Cell Culture in vivo

I. Growth of L-Fibroblast and Sarcoma 180 Cell Lines in Diffusion Chambers in vivo*

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In vivo cell culture has been known for several years. The earliest attempts were made by Rezzesi (18) and Biscieglie (6). These investigators reported that Ehrlich carcinoma of mice survived for 12 days in the peritoneal cavity of guinea pigs when cultured in collodion dialysis sacs. More recently Prehn, Weaver, and Algire (16, 17) introduced the diffusion chamber technic using Millipore Filters for the porous membranes of the chamber. These diffusion chambers separate the cell culture from host cells but provide free diffusion of all nutrient materials, including protein, needed for growth. In such a system the cells in culture should be subject to all the noncellular growth-controlling influences of the host.

Since 1954 many reports have appeared on the use of these technics to culture cells in vivo (1-5, 12, 15, 21, 22). The methods used by these investigators (except [5]), although useful, did not permit quantitative measurement of cell growth; growth was demonstrated by the presence of mitotic indices in stained preparations and by gross appearance. In general, the inoculum sizes were large (perhaps exceeding the capacity of the chamber to support growth) or consisted of small bits of tissue containing unknown numbers of cells. This report is intended to present methods for quantitative measurement of cell growth in vivo and to demonstrate the type of growth curves obtained by such a method. The cell lines chosen for the initial experiments were the L-fibroblast (originally derived from normal connective tissue of the CSH mouse) and the Sarcoma 180, a malignant cell line of mesodermal origin.

MATERIALS AND METHODS

The diffusion chamber.—L-Fibroblasts and Sarcoma 180 cells in culture were found to adhere strongly to the Millipore Filter (M.F.) membranes of the chamber. Quantitative removal of these cells for analysis was not possible. Therefore, it was necessary to subject the whole chamber system to analysis. Host cells were also found to adhere strongly to the M.F. membranes of diffusion chambers in vivo. Their easy removal from the chamber system prior to analysis was accomplished by the chamber design shown in Chart 1, the three-compartment diffusion chamber (see also Experimental Procedure).

Many adhesives were tested for sealing the M.F. membranes to various materials used for constructing the ring. Of the many combinations tested (not listed here) the most satisfactory with respect to producing a cell-tight chamber was constructed from Lucite and HA Millipore Filters sealed with a combination of adhesives. The adhesives were 1 per cent Lucite in acetone and Acryloid B-7. Diffusion chambers were assembled as follows:

a) The inner seal (see Chart 1): The inner M.F. membrane was fastened with a 1 per cent Lucite in acetone mixture and allowed to dry. A coating of Acryloid B-7 resin was then applied to strengthen the seal.

b) The outer seal (see Chart 1): At the same time Acryloid B-7 was applied to the inner seal it was applied to the Lucite ring in the region of the outer seal, and the outer M.F. membrane was fixed in place. After 1 hour was allowed for drying, the 1 per cent Lucite in acetone mixture was applied to the outer edge of the M.F. membrane until the seal became transparent. After

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1 Direct counting methods, applicable only to ascites cells, provided they grow in free suspension within the diffusion chamber, were used by these authors.
drying, the outer seal was given a final coat of Acryloid B-7 to add strength and durability.

Sterilization procedure.—Sterilization of diffusion chambers was carried out by heating (dry) at 77°–79° C. for 72 hours. This treatment produced satisfactory sterilization without any ill effects on the chamber (the distortion point of Lucite is 80° C.) or danger of residual toxicity. Autoclaving distorted the chambers so that they were no longer cell-tight. Gassing with ethylene oxide was found to leave a residual toxicity associated with the Lucite component of the chamber when tested in vitro.

Chemical analysis of cell growth.—Various chemical methods were tested to determine growth (cell number). Direct counting methods were not feasible, because the cells to be studied could not be removed quantitatively from the M.F. membranes of the chamber. An assay for the enzyme lactic dehydrogenase (LDH) (14) met the requirements of sensitivity² and provided a low blank for the chamber components which must be subjected to the chemical procedure.

The level of enzyme activity was determined by measuring the rate of formation of reduced DPN by the following DPN-linked reaction:

\[
\text{LDH} \\
\text{Lactate} \rightarrow \text{pyruvate} \\
\text{DPN} \rightarrow \text{DPNH}
\]

DPNH was determined fluorometrically.⁴ For samples containing less than 50,000 cells, the following incubation medium was found satisfactory: 5.00 ml. (0.1 M) glycine buffer, pH = 9.8; 0.30 ml. (5.6 M) lactate, pH = 8.5; 0.20 ml. (0.3 M) DPN. For larger numbers of cells the capacity of the incubation medium was increased by in-

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³ Low inoculum sizes seemed desirable if diffusion of nutrient materials through the chamber was not to be the growth-limiting factor.

⁴ A Farrand Fluorometer Model A was used in this laboratory. The primary filter was Corning Glass Nos. 5860 and 5970; the secondary filter was comprised of Corning Glass Nos. 4308, 5562, and 3389.
formed per minute vs. number of cells were determined for the different cell lines under investigation (Chart 2).

The LDH per cell for cell lines grown in different environments (Table 1) and sampled at various phases of growth (Table 2) was shown to be relatively constant as compared with the 20- to 100-fold increases measured for growth experiments.

Staining procedure.—M.F. membranes were stained with hematoxylin and eosin for direct visualization of the cell culture.

Cell lines.—

a) L-Fibroblasts were derived from clone 929, originally isolated by Sanford et al. (19). These cells were maintained for the past 18 months on the defined medium described by Eagle (7) supplemented with 10 per cent beef serum.

b) An in vitro culture of Sarcoma 180 cells was obtained from Foley (10) and was originally derived from a sarcoma carried in mice for many years. This cell line was maintained on the medium described above for L-fibroblasts since March, 1958.

Animals.—Male white mice of the Swiss-Webster strain, weighing 15–25 gm., were used for all diffusion chamber experiments. Male C3H/Cgri mice weighing 15–25 gm. were used in some experiments to test the tumor-producing capacity of L-fibroblasts and S-180 cells.

Experimental procedure.—Cells were suspended in normal nutrient medium, and the concentration was determined by counting in a hemocytometer (±5 per cent). The suspension was then diluted with nutrient medium so that a 0.2-ml. aliquot contained the desired number of cells for inoculation of each chamber. 0.20 ml. of such a suspension was placed in the inner compartment of the diffusion chamber (sterile technic) through the small hole in the ring (see Chart 1), the hole sealed by touching the surrounding area with a hot glass rod, and the chamber inserted into the peritoneal cavity of a mouse (ether anesthesia, ventral mid-line incision, sterile technic). On removal of the diffusion chamber from the animal, the outer M.F. membranes were cut off and discarded. The rest of the chamber was washed with saline and carefully wiped dry. The outer surface of the Lucite ring was then coated with a 1 per cent Lucite in acetone mixture to prevent host cell contamination from this component. The inner membranes (free of host cells) were then removed.

Table 1

<table>
<thead>
<tr>
<th>Medium (6 days)</th>
<th>Total cell number X 10^6 (day 6)</th>
<th>DPNH (moles/min/cell X 10^-15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% beef serum</td>
<td>1.6</td>
<td>7.6</td>
</tr>
<tr>
<td>10% beef serum, heated (68°C for 30 min.)</td>
<td>1.0</td>
<td>6.7</td>
</tr>
<tr>
<td>10% ascites fluid</td>
<td>1.1</td>
<td>8.1</td>
</tr>
<tr>
<td>5% ascites fluid</td>
<td>1.2</td>
<td>6.6</td>
</tr>
<tr>
<td>0.5% baetopeptone</td>
<td>1.6</td>
<td>6.8</td>
</tr>
</tbody>
</table>

L-Fibroblasts were grown from an inoculum of 1 X 10^6 cells in the basic nutrient medium described by Eagle, supplemented with protein as indicated above. After 6 days the cells were washed thoroughly while attached to the glass and then scraped into a small volume of saline. Cells from each source were assayed for LDH activity in duplicate and the results reported in terms of moles DPNH formed per cell per minute. Duplicate results for cells from the same culture agreed within ±2 per cent (note that more variation was encountered when cells from different cultures were compared, even though the cultures were maintained under the same conditions. See Table 2).

1 Obtained from Diablo Animal Laboratories, 1390 8d Street, Berkeley 10, Calif.
2 Obtained from Cancer Genetics Laboratory, University of California, Berkeley, Calif.
washed thoroughly (gentle shaking in 20 ml. buffered saline for 30 minutes), and together with the Lucite ring subjected to analysis for lactic dehydrogenase activity. Such treatment was shown not to alter the LDH content of cells. The fluid contents of the chamber were collected and added to the washings of the inner M.F. membranes. The washings were then centrifuged (2000 r.p.m. for 15 minutes) to collect any cells which might have separated from the M.F. membrane. The few cells so obtained were washed again with saline and transferred quantitatively to the incubation flask for analysis. With the above washing procedure, control chambers (no cells) gave low blanks (see Table 3).

At least one chamber from the group removed at each interval was set aside for staining. The inner M.F. membranes from such chambers were stained directly without being subjected to the washing procedure or LDH determination. The stained preparations were scanned carefully to detect host cells (host cells are much smaller than either of the S-180 or the L-fibroblast and are more deeply stained). The presence of cells on the outside of the inner M.F. membrane indicated a defective chamber. The stained preparation was also used for a gross determination of growth and to note the general condition of the cells under investigation.

**TABLE 2**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inoculum size</th>
<th>Days after inoculation of culture</th>
<th>LDH (moles/min/cell x 10^-13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA (in vivo)</td>
<td>1 x 10^7</td>
<td>3 (log phase)</td>
<td>6.5</td>
</tr>
<tr>
<td>EA (in vivo)</td>
<td>1 x 10^7</td>
<td>6 (log phase)</td>
<td>6.3</td>
</tr>
<tr>
<td>EA (in vivo)</td>
<td>1 x 10^7</td>
<td>9 (stationary phase)</td>
<td>6.2</td>
</tr>
<tr>
<td>EA (in vivo)</td>
<td>1 x 10^7</td>
<td>12 (stationary phase)</td>
<td>6.2</td>
</tr>
<tr>
<td>S-180 (in vitro)</td>
<td>1 x 10^6</td>
<td>3 (log phase)</td>
<td>8.6</td>
</tr>
<tr>
<td>S-180 (in vitro)</td>
<td>1 x 10^6</td>
<td>4 (log phase)</td>
<td>6.5</td>
</tr>
<tr>
<td>S-180 (in vitro)</td>
<td>1 x 10^6</td>
<td>6 (log phase)</td>
<td>10</td>
</tr>
<tr>
<td>S-180 (in vitro)</td>
<td>1 x 10^6</td>
<td>9 (stationary phase)</td>
<td>10</td>
</tr>
</tbody>
</table>

Ehrlich ascites cells from the clone originated by Gross and Furst (10) were used. Groups of mice were given inoculations of the standard inoculum of 1.0 x 10^7 EA cells at intervals of 3 days so that on the 12th day mice were available with 3-, 6-, 9-, and 12-day tumors. One mouse from each group was selected and a suspension of EA cells prepared. The suspensions were counted, diluted, and a known number of cells from each suspension was assayed for LDH activity. According to Gross and Furst (10) no increase in total cell number occurs later than the 8th day in these animals after inoculation of 1 x 10^7 cells.

S-180 cells were studied in vitro. Flasks were inoculated with 1 x 10^4 S-180 cells. Two such flasks were used as a source of cells for each analysis. The cells were washed thoroughly with saline while attached to the glass and scraped into a small volume of saline and combined. The cells so obtained were counted, diluted, and a known number subjected to LDH assay. Cell counts indicated no increase in cell number after day 6.

All M.F. membranes subjected to LDH analysis were stained after determination of enzyme activity, allowing an additional check for leaks on individual chambers.

**RESULTS**

In vitro.—Eighteen diffusion chambers were inoculated, each with 1 x 10^4 L-fibroblasts, and

Saline was buffered with 0.1 gm. Na_2PO_4 and 0.04 gm. NaH_2PO_4/l. This type of saline was used throughout these experiments.

The centrifuging of mammalian cells causes some loss of cellular protein including lactic dehydrogenase. Fortunately very few cells were found to be present in this fraction.
placed in 125-cc. Erlenmeyer flasks (three chambers per flask) containing 40 ml. nutrient medium. The flasks were placed on a rotary shaker at 120 r.p.m. in a constant-temperature room at 37°C. One such flask was removed every other day for 15 days. The LDH level for two chambers from each flask was determined separately; the third chamber was stained for visual examination. The nutrient medium was changed every other day for the duration of the experiment.

An identical experiment was carried out with Sarcoma 180 cells. The results of the LDH assays for the two experiments are shown in Chart 3. Approximately a 20- to 100-fold increase in LDH level of these cell cultures was noted over the 12-day period. Control chambers (no cells) had no enzyme activity. These results were confirmed by stained preparations which showed a concurrent increase in the density of the cell population (see Fig. 1).

**In vivo.**—Chambers were inoculated with L-fibroblasts or S-180 cells and implanted intraperitoneally in mice. The increase in LDH content of the individual L-fibroblast cultures with time is shown in Chart 4 for inocula of 5 x 10^5 and 1 x 10^4 cells. At the lower inoculum size some cultures did not grow—or perhaps got a poor start—as evidenced by the low points. The higher inoculum size gave more consistent results. Stained preparations confirmed growth (or no growth) in all cases. The apparent mean generation time was about 2½ days for both experiments.

The results for S-180 cells are shown in Chart 5. Stained preparations showed an increase in the density of the cell population confirming growth. The apparent mean generation time closely approximated that obtained for the L-fibroblast cultures, i.e., about 2½ days.

**Tumor-producing capacity of L-fibroblasts and S-180 cells.**—L-Fibroblasts and S-180 cells were injected into C3H and Swiss-Webster mice as indicated in Table 4. No tumors were detected within a 60-day period in either strain after inoculation with L-fibroblasts. In contrast, over 90 per cent of the mice receiving S-180 cells developed tumors. Subcutaneous tumors were usually detected within 1–2 weeks. Intraperitoneal tumors were detected on autopsy (death usually occurring about 30 days after inoculation with S-180 cells).

**DISCUSSION**

By several criteria such as morphology and rate of growth, cell lines derived from normal tissue are similar to cell lines derived from malignant tissue when compared *in vitro*. One conceivable hypothesis to explain these observations is that
the cells derived from normal tissue when placed in vitro have escaped from the normal physiological control limiting cell division. Conceivably when placed back in vivo they might respond again to this growth-controlling factor(s).

A comparison of growth curves obtained for the L-fibroblast and Sarcoma 180 cell lines in the in vivo diffusion chamber system (Charts 4 and 5) shows the apparent mean generation time to be approximately the same for both cell lines (about 89 days). However, the L-fibroblast cell line used in this laboratory (derived from clone L-929 and originally isolated from normal connective tissue of a C3H mouse) does not produce tumors when injected subcutaneously or intraperitoneally in either Swiss-Webster or C3H mice, whereas S-180 cells produce tumors in over 90 per cent of such animals (Table 4).

Therefore, if one assumes that the L-fibroblast is able to respond to a physiological control limiting cell division when injected directly, it can be concluded that such a factor is associated with cells and excluded from the diffusion chamber.

Another possible interpretation of the data could be that the L-fibroblast is no longer capable of responding to such a growth-inhibiting factor.

In this case, the fact that L-fibroblasts do not grow to produce tumors when injected subcutaneously or intraperitoneally may be explained by invoking the idea of an immunological interaction. It is well known that transplantable tumors are usually strain-specific and often subline-specific; the C3H subline used in these experiments is different from the C3H/An subline from which the L-fibroblast was derived. Further, Algire et al. (4) have reported that such a mechanism requires cellular interaction and is excluded from the diffusion chamber. It should be noted, however, that recent evidence (11) indicates that strain-specificity may be lost after in vitro culture.

Because of the long in vitro history of the L-fibroblast, which includes periods during which these cells were known to produce tumors (8), and the more recent experiments of Sanford et al. (90), showing some incidence of tumor production under conditions which reduce host resistance, the L-fibroblast may no longer be representative of cell lines immediately derived from normal tissue.

**TABLE 4**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Strain</th>
<th>No. mice</th>
<th>Inoculum</th>
<th>No. mice with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-180</td>
<td>Swiss-Webster C3H</td>
<td>30</td>
<td>0.5-8.5 x 10^6</td>
<td>26</td>
</tr>
<tr>
<td>L-Fibroblast</td>
<td>Swiss-Webster C3H</td>
<td>32</td>
<td>1-2 x 10^6</td>
<td>0</td>
</tr>
</tbody>
</table>

L-Fibroblasts and S-180 cells from in vitro culture were washed, suspended in saline, and injected into adult mice.

Perhaps a better choice for a cell line derived from normal tissue would be one which was derived from a highly inbred strain and with a very short or no in vitro history. Such experiments are now under way in this laboratory.

The advantages of the three-compartment diffusion chamber are worthy of emphasis. These include: (a) a method for separating host cells from the chamber system containing the experimental cell culture, making analysis for growth possible; (b) means for inoculation of a sterile culture into a completely assembled, sterile chamber; and (c) a method of checking each individual chamber of an experiment for cell leakage. This last advantage is particularly important when chambers are left in vivo for long periods.
SUMMARY
A leakproof diffusion chamber capable of supporting growth of L-fibroblasts and S-180 cells in vitro and in vivo (Swiss-Webster mice) has been described. Evidence has been presented indicating that the LDH level of a cell culture may be used as a measure of cell number for a growing population of a homogeneous cell culture.

With the LDH activity used as a measure of cell number, growth curves were obtained for the L-fibroblast and S-180 cell lines grown in diffusion chambers in vitro and in vitro. The apparent mean generation time for both cell lines in diffusion chambers in vitro was about 1-1.5 days; in vivo the apparent mean generation time was about 2-3 days for both cell lines.

L-Fibroblasts did not produce tumors in C3H or Swiss-Webster mice under conditions in which S-180 cells produced a very high incidence of tumor.

Whatever inhibits the growth of L-fibroblasts injected directly into these mice did not inhibit their growth in diffusion chambers.

ACKNOWLEDGMENTS
The authors wish to express their appreciation to Dr. Avram Goldstein for his support, cooperation, and advice.

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17. ------. The Diffusion Chamber Technique Applied to a Study of the Nature of Homograph Resistance. Ibid., pp. 509-17.
Cell Culture

In Vivo

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Cell Culture in Vivo I. Growth of L-Fibroblast and Sarcoma 180 Cell Lines in Diffusion Chambers in Vivo

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