Differences in Cell Density Associated with Differences in Lung-colonizing Ability of B16 Melanoma Cells

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ABSTRACT

The B16 melanoma-derived low lung-colonizing variant B16-F1 and the high lung-colonizing variant B16-F10 retained their differential lung-colonizing abilities throughout at least 35 serial s.c. transplant generations. The majority of the cells originating from solid B16-F1 tumors had a higher density than did cells originating from solid B16-F10 tumors. Cell suspensions of unselected solid B16 melanomas contained two major subpopulations differing in their cell density. The subpopulation with the lower cell density was more efficient in lung tumor colony formation, following i.v. administration, than was the high-density subpopulation. Cloned tumors from low-density B16 cells were more efficient in lung colony formation than were cloned tumors from high-density cells.

INTRODUCTION

The selective localization and retention of certain tumor cells at particular organ sites is a well-known but poorly understood phenomenon. The characteristics of the tumor cells and host factors probably contribute to the fact that some tumor cells settle and proliferate in a particular distant organ, whereas others do not (8).

Fidler (5, 6) has selected 2 variants from the spontaneously metastasizing B16 mouse melanoma. Whereas one variant [the B16-F1 (F1)] produces but a few lung colonies after i.v. inoculation, the other variant [B16-F10 (F10)] has a considerably greater colonizing ability. The availability of this experimental system enables the comparison between a high and a low lung-colonizing tumor while factors associated with individual variability of 2 different tumors are minimized. Quite a few differences between these 2 variants were detected (1, 2, 12, 13). In this study, we report that there is a difference in the cell density between the 2 variants. We suggest that this density difference may be partially responsible for their different lung-colonizing capacities.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old female C57BL/6 mice were used throughout this study. The mice were purchased from the Weizmann Institute of Science, Rehovot, Israel.

Tumors. Cultured F1 cells and F10 cells were kindly supplied by Dr. I. Fidler, the Frederick Cancer Research Center, Frederick, Md. Cultured lines were inoculated s.c. and then passaged by s.c. inoculation of 10^6 cells/mouse for 4 passage generations.

Preparation of Gradient Material. Nine parts of Percoll (Percoll TM; Pharmacia Fine Chemicals, Uppsala, Sweden), a gradient medium based on colloidal silica, were mixed with one part of 10-fold-concentrated Dulbecco’s Phosphate-buffered Saline “A” (Oxoid Limited, England). From this starting solution 0.25% trypsin (Bio–Lab, Jerusalem, Israel) and 0.25% DNase I (DNase; Sigma Chemical Co., St. Louis, Mo.) at 37° for 20 min. The suspended cells were passed through an 80 mesh stainless steel screen, suspended in HBSS with 10% FCS (Grand Island Biological Co., Grand Island, N. Y.), and washed 3 times (5 min at 200 x g). Viability was determined by trypan blue exclusion and was shown to be about 60% in most experiments. The variation in viability from experiment to experiment ranged from 48 to 86%.

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Gradients and Centrifugations. Linear density gradients from 10 to 70% of the starting Percoll solution were constructed with a 2-chambered gradient maker in 5-ml serological glass tubes (Kimble, Toledo, Ohio) or 12-ml nitrocellulose tubes (331101; Beckman Instruments, Inc.). Either 5 x 10^6 or 1.2 x 10^7 cells were layered on top of the 5-ml and 12-ml gradients, respectively. Sedimentation was performed for 15 min.
at 4° at a centrifugal force of 250 × g measured at the middle of the gradient tube.

**Gradient Fractions.** Fractions (200 or 500 μl) were collected manually from the top of the 5- and 12-ml gradients, respectively, with a Finn pipet. The refractive index of the fractions was measured with a model 87958 refractometer (Carl Zeiss, West Germany). Cell viability in all fractions was determined in hemocytometer chambers by the trypan blue exclusion test. Based on recovery data of viable cells, it was established that the gradient separation did not affect cell viability. In Percoll density gradient, the dead cells accumulate at the top fraction of the gradient, and this fraction was routinely excluded from all experiments.

**Lung Colony Assays.** Fractions comprising a single band were pooled. Cells present in the pools were washed twice with HBSS. Viable cells (10⁵) from every peak were injected i.v. to each of 4 C57BL/6 mice. Fourteen days later, the mice were sacrificed, and their lungs were removed into Petri dishes. Tumor colonies were counted.

**Cloning of Melanoma Cells Differing in Their Density.** C57BL/6 thymocytes serving as feeder cells were suspended at a concentration of 10⁶ cells/ml of minimum essential medium (Bio-Lab) containing 20% FCS. Samples (0.1 ml) of this suspension were added to each well. The cell separation profiles of the 2 tumors revealed a density difference between them (Chart 1).

Whereas the majority of the F1 tumor cells formed a sharp peak at the density of 1.073, the majority of the F10 cells sedimented at a lower density of 1.067.

The cell fractions comprising the major cell population (the F1 fractions with densities of 1.070 to 1.078 and the F10 fractions with densities of 1.065 to 1.070) were pooled and washed. Cells (10⁵) were injected i.v. into C57BL/6 mice. The separated major population of the F1 and F10 cells maintained the difference in the colonizing capacities of the unseparated F1 and F10 tumors.

In another series of experiments, fractions with the same density from both tumors were compared for their lung-colonizing ability. The results presented in Table 2 show that the number of lung colonies produced by low-density F10 cells was higher than that produced by high-density F10 cells. The

### RESULTS

**F1 and F10 Maintain Their Different Lung-colonizing Capacities during s.c. Transplantations.** Cell suspensions were prepared from s.c. transplanted tumors of similar size of both F1 and F10 variants. Cells (10⁶) were injected i.v. into normal C57BL/6 mice. Fourteen days later, the lungs of the inoculated mice were examined for lung colonies. The F1 cells produced a considerably lower number of lung colonies than did the F10 cells (Table 1).

**Isopyknic Sedimentation of F1 and F10 Tumor Cells.** Cell suspensions of solid F1 and F10 tumors growing s.c. for 15 to 17 days were prepared as described above. Washed cells of each tumor were layered separately over Percoll isopyknic gradients (10 to 70%). After a 250 × g sedimentation for 15 min, 24 fractions were collected. The cell separation profiles of the 2 tumors revealed a density difference between them (Chart 1).

The cell fractions comprising the major cell population (the F1 fractions with densities of 1.070 to 1.078 and the F10 fractions with densities of 1.065 to 1.070) were pooled and washed. Cells (10⁵) were injected i.v. into C57BL/6 mice. The separated major population of the F1 and F10 cells maintained the difference in the colonizing capacities of the unseparated F1 and F10 tumors.

In another series of experiments, fractions with the same density from both tumors were compared for their lung-colonizing ability. The results presented in Table 2 show that the number of lung colonies produced by low-density F10 cells was higher than that produced by high-density F10 cells. The

### Table 1

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>No. of mice</th>
<th>Median Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16⁵</td>
<td>28</td>
<td>45–1193</td>
</tr>
<tr>
<td>F1</td>
<td>16</td>
<td>28–167</td>
</tr>
<tr>
<td>F10</td>
<td>17</td>
<td>89–231</td>
</tr>
</tbody>
</table>

* The median number of lung colonies was combined from the results of 5 separate experiments using F1 and F10 cells and of 7 separate experiments using B16 cells. The difference between the number of F1 and F10 colonies was statistically significant (p < 0.01).

* B16 cells from Stockholm, Sweden, were used in these experiments.

### Table 2

Number of lung colonies in mice given i.v. injections of 10⁵ F1 or F10 cells with similar densities

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Density</th>
<th>No. of mice</th>
<th>Median Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Low</td>
<td>13</td>
<td>70–2–140</td>
</tr>
<tr>
<td>F10</td>
<td>Low</td>
<td>13</td>
<td>107–17–437</td>
</tr>
<tr>
<td>F1</td>
<td>High</td>
<td>16</td>
<td>21–1–118</td>
</tr>
<tr>
<td>F10</td>
<td>High</td>
<td>15</td>
<td>34–7–306</td>
</tr>
</tbody>
</table>

* Four different separation experiments were carried out. The cell densities for these experiments were:

<table>
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<th>Experiment</th>
<th>Density</th>
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<tbody>
<tr>
<td>1</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
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<tr>
<td>3</td>
<td>Low</td>
</tr>
<tr>
<td>4</td>
<td>Low</td>
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<table>
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<th>Experiment</th>
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<tbody>
<tr>
<td>1</td>
<td>1.063–1.074</td>
</tr>
<tr>
<td>2</td>
<td>1.082–1.110</td>
</tr>
<tr>
<td>3</td>
<td>1.105–1.132</td>
</tr>
<tr>
<td>4</td>
<td>1.111–1.137</td>
</tr>
</tbody>
</table>

* The median number of lung colonies was combined from the results of 4 separate experiments.
same correlation between the density and the colonizing ability was evident for the F1 cell subpopulations. However, both high- and low-density F10 cells produced a higher number of lung colonies than did the corresponding fractions of F1 cells.

These results suggest that a relatively low cell density is associated with a high lung-colonizing capacity and vice versa, but it seems that cell density is not the only parameter responsible for this biological behavior.

Isopyknic Sedimentation of B16 Tumor Cells. Fidler and Kripke (9) have demonstrated that cells with high or low lung-colonizing capacities preexist in unselected B16 tumors. Since the results of the previous section suggested that cells exhibiting a high lung-colonizing capacity had a lower density than did cells with a low lung-colonizing capacity, we asked the question of whether or not we could segregate cells of high and low lung-colonizing capacities by separating cells from the original unselected B16 tumor according to their density.

In several different experiments, cell suspensions of solid Stockholm B16 tumors growing s.c. for 15 to 17 days were prepared and separated on Percoll gradients as described in the previous section.

Two major separation patterns were obtained. The first was characterized by the emergence of 2 major subpopulations differing in their densities (Chart 2). The other was characterized by a broad distribution of densities but without a division into distinct peaks (Chart 3).

The fractions comprising the low-density (Chart 2, 1.059 to 1.063; Chart 3, 1.046 to 1.064) cell subpopulation were pooled as were the fractions comprising the high-density (Chart 2, 1.075 to 1.090; Chart 3, 1.076 to 1.091) subpopulation. Cells (10^5) from these cell suspensions were then injected i.v. into normal C57BL/6 mice. As a rule, the cell subpopulation with the lower density exhibited about a 3-fold-higher lung-colonizing capacity than did the cells with the higher density. The summary of 9 such experiments is presented in Table 3. These results support the suggestion raised above that a high lung-colonizing capacity of B16-derived cells is associated with cells having a relatively low density and vice versa.

Since B16 melanoma cells originating from the Karolinska laboratory might have different properties from those originating in Frederick, we used in 4 separate experiments B16 tumors originating in the latter laboratory. Table 4 shows that cells originating from the Frederick B16 tumor can also be separated into 2 subpopulations differing in their density and in their colonizing capacity.

The results presented in this section support the conclusions of Fidler and Kripke (9), which were based on a different methodology, that cells exhibiting a high lung-colonizing capacity as well as those exhibiting a low capacity preexist in B16 tumors.

Cloning Experiments. We have cloned cells from the 2 major B16 (Stockholm) subpopulations differing in their densities. Cells (10^5) from each of 18 randomly chosen clones originating in low-density fractions and cells (10^5) from each of 14 randomly chosen clones originating in high-density fractions were injected i.v. into 4 to 6 normal C57BL/6 mice. Fourteen days after the injection, the number of lung colonies was determined. The results presented in Table 5 show clearly that cloned tumors from low-density B16 cells tend to produce a higher
number of lung colonies than do cloned tumors from high-density cells. The differences were highly significant ($p < 0.01$).

**DISCUSSION**

It would be highly advantageous for the clinician as well as for the researcher if they could predict, on the basis of a marker expressed on certain cells lodging in the local tumor, whether or not this tumor is or will be metastasizing; to what sites it is or will be spreading; and if cells expressing or lacking this marker are those exhibiting metastatic potential.

The results obtained in this study suggest that the density of cells in a particular local, unselected B16 tumor is related to the capacity of the cells to form lung colonies. Although the absolute density values may vary from experiment to experiment (due probably to experimental variation and to differences between individual solid tumors), we have shown that, in every B16 tumor tested, the cells having a relatively low density tend to produce a higher number of colonies (approximately 3-fold) than do cells with the relatively high density.

If, indeed, high and low lung-colonizing ability is correspondingly related with low and high metastatic potential, as suggested from the work of Fidler (7), then the relative density of cells in a certain tumor could serve eventually as a marker for metastasis.

In conformity with the results of the B16 tumors, we demonstrated that the majority of cells in the low lung-colonizing F1 variant, grown as a solid local tumor, had a relatively higher cellular density than did the F10 variant with the high lung-colonizing capacity.

These results confirm those of Grida et al. (10) who showed that high-density cells from methylcholanthrene-induced mouse tumors had a reduced capacity to induce lung colonies compared to low-density cells derived from the same tumors. These authors concluded also that neither cell size nor the position of the cells in the cell cycle played an important role in this ability. However, in another study, Suzuki et al. (15), working with the same type of tumors, showed that the position in the cell cycle had a marked influence on lung-colonizing activity. From this discrepancy, it may be concluded that various parameters may influence lung-colonizing ability.

The reason for the higher lung-colonizing capacity of the low-density B16 cells is unknown. We are at present analyzing various surface properties of the high- and low-density cell populations. It is possible that separation according to density segregated between 2 populations with distinct, but as yet unidentified, surface properties.

It is possible that the low-density cells have a higher proliferating ability than do the cells with a higher density. Alternatively, or in addition, it is possible that the low-density fraction of the B16 tumor is enriched for tumor cells *per se* while the high-density fraction is relatively richer in infiltrating host-derived cells, such as macrophages (4) or other immunocytes (11). Host immunocytes present in the latter fraction could control the formation of metastasis by an active process as suggested by Eccles and Alexander (3). These researchers found that a high content of macrophages within local rat tumors was correlated with a low incidence of metastasis and *vice versa*. On the other hand, postulating a passive mechanism, it is not improbable that a subpopulation relatively depleted of host cells shows higher lung-colonizing efficiency simply on account of, and corresponding to, the tumor cell enrichment factor in this population.

The possibility that host-derived cells may be actively or...
passively involved in inhibition of lung colonization is, however, not supported by the outcome of the experiment using cloned tumors and by preliminary results (not shown), indicating that the high-density fractions of B16 tumors contain approximately the same negligible percentage of macrophages and other immunocytes as do the low-density fractions.

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REFERENCES

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