Cultivation and Characterization of Cells from a Malignant Lymphoma in an African Child*

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SUMMARY

In the course of studies of factors responsible for the malignant lymphoma of children in Africa, cells derived from a maxillary tumor of an 8-year-old male patient in Kenya have been serially cultured in this laboratory. Fragments, prepared from tumor tissue, became attached to the glass, and cellular outgrowth was observed after 9 days of incubation. After 15 months of serial passage morphologic features remained similar to those of the early passages. The major part of the dividing cells were diploid, although great variation of diameters of interphase nuclei suggested the presence of cells with higher chromosome numbers. Attempts to produce tumors in newborn hamsters and in human cancer patients have failed. In one female patient, after subcutaneous inoculation of the cultured cells, a nodule developed, but sections failed to reveal tumor cells. General cytological properties of the cells cultivated from the lymphoma include considerable cytoplasmic motility and an apparent capacity to phagocytize, observed in time-lapse movie studies; lysosomes were frequently observed by electron microscopy.

The cultivated cells resembled certain histiocytes which are characteristically present in this type of lymphoma; they contained periodic acid-Schiff-positive intracytoplasmic granules and were positive for acid phosphatase and for nonspecific esterase. Conversely, the predominating tumor cells in the malignant lymphoma resembled lymphoblasts and showed none of the above features.

The results strongly suggest that the cultivated cells have arisen from histiocytes ("waterpot cells") in the tumor, rather than from the predominating lymphoblasts. Although obscure, the role of the histiocytic cells may be important.

The present report describes the cultivation and certain characteristics of cells derived from a malignant lymphoma of the maxilla of an African child (5). The study was undertaken as part of a general inquiry into the nature of such tumors, which have attracted much attention in East Africa (3, 4, 11) because of the frequency of conspicuous, early involvement of the jaw bones and the unusual geographic distribution of the patients.

The cells have now been transferred serially for 15 months. The method of cultivation, their general appearance and staining characteristics, chromosome number, ultrastructure, and attempts to produce tumors in animals and human cancer patients are being reported as part of a description of the observations made of the disease in Kenya.

MATERIALS AND METHODS

SOURCE OF THE CELLS

The cells were cultivated from portions of a maxillary tumor removed surgically from an 8-year-old male patient, a member of the Msaba tribe of Western Kenya. He was born and had always lived on the island of Mfangano, which lies near the coast of Kisii District in Lake Victoria. This is an area in which the disease was found to be relatively common. He was taken to the Kendu Mission Hospital where the nature of the tumefaction was identified, and from there he was transferred to the King George VI Hospital in Nairobi for treatment.

The tumor was the size of an orange on admission. It greatly displaced the eye, filled the right nostril, and infiltrated the palate and right alveolar arch. Submandibular lymph glands were found to be enlarged, as were the liver and spleen. His hemoglobin was 13.3 gm.
per cent, white blood cells numbered 11,500, and platelets 140,000 per cu. mm. His blood urea nitrogen was 32 mg. per cent and alkaline phosphatase 8.8 Bodansky units.

The tumor was soft and friable and contained many areas of necrosis. Histologic examination showed a uniform structure of unorganized, large, immature lymphoblasts with many scattered histiocytes.

**Cultivation of Cell Strain**

Tumor tissue obtained on March 1, 1962, in Nairobi, Kenya, was suspended as small pieces in LY medium (7) and carried to New York at close to body temperature. On March 3 it was prepared for cultivation as follows: the tumor tissue was minced into fragments less than 1 cu. mm. with scalpels. The fragments were distributed into a number of T-15 flasks containing different media with various concentrations of human and/or fetal bovine serum. In some of the culture flasks, attachment of the explant occurred after several days of incubation at 37° C., but definite cellular growth was observed only after 9 days of incubation and only in one culture containing the tumor cells in Puck's (14) medium N-16, 40 per cent; Evans' NCTC-109, 4 per cent; saline F, 41 per cent; fetal bovine serum, 15 per cent; glutamine, 8 mg. per cent; added penecillin and streptomycin. The outgrowth consisted of cells growing radially from the attached explant. Ten days later a nearly confluent sheet of cells had grown across the culture flask, and at this stage cells were transferred, after treatment with 0.25 per cent trypsin, into several other culture containers. The appearance of a cell of the first subculture is shown in Figure 1, and the growth pattern of an established cell sheet, after several transfers of the cell strain, is seen in Figure 2.

Parallel cultures were inoculated into an x-radiated sheet of primary human amnion cells (3000 r). Although a temporary growth stimulation was observed, cultivation with the feeder layer was not essential for long-term maintenance. Several weeks after initiation of the culture and after four passages the growth rate decreased, and the cells appeared granular. At this time the medium was changed to consist of: basic medium, 60 per cent; fetal bovine serum, 20 per cent; and human serum, 20 per cent—which enhanced the growth of the cultured lymphoma cells. Several weeks later the fetal bovine serum concentration was reduced to 15 per cent, and in this medium the cells have continued to divide slowly. In the early stages weekly transfers of one culture into at least two cultures were possible; later the growth rate decreased, permitting a total of only twenty transfers during the first year of cultivation.

**Methods of Characterization of the Cultured Cells**

*Chromosome analysis.*—To establish counts of the chromosomes cells grown on coverslips in Leighton tubes were treated with 0.00025 per cent colchicine solution for 1 hour and then with 0.9 per cent sodium citrate followed by air drying and fixation in 3:1 alcohol-acetic acid fixative. They were stained with 2 per cent acetic orceine. After dehydration preparations were mounted in Permount. Counts were made by means of a drawing apparatus.

*Electron microscopy.*—For micromorphologic studies scraped cell sheets were fixed in 1 per cent phosphate-buffered osmium for 35 minutes, post-fixed in 10 per cent neutral isotonic formalin, and dehydrated in a graded series of ethanol.

The material was embedded in butyl-methacrylate. Sections, 60–100 μm in thickness, were cut with a Porter-Blum microtome, with a glass knife, and collected on collodion-carbon-coated grids. Contrast was enhanced by "staining" with lead hydroxide.

The specimens were examined, and electron micrographs were taken at an instrument magnification of 7,200 with an RCA-EMU-3FF electron microscope and the use of a 250-μ condenser aperture and a 50-μ objective aperture.

*Microcinematography.*—For cine records, an Emdeco time-lapse unit combined with a Zeiss inverted microscope with a 40 × phase-contrast objective was used to record cells inoculated into a Sykes-Moore culture chamber. The speed was four frames per minute. Maintained at 37° C., the culture fluid was changed every 24 hours throughout the 72-hour sequence.

*Histochemical studies.*—The materials studied included cultivated cells grown as monolayers on coverslips and, for comparison, stored and frozen (−20° C.) solid tissue from the parent tumor which had given rise to the cultivated strain.

For routine histological examination the tissues were stained with hematoxylin and eosin after fixation in a mixture of 10 per cent formalin, 80 per cent ethanol, and 10 per cent distilled water. After similar fixation the methyl green-pyronin (9) stain was utilized for studying the distribution of DNA and RNA, the periodic acid-Schiff (PAS) (16) reaction for polysaccharides, and an oil red O (16) stain for lipide in the cultivated cells. The acridine orange stain (1) was also utilized to demonstrate DNA and RNA after fixation of the cultivated cells in a mixture of 90 per cent ethanol and 10 per cent glacial acetic acid, and the Feulgen stain (10) was employed for detection of DNA after fixation in a mixture of 1 part glacial acetic acid and 3 parts absolute ethanol. The Gomori cobalt sulfide method (8) was used for alkaline phosphatase, the Gomori lead sulfide method (8) for acid phosphatase, and the alpha-naphthyl acetate method (12) for non-specific esterase in cultivated cells which had been fixed for 20 minutes in cold calcium-formal solution and in parent tumor tissue which had been fixed overnight in cold calcium-formal and then sectioned with a freezing microtome.

**Results**

*Appearance of cultivated cells.*—From the earliest passages, the cultivated cells exhibited a considerable degree of morphological heterogeneity. Characteristically, the cells were large and bizarre and contained large, often irregularly shaped nuclei with prominent nucleoli. Many cells had multiple stellate processes; they tended to

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1 10 per cent formalin with 0.4 gm. per cent calcium chloride added and brought to pH 7 by addition of 0.1 M sodium carbonate.
overlap and intertwine (Fig. 4), and their growth pattern did not simulate that of fibroblasts or epithelial cells. A striking amount of extracellular eosinophilic debris was often seen scattered about in the vicinity of the cell aggregates. The nuclei varied in diameter from 12 to 80 μ, and often presented an irregular lobulated outer margin (Fig. 5). This typical morphological appearance has remained relatively unchanged during the entire period of cultivation.

The appearance of the monolayer of cells depended strongly on the amounts and relative concentrations of human and fetal bovine serum. In medium containing 20 per cent human serum the growth was poor. An addition of 5–10 per cent fetal bovine serum improved the cell sheet only slightly. The most uniform-appearing cell sheets with well spread out cells of high density were observed in cultures containing 15 per cent or more fetal bovine serum, and an addition of human serum appeared advantageous (Fig. 3). Results obtained after several transfers showed, as mentioned, that the optimal serum levels for sustaining cell division were 15 per cent fetal bovine serum and 20 per cent human serum, although the cells under these conditions formed a less uniform growth pattern.

A microcinematographic observation of the cells for a period of 72 hours after a transfer revealed an unusual degree of cytoplasmic activity. Compared with other human cell types studied in our laboratory by this technic (primary human amnion cells and FL cells), the cultured lymphoma cells exhibited very fast movements in all directions in irregular patterns on the glass surface. The most startling movements of some cells in the culture included surface blebs which, due to constant protrusion and retraction in a rhythmic fashion, gave the impression of a rotary movement, clockwise or counter-clockwise, of the peripheral parts of the cytoplasm. In other cells in the field, however, the blebbing appeared in a nonrhythmic manner, and such cells constantly changed shape with violent motions. Some cell borders showed only undulating movements. The nuclear activity appeared to be less than that of the cytoplasm. Extracellular debris, often appearing as spherical cell fragments, seemed to be attracted to the cell surface, and occasionally such fragments appeared to be phagocytized.

Histochemical studies.—Three distinct classes of intracytoplasmic granules have been identified within the cultivated cells (Table 1): (a) large eosinophilic granules (Fig. 6) which varied considerably in diameter (1.5–18.5 μ) and in shape. These stained moderately positive with Feulgen, acridine orange, and methyl green-pyronin. They were often surrounded by a halo. (b) Small eosinophilic intracytoplasmic granules (Fig. 6) which varied less than the large granules in diameter (0.5–1.0 μ) and tended to be uniformly rounded. These stained strongly PAS-positive, were negative to Feulgen, acridine orange, and methyl green-pyronin, and often did not appear to be surrounded by a halo. (c) Micronuclei (Fig. 7) were occasionally encountered. They varied in diameter from 3.5 to 14 μ, were oval in shape, appeared to contain chromatin and nucleoli, and were bordered by an intact nuclear membrane. They were Feulgen-positive and fluoresced like intact nuclei when stained with acridine orange and examined in ultraviolet light.

Other cytoplasmic constituents have been identified which were separate and distinct from the three types of granules described above. Acid phosphatase, which is demonstrable as precipitated insoluble lead sulfide in the Gomori technic, appeared in the cytoplasm as numerous, small, localized black deposits (Fig. 8) measuring less than 1 μ in diameter. Scattered, oil red O-positive lipide granules were also present in the cytoplasm.

The cultivated cells showed diffuse faint activity when stained for nonspecific esterase and were largely alkaline phosphatase-negative.

The original tumor had histologic features typical of a malignant lymphoma (Fig. 9) and was consistent in appearance with those East African lymphomas described by O’Conor and Wright (11, 17). The predominating cells resembled lymphoblasts, with moderately large, uniform, centrally located oval-shaped vesicular nuclei, and with a scant to moderate amount of amphophilic, nongranular cytoplasm. Scattered throughout the tumor were seen large, benign-looking histiocytes, with abundant cytoplasm containing granular material. The distribution of these cells created a typical “starry sky” appearance (Fig. 9), and they were undoubtedly the cells which have been given the name of “waterpot.”

The predominating tumor cells failed to show acid phosphatase, alkaline phosphatase, and nonspecific esterase activity. However, acid phosphatase- and nonspecific esterase-positive cells were seen scattered throughout the tumor in precisely the distribution of the histiocytes described above (Figs. 10, 11). These latter cells were alkaline phosphatase-negative.

Chromosome analysis.—The first chromosomal counts were obtained from cells of the second passage of cultivation. To permit exact counting, cells were selected in which the chromosomes were well spread out (Fig. 12).

As seen in Table 2, the major part of dividing cells were diploid. Of 100 mitoses scored for diploidy (or near-diploidy) or tetraploidy (or near-tetraploidy), 89 per cent belonged to the first group and 11 per cent to the second group. This ratio probably represents more nearly the actual distribution than that obtained from the exactly

<table>
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<th>TABLE 1 STAINING CHARACTERISTICS OF THREE TYPES OF INTRA-CYTOPLASMIC PARTICLES IDENTIFIED IN CULTIVATED CELLS, DERIVED FROM A MALIGNANT LYMPHOMA</th>
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<td><strong>Particle</strong></td>
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<tr>
<td>Large intracytoplasmic granules</td>
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<td>Small intracytoplasmic granules</td>
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<td>Micronuclei</td>
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counted 33 cells. The quality of the preparations was not sufficient to determine whether the diploid cells were truly euploid or whether abnormal chromosome rearrangements were present, not affecting the total chromosome number (quasi-diploidy).

A subsequent chromosome study was made on cells of the fifteenth-passage level. In these preparations mitosis was rare, and reliable counts were obtained from only four cells, of which three contained 46 chromosomes and one contained 92 chromosomes. In each of these three diploid cells, five small acrocentrics could be distinguished, and the cultured cells, therefore, could be recognized as being of male origin. In the tetraploid cell a well illustrated endoreduplication with 46 pairs of chromosomes, or a total of 92 chromosomes, was observed. The number of small acrocentrics in that cell (10) was also consistent with the male origin of the cells.

The frequent observation of cells with lobated and multiple nuclei and the great variation in nuclear size indicate a greater variation in chromosome number than that encountered from the counts. It is probable that polyploid or even heteroploid cells were present in a greater proportion than apparent from the data, since such cells may divide much less frequently than diploid cells.

Attempts to produce tumors in heterologous and homologous hosts.—To further test for possible malignant characteristics of the cultured cells, newborn Syrian hamsters and terminal human cancer patients were selected as the recipients most likely to develop tumors after inoculations of these cells. The amounts of available cells limited these inoculation experiments to one attempt with twelve recipients most likely to develop tumors after inoculations. The survival of the cells through multiple serial passages made possible their detailed characterization. During the long period of cultivation no evidence of morphologic change has been observed. This appears to exclude in vitro transformation, and the different characterization studies, which, owing to the low rate of division, had to be performed at many passage levels, are

**TABLE 2**

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<tr>
<th>No. cells</th>
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<td>1</td>
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<td>49</td>
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<td>1</td>
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...swelling was too small for biopsy, and the attempt with this patient was therefore considered negative. The third trial resulted in the development of a palpable nodule in one of two sites of inoculation of the cultured cells on the forearm of a 33-year-old female suffering from adenocarcinoma of the cervix. The nodule was excised 15 days after inoculation, and microscopic sections of the biopsy failed to reveal tumor cells; however, a number of histiocytes were seen at the inoculation site. A simultaneous injection into this patient of H.Ep. #2 cells resulted in a larger swelling.

From the excised piece of tissue, cultures were established in our laboratory. Cells of these cultures showed some resemblance to the originally cultured cells (Fig. 13), and serial transfers were possible. A chromosome analysis of the recultured cells showed them to be of diploid chromosome number, but of female sex. It may, therefore, be inferred that the latter cells originated from the recipient of the experimental inoculation, since this patient was female, whereas the African patient was male.

Attempts to produce changes in various types of tissue culture (amnion cells, primary cultured or of the FL line, cells of Wistar diploid human embryonic lung strain WI 26, the RECL-1 and RECL-6 strain of rat embryo cells [13], and primary mouse embryo cells) all gave negative results. No positive results were obtained by inoculating newborn hamsters or chicken chorioallantoic membrane, either directly with lymphoma culture supernatant or with supernatants from "blind passages" in the other types of cells.

Electron microscopy.—Electron micrographs of the cultured lymphoma cells (Figs. 14-16) showed irregular cell surfaces, rather large and irregular nuclei, and nucleoli appearing as irregular, coarse, granular, or filamentous masses (Fig. 14). In the cytoplasm of many cells, the endoplasmic reticulum was prominent (Fig. 15); the associated RNP granules appeared to be of normal size and density. Lysosomes and lipid bodies were frequently seen throughout the cytoplasm (Fig. 16).

Several different types of small vacuoles or granules were observed in the cytoplasm, but no structures of definite viral morphology were found either in the cells or in the intercellular spaces.

**DISCUSSION**

The above represents the first reported cultivation of cells from the malignant lymphoma of children in Africa. The survival of the cells through multiple serial passages has made possible their detailed characterization. During the long period of cultivation no evidence of morphologic change, preferential outgrowth of a specific cell type, or chromosome change has been observed. This appears to exclude in vitro transformation, and the different characterization studies, which, owing to the low rate of division, had to be performed at many passage levels, are

2 Fragments of two additional lymphomas were sent to The South African Institute for Medical Research and were cultivated by J. H. S. Gear and J. M. Spence. One yielded cells which, in their opinion, resembled fibroblasts. One of us (G. D.) was privileged to examine these cells and found them more uniform and lacking certain features of our cells.
therefore considered comparable. Further characterization of this cell strain does not appear to be possible, since cell division has slowed markedly during the past few weeks.

So far no evidence has been obtained to prove the malignant nature of the cultivated cells. There was no morphologic evidence of the presence of virus on electron microscopic examination.

The cultured cells had in their cytoplasm large and small eosinophilic granules which contained polysaccharide but appeared not to contain DNA and RNA. These granules could have arisen endogenously. However, some of the eosinophilic bodies were surrounded by halos, and, since the cultures were characterized by a large amount of eosinophilic extracellular debris, it is also possible that some of this material could have been phagocytized. Although the presence of phagocytic activity in these cells has not been conclusively established, movements in time-lapse cinematography suggestive of activity in these cells has not been conclusively established, movements in time-lapse cinematography suggestive of phagocytosis have been observed. It should also be pointed out that acid phosphatase is often seen in the cytoplasm of macrophages which are fully capable of phagocytosis, and, as mentioned above, this enzyme was histochemically demonstrable in the cytoplasm of the cultured lymphoma cells.

The cultured cells also contained micronuclei. It is known that x-ray treatment of cultured cell strains can produce similar structures (6) and that occasionally non-irradiated cells have shown a tendency to produce micronuclei (13) when carried in continuous culture.

Histologic examination of the malignant lymphoma from which the cells were cultivated revealed two types of parenchymal cells. The predominating one was apparently lymphoblastic, but numerous scattered histiocytes were also seen. The cultured cells showed several features associated with histiocytes—i.e., abundant cytoplasm, vigorous cytoplasmic motility, apparent phagocytosis, and lysosomes. These are features which are usually not seen in cells of the lymphocytic series, nor are they characteristic of cultures of fibroblasts. Acid phosphatase and nonspecific esterase have been shown histochemically to be present in the cultivated cells and in cells that appeared to be benign histiocytes within the parent tumor. Since the predominating tumor cell did not appear to contain either of these enzymes, it is more likely that the cultured cells arose from histiocytes present in the tumor rather than from the tumor cells per se (2).

The role of these histiocytic cells, considered to be benign by O'Connor (11) and Wright (17), remains obscure. Their characteristic presence in the tumors may indicate that they play an important part in the tumor process.

We believe that these results demonstrate the importance of caution in drawing conclusions about the origin of cells cultivated from malignant tissues.

ACKNOWLEDGMENTS

For help in supplying human serum for these studies, we are indebted to the American Red Cross.

REFERENCES

FIG. 3.—Series of T-15 flasks demonstrating variation in appearance of the cell sheet with changes in serum content in the culture medium. Concentrations of fetal bovine and human serum in per cent for the different cultures, from bottom to top, was as follows (fetal bovine/human): 25/10; 30/5; 35/0; 15/15; 10/25; 15/0; 10/20; 5/30; 5/20; 0/20.

FIG. 4.—Hematoxylin and eosin stain of cultured lymphoma cells growing on a coverslip. Note the irregular cell shapes and abundant cytoplasm which extends from the nucleus to form intertwining stellate processes. Also darkly staining granular extracellular debris is present. Mag. ×340.

FIG. 5.—Feulgen stain of cultivated cells showing marked nuclear lobulation. Mag. ×1085.
Fig. 6.—Periodic acid-Schiff stain of cultivated lymphoma cells showing positively staining large intracytoplasmic granules, surrounded by halos, and also small intracytoplasmic granules. Mag. X850.

Fig. 7.—Micronuclei are seen in the cultivated cell in the center, stained with hematoxylin and eosin. Mag. X1350.

Fig. 8.—Gomori preparation for acid phosphatase demonstrating considerable enzyme activity, represented by black intracytoplasmic granules of lead sulphide, in the cultivated lymphoma cells. Mag. X850.

Fig. 9.—Low-power view of the lymphoma from which the cultivated cells originated shows predominating lymphoblastic cells with interspersed histiocytes creating a “starry sky” appearance (hematoxylin and eosin stain). Mag. X212.
Fig. 10.—Acid phosphatase stain of the parent tumor shows enzyme activity restricted to the darkly staining histiocytes. Mag. ×270.

Fig. 11.—Darkly staining, nonspecific esterase also appears to be exclusively in the histiocytes. Mag. ×270.

Fig. 12.—Typical metaphase plate with 46 chromosomes from cultured lymphoma cells. Mag. ×2120.
Fig. 13.—Cells cultured from biopsy of nodule resulting from injection of the cultured lymphoma cells into a terminal human cancer patient. Mag. $\times 340$.

Fig. 14.—Electron micrographs of thin sections of cultured lymphoma cells. Mag. $\times 18,000$. Large nucleus with lobes and incisions containing rather large, coarse, granulated nucleoli. Cluster of small vesicles at the cell border.
Fig. 15, 16.—Electron micrographs of thin sections of cultured lymphoma cells. Mag. X18,000.
Fig. 15.—Perinuclear concentric layers of rough endoplasmic reticulum tubules.
Fig. 16.—Lysosomes (ly), small vesicles, and unidentified membrane-limited, fine, granular body in the perinuclear area (b).
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