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Kinetics of Clonogenic Melanoma Cell Proliferation and the Limits on Growth Within a Bilayer Agar System

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Accurate descriptions of the kinetics of cell growth in semi-solid agar clonogenic systems have been difficult because the number of cells in colonies of different sizes is largely unknown. We stained and removed tumor cell colonies from agar, directly counted their cells, and established equations to quantitate the number of cells within colonies of different sizes. We used these equations to quantitate, in terms of cell number and volume, the total amount and kinetics of clonogenic cell proliferation from biopsies of human melanoma and cell lines of several different tumor types. Daily observations of cells in agar and serial photography indicated a 0- to 4-day delay in the onset of proliferation in agar followed by rapid growth and then abrupt cessation of proliferation. We quantified the extent of proliferation of cells from melanoma biopsies of seven patients and 11 cell lines after they were allowed to proliferate in agar until they stopped. Approximately 10% of cells divided one to five times while only 0.01% divided six to nine times. The total number of cells within the colonies at the end of growth was different while the total volume of cells within the colonies per plate was similar; approximately 10^9 μm^3 cellular volume per plate represents an upper limit for proliferation within the closed, nonrefed bilayer agar system. Previous replating studies using the same biopsy cells have shown that clonogenic melanoma cells can self-renew and have more proliferative capacity than that expressed during primary colony formation. Thus, the clonogenic assay only measured initial proliferative capacities. Furthermore, variable delays in the onset of proliferation may contribute to the heterogenity of colony size within clonogenic assays.

The stem cell model provides a paradigm for the characteristics of normal and neoplastic renewing cell populations. The model proposes a hierachy of cellular proliferative capacities, from transit cells with limited capacity for cell division, to stem cells; those with extensive proliferative capacity which can renew the entire populations of cells and themselves through "self-renewal" (McKillop et al, 1983; Tannock, 1983; Steel, 1977). This stem cell model has been used extensively and successfully in studies of normal and malignant hematopoietic cells (reviewed by Till and McCulloch, 1980; and Metcalf, 1977) and more recently in studies of solid tumor cell biology. Several characteristics of human tumors are consistent with the stem cell model (Tannock, 1983; Selby et al., 1983) including that the fractionation of human tumor cells by density and volume can separate subpopulations of varying proliferative activities and clonogenic potentials (Mackillop et al., 1983). Stem cells may be responsible for the cellular maintenance of tumors and their regrowth after subcurative therapy (Steel 1977; Tannock, 1983) and thus are central cells for the study and therapy of tumors.

Clonogenic assays measure cells capable of forming a multicellular group of descendants, a colony, either in vivo or in vitro. Several in vitro clonogenic assays which attempt to measure human tumor stem cells are in use and were developed from extensive experience with cell lines, hematopoietic cells, animals tumors, and human tumor xenografts (reviewed by Selby et al., 1983). Clonogenic cells are defined by their ability to form a specified size of colony within an assay. Tumor stem cells by definition are able to produce a tumor and have self-renewal capacity. The extent that clonogenic cells measure tumor stem cells is largely unknown; however, data suggest that at least some clonogenic cells are stem cells. Human leukemic blast (Buick et al., 1979), ovarian (Buick and Mackillop, 1981), and melanoma (Thomson and Meyskens, 1982) clonogenic cells form primary colonies which contain cells with the capacity to form secondary colonies upon replating; that is, some clonogenic cells can self-renew. Furthermore, clonogenic cells from oat cell lung carcinoma and metastic melanoma cancers (Carney et al., 1981; Meyskens and Thomson, 1984) formed primary colonies which contained cells capable of producing tumors when injected into athymic nude

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mice. These data suggest that clonogenic tumor cell assays may be useful in studies of human tumor stem cells.

In a recent critique of clonogenic assay Selby et al. (1983) described several significant developmental problems that should be solved to increase the utility of clonogenic assays for studies of human tumor biology and chemosensitivity testing. One problem was the unusual radiation survival curves our laboratory reported (Meyskens, 1980) for melanoma clonogenic cells assayed by the widely used Hamburger-Salmon technique (Hamburger and Salmon, 1977). We have recently shown that the unexpected plateaus were artifactual and can be avoided through the use of controls which assess cellular aggregates within the cell suspensions prepared from tumors (Meyskens, 1983). Another significant developmental problem has been the inability to accurately quantitate clonogenic growth in terms of cell numbers. In all but relatively small colonies the cells overlap and stack up on each other within the colony, which prevents accurate counting of the cells. Thus, little is known about the rate and extent of cell formation within clonogenic agar systems. In this study we removed colonies from the agar, directy counted the cells, and developed equations relating colony diameter to the number of cells within the colony. Then we used these equations to define the kinetics and extent of proliferation of melanoma clonogenic cells in agar. We found a variable delay in the onset of exponential growth and showed that the bilayer agar system can only support growth to an upper limit of approximately 10^9 μm^3 cellular volume.

MATERIALS AND METHODS

Preparation of melanoma biopsy cell samples

Tumor tissue was obtained under aseptic conditions from patients with metastatic malignant melanoma (Protocol approved by the University of Arizona Committee on Human Subject). Tumor tissue was cut free of necrotic and normal tissue and minced into 1-mm^2 pieces or less by extensive slicing with scissors. The tissue was placed into a 50-ml conical tube containing Ham's F-10 medium (Gibco) with 10% heat-inactivated fetal bovine serum, penicillin (100 mg/ml), and streptomycin (100 units/ml), and was inverted several times. Tumor tissue 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 single cells was aspirated. Tumor pieces were resuspended in media and the process was repeated several times until the supernatant was clear. Cells were washed, pooled, counted, and tested for viability by exclusion of 0.4% trypan blue. Some of these cells were immediately cultured in agar and the remaining cells were cyropreserved. The seven cyropreserved cell samples used in this study were chosen out of over 100 processed tumors because they were true single-cell suspensions which grew well in agar.

Quantitative methods

Groups of cells which arose from single cells were generically designated as growth units for the purpose of this investigation, instead of using clusters and colonies to define large and small cell groups. We used three samples from biopsies and a mouse melanoma cell line (Cloudman 53.1, CCL) to quantitate the number of cells per growth units of different sizes. In brief, growth units from 30 to 250 μm in diameter were removed after staining with toluidine blue, placed on a slide, and overlaid with a coverslip. This allowed direct counting of the cells within each specific colony. The natural logarithm of diameter and number of cells in the growth units of each sample were mathematically related using linear regression coefficients; their standard error and the multiple correlation coefficient was also calculated (Draper and Smith, 1981). These procedures have been described in detail for a concurrent study that analysed the number of cells per growth unit from several tumor types (Meyskens et al., 1984a). This analysis of growth units from 38 samples established this general equation: no. of cells/growth unit = 2.40 (growth unit diameter)$^{2.378}$/(cell diameter)$^{2.804}$. This equation was used to calculate the number of cells per growth units of different sizes from the samples in the current study.

The frequency of growth units was determined by direct microscopic enumeration of growth units of all sizes within randomly chosen areas of 1 mm^2, calculating the mean $\pm$ S.E., and multiplying by 909 mm^2/35 mm plate to obtain the mean $\pm$ S.E. number of growth units/plate. Size distributions were obtained by measuring the diameter of 100 to 200 consecutive growth units in agar to the nearest 10 μm size class using a micrometer scale, e.g., a 67-μm diameter growth unit would be classified as 70, an 82 as an 80-μm, etc. The frequency of growth units of different sizes was calculated as the product of the relative frequency, obtained from the size distributions, and the total number of growth units per plate. We used an automated image analysis system (Salmon et al., 1984) to obtain the number of size of growth units from the two biopsy samples used in the study of proliferation kinetics. The total number of cells formed per plate was calculated as the product of the number of growth units of each size per plate and the number of cells within those growth units, as calculated using the above equation. The total volume of cells was calculated as the product of the number of cells formed per plate and the cell volume. Cell volumes were calculated using $4/3\ \pi r^3$, with r derived from the mean diameter of cells within the growth units, which was specific for each source of cells. Cloning efficiencies were calculated by dividing the number of growth units by the number of viable tumor cells. (Thomson and Meyskens, 1982).

Kinetics and extent of proliferation in agar

The initial analyses were daily microscopic examinations for the presence of growth, two cell groups or larger. Subsequently, serial photography of the same field was done each day on three cell samples from melanoma biopsies. Detailed kinetic studies were performed on two cell samples from melanoma biopsies and cells from a murine melanoma cell line. We placed a large group of control plates in the incubator on day 0 and sequentially removed three to four plates until growth stopped. We enumerated the number and sizes of growth units and calculated the total number of volume of cells formed within the growth units at each time point. The upper limit to clonogenic cell proliferation for cell samples from seven melanoma biopsies and 11 cell lines was determined by allowing the plates to incubate until no further growth was seen, enumerating the number of size of the growth units, and then calculating the total number of volume of cells within the

growth units. Refeeding experiments were done by adding an extra amount of the original media formulation on top of the bilayer agar after 7 to 12 days of incubation and enumerating growth units after growth stopped.

Culture of cells in bilayer agar

The conditions for culture have been extensively described elsewhere as a modification of the method of Hamburger and Salmon (1977). We used a simplified medium in a bilayer agar system constituted in 35 mm-diameter plastic Petri (Falcon) dishes (Meyskens et al., 1983). The underlayer consisted of 1.0 ml 0.5% agar (Bacto) in standard medium [Ham's F-10] containing 10% heat inactivated fetal bovine serum with penicillin (100 μg/ml) and streptomycin (100 units/ml). The plating layer consisted of 1.0 ml 0.30% agar in standard medium with freshly added nutritional supplements of pyruvate 0.34 mg/ml, glutamine 0.45 mg/ml, and insulin 1.54 units/ml and 300,000 to 500,000 nucleated cells from the melanoma biopsies.

The following cell lines were also cultured in the bilayer (0.3% over 0.5%) agar system using the same kinds of media the cells grew in as monolayer cultures and supplemented with 10 to 15% fetal bovine serum. Human tumor cell lines: ovarian, in Ham's F-10, from R.N. Buick, Toronto, Ontario; Colon, Widr in Ham's F-10 from American Tissue Type Collection (ATTC); oat cell lung, NCI 417; in Ham's F-10, from D.N. Carney, NCI; lung cancer, P3P37, in CMRL from D. Kern, UCLA; breast, MDA, in Ham's F-10, from B. Soehnlen; pancreatic, in CMRL; myeloma, 8226, in Ham's F-10 from ATTC; endometrial Hec 1A from ATTC ZR75 breast in Ham's F-10; MCF-7 breast in McCoy's 5A, from C.M. McGrath, Detroit, MI. Murine melanoma cell line: cloudman S91, 53.1. All plates were incubated in a humidified incubator with a 5% CO_2 95% air atmosphere.

RESULTS

Quantitation of the number of cells within growth units

The number of cells per growth unit increased geometrically as the diameter of the growth unit increased from 30 to 250 μm. The linear regression equation ln (No. cells in growth unit) = A + B (ln growth unit diameter) was used to relate the number of cells in a growth unit and growth unit diameter and had a good fit (Table 1). The data were linear on a log-log scale (Fig. 1), with no systematic deviations from the equations. Note that the number of cells per growth unit was inversely related to the diameter of the cells within the growth units (Fig. 1, Table 1). In analysis detailed elsewhere (Meyskens et al., 1984a) growth units from 38

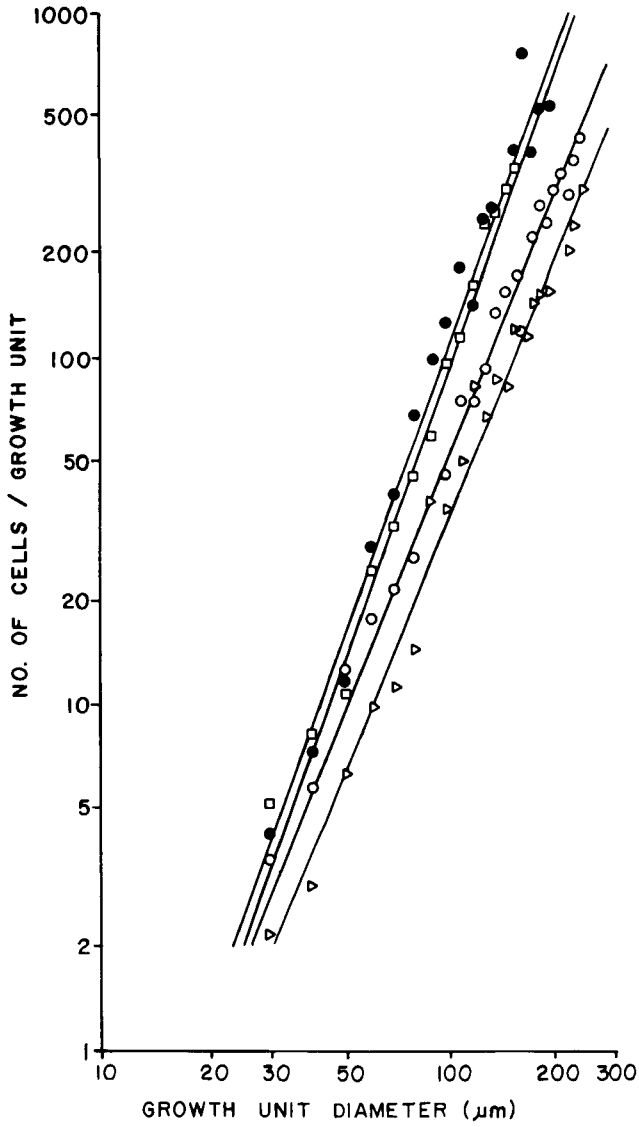

Fig. 1. The number of cells within growth units from 30 to 250 μm in diameter. We used four sources of melanoma cells: biopsies, □, patient 80-62; ○, patient 81-30; △, patient 80-56; and ●, the murine melanoma cell line.

TABLE 1. Relation between number of cells per growth unit and growth unit diameter: Parameter estimates for the linear regression equation[1]

Melanoma source	Cells diameter (μm)	A + SE (A)	B + SE (B)	Multiple correlation coefficient (r)
Patient 80-56	20.23 ± 1.10	−7.42 ± .23	2.37 ± .05	.983
Patient 81-30	16.44 ± 0.46	−7.09 ± .22	2.39 ± .04	.974
Patient 80-62	13.0 ± 0.45	−8.03 ± .23	2.73 ± .05	.987
Murine melanoma cell line	13.35 ± 0.36	−7.93 ± .23	2.73 ± .05	.975

[1] ln (no. cells/growth unit) = A + B [ln (growth unit diameter)]

samples of tumor cells confirmed this relationship and established this generally applicable equation: 1n (no. cells/growth unit) = 0.874 + (−2.804) (1n cell diameter) + (2.378) (1n growth unit diameter). These data allowed the calculation of the number of cells per growth units of various diameter for the other tumor cell samples.

This quantitative description of the relationship between the number of cells per growth unit and the cell and growth unit diameters permitted the calculation of the frequency of growth unit containing the number of cells corresponding to population doublings, i.e., 2, 4, 8, 16 . . . etc. This was done by solving the equation for growth unit diameter at the specific cell numbers per growth unit, 2, 4, 8 . . . etc; and extrapolating from a graph of cumulative number of growth unit versus growth unit diameter (Fig. 2). For example, for a growth unit of 32 cells, the murine melanoma cell line had a diameter of 65 μm and, by extrapolation from the cumulative growth unit curve, there were approximately 7,000 growth units per plate. The frequency of different-sized growth units was calculated in a similar fashion using specific cumulative number of growth unit curves obtained for each sample.

Kinetics of proliferation of growth units in agar

Daily observations of cells from melanoma biopsies in agar showed that proliferation often occurred after a variable delay of a few days. A representative case is shown in a sequence of serial photomicrographs from a single inverted microscopic field (Fig. 3). Little proliferation was observed until day 4, when there was a large increase in the frequency of cell doublets (Fig. 3A). These four doublets proliferated from eight cells to more than 32 in three days (Fig. 3A–D), a doubling time approximately 1.5 days, before stopping on day 9 (Fig. 3E) and then disintegrating (Fig. 3F). In general, similar results were seen for cells from other melanoma biopsies, while cells in agar from cell lines and colonies used for replating studies had less of a delay before the initiation of proliferation.

To quantitate these kinetics more completely we made detailed measurements of the changes in the number and size of growth units in agar over time using a murine melanoma cell line and cells from biopsies of melanoma from two patients. Cells from the murine melanoma (Fig. 4A) had little delay in the initiation of proliferation; approximately 40% of the cells plated had divided once within the first day. There was a sequential increase in the frequency of growth units of increasing size until proliferation stopped on day 9. Note that virtually all the cells that completed one division completed two or more divisions and that over 50% of the cells plated completed six divisions. Thus, when proliferation stopped there were few small growth units in the agar.

In contrast, the proliferation of cells from the melanoma biopsies (Fig. 4B,C) started and stopped later than cells from the cell line. Significant numbers of doublets were not seen until day 3 for the biopsy cells instead of after only 12 hours for the cell line cells (Fig. 4A). Furthermore, the appearance of growth units of all sizes was delayed 2–3 days for the biopsy cells compared to the cell line. For example, significant increases in the number of growth units with eight cells were initially observed on day 7 for the biopsy cells rather than day 4

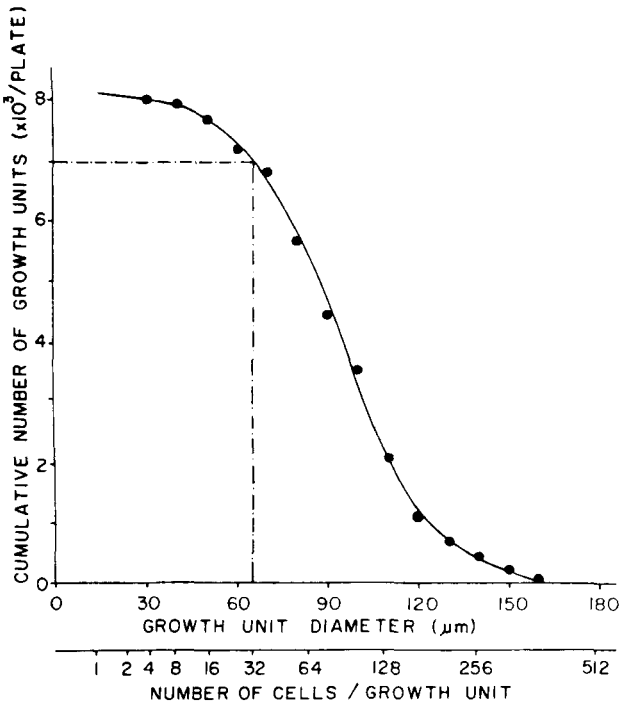

Fig. 2. The cumulative number of murine melanoma growth units of different sizes. An example of the curves used to calculate the frequency of growth units containing numbers of cells corresponding to cell population doublings. The number of cells per growth unit (abcissa) was calculated using the equation relating diameter of the growth unit to the number of cells per growth unit and would be different for growth units with cells of a different diameter than the murine melanoma. The frequency of growth units of various sizes were extrapolated from similar curves, e.g., frequency of 32 cell growth units was approximately 7,000/plate in this case.

for cells from the cell line. The cumulative frequency of smaller growth units increased for the biopsy cells throughout the period of proliferation in contrast to the smaller growth units of the cell line which increased in frequency initially and then remained the same, while the frequency of larger growth units continued to increase. This resulted in a decreased proportion of larger growth units for the biopsy cells when proliferation stopped. For example, approximately 50% of the cell line cells that formed growth units with eight cells also formed growth units of 64 cells compared to 10% or less of the biopsy cells (Fig. 4A–C).

We also calculated the change in total number and volume of cells within the growth units during incubation using the frequency and size of the growth units formed and the number of cells per growth unit equations (Table 1). There were exponential increases in the total number of cells within the growth units from the biopsy and cell line cells during incubation (Fig. 5A). Note that the increases in total cell number for the murine melanoma cell line started approximately 3 days before the biopsy cells and that the increases in all samples stopped abruptly as proliferation ceased. Furthermore, the total number of cells at the end of proliferation for patient 80-56 was less than 30% that of patient 80-62. However, cells from patient 80-56 had a larger cell diameter (Table 1) so that the total volume of

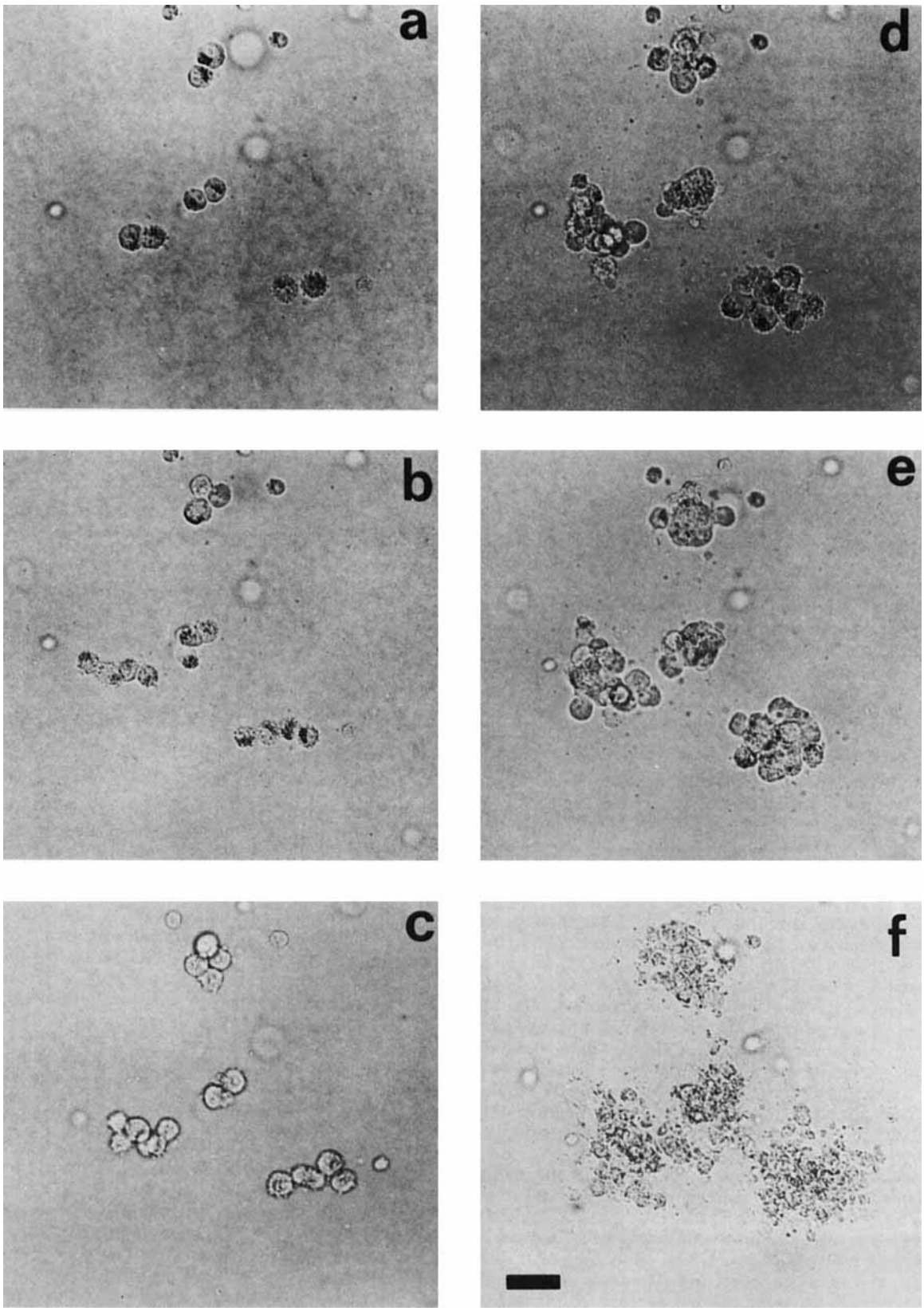

Fig. 3. Proliferation of melanoma clonogenic cells in agar. Cells from a biopsy (patient 80-62) were plated and observed daily. Little proliferation occurred until day 4; then we serially photographed a representative microscopic field on days 4 (a), 5 (b), 6 (c), 7 (d), 9 (e), and 28 (f). Bar = 30 μm.

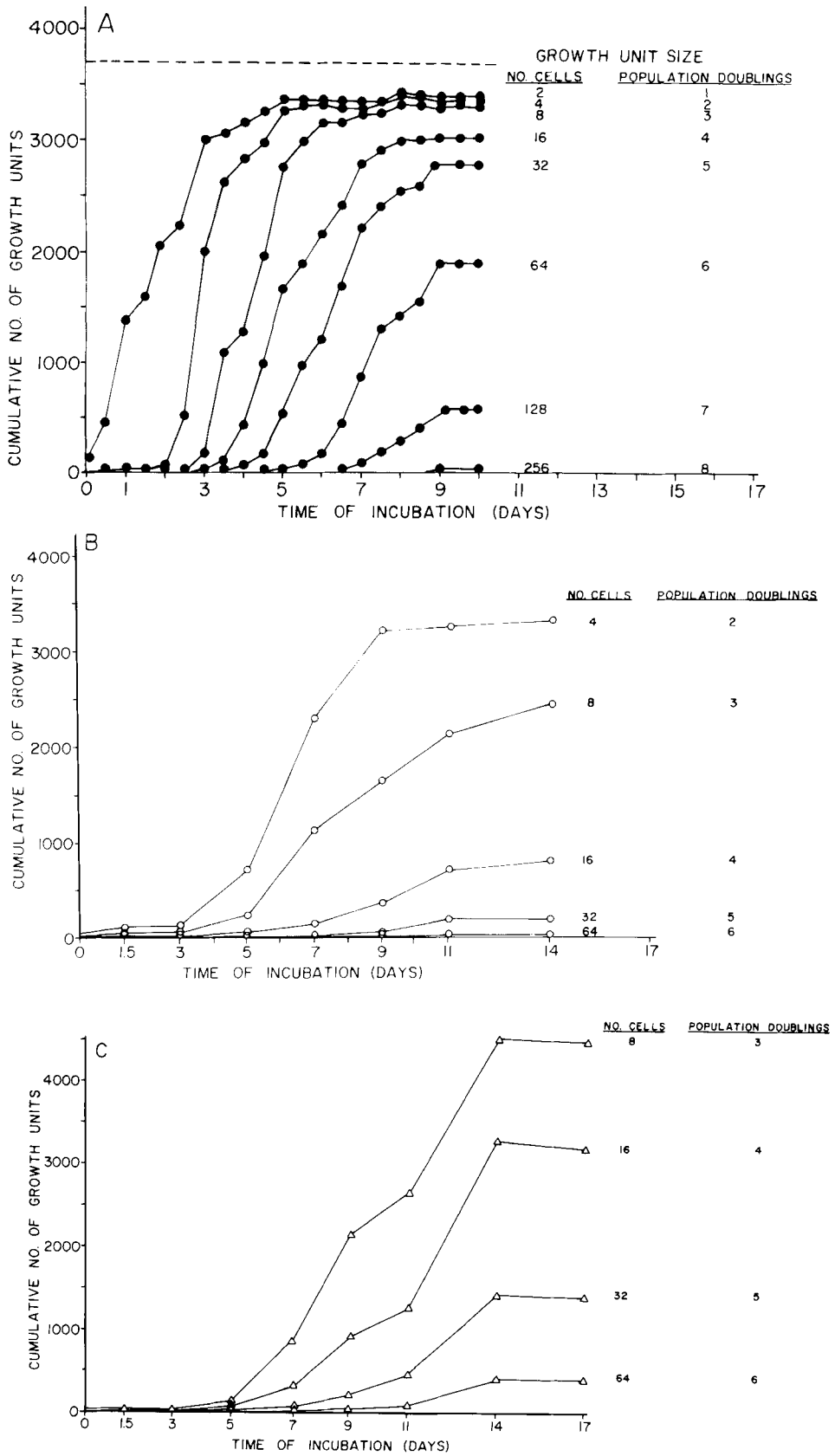

Fig. 4. Kinetics of the formation of growth units containing numbers of cells corresponding to population doublings. Cumulative frequency of growth units by size are shown. Data are from serial observations of cells from the murine melanoma cell line, panel A; biopsy of patient 80-56, panel B; and biopsy of patient 80-62, panel C. Dashed line in A represents the total number of single cells plated, 3,700.

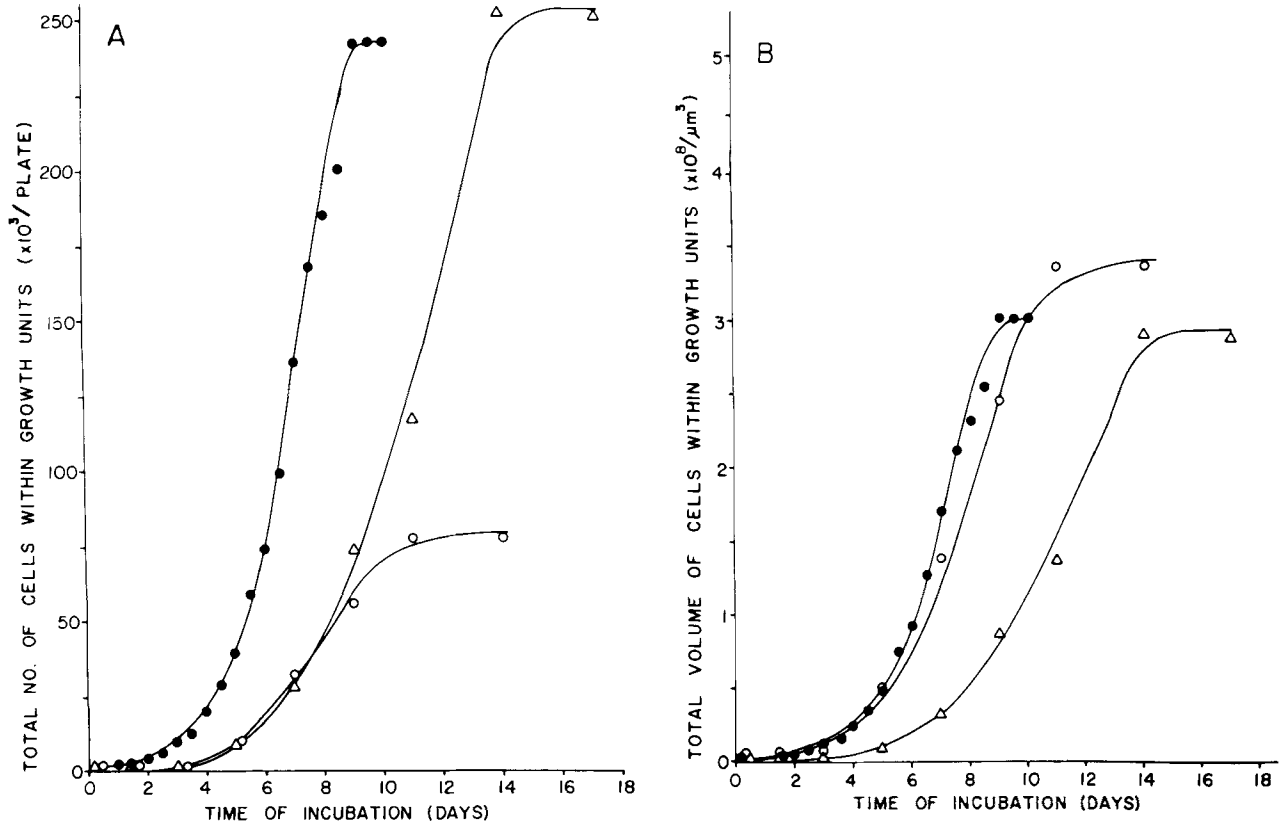

Fig. 5. Kinetics of the formation of the total number of volume of cells within growth units. Panel A: total number of cells within the growth units from the murine melanoma and patient biopsy cells. From kinetic study shown in Figure 4: ●, murine melanoma cell line; △, patient 80-62; and ○, patient 80-56. Panel B: total volume of the cells within the growth units from the same cells, symbols as in A.

cells within the growth units at the end of proliferation was similar for all the samples (Fig. 5B).

Extent of proliferation in agar

To further define the extent of proliferation within the closed 2-ml agar system, we determined the cloning efficiency for growth units of different sizes after cells completed proliferating. For the melanoma biopsy cells from seven patients, the cloning efficiencies for small growth units were high, but decreased rapidly for the larger growth units (Fig. 6A). The pattern was similar for cells from ten different cell lines (Fig. 6B), but the cloning efficiencies were generally higher for all sizes of growth units. These data confirm that only a small proportion of cells that divided once continued to proliferate into large growth units.

We also calculated the total volume of the cells within the growth units at the end of proliferation within the "closed" 2-ml agar system and compared it to the volume of the tumor cells plated. Although the initial volume of cells plated for the biopsy cells was generally higher than the cell lines, the volume of cells within the growth units at the end of proliferation was similar for cells of different sources grown in various media (Fig. 7). Futhermore, for several concentrations of the murine melanoma cells that were plated at different initial numbers and volumes, similar total volumes of cells within growth units were obtained (Fig. 7).

Refeeding and the number of cells plated: effects on proliferation

The effect of increasing the 2-ml total volume of the system was examined by adding more media to the agar during incubation. Refeeding increased the total volume of cells within the growth units in a dose-dependent manner after growth ceased for cells from the five cell lines and four patient biopsies examined (Fig. 8). Refeeding delayed the cessation of proliferation and increased the size of growth units by increasing the number of cells within existing growth units rather than inducing single cells to form new growth units.

Plating murine melanoma cell lines cells over a concentration range from 100 to 150,000 cells per plate caused a dose-dependent shift in the cloning efficiency of different sizes of growth units (Fig. 9). For example, at 150,000 cells per plate, only 1% of the cells proliferated into growth units of 32 or more cells, while at 100 cells per plate almost all the cells proliferated into growth units of 32 or more cells (Fig. 9). The different concentrations of murine melanoma cells also proliferated over different lengths of time to produce the same total volume of cells within the growth units (Fig. 7). When higher concentrations of cells were plated many small growth units were formed and stopped proliferating sooner than when lower concentrations of cells were plated, which continued proliferating into larger growth

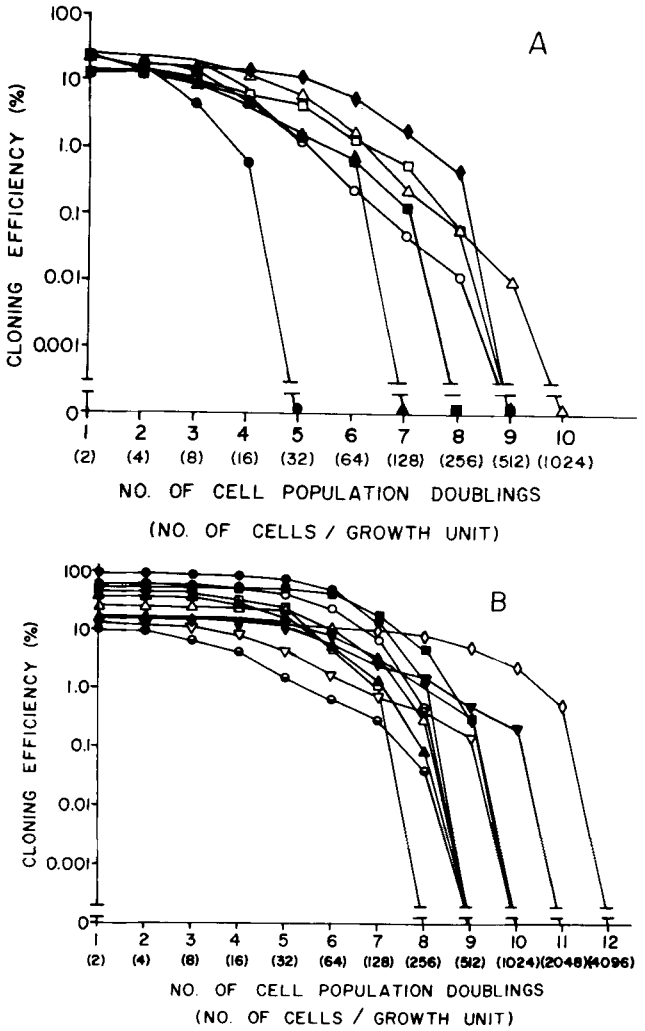

Fig. 6. Extent of proliferation in agar as measured by the cloning efficiency of different sizes of growth units. Cells were plated in agar and allowed to proliferate until no further increase in growth unit size occurred usually after 21 days of incubation. We then determined the size and frequency of growth units. Panel A: proliferation of cells from seven patient biopsies: ○, patient 80-56; ▲, patient 80-62; ●, patient 82-6; ▲, patient 82-7; ■, patient 81-46; □, patient 81-57; ◆, patient 80-54. Panel B: proliferation of cells from 11 cells lines: ●, murine melanoma; ○, ovarian; ■, colon (WIDR); □, oat cell lung (NCI417); ▲, lung (P3P37); △, breast (MCF-7); ◆, pancreatic; ◇, myeloma (8226); ▼, endometrial HEC 1A; ▽, breast (MDA); and ●, breast (ZR-75).

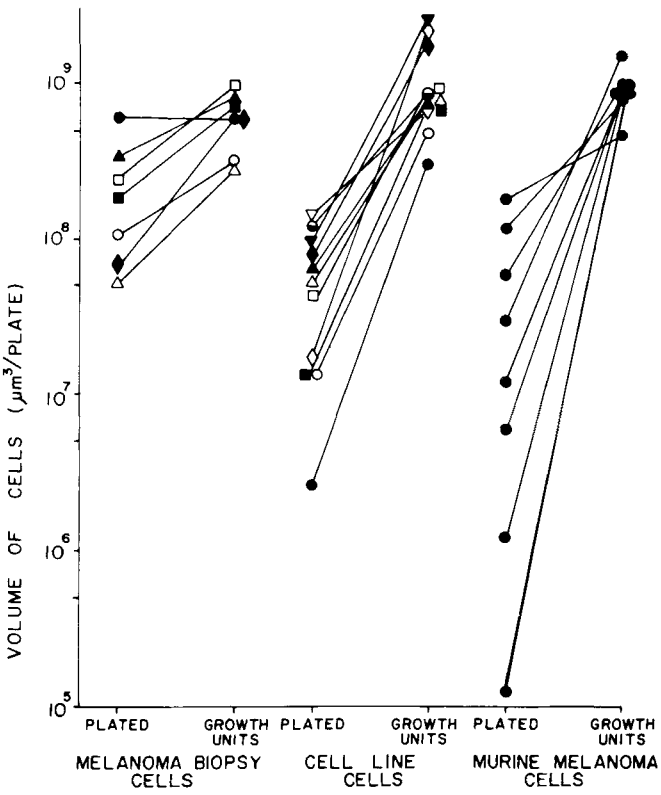

Fig. 7. Volume of tumor cells plated and of cellular volume of the growth units after cessation of proliferation. Shown are cells from seven patient biopsies, 11 cell lines, and a murine melanoma cell line plated at different concentrations. Symbols are as in Figure 6.

units. At 150,000 cells/plate proliferation stopped after 4 to 5 days while at 100 cells/plate proliferation stopped after 18 to 20 days. These data suggest that slightly less than 10^9 μm^3 of cellular volume is a common maximal potential and upper limit for growth within the 2-ml agar system and that the duration of proliferation and size of growth units can be increased by plating fewer cells or refeeding.

DISCUSSION

Precise quantitation of proliferation within clonogenic assays in terms of cell numbers has been limited because direct enumeration of the number of cells per colony has been prevented by the tight packing and stacking of cells within all but very small growth units. Removal and direct counting of the number of cells in growth units allowed us to establish equations that related growth unit diameters with the number of cells within the growth units accurately, with correlation coefficients ≥ 0.97. Subsequent observations (Meyskens et al., 1984a) have established a general equation showing that the number of cells per growth unit is exponentially related to growth unit diameter and inversely related to growth unit cell diameter. These equations allowed calculation of the total number and volume of cells within growth units in a plate and the frequency of colonies containing specific numbers of cells. These measurements give more complete descriptions of clonogenic cell proliferation than cloning efficiencies which only measure the frequency of growth units larger than a particular size.

Our studies showed either no or a variable delay in the onset of clonogenic cell proliferation which caused significant variation in the size of growth units formed. Cells from cell lines started proliferation quickly after plating, formed a maximal number of small growth units after 2 to 3 days and produced mostly large colonies at the end of growth. In contrast, the cells from biopsies had a variable delay before starting to proliferate. Some cells started after 4 or 5 days, while others within the same dish started later, as illustrated by the increasing frequency of small growth units for 7 to 9 days rather than the 2 to 3 days for cell line cells. Thus many small growth units were formed from cells that started prolif-

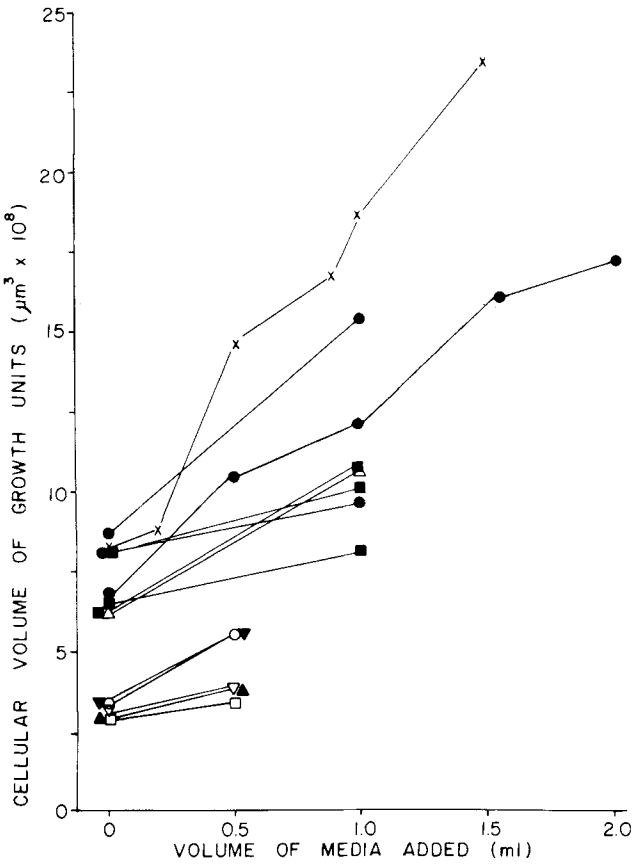

Fig. 8. The effect of adding media to the 2-ml bilayer agar system. Note that additions of the original formulation of overlayer media to the second bilayer system during incubation gave dose-dependent increases in cellular volume. The total number and volume of the cells within all the growth units on the plates with and without additions was determined after cessation of proliferation.

erating late during the incubation period. Other small growth units may have been formed from cells that stopped proliferating or had slower cycling times. This led to a mixture of small and large colonies at the end of growth.

An implication of these kinetic results is that the size of growth units may in part depend simply on the variation in the onset of proliferation. Thus clonogenic cells with extensive proliferative capacity may form variable-sized growth units simply because of a variable delay before initiation of proliferation. For example, some clonogenic cells started to proliferate on day 10 and could only divide a few times before all the growth stopped. Thus some small growth units may represent clonogenic cells with extensive proliferative capacity rather than a group of end cells formed from a transit cell with limited proliferative capacity (McKillop et al., 1983). The general observation of heterogeneity of growth unit size in may clonogenic assays may be due to a number of factors, including 1) variation in the initiation of clonogenic cell proliferation, 2) differences in the rate of cell division, 3) variation in the culture requirements for clonogenic subpopulations, and 4) inherent and variable limits on proliferative capacity due to the variable position of clonogenic cells within the stem cell hierachy.

Another implication is that the kinetics of growth within clonogenic systems could affect assays of regulatory factors or drug sensitivity testing. For example, 1-hr incubation of clonogenic cells with a cell-cycle-dependent drug may not affect the cells because many cells do not begin to cycle until several days after the drug is gone.

The kinetic studies also showed that proliferation stopped abruptly at a similar total volume of cells within the growth units rather than at a similar total number

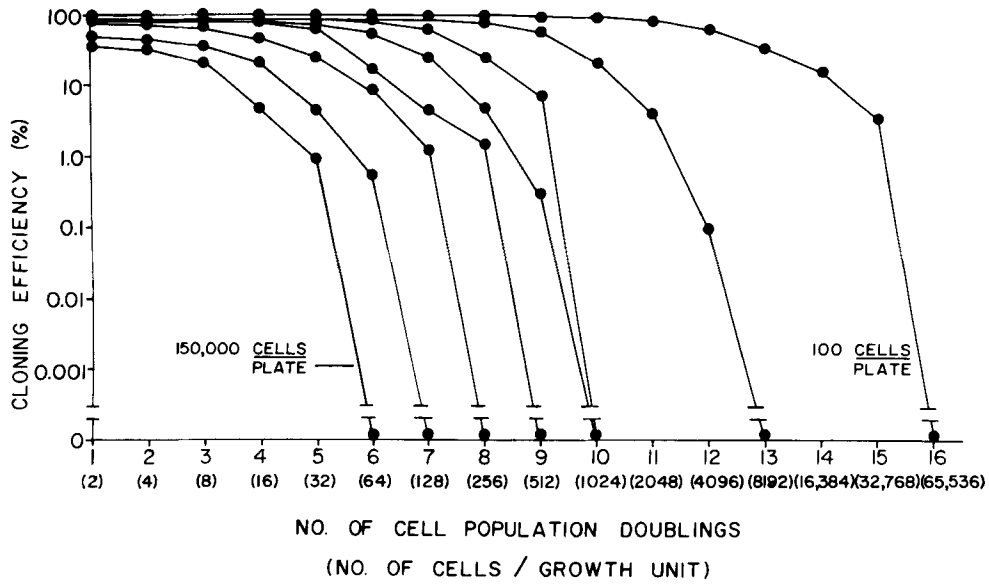

Fig. 9. The effect of the number of cells plated on the cloning efficiency of different-sized growth units. Curves represent murine melanoma cell line growth units formed from plating, left to right: 150, 100, 50, 25, 10, 5, 1, 0.1 $\times$ 10^3 cells/plate.

of cells. Cells from seven biopsies and 11 cell lines which were allowed to grow until they stopped showed that proliferation ceases at a similar cellular volume, approximately 10^9 μm^3. The proliferation of the clonogenic cells and total cellular volume at the end of growth was extended by refeeding with fresh media. These data suggest that 10^9 μm^3 of cellular volume represents the maximum potential or upper limit for growth within the closed non-refed 2-ml bilayer agar system, probably due to exhaustion of nutrients or build-up of inhibitory substances.

The extent of individual clonogenic cell proliferation was also dependent on the number of cells plated. This would be expected from our data that defines an upper limit for proliferation. Thus, plating relatively high concentrations of clonogenic cells produced only small growth units because cells only needed to divide a few times to reach the cellular volume limit. Conversely, plating few clonogenic cells produced larger colonies because the cells had to proliferate extensively to reach the limit on total volume of cells within the growth units. Studies have shown that the number of clonogenic cells plated varies over a considerable range for cells derived from tumor tissue and plated at 5×10^5 nucleated cells/plate. Furthermore, frequently there are many growth units smaller than the ones used to define a colony. This raises the possibility that cloning efficiences are underestimated because the small growth units could not express their true proliferative capacity. Thus, because of the limit on the proliferative capacity imposed by the 2-ml system, plating several cell concentrations, refeeding or defining colonies as small growth units may be required to accurately estimate the number of clonogenic cells within tumor cell samples. Other studies (Tveit et al., 1982; Page et al., 1983) have shown that decreasing the concentration of cells plated can increase cloning efficiency in agar.

The results suggest that clonogenic cells plated in a 2-ml bilayer agar system can only express their initial proliferative capacity. In previous studies we have replated colonies formed from the same biopsy cells used in this study (Thomson and Meyskens, 1982). We found extensive self-renewal and proliferative capacity which was several times greater than that expressed in the first plating of the cells. Furthermore, we have found that self-renewal capacity was similar for growth units containing sixteen to more than 512 cells (Meyskens et al., 1984b). Thus, many clonogenic cells may stop proliferating simply because the system can only support the formation of approximately 10^9 μm^3 of cellular volume within growth units rather than because of an inherent limitation which would be expected if the cells were transition cells of the stem-cell hierachy model recently summarized by McKillop et al. (1983).

The finite upper limit on proliferation within the bilayer agar system can have marked effects on both assay linearity and survival curves. We have recently shown that the range of linearity between the number of cells plated and number of colonies formed is inversely related to the size of the growth unit used to define a "colony" (Meyskens et al., 1983; Thomson et al., 1984). Small growth units have wider ranges of linearity because they can form when relatively high concentrations of cells are plated. However, the large number of small growth units only proliferate until the 10^9 μm^3 upper limit is reached so that larger growth units can not form, thus restricting their range of linearity.

The finite limit on proliferation also affects the survival of growth units after treatment with cytotoxic agents. Low doses of cytotoxic agents can kill a proportion of cells and allow the remaining cells to proliferate more than control cells to produce an apparent stimulation (Meyskens et al., 1983). The stimulation is more pronounced if relatively high numbers of cells are plated or large growth units are used to define a colony. Thus paradoxical "stimulation" of clonogenic growth by cytotoxic agents has been observed (Thomson et al., 1984).

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