal volunteers and patients with various neoplasms with known bone marrow involvement. Malignant ascites was collected (in heparin) by paracentesis. Red blood cells were removed from the bone marrow samples by sedimentation in 3.0 percent dextran-saline, and the supernatant bone marrow cells were collected and then washed three times in Hanks balanced salt solution with 10 percent fetal calf serum that had been inactivated by heat. The number of viable cells was determined by hemocytometer counts with trypan blue.

Cells to be tested were suspended in 0.3 percent Bacto agar (Difco) in CMRL 1066 supplemented with 20 percent horse serum, penicillin (100 unit/ml), streptomycin (2 mg/ml), glutamine (2 m*M*), $CaCl_2$ (4 m*M*), insulin (3 unit/ml), asparagine (0.1 mg/ml), and DEAE dextran (0.5 mg/ml) to yield a final concentration of 5×10^5 cell/ml. 2-Mercaptoethanol was added at a concentration of $5 \times 10^{-5} M$ immediately before the cells were plated (*9*). A portion (1 ml) of the resultant mixture was pipetted onto a 1.0-ml feeder layer (which combined 0.2 ml of conditioned medium in 0.5 percent Bacto agar) in 35-mm plastic petri dishes. Cultures were incubated at 37°C in 5 percent

CO_2 in a humidified atmosphere for approximately 3 weeks with no additional feeding.

With respect to myeloma, clusters of 8 to 40 cells appeared 5 to 10 days after plating, whereas colonies (defined as collections of more than 40 cells) appeared 14 to 21 days after plating. Colonies consisted of 40 to several hundred large (> 20 μm) round cells. Cells in myeloma colonies appeared to pile up on one another (Fig. 1B), as compared to cells in rare contaminating granulocyte colonies in which cells were loosely aggregated. [The number of granulocyte colonies in different cultures varied, but never exceeded 10 percent of the total number of colonies. Since granulocyte colony growth occurred more frequently when 10^6 nucleated cells were plated in a culture dish, we chose a standard concentration of 5×10^5 marrow cells per dish to minimize stimulation of granulocyte colony formation (*10*).] The number of myeloma colonies was 5 to 500 per plate, yielding a plating efficiency of 0.001 to 0.1 percent, which compared favorably with analogous systems (*4*, *5*). The number of myeloma colonies was proportional to the number of cells plated between concentrations of 10^5 to 10^6 cells per plate, and the plot could be extrapolated back to zero, suggesting colony origin from a single monoclonal plasma cell. In order to enrich the myeloma stem cell fraction and obtain higher plating efficiencies, we are applying a combination of velocity gradient sedimentation (1*g*) and adherent depletion of nonmyeloma cells. In initial experiments we have obtained a 20-fold increase in plating efficiency with the combination of these techniques.

Cells from individual colonies plucked from the agar with a pipette appeared to be plasma cells when examined by light microscopy after staining with Wright-Giemsa and methyl-green pyronine. They were peroxidase negative, incapable of phagocytosis of neutral red or latex particles, and positive for plasma cell acid phosphatase. Immunofluorescence studies demonstrated that 60 to 80 percent of the myeloma plasma cells contained intracytoplasmic monoclonal immunoglobulin of immunologic specificity qualitatively identical to the type present in the serum or urine of the patient studied. Colony growth has been obtained in more than 86 percent of the total myeloma cases and in more than 89 percent of previously untreated myeloma patients (Table 1). In contrast, when a series of bone marrow samples from ten normal volunteers was tested in the same culture system, only a rare granulocyte colony would form and undergo deterioration within 10 days (*10*) (Table 1). Our system was not optimized for granulocyte colony formation.

We also found that the same conditioned medium feeder layer supported tumor colony growth by a variety of metastatic cancers. Table 1 summarizes our experience. In addition to myeloma and the related disorder, Waldenström's macroglobulinemia, tumor colony growth occurred from bone marrows of patients with disseminated lymphoma, neuroblastoma, and oat cell carcinoma of the lung. Tumor colony growth was also obtained from malignant ascites from patients with ovarian adenocarcinomas (Table 1). Histological studies indicated that the colonies were derived from the tumor of origin.

Colony morphology, colony growth kinetics, and plating efficiency varied with the different tumors tested, but these criteria were constant for each individual tumor type (Table 1). Colonies from bone marrows of patients with lymphoma appeared 4 days after plating, reached a peak size 7 days after plating, and degenerated after 3 weeks in culture. Individual cells were smaller than those of myeloma stem cell colonies (approximately 10 μm), but the number of cells per colony reached several thousand—greater than that seen in myeloma colo-

Table 1. Growth of tumor stem cell colonies from various human neoplasms.

Type of tumor (source of sample)*	Subjects with positive cultures per total subjects tested†	Colonies per 5×10^5 cells plated in positive cultures	Required incubation time (days)
Normal volunteers (controls) (M)	2/10‡	1–4‡	7
Multiple myeloma (M)	56/63	5–500	21
Non-Hodgkin's lymphoma (M)	9/18	41–150	14
Hodgkin's disease (M)	0/3		
Waldenström's macroglobulinemia (M)	3/3	50–150	21
Chronic lymphocytic leukemia (M) (N)	2/9	20–200	21
Oat cell carcinoma of the lung (M)	1/1	100	14
Adenocarcinoma of the ovary (A)	8/8	100–800	12
Melanoma (N)	2/3	80–150	21
Neuroblastoma (M)	1/1	250	7

*M, bone marrow aspirate; N, lymph node; A, malignant ascites collected by paracentesis. †In some of the tumor categories, failure to obtain colony growth from a particular sample may be due to prior cytotoxic chemotherapy or specific histological subtype (such as in the non-Hodgkin's lymphomas) where there may be differences in growth requirements. ‡Granulocyte-macrophage colonies.

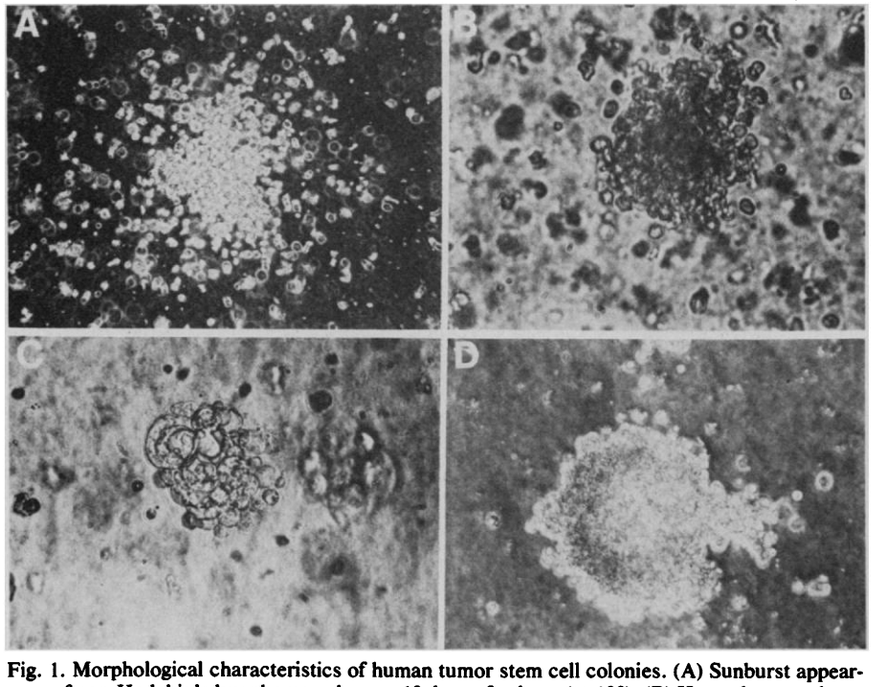

Fig. 1. Morphological characteristics of human tumor stem cell colonies. (A) Sunburst appearance of non-Hodgkin's lymphoma colony at 13 days of culture (× 100). (B) Heaped-up myeloma colony at 13 days (× 200). (C) Fourteen-day-old ovarian carcinoma colony (× 200). The morphology is consistent with a mucin-secreting adenocarcinoma. (D) Spherical neuroblastoma colony at 21 days of culture (× 200).

nies (Fig. 1A). Tumor colonies from the bone marrow of a patient with neuroblastoma grew as a sphere of large (> 25 μm), round, tightly packed cells (Fig. 1D). These colonies grew rapidly and continued to grow for 5 weeks. Ovarian adenocarcinoma cells retained their epithelial morphology (Fig. 1C), and the plating efficiency of these was high enough to indicate linear increase in colonies with increasing numbers of cells plated above 10^3 cells. The characteristic morphology and individual growth kinetics of each colony type have enabled us to distinguish between stem cell colonies of different tumor types and between tumor stem cell colonies and the occasional colonies of normal granulocyte-macrophage precursors. To date, we have studied the behavior of tumor stem cells from metastatic sites only. Clearly, studies of stem cells from the site of origin of the tumor and comparison to metastatic clones will be important and perhaps will elucidate the metastatic process.

We believe that application of such simple in vitro culture techniques for studies of human tumor stem cells from primary explants will prove of clinical importance. First, the technique permits characterization of many of the biophysical properties of tumor stem cells, such as sedimentation velocity, fraction in the S phase as determined by cell death as a result of treatment with tritiated thymidine, and surface antigenic features. Second, formation of in vitro colonies may prove a more sensitive indicator of occult metastatic disease than standard pathological studies. Third, such an assay could potentially be applied to develop individualized predictive trials of anticancer drugs in a manner analogous to techniques used for selection of antibacterial agents. Finally, full realization of the clinical application of bioassay of human tumor stem cell colonies with regard to their sensitivity to drugs, hormones, immunological agents, heat, and radiation could lead to major advances in clinical oncology.

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8. The techniques applied to provide stimulation of human myeloma stem cell colony formation deserve some comment. Our technique for preparation of conditioned medium was an extrapolation of the conditions described for primary induction of myeloma in vivo in BALB/c mice with intraperitoneal injection of mineral oil [M. Potter, *Physiol. Rev.* **52**, 631 (1972)]. In addition, Namba and Hanoka (*7*) have demonstrated that long-term culture of mouse myeloma MOPC 104 E cells could be established only by culturing cells with phagocytic cells or media conditioned by these cells.
9. A thiol such as 2-mercaptoethanol was necessary for tumor colony growth in approximately 80 percent of the experiments. We therefore incorporated it routinely in the final culture medium.
10. Colony growth of normal human granulocyte-macrophage progenitors is dependent on the presence of a specific humoral stimulus [colony-stimulating factor (CSF)] [T. Bradley and D. Metcalf, *Aust. J. Exp. Biol. Med. Sci.* **4**, 287 (1966); D. Pluznik and L. Sachs, *Exp. Cell Res.* **43**, 553 (1966); B. Pike and W. Robinson, *J. Cell. Physiol.* **76**, 77 (1971)]. Although no exogenous source of CSF is supplied in our culture system, adherent bone marrow cells can elaborate endogenous CSF. Depletion of these CSF-producing cells, before plating, by allowing adherence to plastic or uptake of carbonyl iron, did not reduce the number or size of myeloma colonies. In addition, antibody to CSF did not appear to reduce the number of myeloma colonies. Therefore, we conclude that colony growth in our system is not dependent on CSF and that the contamination of myeloma colonies by granulocyte colonies is minimal.
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Harvesting Natural Populations in a Randomly Fluctuating Environment

Abstract. *As harvesting effort and yield are increased, animal populations that are being harvested for sustained yield will take longer to recover from environmentally imposed disturbances. One consequence is that the coefficient of variation (the relative variance) of the yield increases as the point of maximum sustained yield (MSY) is approached. When overexploitation has resulted in a population smaller than that for MSY, high effort produces a low average yield with high variance. These observations accord with observed trends in several fish and whaling industries. We expect these effects to be more pronounced for a harvesting strategy based on constant quotas than for one based on constant effort. Although developed in a MSY context, the conclusions also apply if the aim is to maximize the present value of (discounted) net economic revenue.*

The conventional theory of harvested populations (*1–5*) is based on equations in which the various environmental and biological parameters are treated as constants. But environmental randomness can have important effects on the dynamics of animal populations (*6, 7*).

Most animals that are harvested have a net population growth rate that is density dependent. In the unharvested state, the population is maintained around an equilibrium value, K, at which gains from recruitment balance losses from natural mortality. Harvesting constitutes an additional source of mortality; if the harvesting rate is steady and not too high, the population will settle to a new equilibrium value, $N^* < K$, at which the increased intrinsic growth rate balances the losses resulting from harvesting. To maximize this sustained yield, one seeks to determine the intrinsic population growth curve and to harvest at the rate that keeps the population at the maximum of the curve.

As a deliberately oversimplified example (*1, 3, 4*), consider a population $N(t)$ for which the intrinsic net growth rate is logistic, and where the rate of harvesting (the yield per unit time) is EN; here E represents the harvesting effort, and it is assumed that the catch per unit effort is linearly proportional to N. The net growth rate is then

$$dN/dt = rN(1 - N/K) - EN \quad (1)$$

This is illustrated in Fig. 1, in which the intrinsic growth rate rises and falls parabolically as N increases from 0 to the carrying capacity (K), while the harvesting losses increase linearly. For a strategy that keeps effort constant, the equilibrium population is

$$N^*(E) = K(1 - E/r) \quad (2)$$

and the sustained yield (Y) as a function of E is

$$Y(E) = EK(1 - E/r) \quad (3)$$

The maximum sustained yield (MSY) is attained for $E = r/2$, at which point $Y_{MSY} = rK/4$ and $N^* = K/2$. The logistic growth curve in Eq. 1 is only one of many broadly similar forms that have