Correlation of Growth Morphology and Clonability with Malignancy of WEHI-7 T-Cell Lymphoma Sublines

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ABSTRACT

This study examined the development of subclones of different proliferative capacity and malignancy in the WEHI-7 tumor. The mouse T-cell lymphoma WEHI-7 can be clonable in agar with a cloning efficiency of 40 to 50%. On the basis of growth morphology, two types of colonies were distinguished. Most colonies were compact, but a few, no more than 5%, were diffuse. Sublines of the two colony types were established. The cloning efficiency in agar was 40 to 50% for the compact and 15 to 25% for the diffuse sublines. The cloning efficiency did not change for compact colonies. In contrast, the cloning efficiency of diffuse colonies decreased with repeated reclonings. The mice died within 24 to 34 days of i.v. injection of 10⁴ WEHI-7 cells. The same number of cells of the individual sublines administered to mice resulted in improved survival. Injection of compact subline cells resulted in death in 24 to 48 days, while diffuse subline cells resulted in death in 30 to 75 days. The sublines were indistinguishable by light and electron microscopy. Both subline types were negative for terminal deoxynucleotidyl transferase and positive for Thy 1.2. Most cells from the two types of sublines were Lyt-1 and Lyt-2 positive. The doubling time was 10 hr for compact and 14 hr for diffuse sublines. Colony morphology was conserved after passage in vivo and after more than 10 transfers in vitro in liquid and agar media. In conclusion, the different growth morphology in vitro distinguished the subclones of different malignancy in the WEHI-7 tumor cell line.

INTRODUCTION

Tumor cell populations are heterogeneous. Although the origin of most tumors is probably monoclonal, the high rate of mutation results in continuous production of different clones of cells (17). Thus, tumors may express immunological (18) or metastatic (8, 12, 21) heterogeneity or heterogeneity with respect to any of a variety of phenotypes. However, efforts to find qualitative differences, e.g., of surface proteins (22, 23) or secretion of proteolytic enzymes (6), between cells with different malignant potentials have been disappointing.

In the present study, I tried to distinguish subclones of the WEHI-7 tumor cell line of different proliferative capacity and malignancy by a single agar cloning method.

MATERIALS AND METHODS

Animals. Inbred female BALB/c mice, 2 to 3 months old, were obtained from the Panum Institute stock.

Culture Technique in Liquid Medium. The murine T-cell lymphoma cell line WEHI-7 (11) and compact and diffuse sublines derived from it were cultured in RPMI 1640, containing 10% fetal calf serum and 8 mM fresh glutamine, at 37°C in a fully humidified mixture of atmospheric air and 8% CO₂. Cultures were maintained by change of medium 3 times a week; the WEHI-7 cells were adjusted to 10⁶ viable cells/ml, and cells from the sublines were adjusted to 10⁵ viable cells/ml.

Injection of the Tumor Cells. Prior to injection, the cells were washed in serum-free medium; then between 10⁶ and 10⁷ cells in 0.2 ml of RPMI 1640 were injected into the tail vein of the mouse. Normal thymocytes were added to bring the total cell number to 10⁶ cells per injection. When the cells were administered s.c., 10⁴ to 10⁵ tumor cells were injected per mouse.

Culture Technique in Semisolid Medium. At various intervals after s.c. tumor cell injection, the animals were killed, and the regional lymph node, spleen, and s.c. tumor were removed under sterile conditions. Single-cell suspensions were prepared by teasing the organs in RPMI 1640, using fine needles. The medium for the desired number of cells was adjusted to 0.2 ml and added to an equal volume of double-strength Dulbecco’s modified Eagle’s medium and 0.6% Difco Bactoagar with 0.3% SRBC. One ml of this mixture or one ml of 1% methylcellulose, prepared as described by Iscove and Shreir (13), was incubated in 35-mm plastic dishes at 37°C in a fully humidified mixture of atmospheric air and 8% CO₂. The same procedure was used to generate compact and diffuse colonies from liquid cultures of the sublines. Seven days later, 3% acetic acid was added to the cultures to lyse SRBC, and tumor colonies were scored under a Nikon dissecting microscope.

WEHI-7 Tumor Cell Sublines. Sublines derived from clonable WEHI-7 cells were established from Day 7 agar cultures. One ml of a 0.17 M NH₄Cl solution was added to lyse SRBC. The culture dishes were washed in RPMI 1640, and colonies of compact and diffuse morphology were picked up under sterile conditions with Pasteur pipets using the dissecting microscope. Individual colonies were transferred to flat-bottomed microplates (Nunc, Roskilde, Denmark), which contained 10³ irradiated normal thymocytes per hole. To disperse the agar from the cells, each colony was resuspended in RPMI 1640 by being sucked up and down in the Pasteur pipet 20 times before the medium was adjusted to its final concentration of 10% fetal calf serum and 8 mM glutamine. The growth of the colonies was checked daily under the inverted microscope. After 3 to 6 days, the cells were transferred to tissue culture flasks (Nunc). When cell density permitted, the cells were transferred to tissue culture flasks (Nunc) in concentrations of 10⁴/ml.

Recloning Assays. Agar cultures of cells from compact and diffuse sublines were incubated between 5 and 9 days. Each day, 10 to 25 individual colonies of the different sublines were picked up under sterile conditions. Colonies from the same subline were pooled, and after resuspension, the colony cells were washed in RPMI 1640. The cells were counted in a hemocytometer, using the eosin dye exclusion test, and viable cells were adjusted to 10⁶, 500, and 250 cells per dish before they were seeded in agar medium.

TdT. Methanol-fixed cells from the sublines were incubated for 30 min at 20°C with rabbit anti-TdT (Bethesda Research Laboratory, Bethesda, Md.), washed, and reincubated 30 min at 20°C with fluorescein-labeled goat anti-rabbit IgG (Bethesda Research Laboratory). Positive

1 The abbreviations used are: RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; SRBC, sheep red blood cells; TdT, terminal deoxynucleotidyltransferase.

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controls were normal bone marrow cells. Cells were examined in a fluorescence microscope at X400.

Membrane Fluorescence. Monoclonal fluorescein isothiocyanate-conjugated rat anti-mouse anti-Thy 1.2, anti-Lyt-1, and anti-Lyt-2 sera (Becton, Dickinson, Rutherford, N. J.) were used in a direct immunofluorescence assay. Stock solutions of the antibodies were 0.5 mg/0.5 ml. From a 20-fold dilution of Thy 1.2 and Lyt-2 and a 4-fold dilution of Lyt-1, 20 µl were used per 10^6 cells. The cells were incubated with the antibodies (45 min at 0°C) and washed 3 times before they were examined under a Leitz Ortholux II fluorescence microscope.

Light and Electron Microscopy of the Sublines. The cells were washed, fixed in absolute methanol, and stained with May-Grünwald-Giemsa. For electron microscopy, the cells were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 2 hr. Postfixation was performed in 2% OsO4 in 0.1 M phosphate buffer for 2 hr at 37°C. Ultrathin sections were examined in a Phillips EM 300 microscope.

Statistics. To analyze the survival data statistically, the Wilcoxon rank sum test for 2 samples from continuous distributions was used. Significance limits for the rank sum are given in the text.

RESULTS

Establishment of WEHI-7 Sublines of Compact and Diffuse Morphology. Individual Day 7 agar colonies of compact and diffuse morphology were recloned in agar. The recloning was repeated 4 times. By this method, it was impossible to obtain colonies of 100% purity: colony cells from compact colonies gave rise to approximately 95% compact colonies; cells from diffuse colonies resulted in 10 to 75% diffuse colonies. The proportion of diffuse colonies showed a tendency to increase by the number of reclonings. Two reclonings in agar, followed by mass harvesting of the colonies, seeding in methylcellulose, and subsequent recloning in agar, made it possible to generate compact and diffuse colonies of 100% purity. Sublines from individual colonies were established and propagated as described in "Materials and Methods." Fig. 1 illustrates the different morphology in agar of the compact and diffuse colonies.
Growth of Compact and Diffuse Colonies in Agar. Both types of colonies started to develop at Days 4 to 5 of culture and increased in size until Days 7 to 8. About Day 8, the cells in the colonies began to die. The number of cells per colony was higher in compact than in diffuse colonies (Table 1). Compact colonies usually contained about 800 cells on Day 5, which was about 5 times more than diffuse colonies contained. After 8 days of culture, compact colonies contained almost $5 \times 10^5$ cells, and diffuse colonies contained about 10 times fewer cells.

Proliferation of the Sublines in Liquid Medium. The doubling time in liquid medium of cells from compact sublines was 10 hr compared to 14 hr for diffuse sublines. The calculations were based on cells seeded in low density ($10^4$/ml) (see Chart 2) in exponential growth, i.e., within the first 48 hr after medium shift. Chart 2 also illustrates the growth rate of the sublines when cultured in high cell density ($5 \times 10^5$/ml). During the first day of culture, the doubling time was then calculated to be 13 hr for “compact” cells and 17 hr for the “diffuse” cells. After the first 24 hr, the proportion of dead cells increased significantly. The percentage of dead cells was highest in cultures of diffuse sublines (data not shown).

Surface Markers of the Tumor Cells. The antigenic phenotype of WEHI-7 cells and of the different sublines was studied using monoclonal antibodies in a direct immunofluorescence assay. Normal thymocytes were included for comparison (Table 2). Between 85 and 100% of the cells from a freshly extirpated WEHI-7 tumor were positive for the Thy 1.2, Lyt-1, and Lyt-2 antigenic markers. The number of Lyt-1-positive cells decreased with increasing time in culture. After 5 days or more of culture, it was about 25%. In contrast, about 75% of the 2 types of sublines demonstrated antigenicity for the Lyt-1 and Lyt-2 antibodies. The sublines were derived from the clonable fraction of WEHI-7 cells, indicating that all Lyt-1-positive WEHI-7 cells must be clonable. No phenotypic differences were found between the different types of sublines; all were Thy 1.2 positive.

Light and Electron Microscopy of the Sublines. Fig. 2 shows cells from compact and diffuse sublines in May-Grünwald-Giemsa-stained cytocentrifuge preparations. Both types of cells were mononuclear, medium to large lymphocytes. The nucleus/cytoplasm ratio was approximately 1/1. The cytoplasm was basophilic with a distinct negative picture of the Golgi apparatus. Electron microscopy showed cells with irregularly displaced chromatin and an abundance of free ribosomes; only a minority of ribosomes were membrane bound. Cells from compact and diffuse sublines appeared identical.

Malignancy of WEHI-7 Cells and the Different Sublines. The malignancy of WEHI-7 cells and of cells from compact and diffuse sublines was investigated by i.v. injections of the cells in groups of animals. Doses of $10^2$ to $10^6$ tumor cells were administered to determine the dose necessary to kill the animals. A dose of $10^4$ cells was the smallest to result in 100% mortality. Approximately 80% of the mice survived injection of $10^2$ and $10^3$ cells.

### Table 1

<table>
<thead>
<tr>
<th>Colony type</th>
<th>No. of viable cells at following culture times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
</tr>
<tr>
<td>Compact colonies</td>
<td>797 ± 210*</td>
</tr>
<tr>
<td>Diffuse colonies</td>
<td>162 ± 38</td>
</tr>
</tbody>
</table>

*Mean ± S.D. of 50 to 75 colonies from 2 to 4 different sublines.

### Table 2

Antigenic phenotype of thymocytes, WEHI-7 cells, and compact and diffuse sublines

<table>
<thead>
<tr>
<th></th>
<th>% of immunofluorescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy 1.2</td>
</tr>
<tr>
<td>Normal thymocytes</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>WEHI-7 cells</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>WEHI-7 cells after 5 days of culture</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>Compact sublines</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>Diffuse sublines</td>
<td>86 ± 6</td>
</tr>
</tbody>
</table>

*From fresh tissue.

** Chart 2. Growth rate of cells from compact and diffuse sublines cultured in liquid medium. The cells were set up in high density ($5 \times 10^5$ cells/ml) and low density ($10^4$ cells/ml), and viable cells were quantitated daily by the eosin dye exclusion test. Points, mean of 4; bars, S.E.

** Fig. 2. Cells from liquid cultures of compact (A) and diffuse (B) sublines. May/Grünwald/Giemsa, x 1200.
The mice died within 24 to 48 days after injection of 10⁴ WEHI-7 tumor cells. The results were identical for WEHI-7 cells and cells. Mice given injections of cells from diffuse sublines survived longer (p < 0.05) than did mice receiving cells from compact sublines. Survival of mice given injections of compact and diffuse sublines in a mixture similar to the in vivo situation was equal to the survival of mice that received nonseparated WEHI-7 cells (data not shown).

Effect on Colony Morphology in Agar after in Vivo Passage of Compact and Diffuse Sublines. To examine the stability of the colony morphology in agar after in vivo passage of the tumor cells, 10⁴ cells from pools of diffuse and compact sublines were injected s.c. into mice. After 9, 15, and 22 days, the mice were killed, and tissue from the site of injection, the regional lymph node, and the spleen were extirpated. Single-cell suspensions were prepared. Before seeding in agar, the number of cells from the organs was adjusted to 2.5 × 10⁴ and 5 × 10⁴ cells per dish. Cells from the primary tumor were cultured in groups of 250 and 500 per dish. After a culture period of 7 days in agar, the morphology of the colonies was evaluated. The colonies consisted of colonies of either compact or diffuse morphology (data not shown).

DISCUSSION

Previously, cloning in soft agar was used to quantify proliferating WEHI-7 tumor cells after dissemination within an organism (9). The present study demonstrated that it was possible to use this simple procedure to distinguish between subclones of different malignancy within the WEHI-7 T-cell lymphoma cell line. The correlation of morphology with malignancy can be applied in general to the WEHI-7 tumor system. The compact and diffuse sublines constitute the clonable fraction of the WEHI-7 tumor. Therefore, the present study is not merely an investigation of 2 subpopulations selected randomly.

The correlation will not necessarily be true for other tumor systems. A study by Cifone (4) demonstrated a similar correlation, but investigation is needed to determine if the findings apply to all tumor systems. In the present study, I used the different colony morphology of subclones derived from the WEHI-7 tumor cell line to isolate highly malignant cells from less malignant subpopulations within this tumor. Since the isolation proved possible, my next aim was to try to identify properties of cancer or characteristics specifically correlated with cancer.

A selective overgrowth of compact colonies occurred among the agar-clonable cells. Only a minority of the cells grew in diffuse colonies. It proved possible to isolate individual compact and diffuse colonies and to establish these as sublines. The isolation and conservation of the original growth pattern of the cells in agar following numerous reclonings in agar and in vivo passages suggested that the differences did not result from adaption but represented an expression of preexisting subclones within the tumor. When the study was initiated, the tumor cells were injected s.c., and the appearance and growth of the tumor and the status of the mice were followed. No differences were revealed between mice receiving different tumor cells. Also, the lengths of survival were identical (data not shown). This observation may be explained mechanistically. To reach the circulation, the tumor cells must migrate through the s.c. tissue and penetrate the wall of a vessel. In the case of the WEHI-7 tumor cells, the individualities of the sublines may have been obscured by these obstacles. These tumor cells needed another environment to expose their differences, perhaps because the s.c. environment is unnatural to lymphoma cells. The fact remains that the sensitivity of the system was increased by i.v. injection of the tumor cells. By the i.v. route of administration, 10⁴ tumor cells resulted in 100% mortality, while the same number when injected s.c. gave rise to no tumors. Differences in survival of mice after i.v. injection of cells from compact and diffuse sublines demonstrated clearly that variations of biological activity exist within the WEHI-7 tumor cell line. In this study, the WEHI-7 tumor cell line was more malignant following i.v. injection than were any of the sublines derived from it. Injections of compact and diffuse sublines in a mixture imitating the in vivo situation turned out to be more malignant than were the sublines given separately. Survival of these mice equaled that of mice receiving the original WEHI-7 tumor cells. This observation agrees with the finding of Poste et al. (19) that the various subclones in a polyclonal tumor may interact with one another to stabilize their relative proportions within the tumor.

The proliferative potential in both liquid and agar media was greater for compact than for diffuse sublines. In cultures of cells from diffuse sublines, the percentage of dead cells was always higher than in the cultures of compact sublines, suggesting...
increased fragility of these cells (data not shown). Together with the poor recloning capacity of diffuse sublines, the increased fragility may explain why the diffuse sublines were less malignant than were the compact sublines. For many cell types, it has been shown that regulation of proliferation is mediated by coupling of growth arrest and differentiation (10, 15). Accordingly, cells from the diffuse colonies may represent cells that are more differentiated than cells from compact colonies. The investigation of conventional differentiation markers, however, demonstrated identical profiles. All cells were negative for TdT, a differentiation enzyme found on immunologically immature T-lymphocytes (14). Likewise, the cells were Thy-1.2 positive, and about 75% of cells from both cell types were Lyt-1 and Lyt-2 positive. In normal T-cell differentiation, cells of Lyt-1.2.3 phenotype represent a precursor pool that can give rise to Lyt-1 and Lyt-2 progeny (3).

The sublines were derived from the clonable fraction of WEHI-7 cells. The data summarized in Table 2 indicate that all Lyt-1-positive WEHI-7 cells and some Lyt-2-positive cells gave rise to colony formation. The question of why the clonable WEHI-7 cells were Lyt-1 positive while the nonclonable fraction was Lyt-1 negative may be raised, but it remains unanswered.

Preliminary experiments with the sublines demonstrated responsiveness to the mitogen concanavalin A; and investigations are now in progress to test whether the sublines, before or after stimulation by concanavalin A or phorbol esters, can be induced to produce factors like the colony-stimulating factor (1, 16) or the T-cell growth factor (IL-2) (25). Farrar et al. (7) demonstrated recently that the thymoma cell line (EL-4) can be induced to produce IL-2. Likewise, colony-stimulating factor, a regulator of normal hemopoiesis, was shown to induce nonmalignant differentiation of the lymphomyelocytic cell line WEHI-3 (2).

Many experimental animal tumors contain subpopulations of cells of different malignancy (20). Studies by other investigators (4, 5) have also demonstrated a correlation between in vitro anchorage-independent growth to tumor cells and their ability to produce metastasis. However, the present WEHI-7 tumor cell line provided additional information. The WEHI-7 tumor is a T-cell lymphoma cell line. This makes an investigation of the tumor cells by lymphocytic antigenic markers very interesting. Subclones of different malignancy may be correlated to different stages of cancer. Morphological, enzyme-histochemical, and conventional phenotypic markers did not reveal differences among the WEHI-7 subclones. The ever-expanding panel of lymphocytic antigenic markers, however, may eventually shed light on the differentiation and interrelationship of the WEHI-7 subclones. Similar information cannot be obtained by studying the cell line used by Cifone and Fidler, because no differentiation pattern has been established for fibrosarcoma cells.

The WEHI-7 sublines of different malignancy preserve their colony morphology upon recloning, in contrast to the fibrosarcoma sublines (4, 5). This characteristic of the WEHI-7 sublines, as well as the fact that the cells can be expanded endlessly in vitro, sets no quantitative limits to the tests that can be performed. The WEHI-7 lymphoma cell line gives rise to subclones which produce T-cell growth factor (II-2) (24). About 50% of the WEHI-7 subclones in that study produced II-2. This fact makes this tumor line unique. The differences of II-2 production, a factor essential for T-cell proliferation, offer yet another opportunity to study the significance of intratumor heterogeneity. II-2 may turn out to have an intratumor-regulatory effect. In addition, it may be crucial to host defenses against the growing tumor.

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