High-Frequency Variation and Population Drift in a Newly Transformed Clone of BALB/3T3 Cells¹

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ABSTRACT

During repeated passage of BALB/3T3 cells and testing for anchorage-independent growth, a single transformed clone was isolated from agar, and five subclones were derived from it. These subclones differed from one another in morphology on a solid substratum, efficiency and size of colony formation in agar, and rate of tumor formation in nude mice. With weekly passage over a period of 6 months, the differences in morphology and growth in agar gradually decreased. The subclone which produced the fastest-growing tumors in nude mice after 4 weeks of culture produced the slowest-growing tumors after 18 weeks, and a change in the opposite direction was made by another subclone. There was no difference among the subclones in growth rate on plastic. The distribution of chromosome numbers was heterogeneous but overlapping in all the primary subclones at 16 and 24 weeks, with no statistically significant difference in the mean number of chromosomes per subclone. An extremely high degree of variation must have occurred to produce the multiple differences between the subclones, and the same type of variation could have been responsible for the subsequent changes with repeated passage. The high frequency and graded nature of the changes and the concurrent involvement of several traits suggest an epigenetic basis for the variation.

INTRODUCTION

The ability of many animal cell lines to form colonies when suspended in semisolid medium (anchorage independence) is strongly correlated with their ability to produce tumors when inoculated into animals (1, 4, 25). During the repeated passaging of BALB/3T3 cells on plastic, they were repeatedly tested for their capacity to form large colonies when suspended in soft agar. In one such colony, which appeared after several months of passaging, the anchorage-independent, transformed cells were isolated and maintained in continuous passage on plastic (19). Passage of the anchorage-independent nontransformed parental population on plastic was continued for 5 more months before another colony arose that was capable of producing large colonies in agar. Because the transformed cells multiply much more rapidly than do the nontransformed cells, the presence of even a single transformed cell in the parental culture would soon be detected. Therefore, it is likely that the cell which initiated the first agar colony was the only one in the routinely passaged population and that it had become transformed at just about the time it was seeded in agar. Subclones were made of the transformed clone to observe the extent of variation in a newly transformed population of cells. It was found that each of 5 subclones differed from the others in morphology, CFEag, and rate of tumor formation in nude mice. The properties of the subclones changed with repeated passaging. The changes in CFEag occurring during the first 6 weeks of culturing the subclones and secondary subclones have been described (19). In the present work, we describe further changes which occurred in the subclonal populations during a 6-month period, including changes in morphology and tumor-producing capacity as well as CFEag. The results are discussed in terms of their possible significance for understanding the genesis of heterogeneity and its role in progression of tumors during their early development.

MATERIALS AND METHODS

Cells and Culture Methods. The cells used in these experiments were derived by 2 cycles of redenuding from the AS1 clone of the BALB/3T3 line of mouse fibroblasts. They were transferred weekly at a concentration of 10⁶ cells/50-mm Falcon plastic tissue culture dish. The growth medium was MCDB402 (23) plus 10% calf or fetal calf serum. The medium was always changed the day before transfer, and 2 x 10⁶ to 10⁷ cells were assayed every 2 weeks for their capacity to produce large colonies in agar. A single colony arose during one such assay of 2 x 10⁶ cells and was isolated, grown on plastic, and reseeded in agar where CFEag was 9.2%. Five of the agar colonies were isolated from a dish containing 9 such colonies which arose from 100 seeded cells, and these are the primary subclones described here. The cells in all the primary subclones had the rounded morphology representative of transformed cells, although there were significant differences in appearance among the subclones. They also multiplied about 3 times faster than did their nontransformed progenitors and metabolized glucose at a much faster rate. Since the agar colonies arise from single cells, the original transformed colony and the 5 colonies derived from it are considered pure clones. No transformed cells appeared in the parental nontransformed culture during a further 6-month passaging schedule. If there had been any other transformed cells in the culture at the time the original colony appeared in agar, they would have soon become apparent because of their selectively fast growth rate. We therefore assume that the transforming event occurred about the time the cells were seeded in agar. The primary subclones were therefore obtained within about 3 weeks after the initial transforming event (2 weeks for development of the original colony and 1 week of growth on plastic after its isolation before reseeding in agar).

After the 5 primary subclones were isolated, they were trypsinized weekly and transferred at a concentration of 5 x 10⁶ or 10⁷ cells/50-mm dish, with a medium change the day before transfer. When 5 x 10⁶ cells were transferred, there was a weekly increase of 500- to 800-fold in cell number. They were also assayed in agar every week during the first 6 weeks and every week or 2 thereafter. At the second week, a second generation of subclones was initiated from 3 of the 5 primary subclones.

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by seeding in agar. Another second generation of subclones was initiated from the same primary subclones at 8 weeks.

Colony Formation in Agar. Cells from the routine weekly transfers were used for the agar assay 18 to 20 hr after a medium change. A base layer of 7.0 ml containing 0.9% Difco Bacto-Agar in growth medium was made in 60-mm bacteriological dishes. It was gelled at room temperature and then brought to 37°C before adding the top layer containing cells (21). Usually, 500 cells were suspended in 0.5 ml of medium at 37°C, mixed with 1.0 ml of 0.6% of melted agar at 43°C, and immediately layered over the base layer. After gelling of the top layer at room temperature, the cultures were sealed with paraffin, and incubated at 37°C for 2 weeks to allow development of colonies. The colonies were counted on a grid at ×25 in an inverted microscope. An ocular micrometer was used to measure colony diameter. Usually, the diameters of 100 colonies were measured, and the mean and standard error were calculated.

Chromosome Preparation. We used an adaptation by Walen (26) of a standard method for preparing chromosomes (22). Medium was changed the day before processing cultures for chromosome analysis. They were treated with 10^{-4} M Colcemid for 4 hr. Briefly, they were then trypsinized, the cells were centrifuged, and the pellet was treated with hypotonic medium. The cells were fixed in methanol:glacial acetic acid (3:1) and dropped onto a slide. After at least 24 hr aging, the slides were stained with Giemsa solution in phosphate buffer at pH 6.8, rinsed, and dried; and a coverslip was mounted on the slide. The chromosomes were counted either directly with the aid of an ocular grid or in photographs. As a control on technique, a single clone derived from a tumor of subclone 1 was prepared for counting 2 weeks (about 20 cell divisions) after initiating a colony on plastic with a single cell. The distribution of chromosome numbers in 25 cells was 74(1); 78(2); 79(6); 80(5); 81(9); 82(2). The average was 79.9 ±1.7 (S.D.).

Tumor Formation. Cells were used for tumor formation in mice at the same time that aliquots were used for transfer and for assay in agar. They were suspended in growth medium, and 5 × 10³ or 1 × 10⁴ cells in 0.5 ml were inoculated under the skin between the shoulder blades of young adult N:NIH(S)xnu/nu mice raised at the California Department of Health Services. The inoculation sites were inspected weekly, and tumor sizes were measured after they had reached 2 mm. In the second series of inoculations, ear bands were used to identify individual mice.

RESULTS

Cell Morphology. When first isolated, the 5 subclones differed from each other unambiguously in appearance. Subclone 1 was particularly distinctive, since it formed spherical clumps which readily detached from the surface of the culture dish, while the others formed discrete, flat islands of cells which remained attached. With repeated passage, however, subclone 1 took on the colonial morphology of the others (Fig. 1; Ref. 19), which in turn became more like one another (not shown).

CFEag and Colony Size in Agar. The sequential changes in CFEag up to the sixth week after routine passaging of the subclones was begun were as described previously (19). These and subsequent changes are now represented in Chart 1. The 5 subclones differed from each other in the early assays, and their CFEag followed different and erratic paths thereafter. However, subclones 1 and 3 followed the same path after the ninth week. They were joined by subclone 4 at the 18th week and by subclone 5 at the 22nd week. A distinct shift of a major peak of subclone 1 to a larger size is also evident.

The average colony sizes of the 5 subclones during the first 8 weeks of passaging are shown in Chart 2. Although the subclones differed in average diameters in the first few weeks of passaging, they tended to converge and by the eighth week were not significantly different from one another. Their sizes were again determined between the 22nd and 26th week, and no consistent differences between clones were found (data not shown). In effect, the size of colonies of subclone 3 which had the largest diameter initially remained constant, while the colony sizes of the smaller subclones gradually increased to the level of subclone 3.

Profiles of the size distribution of the 5 subclones were drawn for each of the first 6 weekly assays. For the sake of clarity, the profiles of only the first and sixth weeks of subclones 1 to 4 are shown in Chart 3. The most obvious change occurred in subclone 2 which initially had only small colonies. By the sixth week, a considerable fraction of larger colonies had appeared in the subclone 2 population. A distinct shift of a major peak of subclone 1 to a larger size is also evident.

CFEag among Secondary Subclones. Secondary subclones were made from the primary subclones 1, 2, and 3 at the second and ninth weeks of passage. The CFEag in the first agar assay of the secondary subclones is shown in Chart 4. The average CFEag of the first set of secondary subclones of primary subclone 3 was distinctly higher than that of the secondary subclones of primary subclones 1 and 2. This might be expected since they were assayed at a time that subclone 3 itself had the
cells from each of the primary subclones were inoculated into nude mice at the 4th and 18th weeks of passage. Cells of the nontransformed progenitor line ($10^6$) were also inoculated into mice at about the same times. All mice inoculated with the transformed cells developed tumors within 3 weeks, whereas no mice inoculated with the nontransformed cells developed tumors within a period of more than 5 months. The rates of tumor development are shown in Chart 5. Table 1 shows the number of mice surviving the inoculation of the 4- and 18-week cells. The cause of death between 4 and 5 weeks after inoculation of the 4-week cells is unknown. In the first assay, subclone 3 produced the most rapidly growing tumors, and subclones 4 and 5 produced the slowest. In the later assay, the situation had been strikingly reversed for subclones 3 and 4, since the former now produced the slowest-growing and the latter produced the highest CFEag of the 5 primary subclones. This situation reversed itself in the later set of secondary subclones, when the CFEag of primary subclone 3 had decreased. There was considerable heterogeneity in CFEag even within families of the secondary subclones.

Tumor Production by the Primary Subclones. Five $\times 10^5$
fastest-growing tumors. Subclone 5 also grew at a much faster rate in the later assay. There was no consistent quantitative correlation of tumor growth rate with CFEag, since subclone 4 had a slightly higher CFEag at 4 weeks than subclone 3 with the same cells that produced much slower-growing tumors. As noted previously (18, 20), there is an excellent qualitative correlation, among the populations of the BALB/3T3 cells used here, between capacity for colony formation in agar and tumor production in nude mice: all clones and subclones which produce a significant fraction (≥1%) of colonies in agar (≥0.14 mm in diameter) form sarcomas in nude mice; all those which fail to produce colonies of this size in agar also fail to form tumors.

**Growth Rates and Chromosome Counts of the Primary Subclones.** Spot checks of the growth rates of the cells on plastic were made by counting cells at each weekly transfer. No consistent difference in weekly cell yield between clones was found in the 6 months of observation (data not shown). Daily counts of the primary subclones were made for 7 days after their transfer on plastic at the sixth week (Chart 1). The growth rates of the 5 subclones were indistinguishable from one another (Chart 6), although there was more than a 3-fold difference in CFEag among the subclones at this time (Chart 1).

The distributions of chromosome numbers for the 5 primary subclones at Weeks 16 and 24 are shown in Chart 7. There was a heterogeneous distribution of chromosome numbers with considerable overlap at both times among all the subclones. In most cases, there was a clustering of frequencies at about 80 chromosomes/cell, and there was no significant difference in the mean number of chromosomes among the subclones. Given the heterogeneous distribution and degree of overlap, no conclusions can be drawn about the role of chromosomal change in producing the observed changes in cell behavior.

**DISCUSSION**

The results presented here indicate that variation occurs at a much higher rate in animal cells than can be accounted for by somatic mutations. This was most clearly evident in the multiple differences between the 5 primary subclones obtained from the original transformed clone soon after its transformation, the subclones differed in morphology, frequency, and size of colonies produced in agar suspension and in the rate of tumor formation in nude mice. Each of these characters changed with time as the subclones were serially passaged. The tendency toward a common morphological type was most clearly evident in subclone 1,
which originally multiplied to form localized clumps on the surface of the dish but eventually took on the quasi-monolayered appearance of the other subclones. In the first agar assay, there was more than a 6-fold spread in CFEag; but by 23 weeks, 4 of the subclones were grouped at the same CFEag level; and the fifth was only slightly higher (Chart 1). The subclone which produced the fastest-growing tumors at 4 weeks produced the slowest-growing at 18 weeks, and one of the slowest tumor producers at 4 weeks became the fastest at 18 weeks. There was no indication that the changes in morphology, growth in agar, and tumor production were coordinated with one another. There was no difference among the subclones in their growth rates on plastic, nor was there any change in weekly cell yields during the course of passageing. The high frequency of the initial variation, the involvement of multiple characters, and the lack of evidence for selective overgrowth tend to support an epigenetic basis for the observed changes. Parsimony leads us to propose that the changes in cell appearance and behavior which occurred during the long-term culture arose from the same type of event as that responsible for the heterogeneity which was expressed when the subclones were first isolated. Indeed, the question arises whether the initial transforming event was not of the same type. It is known that cells in spontaneous tumors become frankly malignant only after they have undergone a complex, generally unpredictable set of changes, subsumed under the rubric of progression, and these changes continue as the cells become more autonomous (8). Evidence has also been presented for the progressive nature of neoplastic transformation in culture (2). The changes in appearance and behavior observed in serial passage of our cells may be likened to progression, particularly since the behavioral changes include the tumor-forming capacity itself. Four of our subclones produced faster-growing tumors at 18 weeks than at 4 weeks, while one produced slower-growing tumors. Although increased speed of tumor formation would ordinarily be associated with progression, there are other reports of loss of tumor-forming capacity in culture (15, 17), and both types of change may have a similar basis.

There have been many reports of heterogeneity in tumors in recent years involving a wide variety of cellular properties. Our report is the first one of our knowledge to trace the origin of heterogeneity to the initial transforming event. Heterogeneity, of course, implies heritable change in cells. It has been estimated previously that the events which lead to metastatic heterogeneity occur at a rate of about $10^{-6}$/cell division (11). The heterogeneity described here must occur at a much higher rate, since all 5 of the primary subclones differed from each other, and they were obtained shortly after the cloning of the original transformed cells. Indeed, it is not inconceivable that every cell in the initial clone differed in one respect or another from every other cell.

It would be useful to obtain estimates of the rates of variation for CFEag, which is the most accurately and frequently measured of our 3 characters. The usual method for doing so is to apply fluctuation analysis (16). This, however, is most suitable for rare, random, stable mutations that tend to be of an all-or-none type (14). The changes in CFEag are so common that we have not obtained cultures without variants, which makes calculation of rates by fluctuation analysis impossible. A further difficulty for fluctuation analysis is the quantitative, graded nature of the changes in CFEag. Assuming that more sensitive methods can be found for detecting variants, it is not impossible that the rate of variation will approach unity.

In contrast to the instability of CFEag, the clones maintained their generic identity as transformed, tumor-producing cells, and in some traits such as growth rate on plastic they were indistinguishable from one another. In addition, they tended to converge in CFEag during long-term culture. It seems likely, therefore, that each subclone rapidly became a mosaic of cells with somewhat differing properties as it grew into a large population. This suggests a similarity to the mosaicism described for the ability of Chinese hamster ovary cells to multiply in selective HAT medium after they had been subject to prolonged culture in bromodeoxyuridine (13). It also recalls the great variation in content of several enzymes observed among clones and subclones of normal human genital fibroblasts (10). The extremely high frequency and the broadly graded variation in both these cases are suggestive of an epigenetic origin. The evidence for an epigenetic basis for the ability to grow in HAT medium is reinforced by the ability of 5-azacytidine to induce a massive and rapid restoration of the ability of cells to grow in the selective medium (12). This, of course, implies that DNA methylation governs the capacity to grow in HAT medium, but the implication is compromised by the observation that unrelated compounds produce a similar effect.

DNA methylation has also been proposed as an epigenetic mechanism for generating tumor cell heterogeneity (9). This is but one of many explanations that have been offered for the occurrence of variation at rates which are much higher than the rates for clearly established somatic mutations. A nonexhaustive list would include gene amplification (14), chromosomal rearrangements (3), and the random distribution of scarce generator molecules (24). Any one, or combination of them, might be involved in the multifaceted variation described here. None of them predict the present findings that many characters differ among a high proportion of progeny in a clone and do so in an inheritable and uncoordinated manner. A radically different type of analysis which anticipated the initial observations of tumor cell heterogeneity by 2 decades has been developed by Elsasser (5–7). On the basis of the enormous molecular complexity of living cells, he concludes that the number of existing cells is only an immensely small fraction of the number possible according to combinatorics and quantum mechanics. This then leads to the further conclusions that each cell is unique and that causal chains of processes which determine morphology and behavior cannot be traced beyond a terminal point. The latter conclusion appears similar to the uncertainty principle of quantum mechanics but is based on the unfathomable complexity of the cell rather than on the perturbing role of the observer. The steps in development of this theory follow the methodology of logical positivism introduced into physical science by Ernst Mach. This rigorous mode of analysis avoids contradictions and deals only with observables, making no inferences about unknown links to underlying causal processes. It is a type of theory building which led to the 2 great modern revolutions in physics of relativity and quantum mechanics but is unfamiliar in biological thinking. It has the virtue, in the form of Elsasser’s theory, of imaging all the findings described here in a self-consistent manner.

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REFERENCES


Fig. 1. Morphology of subclone 1 at early and late stages in its passage history. Cells \((5 \times 10^5)\) were transferred and photographed at 6 days. The cells had been passaged for 4 weeks (a) and 22 weeks (b). Bar, 50 \(\mu\)m.
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