Population Density as a Factor in the Evolution of Neoplastic Cell Lines

William G. Taylor,1 Richard F. Camalier, Robert W. Tucker,2 and Katherine K. Sanford

ABSTRACT

The influence of population density in the progression from the nonneoplastic to the neoplastic state has been reassessed. Two twice-cloned, nonneoplastic mouse lines, NCTC 7914 and 7915, were transferred each 3 to 4 days at inoculum sizes selected to minimize or maximize cell-cell contact, $1 \times 10^5$ or $4 \times 10^5$ cells/T-15, respectively. As tested by in vivo assay, the regime designed to minimize cell-cell contact did not reproducibly delay transformation, and tumor production was observed in all lines, irrespective of inoculum size. Also, results of tumorigenesis assays correlated with blind evaluation of morphological and cytological alterations, growth in agarose, and susceptibility to killing by activated macrophages. Generally higher saturation densities were seen as a function of period in culture, and no significant differences in glucose utilization or lactic acid production were observed between nonneoplastic and neoplastic cell populations.

MATERIALS AND METHODS

Experimental Design. Clones NCTC 7914 and 7915 (11) were recovered from liquid nitrogen storage. These were grown in Pyrex T-15 flasks with 3 ml antibiotic-free Dulbecco-Vogt's medium (Schwarz/Mann, Orangeburg, N. Y.) containing 10% fetal bovine serum (Flow Laboratories, Rockville, Md.). Orientation studies determined the lowest initial inoculum size that allowed cell survival and minimized cell-cell contact, and at 3 to 4 days yielded a population ≤50% of saturation density. This value was $\sim10^5$ cells/T-15 flask. To enhance cell-cell contact, the high inoculum was set at 4 times the low inoculum size, as in the earlier studies (1, 13).

A fresh ampule of each clone was thawed and 2 cell lines were initiated from each clone, and these were carried at the low (NCTC 8282 and 8283) and high (NCTC 8284 and 8285) inoculum sizes. After each 3- or 4-day growth period, cells were dispersed with 0.25% trypsin, a sample of the cell suspension was counted with a Coulter Counter, the cell suspension was diluted to the appropriate inoculum size, and new cultures were prepared. The initial inoculum sizes and subculture protocol remained fixed throughout the course of the experiments. Cultures used for serial transfer were not selected on the basis of cell morphology.

Assays for Neoplastic Transformation. In vivo assays were made at approximately 3-month intervals, and with 3 exceptions all were intraocular implantations in syngeneic C3Hf/HeN mice. The cell sheet was dislodged from the growth surface with a silicone rubber policeman, and clumps of compacted cells were transferred to a Petri dish and cut to uniform size (~1 cu mm) for insertion into the anterior chamber as described in (3). Animals were examined weekly for intraocular growth, and animals in which tumors failed to develop were held for 12 months. All eyes implanted were fixed in Zenker-formol, sectioned, and stained with hematoxylin and eosin for microscopic examination. One tumor arising from each line was carried i.m. for 2 additional generations in vivo to test for continued progressive growth. The first in vivo assays of NCTC 8282 and 8283 and the final in vivo assay of 8282 were i.m. implantations of $10^5$ cells (first assays) and $10^5$, $10^4$, and $10^3$ cells per X-irradiated (425 R) syngeneic animal. The tumor latent period is defined as the interval between implantation of cells and appearance of tumors. Methods for examination of colony morphology (5, 14), growth in agarose (14), and sensitivity to BCG-activated macrophages (9) are detailed elsewhere.
RESULTS

Influence of Inoculum Size on Growth and Neoplastic Transformation. Inoculations i.m. of NCTC clones 7914 and 7915 showed that these cells were nontumorigenic prior to use in this study (11). Early in the experiments, sublines 8282 and 8283 showed a morphology typical of nonneoplastic cells (Fig. 1), and no tumors arose after i.m. inoculations into a limited number of X-irradiated syngeneic hosts (Table 1). In addition, cells of each parental clone were thawed, grown as confluent sheets on 0.5-sq cm polycarbonate plates (~7 to 8 x 10^4 cells/plate) and implanted s.c. into syngeneic hosts (2). No tumors were observed after 11 months in 7 animals implanted with 7914 or 11 animals implanted with 7915 cells.

Initially, sublines of both clones were exceedingly adherent to the growth surface and were difficult to remove by trypsinization. As a result, cell yields were erratic during the first 10 to 15 weeks of this study (Charts 1 and 2), and a longer incubation period was sometimes required to obtain sufficient cells for subculture. This operational problem necessitated use of intraocular implantation rather than i.m. injection of a trypsinized cell suspension for assay of neoplastic potential. After the initial 10- to 15-week period, the cell yield/flask tended to stabilize at ≤50% of saturation density (Chart 3). Although cell yields frequently were similar at the end of the 3- to 4-day growth period, the use of different inoculum sizes resulted in 2 distinct patterns of growth kinetics. Sublines carried at the low inoculum size underwent several population doublings before approaching confluence near the end of the growth period. In contrast, sublines carried at the high inoculum size formed confluent monolayers within 48 hr of subculture, achieved a greater degree of cell-cell contact during the remaining incubation time, and underwent relatively fewer population doublings (Chart 4). Nonadherent cells, that is, cells floating in the growth medium, were not observed in cultures planted at either inoculum size after the 3- or 4-day growth period. As seen in Charts 1 and 2 and Table 1, frequent subculture of nonneoplastic clones at the low inoculum size failed to prevent neoplastic transformation.

Clones carried at the high inoculum size also produced tumors in vivo (Charts 1, 2, and 4). After identical periods in culture, sublines carried at the high inoculum size produced tumors with significantly shorter latent periods than those carried at the low inoculum size (p = 0.004). Thus, use of the lower inoculum size did not prevent neoplastic transformation, but the tumors had longer latent periods.

Cellular Changes during Transformation

Cells obtained during the course of the experiment and also cryopreserved cells of each parent clone and from early (17th to 25th) and late (61st to 63rd) passage (Charts 1 and 2) were used to assess the relationship between neoplastic transformation and changes in morphology, growth in agarose, susceptibility to BCG-activated macrophages, and saturation density.

Morphology. Cytological diagnoses for transformation (5, 14) were made on the parent clones at the beginning of the experiment. Since cells of derivative sublines exhibited poor colony development, evaluation of colony morphology could not be routinely performed during the course of the experiment. The cytology of the parent clones was characterized by parallel cell-cell orientation, low nuclear-cytoplasmic ratio, uniform cell and nuclear size and shape, and a relatively flat, spread cytoplasm (Fig. 1, Table 1). By passage 55 (474 days in vitro), cells exhibited increased cytoplasmic basophilia, an increased nuclear-cytoplasmic ratio, heterogeneity in size and shape of cells and nuclei,

<table>
<thead>
<tr>
<th>NCTC designation</th>
<th>Colony morphology</th>
<th>Growth in agarose colonies/10^5 cells avg. (range)</th>
<th>Cells/animala</th>
<th>Sarcomas/animals implanted</th>
<th>Tumor latent period in days avg. (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7914</td>
<td>Colony morphology</td>
<td>Total examined</td>
<td>% diagnosed neoplastic</td>
<td>10^6</td>
<td>5/5</td>
</tr>
<tr>
<td>7915</td>
<td>Colony morphology</td>
<td>Total examined</td>
<td>% diagnosed neoplastic</td>
<td>10^6</td>
<td>5/5</td>
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<td>7915</td>
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<td>Total examined</td>
<td>% diagnosed neoplastic</td>
<td>10^6</td>
<td>5/5</td>
</tr>
</tbody>
</table>

a Inoculum sizes are for animals given implants i.m.
b Morphology diagnosed from 24-hr coverslip preparations (11); all other diagnoses from plated cells.
and a reduced spreading on the growth surface. In both clones, 85 to 100% of colonies were diagnosed as neoplastic, and the cells ultimately grew as invasive sarcomas in vivo (Fig. 2).

Assessment of 3 sublines of clone 7914 recovered from liquid nitrogen storage showed an increasing frequency of cytologically neoplastic colonies with time in culture (Table 2; Figs. 3 and 4). The lower percentage of colonies diagnosed neoplastic in thawed subline 8609 as compared with the progenitor subline 8282, examined after comparable periods in culture, suggests a selective pressure exerted by cryopreservation and thawing. With 3 analogous sublines of clone 7915, a similar pattern of reduced cytoplasmic spreading was observed, and in a separate study measurements of the projected area of attached cells showed significant reduction after neoplastic transformation (4).

In conclusion, the morphological changes observed in these clones accurately predicted tumorigenicity.

Growth in Agarose. Assays for growth in agarose were performed only after sublines 8282 or 8283 were diagnosed neoplastic by the cytological criteria discussed above. By 539 days in vitro, all sublines grew in agarose as colonies with >0.1 mm diameter, but the number of colonies observed was greater for sublines routinely grown at the higher inoculum size, 8284 and 8285, when compared with sublines 8282 and 8283, respectively (Table 1).

In subsequent studies with cells cryopreserved during the course of the experiment, neither parent clone 7914 nor
Population Density and Neoplastic Transformation

Chart 3. Growth and glycolysis of clones NCTC 7914 and 7915 after serial subculture at 10^5 cells/T-15 flask. Sublines were derived from aliquots of each clone cryopreserved after 286 (NCTC 8560, 8659), 330 (NCTC 8608, 8860) and 519 (NCTC 8609, 8661) days in vitro. As reflected in the increased saturation density values, sublines 8609 and 8661 were extremely crowded after 6 to 8 days of incubation; however, both sublines continued to adhere to the growth surface, and no floating cells were observed. Arrow, time at which spent culture medium was replaced with fresh growth medium; *t* sub., population doubling time during logarithmic phase growth; SD, saturation density in cells/sq cm. Variation bars, actual differences in yield seen in 3 to 4 replicate T-15 cultures; solid bar, glucose used; hatched bar, lactic acid accumulated.

Chart 4. Rate of population doublings and tumorigenicity in sublines of clones NCTC 7914 and 7915. Population doubling level is plotted against number of subcultures. Top, the corresponding number of days in vitro is indicated for each subline.

7915 (sublines 8560 and 8659, respectively) formed colonies in agarose (Table 2). After 358 days in vitro, subline 8660 formed many colonies <0.1 mm in diameter; the parent line was tumorigenic at 376 days, but tumor latent periods were prolonged (Table 1, subline 8283). Sublines 8609 and 8661 formed colonies as did their progenitor sublines 8282 and 8283, respectively, at a comparable time in culture.

Thus, colony development in agarose was observed only with tumorigenic cells, but not all tumorigenic cells developed colonies with a diameter >0.1 mm. Sublines continuously grown at the high inoculum size produced a greater number of colonies in agarose than did sublines continuously grown at the low inoculum level.

**Susceptibility to BCG-activated Macrophages.** The degree of cytotoxicity in the presence of activated syngeneic macrophages, as expressed by release of tritiated thymidine from prelabeled target cells (9), is shown in Chart 5. Sublines 8560 and 8659, derived from the nonneoplastic parent clones, released only a small percentage of isotope, as did subline 8608; subline 8608 was predominantly non-neoplastic by cytological criteria and did not grow in agarose (Table 2). In contrast, the sublines derived from 8282 and 8283 cells after neoplastic transformation (8609 and 8661, respectively) were killed by activated macrophages *in vitro* and released large amounts of isotope. Cultures of subline 8660 showed a percentage of released isotope intermediate between the nonneoplastic parent 8659 and the late passage, neoplastic 8661. Possibly the variation observed with 8660 results from a mixture of neoplastic and nonneoplastic cells in the population since implantation of the progenitor cells (8283) at a comparable time (376 days) gave rise to tumors with long latent periods.

Thus, cells derived from a neoplastic population are susceptible to BCG-activated macrophages and the degree of susceptibility increases as the tumor latent period decreases. In contrast, sublines derived from the nonneoplastic parent clones were not killed by BCG-activated macrophages.

**Saturation Density and Glycolysis.** Chart 3 shows the growth and glycolysis data for the sublines described. The population doubling time decreased with time in culture, and the saturation densities of nonneoplastic sublines 8560 and 8608 were less than that of 8609. No saturation density value was calculated for subline 8659, since it was not clear that a plateau had been reached. Though 8660 formed colonies in carboxymethylcellulose (Table 2) and was susceptible to activated macrophages (Chart 5), the yield after 8 days of incubation appeared very similar to that of the nonneoplastic parent clone. The saturation density of 8661, derived from a neoplastic population, was about 2-fold greater than that of 8660. Thus, with both clones, saturation density increased with period in culture and population...
Table 2
Colony morphology and growth in agarose of sublines of NCTC clones 7914 and 7915a

<table>
<thead>
<tr>
<th>NCTC designation</th>
<th>Clone</th>
<th>Subline</th>
<th>Days in vitro</th>
<th>Total examined</th>
<th>% diagnosed neoplastic</th>
<th>Growth in agarose Colonies/10^5 cells</th>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>7914</td>
<td>8560</td>
<td>338</td>
<td>250</td>
<td>0.8 (0-2)</td>
<td>0</td>
<td>330 (248-408)</td>
</tr>
<tr>
<td></td>
<td>8606</td>
<td>352</td>
<td>204</td>
<td>7 (0-14)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8609</td>
<td>541</td>
<td>848</td>
<td>39 (31-50)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7915</td>
<td>8659</td>
<td>282</td>
<td>– b</td>
<td></td>
<td>0 c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8660</td>
<td>358</td>
<td>–</td>
<td></td>
<td>0 (0-1) c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8661</td>
<td>544</td>
<td>–</td>
<td></td>
<td>11 (2-39) c</td>
<td></td>
</tr>
</tbody>
</table>

a For origin of sublines, see legend to Chart 3.
b See text and Chart 1 in (4).
c When the same cell suspensions were plated in carboxymethylcellulose, colony numbers were: 8659, 0; 8660, 0 with many colonies <0.1 mm in diameter; and 8661, 115 (99 to 133).

Several experimental observations prompted us to reasess population density as a determinant of neoplastic transformation in vitro. Nonneoplastic rat embryo cells, maintained for approximately 3 years with regular fluid renewals but with limited or no subculturing, formed dense multilayered sheets in vitro, but did not grow as sarcomas in vivo. In contrast, cells initiated from the same suspension of embryo cells, but maintained at a lower population density by repeated subculture became tumorigenic (6). In a second study, 2 sublines of rat embryo cells passaged at a low inoculum size (1.5 x 10^6 cells/60-mm dish) exhibited a trend toward earlier neoplastic transformation than did 3 sublines carried at high inoculum size (6 x 10^6 cells/dish) (7). Moreover, a variety of studies suggested that extensive cell-cell contact and physical crowding, decreased population doublings times, and increased saturation density generally change with increasing time in culture and were not necessarily associated with neoplastic transformation (11). For example, in primary culture rodent embryo cells form multiple cell layers, although neither the source tissue nor the cell population in vitro is neoplastic. Similarly, normal human WI-38 cells form multiple cell layers when perfused continuously with fresh growth medium (8).

There are at least 2 alternative interpretations regarding the influence of cell density on the acquisition of malignant potential by mouse cells during culture. Cell density may determine the transformation frequency, or alternatively, selective pressures inherent in the microenvironment and/or culture techniques used may promote the evolution of neoplastic and nonneoplastic cell lines while the transformation frequency remains independent of population density. For example, maintaining cultures at high population density selects for cells that continue to proliferate under conditions of both extensive cell-cell contact and physical crowding. Such cells exhibit less anchorage dependence, since less area of inert glass or plastic growth surface is available per cell; of necessity, a population emerges which can form multilayered foci on monolayers of other cells. These properties are diagnostic of neoplastic cells of fixed tissue origin (5, 14). In contrast, maintaining cultures at low population density may select for cells able to survive and grow with a high cloning efficiency, but need not necessarily exert a selective pressure for either nonneoplastic or neoplastic cells. Moreover, if several population doublings are required to “fix” a lesion associated with or responsible for transformation, it is plausible that a culture regime which enhances proliferation rate will enhance the probability of transformants arising in the population. This study shows that clones carried at low inoculum size undergo more population doublings per unit time than those carried at high inoculum size (Chart 4) and become tumorigenic at...
the same time or somewhat later. Parenthetically, when colonies of flat, well-spread cells are deliberately selected for further study, the probability of maintaining a nonneoplastic cell population is enhanced, since these morphological features are characteristic of nonneoplastic cells (5, 14).

Our experimental results support a “selective” rather than “inductive” interpretation. Sublines carried at the high inoculum size became tumorigenic earlier, although they had undergone fewer population doublings during the same time interval in vitro (Chart 4). These sublines became less dependent on attachment and spreading upon an inert glass or plastic growth surface in that they acquired the capacity to form colonies when suspended in agarose or carboxymethylcellulose. Cytologically, the attached cells became less spread with an increased ratio of nucleus to cytoplasm. However, the sublines grown at low inoculum size and without deliberate selection of colonies with flat well-spread cells also transformed although at a slightly later time in culture. Initially they formed fewer colonies in agarose and grew into tumors after a longer latent period than the sublines carried at the high inoculum size. We conclude that carrying mouse cells at high cell density may accelerate the evolution of a neoplastic population; however, carrying cells at low cell density with limited cell-cell contact is insufficient to prevent transformation, and deliberate selection of colonies or cultures of well-spread, cytologically normal cells is necessary to maintain a nonneoplastic population. Moreover, these data show that certain cytological changes, loss of anchorage dependence (or capacity to grow in agarose suspension), and cytotoxic response in the presence of activated macrophages are associated with the neoplastic change and can be used as phenotypic markers. The lesions and mechanisms underlying these morphological and physiological manifestations are present unknown.

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esis, for carrying out the tumorigenicity assays of NCTC 7914 and 7915 with polycarbonate plates; Dr. Robert E. Tarone, Biometry Branch, for statistical analyses of the data; and Floyd M. Price and Avery Kerr for assistance during the study. We also thank Margaret Faile for her help in the preparation of the manuscript.

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