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Method for Measurement of Self-Renewal Capacity of Clonogenic Cells from Biopsies of Metastatic Human Malignant Melanoma[1]

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ABSTRACT

A procedure was developed to directly measure the self-renewal capacity of clonogenic cells from biopsies of metastatic human malignant melanoma. A culture of colony-forming cells was performed with bilayer agar in microtiter wells. The number of live tumor cells from biopsies of melanoma tissue was determined and was used to calculate plating efficiencies. Sequential photography showed that cells did not migrate in agar, thereby documenting that all the cells within colonies were direct descendants of clonogenic cells. A calibrated pneumatically controlled micropipet attached to a micromanipulator was used to quantitatively remove melanoma colonies without removing adjacent cells or agar. Plucked primary colonies were mechanically disaggregated into single cells; viability was greater than 95% as determined by trypan blue dye exclusion. Dose-related formation of secondary colonies was observed after replating of cells from pooled primary colonies. Cells from individual colonies were replated, and secondary colonies formed. These techniques allowed a simple and direct assessment of the self-renewal capacity of colony-forming melanoma cells.

INTRODUCTION

Self-renewal capacity is a distinguishing property of stem cells (9, 20, 23). Normal stem cells are capable of extensive self-maintenance through self-renewal and produce cells which maintain tissues that have physiological or accidental removal of cells (9, 19). Animal tumor stem cells have self-renewal capacity and are responsible for the growth and regrowth of tumors after subcurative therapy (20). Studies of human tumor stem cells cannot use *in vivo* models, so that clonogenic assays have been developed to estimate the frequency and properties of human tumor stem cell populations (14, 18). These assays measure the ability of tumor cells to form multicellular colonies in semisolid medium, and the extent to which clonogenic assays measure tumor stem cells is largely unknown. However, if the capacity for self-renewal was detected in the direct descendants of clonogenic cells, it would suggest that at least some clonogenic cells are tumor stem cells.

There are a limited number of studies on the self-renewal capacity of human clonogenic cells, and they have used methylcellulose as the semisolid support. Normal human hemopoietic stem cells have been shown to have at least a limited capacity for self-renewal (1). Human leukemic clonogenic cells have also been shown to have capacity for self-renewal, and secondary plating efficiency was correlated with successful

remission induction in acute myeloblastic leukemia (2, 5). Another study concluded that some human ovarian tumor clonogenic cells have a self-renewal capacity (4). However, the replating techniques used in these studies to assess self-renewal capacity were indirect because the harvesting procedure also included cells which were not the direct descendants of clonogenic cells. Consequently, the number of direct descendants of clonogenic cells replated could not be determined precisely, and thus the proportion of cells within the colonies with self-renewal capacity was unknown.

This investigation describes an approach and method for a more direct assssessment of the self-renewal capacity of human clonogenic melanoma cells. We used 0.3% agar in the plating layer which prevented migration of cells. Direct quantitative removal of colonies from the agar with a calibrated pneumatic micropipet allowed replating of colony cells without contamination by non-colony cells. The replating of cells from colonies gave reproducible, dose-related formation of secondary colonies.

MATERIALS AND METHODS

Preparation of Cells from Biopsies. Tumor tissue was obtained under aseptic conditions by excisional biopsy of s.c. nodules from patients with metastatic malignant melanoma (protocol approved by the University of Arizona Committee on Human Subjects). Tumor tissue was cut free of necrotic and normal tissue and minced into 1-sq mm pieces or less by extensive slicing with scissors. The tissue was placed into a 50-ml conical tube containing Ham's F-10 medium (Grand Island Biological Company, Grand Island, N.Y.) with 10% heat-inactivated fetal calf serum (KC Biologicals, Inc., Lenexa, Kans.), penicillin (100 μg/ml), and streptomycin (100 units/ml; Eli Lilly, Indianapolis, Ind.) and inverted several times. Tumor pieces and macroscopic clumps were allowed to settle to the bottom of the tube for 5 to 10 min at unit gravity, and the supernatant containing the single cells was aspirated. Tumor pieces were resuspended in media, and the process was repeated several times until the supernatant was clear. Cells were pooled, counted, tested for viability by exclusion of 0.4% trypan blue (Grand Island Biological Co.), and stored in liquid nitrogen in 10% dimethyl sulfoxide (spectrophotometric grade; Aldrich Chemical Co., Milwaukee, Wis.) in Ham's F-10 medium containing 10% heat-inactivated fetal calf serum. Cryopreserved cells were used in these experiments. Previous work using clonogenic human myelogenous cells has shown similar characteristics of replating using fresh or cryopreserved cells (5).

Estimation of Live Tumor Cells. Viability of fresh and cryopreserved cells derived from biopsies of human malignant melanoma was determined using exclusion of 0.4% trypan blue dye. Cells that excluded the dye were considered as viable. Viable cells were divided into 2 groups based on size because morphological identification of the cells after cytocentrifugation and Papanicolaou staining showed that all the small nucleated cells were mature lymphocytes (Chart 1). Thus, the groups were defined as cells which were 4 to 7 μm in diameter (corresponding to mature lymphocytes) and those greater than 7 μm in diameter. The group of larger cells included both tumor cells and normal large cells such as macrophages. The morphology of cells was
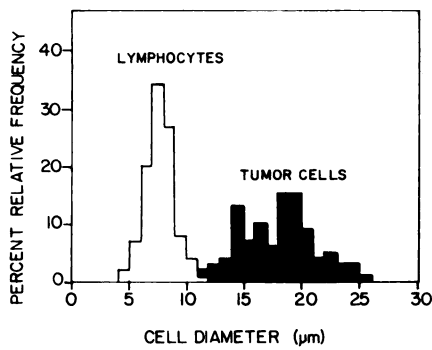
Chart 1. Size of lymphocytes and tumor cells. Cells were obtained from a biopsy of melanoma, cytocentrifuged, fixed, and Papanicolaou-stained, and 100 lymphocytes and tumor cell diameters were recorded. Data are from a representative patient.

examined by light microscopy after Papanicolaou and Wright-Giemsa staining. Large host and malignant melanoma cells were clearly distinguished on the basis of the amount of cytoplasm, nuclear characteristics, and cellular staining pattern. It was difficult routinely to further distinguish the types of large viable cells as either melanocytes or macrophages because both cell types can contain pigment granules and both have the ability to phagocytize and adhere (15). Thus, in some cases in which normal large viable cells were present, the number of viable tumor cells may have been overestimated. However, the estimate of large viable tumor cells excluded the dead cells and viable mature lymphocytes and thus was a better estimate of the cells that could form colonies than was the number of total nucleated cells.

Bilayer Agar Microtiter Culture System. Our clonogenic culture system was a modification of the agar system developed by Hamburger and Salmon (7) and described extensively elsewhere (14). We have simplified the culture conditions for clonogenic melanoma cells and performed the assay in microtiter wells. The underlayer was 0.5% agar (Bacto; Difco Laboratories, Detroit, Mich.) in Ham's F-10 medium which contained 10% heat-inactivated fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 units/ml). The plating layer was 0.3% agar in the same medium as the underlayer, with freshly added animal-derived insulin (1.54 units/ml; Eli Lilly), glutamine (0.45 μg/ml), pyruvate (0.34 μg/ml), and mercaptoethanol (0.77 mM; Grand Island Biological Co.). Only a limited number of cells from colonies were available for replating; therefore, we reduced the number of cells needed by using 0.075-ml volumes of underlayer and plating layer in microtiter wells in both the primary and the secondary platings. We placed 0.075 ml of the 0.5% agar underlayer into the 60 interior wells of a standard 96-well microtiter tissue culture tray (Nunc; Grand Island Biological Co.), and 0.3 ml of Ham's F-10 medium into the 36 outside wells to preserve humidity. The microtiter trays were incubated in a 5% CO_2–95% air atmosphere at 37° which was humidified by placing water in the bottom and in a tray on the top shelf of the incubator and turning the door heater off. Colonies greater than 60 μm in diameter were enumerated after 16 to 18 days in culture. We have previously shown by light microscopy, transmission electron microscopy, and karyology (14, 16) that the cells within the colonies were human malignant melanoma.

Removal of Colonies with a Micromanipulator. Colonies were removed aseptically at room temperature by placing the inverted microscope and micromanipulator inside an isolation box sterilized by UV. This provided a dead-air space and avoided the vibrations associated with the use of HEPA filtered clean air hoods. Colonies greater than 100 μm in diameter were removed after 16 to 18 days of growth. Micropipets were hand made by drawing 100-μl measuring capillary pipets (Kimble 71900; Owens-Illinois, Toledo, Ohio) to a fine point, using a flame and breaking off the end. Tips with different-sized apertures were obtained, and those from 180 to 200 μm in diameter were selected and autoclaved. The micropipets were pneumatically

connected via autoclaved Tygon tubing (VWR Scientific, Phoenix, Ariz.) to a microsyringe (Gilmont, S-1100; VWR Scientific) equipped with a Luer joint and a 23-gauge needle. This microsyringe had a total capacity of 0.2 ml and could easily and slowly aspirate μl amounts of media. The micropipets were attached to a micromanipulator (M33; Stoelting Co., Chicago, Ill.) which was capable of precisely and smoothly moving the tip of the micropipet within the μm range required for dissection of the colonies out of the agar. The micropipets were held at an approximated 30° angle so that colonies would collect near the bend of the pipet and not travel all the way up the pipet with the media. This made it easier to rinse them out by expelling the media. The set-up of these components is shown in Fig. 1.

Before the colonies were removed, 0.150 ml of fresh plating media minus the agar was added to each well. The tip of the micropipet was placed into the well and prefilled with 5 μl of medium by aspiration with the microsyringe. The tip was moved down through the agar to the side of the colony with the micromanipulator while observing through an inverted microscope (×40 to ×100). Next, the tip was moved completely around the edge of the colony, thus freeing it from the agar. The aperture of the micropipet was placed directly above and over the colony. Finally, each colony was pulled slowly into the micropipet by aspiration of medium (less than 5 μl) with the microsyringe. Individual colonies were stored in the micropipet while other colonies were collected until 20 were collected. They were then expelled into a plastic 12 x 75 mm tube (Falcon 2054). After 80 colonies were collected, the tube was tapped vigorously with a finger 25 times, and a single-cell suspension was obtained. A portion of the cells were counted and used to determine trypan blue viability before replating the cells in the microtiter tray at concentrations from 37 to 1500 tumor cells/well. Alternatively, for some experiments, single colonies were collected in the pipet and transferred for replating studies as will be described separately below.

Plating and Culture of Cells. Cells were suspended at different concentrations in 0.275 ml of plating medium minus the agar. For each different concentration of cells, 0.3 ml of 3.0% liquid agar stored in a 50° water bath was added to 1.2 ml of plating layer and mixed. Next, 0.275 ml of this 0.6% agar plating layer was added to the 0.275-ml cell suspension and mixed, and then 0.075 ml of the complete plating layer was placed into each of 6 microtiter wells. Each different concentration of cells was plated separately and quickly because once the 3% agar is removed from the 50° bath it must be diluted, added, and mixed before it gels prematurely. The serial transfer experiments were done using the procedures above with cells from secondary colonies, tertiary colonies, etc.

Transfer and Culture of Cells from Individual Colonies. There were very few cells available from individual colonies; therefore, another procedure was used to plate them in separate wells. When individual colonies were removed, the micropipet was prefilled with 35 μl of plating medium. Thus, when an individual colony was removed with 5 μl of medium, a total of 40 μl was available to rinse the colony into a 6- x 50-mm glass tube. The tube was tapped vigorously 25 times with a finger to obtain a single-cell suspension. Next, 0.040 ml of a 0.6% agar plating layer solution was made by adding 0.3 ml of 3.0% agar to 1.2 ml of plating layer medium. This was added to the tube containing the cell suspension and mixed. This plating layer with cells was quickly transferred to a well. The plating of cells from each colony was completed before starting to plate cells from other colonies.

RESULTS AND DISCUSSION

Agar was used as the semisolid support media to prevent the migration of cells which occurs when methylcellulose is used (12). Fig. 2 shows the sequential development of colonies. Groups of 2 cells were identified after 3 days of culture (Fig. 2a), and growth continued after 6 days (Fig. 2b) and 9 days (Fig. 2c). Partial decay of colonies was evident after 35 days

(Fig. 2d). The nonclonogenic cells in the background (arrows) did not migrate during colony formation and decay. Thus, the cells within the colonies were direct descendants of the clonogenic cells. Metcalf et al. (13) have reported similar observations by noting the exact location of bone marrow cells during culture in 0.3% agar. They saw no evidence of movement. This suggests that cells in agar do not migrate into groups of cells to form pseudocolonies. This is an important advantage of the bilayer agar system compared to methylcellulose in which colonies may also contain cells that migrated into the colonies (12). A disadvantage of the agar system is that the colonies cannot be recovered as easily as can the colonies in methylcellulose which can be recovered for replating by simple dilution (5).

Plating efficiencies are generally calculated by dividing the number of tumor colonies formed by the number of nucleated cells plated (11, 14). Using this approach, comparisons of primary and secondary plating efficiencies are tentative as different populations of cells are plated. Primary colonies are formed from cell populations derived from biopsies of tumor tissue which often contain many dead cells and mature lymphocytes, neither of which are expected to form colonies. Secondary colonies are formed from a cell population which is essentially 100% viable tumor cells derived from primary colonies. Thus, to make more meaningful comparisons, we have estimated the proportion of viable tumor cells in the nucleated cell population derived from the biopsies.

Estimates of the proportion of viable tumor cells were obtained by categorizing the cells as dead, small viable, or large viable. Fig. 3 shows a dead cell which took up trypan blue, a 6-μm-diameter small viable cell, and a 14-μm-diameter large viable cell. Several studies (17, 21, 22) have shown that the ability of cells to attach, proliferate, or incorporate amino acids was lost before the ability to exclude trypan blue, and thus cells that cannot exclude dye would not be expected to form colonies. Examination of Papanicolaou-stained cytocentrifuge preparations of cells from biopsies of melanoma showed that melanoma tumor cells were much larger than lymphocytes and can be distinguished from them entirely by size. Chart 1 shows the frequency of lymphocytes and tumor cells by size from a representative patient. The mean diameter in cell preparations from 11 patients was 7.5 ± 0.15 (S.E.) μm for lymphocytes and 18.2 + 1.4 μm for tumor cells. The only small nucleated cells present were mature lymphocytes; therefore, the small viable cells in the trypan blue preparations can be considered lymphocytes. The proportion of viable tumor cells can be overestimated because normal large viable cells can also be present. Estimates of the proportion of large viable cells from biopsies were low; medians from 11 samples of fresh and cryopreserved cells were 6.2 and 7.0%, respectively (Table 1). Thus, the absolute or tumor plating efficiency (the number of tumor colonies per number of viable tumor cells plated) averaged about 20 times higher than the relative plating efficiency which uses the number of total nucleated cells as the denominator. The absolute plating efficiency may be still higher if there are significant numbers of normal large viable cells present. These results are in contrast to those of Buick et al. (3), who reported mean tumor cell percentages of 48.2% in 37 malignant effusions (20 ovarian, 11 breast, and 6 other cancers); however, the viability of cells in these effusions were over 90%, while the mean viability of cells from biopsies of melanoma

Table 1
Percentage of dead, small viable, and large viable cells in fresh and cryopreserved melanoma tumor cell preparations

Fresh cells				Cryopreserved cells			
Patient code	Dead	Small viable	Large viable	Patient code	Dead	Small viable	Large viable
81.48	49.2	4.5	46.3	80.54	82.5	12.6	4.9
81.49	89.2	3.8	7.0	80.62	87.6	0.8	9.6
81.50	98.3	0.7	1.0	81.13	46.6	46.8	5.6
81.51	70.0	26.6	3.4	81.30	87.3	2.7	10.0
81.52	27.0	69.0	3.8	81.38	92.5	0.5	7.0
81.54	63.0	31.0	6.0	81.46	92.8	2.2	5.2
81.55	53.0	0	47.0	81.47	68.0	24.3	7.6
81.58	82.6	2.8	14.0	81.58	91.0	4.7	4.3
81.59	92.2	0	7.8	81.56	67.1	21.1	11.8
81.61	91.8	1.8	6.2	81.44	95.7	2.3	2.0
82.1	95.0	0.8	4.2	81.61	86.6	1.0	12.4
Median	82.6	3.8	6.2		87.3	2.2	7.0

tissue was, respectively, 17.4 and 12.7% from fresh and cryopreserved samples. The fact that relative plating efficiency was lower than the absolute plating efficiency is in agreement with the analysis of Metcalf (11), who showed that current estimates of plating efficiency tend to be underestimates because they are calculated as the frequency of colonies per number of starting cells rather than per the number of cells of a known, uniform type.

Previous procedures have generally utilized hand-held finely drawn Pasteur pipets to remove colonies from agar with a gentle scooping action (1, 6, 8, 11, 12). This seemingly simple procedure is in practice difficult to perform because of the small size of the colonies, usually only 0.10 to 0.30 mm in diameter. Hand movements are not steady enough to guide the pipet and consistently remove colonies from the agar without also removing chunks of agar and adjacent cells.

We modified these procedures by attaching a finely drawn 100-μl calibrated pipet to a micromanipulator. Micromanipulators are readily available and can easily and precisely position and move the pipet in the μm range required for consistent removal of colonies from agar. Another important modification was the use of a microsyringe which was pneumatically connected with flexible tubing to the finely drawn pipet (Fig. 1). This approach isolated vibrations and allowed controlled aspiration of μl amounts of media so that colonies could be drawn up to the pipet slowly and expelled into another container or onto a slide as needed. This technique prevented the nonspecific removal of adjacent agar and non-colony cells which occurred with conventional techniques which use larger aspiratory forces. The melanoma colonies seemed to push back the agar as they grew, forming small pockets, so that when the pipet tip was moved around the edge of the colonies it readily dislodged the colonies from the agar. Fig. 4 shows a typical colony removal sequence. The culture plate was moved to position a colony in the center of field of the inverted microscope (Fig. 4a). The micromanipulator was used to move the pipet tip through the agar over and around the colony (Fig. 4b), which freed it from the agar. The pipet tip was then positioned directly over the colony which was pulled into the tip by aspirating 2 to 3 μl of media with the microsyringe (Fig. 4c). This procedure easily and quantitatively removed the colonies without removing the adjacent cells or agar (Fig. 4d, arrows). Colonies can be removed aseptically by an experienced oper-

ator at the rate of approximately 30/hr.

Cells from patients which grew colonies greater than 100 μm in diameter were used. Approximately 80 primary colonies were collected for each transfer. Repetitive tapping of the tube containing the colonies was sufficient to disaggregate the colonies into single-cell suspensions. Trypan blue dye exclusion was done after counting the cells and viability was greater than 95% in all cases. Cell loss for pooled colonies during transfer was less than 5.0%. Chart 2 shows the results from a representative patient (Table 2, Patient A). Primary colonies were formed in a linear fashion according to the number of

viable tumor cells plated. Primary colonies were removed after 18 days and secondary colonies formed which were similar in size and morphology to the primary colonies. The secondary, tertiary, and later colonies were formed in a linear fashion according to the number of cells plated and with similar frequency as primary colonies until the ninth transfer (Chart 2). Then, the frequency of colony formation was significantly lower. No colonies formed after the 10th serial transfer in this case.
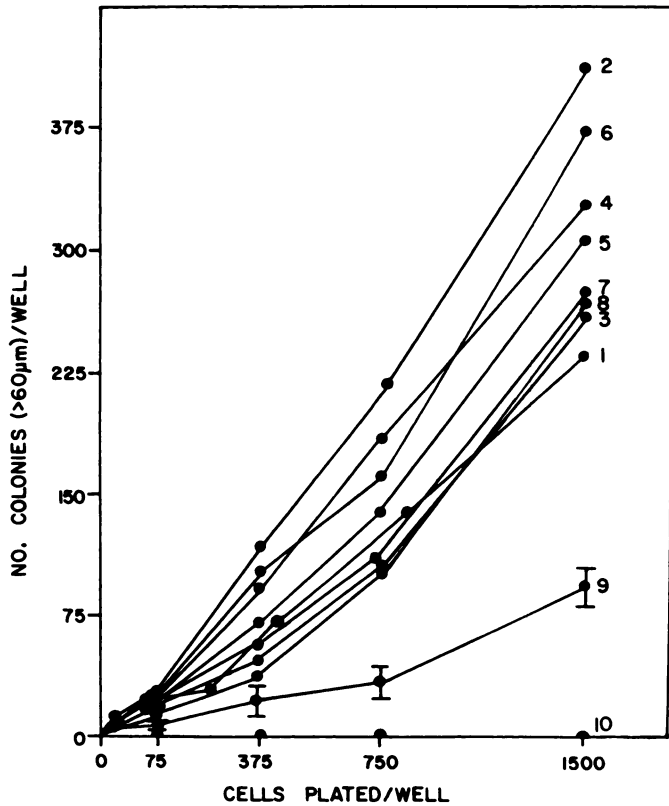

Chart 2. Melanoma colony formation from cells obtained from melanoma tissue (*Curve 1*) and serial transfers of cells from pooled colonies (*Curves 2* to *9*). No colonies were formed after the ninth transfer. Data are the means of 6 replicates. The standard errors averaged 11% and were omitted for clarity except for colonies from the ninth transfer.
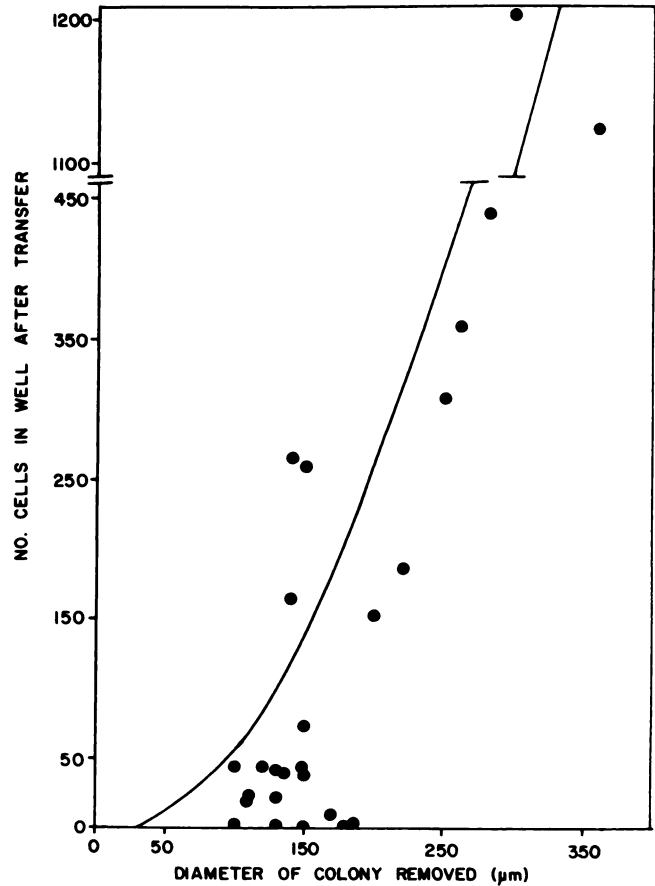

Chart 3. Efficiency of transfer of individual colonies. Colonies of different diameters were removed and replated into separate wells. The number of cells in each well was obtained and compared to the number of cells expected (————) for a colony of corresponding size. Cell loss was less than 50% in 14 of 27 transfers.

Table 2

Cloning efficiency of human melanoma cells in soft agar with serial transfers in agar

Patient	% of cloning efficiency[a]									
	Plating 1[b]	Plating 2	Plating 3	Plating 4	Plating 5	Plating 6	Plating 7	Plating 8	Plating 9	Plating 10
A-1 (80–56)[c]	16.2	28.3	15.4	23.3	19.1	24.0	15.4	16.2	7.7	0
A-2 (80–56)	20.9	—[d]	—	—	—	—	—	—	—	—
B (80–54)	0.10	3.7	4.8	4.4	2.2	0	—	—	—	—
C (81–6)	2.23	4.1	6.4	7.6	13.4	13.9	5.2	11.2	11.7	11.5
D-1 (80–62)	10.0	0	—	—	—	—	—	—	—	—
D-2 (80–62)	9.3	0	—	—	—	—	—	—	—	—

[a] Cloning efficiency represents the absolute (or tumor) cloning efficiency as discussed in "Materials and Methods" and is calculated from the mean of close response curves from 38 to 1500 viable tumor cells plated per microtiter well.
[b] Primary plating (1) represents the culture of cells from the original tumor specimen. Serial transfer (2, 3, . . .) of cells in colonies was accomplished by direct removal of colonies using a micromanipulator.
[c] Numbers in parentheses, code number.
[d] —, not done.

We have also studied the replating characteristics of pooled primary colonies from an additional 3 patients (Table 2). Cells from Patient B increased their cloning efficiency in the tertiary and 4° colony formation, but with the 5° colony formation the cloning efficiency decreased markedly (from 4.4 to 2.2%) and nonlinearity below 750 cells plated was evident. Cells replated from 5° colonies failed to form colonies at even the highest (1500) number of tumor cells plated. Cells from Patient C demonstrated a gradual increase in cloning efficiency with successive replatings (4.1% in 2° colonies to 13.9% in 5° colonies). A decrease in cloning efficiency to 5.2% was noted
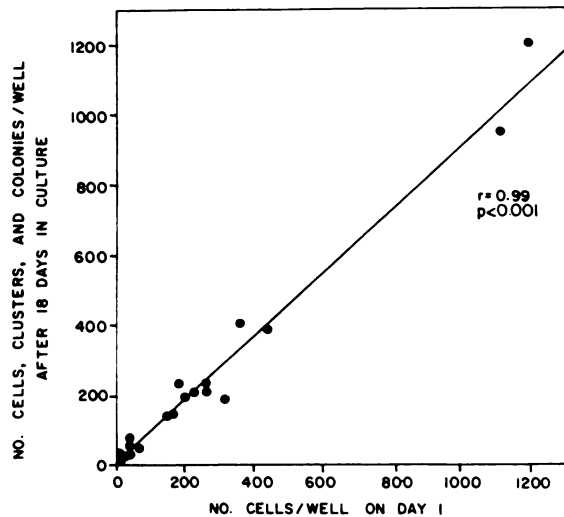
Chart 4. Number of cells in each well after individual colony transfer compared to the total number of cells, clusters, and colonies after 18 days of incubation.
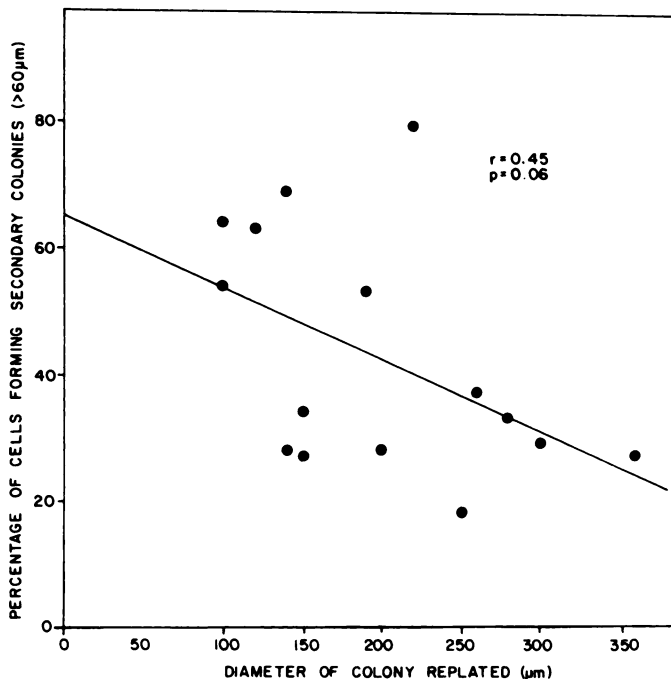

Chart 5. Secondary colony formation related to the size of the colony removed and replated. The rate of formation of secondary colonies may have decreased for the larger colonies.

in the 7° colonies, but nonlinearity at low cell numbers plated did not occur. The cloning efficiency of replated cells has remained at about 11% from the 8° to 10° colonies. Cells from primary colonies of Patient D did not form secondary colonies.

In addition to our investigations with pooled primary colonies, we have studied the replating characteristics of cells from individual colonies which were removed, dispersed into single cells, and plated separately into microtiter wells. The collection and transfer of cells from single colonies were less efficient than those of pooled colonies. The efficiency of transfer was measured by counting the number of cells in the well immediately after plating and comparing it to the number expected for a colony of that diameter calculated from a nomogram that we have developed which relates colony diameter, cell diameter, and the number of cells within a colony.[3] In 14 of 27 cases of individual colony transfer, the loss of cells was less than 50% (Chart 3). The cases in which more than 50% of the cells were lost during the transfer procedure were excluded from subsequent analysis as selective loss of cell subpopulations may have occurred. The cells plated from each primary colony remained in the agar as single cells or formed clusters and colonies. Chart 4 shows that the number of cells in the well on Day 0 was very nearly the same as the number of cells, clusters, and colonies on Day 18 ($r = 0.99$, $p = 0.001$). The cells did not lyse during incubation, and the ratios of cells and clusters were therefore accurate. Secondary colonies were formed from cells of each colony in all cases (Chart 5). Linear regression analysis gave $r = 0.45$ and $p = 0.06$ which suggests an inverse relationship between secondary colony formation and size of the primary colony. These data, from the same patient shown in Chart 2, suggested that the secondary colony formation seen with the cells from pooled colonies was not due to a few colonies which contained a very high proportion of cells with the ability to form secondary colonies. The ability to transfer single colonies will allow us to directly compare the chemosensitivity of clonogenic and self-renewing units and to analyze for preadaptive and postadaptive acquisition of drug resistance utilizing the method of fluctuation analysis as originally proposed by Luria and Delbruck (10).

The techniques that we have described allowed the direct assessment of the self-renewal capacity of melanoma colony-forming cells. We used 0.3% agar and quantitative removal of colonies with a calibrated pneumatic micropipet attached to a micromanipulator to assure the replating of only the direct descendants of clonogenic cells. The experiments discussed here and our ongoing studies suggest that at least some clonogenic cells have the self-renewal capacity of tumor stem cells. The quantitative removal of colonies has also allowed us to determine the number of cells within colonies of different sizes in detail, and we plan to use these data to begin to describe the kinetics of cellular proliferation of the clonogenic compartment of metastatic melanoma tumors.

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[3] F. L. Meyskens, Jr., and S. P. Thomson, unpublished observations.

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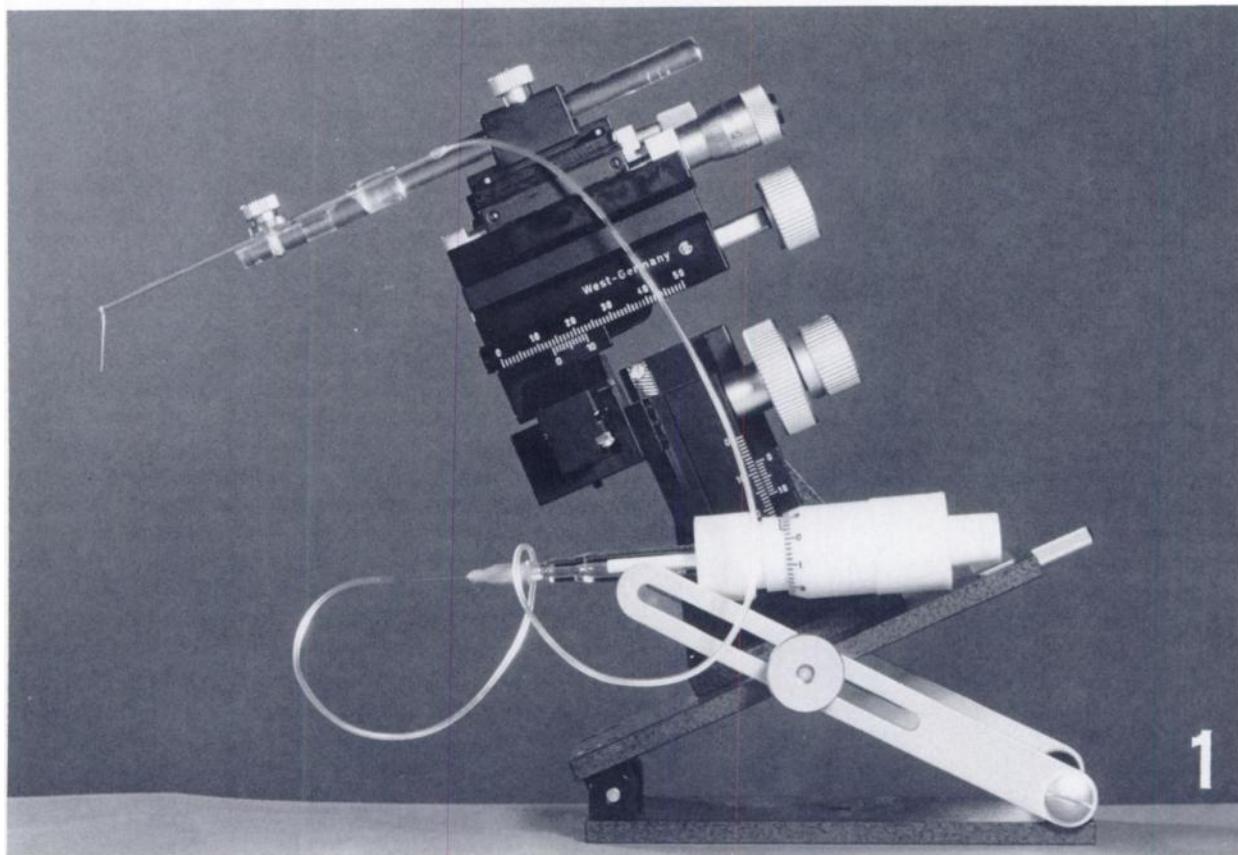

Fig. 1. Micromanipulator holding a microcapillary pipet. The micromanipulator allows precise movement of the pipet in X, Y, and Z axes. The calibrated pipet is connected pneumatically via Tygon tubing to a microsyringe which is hand held during use.
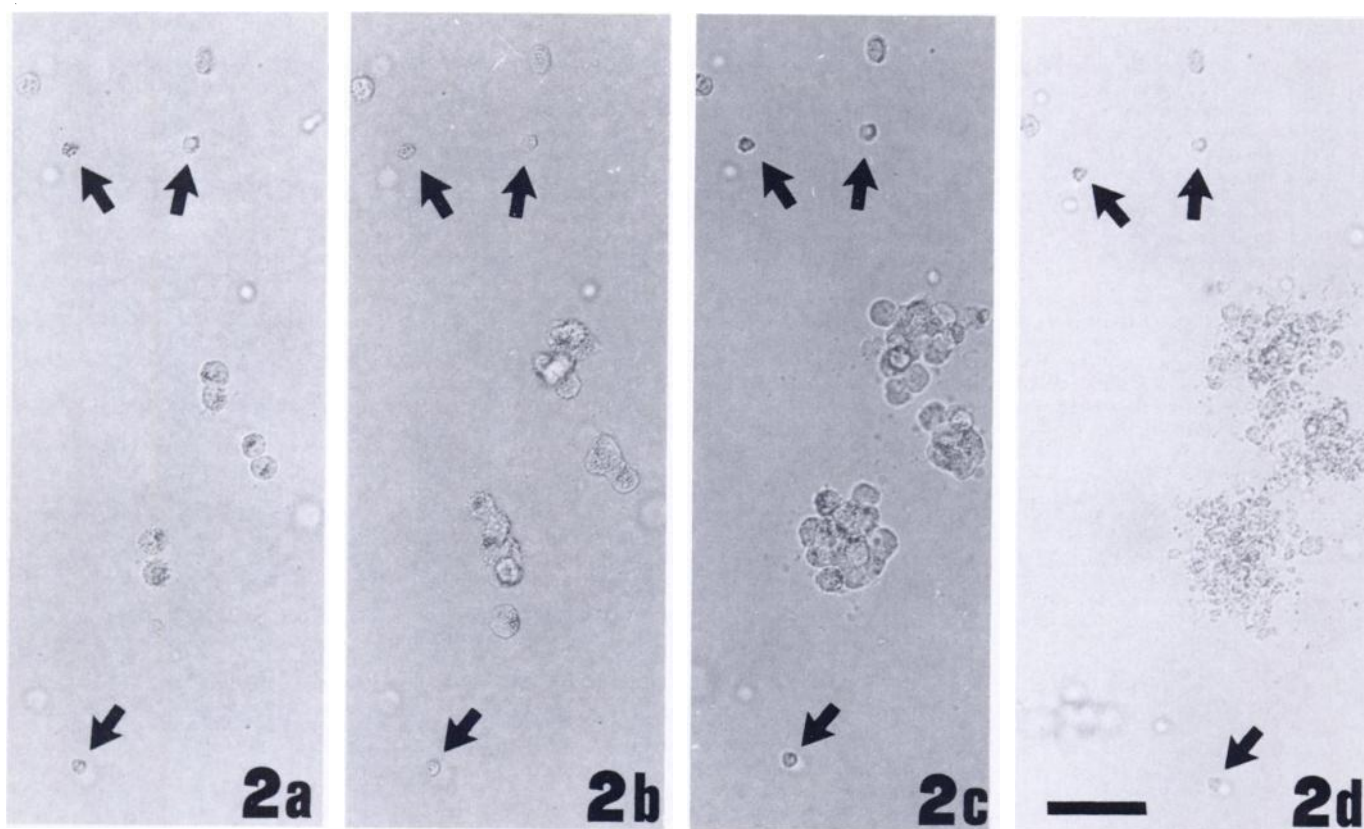
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Fig. 2. Sequential development of colonies in agar. Groups of 2 cells appeared after 3 days (a). Growth continued after 6 days (b) and 9 days (c). Partial decay of the colonies was seen after 35 days of incubation (d). Note the surrounding cells (arrows) which did not migrate during colony development. Bar, 70 μm.
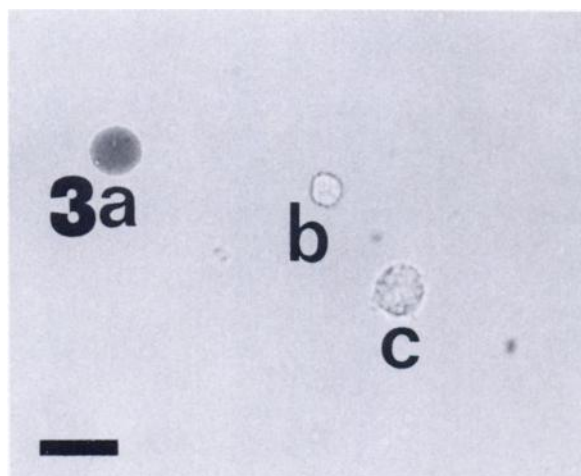

Fig. 3. Typical morphology of cells during the test for exclusion of trypan blue. a, dead cell; b, small viable cell; c, large viable cell. Bar, 20 μm.
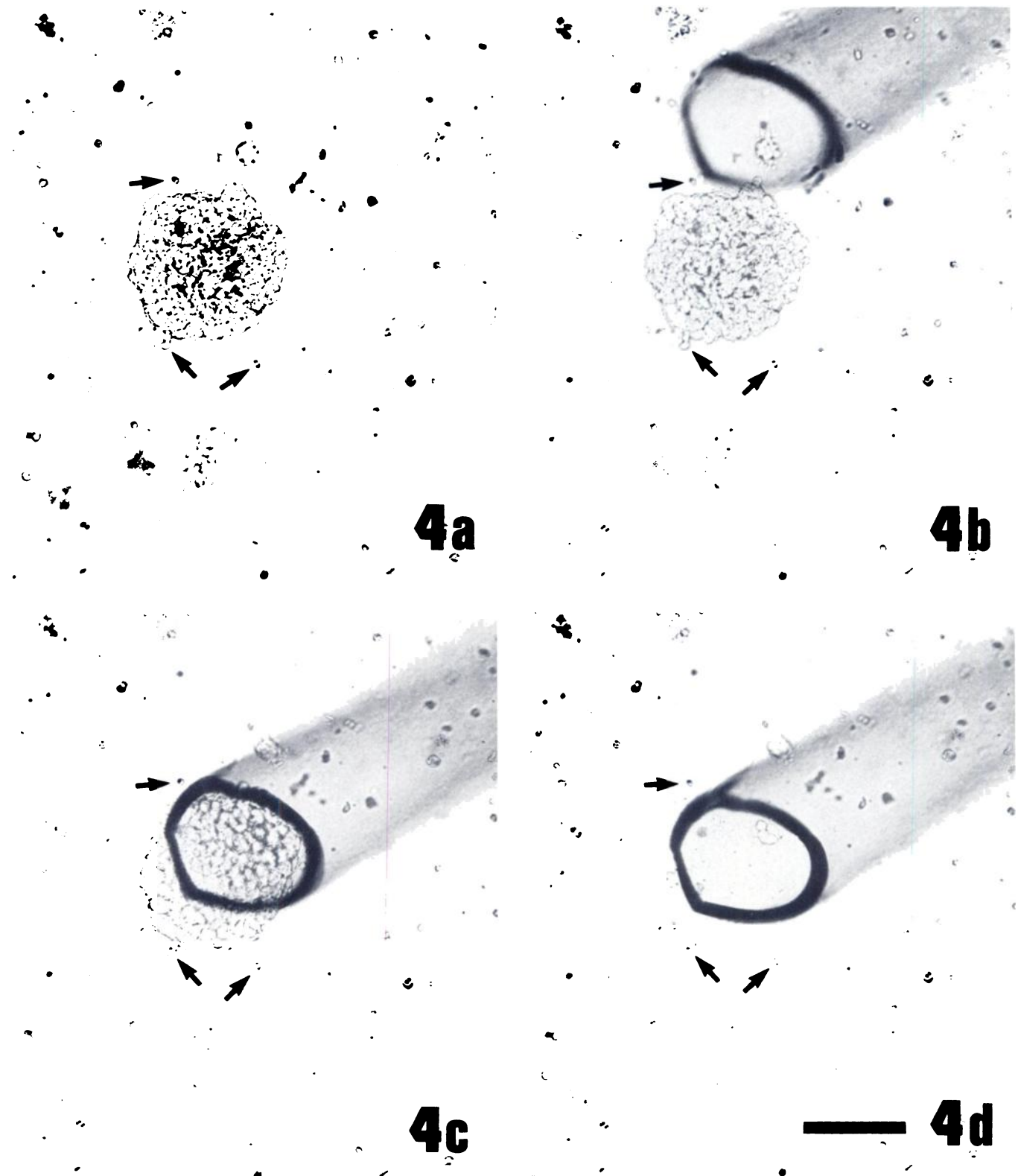
Fig. 4. Melanoma colony removal sequence. A 200-μm diameter colony was positioned in the center of the field (*a*). The micropipet was moved through the agar around the edges of the colony (*b*). This freed the colony, and the pipet aperture was placed directly over the colony (*c*). Aspiration of 1 μl of media pulled the colony into the pipet (*d*). Note that the adjacent cells (*arrows*) and agar were not removed with the colony. *Bar*, 200 μm.

Cancer Research

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