Tumor Production in X-Ray- and Cortisone-treated Rats Given Injections of Human Cells in Continuous Cultivation*

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Toolan's (10) technics allowing growth of tumors in x-ray- and cortisone-treated animals opened a new field for the study of human cells grown in heterologous hosts. Toolan and other investigators (8, 9, 11) extended this work to the study of tissue culture-grown human cells of both malignant and normal origin.

A technic similar to the one described by Suskind et al. (9) was applied in our laboratories as part of a study of the FL strain of cells (4) derived from normal human amniotic membrane. This report presents data in which the FL strain is compared with other cell strains of malignant or normal tissue origin maintained in continuous culture. Primary tissue (amnion) has been studied by the same technic.

MATERIALS AND METHODS

Cell strains.—The following human cell strains in continuous cultivation were examined:

1. Cells of malignant origin. This was the strain of HeLa cells (6) derived from a cervical carcinoma (our strain HeLa2).
2. Cell strains derived from nonmalignant tissues.
   a. Chang's cells (3) derived from a conjunctival membrane (designated Chang).
   b. FL cells (4) derived from an amniotic membrane.
3. Four strains of cells characterized as questionable with respect to malignant or nonmalignant origin:
   a. Detroit-6 cells (2) derived from presumably normal sternal bone marrow of a patient with carcinoma of the lung (designated D6).
   c. MF cells, isolated December, 1955.

The AH, MF, and 14-87 cell strains, which have not been previously described, were established (by J. Fogh and R. Backus) in the following manner. The primary amnion cells were grown in 5-ml. cylindrical glass cells filled with nutrient medium. A millipore filter (type HA, Millipore Filter Corp.), rimmed with wax, was placed on top of the container with a drop of suspended HeLa cells (approximately 200,000 cells) in the center. Thus, the HeLa cells came in contact with the amnion culture medium through the moistened filter. Placed in a tall petri plate covered by glass, the whole was incubated at 37° C. in a 5 per cent CO2 atmosphere for 48 hours. Following trypsinization and transfer of the amnion cells to new containers, these cells have subsequently been maintained in continuous culture. The AH and MF strains were derived from exposed primary amnion cells, and the 14-87 strain from similarly treated cells of the FL strain.

The filters were found to retain a suspension of Escherichia coli, strain B. It is extremely unlikely that the HeLa cells could migrate either through or around the edges of the filter, which extended 12 mm. beyond the glass. Microscopy and cultivation experiments failed to reveal cells on the lower side of the filter; also, no cells were observed at an appreciable distance from the area of the drop placed on the upper side of the filter.

Preparation of cells.—Normal human amniotic cells were prepared in suspension in one of two ways: (a) the membrane was trypsinized and the cells were resuspended in medium for direct injection into animals; (b) the cells were grown in primary culture before they were suspended for injection. The latter cultures were grown in Earle's balanced salt solution containing 20 per cent ox serum.

The LY-medium (4) was used for propagation of all the cell strains that were maintained in continuous culture, with the exception of the D6 cells, which were grown in medium 199; all the media contained 20 per cent human serum. When rat serum was used in cultures of D6 cells, the serum was obtained by heart puncture of mature rats. For inoculation into animals, cells were grown in Povitsky bottles for 2-3 days. After decanting, the scraped cells were dispersed by gentle agitation to facilitate counting. The suspension was then centrifuged, and the cells were resuspended in their growth medium to the desired concentration. Maintenance of cultures and methods for counting have been described (4).

Preparation of animals.—Unless otherwise specified, Long-Evans weanling rats of both sexes, weighing 40-60 gm., were used. They were irradiated from a Phillips "Deep Therapy
Apparatus. Type 11645, with beryllium window and inherent filtration = 3 mm. Al. It was operated at 245 kv and 15 ma. filtered with 0.6 mm. Cu and 1.0 mm. Al; the tube distance was 65–70 cm. The dose was recorded with a Victoreen r-meter. Ten rats at a time were placed in a rotating stage covered with a Lucite lid. The actual total-body dose was 400 r. On the 1st and 3d days after irradiation, each rat received 5.0 mg. cortisone acetate (Sharpe and Dohme) subcutaneously and 0.1 ml. "Combiotic" (Pfizer) intramuscularly. On the 4th day, the cell suspension was injected intraperitoneally, each rat receiving 6–10 million cells in 1 or 2 ml of medium. Another injection of "Combiotic" was given 3–4 days after cell implantation. Under these conditions, approximately 25 per cent of animals died within the first 10 days.

Growth of tumors in the animals was estimated by gentle bimanual, abdominal palpation. In experiments in which measurements were of importance, the animals were starved overnight and palpated under ether anesthesia. During the first 10 days, they were usually left undisturbed. Those that died were examined for the presence or absence of tumors. All cages were steam-sterilized after use.

For surgical procedures, the abdomen was shaved, and the animals were anesthetized with ether. With aseptic technic, a median incision was made exposing the tumor and the abdominal viscera. This exposure was found useful for direct examination of tumors at various stages and for direct inoculation of virus suspensions into tumors, as shown in Figure 1. There was almost no operative mortality.

To re-establish the cells in tissue culture, the tumor was cut into small pieces and treated with 0.1 per cent trypsin solution. The cells were then resuspended in the growth medium and planted in the usual manner.

RESULTS

1. Growth of tumors was observed with cells of the FL strain and with all the other six cell types that were grown in continuous culture. Although earlier passages of the FL strain failed to produce tumors (4), successful tumors were obtained after 11 months in culture with the 33d and 35th passages, February, 1957. The other strains, when examined for tumor growth, had been in continuous culture for the following approximate periods: HeLa, 5 years; Chang, 3 years; D6, 2½ years; AH and MF, 1 year; 14–87, ½ year.

2. Primary amnion cells failed to develop into tumors. In three different experiments, a total of 40 rats, 24 Long-Evans and sixteen Sprague-Dawley, were given injections of primary amnion cells. In this set of experiments, an increased number of cells was injected: fourteen Long-Evans rats received 20 million cells each, ten Long-Evans rats received 30 million cells, and sixteen Sprague-Dawley rats received 25 million cells. The cells were pooled from several amnion membranes. In control groups injected with HeLa, D6, and FL cells, tumor development was observed with the usual frequency.

3. The incidence of tumors (defined as the percentage of those animals which had produced tumors and had survived beyond the 10th day) is shown in Table 1. It was highest for the HeLa (56 per cent) and D6 cells (54 per cent); the FL cells showed a distinctly lower response (27 per cent). The four remaining cell types were examined less extensively.

4. The sizes of tumors varied at any one given time (Chart 1). The variation in tumor develop-

### Table 1

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No. Experiments</th>
<th>No. Animals inoculated</th>
<th>Died before 10th day</th>
<th>Died on killed after 10th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>9</td>
<td>89</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>D6</td>
<td>10</td>
<td>120</td>
<td>49</td>
<td>33</td>
</tr>
<tr>
<td>FL</td>
<td>11</td>
<td>140</td>
<td>45</td>
<td>69</td>
</tr>
<tr>
<td>AH</td>
<td>5</td>
<td>36</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>MP</td>
<td>2</td>
<td>13</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>14–87</td>
<td>1</td>
<td>12</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Chang</td>
<td>2</td>
<td>13</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

From data compiled in Chart 2, it is apparent that at any one day the tumor sizes fall into a wide range. At the 11th and 13th days all tumors were relatively small. The very large tumors were observed on the 17th and 19th days, with a tendency toward a decrease in mean size on the 19th day.

Although the incidence of the largest tumors was highest with HeLa cells, the D6 and occasionally the FL tumors grew to similar sizes.

5. The gross appearance of tumors showed considerable variability and no relation to cell type.
On the whole, tumor growth was confined to one place, near the greater curvature of the stomach; many of the tumors were recognized as being in the omentum. Most of the tumors tended to stretch their attachments into one or several slender pedicles, thus becoming more or less pendulous in the peritoneal cavity and readily mov-

able. Some tumors appeared to be more intimately connected to the mesentery. The tumors were rounded or lobulated, of soft to rubbery consisten-

cy when growing progressively, but showing an increasingly firm consistency with advancing stages of regression. Some were solitary, but many were multinodular. The color was usually greyish white, although varying from dark red to pearly white and accompanied by various amounts of vascularization. In an occasional animal, and in some experiments in a large number of animals, a considera-

ble amount of peritoneal fluid, sometimes hemorrhagic, was noted. None of these characteristics could be related to age and size of tumor, or to cell type.

6. Regression of tumors is exemplified in Chart 1. A number of animals in all three groups, particularly in the FL group, showed tumors that could definitely be established by palpation. As may be seen in Chart 1, these tumors decreased in

size after a certain period of time (17–25 days), and after 40 days they were no longer palpable. In several animals, the disappearance of tumors was confirmed by autopsy. This tendency toward disappearance of tumors was most pronounced in the FL group. Several of the animals survived for 3–4 months after completion of the experiment; none of them showed signs of tumor at autopsy. In our experience, the tumors in animals surviving beyond the 1st month after cell injection diminished in size. This was true of all the cell strains studied.

7. No metastasis has been observed. In a number of animals, the lungs, liver, kidneys, spleen, heart, and the tissues adjacent to the tumor were examined microscopically (5).

In a limited number of animals, HeLa tumors were surgically removed. The animals were main-
tained on cortisone and observed for periods of weeks to months. Examination of animals that were later sacrificed failed to reveal recurrence of tumor growth. Again, there was no evidence of metastasis.

8. Cells derived from tumors could be established in tissue culture and showed characteristics of the original cell strain. Stained coverslip preparations of cells derived from the D6 and FL tumors were indistinguishable from the respective stock cultures. Primary tumor cultures contained remnants of fibroblasts, but these did not grow and disappeared completely upon subculture. The D6 tumor-derived cells gave growth in rat serum comparable to growth in human and calf serum. They were maintained in rat serum through 30 subcultures.

Attempts to establish D6 tumors in cortisone-treated, nonirradiated rats.—The D6 cell strain was chosen for this experiment. The source of cells for implantation consisted of: (a) stock tissue cultures, (b) D6 tumors of varying ages, minced or trypsinized, and (c) cells derived from tumors and subsequently grown in tissue culture (for three to 29 passages). The cellular material was implanted either subcutaneously or intraperitoneally. The animals received several injections of cortisone before and after implantation.

Results from 36 attempts to establish tumors were almost entirely negative. Temporary growth of palpable tumors occurred in four rats (two injected subcutaneously and two intraperitoneally). Sections from one such tumor revealed small pockets of typical D6 tumor cells, suggesting that the four tumors were not due to irritation at the site of implantation. In each of the four rats, the implanted material was derived from tumors previously established in cortisonized and x-irradiated rats; no signs of tumor formation were observed when stock tissue culture cells were injected. Attempts to transfer three of these tumors into other cortizonized rats were unsuccessful.

DISCUSSION

Moore, Southam, and Sternberg (8) showed that Chang's liver, conjunctiva, and kidney cells (8), derived from normal human tissues and grown in continuous cultivation, produced tumors when injected into treated rats. The cells had been grown in culture for more than 50 passages at the time of examination. We confirmed the tumor-producing ability of Chang's conjunctiva cells.

When first examined (4), FL cells, originating from normal human amnion cells, did not produce tumors after intraperitoneal injection in their 23d and 25th transfers under conditions in which HeLa cells produced significant tumors. This observation might possibly be explained by a less adequate conditioning technic in our preliminary experiments, although there is the possibility that earlier transfers might not have had the tumor-producing capacity as did the later transfers. These findings clearly demonstrated a difference in the tumor-producing ability of HeLa and FL cells. The first tumors were observed in the 33d transfer of the FL strain. In later experiments in which HeLa cells showed a higher tumor incidence than occurred in the preliminary experiments, the differences between groups of rats injected with HeLa and FL cells were noted as being differences in incidence and tumor sizes.

Since attempts to reproduce such effects with primary amnion cells were completely negative, the FL cells can no longer be characterized as normal cells. This is in agreement with the opinion of Moore and coworkers (7, 8) based on their observation of Chang's cells. Other studies (1, 5) support this statement regarding FL cells.

Strains AH, MF, and 14-87, all derived from amnion cells, have periodically shown a high incidence of tumors. They have not been examined to the extent necessary to show significant differences.

Since no tumors were produced in untreated animals by cells that grew into tumors in treated animals, the treated rats could be considered as a "conditioned medium." Cells would or would not grow into tumors, depending upon the variation in the conditions of the host and the injected cells. Both methods of conditioning—x-radiation and cortisone treatment—were necessary for successful tumor growth. Toolan (10) reported success in obtaining tumors with neoplastic human biopsy material in cortisonized animals. We found that unirradiated, cortisonized animals retained their defense mechanism to the extent that growth of the heterologous cell strains into tumors was almost entirely excluded. Even a 10 per cent decrease in the dose of x-ray markedly reduced tumor appearance in cortisonized animals. X-ray treatment without cortisone treatment was studied only to a minor extent, but tumors were not observed under such conditions.

The exact mechanism of the effects of x-rays and cortisone is unknown; the suppression they cause appears to be only temporary, at least under our conditions. Regression of the tumors was noted with each tissue culture strain, although with varying incidence. It has been observed that tumors could be produced only in young animals, which indicates that a maturation process is possibly concerned with resistance. Thus, it is conceiv-
able that one of the explanations for regression would be concerned with this maturation process. Inasmuch as, on occasion, sera from tumor-bearing animals were toxic for tissues in vitro, it is possible that an immunological mechanism is involved. When D6 cells were grown in tissue culture in media containing 5–10 per cent serum from tumor-bearing rats, the growth was minimal, and the cells became necrotic within 24 hours. No such effects were seen in control cells grown in normal rat serum; the cells remained normal in appearance, both initially and during subsequent passages. It would seem that tumor-bearing animals had acquired an immunity against the tumor cells, which was not present in tumor-free animals. It is logical to assume that this immunity was part of the rebuilt defense mechanism against further tumor growth.

Whether or not a tumor will result from the cells injected into the animals, a tumor will grow and later regress, or a tumor will continue to grow and to develop into a full picture of cancer with invasion, and metastases must, therefore, depend upon the "condition" of the cells and the "condition" of the host. The role of the cell was demonstrated by the fact that primary amnion cells failed to produce tumors, whereas strains derived from primary amnion cells acquired the tumor-producing capacity.

Upon macroscopic examination, it occasionally appeared that the tumor was invading the surrounding tissues, for example, the liver. This behavior could not be characterized as invasion by microscopic examination of sections (5). The usual observation was to find the tumor as one mass, and very often located at the same place intra-abdominally. At present, no reasonable explanation is given or can be given for this fact. With 6 to 10 million cells injected intraperitoneally, one could expect growth to be spread over the peritoneal cavity. Further exploration is needed to determine if a lower resistance favors tumor growth in a particular location or if a single cell or an agglutinate of cells initiate the growth. Studies on the early fate of the injected cells and on the effects on tumor production of a variation in the number of cells inoculated should elucidate this problem.

It is possible that certain differences in our procedures, compared with those reported by Suskind et al. (9), may explain the extended period necessary for tumor development and the overall lower incidence for the HeLa strain. No technique, however, can be considered optimal, when an inoculum of 6 million cells or more is necessary to initiate tumor growth. Attempts should be made to establish conditions under which tumors could be produced from fewer cells and even from single cells.

The principal purposes of the present study were to compare the tumor-producing ability of the FL strain with the abilities of the six other cell strains to produce tumors for comparative histological and cytological studies of the seven strains, and to evaluate their malignant potencies (5). A larger number of tumors than is here presented has been analyzed (for instance, 156 tumors of the D6 cell type), but in this report only results from experiments with parallel conditions are presented.

Two main points stand out as a result of our studies: (a) The tumors produced by all the seven examined cell strains were macroscopically indistinguishable, although differences in incidence and in size related to time were indicated. (b) A great deal of variation (time of death and number of animals which died, incidence, tumor size and appearance, etc.) has been observed from one experiment to another, in spite of attempts to maintain parallel conditions. This would make statistical analysis of quantitative differences difficult.

SUMMARY

Data are presented on tumor production in x-ray- and cortisone-treated rats (strain Long-Evans) by seven tissue-cultured strains of human cells in continuous cultivation. The FL strain, derived from a normal human amnion, produced tumors when examined in its third transfer. Incidence, tumor size, and appearance are reported and discussed for the seven strains, of which one (HeLa) was of malignant origin, two (FL and Chang's conjunctiva) were of normal origin, and four strains (Detroit-6, AH, MF, and 14-87) are characterized as questionable in reference to malignant or normal origin. Regression of tumors has been observed for all the strains. No metastases were seen. Cells derived from tumors could be established in tissue culture and showed characteristics of the original cells. Attempts to establish tumors in nonirradiated rats were negative. Primary cells from the normal human amniotic membrane, whether isolated directly or grown in culture in one passage, failed to support tumor formation. The technic was sensitive but showed a great deal of variation. The data are part of a study of human cells in continuous cultivation.

ACKNOWLEDGMENTS

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FIG. 1.—Tumor derived from FL cells, 17 days after implantation
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