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Tumor cell heterogeneity: Divided-colony assay for measuring drug response

(*HRAS* human oncogene/chemotherapy/human tumor cloning system/random walk/multitype model)

THOMAS KUCZEK*† AND DAVID E. AXELROD‡

*Department of Statistics and ‡Waksman Institute of Microbiology, Rutgers–The State University of New Jersey, Piscataway, NJ 08855-0759

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ABSTRACT **_In vitro_ tests for predicting the response of tumors to chemotherapeutic agents might be improved if they were modified to take into account tumor-cell heterogeneity. We have studied the heterogeneity of cellular growth rate and drug response in mouse fibroblast NIH 3T3 cells and in NIH 3T3 cells transformed with the human *HRAS* gene (homologue of the Harvey sarcoma virus oncogene v-Ha-*ras*) from the EJ human bladder carcinoma cell line. Growth-rate heterogeneity was detected as a broad distribution of numbers of cells per colony. In spite of this heterogeneity, secondary colonies have numbers of cells per colony that resemble that of the primary colony from which they were derived. The variance between unrelated secondary colonies is increased by $HRAS^{EJ}$. Colony-size measurements are reliable because primary colonies divided in half formed two groups of secondary colonies (on two separate plates) that had indistinguishable mean colony sizes. Based on these observations, a divided-colony procedure was devised to detect the drug response of heterogeneous cell populations. Primary colonies are divided into two groups of cells, one of which is treated with a drug and the other is left untreated as a control. The size distribution of treated secondary colonies is then compared to that of the untreated control and to that of the primary colony from which it was derived. The divided-colony procedure is proposed as a modification of the human-tumor-cloning system to increase the sensitivity and reliability of *in vitro* procedures used to determine the drug response of heterogeneous tumor-cell populations.**

One standard method of assaying a population of tumor cells for drug sensitivity involves plating an equal number of cells with and without the drug and then comparing the two groups on the basis of the number of colonies that have grown to 50 or more cells. The technique, while useful, fails to take into account clonal heterogeneity of growth rate and drug response in the population of cells. It would be helpful to develop a reliable assay that accounts for heterogeneity.

Tumors are generally composed of subpopulations of cells that are heterogeneous for many characteristics such as growth rate, metastasis, karyotype, antigenicity, biochemical properties, and sensitivity to chemotherapeutic agents (reviewed in refs. 1–3). Even single-cell-derived tumors may be heterogeneous. The heterogeneity of growth rate and of sensitivity to chemotherapeutic agents has made it difficult to devise effective *in vitro* predictive tests and has complicated designs of effective treatment protocols. A better understanding of tumor heterogeneity could have clinical applications for improved chemotherapy.

When cells in a population are dispersed and allowed to form clones, two different results may be obtained. The clones may be similar to each other, or they may be different. If cells from a population form clones that are different from each other, then the population exhibits clonal heterogeneity. For clonal heterogeneity of growth rate to exist, there must be a tendency for the growth rate to diversify as well as a tendency for the growth rate to persist. In this report, diversification of growth rate was observed as a broad distribution of primary-colony sizes. Persistence of growth rate was observed as a similarity of sizes of primary colonies and their subclones. Although primary colonies differed from each other, subclones derived from the same primary colony were similar to each other. This last observation suggests a divided-colony procedure as an assay for measuring drug sensitivity in heterogeneous tumor-cell populations.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. NIH 3T3 is a nontransformed mouse fibroblast cell line, and NIH 3T3($HRAS^{EJ}$) is a transformed derivative containing a 6.6-kilobase *Bam*HI DNA fragment with the human *HRAS* gene (homologue of the Harvey sarcoma virus oncogene v-Ha-*ras*) from the EJ human bladder carcinoma cell line (4), kindly provided by G. Cooper (Dana–Farber, Boston). These cells were routinely grown in Dulbecco's modified Eagle's medium with pyruvate (GIBCO 430-1600) with 10% (vol/vol) fetal calf serum (DME10 medium) in 7.5% CO_2/92.5% air and maintained in an actively grown state by dilution twice weekly.

Colony Size and Subcloning. The number of cells per colony was routinely determined by plating 20 cells in 60-mm tissue culture dishes in 4 ml of DME10 medium, incubating for 4 days, staining colonies (10% formaldehyde/0.1% crystal violet), and observing (×25–50 magnification) under a dissecting microscope fitted with an eyepiece reticle. Individual cells in isolated colonies (of both transformed and nontransformed cell lines) could be easily visualized and counted when the total number of cells did not exceed about 150 cells per colony. For subcloning, colonies were not fixed or stained, and the number of live cells in each primary colony was determined at ×60 magnification with an inverted phase-contrast microscope fitted with an eyepiece reticle. Each primary colony was surrounded with a 0.5-cm porcelain cylinder and exposed to trypsin. Live cells were removed with a Pasteur pipet, resuspended in 4 ml of fresh DME10 medium, replated in a fresh dish, and incubated for 4 days. At that time cells in each secondary colony were fixed, stained, and counted. For the divided subclone procedure, cells from primary colonies were resuspended in 8 ml of fresh DME10 medium, and 4 ml was added to each of two 60-mm dishes. In some experiments cycloheximide was added to one of a pair of dishes. Cycloheximide (Sigma C-6255) was dissolved in DME10 medium (1 mg/ml), filter-sterilized, and diluted to a final concentration of 0.01 μg/ml in DME10 medium. After 4 days of incubation, secondary colonies were fixed, stained, and counted.

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†Present address: Department of Statistics, Purdue University, West Lafayette, IN 47907.

Data Analysis. The rate of growth of primary and secondary colonies was determined as the logarithm of the number of cells per colony. The growth-rate difference between secondary colonies and their primary colony is defined as the growth rate of the secondary colony minus the growth rate of the primary colony, divided by the growth rate of the primary colony. As defined, growth-rate difference is unitless, does not depend on the base of logarithm used, and quantifies differences in growth rate between primary and secondary colonies. A growth-rate difference of zero indicates that the secondary colony has the same number of cells as the primary colony. A negative growth-rate difference indicates that the secondary colony had fewer cells than the primary colony, while a positive growth-rate difference indicates that the secondary colony had more cells than the primary colony. Secondary colonies are considered to be of the same clonal line when they are derived from the same primary colony. Figures of cumulative frequency plots of response variables such as colony size or growth-rate difference are graphed as percentile vs. response variable. Statistical evaluations of growth-rate differences between groups as well as variance component estimates were made by standard techniques for analysis of variance (5) with SAS software (6).

RESULTS

Colony-Size Distribution. Actively growing populations of NIH 3T3 and NIH 3T3(*HRAS*^{EJ}) cells were diluted to suspensions of nonaggregated cells, plated and incubated for 4 days, and the number of cells per colony was determined. Fig. 1 displays the colony-size distribution of NIH 3T3 cells, while Fig. 2 displays the colony-size distribution of NIH 3T3(*HRAS*^{EJ}) cells. These are plotted as cumulative frequency distributions, which allow direct comparison of distributions based on different numbers of observations and allow smooth curves to be drawn when the number of observations is limited (7). Note that each colony-size distribution is broad, consistent with heterogeneity of growth rate in each population.

Two factors in addition to growth rate that might contribute to broad colony-size distributions are asynchrony of the initial cells and cell death. Asynchrony of initial cells alone, provided all the cells have the same generation times, could only account for colony-size differences of a factor of 2, whereas colony sizes actually ranged from 2 to >50 cells. Cell death was not extensive because colonies could be recloned to give secondary colonies of similar size, as discussed below. Comparison of Figs. 1 and 2 shows that differences in the distributions are small relative to the breadth of the distributions. Therefore, differences that may exist between these cell lines might not be easily detected by comparing primary colony-size distributions.

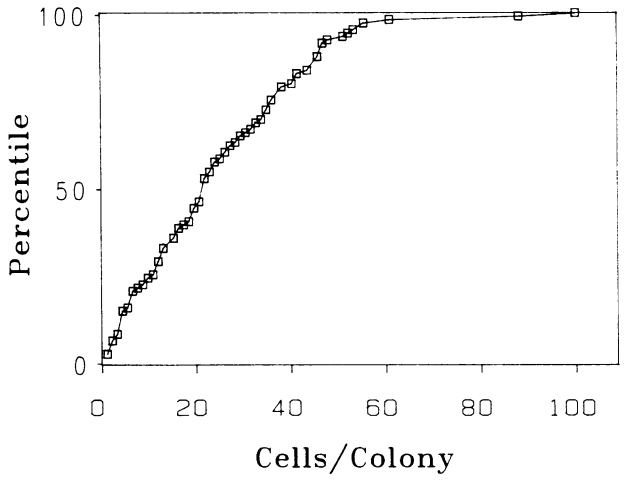

FIG. 2. Colony-size distribution of cell line NIH 3T3(*HRAS*^{EJ}) incubated for 4 days.

Subcloning. To compare sizes of primary and secondary colonies, cells in primary colonies were counted, dispersed, and allowed to form secondary colonies. Figs. 3 and 4 display cumulative frequency plots of growth-rate differences between primary and secondary colonies for NIH 3T3 and NIH 3T3(*HRAS*^{EJ}) cells lines, respectively. For NIH 3T3 cells, secondary colonies varied in the rate of growth: some rates decreased compared to rates of parental colonies, whereas others increased. Nevertheless, the growth-rate differences centered about zero, indicating that secondary colonies resembled parental colonies. This observation is consistent with the notion of persistence of growth rate of cells in primary and secondary colonies. For NIH 3T3(*HRAS*^{EJ}) cells also, secondary colonies varied in growth rates: some rates decreased compared to rates of parental colonies, whereas others increased. However, when NIH 3T3(*HRAS*^{EJ}) cells were compared to NIH 3T3 cells, there appeared to be a greater range of growth-rate differences between primary and secondary colonies. This observation is consistent with the general observation that tumorigenic cells are more heterogeneous than nontumorigenic cells.

To determine if there were statistically significant differences between the clonal lines, mean growth-rate differences were compared by using an F test. For NIH 3T3 cells, P = 0.042 based on six clonal lines; for NIH 3T3(*HRAS*^{EJ}) cells,

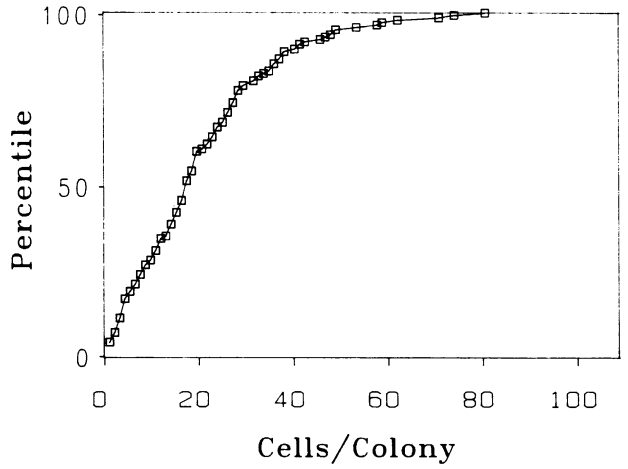

FIG. 1. Colony-size distribution of cell line NIH 3T3 incubated for 4 days.

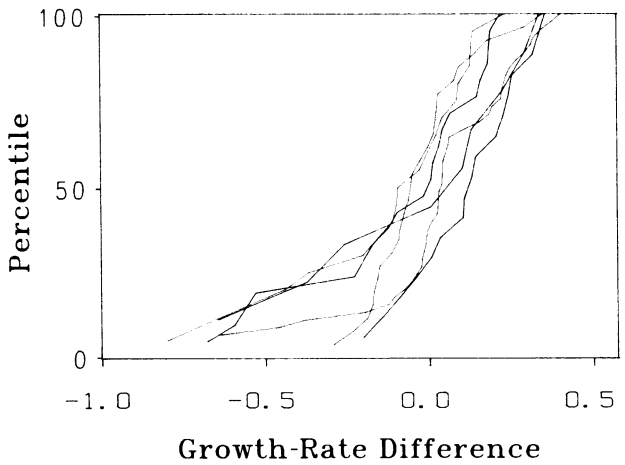

FIG. 3. Growth-rate differences between primary and secondary colonies of cell line NIH 3T3.

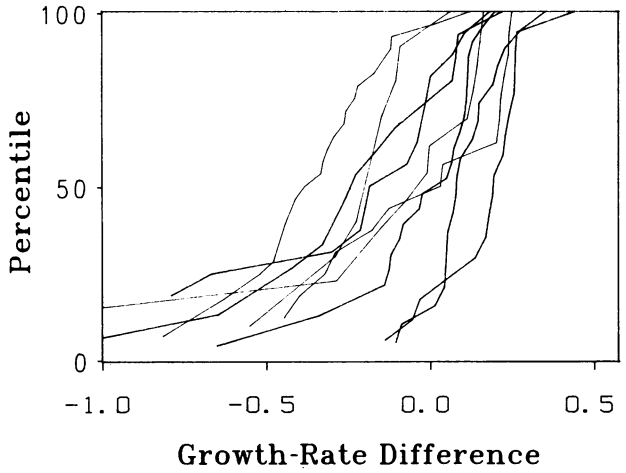

FIG. 4. Growth-rate differences between primary and secondary colonies of cell line NIH 3T3(*HRAS*^{EJ}).

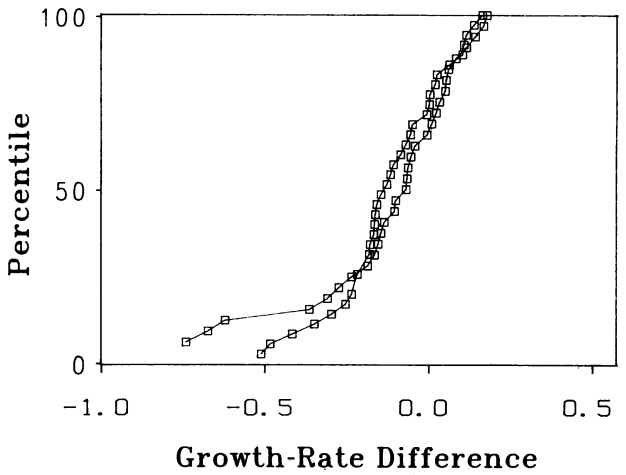

FIG. 5. Growth-rate differences between one primary colony of NIH 3T3(*HRAS*^{EJ}) and two groups of secondary colonies obtained from it. The primary colony was divided in half and plated onto two plates with the same medium.

$P \leq 0.001$ based on nine clonal lines. Each of these small P values indicates that there were significant differences between clonal lines.

The variances of mean growth-rate differences were calculated to determine if the range of growth-rate differences was significantly greater for clonal lines of NIH 3T3(*HRAS*^{EJ}) cells than for clonal lines of NIH 3T3 cells. The variance between clonal lines for NIH 3T3(*HRAS*^{EJ}) cells was estimated to be 0.0281 and for NIH 3T3 cells, to be much less, 0.00329. The variances within clonal lines were similar: 0.0566 and 0.0523, respectively. These statistical analyses confirm the conclusion drawn from inspection of the plots—that there is a greater clonal heterogeneity among the *HRAS*-transformed NIH 3T3 cells than among nontransformed cells with respect to shifts in growth rate.

Divided Colonies. If subcloning is to be used to evaluate the effect of a drug, it is important to estimate the effect of the experimental procedure of subcloning, if any, on secondary colony growth rates. The notion of persistence suggests that divided primary colonies should produce subsets of secondary colonies with comparable growth-rate shifts. To observe secondary colonies, we divided the cells from each primary colony into two aliquots and plated each onto separate plates to form secondary colonies. Growth-rate differences were then compared for the secondary colonies on the two separate plates. Fig. 5 displays cumulative frequencies of the two sets of secondary colonies derived from a single divided primary colony of NIH 3T3(*HRAS*^{EJ}) cells. The two distributions appear to be similar—namely, the difference between the means is small compared to the breadth of the distributions. This similarity was tested statistically by calculating an F statistic. The means of growth-rate differences were compared for sets of secondary colonies derived from 5 primary colonies of NIH 3T3 cells ($P = 0.573$) and derived from 11 primary colonies of NIH 3T3(*HRAS*^{EJ}) cells ($P = 0.974$). Each of these large P values indicates that there is no significant difference between the means of two sets of secondary colonies derived from the same primary colony. This result suggests that one set of secondary colonies could provide an effective control for the other set of colonies. The divided-colony procedure would be useful when determining the drug response of a population of cells where clonal heterogeneity is present.

Divided Colonies as a Treatment Control. The broad heterogeneity of growth rate that exists in populations of cells introduces an element of confusion when such populations are tested for drug sensitivity. When primary colonies are plated out in the presence of a drug, a general decrease in colony-size distribution may be observed, but two types of errors may occur in interpreting results for the treated primary colonies. A colony may be completely resistant to the drug but could seem affected if it were growing at a relatively slow pace. A colony of quickly growing cells may be sensitive and grow more slowly in the presence of a drug, but perhaps might seem resistant if a relatively large colony were produced. The alternative of testing subcolonies by using primary colonies as a control presents the same type of problem. An ineffective drug may seem effective if the clonal line were shifting down in growth rate, while an effective drug may seem ineffective in slowing growth rate in a clonal line that was shifting up.

Secondary colonies from the same primary colony have statistically indistinguishable mean growth rates. This resolves the problem presented by clonal heterogeneity of growth rate and/or growth rate shift. Dividing a colony provides a controlled method to test for drug sensitivity or resistance in clonal lines. Fig. 6 shows frequency distributions of the growth-rate difference of two sets of secondary colonies, each set being derived from half of a divided primary colony—one a control and the other treated with a low concentration (0.01 μg/ml) of cycloheximide. Even at

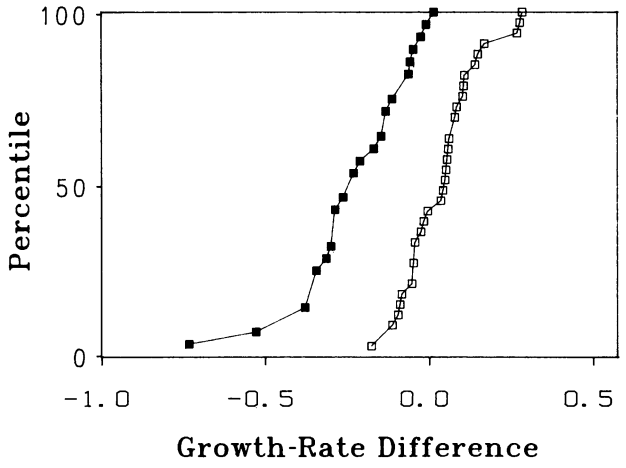

FIG. 6. Growth-rate differences between one primary colony of NIH 3T3(*HRAS*^{EJ}) and two groups of secondary colonies obtained from it. The primary colony was divided in half and plated onto two plates, one with cycloheximide at 0.01 μg/ml (■) and the other without cycloheximide (□).

such a low concentration of the drug, the effect on growth rate is clear.

To determine if the difference between the treated and control secondary colonies was statistically significant, the mean growth-rate differences of secondary colonies from 12 primary colonies were compared by using an F test. The result, $P \leq 0.0001$, indicates a highly significant difference between the treated and control secondary colonies.

It should be noted that this concentration of cycloheximide would be considered negative in the human-tumor-colony assay (8, 9) because it allows >90% survival of colonies of 50 or more cells, which is greater than the 30% threshold criterion used in the human-tumor-colony assay. However, this concentration of drug would be considered positive by the divided-colony procedure because growth-rate differences between treated and untreated secondary colonies are highly significant. Therefore, the divided-colony procedure is more sensitive than the traditional assay under these conditions.

In addition to being sensitive, the divided-colony procedure is also reliable in the sense that growth-rate differences between two sets of untreated secondary colonies from the same primary colony are not significantly different. Therefore, any significant differences that are observed between untreated and treated secondary colonies could be attributed to treatment effects rather than to clonal heterogeneity.

DISCUSSION

We have observed that NIH 3T3 and NIH 3T3(*HRAS*^{EJ}) cell lines contain cells that give rise to colonies with a broad distribution of sizes. This implies that each of these populations is heterogeneous with respect to cell growth rate. When primary colonies of different sizes are subcloned, the size of secondary colonies resembles the size of the primary colony from which they were derived. This implies that growth rates persist. This persistence is stronger for NIH 3T3 cells than for *HRAS*^{EJ} oncogene-containing cells. If cells from a primary colony are divided and placed into two separate plates, then the secondary colonies on the two plates have average sizes that are indistinguishable from each other. The similarity between secondary colonies holds for both NIH 3T3 and for NIH 3T3(*HRAS*^{EJ}) cell lines, in spite of the heterogeneity of growth rates in each cell line and in spite of the greater drift in growth rates in the *HRAS*-oncogene-containing cell line. The divided-colony procedure provides two similar sets of cells from a heterogeneous population. One of these sets of cells can be treated with a drug, and the other can be left untreated as a control. The divided-colony procedure is a method for detecting drug-sensitivity that accounts for clonal heterogeneity of growth rate and for growth-rate shifts.

Clonal heterogeneity of growth rates has been reported previously. The short-term inheritance of cell growth rates has been noted in populations of bacteria (10–13) and in eukaryotic cells (14, 15). In contrast, the diversification of growth rates has been observed in subclones after irradiation (16). Heterogeneity of drug resistance in cell lines derived by subcloning tumor-cell populations has been repeatedly observed (17–20). Subcloning has also been used to study stem-cell population dynamics, especially of hematopoietic colonies (21–27).

A heuristic model would be useful for analyzing an experimental situation in which opposing tendencies of diversification and persistence of growth rate are present. A natural approach to modeling the diversification of growth rate in clonal lines would be to assume that cells in a particular line of ancestry take a random walk on a rate space—i.e., a model in which growth rates diffuse. This sort of model falls under the general heading of multitype branching processes, which are often used to model uniparental populations that are heterogeneous with respect to some characteristic(s). An extensive literature exists on the mathematical development of such models, and standard techniques for their analysis are available (28–30). Some multitype models whose purpose is to describe the short-term persistence of growth rate have been presented (31, 32). Multitype branching models, with specific relevance in the study of cancer chemotherapy, have been reported (17–19). Multiple models with random walk also have been presented (33–35). Similar models that consider distributions of generation times have been developed (36–38).

The subcloning procedure detected a greater heterogeneity in growth rates of secondary colonies for cells containing the human *HRAS*^{EJ} oncogene than for cells not containing this oncogene. This may have clinical significance because activated *RAS* oncogenes have been detected in human tumor cells from many tissues (26, 27, 39–43) and have been implicated in an increased metastatic phenotype (44–49). In spite of this heterogeneity, the divided-colony procedure can be used to determine the response to drugs of such tumor-cell populations.

The divided-colony procedure provides a useful control for growth-rate heterogeneity in drug testing. It could be used to improve the human-tumor-cloning system (8, 9, 50) that has been proposed as an *in vitro* predictive test for determining the drug response of tumors. The interpretation of results from such tests is presently compromised by the observations that tumor-cell populations frequently contain subpopulations with various growth rates (16) and drug responses (51–55). The tests could be modified to include the divided-colony procedure in the following manner. One-half million cells from a tumor biopsy could be plated in semisolid medium to produce roughly 100 colonies of 50 or more cells. These colonies of self-renewing "stem cells" would be the primary colonies to be divided and subcloned to form two sets of secondary colonies. One set of colonies would be treated with a drug, and the other set would be left untreated as a control. Applied in this way, the divided-colony procedure would provide a sensitive and reliable assay for determining the drug response of the tumor, even when clonal heterogeneity of growth rate and drug response is present within the tumor-cell population.

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