The Ability of Tumor Cells of the Lymphoreticular System to Grow in Vitro

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SUMMARY

Fifty-eight cultures of lymphoreticular cells (7 normal, 51 pathological) were studied. While all lines, regardless of origin or diagnosis, were able to grow in vitro for short terms, cells derived from malignant tumors had the greatest ability to grow for long periods. This proliferative capacity was also present in a mixed lymphocytic culture derived from two healthy donors and in lymph nodes containing metastatic tumors. Four lines were established in continuous culture. Two of these synthesize immunoglobulin and have shown evidence of viral particles. All of the long-term cultures have aneuploid karyotypes and similar ultrastructural characteristics. It is suggested that the ability to grow in vitro is related to the number and quality of cells with unlimited proliferative capacity present in the original explant. Unlimited proliferative capacity is probably conferred by malignant transformation or antigenic stimulation of the lymphocytic elements.

INTRODUCTION

In 1964, Woodliff (53) indicated that human lymphocytic cells had not yet been grown in continuous cultures for significant periods. Since that time, a host of human cell lines derived from lymphopoietic organs, bone marrow, and peripheral blood have been established (9, 17, 18, 25, 35, 36, 41, 47, 50). The vast majority of these lines were obtained from patients with various diseases, mostly malignant lymphomas and leukemias, whereas normal cells grew very poorly or not at all (33). Although the precise cellular origin of the cell lines could not be established with absolute certainty, it was generally accepted that they originated from malignant cells (8).

In studying the proliferation kinetics, Rosenfeld et al. (42) categorized lymphoid cultures according to survival as (a) populations that die rapidly, (b) populations with a limited life-span, and (c) established lines. Lymphoid cells have precise nutritional requirements for growth in vitro (19) which are highly population dependent (20). Some cultures of lymphoreticular cells can be maintained for prolonged periods by the addition of blastogenic inducers to the nutrient medium of certain stimulatory agents that produce characteristic morphological and biochemical changes in the lymphoid cells, an event called “blastic transformation.” Such transformation can be induced by a variety of agents such as phytohemagglutinin, pokeweed mitogen, pyogenic endotoxins, histocompatibility antigens, etc.

In other cultures, lymphocytes may undergo “spontaneous” blastic transformation; this is sometimes more easily accomplished by supplying the medium with heterologous rather than homologous serum (5). Spontaneous transformation can also occur from cocultivation of lymphoid cells obtained from 2 healthy donors (2, 32), due to antigenic stimulation by histocompatibility antigens. However, in the vast majority of cultures of lymphoreticular origin that grow for prolonged periods, the cause of such a spontaneous blastic transformation cannot be deduced.

The present study encompasses a 5-year systematic exploration by our tissue culture laboratory of the capacity of cells of the lymphoreticular system to grow in vitro without the addition of blastogenic inducers to the nutrient medium.

MATERIALS AND METHODS

Biopsies. Fresh tissue biopsies were utilized for the primary explants. The material was obtained from lymph nodes (45 cases) and peripheral blood (7 cases). Hemopoietic tissue came from bone marrow (5 cases) and peripheral blood (1 case). Material from pretreated patients was rejected, as were samples that could not be seeded immediately after they were obtained. The tissue was finely minced, and small explants were set up in T-30 flasks under a perforated cellophane membrane, as previously described (49). In some instances, loose cells were suspended in medium and cultured by the spinner flask method (34). The cells were maintained in Ham's F-10 medium supplemented by 20% fetal calf serum, vitamins, glutamine, and penicillin (100 units/ml). Feeding was performed initially when needed and afterwards regularly once or twice a week. Serial propagation was carried out by previously described methods (14).

Bone Marrow. One-half to 1 ml of heparinized bone marrow material was placed in a Petri dish containing a few ml of fresh medium. Particles were selected with a Pasteur pipet and placed in T-30 flasks under a perforated cellophane membrane. The medium was changed frequently to remove red cell contaminants.

Peripheral Blood. Leukocyte-containing plasma from 2 pints of blood, each from 2 healthy donors (groups O and A) was
centrifuged, and the leukocytes were mixed and resuspended in 12 ml of plasma. The cell concentration was adjusted to 2.2 \times 10^7 cells/ml. Two spinner flasks each were seeded with 6 ml of the leukocyte suspension, and nutrient medium was added to a final volume of 100 ml. Feeding was carried out twice weekly by the removal of part of the used medium and the addition of fresh medium.

Cytological Studies. These studies were carried out both on the original explants and after continuous culture was achieved. Phase microscopy was performed at least once weekly with the aid of an inverted microscope. To stain the cells, we grew cultures on slides for 1 week. They were then removed, rinsed in 0.9% NaCl solution, and air dried. Stains included Giemsa, Wright's periodic acid-Schiff, methyl green-pyronin, Wielder's reticulin, trichrome, and Alcian blue.

Electron Microscope Studies. A pellet that we obtained by harvesting a single culture and centrifuging the cell suspension at 3000 rpm for 5 min was fixed in 2.5% glutaraldehyde at pH 7.2 for 45 min and postfixed in 1% osmium tetroxide for 1 hr. The cells were embedded in Epon 812. Sections were cut with a Porter-Blum MT-1 ultramicrotome, stained with 1% uranyl acetate and Reynold's lead citrate, and finally examined in a Siemens-Elmiskop IA electron microscope. In addition, round and fusiform-like cells were located by light microscopy on coverslips placed in tissue culture vessels of logarithmically growing cells. Flat embedding techniques were utilized to examine selectively these separate configurations at the electron microscopic level.

Chromosome Studies. Actively growing cultures were incubated with Colcemid (0.02 \mu g/ml) for 6 hr. The cells were harvested, and “squash” preparations were made according to standard techniques. The preparations were observed under a phase microscope, and suitable cells in metaphase were photographed for routine karyotype analysis. These studies were repeated at various intervals, usually at 3, 6, 8, 12, 14, and 18 months.

RESULTS

As soon as the cellular growth pattern was relatively stable, usually after 6 months of continuous culture, samples were frozen for use at a later time. Currently, 34% of the original cultures are maintained in liquid nitrogen refrigeration at \(-193^\circ\). The recovery rate has been excellent.

For operational purposes, the growth span of the cells was arbitrarily classified as short (less than 2 weeks), early (2 weeks to 6 months), and long (over 6 months). A line was considered established when the cells had been maintained in a continuous, actively growing culture for over 1 year. From the 58 cases collected, 96.5% grew rather easily as short-term cultures, and 84.5% progressed to the stage of early cultures. However, only 55.5% were able to grow for longer periods (Table 1). The loss of proliferative capacity occurred in a similar pattern for all dying cultures, regardless of origin or culturing method; the pattern consisted of an increase of the time needed to obtain a confluent monolayer or a large concentration of cells in suspension cultures, appearance of numerous round cells with pycnotic nuclei, and marked morphological aberrations of the cellular configurations. Some cell lines could be reinitiated from frozen samples, but these were not included in the table since they do not represent continuously growing cultures.

Although about one-half of the cultures were able to grow in continuous serial passage for over 6 months, the vast majority began to decline after 8 to 10 months, and the cultures were subsequently lost.

Four lines in this latter series, designated as T1, T3, T4, and T5, have been established as permanent cultures. These have been maintained in continuous growth for at least 2 years, and 1 culture (T1) has been maintained for over 6 years. T1 was derived from the lymph node of a patient with lymphocytic lymphoma and is an immunoglobulin-producing cell line (12). T5 was derived from the lymph node of a patient with undifferentiated lymphoma. T4 was obtained from the bone marrow of a patient with acute leukemia and has occasionally demonstrated immunoglobulin synthesis. Line T2 was derived from a mixed lymphocytic culture from healthy blood donors.

As a general rule, the cells grew out from the explants as long, slender elements with rare admixtures of round cells. The round cells became more abundant with the passage of time (Fig. 1) until the established pattern was that of a mixed population of round, elongated, and polygonal cells (Fig. 2). Stained preparations of these cells revealed morphological features of lymphocytoid, plasmacytoid, and reticulum cells (Fig. 3). In many instances, the round cells were intensely periodic acid-Schiff and pyronin positive. No reticulin- or collagen-like material was ever detected.

The cells, especially the fusiform ones, attached to the glass rapidly, but occasionally would grow primarily in suspension.

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of cell samples</th>
<th>Short</th>
<th>Early</th>
<th>Long</th>
<th>Established</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>45</td>
<td>44</td>
<td>37</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>Spleen</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>56</td>
<td>49</td>
<td>32</td>
<td>(96.5%)</td>
</tr>
</tbody>
</table>

* See "Results."
The spinner flask cultures proliferated for very long periods as suspended, round cells, but invariably large numbers of cells attached to the walls of the culture vessel, revealing a mixed morphological population. The passage of these cells into regular T-flasks resulted in monolayer growth with the characteristics described for the cultures initiated as explants. These growth patterns were observed in all surviving lines, regardless of their origin.

Cytogenetic studies revealed that most of the primary cultures had diploid or near-diploid karyotypes with occasional deletions or supernumerary chromosomes. When the line surpassed 6 months to 1 year of continuous growth, aneuploid cells in the near-tetraploid range began to emerge. With the passage of time, the aneuploid cells predominated. In some cell lines, the karyotype even changed to a near-triploid modal number. Five lines in the long-term culture group never changed ploidy and were lost between 8 to 10 months after the initial culture. All of the remaining 25 lines exhibited the above-mentioned karyotypic changes. Parallel cultures reinitiated from frozen samples revealed an initial diploid or near-diploid karyotype but, eventually, these also changed to an aneuploid range if maintained in continuous growth. All of the established cell lines exhibit aneuploid karyotypes (Fig. 6).

Electron microscope studies disclosed several noteworthy characteristics. The cell lines that grew for short periods maintained the ultrastructural attributes of small lymphocytes—a large N/C ratio, clumped chromatin, very few organelles, and sparse granules and vacuoles (Fig. 5). The lines that did grow out and that finally became established had common characteristics, regardless of their origin. The N/C ratio was small, the chromatin was finely distributed, large nucleoli were present, and there were numerous cytoplasmic organelles, especially mitochondria and endoplasmic reticulum profiles. The polygonal and fusiform cells presented similar ultrastructural characteristics. However, no specific cytoskeleton ever was demonstrated that could differentiate the 2 main morphological entities. The immunoglobulin-producing cell lines contained, in addition, abundant polyribosomes dispersed in the cytoplasm (Figs. 7 and 8). Immunelectron synthesis was assessed by direct and indirect immunofluorescent methods (Fig. 4).

Two of the cell lines (T₁ and T₄) revealed, in the 1st few passages, the presence of viral particles. T₁ contained elementary bodies resembling C-type particles, and T₄ revealed intranuclear herpes-like viral particles. However, subsequent cultures failed to exhibit the viral bodies.

Arranging the life-span of the cultures according to diagnosis (Table 2) reveals that cultures derived from malignancies had a vigorous capacity to grow for extended periods. This is evident in the lymphomas (14 of 19 long-term cultures), reticulum cell sarcomas (5 of 8), leukemias (3 of 4), and malignant reticuloendotheliosis (2 of 2). Cells derived from Hodgkin’s tumors, on the other hand, presented poor survival capabilities (1 of 8). Lymph nodes with metastatic carcinomas also presented the ability to grow for long periods (3 of 4). These latter cultures presented similar morphological patterns to the rest of the lymphoid cultures, but cells with the characteristics of carcinoma were never identified.

Cultures derived from normal lymphoreticular tissues had a much lower capacity to grow for extended periods (3 of 8). In fact, 2 of the 3 long-term cultures of normal cell origin were lost at about 8 months after the initial explant. However, 1 line (T₁) was established from the cocultivation of the peripheral lymphocytes of 2 different healthy blood-bank donors. Lymphoreticular tissues with inflammatory diseases (cat scratch disease, lymphadenitis, and reticulum cell hyperplasia) also failed to thrive in vitro for significant periods (1 of 5).

**DISCUSSION**

Lymphoreticular cells, considered in this investigation, demonstrated a similar pattern of outgrowth, regardless of their origin. In general, small sheets of fusiform to polygonal cells were the main features, with rare round cells. After several weeks, larger aggregates of round cells began to appear. These findings are in accord with the experience of others (3, 5, 6, 39, 40, 45), and the event has been named “lymphoblastoid transformation.” The single exception was line T₁, which had both morphological forms in the initial explant. In numerous instances, the polygonal cells possessed vacuoles which sometimes contained various debris suggestive of phagocytic activity. It has been postulated for a long time that lymphoid cells can give rise to macrophages (30); such a transformation has been proven in vitro (20, 26, 27) and in
vivo (23). Thiele and Stark (48) postulate that the origin of macrophage cells in peripheral blood cultures is in the monocytic cells and not in the lymphocytes.

The significance of the elongated cells in lymphoreticular cultures has been the subject of much study. Sinkovics et al. (45) claimed that these cells may behave as a "feeder layer," furnishing essential metabolites for the propagation of the culture. A large number of investigators have used the terms "fibroblast-like" and "fibroblastoid" to describe these cells; however, the cells have only a morphological resemblance to fibroblasts in vivo and do not really exhibit the growth pattern of fibroblasts in culture. Fibroblast cultures are characterized by the rapid growth of sheets of very long, slender, mononuclear cells, where very few cells adopt a round configuration; these are generally cells in mitosis or dying cells. The polygonal and fusiform cells in lymphoreticular cultures are sometimes called fibroblasts, although no investigations to prove this point were undertaken. Studies involving the uptake of radiolabeled hydroxyproline have not been reported, nor has collagen or reticulin material been demonstrated in these cells. At the ultrastructural level, no procollagen units have been noted. Morphological diagnosis of the cellular type of in vitro cells is precarious (52). Therefore, tissue culturists resort to more "fundamental" functional characteristics to categorize in vitro cells, since it has been noted that functional properties often persist in vitro more than do morphological properties (13). However, the so-called fibroblastic cells of lymphoreticular cultures present neither morphological nor functional properties of fibroblasts. Hence, one can hardly apply this term, which connotes a specific cell with a specific function, to cells that have only a remote morphological similarity to fibroblasts.

Cultures of mesenchymal origin may sometimes transform into fibroblastic elements, and the event was called "homicoplastia" (10). However, this transformation was studied in cultures of meningiomas that contain abundant fibroblasts; these are the most likely source of the fibroblasts overtaking the culture. In a later publication (38), the same authors consider preexisting fibroblasts as the source of the fibroblastic transformation of nerve tissue culture. In all fairness, it should be pointed out that Petrakis et al. (37) have reported collagen production, assessed by histochemical methods, in peripheral blood cultures. These findings, however, have not been repeated.

We have documented, by means of time-lapse photography, that lymphoid cells can undergo a cyclic morphological interconversion from round to fusiform and back to round, each morphological phase being the expression of a functional state (15). Some of the fusiform elements can certainly be confused as fibroblasts, but they are not true fibroblast cells. It is very appealing to postulate that lymphoid cells, in order to adapt to in vitro growth, adopt a fusiform or polygonal shape with little mobility and maximum stickiness; once adaptation has occurred, round lymphoid cells, with full expression of functional capabilities, reappear.

The chromosomal studies revealed an instability of the karyotype in vitro. The cells of established cultures presented aneuploid karyotypes in the triploid or tetraploid range. This is similar to the findings in other continuous mammalian cultures, in which the cells, in order to survive in a new environment, must undergo metabolic changes that may lead to an altered karyotype (7, 44). That is, many cell lines, in order to survive in vitro conditions, transform (22) or rearrange the karyotype (1, 43). Such an abnormal karyotype does not seem to interfere with most of the normal functions of these cells, i.e., immunoglobulin synthesis (44).

The ultrastructural characteristics demonstrated by the established lines in this series correspond exactly to those encountered in blast-transformed lymphocytes induced by phytohemagglutinin (11, 16). Evidence indicates that the capacity of reverting to a relatively immature stage and the capability of indefinite growth of lymphoreticular cells can be induced by a variety of agents; moreover, this transformation proceeds by a precise sequence of events, regardless of the inducing agent. The morphological features of these events are characteristic for lymphoid cells and are marked by a small N/C ratio and a quantitative increase in cytoplasmic organelles. The immunoglobulin-producing cell lines did not present a well-developed system of endoplasmic reticulum, which has been considered the ultrastructural evidence of immunoglobulin synthesis (4). Even cells with morphological characteristics of plasmacytoid elements demonstrated only a few endoplasmic reticulum profiles. Harris et al. (21), on the basis of extensive ultrastructural studies on immunoglobulin-producing cells, indicate that the endoplasmic reticulum is not the chief organelle for protein synthesis but merely represents the organ of storage of such proteins. In fact, immunoglobulin synthesis is carried out by free ribosomes in the cytoplasm.

The growth potential of lymphoreticular tissue is apparently dependent on the types of cells seeded. Imamura and Moore (24) have pointed out that colony-forming ability is directly related to the malignant properties of the clonogenic elements, and Moore et al. (32) have suggested that survival of leukocytes in vitro might be related to the presence of herpes-type viral particles. Iwakata and Grace (25) noted viral particles in the myeloblasts in a successful culture of peripheral leukocytes in a case of acute leukemia.

We have encountered viral particles in 2 of our established cell lines: in the early cultures of line T1, where elementary bodies resembling C-type particles were noted (50), and in the culture of a lymph node of a patient with undifferentiated lymphoma (line T2), where herpes-like particles were observed. In the present series, malignant cells, presumably with unlimited capacity for division, presented the greatest potential for extended proliferation. Such potential for in vitro growth was noted particularly in the lymphomas, reticulum cell sarcomas, leukemias, and malignant reticuloendotheliosis. It may be argued that, although the cultures derive from malignant tissues, it is the normal lymphoid cells that proliferate in vitro. However, it is difficult to visualize how these normal lymphoid cells could overtake a culture from explants in which the immense majority of cells are neoplastic as assessed by histological examination. Furthermore, in an extended discussion of the cellular origins of lymphoreticular cultures, Clarkson (8) pointed out that the most likely source, in malignant explants, is the neoplastic cell. Unfortunately, we do not have karyotypes performed on the original tissues which might have presented marker chromosomes helpful in elucidating the true origin of our cultures (9). However, at least 1 of our lines (T1) has
demonstrated the presence of a presumably tumor-specific antigen common to lymphomas (51).

Cells derived from Hodgkin's tumors had very poor survival. Further investigation revealed that all of them were initiated from lymph nodes diagnosed as either nodular sclerosis (2 cases) or lymphocytic depletion, diffuse fibrosis type (6 cases) (29). In both types, but especially in the latter, tumor cells and even the immunologically committed normal lymphocytes (46) are very rare (J. J. Butler, personal communication). It appears, then, that the failure of our Hodgkin's tumor expiants to thrive in vitro is due to the paucity of both malignant cells and antigenically stimulated lymphocytes. The observation of Meier et al. (31) of Paltauf-Reed-Sternberg cells in these cultures (31) has not been repeated in this laboratory.

Normal lymphoid cells demonstrated a vigorous early growth which diminished after 4 to 5 months, and the cultures were subsequently lost. Two cultures were able to proliferate for at least 8 months before declining. The single case in which a continuous normal lymphoid cell culture was established should be attributed to the fact that it resulted from a mixed lymphocytic culture which possesses allogeneic stimulatory properties (2). This same reasoning could apply to the prolonged cultures derived from lymph nodes bearing metastasis in which tumor antigens may serve as the stimulating factors. The category of miscellaneous diagnosis comprised lymphadenitis, cat scratch disease, and reticulum cell hyperplasia. These lacked the ability to grow in vitro for any significant period.

The present series indicates that cells from the lymphoreticular system are almost invariably able to grow in vitro for short periods. However, long-term growth ability is apparently related to the number and quality of lymphoid elements capable of unlimited proliferation present in the original explants. Tissue culture systems provide an invaluable tool for the study of physiological and kinetic properties of individual tumor cell lines in a controlled environment. This type of investigation could be extended to the study of single cell response to chemo- and radiotherapy, thus providing an individualized protocol for rational treatment of lymphoreticular tumors.

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REFERENCES


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