Potentiation of Fibroblast Growth by Nodular Sclerosing Hodgkin’s Disease Cell Cultures

By Samuel R. Newcom and Leslie O’Rourke

Cell cultures were established from 8 lymph nodes replaced by nodular sclerosing Hodgkin’s disease. Serum-containing and serum-free conditioned media from these cultures potentiated fibroblast growth and were found to be consistently more potent than fibroblast growth factor, 100 ng/ml, every other day. Both a proliferative response and transformation-like growth were observed using BALB/c 3T3 cells, human diploid fibroblasts, and human embryonic fibroblasts as target cells. The Hodgkin’s disease growth factor(s) was not produced by fibroblasts or lymphocytes in the Hodgkin’s cultures and was most potent when the Hodgkin’s cultures had been enriched with Hodgkin’s giant cells. Removal of normal macrophages decreased the proliferative activity but did not eliminate it or nonadherent growth of 3T3 cells in agar. Control cultures of 6 nonmalignant lymph nodes, a Lennert’s lymphoma, a mixed cellularity Hodgkin’s disease lymph node, and a malignant histiocytosis cell line suggested that among lymph node disorders, this feature may be relatively specific for nodular sclerosing Hodgkin’s disease.

RECENT WORK demonstrates that transformed cells can produce mitogenic agents capable of stimulating fibroblast proliferation. Also, work with normal cells and tissue has demonstrated that macrophages and platelets contain and elaborate potent mitogens capable of stimulating fibroblast proliferation.

There is considerable evidence that the Hodgkin’s cell (Reed-Sternberg cell and mononuclear variants) is a malignant macrophage. It was hypothesized in the present study that during malignant transformation, the Hodgkin’s cell from nodular sclerosing Hodgkin’s disease acquires or greatly increases its ability to produce a growth factor(s) for fibroblasts.

The experiments reported here were designed to test this hypothesis utilizing short-term cell cultures of nodular sclerosing Hodgkin’s disease from which conditioned medium was obtained. Hodgkin’s cells and normal macrophages from one specimen were successfully enriched. Phagocytic macrophages were removed from four cultures and nonadherent lymphocytes were established separately in two cultures. Fibroblasts were cloned from one culture. The growth of BALB/c 3T3 cells, human diploid fibroblasts, and human embryonic pulmonary fibroblasts was analyzed in response to the soluble products of these cultures (termed here Hodgkin’s disease growth factor(s)—HDGF and compared to the proliferation achieved with fresh medium and calf serum (as a source of platelet-derived growth factor—PDGF and with complete medium plus fibroblast growth factor (FGF), 100 ng/ml, every other day.

MATERIALS AND METHODS

Materials

Plain tissue culture dishes were from Falcon Labware, Division of Beckton, Dickinson & Co., Oxnard, Calif., and 35-mm dishes with grids were from Lux Scientific Corporation, Newbury, Calif. Gentamicin was from Schering Corporation, Kenilworth, N.J. Dulbecco’s modified Eagle’s (DME) medium, trypsin, 1-glutamine, amphoterin, and calf serum were from Grand Island Biological Co., Grand Island, N.Y. Lidocaine (200 mg/ml) without bacteriostat was from Abbott Laboratories, North Chicago, Ill. Beef lung heparin (1000 U/ml) was from The Upjohn Co., Kalamazoo, Mich. FGF was a generous gift from Dr. Denis Gospodarowicz, Cancer Research Institute, University of California, San Francisco.

Source of Hodgkin’s Cells

Involved lymph nodes were obtained fresh from eight patients with nodular sclerosing Hodgkin’s disease. One-half of the node, not needed for histopathology, was utilized for cell culture.

Controls

Lymph nodes from 6 patients with normal (4) or reactive (2) changes were obtained, as well as 1 lymph node replaced by mixed cellularity Hodgkin’s disease, 1 with Lennert’s lymphoma, and a malignant histiocytosis cell line (graciously provided by Marshall Kadin, M.D., University of Washington, Seattle, Wash.)

Patient’s Plasma

One-hundred milliliters of whole blood was drawn from each patient or control prior to lymph node biopsy. Anticoagulation of the blood was with 20 U of heparin/ml. The heparinized blood was centrifuged at 1250 g for 15 min in 50-ml capped conical tubes. The plasma was centrifuged a second time at 2000 g for 15 min and filtered to 0.22 micron. Absence of platelets was documented by Wright-giemsa staining. Storage was at −20°C in 10-ml aliquots.

From the Osgood Memorial Laboratory and the Department of Medicine, Oregon Health Sciences University, Portland, Ore.

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Address reprint requests to Samuel R. Newcom, M.D., Section of Medical Oncology, Oregon Health Sciences University, 3181 S. W. Sam Jackson Park Road, Portland, Ore. 97201.

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Preparation of Tissue Explants and Cell Suspensions

All procedures were carried out in a laminar air flow hood. Specimens were transferred to DME medium supplemented with 10% calf serum, 1% 200 mM L-glutamine and 50 μg of gentamicin/ml (complete medium). Tumor nodules were minced with scissors to small fragments (1–2 mm in diameter). A cell suspension was prepared by disaggregation of small fragments with the tips of needles and, following vigorous agitation of the supernatant, aspiration of the suspension through progressively smaller needles (nos. 21, 23). Cytocentrifuge preparations of the cell suspension were prepared on microscope slides at 1000 rpm for 5 min and stained with Wright-Giemsa to confirm the presence of Hodgkin’s giant cells. Viability was measured by 0.1% Trypan blue dye exclusion at 5 min. Cell counts were by hemacytometer.

Hodgkin’s Tissue Culture Conditions in Serum-Containing Medium

Sixty and 100 mm Petri dishes were coated with a thin layer of the patient’s plasma (0.5–2 ml). Excess plasma was removed. Explants (6–10) were placed in each coated dish with forceps. The cultures were maintained in a double-jacketed CO2 incubator with 100% humidity and 7.5% CO2 at 37°C. After 18–36 hr, the plasma, tissue fragments, and nonadherent cells were washed free with complete medium. The adherent cells (Hodgkin’s cells, a few lymphocytes adherent to the Hodgkin’s cells, monocyte-macrophages, and fibroblasts) were then cultured in 5 ml (60-mm dishes) or 15 ml (100-mm dishes) of complete medium, which was changed weekly. Cultures were examined daily with an inverted photomicroscope with phase optics. In one set of cultures (HD no. 13), lymphocytes were washed free at 7 days, fibroblasts were depleted by removal with trypsin (0.25% for 6 min at 37°C), and viable Hodgkin’s cells and macrophages were successfully removed with lidocaine (2 mg/ml for 30 min at 20°C). The Hodgkin’s cells and macrophages were concentrated to 10⁵/ml and replated in complete medium. Serum-containing conditioned media from the 4 nodular sclerosing Hodgkin’s lymph nodes, 2 normal lymph nodes, a Lennert’s lymphoma, a malignant histiocytosis cell line, and a cloned line of Hodgkin’s fibroblasts were prepared by aspirating the medium every 7 days, centrifugation at 2000 g for 15 min, and filtration of the supernatant through a 0.22-μ filter. Amphotericin (2.5 μg/ml) was added. Storage was at −20°C.

Preparation of Macrophage-Depleted Serum-Free Conditioned Medium (CM)

The cell suspensions from 4 nodular sclerosing Hodgkin’s disease lymph nodes, 4 control lymph nodes (2 normal, 2 reactive), a mixed cellularity Hodgkin’s lymph node, and the nonadherent cells from 2 Hodgkin’s lymph nodes were divided into 2 equal portions in 10 ml of unsupplemented DME. Cell counts were 0.5–2 × 10⁶/ml aliquot, and viability was routinely greater than 90%. Macrophages were identified and enumerated by alpha-naphthyl butyrate staining of cytospins and differential counting. The presence of Hodgkin’s cells was confirmed by Wright-Giemsa staining. Macrophages were removed by incubation of a 10-ml aliquot with 10 mg carbonyl iron. Both aliquots (with and without iron) were incubated for 1 hr at 37°C (7.5% CO2) with resuspension every 20 min. Iron phagocytes were then removed by repeated exposure to a magnet. The resultant cell suspension was usually found to contain less than 10% of the original number of esterase-positive cells. The macrophage-depleted and control cultures were then pelleted and resuspended in 1.0 ml human AB plasma for plating in 10-cm dishes. After 18 hr, cultures were vigorously washed 3 times with plain DME (37°C) and the nonadherent cells removed. The adherent cells were then cultured in 15 ml DME for 24 hr. The CM was removed, centrifuged at 1200 g, filtered to 0.22 μm, and stored at −20°C.

Test Cells

Test cell lines included BALB/c 3T3 cells (Flow laboratory, Inglewood, Calif.), human diploid fibroblasts (initiated from a newborn skin specimen), and human embryonic pulmonary fibroblasts (a gift from Wallace Iglewski, Department of Micorbiology). All cell types are routinely cultured in complete medium supplemented with 2.5 μg/ml amphotericin. Cell stocks are passaged every 7–10 days with a split ratio of 1:64 and maintained in 7.5% CO2 humidified incubators at 37°C.

Cell Growth Measurements in Serum-Containing Medium

To determine the growth response of fibroblasts to Hodgkin’s CM, 0.5–1.0 × 10⁴ BALB/c 3T3 cells, human diploid fibroblasts, or human embryonic fibroblasts were plated per triplicate 35-mm dish in 2 ml of complete medium with 2.5 μg amphotericin/ml. In the experiments utilizing CM from HD nos. 6, 7, and 10, the Hodgkin’s CM was renewed every other day. In the experiments with CM from HD no. 13, renewal was weekly. Dilutions of HD 13 CM made in fresh medium were also evaluated. Controls included complete medium changed weekly, or every other day, and FGF 100 ng/ml added every other day.

Triplicate plates were prepared for each determination. Dishes were tripinized (0.25%) every 2-4 days and counted with a Coulter counter. Sample dishes were fixed in formalin (10%) and stained with methylene blue for correlation.

Cell Growth Measurements in Serum-Free Medium

Serum-free Hodgkin’s CM was supplemented with calf serum (10%) for one experiment, calf serum (0.5%) for two experiments, or with bovine serum albumin (0.5%) for one experiment. Triplicate monolayer fibroblasts cultures (10⁴ cells/35-mm dish) were established in complete medium. After 18 hr, when the cells had become adherent, they were brought into rest by changing the medium to DME supplemented with 0.5% calf serum for 24 hr. While at rest, the fibroblast monolayer was aspirated free of medium and replenished with 2 ml test or control medium. Cell counts were performed every 3–8 days until no further growth was observed. Sample dishes were fixed with formalin (10%) and stained with methylene blue for correlation.

Nonadherent Cell Growth

BALB/c 3T3 (4 × 10⁴) cells or skin fibroblasts were plated in 1 ml of 0.3% agar in Hodgkin’s CM over 1 ml of 0.5% agar in complete medium in 35-mm dishes. Controls were complete medium and complete medium supplemented with FGF 100 ng/ml. Incubation was at 37°C with 100% humidity. A single cell suspension was identified with an inverted microscope on day 1 and colonies (50 or more cells) were looked for weekly. All plates were scored weekly × 3.

RESULTS

Cell Suspension

All cell suspensions had an initial viability greater than 95% by Trypan blue dye exclusion. For Hodgkin’s lymph nodes, cytocentrifuge preparations confirmed the presence of large cells with characteristic nuclei
and gigantic nucleoli. Lymphocytes, adherent to the Hodgkin's cells, were usually present. Normal macrophages were less than 10% of the cell population in both Hodgkin's and normal or reactive lymph nodes.

**Hodgkin's Cell Culture**

A collection of multinucleated giant cells was identified in cultures from all Hodgkin's specimens. Trypsin (0.25%) was successful in removing fibroblasts but was ineffective in removing Hodgkin's cells for subculture and frequently killed them if left on the monolayer for 10 min or longer. Lidocaine (2 mg/ml) was successful in removing viable Hodgkin's cells and normal macrophages in HD no. 13 (viability 48%–85% by Trypan blue dye exclusion) (Fig. 1) and allowed subcultivation of enriched Hodgkin's cells (Fig. 2). The cells resulting from the HD 13 subcultivation were used as a source of Hodgkin's CM. Hodgkin's cells were identified in macrophage-depleted cultures after 48 hr of serum-
free culture by removal with lidocaine, cytospin, and Wright-Giemsa staining (Fig. 3).

**Serum-Containing Hodgkin's CM Applied Every Other Day**

Hodgkin's CM changed every other day was compared to FGF 100 ng/ml added every other day. Three CM were tested in this manner. Both mitogens resulted in proliferative responses, eventuating in large numbers of cells in each dish as compared to DME medium with 10% calf serum. Packing and overlapping of cells was seen in response to Hodgkin's CM. In 9 days, BALB/c 3T3 cells underwent 7 doublings in response to HDGF, whereas calf serum (10%) weekly resulted in 5 doublings and FGF in 5.5 doublings (Fig. 4). In 12 days, human diploid fibroblasts also underwent 7 doublings in response to HDGF, whereas calf serum (10%) resulted in only 3 doublings and FGF in 5 doublings. Therefore, HDGF produced 2–3 times the number of cells resulting from FGF stimulation and 10 times the number achieved in DME with 10% calf serum alone. These results were confirmed using CM from two additional nodular sclerosing cell cultures. Figure 5 illustrates the results with HDGF10.

![Figure 3. Binucleate Hodgkin's giant cell after 48 hr of serum-free culture of lymphocyte and macrophage-depleted cell population. Cytoplasmic processes have retracted with lidocaine (2 mg/ml) and there is a small amount of culture debris in the cytoplasm. A degenerating adherent lymphocyte is noted. Wright-Giemsa (x1000).](image1)

![Figure 4. Configuration of the monolayers of BALB/c 3T3 fibroblasts after 9 days in culture. Cells were plated at 10,000 cells/3.5-cm dish in complete medium and amphotericin (2.5 µg/ml). (A) Appearance of contact-inhibited cells grown in weekly changes of fresh medium. Cells are cuboidal, loosely arranged, and orderly. (B) Appearance of culture supplemented with FGF 100 ng/ml every other day. Cells are more tightly packed but remain orderly in appearance. (C) culture in HDGF every other day. Cells are less distinct and heaped up. There is transformation-like overgrowth. Changes were reversible when cells were recultured in DME medium with 10% calf serum. Phase microscopy through plastic (x300).](image2)
Response of BALB/c 3T3 Cells, Adult Human Diploid Fibroblasts, and Embryonic Human Fibroblasts to Serum-Containing Hodgkin's CM Renewed Weekly

In contrast to the previous experiments, the BALB/c 3T3 experiment with HD no. 13 compared Hodgkin's CM from enriched Hodgkin's cell cultures changed weekly to FGF 100 ng/ml added every other day and DME with 10% calf serum changed weekly or changed every other day. In addition, HDGF₁₃ was diluted 50:50 with DME supplemented with 10% calf serum and changed weekly. The most marked proliferation was seen with the 50% dilution of HDGF₁₃, which induced 7 doublings in 6 days and was as effective weekly as fresh 10% calf serum in DME medium changed every other day and more potent than FGF 100 ng/ml added every other day.

The human diploid fibroblast experiment (Fig. 6) demonstrated weekly 25% and 50% HDGF₁₃ to be approximately equally potent and to yield tightly packed overlapping and criss-crossing cells at 12 days, with 7.5 doublings having occurred as compared to 6 doublings with FGF every other day, 5 doublings with Lennert's lymphoma, normal lymph nodes no. 1 and no. 2, 4 doublings with DME with 10% calf serum, and 3 doublings with nodular sclerosing Hodgkin's disease fibroblast CM (see Fig. 7 for correlation).

In the experiment with human embryonic pulmonary fibroblasts, HDGF₁₃ was used at concentrations...
media dilutions were in DME medium with control serum (0.5%) of 8% and 4% and compared to control medium (DME example of the growth curves obtained with this test Serum-Free CM control media were replenished weekly. Four percent HDGF, yielded 4 doublings in controls. other day to DME medium with 0.5% calf serum. with 10% calf serum) at concentrations of 8% and 4% yielded 3 doublings in 18 days, as did the yield in HDGF, and 8% HDGF, yielded 5 doublings. Test and control media were replenished weekly.

Response of Fibroblasts to Macrophage-Depleted Serum-Free CM

For 3 nodular sclerosing Hodgkin's CM, the CM was supplemented with calf serum (10% or 0.5%). An example of the growth curves obtained with this test medium changed weekly on human diploid fibroblasts is illustrated in Fig. 9. BALB/c 3T3 cells also responded in a similar way. Figure 10 demonstrates the morphology of human diploid fibroblasts in HD no. 35 CM produced by a macrophage-depleted culture. Removal of macrophages tended to reduce the activity but did not eliminate it.

Nonadherent cells were removed and cultured in serum-free DME for 24 hr in 2 specimens. These cells, essentially 100% normal lymphocytes, did not produce a growth factor for fibroblasts, and the lymphocyte CM was either partially exhausted or contained an inhibitor when compared to fresh medium and FGF.

For one nodular sclerosing Hodgkin's CM, the DME was supplemented only with 0.5% bovine serum albumin (BSA). One doubling of cell number and sustained viability at 18 days was seen in Hodgkin's CM as compared to control medium and FGF 100 ng/ml every other day, which resulted in no proliferation and cell death over a 5–7 day period.

Nonadherent Transformation-Like Growth of Fibroblasts

A single cell suspension was documented on day 1 in all dishes after plating in agar. Clusters (20–50 cells) and colonies (greater than 50 cells) were noted on day 14 in the cultures of BALB/c 3T3 cells in 30% Hodgkin's CM (Fig. 11). Occasionally, colony or cluster formation was noted in FGF or calf serum, but in most experiments the cells remained as a single cell suspension. No colony formation was noted by human diploid fibroblasts. Data are presented in Table 1. A slight decrease in 3T3 cell colony-stimulating activity was noted with removal of macrophages.

DISCUSSION

Reticulin fibrosis, collagen formation, and fibroblast proliferation are seen in several human malignan-
cies including breast cancer, myelofibrosis with myeloid metaplasia, and most notably, nodular sclerosing Hodgkin’s disease. The fibrosis in these states has been postulated to represent a host response.  

Recently, however, work has centered on the production of growth factors from murine sarcoma virus (MuSV) transformed cells. These studies suggest that MuSV-transformed cells produce growth factors that stimulate cell division in normal murine fibroblasts, interact with the membrane receptors for epidermal growth factor (EGF), and appear to be responsible for reversible transformation-like growth of untransformed cells.  

The partial purification of this family of growth-stimulating polypeptides, sarcoma growth factors (SGFs), from the supernatant fluids and cells of the Moloney MuSV-transformed mouse 3T3 cell cultures has been described. SGFs not only stimulate fibroblasts to divide and overgrow in monolayer cultures, but also produce a profound morphological alteration in the cells and allow them to form progressively growing colonies in soft agar, a property closely associated with the transformed phenotype. Similar growth patterns of BALB/c 3T3 cells were identified with concentrations of nodular sclerosing Hodgkin’s CM as low as 25% replenished weekly. A proliferative
response of embryonic human fibroblasts greater than achieved with FGF 100 ng/ml every other day was noted with 4% Hodgkin’s CM renewed weekly. In addition, anchorage independent growth was seen in semisolid agar with the 3 CM tested, 2 of which were produced in serum-free conditions.

In the present study, production of a potent mitogen for fibroblasts by 8 nodular sclerosing Hodgkin’s disease cell cultures is demonstrated. The cell of origin was not the fibroblasts and appeared not to be the lymphocytes that had been depleted from the cultures as well as cultured separately. The remaining adherent cells were predominantly the multinucleated and mononuclear Hodgkin’s giant cells and normal macrophages. Potency was high following partial purification of the Hodgkin’s cells and normal macrophages. Control cultures with similar numbers of normal macrophages (normal lymph nodes) did not demonstrate significant activity nor did a culture with a large number of normal macrophages associated with lymphoma (Lennert’s lymphoma). Removal of normal macrophages decreased the activity but did not deplete it. Activity was produced in serum-free cultures and did not require the presence of serum for its mitogenic action on fibroblasts. Although presence of the monoionic, Interleukin-1 (IL-1), must be considered a possible explanation for our observations, IL-1 has not been reported to induce transformation-like growth properties. It appears unlikely that IL-1 is solely responsible for our observations, and, as measured by mouse thymocyte stimulation, we have found IL-1 in only 2 of our 8 Hodgkin’s CM and only 1 after removal of macrophages.

Normal macrophages are known to be a source of mitogens for other cells in several settings. Macrophages are essential for the normal wound healing process. When they are eliminated from a wound, fibroblast proliferation is reduced and healing at all stages is severely inhibited. In addition, it has been shown that macrophages can stimulate the proliferation of fibroblasts in vitro, while others have reported their growth-promoting activity for stem cells. The ability of wound-derived macrophages and wound fluids to stimulate the proliferation of vascular endothelial cells and vascular smooth muscle cells maintained in tissue culture has also been reported. A recent review summarizes these and other observations regarding the macrophage as an effector cell. However, transformation-like fibroblast growth has not been reported with normal macrophage-derived growth substances suggesting that a malignant cell is more likely to be the source of the growth factor(s) reported here.

Table 1. 3T3 Colonies in Agar at 14 Days

<table>
<thead>
<tr>
<th>Medium</th>
<th>Colonies/35-mm Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>DME + 10 CS</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>DME + 10 CS + FGF</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>NSHD #31 (serum-free)</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>NSHD #31 (serum-free, macrophage-depleted)</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>NSHD #35 (serum-free)</td>
<td>9,581 ± 20</td>
</tr>
<tr>
<td>NSHD #35 (serum-free, macrophage-depleted)</td>
<td>6,834 ± 26</td>
</tr>
<tr>
<td>NSHD #13</td>
<td>300 ± 10</td>
</tr>
<tr>
<td>Nonmalignant nodes (#3-6)</td>
<td>0</td>
</tr>
<tr>
<td>Malignant histiocytosis cell line</td>
<td>0</td>
</tr>
<tr>
<td>HD #38 (mixed cellularity HD)</td>
<td>0</td>
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</tbody>
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Characterization of the Hodgkin's cell as a malignant macrophage led to our hypothesis that a growth factor(s) might be produced in the nodular sclerosing subtype, which resulted in an abnormal fibroblast proliferation. The Hodgkin's cell does not have T-cell markers.\textsuperscript{24,25} Intracytoplasmic immunoglobulin and surface immunoglobulin have been reported in some cases, suggesting a B-lymphocyte origin\textsuperscript{26,27} supported by the lack of abundant nonspecific esterase in fresh cells.\textsuperscript{28,29} However, the intracytoplasmic immunoglobulin has recently been demonstrated to be polyclonal in nature and to be internalized by the cell.\textsuperscript{12} Correlating with the endocytosis of immunoglobulin has been the observation of phagocytosis of India ink particles in cultured Hodgkin's cells\textsuperscript{10} and the demonstration of lysozyme production.\textsuperscript{11} However, these observations were of cultures with normal macrophage contamination. These macrophage characteristics have been complemented by the observation of surface "ruffles" by scanning electron microscopy (EM)\textsuperscript{30} and intracytoplasmic macrophage-like organization by transmission EM in some studies,\textsuperscript{13} but not in others.\textsuperscript{31} The observation of glass and plastic adherence, as noted in the present study, is most characteristic of the macrophage. Cell surface marker results have also been most consistent with a macrophage nature and include IgG-EA rosette formation as well as a complement receptor for C3b but not C3d.\textsuperscript{10} The summation of these observations indicates a probable macrophage nature for the Hodgkin's cell and a possible role as an effector cell.

The observation reported here, of transformation-like fibroblast proliferation in response to the serum-containing and serum-free conditioned media from 8 nodular sclerosing Hodgkin's disease cell cultures, conclusively demonstrates the production of a potent mitogen for fibroblasts by these cultures. This activity was soluble, withstood freeze-thawing, and passed through a 0.22-\mu filter. The activity was more potent than FGF and, in contrast to FGF, consistently induced transformation-like growth of fibroblasts. The Hodgkin's cell, possibly a transformed macrophage, appeared to be the source, although conjoint production of IL-1 by normal macrophages may have potentiated this activity in some cases. A well characterized cloned Hodgkin's cell line or a large number of pure fresh Hodgkin's cells will be necessary to further confirm our observations and conclusions. It has been reported that transforming growth factors are found in several different types of human malignancies.\textsuperscript{2,22,23} In addition, transforming growth factors may be isolated from normal tissues if their activity is potentiated by other normal growth factors,\textsuperscript{24} a phenomenon that could be present in nodular sclerosing Hodgkin's disease. Isolation, purification, and further characterization of the Hodgkin's growth factor will be necessary to determine its biochemical features, its role in the pathogenesis of the disease, and its possible usefulness as a tumor marker.

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SR Newcom and L O'Rourke