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ORIGINAL ARTICLE

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Ras oncogene-transformed and nontransformed cell populations are each heterogeneous but respond differently to the chemotherapeutic drug cytosine arabinoside (Ara-C)

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Abstract In order to determine whether the growth of *ras* oncogene-transformed cells and nontransformed cells was inhibited differently by the chemotherapuetic drug cytosine arabinoside (Ara-C) their growth was analyzed by a novel colony-based assay that is sensitive and appropriate for heterogeneous cell populations. Colonies of nontransformed NIH3T3 cells, or *ras* oncogene-transformed NIH(*ras*) cells, were grown in the absence of drug and then divided into subclones. Subclones were allowed to continue to grow in the absence or presence of drug. Growth inhibition was determined by comparing the growth of drug-treated subclones with the growth of related untreated subclones. Colonies of nontransformed cells grown in the absence of the drug displayed a large variation in growth, and when grown in the presence of the drug displayed a large variation in growth inhibition. Colonies of transformed cells also displayed a large variation in the absence and presence of the drug. For each cell line, related subclones were more similar to each other than to unrelated subclones, implying inheritance of growth rates and drug response. For NIH3T3 cells, the growth of subclones in the presence of drug was highly correlated with the growth of related subclones in the absence of drug. However, for NIH3T3(*ras*) cells the growth of subclones in the presence of drug was not correlated with the growth of related subclones in the absence of drug. Therefore, *ras* oncogene-transformed and nontransformed cell populations differ in their response to Ara-C.

Key words Cytosine arabinoside · *Ras* oncogene · Heterogeneity

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Introduction

Populations of tumor cells may be heterogeneous for many characteristics, including growth in the absence of drugs [1], and growth inhibition in the presence of chemotherapeutic drugs [19]. In single tumors it is possible to detect subpopulations that differ in drug sensitivity [7, 13, 29, 31]. Also, the drug sensitivity of different metastases from the same primary tumor may differ from each other and from the primary tumor from which they were derived [23, 26, 29].

The heterogeneity of response of tumor cell populations to cancer therapeutic agents has contributed to the difficulty in designing tests to screen for new agents [24] and tests that are useful as in vitro predictive protocols [22, 27, 28]. These difficulties cannot be easily overcome by application of statistical parameters which describe properties of mixed populations [5] or experimental procedures that improve the conditions for culturing cells or detecting cell growth [14]. However, the difficulties arising from heterogeneity might be alleviated by techniques that analyze individual subclones within the heterogeneous population.

In addition to these practical problems posed by heterogeneity of cell populations there are also important fundamental questions about the sources of

diversity and persistence of traits within heterogeneous cell populations [4, 12, 20, 21]. In order to help to answer such questions we have developed experimental and analytical procedures for characterizing clones within heterogeneous populations. It has been determined by measuring the growth of individual colonies by counting cells per colony that there is a broad distribution of colony growth rates [3]. This clonal heterogeneity can be obscured by pooling results from diverse clones within a heterogeneous population. This has been shown by statistical analysis of cell cycle times determined by time-lapse photography of cell pedigrees (clones) [17]. Computer modeling of experimental results suggests that the growth rate of cells within each clone persists for many generations [10] and regresses to the mean slowly [8]. The persistence of the growth rate of cells within clones continues even if the clones are dispersed and individual cells are allowed to form secondary colonies [3]. Persistence of the growth rate of clones within heterogeneous populations has also been reported by others for populations of mammalian cells [9] as well as for populations of lower eukaryotic cells [30] and bacteria [15]. The persistence of the growth rate of related subclones of tumor cells has been used as the basis for an assay designed to determine the response of a heterogeneous tumor population to chemotherapeutic drugs – the divided colony assay [18].

In the study reported here we investigated the growth of *ras*-transformed tumor cell populations and nontumor cell populations in the presence of the chemotherapeutic drug cytosine arabinoside (Ara-C). Clonal analysis revealed that *ras*-transformed and nontransformed cells respond differently to Ara-C.

Materials and methods

Cell lines and culture conditions

NIH3T3 is a nontransformed mouse fibroblast cell line derived from the NIH strain of mice [16]. NIH3T3(*ras*) is a morphologically and malignantly transformed derivative containing a 6.6-kb *Bam*HI DNA fragment with the human Ha-*ras* oncogene (cellular homologue of the Harvey sarcoma virus *ras* oncogene) from the EJ bladder carcinoma cell line [6] with an activating mutation in the 12th codon [25]. These cell lines were previously referred to as NIH and N(EJ MboI)2′ and were kindly provided by Dr. G. Cooper (Dana-Farber, Boston, Mass.). Transformation was confirmed by cell morphology and anchorage-independent growth. Cells were routinely grown at 35 °C in Dulbecco's modified Eagle's medium (low glucose) with pyruvate (GIBCO 430-1600) with 10% (vol/vol) fetal bovine serum (DME10 medium) in an atmosphere of 7.5% CO_2/92.5% air and maintained in an actively growing state by dilution twice weekly.

Semiautomated divided colony assay

The semiautomated divided colony procedure is illustrated in Fig. 1. Primary colonies were obtained as follows: a well-separated suspension of cells from an actively growing culture was diluted to 1.5 cells per ml of DME10, 0.2 ml was distributed into each of 96 wells of flat bottom multiwell dishes (Linbro 76-003-05), and incubated for 10 days (NIH3T3 cells) or 8 days (NIH3T3(*ras*) cells). This resulted in 15–20 single-cell-derived colonies per multiwell plate, the remainder of the wells having no cells. Secondary subclones were obtained as follows. Wells with colonies were located with the aid of a phase contrast microscope at 6× magnification. Each well was marked, cells were separated with trypsin, resuspended into 0.25 ml DME10, and 0.05 ml was distributed into each of four wells of a new plate. Then 0.15 ml fresh DME10 was added to two wells (controls) and DME10 with a final concentration of 0.03 μg/ml Ara-C was added to two wells (treated). Ara-C (cytosine-β-D-arabino furanoside hydrochloride crystalline, Sigma C-6645) was dissolved in DME10 (100 μg/ml), filter-sterilized, and diluted to a final concentration of 0.03 μg/ml. Subclones were incubated for 6 days (NIH3T3 cells) or 7 days (NIH3T3(*ras*) cells). The number of cells per well (actually cell mass) was determined by staining and reading optical density of each well in a microplate reader as follows. Cells were fixed by adding 0.1 ml cold 30% trichloroacetic acid to the medium in each well and refrigerating the dish for 1 h. Medium and precipitate were removed by washing in tap water and dishes were dried in air. Cells were stained for 20 min by adding 0.1 ml per well of 0.2% bromophenol blue (Fisher B392) in 10% acetic acid, and then unbound stain was washed out with excess 10% acetic acid. Stain bound to cells was solubilized, and color was developed by the addition of 0.1 ml per well of 25 m*M* unbuffer Tris base. After incubation for 15 min at room temperature, the optical density of each well was measured using a microplate reader (Model 580; Dynateck Laboratories, Alexandria, Va.) set for transmission at a wavelength of 570 nm and reference at 405 nm. Optical density readings between 0.14 and 1.2 were proportional to cell number. The contents of wells with readings greater than 1.2 were diluted in 0.1 ml 25 m*M* unbuffered Tris base and read again. Blank wells had medium but no cells. Data was collected on paper tapes, or directly into computer files by interfacing the miroplate reader to a Macintosh computer with Mac I/O software (Dynatech Laboratories, Alexandria, Va.). We are indebted to Dr. P. Skehan, Frederick Cancer Center, for the staining procedure and to Dr. R. Simpson, Rutgers University, for use of the microplate reader.

Simulation and statistical analysis

GPSS/VX (Simulation Software Design and Development, London, Ontario) software was used to develop programs for simulation on a VAXcluster (Digital Equipment Corp., Maynard, Mass.) and StatView (Abacus Concepts, Berkeley, Calif.) was used for statistical analysis on a Macintosh IIci (Apple Computer, Cupertino, Calif.). The significance of differences between means was determined by the unpaired *t*-test. Tests of differences between regression coefficients and between simple product moment correlation coefficients were programmed in Excel version 3.0 (Microsoft Corp., Redmond, Wash.) based on algorithms in Zar [32]. Files of experimental data for the semiautomated divided colony experiments are available upon request: disk name, DATA2.AX. NIH3T3; cell line file name, MD070191; NIH3T3(*ras*) cell line filename, MD050391.ALL.

Results

Measuring drug response with the semiautomated divided colony assay

Clonal heterogeneity of drug response can be obscured by assays that characterize a population of cells by a single parameter, for instance comparing the growth

of a population of cells in the presence and absence of a drug. We have previously devised an assay that measures the drug response of individual clones and is suitable for clonally heterogeneous populations [18]. We have now developed a semiautomated version of the divided colony assay. It is illustrated in Fig. 1 and technical details are given in the Materials and methods section. Briefly, single cells are allowed to form primary colonies in wells of a multiwell dish, then each primary colony is divided into four equal parts and each part allowed to grow into a subclone. Two subclones from each primary colony are grown in the absence of a drug (controls), and two subclones are grown in the presence of a drug (treated). The numbers of cells in subclones is determined by staining cells and measuring the optical density in wells with an automated multiwell reader. The reliability of the assay is determined by comparing pairs of related control subclones with each other, and by comparing pairs of related treated subclones with each other. The effect of a drug is determined by comparing pairs of control and treated subclones. Typically, 50 to 100 primary colonies are analyzed. This procedure allows a large amount of reliable data to be collected in a reasonable time with a reasonable effort. It has proved suitable for analysis of response of heterogeneous cell populations to low doses of drugs.

Response of NIH3T3 and NIH3T3(*ras*) cells to Ara-C

The response of populations of NIH3T3 and NIH3T3(*ras*) cells to Ara-C was determined in the semiautomated divided colony assay. For each cell line a broad distribution of sizes of untreated subclones was observed (Fig. 2) as previously detected using a manual version of the assay [18]. Nontumor and tumor cell lines are each heterogeneous. Nevertheless, for each cell line the sizes of untreated subclones were highly correlated with the sizes of paired untreated sublclones (Fig. 2, Table 1). Also for each cell line the sizes of drug-treated subclones were highly correlated with the sizes of paired treated subclones (Fig. 2, Table 1). This indicates that in spite of the differences in sizes between subclones from different primary colonies the assay was able to detect the similarity of cells within each clone.

The response of cells to the drug was determined by comparing the size of control subclones with the size of related subclones treated with drug. This is a novel aspect of the divided colony assay that can provide new information about heterogeneous cell populations. Clonal analysis revealed an interesting difference between NIH3T3 and NIH3T3(*ras*) cell populations. For NIH3T3(*ras*) cells the correlation coefficient of control vs treated subclones was low ($r = 0.098$) and not significantly different from zero (Table 1, Fig. 3). In contrast, for NIH3T3 cells the correlation coefficient of control vs treated subclones was high ($r = 0.851$) and significantly different from zero. This difference between *ras*-transformed and nontransformed cells was not detectable by the usual procedure of comparing the ratios of growth in the presence of the drug to growth in the absence of drug. This is because for NIH3T3 cells the ratio of the sum of treated clones to that of untreated clones was 0.42, and for NIH3T3(*ras*) cells the ratio was 0.40. These values are similar to each other when compared with the broad distribution among clones (Fig. 3). Although growth inhibition of the two cell populations appeared to be similar (about 40%) when clones within the heterogeneous populations were pooled, analysis of growth inhibition of individual clones revealed that the two populations responded differently to the drug. Pooling data from individual subclones within a heterogeneous population may obscure differences when two heterogeneous populations are compared.

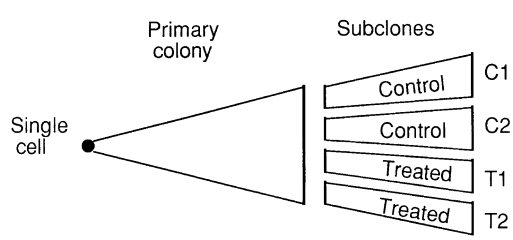

Fig. 1 Divided colony procedure. Single cells grow into primary colonies in individual wells of a multiwell dish, then primary colonies are divided into four subclones in separate wells and allowed to grow again. Two subclones are treated with a drug and two control subclones are not treated. The relative number of cells in each subclone is determined by measuring the optical density of stained cells using an automated multiwell dish reader. Comparison between the two control subclones (C1 vs C2) , and between the two treated subclones (T1 vs T2) from the same primary colony indicates the reproducibility of the procedure. Comparison between control and treated subclones (C1 vs T1, etc.) from the same primary colony indicates the inhibition of cells in the presence of the drug. Comparisons are made by calculating correlation coefficients for 50 to 100 single cell-derived colonies from a population of cells

Discussion

The results of this study indicate that the cell populations were heterogeneous since individual cells generated clones of different sizes and different drug response. In spite of this heterogeneity between clones, cells within each clone were similar in growth ability and response to drug. When the effects of interclonal variability were reduced by the divided colony procedure, a difference in drug response was revealed between *ras* oncogene-transformed and nontransformed cells.

These observations bring up two questions about cells in clones. First, how is the cell cycle time of a cell

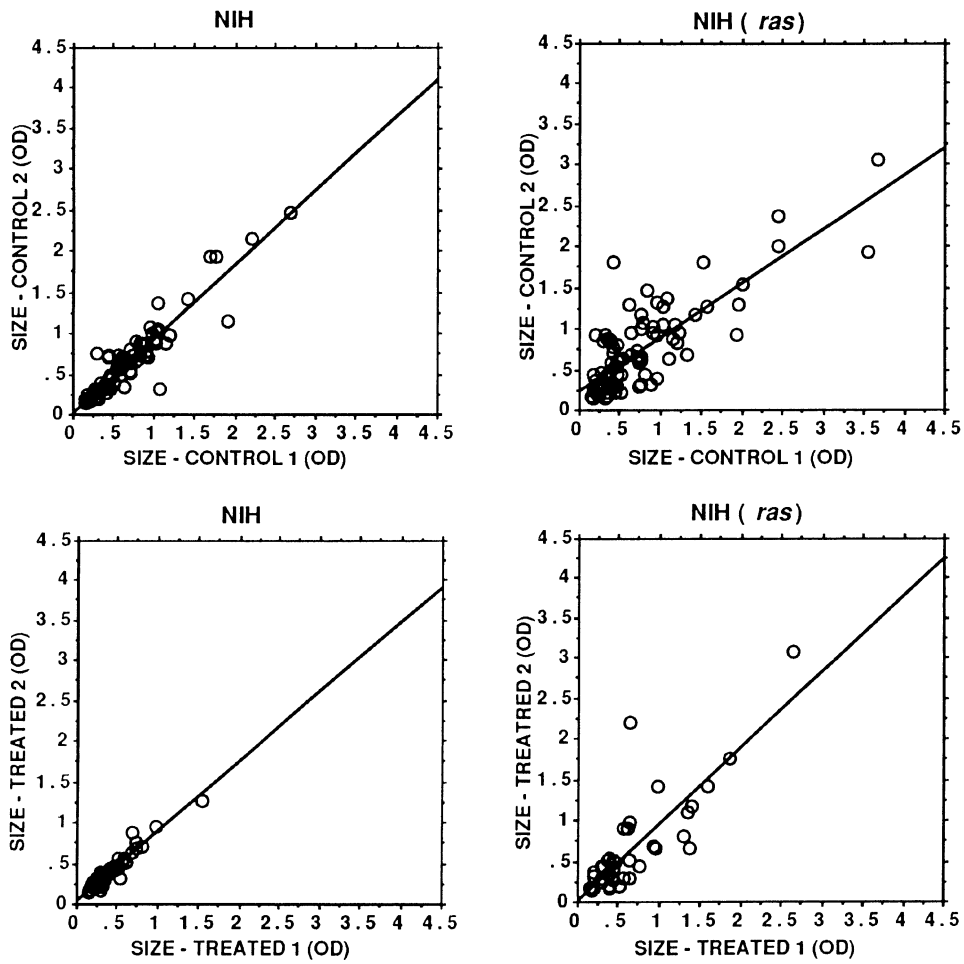

Fig. 2 Reproducibility of the divided colony procedure. The size of sibling subclones is compared by plotting the size of paired control or paired treated subclones for the cell lines NIH3T3 and NIH3T3(*ras*). There is a range of sizes of subclones, but the sizes of sibling subclones are similar, as indicated by the values of the correlation coefficients greater than 0.8 (Table 1)

Table 1 Correlation coefficients between subclones in the divided colony assay (*r* correlation coefficient, *n* number of observations). For modeling, the average cell lifetime was assumed to be 16–18 h in the absence of drug. A formal description of the clonal inheritance model, and of linear and nonlinear response to drug, is given in the Discussion

	Control 1 vs control 2		Treated 1 vs treated 2		Control vs treated	
	r	*n*	*r*	*n*	*r*	*n*
Observed (cell lines)[a]						
NIH	0.931	86	0.961	65	0.851	62
NIH(*ras*)	0.804	95	0.821	42	0.098	49
Simulated (clonal inheritance model)[b]						
Linear response	0.98	79	0.97	79	0.97	79
Nonlinear response	0.95	79	0.97	79	0.027	79

All correlation coefficients are significantly different from zero, except 0.098 and 0.027
[a] One of four nearly identical values for correlations of control vs treated are given
[b] Other models of inheritance give correlations of control vs treated greater than zero, even if the response was assumed to be nonlinear (see Discussion)

related to the cell cycle time of its progenitor cells? Second, how do cell cycle times change in the presence of a drug? In order to explore these, and other, questions we have developed several models of inheritance of cell cycle times and investigated their behavior by computer simulation. When the simulation results were compared to experimental observations on inheritance of colony sizes in recloning experiments, in the absence of drugs, it was found that a model in which cell cycle times were clonally inherited was adequate to reproduce experimental results by several criteria. These criteria included the variance of cell numbers in different clones, and the correlation coefficient between numbers of cells in primary colonies and numbers of

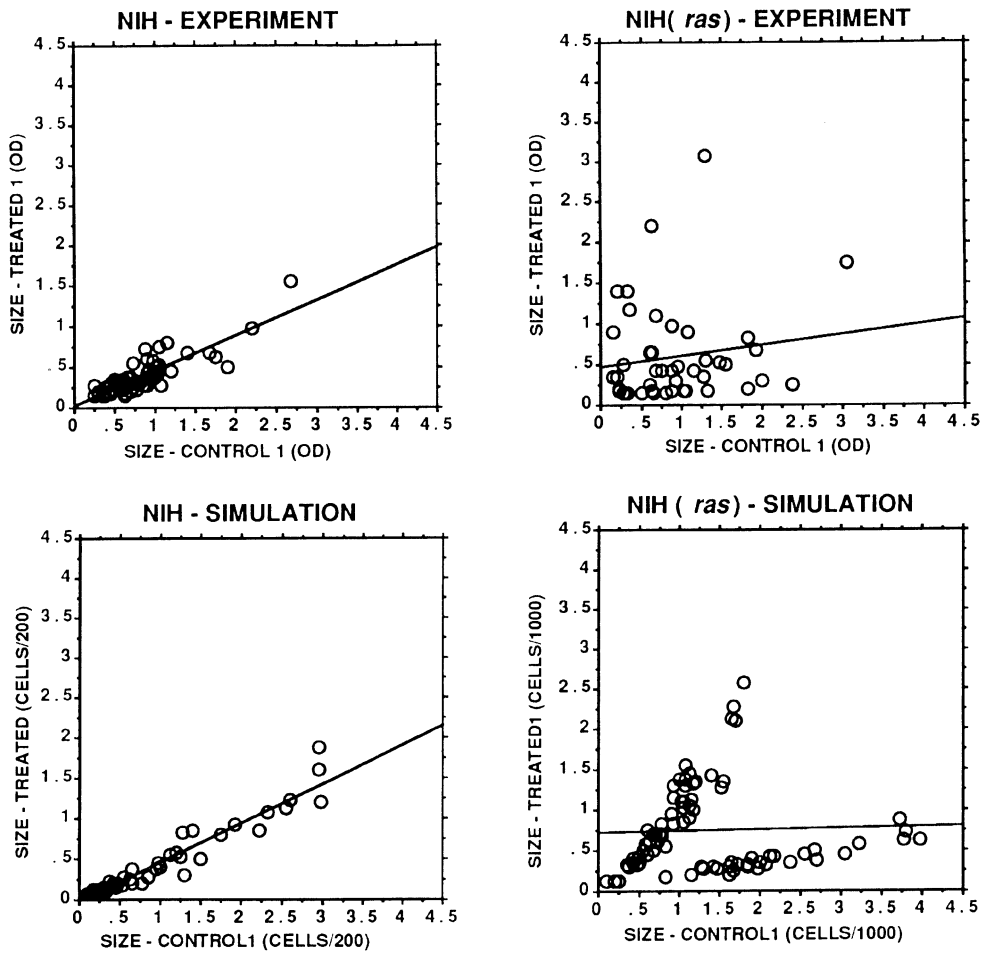

Fig. 3 Response of cells to drug treatment. *Top* The size of sibling subclones is compared by plotting the size of treated vs control subclones for the cell lines NIH3T3 and NIH3T3(*ras*). For NIH3T3 $r = 0.851$, and for NIH3T3(*ras*) $r = 0.098$. *Bottom* The size of sibling subclones is compared by plotting the size of treated vs control subclones for the model of clonal inheritance, assuming linear response for NIH3T3 and nonlinear response for NIH3T3(*ras*). For the clonal inheritance model with linear response $r = 0.97$, and for nonlinear response $r = 0.027$

cells in secondary colonies derived from cells in the primary colonies [3, 11, 12]. Three other models of inheritance of cell cycle times were not consistent with experimental observations; these models are no inheritance, exact inheritance, and mother-to-daughter inheritance. The clonal inheritance model describes the cell cycle times of cells in each clone as deviating from the cell cycle time of the cell which initiates each clone.

The clonal inheritance model of cell cycle times was then used as a basis to model the size of sibling subclones observed in the divided colony assay. By comparison of experimental and modeling results additional information was obtained about the growth of cells in the absence, and in the presence, of the drug, and about how the *ras* oncogene altered the growth in the presence of the drug.

It was observed that there were high correlation coefficients between the sizes of sibling subclones that grew in the absence of the drug. The clonal inheritance model, as well as the other models, generated high correlation coefficients between the sizes of unperturbed cells within each colony. Also observed were high correlation coefficients between sizes of treated subclones. High correlation coefficients between sizes of treated subclones could be simulated using various forms of drug-induced delay of cell cycle times, so that the values of these correlation coefficients were not sufficient to decide how drug delayed cell cycle times.

The values of the correlation coefficients between sibling control and treated subclones were observed to be significantly different in NIH3T3 and NIH3T3(*ras*) cell populations. These differences are informative in distinguishing different possible modes of drug response in the two types of cell populations. The numbers of cells in single cell-derived clones were simulated using the previously established clonal inheritance model of cell cycle times:

$$T_{i=}(T_0 + R_i) \qquad i = 1, 2, 3, \ldots$$

where T_i is the cell cycle time of a progeny cell in a clone, T_0 is the cell cycle time of the cell initiating the clone, and R_i is the value of a random variable which is different for each cell.

The effect of drug was first modeled as an additive delay of cell cycle times:

$$T_{i,\mathrm{drug}} = (T_0 + R_i) + T_{\mathrm{drug}}$$

where T_{drug} is an additional time in the presence of the drug. Based on previous time-lapse data [17] the cell cycle times of initial cells were assumed to be 16–18 h,

and based on the observed inhibition the delay due to drug was assumed to be 8–10 h. The assumption that the drug causes an additive delay of cell cycle time reproduced the high values of correlation coefficients of control vs control, treated vs treated, and control vs treated subclones observed for NIH3T3 cells (Table 1, Fig. 3). However, the assumption of simple additive delay could not reproduce the low values of correlation coefficients of control vs treated subclones observed for NIH3T3(*ras*) cells.

For NIH3T3(*ras*) cells the correlation coefficients between the sizes of control and control, and between treated and treated subclones were observed to be high, but the correlation between control and treated subclones was observed to be low. Models incorporating additive delay of cell cycle times were not able to simulate low correlation coefficients, so other forms of delay were considered. Based on the biological hypothesis that fast-growing cells might be more sensitive to drug than slow-growing cells, the possibility was considered that a cell growing in the presence of drug would have an increase in lifetime that was inversely proportional to its clonally inherited lifetime T_0:

$$T_{i,\mathrm{drug}} = (T_0 + R_i) + T_{\mathrm{drug}}(k/T_0)$$

where k is the mean lifetime of cells initiating clones. Simulations based on this model were able to reproduce the high correlation coefficients observed between control and control and between treated and treated. In addition, simulations based on this model were able to reproduce the low correlation coefficients for control vs treated observed for NIH3T3(*ras*) cells (Table 1, Fig. 3). It is also noteworthy that this low value for the correlation coefficient between control and treated subclones can occur even in the presence of inheritance of cell lifetimes.

From comparisons between these simulation results and the experimental observations we conclude that cell cycle times are clonally inherited for both NIH3T3 and NIH3T3(*ras*) cells. In the absence of drug, progeny cells in a clone have cell cycle times that deviate from the cell cycle time of the cell initiating the clone. In the presence of a specific concentration of drug, NIH3T3 cells have cell cycle times that are increased by a specific amount, whereas NIH3T3(*ras*) cells have an increase that depends also upon their clonally inherited cell cycle time.

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