Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6226–6230, October 1981
Cell Biology

Interactions among clonal subpopulations affect stability of the metastatic phenotype in polyclonal populations of B16 melanoma cells

(cancer/cellular interactions/phenotypic regulation/growth control)

GEORGE POSTE*†, JOHN DOLL*, AND ISAIAH J. FIDLER‡

*Department of Tumor Biology, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101; †Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104; and ‡Cancer Metastasis and Treatment Laboratory, National Cancer Institute–Frederick Cancer Research Center, Frederick, Maryland 21701

Communicated by Peter C. Nowell, May 4, 1981

ABSTRACT **Analysis of the metastatic properties of clones isolated from mouse B16 melanoma cell lines (B16-F1 and F10) shows extensive cellular heterogeneity and the presence of subpopulations that have widely differing metastatic abilities. This pattern of metastatic heterogeneity is maintained during serial passage *in vitro* and *in vivo*. In contrast, even a short serial passage of individual clones isolated from these heterogenous parent lines results in rapid emergence of variant subclones that have different metastatic properties. If several clones are mixed and cocultivated, this instability is not expressed. These data suggest that, in polyclonal populations, the various clonal subpopulations somehow interact with one another to "stabilize" their relative proportions within the population. Restriction of clonal diversity by selective killing of the majority of clones in a polyclonal population eliminates the stabilizing restraints and stimulates rapid emergence of new subpopulations to create heterogenous populations containing a new panel of phenotypically diverse subpopulations that then reach stable proportions until the next selection pressure(s) is encountered.**

Recent studies have shown that many experimental animal tumors contain subpopulations of cells that differ in their ability to form metastases (for review, see ref. 1). Isolation and comparison of tumor cell clones (2–6) or sublines (7–15) that have different metastatic abilities offers new opportunities for correlating specific cellular alterations with the metastatic phenotype. The success of this approach will depend, however, on the stability of the metastatic phenotype in these subpopulations during serial passage *in vivo* or *in vitro*. In this paper, we examine the effects of cultivation *in vitro* and serial transplantation *in vivo* on the metastatic properties of a series of B16 melanoma clones of defined metastatic potential. We report that the metastatic phenotype is highly unstable in clones passaged in isolation. In contrast, the metastatic profile of heterogenous uncloned tumor cell lines or polyclonal mixtures created by deliberate mixing of cloned lines is stable over long period of transfer. The marked difference in the stability of the metastatic phenotype in cloned and uncloned populations suggests that individual subpopulations may be interacting to "stabilize" expression of the metastatic propensity of the population as a whole. This concept, if valid for other tumor systems, has important implications for experimental efforts to analyze the malignant phenotype and for the therapy of tumors *in situ*. In the latter, the therapeutic elimination of a major fraction of the subpopulations may destroy the stabilizing equilibrium that may exist between the subpopulations and lead to enhanced phenotypic instability in the surviving subpopulations. This, in turn, could be responsible for the subsequent generation of new subpopulations that have different phenotypes.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from the Jackson Laboratory, Bar Harbor, Maine and the Animal Breeding Facility of Roswell Park Memorial Institute. Animals were age and sex matched within a single experiment.

Cells. The origin and properties of the murine B16 melanoma cell lines, B16-F1 (low lung metastasis) and B16-F10 (high lung metastasis), have been described (7, 8). Clones were isolated from these lines by replica plating as described (16), and subclones were isolated from cloned lines by the same method. Aliquots of all clones and subclones, together with the parent cell populations from which they were derived, were stored at liquid nitrogen temperature for use as reference stocks. The isolation and properties of cell variants resistant to concentrations of ouabain (Oua^r), trifluorothymidine (TFT^r), and diaminopurine (DAP^r) toxic to wild-type B16 cells will be described elsewhere. All cultures were grown in Dulbecco's modified Eagle's minimal essential medium/10% fetal bovine serum (GIBCO) without antibiotics at 37°C in humidified 5% CO_2/95% air as described (8). Cell cultures were established from subcutaneous (s.c.) tumor nodules by enzymic dispersal of excised tumor tissue using 0.2% collagenase, type I/0.1% trypsin and grown in Eagle's medium/20% fetal bovine serum containing antibiotics.

Experimental Metastasis Formation. Unanesthetized mice were injected intravenously (i.v.) via the tail vein with 2.5×10^4 viable cells as a single-cell suspension in 0.2 ml of Hanks' balanced salt solution. Mice were killed 18 days later; the lungs were removed, rinsed in water, and fixed in formalin; and the melanotic lung tumor colonies (experimental metastases) were counted under a dissecting microscope (3).

Serial Transfer of Tumor Cells *in Vivo*. Mice were inoculated s.c. in the flank with 1×10^6 viable tumor cells. The resulting tumor nodules were excised 14 days later and dispersed to single-cell suspensions as described above. This sequence was repeated at biweekly intervals.

Statistical Analysis. The number of lung metastases produced by individual clones and subclones was compared with that produced by parent-cell populations by using the Mann–Whitney *U* test (17).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: DAP^r, diaminopurine resistant; i.v., intravenous; Oua^r, ouabain resistant; s.c., subcutaneous; TFT^r, trifluorothymidine resistant.

RESULTS

Stability of the Metastatic Phenotype in Uncloned B16 Melanoma Cell Lines. The B16-F1 and B16-F10 cell lines differ significantly in their ability to form lung metastases after i.v. injection (7, 8). The low (F1) and high (F10) metastatic phenotypes exhibited by these two lines are stable, however, even after continuous passage *in vitro* and *in vivo* (Table 1).

The heterogenous F1 and F10 lines have not been cloned and contain subpopulations of cells that have differing phenotypic properties, including metastatic abilities (3, 8, 11, 19). Analysis of the metastatic properties of clones isolated from these two lines during serial passage *in vivo* and *in vitro* shows that both the range of clonal variation and the relative proportion of clones in the population producing more, less, or similar degrees of metastatic disease than the uncloned parent population remains stable (Fig. 1).

Stability of the Metastatic Phenotype in Cloned B16 Cell Lines. To assess the stability of the metastatic phenotype in cloned B16 lines during serial passage, we have examined the metastatic properties of four clones isolated from the B16-F10 line. Clones 5 and 22 have a low capacity to form lung metastases and clones 18 and 42 produce significantly more metastases but not so many as to limit detection of variants with that have greater metastatic ability.

Each clone was cultured *in vitro* for 10, 20, or 40 subcultivations (5, 10, or 20 weeks), at which time a series of subclones was isolated and tested for ability to form lung metastases. The results (Fig. 2) indicate that the metastatic properties of many subclones differ significantly from those of their original parent clone, suggesting that the parental phenotype is unstable. Subclones that have significantly different metastatic properties can be identified after as few as 10 subcultivations *in vitro*. Further cultivation introduces additional variability and, after 20 and 40 subcultivations, the majority of subclones differ from the parent clone. Similar instability has been identified in other clones isolated from the B16-F10 line and in the B16-F1 line (not shown).

The metastatic phenotype is also unstable in cloned lines passaged in vivo via serial s.c. transplantation (Fig. 3).

The instability in the metastatic properties of the clones in Figs. 2 and 3 is in striking contrast to the stability of this phenotype in the uncloned heterogenous B16-F10 line from which they were isolated (Fig. 1).

Although phenotypic instability and the emergence of variant phenotypes was detected in subclones of all four parent clones, greater phenotypic shifts were detected in subclones from the low metastatic clones 5 and 22 as compared with the high metastatic clones 18 and 42.

Table 1. Metastatic properties of B16-F1 and B16-F10 melanoma cell lines after serial passage *in vitro* and *in vivo*

Passage	Passages	Lung metastases B16-F1	Lung metastases B16-F10	Metastasis formation (F10:F1)
In vitro	1	11 (1–53)	102 (14–160)	9.27
	10	12 (1–64)	114 (16–175)	9.50
	20	11 (1–53)	96 (12–165)	8.73
	40	17 (4–89)	126 (37–228)	7.41
	60	12 (2–58)	108 (40–235)	9.00
In vivo	1	8 (2–29)	113 (48–241)	14.13
	10	11 (2–61)	87 (10–180)	7.91
	20	14 (3–74)	123 (35–240)	8.79
	30	22 (1–54)	185 (52–270)	8.41

Cell lines *in vitro* were subcultured at 4-day intervals; cells *in vivo* were passaged s.c. every 2 weeks as single-cell suspensions (1×10^6 cells) obtained by enzymic dispersal of excised tumor tissue from the previous passage. C57BL/6 mice were injected i.v. with 2.5×10^4 cells and lung metastases were measured 18 days later. Results are based on 10 mice per group and represent median values; numbers in parentheses represent ranges of values.

Stability of the Metastatic Phenotype in Cell Populations Produced by Cocultivation of Tumor Cell Clones of Differing Metastatic Potential. The instability of the metastatic phenotype in cloned lines relative to the stable nature of this phenotype in uncloned cell populations suggests that the cellular subpopulations in uncloned lines might somehow be interacting to stabilize the relative proportions of each subpopulation within the entire population. In the absence of other selection pressure(s) favoring elimination of specific subpopulations, this stabilizing mechanism would maximize phenotypic diversity in the population and prevent dominance of a few or even a single population. To test this possibility, we mixed a series of clones of known metastatic potential to create a heterogenous population that was then cultivated *in vitro* to determine whether new variants having altered metastatic properties would rapidly emerge in similar fashion to events during propagation of single clones. To identify the parental origin of individual subclones isolated during serial passage, it was necessary to use parent cells bearing a variety of stable biochemical markers.

For these experiments, drug-resistant variants were isolated from the B16-F10 line and cloned, and clones showing high, intermediate, or low metastatic properties were identified. Freezer stocks of these clones were then mixed in equal numbers with wild-type clones having high, intermediate, or low metastatic properties (Fig. 4*A*).

As shown in Fig. 4*B*, the metastatic properties of subclones isolated from serially passaged mixed clone populations resemble the metastatic profiles of the original parent clones. Although it cannot be excluded that certain wild-type subclones that have high metastatic activity have not arisen from wild-type parent clones that have low or intermediate metastatic activity (or vice versa), this is considered unlikely as the overall profile of metastatic behavior exhibited by the subclones parallels the pattern of high, intermediate, and low metastatic abilities seen when the original clones were first mixed. Moreover, the finding that the metastatic properties of drug-resistant subclones corresponds to that of parent clones that have the same drug-resistant phenotype(s) further suggests that the clones are stable. The stability of the metastatic properties in the latter clones is not imposed by their drug-resistant status as, when propagated as single clones, emergence of variants that have different metastatic abilities is quickly detected (results not shown).

Testing of drug-resistant clones against a range of drug concentrations showed that no significant drift in the drug-resistance phenotype occurred in either clones grown singly or cocultivated with other drug-resistant and wild-type cells (not shown).

The data in Fig. 4*B* therefore suggest that coexistence of several subpopulations of cells that have different metastatic potential somehow exerts a stabilizing effect on the metastatic phenotype. This contrasts with the behavior of single clones, in which phenotypic instability is marked and new variants emerge rapidly (Figs. 2 and 3). The stabilizing effect seen in polyclonal populations is, however, specific for cells from the same tumor. Single clones of B16 melanoma cocultivated with clones from the Lewis lung carcinoma or UV2237 fibrosarcoma showed marked phenotypic instability and rapidly generate subclones that have very different metastatic properties (not shown).

The Effect of Restricting Subpopulation Heterogeneity on the Metastatic Properties of Polyclonal Populations. We next determined if the "stabilization" of metastatic properties seen

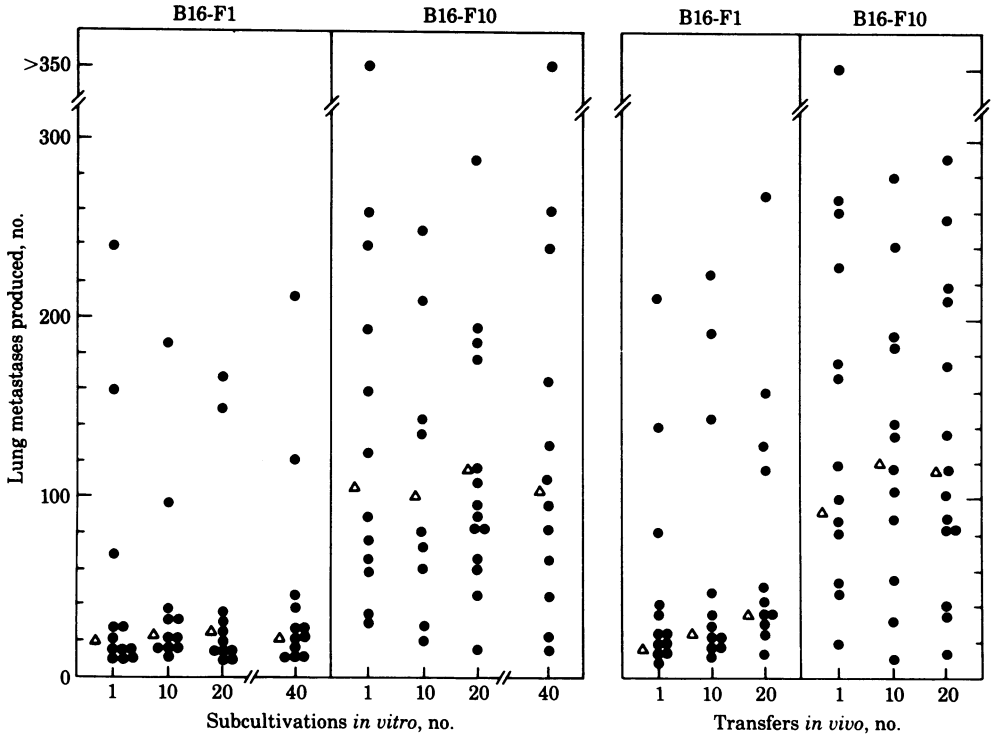

FIG. 1. Comparison between incidence of metastases produced by cells of individual clones (●) isolated from B16-F1 and B16-F10 melanoma cell lines during serial passage *in vitro* and *in vivo* and metastatic activity of the uncloned parent lines (△). Cell lines were passaged and metastatic activity was measured as described in the legend to Table 1, except results are based on 5 mice per group.

in polyclonal populations would change in the face of selection pressure(s) that restrict subpopulation diversity by eliminating unfit subpopulations. Polyclonal populations were prepared by mixing drug-resistant clones with *wt* clones and exposed subsequently to drugs to eliminate the *wt* clones. As shown in Fig. 4*B*, polyclonal cultures containing drug-resistant and *wt* clones show a stable range of metastatic behavior during serial passage in vitro. However, when treated with TFT to destroy susceptible *wt* and Oua^r cells, the surviving TFT^r cells exhibit an unstable metastatic phenotype and generate subclones with different metastatic properties (Fig. 4*C*). This, in turn, establishes a new heterogenous cell population whose overall range of metastatic behavior is stable (Fig. 4*C*). This stability then persists only until a new selection pressure that limits the population diversity is encountered which results in the cycle being repeated. This was demonstrated by adding a clone of DAP^r cells to the heterogenous TFT^r population derived from the first cycle of drug treatment. As shown in Fig. 4, panel D, the metastatic phenotype of the newly added DAP^r variant remains stable during serial passage, as does the overall range of metastatic behavior in the TFT^r population. However, on exposure to DAP to eliminate all cells but the DAP^r clone a new cycle of subpopulation restriction and diversification ensues in which the surviving DAP^r cells rapidly generate a new panel of varients whose overall range of metastatic behavior eventually becomes stable (Fig. 4*E*). Application of a new selection pressure to restrict subpopulation diversity in this population would pre-

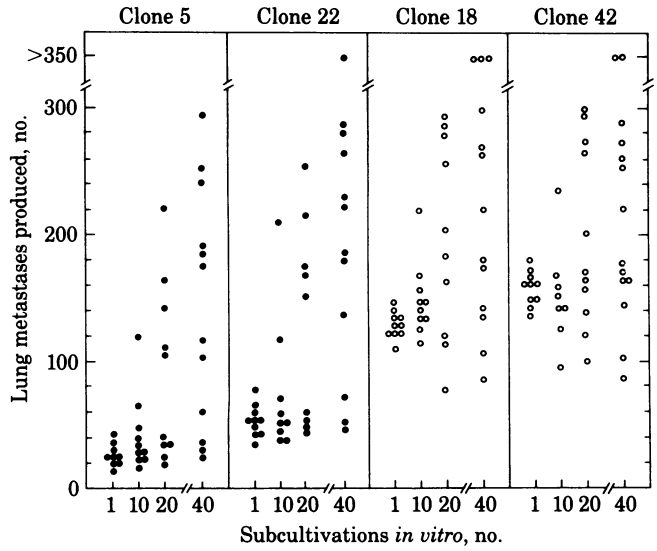

FIG. 2. Comparison of incidence of metastases produced by various subclones isolated during serial subcultivation *in vitro* of individual clones derived from the murine B16-F10 cell line.

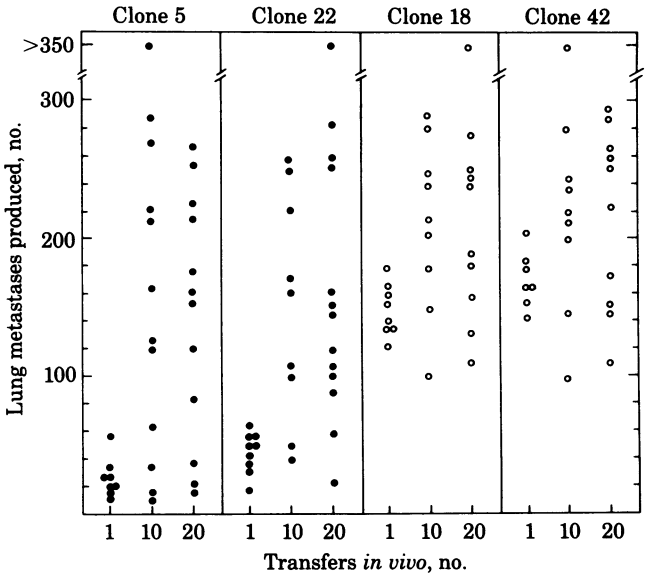

FIG. 3. Comparison of incidence of metastases produced by cells of subclones isolated after s.c. inoculation of individual clones isolated from the B16-F10 cell line and subsequent passage of tumor nodules by serial s.c. transfer.

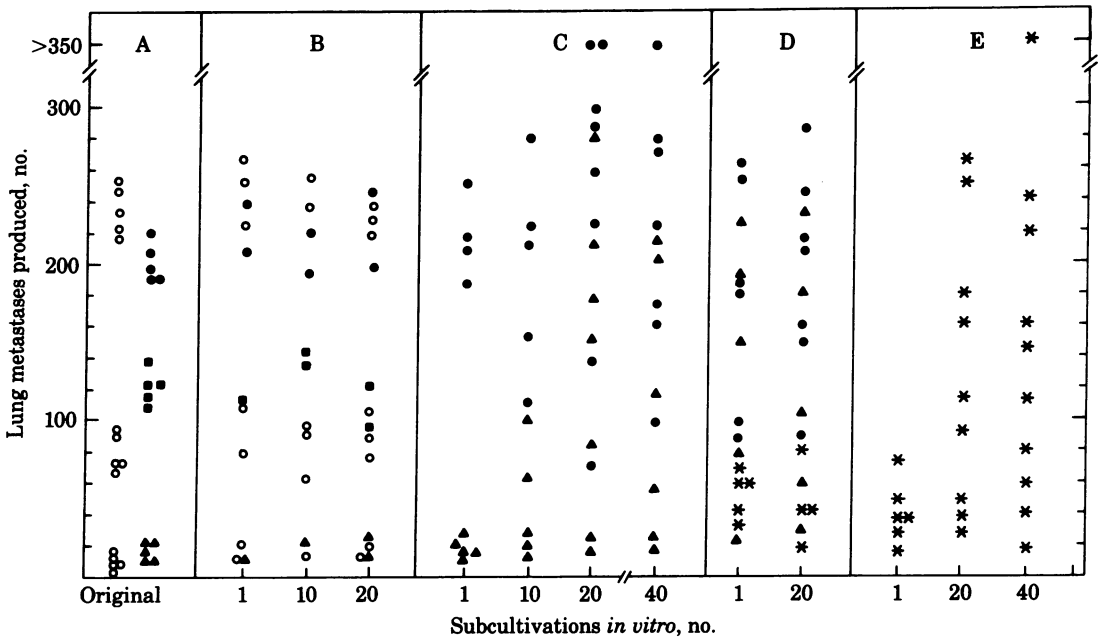

FIG. 4. Stability of the metastatic phenotype in polyclonal cultures prepared by cocultivation of various clones isolated from the B16-F10 cell line. (*A*) Wild-type (○), TFT^r (▲), Oua^r (■), and TFT^r/Oua^r (●) clones having different metastatic properties were mixed and cocultivated. (*B*) Subclones were isolated after a further 10 or 20 subcultivations and assayed for their metastatic properties and drug sensitivities. After 20 subcultivations, the cultures were treated with TFT. (*C*) The surviving cells were passaged and subclones were isolated and tested for metastatic properties and resistance to 2 μg/ml TFT (▲) or to TFT and 1 mM ouabain (●). After 40 subcultivations, a new clone of DAP^r cells (✱) was added. (*D*) Subclones were isolated from this mixed cell population after 20 subcultivations; their metastatic properties and susceptibility to TFT (2 μg/ml), ouabain (1 mM), and DAP (47 μM) were evaluated; and replicate cultures were treated with 47 μM DAP. (*E*) The surviving cells were passaged, and subclones were isolated at the indicated intervals and tested for their metastatic properties and ability to grow in hypoxanthine/aminopterin/thymidine (DAP^r variants grow; TFT^r variants fail to grow).

sumably initiate a new cycle of phenotypic restriction, instability and eventual stabilization.

DISCUSSION

The present experiments have shown marked differences in the stability of the metastatic phenotype in B16 melanoma cell populations passaged *in vivo* or *in vitro* as polyclonal populations containing clones of different metastatic potential compared with their behavior when passaged as single clones. Examination of uncloned B16 cell lines, B16-F1 and B16-F10, which are known to be heterogenous and contain clonal subpopulations that have different metastatic abilities (3, 8, 11, 18), showed that their metastatic activity is stable during prolonged cultivation *in vitro* and s.c. transfer *in vivo*. In contrast, individual clones isolated from these lines show highly unstable metastatic phenotypes and rapidly generate subclones that have very different metastatic properties. This instability is not expressed, however, when the same clones are mixed and cocultivated as a polyclonal population. No significant shift in the range of metastatic activity was detected in subclones isolated from polyclonal populations after as many as 40 subcultivations *in vitro*. This suggests that, in polyclonal populations, some form of "interaction" is occurring between the clonal subpopulations that, in the absence of other selection pressures (see below), stabilizes the relative proportion of each subpopulation in the total population. This type of interaction would conserve maximum phenotypic diversity in the population and thus prevent dominance of the population by a few, or even a single, subpopulation.

Introduction of a new selection pressure can alter the "equilibrium" between different subpopulations and limit subpopulation diversity by eliminating "unfit" clones. As shown in experiments in which drug treatment was used to eliminate wild-type clones from a mixture of wild-type and drug-resistant clones, the reduction in subpopulation diversity imposed by eliminating the large fraction of wild-type cells promotes phenotypic instability in the surviving subpopulations. Under these conditions, the surviving drug-resistant subpopulations rapidly generate a new panel of variant subpopulations that have different metastatic properties, all of which retain drug-resistance markers. The amplification of subpopulation diversity proceeds until it is once again stabilized by establishment of a new equilibrium among the subpopulations. This then presumably persists until the next selection pressure is encountered and the cycle is repeated.

The instability of the metastatic phenotype observed in single clones and in polyclonal populations after restriction of subpopulation diversity via elimination of the majority of clones is not a generalized destabilization phenomenon affecting a wide range of phenotypic properties. As shown here, a variety of drug-resistant phenotypes remain completely stable during development and expression of metastatic instability. Similarly, destabilization of the metastatic phenotype in single clones is not accompanied by detectable changes in cell surface glycolipids and proteins, plasminogen activator production, fibronectin content, or reactivity to plant lectins (unpublished observations). However, acquisition of enhanced metastatic potential after destabilization of the metastatic phenotype in single clones that had an initial low metastatic potential is accompanied by increased ability to grow in 0.6% agar and formation of colonies that have an increasingly pleomorphic morphology (unpublished observations).

The present results introduce a new level of complexity to the question of cellular heterogeneity in malignant neoplasms. As discussed by Nowell (19), the evolution of phenotypically diverse subpopulations of cells in tumors results from emergence and stepwise selection of tumor cell variants that have increasingly unstable phenotypes during tumor progression. In addition to the factors discussed by Nowell (19) that may influ-

ence the genetic and phenotypic stability of tumor cells during tumor progression, the data presented here indicate that cooperative interactions among clonal cellular subpopulations in a tumor may also determine the frequency with which variants emerge.

It is unclear whether the instability of the metastatic phenotype in clones cultivated in isolation and the role of clonal interactions in stabilizing metastatic properties is a peculiarity of the B16 melanoma that may reflect the lengthy cultivation that this cell line has undergone since its original isolation. Another possibility is that this behavior might be a feature of solid malignant tumors on reaching an advanced stage of progression and that examination of tumors at an earlier stage in progression would not show such extensive phenotypic instability. Support for this hypothesis comes from recent experiments similar to those described here using clones isolated from tumors excised shortly after induction with methylcholanthrene or UV irradiation (UV2237; ref. 4), which have shown that the metastatic phenotype in some clones is highly unstable but is stable in others (unpublished observations). Also, in the case of the B16 melanoma cells studied here, metastatic diversity in clonal populations occurred during both *in vivo* and *in vitro* passaging.

In other tumor systems, however, passage *in vivo* of cloned tumor lines led to a rapid emergence of diversity, yet the same tumor lines grown in parallel in culture remain stable (6, 20). Apparently, host selection pressure could have been responsible for this phenomenon (6).

In addition to the present study documenting interaction among tumor cell subpopulations affecting metastatic behavior, recent work by Miller *et al.* (21, 22) has shown that cellular subpopulations isolated from the same mammary tumor exert growth regulatory restraints on each other. The interaction pattern is complex, however, with different patterns of uni- and bidirectional response occurring between different subpopulations. The same subpopulations also exhibit equally complex interactions affecting immunogenicity and sensitivity to cytotoxic drugs (21). Similarly, nontumorigenic clones of B16 melanoma have been reported to suppress the tumorigenicity of other B16 clones when mixed together before implantation into mice (23). The mechanism(s) underlying these fascinating events is not known. The numerous reports showing that the presence of the primary tumor can restrict the growth of metastases in certain tumors (24–31) might also represent an analogous phenomenon.

Independent of the mechanism involved, identification of these interactions among different subpopulations from the same tumor suggests that a full understanding of the role of tumor cell heterogeneity in determining the behavior of tumors and in predicting their response to therapy cannot be achieved by simple analysis of the individual component subpopulations but will require more sophisticated analyses of subpopulation interactions.

1. Poste, G. & Fidler, I. J. (1980) *Nature (London)* **283,** 139–146.
2. Enders, J. F. & Diamandopoulos, G. T. (1969) *Proc. Roy. Soc. B* **171,** 431–443.
3. Fidler, I. J. & Kripke, M. L. (1977) *Science* **197,** 893–895.
4. Kripke, M. L., Gruys, E. & Fidler, I. J. (1978) *Cancer Res.* **38,** 2962–2967.
5. Suzuki, N., Withers, R. N. & Koehler, M. W. (1978) *Cancer Res.* **38,** 3349–3351.
6. Talmadge, J. E., Starkey, J. R., Davis, W. C. & Cohen, A. L. (1979) *J. Supramol. Struct.* **12,** 227–243.
7. Fidler, I. J. (1973) *Nature (London) New Biol.* **242,** 148–149.
8. Fidler, I. J., Gersten, D. M. & Budmen, M. B. (1976) *Cancer Res.* **36,** 3160–3165.
9. Schirrmacher, V., Shantz, G., Claver, K., Domitowski, D., Zimmerman, H.-P. & Lohmann-Mathes, M. L. (1979) *Int. J. Cancer* **23,** 233–244.
10. Liotta, L. A., Vembu, D., Saini, R. K. & Boone, C. (1978) *Cancer Res.* **38,** 1231–1236.
11. Hart, I. R. (1979) *Am. J. Pathol.* **97,** 587–600.
12. Poste, G., Doll, J., Hart, I. R. & Fidler, I. J. (1980) *Cancer Res.* **40,** 1636–1644.
13. Shearman, P. J. & Longenecker, B. M. (1980) *Int. J. Cancer* **25,** 363–369.
14. Kerbel, R. S., Twiddy, R. R. & Robertson, D. M. (1978) *Int. J. Cancer* **22,** 583–594.
15. Griswold, D. P. (1975) *Cancer Chemotherap. Rep.* **5,** 187–204.
16. Poste, G. & Flood, M. K. (1979) *Cell* **17,** 789–800.
17. Siegel, S. (1956) *Nonparametric Statistics for the Behavioral Sciences* (McGraw Hill, New York) pp. 116–127.
18. Cifone, M. A., Kripke, M. L. & Fidler, I. J. (1979) *J. Supramol. Struct.* **11,** 467–476.
19. Nowell, P. C. (1976) *Science* **194,** 23–28.
20. Chow, D. A. & Greenberg, A. H. (1980) *Int. J. Cancer* **25,** 261–266.
21. Miller, B. E., Miller, F. R., Leith, J. & Heppner, G. H. (1980) *Cancer Res.* **40,** 3977–3981.
22. Miller, F. R. & Heppner, G. H. (1979) *J. Natl. Cancer Inst.* **63,** 1457–1463.
23. Newcomb, E. W., Silverstein, S. C. & Silagi, S. (1978) *J. Cell Physiol.* **95,** 169–178.
24. Schatten, W. (1958) *Cancer* **11,** 455–459.
25. Green, H. & Harvey, E. (1960) *Cancer Res.* **20,** 1094–1100.
26. Ketcham, A., Kinsey, D., Wexler, H. & Mantel, N. (1961) *Cancer* **14,** 875–882.
27. DeWys, W. D. (1972) *Cancer Res.* **32,** 374–379.
28. Sheldon, P. W. & Fowler, J. F. (1973) *Br. J. Cancer* **28,** 508–514.
29. Yuhas, J. M. & Pazmino, N. H. (1974) *Cancer Res.* **34,** 2005–2010.
30. Gorelik, E., Segal, S. & Feldman, M. (1978) *Int. J. Cancer* **21,** 617–625.
31. Sugarbaker, E. V., Thornthwaite, J. & Ketchem, A. S. (1977) *Cancer Invasion and Metastasis*, ed. Day, S. B. (Raven, New York), pp. 227–240.