Growth of cultured cells from patients with Hodgkin's disease and transplantation into nude mice

(tissue culture/heterotransplantation)

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ABSTRACT Long-term monolayer cell cultures have been prepared from tumor nodules in spleens removed from 28 patients with Hodgkin's disease and from 84 spleens that did not have tumors from Hodgkin's disease patients, normal adult spleens, and human fetal spleens and thymuses. After 5 to 30 serial passages in culture, cells from nine of the Hodgkin's disease monolayers underwent morphologic change in vitro with transition from a spinle and reticular pattern of replication to polygonal and round cells that propagated in mosaic arrays. Four of such Hodgkin's disease mononayer cell lines were injected subcutaneously into 43 nude, athymic mice. In 36 animals (84%), neoplasms developed at the inoculation site that were locally destructive, capable of pulmonary metastasis, and eventually fatal to the recipients. Transplanted tumors were not observed in 18 athymic mice injected with cultures prepared from normal human adult spleen and fetal spleen and thymus, nor were tumors seen in 16 similar animals that received fresh, noncultured Hodgkin's disease tumor tissue. On microscopic examination, xenografts derived from Hodgkin's disease cultures were pleomorphic malignant neoplasms composed of large, undifferentiated cells, resembling reticulum cell sarcoma. These neoplasms did not involve the lymphoreticular organs of mice. Chromosome studies indicated that the transplanted neoplasms were composed of human cells with an aneuploid karyotype and that mononayer cultures prepared from the heterotransplants contained a karyotype similar to that of the cultured cells prior to passage in mice. The ability of these Hodgkin's disease cell lines to produce invasive tumors with human karyotypes in nude mice is evidence of the neoplastic nature of the monolayer cells and their relationship to the malignant cell of the human disorder.

Experimental studies of Hodgkin's disease are hampered by the cellular heterogeneity of the tumor in vitro and the lack of a morphologically similar counterpart of the lymphoma in animals. Therefore, examination of tissue cultures derived from Hodgkin's disease tumors and transplantation of these cultured cells into experimental animals may be useful in the investigation of this malignant lymphoma. In the present report we describe growth characteristics of primary explants and long-term monolayer cell cultures derived from Hodgkin's disease splenic tumors. Cells from the long-term Hodgkin's disease monolayers were transplanted into nude (athymic) mice. Epstein et al. (1, 2) have recently reported transplanted tumors, in nude mice, produced by intracranial inoculation of cultures of lymphomas other than Hodgkin's disease.

MATERIALS AND METHODS

Monolayer Cultures. Long-term, serially passed, monolayer cultures were obtained from Hodgkin's disease tumors in spleens removed at staging laparotomy. The tumor nodules were dissected from the surrounding uninvolved spleen and minced with scissors; the cell suspensions were washed 3 times with Earl's balanced salt solution. A thin layer of sterile human plasma (obtained courtesy of Dr. Charles Huggins, Jr.) was applied to the culture surface of Falcon 75 cm², 250-ml plastic flasks. This procedure was essential to secure attachment of cells to the flask surface. In general, the buoyant density of the splenic cell population and their surface characteristics interfere with satisfactory attachment without the addition of the plasma as an adhesive. Other agents, such as collagen or agar, have not been useful for this purpose in our hands. A suspension of washed cells was placed on the plasma-coated flasks and fed with Dulbecco's modified Eagle's medium that contained 15% cobalt-irradiated fetal calf serum (Microbiological Associates). After 3 days in culture at 37°C, floating cells were discarded or replanted in new plasma-coated flasks. Within 7–10 days primary explants displayed definite growth, with the appearance of clusters of adherent stellate, round, and spindle-shaped cells. Variable numbers of multinucleate giant cells containing 25 to 50 micronuclei (Fig. 1) appeared, interspersed among the adherent cells, particularly in the cultured nodules from Hodgkin's positive spleens. They rarely appeared in cultures from Hodgkin's negative spleens, and were never seen in cultures of normal spleens. These foci of multinucleated cells generally appeared during the end of the second week of primary culture, reached a maximal number during the third week, and then disappeared. Observation of areas containing many clusters of such multinucleated cells suggested that the giant cells were unable to divide and replicate in vitro and that they underwent degeneration and death within a week of their first appearance. These clusters of multinucleate cells were a hallmark of the early growth characteristics of the positive Hodgkin's spleens in tissue culture under our conditions. Foci of multinucleated cells were observed in primary cultures of 19 out of 28 splenic tumor nodules and in one culture derived from a grossly uninvolved spleen of a patient with Hodgkin's disease. Clusters of giant cells were not detected in monolayers from 72 other negative spleens, or from 12 normal adult and fetal human spleens. Solitary multinucleate cells were occasionally seen in cultures of negative spleens from Hodgkin's disease patients and in one normal spleen culture. After 3 weeks in culture, primary explants were removed with trypsin and passaged to new flasks. Application of plasma to the culture surface was, however, not required or used for adherence of passaged cells. After 8 to 10 serial passages a population of adherent reticular and spindle cells (fibroblast-like) with occasional, scattered binuclear or multinuclear cells persisted in culture. All Hodgkin's disease cultures were passaged 20–30 times with repeated checks for contamination with mycoplasma before cells were used in experiments. No antibiotics were added to the cultures. Nine Hodgkin's disease monolayer cultures have undergone a morphologic alteration in vitro after 3–10 months' propagation in culture, with the appearance of polygonal cells that rapidly replicate in a mosaic pattern (Fig. 2). Cells from four of these Hodgkin's disease cultures (FQ, RB, RY, and SP lines) were inoculated into nude mice.
Monolayer cell cultures used as controls were obtained from normal adult spleens removed for trauma or incidentally during another surgical procedure. Cultures of human fetal thymus and spleen were obtained from the Naval Biomedical Research Laboratories, Oakland, Calif. Primary culture monolayers derived from normal adult spleen were difficult to maintain. The primary explants adhered to plasma-coated flasks, but initial growth of cells from adherent clusters was often sluggish. After four to six passages in culture, normal cells were predominantly spindle and reticular (fibroblast-like) in appearance, without multinuclear giant cells. Our monolayers derived from normal spleen and thymus usually did not survive beyond 18 or 20 serial passages in culture. Cells from four normal adult spleen monolayers and two culture lines each of fetal spleen and thymus were inoculated into nude mice.

Transplantation of Cultured Cells to Nude Mice. Although a larger number of nude mice were inoculated with Hodgkin’s disease cultured cells, only those in which the animals were sacrificed before they died of the disease, and in which complete gross and microscopic pathological examination was done, are included in the series below. Eighty-three nude mice, obtained from Life Sciences Laboratories, St. Petersburg, Fla., were injected with cells. Homozygous (nu/nu) 25- to 35-day-old animals derived from nu/nu males backcrossed with nu/+ NIH Swiss females were used (3, 4). The mice were caged in a separate, isolated room, maintained at 80°F, but without sterile precautions. Forty-three nude mice were administered single, posterior thoracic, subcutaneous inoculations of 10 to 50 × 10^6 cells from Hodgkin’s disease cultures (Table 1). Four animals received Hodgkin’s disease cultured cells intraperitoneally, and two mice were given cells intravenously. Eighteen nude mice were administered similar subcutaneous aliquots of normal human cultured cells, and 16 animals were injected with cells from four different fresh, noncultured Hodgkin’s disease tumors.

Cytogenetic Analysis. Chromosome preparations were made

<table>
<thead>
<tr>
<th>Type of cells, and route of inoculation</th>
<th>No. of mice injected</th>
<th>Tumors at site of injection</th>
<th>Pulmonary metastases</th>
<th>Bone involvement by tumor</th>
<th>Cachexia</th>
<th>Inflammatory liver disease*</th>
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<tbody>
<tr>
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<tr>
<td>Subcutaneous</td>
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<td>36</td>
<td>4</td>
<td>9</td>
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<td>22</td>
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<tr>
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<tr>
<td><strong>Fresh (noncultured) Hodgkin’s disease tumors</strong></td>
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<td>—</td>
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<td>4</td>
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* Inflammatory liver disease refers to non-neoplastic hepatitis (see text).
on Hodgkin's disease cell lines and cultures obtained from the transplanted tumors in mice. Monolayer cells in exponential growth were trypsinized, washed with Earle's balanced salt solution, and suspended in 75 mM hypotonic KCl for 8-10 min. The cells were fixed twice in freshly prepared methanol and glacial acetic acid (3:1, vol/vol) solution for 30 min and stained with Giemsa stain by the method of Moorhead et al. (5).

RESULTS

Of the 43 nude mice administered subcutaneous injections of cells from Hodgkin's disease monolayer cultures, 36 developed tumors at the site of inoculation (Fig. 3; Table 1). Tumor-bearing animals were sacrificed 41-164 days after injection (mean duration of 91 days). The mice were usually cachectic (mean weight of 30 g; range of 25-33 g), particularly those with bulky or locally destructive tumors. The multilobulated subcutaneous masses ranged from 0.3 to 3.6 cm in maximum dimension (mean dimension of 1.6 cm) and were firmly adherent to surrounding skeletal muscle, bone, and skin. Erosion of the lateral rib cage with extension of tumor into parietal pleura was observed on gross examination in six animals. Extensive involvement of the vertebrae by tumor was detected in three mice, two of which were paraplegic due to destruction of the thoracic spinal cord by tumor. Ulceration of skin overlying transplanted tumors was present in only two animals. The four animals that received intraperitoneal inoculations of Hodgkin's disease cultured cells did not develop tumors.

None of the 18 nude mice injected subcutaneously with normal cultured cells developed persistent tumors at the site of inoculation, and at postmortem examination residual injected cells were not observed (Table 1). The 16 animals administered fresh Hodgkin's disease tumor tissue also failed to develop subcutaneous tumors, and none had persistent injected cells at autopsy.

Pathologic Findings. The transplanted tumors involved subcuticular tissues and dermis at the site of inoculation, but did not extend into the epidermis (Fig. 4). Cellular infiltration of skeletal muscle and peripheral nerves was quite prominent in most tumors. Osseous involvement with pathologic fractures of the proximal humerus, scapula, ribs, or thoracic vertebrae was observed in nine mice. Involvement of vertebrae was accompanied by cellular infiltration of dorsal root ganglia in five mice and the spinal cord in three animals. The transplanted tumors were morphologically similar, regardless of the source of the Hodgkin's disease monolayer culture used for subcutaneous inoculation. The randomly disposed cells were pleomorphic microscopically, with eosinophilic cytoplasm, large, round vesicular nuclei, and prominent, eccentrically aligned, basophilic nucleoli (Fig. 5). Scattered ill-formed acinar arrays were surrounded by sheets of undifferentiated polygonal cells in many of the tumors. Mucicarmine, alcian blue, periodic acid-Schiff, and methyl-green pyronine stains of the tumor were negative. Although most of the transplanted tumors contained scattered cells with hyperlobulated nuclei and occasional multinuclear forms, Reed-Sternberg cells were not unequivocally identified. Many of the larger subcutaneous tumors contained foci of central fibrosis with granulation tissue and cystic degeneration. Aggregates of acute and chronic inflammatory cells and mast cells were frequently observed at the interface of infiltrative tumor cells and adjacent normal structures.

Microscopic pulmonary metastases were found in four out of the 36 nude mice with subcutaneous tumors (Table 1). Three animals had subcutaneous nodules 8-10 mm in diameter and

FIG. 3. Nude mouse with a subcutaneous tumor derived from cells of a Hodgkin's disease monolayer tissue culture. The transplanted tumor involves the scapula and distorts the left pectoral girdle.

FIG. 4. Low-power photomicrograph of a transplanted tumor showing a dense cellular infiltrate that has filled the dermis, involved a small dermal nerve, and spared the epidermis (×126).

FIG. 5. Transplanted tumor derived from a Hodgkin's disease culture is composed of pleomorphic cells that have large, round vesicular nuclei with reticulated chromatin and prominent nucleoli (×762).
found to have aneuploid human karyotypes with approximately 80 chromosomes, similar to those of Hodgkin's disease cells prior to murine transplantation (Fig. 6). The spindle cells resembled fibroblasts and contained 41 acrocentric chromosomes characteristic of normal murine cells. No cells with human–mouse hybrid chromosomes were identified.

Negative Transplantation Experiments: Inoculation of Cells from Hodgkin's Disease Cultures to Animals Other Than Nude Mice. Cells from Hodgkin's disease cultures were injected subcutaneously or intravenously into 58 neonatal and adult NIH Swiss mice. Prior to inoculation with cultured cells, approximately half of these mice underwent neonatal thymectomy, lethal irradiation (850 rads), and allogeneic bone marrow transplantation. Hodgkin's disease monolayer cells fused with 23-3T3 murine fibroblasts with inactivated Sendai virus were administered intravenously to 20 thymectomized, irradiated, bone marrow transplanted, NIH Swiss mice. Hodgkin's disease culture cells and cultured cells fused with BHK-2 cells with Sendai virus were injected into 16 baby hamsters. Finally, eight owl monkeys and eight spider monkeys were given intrasplenic inoculations of Hodgkin's disease culture cells, or cultured cells fused with monkey kidney cells (L. Melendez, F. Garcia, and P. Zamecnik, unpublished data). None of the mice, hamsters, or monkeys developed transplanted tumors derived from cultured cells.

**DISCUSSION**

Monolayer tissue cultures derived from Hodgkin's disease tumors are composed of adherent cells that replicate rapidly, can be serially passaged, and, in a number of instances, are capable of propagation for an indefinite period of time. Several of our Hodgkin's disease cell lines have been maintained in vitro for 3–4 years with persistence in culture of a homogeneous population of polygonal and round cells with scattered multinuclear forms. In earlier studies from our laboratory we demonstrated an antigen on the surface and within the cytoplasm of cells from these cultures (6, 7). More recently the antigen has been isolated and partially purified from supernatant medium of Hodgkin's disease cultures by polyacrylamide gel electrophoresis and Sephadex column chromatography (8). Prolonged replication of cells in vitro derived from the tumor has up to the present time been necessary for detection of this Hodgkin's disease culture antigen.

In the present experiments cells from Hodgkin's disease cultures were found to produce malignant tumors when injected into athymic mice, indicating that the monolayers may be derived from neoplastic cells of the disorder. The transplanted tumors destroyed bone, infiltrated the pleura and thoracic spinal cord, and produced pulmonary metastases. Microscopically, the xenografts were undifferentiated, pleomorphic malignant tumors that resembled large-cell lymphoma (reticulum cell sarcoma). The tumors did not involve lymphoid organs in mice and Reed-Sternberg cells were not positively identified, although numerous binucleate and multinucleate cells were seen. The xenografts were composed of cells with human-type chromosomes with aneuploid karyotypes similar to the original Hodgkin's disease monolayers. The transplanted tumors could be reestablished in vitro with propagation in culture of cells that were morphologically and cytogenetically similar to those of Hodgkin's disease monolayers before passage in mice. Cultures derived from the transplants, when re inoculated subcutaneously into athymic mice, produced more rapidly growing, locally destructive tumors than the original Hodgkin's disease monolayers.

Three lines of evidence suggest that the tissue culture cells
under investigation are neoplastic: they are capable of continuous growth in vitro, have aneuploid karyotypes, and produce malignant tumors in athymic mice (9–12). Similar findings have been obtained with tissue cultures derived from other human lymphomas [Burkitt's tumor and histiocytic lymphoma (1, 2, 13)]. It may be mentioned that lymphoblastoid suspension cultures derived from nonmalignant conditions, but carriers of Epstein-Barr virus, which has oncogenic potential, are capable of continuous growth in vitro, may have aneuploid karyotypes, and can be transplanted to experimental animals (14, 15). It should also be noted that while our Hodgkin's disease monolayer cultures readily produced invasive tumors in athymic mice, cultured normal human cells and fresh Hodgkin's disease tumor could not be transplanted.

Despite questions that remain to be answered by future investigations, the ability of these cultured cells with aneuploid karyotypes to produce malignant xenografts capable of pulmonary metastasis in nude mice is strong evidence that the monolayer cells are neoplastic (9). It would appear that tissue culture studies should prove useful in the future investigation of the pathogenesis of Hodgkin's disease. It is of particular interest that clusters of multinucleate cells are commonly seen in certain viral diseases in tissue culture, and were a distinctive feature of our primary cultures from tumor nodules from spleens of patients with Hodgkin's disease.

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