IN VITRO CULTURE AND CLONING OF ASCITES SARCOMA
CELLS OF FRIEND'S DISEASE ORIGIN*1

(Plates XXXIV~XXXV)

Shoichi OBOSHI and Koshi MARUYAMA
(Pathology Division, National Cancer Center Research Institute*2)

Synopsis

A long-term tissue culture cell line was established from the Friend virus ascites sarcoma. The cultured cells grew free in petri dishes showing the same morphology as in vivo and were easily transplantable into ddOM mice. Cultivation from a single cell resulted positive in about one-half of the trials with feeder layer. Both bioassay and electron microscopic examination of the cultured cells revealed virus production in the same degree as in vivo. Metamorphosis of tumor cells into fibroblast-like cells, frequently encountered both in vitro and in vivo, may suggest their innate potency to differentiate along fibroblastic line. Successful cultivation of the deficient virus-producing subline of this tumor may be useful for further analysis of induction phenomenon of infectious virus by ionizing radiation of such cells.

INTRODUCTION

Friend virus ascites sarcoma11) was established in our laboratory in 1962 and has been successively transplanted in the ascites form for over three years. An outstanding characteristic of this tumor is that the cells persistently produce the Friend virus, which is the causative agent of this malignancy, while they themselves have been proliferating as tumor cells. All our attempts to obtain non-virus producing cell lines from the population of the tumor cells proved a failure.12) It may be partly due to the absence of a reliable cloning method for this tumor.

The present paper is concerned with the in vitro cultivation of this ascites tumor, and a simple and efficient method of cloning from the cultured cells. Preliminarily, it also refers to the successful cultivation of a subline of this tumor.

MATERIALS AND METHODS

Cell Source  
FAT: This ascites tumor was derived from the leukemic tissue of a ddOM mouse with advanced Friend disease and it has been described previously.11) In the present study, the tumor ascites of the 181st transfer generation in our laboratory was used as a starting material.

FAT-H: This tumor is a subline derived from FAT by Kobayashi6) and was proved to be a special variant persistently producing a deficient form of the Friend virus. Its details were already reported by the present authors.7,8,15) The culture was initiated from the tumor ascites of the 32nd transfer generation in our laboratory.

*1 This study was partly supported by a Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education.

*2 Tsukiji 5-chome, Chuo-ku, Tokyo (大星章一，丸山孝士).
Medium  Unless otherwise stated, the growth medium consisted of 80% medium 199, 20% calf serum, 0.5% lactalbumin hydrolysate, 0.3% glucose, and a 4-fold concentration of Eagle's vitamins.

Culture Method  For the primary culture, the ascites fluid was harvested with a glass capillary from a tumor-bearing mouse and directly transferred into 4-cm petri dishes containing 2 ml of the medium. The culture medium contained approximately $2 \times 10^6$ cells/ml. All cultures were made and maintained in petri dishes kept at 37° in a humidified incubator with a constant flow of 5% CO$_2$ in air. The pH was adjusted to approximately 7.2. Two days later, the culture medium was agitated by pipetting, and then its 0.5 ml was transferred into a new petri dish containing a fresh medium. Since the cells grew free in the medium, they could be passaged by only pipetting without trypsinization, and all serial passages have been performed by transfer of 0.2~0.5 ml of cell suspensions, that is, $2\sim5 \times 10^5$ cells, into a new petri dish, usually twice a week.

Preparation of Feeder Layer  An irradiated primary culture of ddN mouse embryo tissues was used as a feeder layer. Minced embryos were trypsinized with 0.2% trypsin and then $2\sim4 \times 10^5$ cells/ml were seeded into petri dishes. A few days later, when cultured cells grew to a monolayer, they were washed twice with Hanks' solution and then a fresh medium was added. The cultures were then exposed to X-ray irradiation, 5,000 R of 6 MeV being delivered by a single irradiation, using a linear accelerator of Varian Associates. Dose rate of X-ray was 300 R/min. One-cm thickness of acrylate and the wall of a petri dish can produce the maximum build up at the surface of cultured cells. Within a few days after the irradiation, different inoculum size of cultured tumor cells was explanted.

Cloning Procedure  A microdrop from the cell suspension, prepared to contain $1 \times 10^3$ cultured tumor cells/ml, was placed on a sterilized cover slip with a glass capillary. The cell suspension had been kept in iced water. After being confirmed to contain only a single cell under a microscope, the coverslip itself with a single cell was placed in a petri dish with already prepared feeder layer, and cultured in a CO$_2$ incubator. On the 7th day, 1 ml of fresh medium was added. Cloning was also carried out from the cultured cells pretreated with the antiserum against the Friend virus. After 0.5 to 1.0 ml of antiserum was added to the culture medium and then incubated for 30 mins., diluted cell suspension was prepared in the same manner.

Antisera  C57BL mice were immunized by three consecutive intraperitoneal injections of 0.5 ml of the supernatant of 5%/w/v) homogenate of pooled spleens from the Friend virus-infected Swiss mice. The mice were bled a week after the last injection. The antiserum thus obtained was inactivated and then filtered through a Millipore filter. Antiviral serum of Donryu rats was also obtained by the same manner. Rats, however, received injection of 2 ml of the supernatant.

Virus Titration  The culture medium of 7 to 12 culture days was centrifuged at 1,000 r.p.m. for 10 mins. after the viable cells had been counted. Virus content of the resulting supernatant and precipitated cells was titrated separately. The former was recentrifuged at 5,000 r.p.m. for 20 mins. and its supernatant was used for virus titration of the culture fluid. The latter was resuspended in saline to contain $1 \times 10^5$ cells/ml and then frozen and thawed four times. After centrifugation at 5,000 r.p.m. for 20
mins. to remove the cell debris, the resulting supernatant was used for virus titration of cell lysate. Serial 10-fold dilutions were made, and 0.2 ml of each dilution was inoculated into the adult ddN mice intraperitoneally. After 3 weeks, the mice were sacrificed and the spleens were weighed for each mouse. The mice which exhibited spleen weight of over 0.5 g were judged as infected with the Friend virus. The ID50 was calculated according to the Behrens-Kärber method.

**Electron Microscopy** Cultured tumor cells were washed with chilled saline and then fixed in 4% glutaraldehyde solution followed by 1% OsO4 solution (pH 7.4 with 0.1M phosphate buffer), dehydrated through ethanol series, and embedded in Epon 812. Ultrathin sections were examined with a model HU-11A electron microscope after staining with uranyl or lead salts.

**RESULTS**

**Establishment of Culture of FAT Cells** The ascites primarily explanted contained a small number of macrophages, lymphocytes, and neutrophiles besides the tumor cells. In primary culture, the contaminating macrophages stuck to the glass surface, while the tumor cells proliferated falling to the bottom of the petri dish without any tendency to stick. By serial transfer of suspended cells, the sticking cells were completely removed after the 6th generation. Since then, pure culture of tumor cells was obtained (Photo 1) and has been successively passaged for 37 generations up to date. This course of establishment was proved to be reproducible. Some examples of estimation of cell growth are shown in Fig. 1. Doubling time was determined as 17 hrs.

![Growth curve of cultured FAT line](image)

**Cell Morphology** Smear preparations of centrifuged cultured cells were stained with Giemsa solution. Cultured cells consisted of tumor cells alone without any contaminating element. Morphology of cells was quite identical with that of ascites tumor cells in *vivo*. Mitotic figures were frequently encountered, and chromosome number was approximately 40. Under a phase-contrast microscope, the cultured cells were generally round and frequently had fine protoplasmic protrusions. The nuclei were round to oval, frequently indented, and nucleoli were prominent (Photo 2).
Transplantability Transplantability of the cultured cells was tested by the intra-peritoneal transplantation into ddOM mice in different inoculum sizes. As shown in Table I, the 50% endpoint of transplantability was estimated at $10^{\pm 1.7}$. The results were similar to those of ascites tumor cells.

<table>
<thead>
<tr>
<th>Inoculum size</th>
<th>13th</th>
<th>18th</th>
<th>20th</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^5$</td>
<td>4/4</td>
<td></td>
<td>3/3</td>
<td>7/7</td>
</tr>
<tr>
<td>$1 \times 10^4$</td>
<td>4/4</td>
<td>4/5</td>
<td>2/2</td>
<td>10/11</td>
</tr>
<tr>
<td>$1 \times 10^3$</td>
<td>3/4</td>
<td>3/5</td>
<td>2/2</td>
<td>8/11</td>
</tr>
<tr>
<td>$1 \times 10^1$</td>
<td>0/4</td>
<td>2/5</td>
<td>0/3</td>
<td>2/12</td>
</tr>
</tbody>
</table>

The denominator is the number of mice transplanted and the numerator, the number of positive animals.

Culture from Small Inoculum Size Cultures were carried out from various numbers of inoculum cells of $1 \times 10^1$ to $10^4$ cells. As shown in Table II, inoculation of $1 \times 10^4$ cells was proved to be necessary for cultivation without a feeder layer. However, inoculation of only 10 cells was quite sufficient, when explanted onto the feeder layer. The 50% endpoint of inoculum size indispensable for cultivation was estimated at $10^{2.3}$ without feeder layer, and at $10^{0.07}$ with feeder layer. Explanted cells were scattered and settled on the surface of the feeder cells, and then they proliferated to form variable number of colonies corresponding to the inoculum size. The colonies thus formed were macroscopically distinguishable as grayish white spots after a week (Photo 3). Thereafter, a number of daughter colonies were formed from the cells migrating from the original mother colonies.

<table>
<thead>
<tr>
<th>Inoculum size</th>
<th>18th</th>
<th>23rd</th>
<th>24th</th>
<th>25th</th>
<th>Total</th>
<th>16th</th>
<th>18th</th>
<th>23rd</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^4$</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>20/20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^3$</td>
<td>3/5</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>4/20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^2$</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/20</td>
<td>5/5</td>
<td>5/5</td>
<td>3/3</td>
</tr>
<tr>
<td>$10^1$</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/20</td>
<td>1/2</td>
<td>5/5</td>
<td>3/3</td>
</tr>
</tbody>
</table>

The denominator is the number of dishes inoculated and the numerator, the number of positive dishes.

Cloning Cultivation from a single cell was carried out repeatedly using the cultured tumor cells after the 18th transfer generation. Isolation of a single cell was made from the untreated cultured cells and from the cells pretreated with antiviral sera. As shown in Table III, successful growth was obtained in about one-half of the trials in each group. In successful cases, a single colony formation was confirmed by microscopy at 5 to 7 days after explantation (Photo 4). The subsequent growing process was the same as that in cultivation of a small inoculum size. Thus, growing cells were easily

Table I. Transplantability of Cultured FAT Cells into ddOM Mice

Table II. Cultivation from Small Inoculum Size of Cultured FAT Line
transferable either into mice intraperitoneally or into new petri dishes without a feeder layer.

We have obtained up to date over 30 clones, whose morphological, biological, and virological analyses are in progress. Their details, including the consecutive attempts at cloning, will be reported in another paper.

**Metamorphosis of Cultured Cells**  We have already shown that the pure cultured cells consisted only of tumor cells since the contaminating elements had been removed by serial passages. However, even then, a few fibroblast-like, spindle-shaped cells have been frequently encountered among the population of cultured cells. They stuck to the glass surface of petri dishes, occasionally crowding densely. These cells showed oval or indented nuclei, relatively poor in chromatin, with distinct nuclear membrane. Nucleoli were also prominent with Giemsa stain. Transition of free round-shaped tumor cells into these spindle-shaped cells was observable (Photo 5). They were also encountered in the colonies formed by the inoculation of a single cell and among the cell population of clones which were derived by successive transfer from each colony. Transition from the round-shaped tumor cells was also obvious. They were easily distinguishable from feeder cells in their location, size, and morphology.

Therefore, it was concluded that these fibroblastic cells were not contaminating elements, but a variety of tumor cells under some unknown condition. It might suggest the potency of the tumor cells to differentiate along the fibroblastic line.

**Virus Content**  Virus titration was carried out at and after the 9th culture passage. Both culture fluids and cell lysates contained the Friend virus, as shown in Table IV.

<table>
<thead>
<tr>
<th>Table III. Cultivation from a Single Cell of Cultured FAT Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of inoculum</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>Pretreated with</td>
</tr>
<tr>
<td>antiviral mouse serum</td>
</tr>
<tr>
<td>Pretreated with</td>
</tr>
<tr>
<td>antiviral rat serum</td>
</tr>
</tbody>
</table>

The denominator and the numerator are of the same as in Table II.

<table>
<thead>
<tr>
<th>Table IV. Titer of Virus in Culture Fluids and Cell Lysates from the Culture of FAT Line</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dilution</strong></td>
</tr>
<tr>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>9th</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two ml of culture fluid is regarded as 10<sup>6</sup> dilution.

<sup>b</sup> Two-tenth ml of the lysate of the cell suspension prepared to contain 10<sup>7</sup> cells/ml is regarded as 10<sup>-1</sup> dilution.

<sup>c</sup> The denominator is the number of mice inoculated and the numerator, the number of Friend disease-positive animals.
The ID$_{50}$ of the culture fluids and the cell lysates was estimated as $10^{-0.5}$ and $10^{-0.8}$, respectively. This result was the same as that in vivo. When the ascites at 7 days after the intraperitoneal transplantation of the ascites tumor cells was diluted to contain $2.5 \times 10^6$ cells/ml so as to correspond to the number in vitro, the ID$_{50}$ of the ascites fluid and the cell lysate was $10^{-0.5}$ and $10^{-0.8}$, respectively. These comparative data may indicate that the cultured cells have been producing Friend virus immutably as in vivo.

Culture of FAT-H Cells Repeated attempts to cultivate FAT-H cells by the same method as in the case of FAT cells have resulted in failure. The cells explanted displayed a marked degeneration, even in primary culture, and could never be passaged successively. Only when the culture medium was supplemented with 20% rat serum, instead of calf serum, cultures were successful and transferable. However, the cells showed marked agglutination. When the culture medium supplemented with an equivalent mixture of 10% each of rat and calf sera was used, the cells transferred from the 2nd generation grew without any agglutinating tendency, as constantly as FAT cells did (Photo 6). Thus, it has been passaged successively up to date for over 15 generations in the above medium containing a mixed sera. The growth curve of FAT-H cells is shown in Fig. 2. Preliminary virus assay was negative. The cells have recently been proved to grow in a culture medium supplemented with bovine serum alone, owing to habituation to it.

Electron Microscopy Both FAT and FAT-H cultured cells revealed virus production by budding of the plasma membrane with the same morphology and frequency as in vivo. No electron-dense central nucleoid was formed at the level of the virus bud in both lines (Photo 7). Preliminary examination showed that the majority of virions in FAT consisted of the so-called C-type particles, while those in FAT-H were of the so-called A type-like particles (Photo 8). No typical C-type virion has been encountered in FAT-H, so far as observed to date, as in vivo.
DISCUSSION

Investigations on the Friend virus with tissue culture have been relatively few. They may be divided into two fields. The one concerns the in vitro infection of the Friend virus, and the other the in vitro cultivation of tumor cells induced by virus infection in vivo. Attempts to propagate the Friend virus in vitro were carried out by Moore and Latarjet et al., resulting only in an indefinite or low leveled propagation of the virus. Osato et al. detected virus propagation in cultured cells infected in vitro by the fluorescent antibody technique and, moreover, they obtained a line of in vitro transformed cells, which were transplantable into susceptible mice. Transformed cells contained a Friend virus-specific cellular antigen but no viral antigen detectable by the fluorescent antibody technique. They also described that viral activity of this transformed cell line was detected only when viable cells were inoculated into the susceptible mice, resulting in the induction of fibrosarcoma, although electron microscopic examination revealed the production of virus particles from the cells. Yoshikura et al. have recently obtained cell lines altered in growth rate and morphology of colony from the cultures infected with the Friend virus in vitro.

On the other hand, cultivation of tumor cells induced by the in vivo infection of the Friend virus has been only made by Fields et al. and by Friend and Rossi. Though our present investigation followed their methods, some unique findings have been observed. Fields et al. found that an ascites tumor variant of a Friend virus-induced tumor grew in tissue culture, and after 32 culture passages, the Friend virus could no longer be detected in the cells, nor could it be isolated from serially transplanted tumors during 10 subsequent passages in mice. They claimed that the virus played a small part in the determination of either transplantability or virulence of the tumor, once the original transformation had occurred.

The cell line established by the present authors was also derived from the ascites tumor originating in the leukemic tissue of a Friend virus-infected mouse. It was proved however, that the cultured cells had been producing the Friend virus, detectable either by bioassay or by electron microscopy, in the same amount as in vivo even after 27 transfer generations. Therefore, we have attempted to isolate non-virus producing cells possibly existing in the population of tumor cells by the cloning method. Possible isolation of different variants including non-virus producer by cloning may be useful for the analysis of virus-cell relationships.

The Friend disease is characterized by the proliferation of immature basophilic cells, the so-called Friend cells, and of erythroblasts in the spleen and liver. It has been considered in general, that the neoplastic nature of the Friend disease is represented by the Friend cells, and the Friend disease is a special variant of reticulum-cell sarcoma since the Friend cells originated in undifferentiated reticulum cells around the small vessels. However, it has still remained in question whether the Friend cells could differentiate into erythroblasts or not. It is also suspected that the Friend virus could consist of at least two kinds of viruses, and each of them could propagate reticulum cell and erythropoietic cell line.

Friend and Rossi recently reported that the long-term cultured cell lines established from the transplantable tumors originating from leukemic organs of the Friend virus-
infected DBA/2 mice revealed virus production and differentiation along the erythroblastic line to the level of orthochromatic erythroblasts. The cells of FAT established in our laboratory, however, have never manifested any tendency to differentiate along the erythroblastic line, either in vivo or in vitro. On the contrary, they showed a metamorphosis to fibroblast-like spindle cells in vitro. It was also described in the previous paper that the spindle-shaped tumor cells were often encountered in vivo in the omentum tumors formed by the intraperitoneal transplantation of FAT cells. Such a metamorphosis might suggest an innate potency of the tumor cells to differentiate along the fibroblastic line. These data, including those of Friend and Rossi, might suggest that the Friend virus-induced tumor cells were primitive cells capable of differentiating along both erythropoietic and fibroblastic lines or were a mixture of at least two kinds of immature cells capable of differentiating along each cell line. Such a problem may also be clarified by successful cloning.

FAT-H strain, a subline of FAT, was proved to be a special variant persistently producing non-infectious deficient particles of the Friend virus. The in vitro cultured cells also have produced virus particles of the same morphology as in vivo, and their infectivity has not yet appeared, so far as has been observed. Evidence of recovery of infectivity by the X-ray irradiation of FAT-H cells strongly suggested an induction of infectious virus production. Successful cultivation of FAT-H cells may be useful for further analysis of this induction phenomenon.

The authors are indebted to Mr. T. Seido for valuable technical assistance and to Mr. E. Nishizaki for the photomicrographs.

(Received October 24, 1966)

REFERENCES

5) Kasuga, T., Oota, K., Gan-no-Rinsha, 8, 251 (1962).
7) Maruyama, K., Obohe, S., This Journal, 58, 89 (1967).
14) Obohe, S., Maruyama, K., This Journal, 58, 95 (1967).
EXPLANATION OF PLATES XXXIV~XXXV


Photo 2. Cultured FAT cells (17th generation in vitro). (a) Phase contrast. (b) Giemsa stain.

Photo 3. Colonies grown from $1 \times 10^2$ (upper) and $1 \times 10^2$ cells (lower) inoculated into petri dishes with feeder layer (11th culture day). Giemsa stain.

Photo 4. A colony developed from a single cell inoculation (7th day). Phase contrast.

Photo 5. Fibroblast-like cells sticking to the glass surface (6th culture day of the 22nd generation in vitro). Giemsa stain.


Photo 7. A virus bud at the cell membrane. (a) Cultured FAT cell. $\times 157,000$ (b) Cultured FAT-H cell. $\times 132,000$

Photo 8. Extracellular virus particles in cultured FAT-H line. Note an assembly of A type-like particles. $\times 79,900$