Heterogeneity of Tumor Cells from a Single Mouse Mammary Tumor

Daniel L. Dexter, Henryk M. Kowalski, Beverly A. Blazar, Zuzana Fligiel, Renee Vogel, and Gloria H. Heppner

Departments of Medicine [D. L. D., H. M. K., B. A. B., G. H. H.] and Pathology [Z. F., R. V., R. I. 02908], Medical School, University of California, Berkeley, Berkeley, Calif. They carry MMTV. The tumor was excised, minced, and treated with 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.) in calcium-magnesium-free Earle's balanced salt solution (Grand Island Biological Co.). The resulting cell suspension was plated in Falcon tissue culture flasks (Falcon Plastics, Oxnard, Calif.) when the culture grew to a monolayer, the cells were harvested with 0.25% trypsin in 0.9% NaCl solution and centrifuged for 5 min at 1400 rpm in a Sorvall GLC-2 centrifuge. The pellet was resuspended in Waymouth's medium (Grand Island Biological Co.) supplemented with 15% heat-inactivated fetal calf serum (Grand Island Biological Co.), L-glutamine (2 mg/ml; Grand Island Biological Co.), and gentamicin (20 μg/ml; Schering Corp., Port Reading, N. J.). The cells were plated in 3 Falcon No. 3002 60-mm dishes, 1 of which had previously been treated with 2 ml of a 0.02% gelatin (Difco Laboratories, Inc., Detroit, Mich.) solution for 3 hr at 4° to provide a substrate. All trypsinized cell suspensions were centrifuged for 5 min at 1400 rpm in a Sorvall GLC-1 centrifuge, and pellets were resuspended for plating. All cultures were kept at 37° in a humidified atmosphere of 5% CO₂ in air. Routine maintenance of the cultures was carried out by trypsinization (0.25% trypsin) of confluent cultures and replating, usually at 5 x 10⁶ cells/dish. Colony isolation was achieved through the technique of differential trypsinization (4) or by removing clones from soft agar.

Growth in Agar. Some cultures were grown in semisolid medium. Cells were resuspended in 0.5% agar (Difco) in complete growth medium, and 1 ml of this suspension was layered on a 2-ml base of 1.0% agar in medium in a 35-mm Falcon No. 3001 dish. Growth Rate. For determination of cell-doubling times, replicate culture dishes received inocula of 5 x 10⁶ cells, and cells from 2 dishes were harvested separately each day and counted with a hemacytometer. Results were plotted on semilogarithmic paper, and doubling times were calculated from the curves.

Cloning Efficiencies. Cloning efficiencies on plastic dishes were determined by counting the colonies present in a dish 1 week after seeding. Cloning efficiencies in agar were determined by counting the colonies in a dish 2 weeks after the culture was initiated. In both cases 1 observer counted colonies in duplicate dishes by complete scanning of each dish with the aid of a light microscope.

Saturation Densities. These data were obtained from the results of experiments done to determine doubling times. The numbers of cells present in confluent dishes that
determined the plateau regions of the growth curves provided the maximum cell number per 35-mm dish.

**Karyotype Analysis.** Exponentially growing cultures were incubated with Colcemid (0.2 μg/ml; Grand Island Biological Co.) for 5 hr. Cells were harvested, and chromosome preparations were made according to standard techniques. The preparations were observed under a phase microscope and at least 25 metaphases were analyzed for each cell type.

**Isokinetic Separation of Cells.** A single-cell suspension was prepared from confluent culture dishes by trypsinization and subsequent filtration through a layer of Nytex with a 25-μm pore diameter (Tetko Inc., Elmsford, N.Y.). Twenty million cells were suspended in 7 ml of Joklik's modified minimal essential medium (Grand Island Biological Co.) with 10% fetal calf serum and layered over a linear gradient of Ficoll (Sigma Chemical Co., St. Louis, Mo.) in Joklik's medium. The gradient for isokinetic separation was prepared according to the methods of Pretlow (19, 23); refractive indices varied from 1.3373 at the sample-gradient interface to 1.3435 at the gradient-cushion interface. The cushion was 43% Ficoll solution. Centrifugation was 97 × g, measured at the sample-gradient interface, for 14 min at 4° with an I.E.C. PR-J centrifuge. Except for the first fraction, which consisted of 7 ml, 24 fractions of 4 ml each were collected by displacement with a 55% sucrose solution. Each fraction was diluted with Joklik's medium, centrifuged at 110 × g for 10 min at 4°, and resuspended in 0.5 ml of Waymouth's growth medium. The number of cells in each fraction was determined by hemacytometer counts. The fractions were plated in Waymouth's growth medium.

**Preparation of Papanicolaou-Stained Tissue Cultures.** Cells were grown on No. 4802 Lab Teck (Naperville, Ill.) culture chambers in Waymouth's growth medium. The slides were removed from the bottoms of the chambers, rinsed well with 0.9% NaCl solution, and fixed immediately with 95% ethanol for a wet preparation. Staining was by a modification of the method of Papanicolaou (22). After staining, the slides were dehydrated through ethanol and cleared in xylol, and coverslips were affixed.

**Mammary Tumor Virus Antigen Assay.** The method used was that of Hager and Tompkins (10). Cells were harvested from confluent monolayers by treatment at 37° with 0.25% trypsin; from this point the procedure was carried out at 4° by keeping the cells on ice and incubating in a refrigerator. The cells were harvested as described above. The suspension was centrifuged at 250 × g for 5 min, and the pellet was washed with TBS. The washing was repeated 2 times, and the cells were resuspended in 0.1 ml of a 1:20 dilution in TBS of rabbit anti-C3H MMTV antiserum kindly donated by Dr. J. Gruber, Viral Oncology, NIH. The suspension was agitated every 5 to 10 min for 30 min, the cells were centrifuged, and the pellet was washed 3 times with TBS. The cells were then resuspended in 0.1 ml fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Downingtown, Pa.) diluted 1:4 with TBS. After mixing for 30 min, the cells were centrifuged, and the pellet was washed 3 times with TBS. The cells were resuspended in 3 to 5 drops of Tris-glycerine (glycerol: TBS, 9:1) and added dropwise to a microscope slide. At least 100 cells were counted with a Leitz Orthoplan microscope equipped for fluorescence microscopy. A negative test was one in which no fluorescent cells were seen. In control experiments, nonimmunized rabbit serum was used.

**Tumorigenicity Studies.** Female BALB/cfC3H mice, 6 to 10 weeks old, were used as hosts for cell injections. Cells in culture were trypsinized and centrifuged as above. The pellet was washed twice and suspended in 0.9% NaCl solution to give the appropriate density. Usually, each mouse received a s.c. injection of 1 × 10^5 viable (trypan blue-excluding) cells in a volume of 0.2 ml. In studies designed to compare the in vivo growth capacity of the lines, mice received s.c. inocula of 10^6, 10^5, 10^4, or 10^3 cells.

**Histology.** Morphological studies of tumors were carried out on the original tumor and derived cell lines. All tissue was obtained fresh and was fixed immediately in Bouin's solution. Histological sections were stained with hematoxylin and eosin, Mayer's mucicarmine, PAS, and Alcian blue (at pH 1).

**RESULTS**

**Isolation of Cell Lines**

Several distinct populations of cells were obtained from the original, morphologically heterogeneous culture.

The cells that were replated from a monolayer grown on plastic onto a gelatinized dish (see "Materials and Methods") were cultured on a gelatin substratum for an additional passage and subsequently grown on regular tissue culture dishes. From the time of its culturing on gelatinized dishes, this cell population, which we call "67," has consisted almost exclusively of somewhat elongated, spindle-shaped cells. Indeed after the fourth passage, no epithelial cells have been seen in this population, which has remained morphologically homogeneous through over 40 passages. These cells form a network-like lattice in subconfluent cultures (Fig. 1).

The culture obtained when cells from the primary culture were replated in a second, nongelatinized dish was characterized by a very heterogeneous morphology. An epithelial colony of cells was removed by differential trypsinization and replated in another dish. The culture obtained was again very heterogeneous in morphology. Cell types ranging from epithelial to fibroblastic appeared, and this heterogeneity was maintained through 10 passages, at which time 4 dishes were expanded to 20 dishes to provide cells for isokinetic separation. The heterogeneity in morphology was striking both among and within these dishes. The cells from all 20 dishes were harvested, and isokinetic separation was carried out. The modal population of mouse mammary tumor cells was contained in fractions 16 to 21. All the collected fractions were replated to determine whether growth in culture was affected by the gradient procedure. Cells attached and proliferated in Fractions 14 to 21. Fraction 17 was replated and gave rise to line 66. This cell line was therefore originally isolated by differential trypsinization and then further selected by a gradient cell separation technique. The cells are quite homogeneous in their spindle-shaped morphology and do not form the same net-like lattice characteristic of the 67 cells (Fig. 2). The 66 line has...
retained its homogeneous morphology through more than 30 passages following the gradient separation.

The aliquot of cells from the primary culture that was replated on a third, nongelatinized dish grew out as a population containing both epithelial and fibroblastic cells. One isolated epithelial patch was removed by differential trypsinization at this passage level, and these cells continue to give rise to epithelial-like cells. This line, called "68-H," has maintained its epithelial morphology through more than 20 passages (Fig. 3).

The population from which line 68-H was derived contained only fibroblastic cells after another 2 passages. Injection of these cells produced tumors in syngeneic hosts; a cell culture was established by trypsin treatment of 1 of these tumors. The culture (called "168") obtained was homogeneously fibroblastic in appearance; the cells were morphologically indistinguishable from those of the injected population. This culture has maintained its fibroblastic morphology through more than 20 passages (Fig. 4).

In addition to observation of living cultures, the gross morphology of the 4 lines was studied with Papanicolaou staining. These studies clearly confirmed the distinctive morphologies of the cultures as described above.

In vitro and in vivo growth properties, expression of MMTV cell surface antigen, and karyotype were determined for all 4 of these lines. The results are shown in Table 1 and described below.

In Vitro Growth Properties

It is clear that there are a number of significant differences among the lines. The doubling times of the various lines vary from 12 hr for line 66 to 48 hr for line 68-H. However, these 2 lines are both characterized by a low cloning efficiency in soft agar; lines 67 and 168 clone much better in agar. The cloning efficiencies in agar for all 4 lines are density dependent. Only line 68-H forms very small (50 μm diameter) colonies in agar; the other lines are characterized by larger clones of comparable size (up to 500 μm) regardless of cloning efficiency. The saturation densities of the lines are also different.

Cloning of Cell Lines

Since the 4 lines were obtained with different isolation procedures, it was important to establish that the differences among them are not the result of adaptation to different in vitro circumstances. Accordingly, the lines were all seeded in soft agar to obtain clones by a uniform methodology. Line 68-H failed to produce large enough clones to be successfully propagated. Lines 66, 67, and 168 all produced clones which, when grown up into populations large enough for testing, exhibited morphological and growth characteristics virtually identical with those of their respective lines (Table 1).

Karyotype Analysis

Determination of the modal chromosome number of the 4 cell lines revealed very significant differences in their karyotypes (Table 1). Lines 66 and 168 are diploid or pseudodiploid, whereas line 67 is roughly tetraploid, and line 68-H is very aneuploid. All the lines have a female karyotype. For determination of whether similar karyotypic heterogeneity existed in the parent tumor from which these lines were originally derived, cells from the autochthonous

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Doubling time (hr)</th>
<th>Saturation density (cells/sq cm)</th>
<th>Cloning efficiency in agar (%/no. of cells)</th>
<th>Modal chromosome no.</th>
<th>MMTV cell surface antigen</th>
<th>Tumor potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>12 (clone, 14)</td>
<td>9.6 x 10⁶ (clone, 1.0 x 10⁷)</td>
<td>0.5%/10⁵</td>
<td>39 (39-41)</td>
<td>Negative</td>
<td>6/8 tumors at 3-8 wk with 10⁶ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.005%/10⁴</td>
<td></td>
<td></td>
<td>0/8 tumors after 5 mos. with 10⁶ cells</td>
</tr>
<tr>
<td>67</td>
<td>21 (clone, 21)</td>
<td>3.6 x 10⁶ (clone, 3.0 x 10⁷)</td>
<td>19%/10⁴</td>
<td>74 (70-80)</td>
<td>Negative</td>
<td>6/8 tumors at 4-6 wk with 10⁴ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.03%/10⁴</td>
<td></td>
<td></td>
<td>0/8 tumors after 5 mos. with 10⁴ cells</td>
</tr>
<tr>
<td>168</td>
<td>17 (clone, 15)</td>
<td>4.8 x 10⁶ (clone, 5.2 x 10⁷)</td>
<td>33%/10⁴</td>
<td>41 (39-41)</td>
<td>Negative</td>
<td>7/7 tumors at 4 wk with 10⁴ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5%/10⁴</td>
<td></td>
<td></td>
<td>5/7 tumors after 5 mos. with 10⁴ cells</td>
</tr>
<tr>
<td>68-H</td>
<td>48</td>
<td>2.7 x 10⁶</td>
<td>0.1%/10⁴</td>
<td>130 (110-140)</td>
<td>Strongly positive (&gt;95% of cells)</td>
<td>6/8 tumors at 8-20 wk with 10⁶ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.06%/10⁴</td>
<td></td>
<td></td>
<td>0/8 tumors after 5 mos. with 10⁴ cells</td>
</tr>
</tbody>
</table>

*a Numbers in parentheses, range.
through fourth in vivo passages of that tumor were retrieved from freezing (−80°C), cultured, and immediately analyzed for their chromosomal numbers. Cells with karyotypes ranging from 40 to 130 were easily identified, which suggests that the genotypic heterogeneity observed in the derived lines had its origin in the neoplasm itself.

Expression of MMTV Surface Antigen

BALB/cfC3H mice are infected with MMTV and their tumors are associated etiologically with that agent. Cells from those tumors may express MMTV antigen, detectable by antiviral antibody, on their surface (8). We analyzed our lines for this antigen by the fluorescent antibody sandwich technique, using an anti-C3H MMTV rabbit antiserum. Only the 68-H line was positive for MMTV antigen with over 95% of the cells fluorescing with a 1:20 dilution of serum. A similar study was performed on cells cultured from the parent tumor from which the 4 lines were derived. The percentage of MMTV antigen-positive cells ranged from 2 to 17% with several independently initiated cultures.

In vivo Growth Properties

Tumorigenicity. The tumor potential of these populations (Table 1) can be summarized as follows. Lines 67 and 168 give 100% tumors within 6 weeks at an inoculum of 1 × 10^6 cells. Line 68-H gives approximately 75% tumors at either 1 × 10^6 or 1 × 10^5 cell inocula, with a long latency period of 3 to 4 months in either case, indicating that the tumor potential of line 68-H is much less than that of the population 168, from which it was isolated, or than that of line 67 as well. Line 66 gives tumors with an incidence of 75% to 80% after injection of 1 × 10^5 cells. Thus line 66 has a tumor potential intermediate between line 67 or 168 and line 68-H. The original tumor from which we isolated these lines gave a 100% incidence in an average of 5 weeks following s.c. injection of 1 × 10^5 cells.

Histology of Tumors. The original tumor from which the 4 lines were derived was a differentiated adenocarcinoma showing both papillary and glandular elements (Fig. 5). In addition, a moderately prominent, focal fibroblastic response was noted. No significant inflammatory response was seen. The cytology was fairly uniform and consisted of moderately large cells with oval to round, large nuclei. The chromatin was diffuse and mitotic figures were moderately frequent. The cytoplasm was slightly eosinophilic. This tumor, which was growing as a s.c. implant, was invading the nearby muscle tissue.

Histological examination of the 4 lines revealed much less evidence of adenocarcinoma differentiation than was seen in the parent tumor. Generally, the picture was that of a poorly differentiated neoplasm with a mixed, intermingled, epithelial-sarcomatous pattern (Fig. 6). No definitive evidence of glandular differentiation or papillary or ductal pattern was noted, although scattered glandular structures were present. Further studies are needed to determine with certainty whether their origin is neoplastic. They may represent benign components of the tumor-replaced s.c. tissue.

Plump, round, or oval epithelial cells with round nuclei were admixed with spindle-shaped sarcomatous cells containing elongated nuclei. Most nuclei had a granular chromatin pattern; nucleoli were seen. Pale or slightly eosinophilic staining cytoplasm was present in moderate amount. The mitotic rates were moderate. Scattered within the tumors were occasional giant cells with several large or fused nuclei. Many areas of necrosis accompanied by focal acute inflammation were seen. Special stains with PAS revealed occasional cells containing strongly PAS-positive staining intracytoplasmic material.

In several areas of tumor originating from cell line 67, islands of neoplastic cartilage and osteoid material were present. In other ways there were no striking histological differences among tumors of the 4 lines.

Effect of In Vivo Passage on Culture Characteristics of the Lines

Monolayer cultures were initiated from tumors obtained by s.c. injection of cells from the 4 lines. The morphology of these cultures closely resembled that described above for each of the lines as shown in Figs. 1 to 4. Thus, the culture morphology of the lines remains stable following in vivo cycling, despite the fact that the histology of the tumors that they produce is generally not distinctive.

Stability of Line Characteristics

Since their original isolation all 4 lines have been maintained under identical culture conditions. The growth characteristics of the 4 lines and 3 clones have remained relatively stable in culture for the over 18 months that the lines have been in existence. Further, morphology, expression of MMTV surface antigen, and tumorigenicity, which are monitored frequently, have remained constant over the same period of time.

DISCUSSION

We wish to emphasize 2 results of our study.

1. Although line 168 shows the highest cloning efficiency in agar and is the only line producing tumors at an inoculum of 10^6 cells, no consistent correlations have been observed in this mammary carcinoma system between the tumor potentials of the various lines and any 1 of the in vitro growth parameters studied. Thus, line 66 is the fastest growing line and has the highest saturation density, but its tumor potential is significantly less than those of lines 67 or 168. Although its low cloning efficiency in agar might suggest a correlation between that characteristic and tumorigenicity, it is significantly more tumorigenic than 68-H, which also clones poorly in agar. Line 68-H grows more slowly than the other lines, has the lowest saturation density, provides only very small colonies in agar, and has the poorest tumor potential, but it is also the cell line that expresses MMTV antigen, has a very abnormal karyotype, and presents the epithelioid morphology thought to be characteristic of mammary tumors (5). The lack of predictability of in vitro observations for tumorigenicity of mouse mammary carcinomas has also been demonstrated by Butel et al. (5). Our results, however, do not exclude the possibility that several different in vitro assays, taken together, might prove useful in predicting tumorigenicity in vivo.
2. We have isolated several distinct lines from a single mouse mammary tumor. We feel that our data provide evidence that a single neoplasm can contain a number of genotypically and phenotypically distinct tumorigenic cell types. Indeed there is no reason to believe that we have isolated all the cell types present in the original tumor. Although the lines were isolated by different techniques, it is unlikely that they represent adaptations of a single population to those techniques since they have remained stable for 1.5 years when cultured under identical conditions and following cycling through mice. Further, among them the lines contain the full spectrum of karyotypes exhibited by the cells of the original tumor. Finally, the morphological heterogeneity that we have used to identify the subpopulations was present in the first monolayer culture obtained from the autochthonous tumor, as was the heterogeneity in MMTV expression. The occurrence of several tumor cell types in 1 mammary neoplasm would be consistent with the notorius heterogeneity which for decades has confronted pathologists studying the histology of these tumors, with the earlier observations of Henderson and Rous (13) that discrete colonies of mammary tumor cells obtained by "plating" tumor fragments within the s.c. tissue of the backs and bellies of mice, yield tumors of differing histology, and with recent work of Slijuyser and Van Nie (21) suggesting that hormone-induced mammary tumors of GR mice are mixed populations of estrogen receptor-positive and -negative cells. Our study suggests that this heterogeneity might have its basis in the genotypic heterogeneity of distinct tumor cell subpopulations. Data that suggest the existence of distinct tumor cell subpopulations in other systems have been discussed by Becker et al. (3), using karyotypic diversity and production of marker proteins; by Gray and Pierce (9), using differences in growth rate and melanin production in a hamster melanoma line; by Mitelman, et al. (15), using karyotypic diversity; by Schabel (20), using differences in cell kinetics; by Prehn (18) and Pimm and Baldwin (17), using differences in immunogenicity; by Fidler and Kripke (7), using differences in metastatic potential; and by Barranco et al. (1, 2) and Hakansson and Trope (11), using differences in drug sensitivity. Earlier work along these lines was also reported by the Kleins (14) and by Hauschka and Levan (12). Thus it may be that the type of heterogeneity that we have demonstrated here may be a general phenomenon. If so, it would seem not to be widely appreciated. For example, the great effort currently being made to develop drug screening assays based on the sensitivity of limited cell populations derived from human tumors would seem futile if the cells tested are not representative of the whole cancer. Further, strategies for development of combination therapy protocols are more likely to be based on the delivery of multiple hits to single targets than on the concept of many different targets. Strategies to avoid the emergence of drug-resistant mutants do not consider that substantial resistant populations may exist prior to any therapy. Likewise the development of immunotherapy protocols have thus far assumed an intra-tumor consistency in susceptibility to therapy that the existence of heterogeneous subpopulations denies. We are currently analyzing our subpopulations for sensitivity to various chemotherapeutic agents and for immunological parameters, and we find marked differences among them which, in a clinical setting, would make management of their parent disease quite difficult.

The expression of mammary tumor virus antigen by the highly aneuploid 68-H lines raises an interesting question concerning gene dosage and expression of the antigen. We are in the process of obtaining chromosome banding patterns for each cell line and for normal mouse spleen and fibroblast cells to determine if certain chromosomes are duplicated in the 67 and 68-H lines. We are also planning experiments to see whether viral antigen expression can be induced for the other cell lines. It may be, however, that the absence of viral antigen expression by several tumor cell subpopulations is the result of etiological diversity for this complex tumor which is known to be causally related to endocrine and genetic as well as to viral factors. The existence of composite tumors made up of populations of differing etiology has also been suggested by Mitelman et al. (15).

Clearly, we are unable at this point to say where in neoplastic transformation heterogeneity arose (16). Our data do suggest, however, that spontaneous mouse mammary tumors are, from their first detection, very complex entities, and not simply the result of a single neoplastic cell population. This was eloquently expressed by Dunn 2 decades ago when she wrote, "Analysis of the morphology of mammary tumors disposes forever of the idea that cancer is usually a unit alteration in a single cell, which reproduces itself unchangeably" (6).

ACKNOWLEDGMENTS

We wish to thank Dr. Paul Calabresi for his support of this work, Dr. Jean Hager for help in the fluorescent antibody assay, and Jim Barbosa and Ann Richardson for superb technical assistance.

REFERENCES

Mammary Tumor Heterogeneity


Fig. 1. Living line 67 cells in a subconfluent monolayer culture. Photomicrograph. Phase optics. x 100.
Fig. 2. Living line 66 cells in a subconfluent monolayer culture. Photomicrograph. Phase optics. x 100.
Fig. 3. Living line 68-H cells in a subconfluent monolayer culture. Photomicrograph. Phase optics. x 100.
Fig. 4. Living line 168 cells in an almost confluent monolayer culture. Photomicrograph. Phase optics. x 100.
Fig. 5. Sections from the original tumor showing numerous small, well-defined glands, consistent with adenocarcinoma, which are surrounded by fibrous stroma. H & E, × 375.

Fig. 6. Tumor from cell line 168 showing characteristic pattern of intermingled epithelial (large, pale cells)- and sarcomatous (elongated tightly packed cells)-appearing elements. Occasional cells contain strongly PAS-staining cytoplasmic material. H & E, × 375.
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