FRIEND VIRUS ASCITES SARCOMA: A TRANSPLANTABLE ASCITES TUMOR VARIANT INDUCED FROM FRIEND'S VIRAL LEUKEMIA (Plates VII–IX)

Shoichi OBOSHI,*1 Ken AOKI,*1 Ryuichi SATO,*2 and Tsuneo BABA*1
(Pathology Division*1 and Common Laboratory,*2 National Cancer Center Research Institute*3)

Synopsis

Subcutaneous solid tumor induced by Kasuga and Oota from leukemic tissues of Friend virus-infected ddOM mice was transformed into ascites tumor form and has been successively transplanted in an ascitic form to 30th generation. This tumor has been tentatively designated as Friend virus ascites sarcoma. It is 100% transplantable in ddOM mice but approximately 80% in ddY or ddN mice. The tumor, composed of mononuclear cells resembling immature reticulum cells, is considered to have originated from reticulum cells. Virus content in both tumor tissues and tumor ascites was found to be much smaller than that in spleens by both titration and electron microscopic studies. It is still obscure whether a generalized Friend virus-induced leukemia always following transplantation of tumor ascites is due to tumor cells themselves or not.

INTRODUCTION

Friend’s viral leukemia is a disease which is serially transmissible in adult mice of certain strains by cell-free filtrates from infected animals.3) It is characterized by multicentric proliferation of immature mononuclear cells in organs followed by a marked enlargement of spleen and liver, and an appearance of those cells in the peripheral blood.6,8,9) The neoplastic character of this condition, however, had been disputed because local solid tumor growth could not easily be obtained with the transplants of leukemic tissues from the virus-infected mice.6,8,9) However, this has recently been accomplished by Buffett and Furth1) using Swiss mice as a host, by Friend and Haddad4) using DBA/2 mice, and by Kasuga and Oota7) using ddOM mice. The tumors thus developed have been successively transplanted by each investigator. Buffett and Furth1) are of the opinion that the tumor which they have produced resembles an autonomous nonviral, reticulum-cell sarcoma.

It had been noticed by each of these authors that when these tumors were grafted, generalized Friend virus-induced leukemia also developed simultaneously with the
local tumor growth. This is due to the fact that the tumor tissues contain an appreciable amount of Friend virus. Therefore, it has not yet been determined whether or not the tumor growth is really autonomous and not dependent on the virus. For the investigation of this problem common to viral neoplasms at cell level, transformation of this tumor from solid tumor type into ascites tumor type was desired. This has recently been accomplished in our laboratory employing the serially transplanted solid tumor strain, which had been formerly established from Friend virus-infected ddOM mice by Kasuga and Oota.7)

This paper is concerned with transplantability, morphological features, and the virus content of this ascites tumor variant.

**MATERIALS AND METHODS**

**Source of Tumor** A solid tumor strain of Friend's viral leukemia was obtained in 1962 through the courtesy of Drs. T. Kasuga and K. Oota, Cancer Institute, Tokyo. They induced this tumor in 1961 by successful subcutaneous grafting of the leukemic tissues from Friend virus-infected ddOM mice. Since then, it has been successively transplanted into mice of the same strain as a solid subcutaneous tumor.

**Transformation into Ascites Tumor** The subcutaneous tumor of the 17th generation was removed aseptically and then homogenized in a small amount of physiological saline. The cell suspension was intraperitoneally inoculated into both ddOM and ddY mice. A few days later, smear preparations made from the ascitic fluid showed the presence of free tumor cells in a large number and all the mice of both strains died of tumor within 20 days after inoculation. Thereafter, the tumor cells have been serially transplanted in an ascitic form using ddOM and ddY or ddN mice in our laboratory. At the time of this writing it is in the 30th generation.

**Animals** Both male and female young adult mice of various strains were used. ddY and ddN mice: Albino mice for general purpose. Though both strains were originated from the same source, the former has been maintained at the National Institute of Health, Tokyo, and the latter at the Central Institute for Experimental Animals, Tokyo. They are both susceptible to Friend virus.

ddOM mouse: The mice of ddY line were bred by brother-to-sister mating through 37 generations at Ohmura Veterinary Hospital. The line thus developed was designated as ddOM. It is highly susceptible to Friend virus.

SMA mouse: An inbred strain from Swiss mouse raised at the Central Institute for Experimental Animals. It is also susceptible to Friend virus.

C3H/He mouse: A Friend virus-resistant strain stocked in the Central Institute for Experimental Animals.

C57 Black mouse: A Friend virus-resistant strain stocked in the National Institute of Heredity, Mishima.
Virus Titration  The omental tumors and the spleens to be tested for virus content were removed aseptically from the ddY mice 20 days after intraperitoneal transplantation of the tumor cells. Materials from three animals were pooled. Ten percent (w/v) homogenates were made with physiological saline, and then frozen and thawed three times. The supernatant was obtained after centrifugation at 2,000 r.p.m. for 10 minutes and serial 10-fold dilutions were made. Two-tenth milliliter of each dilution was inoculated into the peritoneal cavity of ddY mice. After 3 weeks, the mice inoculated were sacrificed and the spleen was weighed for each mouse. According to Odaka and Yamamoto,10) 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 method of Reed and Muench.11)

The virus content in the tumor ascites was also measured. The ascitic fluid from three ddY mice 20 days after intraperitoneal transplantation of the tumor cells was pooled. The ascites cells were washed with physiological saline and cell suspensions containing 10⁸ cells/ml were prepared. Then they were treated in the same manner as described above.

Electron Microscopic Examination  Materials for electron microscopic observations were ascites tumor, solid tumor tissues in omentum and peritoneal wall, and spleen from a male ddN mouse 20 days after intraperitoneal transplantation of tumor cells of the 9th transfer generation. A spleen of another male mouse of the same strain 26 days after intraperitoneal inoculation of cell-free filtrate of Friend virus-infected spleen was also observed for comparison.

Small tissue fragments were fixed in 1% OsO₄ solution, adjusted to pH 7.4 with acetate-Veronal buffer, for 2 hours at 4°. They were embedded in a mixture of butyl and methyl methacrylate (9 : 1) with addition of dichlorobenzoyl peroxide-2% methacrylate after dehydration in an ethanol series. Ascitic fluid was fixed in the same solution for 20 minutes and embedded in methacrylate. The sections were cut on a Porter-Blum microtome and subsequently examined with a Hitachi HU-9 electron microscope.

RESULTS

Transplantation Studies

Six strains of adult mice were used for susceptibility test. Strains susceptible to Friend virus appeared to be also susceptible to Friend virus-induced tumor cells (Table I). When approximately 0.05 ml of ascitic fluid obtained from the peritoneal cavity of mice bearing transplanted tumor cells was inoculated intraperitoneally into ddOM mice, the hosts developed ascites containing approximately 10⁸ tumor cells/ml within a week after transplantation and all of them died of tumor within two weeks after transplantation. The tumor cells inoculated into ddY or ddN mice continued
to proliferate until the 12th day after transplantation as did the cells inoculated into ddOM mice. However, they regressed spontaneously in some of the animals. In SMA mice, ascitic tumor cells grew up to the 9th day after transplantation and then regressed in most instances. Tumor cells inoculated into C3H/He and C57 Black mice regressed spontaneously on the 7th day after transplantation. In rats, they also grew up to the 4th day and then regressed. In strains other than ddOM susceptible to Friend virus, only a change due to virus infection which was characterized by marked enlargement of spleen generally remained even after regression of ascitic tumor cells.

It was found that a rather close correlation was present between the number of tumor cells in the inoculum and the mean survival time of the hosts. Thus, an inoculum of $10^6$ cells killed mice within 13 days and an inoculum of $10^4$ cells, in 26 days.

### Morphological Studies

**Ascites Tumor Cells:** Smear preparations of ascitic fluid were routinely stained with May Grunwald-Giemsa solution. Tumor cells were generally spherical and 10–20μ in diameter. The nuclei were round to oval and rarely lobulated. The nuclei of smaller tumor cells had coarse chromatin clumps comparable to those of lymphocytes, but larger tumor cells had distinct nuclear outline, nucleoli, and fine chromatin structure resembling those of reticulum cells. Scanty cytoplasm was deeply basophilic and contained a few fat vacuoles. Neither cytoplasmic inclusion nor phagocytosis was observed. Mitotic figures were numerous. Chromosome numbers are approximately 40 and tetraploid cells were rarely encountered. Supravital staining with Neutral Red revealed a few granules, but no rosette formation. Phagocytosis test with Indian ink and peroxidase reaction was negative.

**Autopsy Findings of Mice which died of Tumor:** Solid tumor growth was seen in the abdominal wall at the sites of transplantation, peritoneal surface, omentum, mesentery, and connective tissues around the kidneys and sex organs. Mesenteric and retroperitoneal lymph nodes were also enlarged. Marked enlargement of thymus as a result of tumor metastasis was characteristic. The heaviest splenic weight was 3.0 g, with an average of 1.2 g. Occasionally, the animals with spleen weighing less

### Table I. Susceptibility of Various Strains of Mice

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. of mice transplanted</th>
<th>No. of mice died of tumor</th>
<th>Tumor death (%)</th>
<th>Survival days of mice dying of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>ddOM</td>
<td>98</td>
<td>98</td>
<td>100</td>
<td>13.4</td>
</tr>
<tr>
<td>ddY</td>
<td>91</td>
<td>70</td>
<td>77</td>
<td>18.2</td>
</tr>
<tr>
<td>ddN</td>
<td>87</td>
<td>70</td>
<td>80</td>
<td>17.6</td>
</tr>
<tr>
<td>SMA</td>
<td>19</td>
<td>4</td>
<td>21</td>
<td>19.0</td>
</tr>
<tr>
<td>C3H/He</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>C57 Black</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>
than 0.1 g were encountered in ddY or ddN mice. However, when transplantation of the tumor ascites from those mice was attempted, the recipients revealed enlargement of spleen characteristic of Friend virus infection.

Histologically, the solid tumors were generally composed of sheets of round to polygonal cells with usually uniform, round, or oval nuclei, distinct nuclear outlines, and prominent nucleoli. The cytoplasm was homogeneous and faintly eosinophilic. Occasionally tumor tissues contained considerably pleomorphic tumor cells with larger, often lobulated nuclei, but multinucleated tumor cells were rare. Mitotic figures were numerous. Tumor cells took stellate form with cytoplasmic processes in some instances, showing a cell arrangement similar to that of human reticulum cell sarcoma. The stroma was generally scanty, but fine reticular fibrils were occasionally demonstrated in older tumors. They were closely associated with individual tumor cells. Intramuscular infiltration of tumor cells was common in tumors of the abdominal wall.

The spleen generally displayed multicentric proliferation of immature mononuclear cells, erythroblasts and megakaryocytes, which is identical to the change induced by inoculation of cell-free filtrate from Friend virus-infected organs. Monotonous proliferation of more anaplastic cells similar to the tumor cells of which solid tumors were made up was occasionally encountered in splenic pulp. These foci are considered as metastases of ascites tumor cells into the spleen. Sections of the liver also revealed intrasinusoidal proliferation of immature mononuclear cells and erythroblasts. Besides, uniform proliferation of tumor cells in lymph spaces of portal area was frequently observed. The former may be regarded as virus-induced change and the latter as tumor metastasis. Pulmonary metastasis was encountered in peribronchial lymph spaces. Metastases were also found in axillary and inguinal lymph nodes which were regional lymph nodes of tumor in abdominal wall, mesenteric, retroperitoneal, and mediastinal lymph nodes. Thymus glands were occupied by tumor cells.

Although of a mild degree, the peripheral blood showed lymphocytosis and erythroblastosis, similar to the change induced by inoculation of cell-free filtrate. An appearance of tumor cells in the peripheral blood was relatively rare.

Titration of Virus Content in Spleen, Tumor Tissues, and Ascitic Fluid

The spleens and tumor tissues of omentum obtained from mice bearing ascites tumor were titrated to determine the virus content in each of these tissues. Although ddOM mice were found to be more uniformly susceptible to Friend virus, the titrations were carried out using ddY mice throughout these studies following a preliminary titration experiment with a small number of ddOM mice, since this strain was unavailable in a large number for its poor propagation. The comparative titers of the virus in spleens and tumor tissues are given in Table II. In these data the
10% homogenate of each tissue is regarded as original dilution. The ID$_{50}$ of spleen and tumor tissue was determined as $10^{-5.1}$ and $10^{-2.3}$, respectively.

Because the number of cells in the 10% suspensions of spleens or tumor tissues was measured as approximately $10^8$ cells/ml, the suspension of ascites cells prepared to contain $10^8$ cells/ml was regarded as nearly the same as the original dilution of tissues in wet weight. Therefore, the ID$_{50}$ of ascitic fluid from mice bearing ascites tumor was determined as $10^{-0.8}$ (Table III). These results of titration showed that virus content in materials such as tumor tissue or ascites, the greater part of which was composed of tumor cells, was much less than that in spleen.

### Table II. Titer of Virus in Spleen and Tumor Tissue from Mice transplanted with Tumor Cells Intraperitoneally

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Spleen$^{b)}$ 6th generation</th>
<th>8th generation</th>
<th>Tumor Tissue$^{b)}$ 8th generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^0$</td>
<td>10/10</td>
<td></td>
<td>6/8</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>10/10</td>
<td></td>
<td>0/8</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>6/10</td>
<td>5/8</td>
<td>0/8</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>10/10</td>
<td>7/8</td>
<td>0/8</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td></td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>ID 50</td>
<td>&lt; $10^{-4}$</td>
<td>10$^{-0.8}$</td>
<td>10$^{-2.3}$</td>
</tr>
</tbody>
</table>

$^{a)}$ The 10% homogenate of each tissue is regarded as $10^0$ dilution.

$^{b)}$ The denominator indicates the number of mice inoculated and the numerator, the number of positive animals.

On the other hand, intraperitoneal injection of 0.02 ml of ascitic fluid obtained from mice inoculated with cell-free filtrate of virus-infected spleen produced splenomegaly of over 0.5 g in 5 out of 6 mice thus treated. In the ascitic fluid only macrophages and lymphocytes were present in a small number.

### Electron Microscopic Findings

#### Solid Tumor Tissues in Omentum and Peritoneal Wall:

The tumor cells showed a marked pleomorphism. Two types of cells were observed, one relatively small, round
or oval in shape, and the other large, elongated or irregular in outline. Nuclei of both types of cells displayed indentation of nuclear membrane in a varying degree and marginally distributed chromatin. One large nucleolus or few rather small ones were quite distinct in some of the cells, while obscure in the others. Cytoplasm usually appeared to be electron opaque, but slightly less in the larger cell type, due to diffusely distributed, numerous, free ribosome particles. The degree of development of various cytoplasmic components was also strikingly different in each individual cell. Mitochondria were numerous in a majority of the cells and often found gathering in one side of the cytoplasm. The rough-surfaced endoplasmic reticulum in the larger cells appeared to be highly developed together with varying sized vesicles or vacuoles and granules of different electron density. In the smaller cells, however, these structures were not so well differentiated, but showed various lengths of elongated endoplasmic reticulum and occasional vesicles or granules. These cells of both types were intimately related with and often embraced by collagen-like substances. Nothing definitely identified as virus particles was observed except for some unusual spherical particles of moderate electron density, 70 m\(\mu\) in diameter, encountered separately or in groups within degenerating cells.

Tumor Ascitic Fluid: The cells in the ascitic fluid were found to be relatively less pleomorphic than those in solid tumor tissues. Majority of the cells showed characteristics of the above-described smaller cell type in tumor tissues and none of them showed high development of cytoplasmic organelles. One of the characteristics worthy of special comment was one to three well-developed Golgi apparatuses. Besides these tumor cells, peritoneal macrophages were also often encountered. In spite of careful observations with expectation of finding the virus particles, nothing was definitely identified as such in the proliferating tumor cells. On the contrary, mature virus particles with nucleoid bodies or immature particles without nucleoid bodies were frequently observed within the intracytoplasmic vacuoles of or on the cell surface of the macrophages.

Spleens from the Tumor-bearing and Virus-infected Mice: Nothing new could be added to the findings previously described by de Harven and Friend.\(^2\) Virus particles, 77~92 m\(\mu\) in diameter, with or without nucleoid bodies, were demonstrated in the interspaces between the proliferating pulpa and sinus endothelial cells, as in the intracytoplasmic vacuoles.

**DISCUSSION**

Some virus-induced tumors are easily transplantable. This phenomenon has generally been considered as one of evidences that viral tumors also belong to a group of true autonomous neoplasms. For example, it has been well known that mammary carcinoma or Gross leukemia of mice is easily transplantable into subcutaneous
tissue of the same strain of mice in which these tumors originated and even transformation into ascites tumor form can easily be carried out in the latter.\textsuperscript{5) Transplantable tumor strains produced from Friend's viral leukemia are merely one of the transplantable virus-induced tumors. However, in these tumors newborn mice must be used as recipients and long latent period of several months is required for bioassay of viruses. On the contrary, Friend's viral leukemia is characterized by successful transmission of the virus using adult mice and in extremely short period of incubation of a few weeks. This is very convenient for bioassay of virus. Therefore, the transplantable tumor-variant induced from Friend's viral leukemia may be considered as a very convenient material to study whether cell proliferation induced by virus infection is truly autonomous and virus-independent or not. For the investigation of this interesting problem common to viral neoplasms at cellular level, transformation of this tumor into ascites tumor form was desired and was accomplished in our laboratory. This newly established ascites tumor has been tentatively designated as Friend virus ascites sarcoma.

Immature mononuclear cells which proliferate in Friend virus-infected spleen or liver are generally regarded as immature reticulum cells.\textsuperscript{6,8,9) Tumor cells of transplantable tumor variants induced from Friend virus-infected spleen or liver in several laboratories were also considered as reticulum cells by each worker.\textsuperscript{1,7) Some of the tumor cells in Friend ascites sarcoma revealed lymphocyte-like nuclear structure, while others displayed nuclear structure comparable to that of reticulum cell. Histological pattern of tumor tissues is characterized by cell arrangement similar to reticular tissue and by reticular fibrils closely associated with individual tumor cells, though not abundant, quite resembling that of human reticulum cell sarcoma. Electron microscopic examination of tumor cells demonstrated well-developed rough-surfaced endoplasmic reticulum and vacuoles or vesicles in cytoplasm. Therefore, the origin of tumor cells of Friend virus ascites sarcoma may be considered as immature reticulum cells, as Buffett and Furth, or Kasuga and Oota formerly stated.

Metcalf, Furth, and others\textsuperscript{8) expected that Friend virus-induced tumor cells, if serial passage had been carried out for a long period, would have been altered into virus-free autonomous tumor cells. Buffett and Furth\textsuperscript{1) considered that the transplantable tumor induced from Friend virus-infected liver in their laboratory resembled autonomous reticulum cell sarcoma since it exhibited a cellular pleomorphism not noted in the leukemic organs of mice inoculated with cell-free filtrate. However, they could not exclude the possibility that the causative virus might still be necessary for the growth of tumor. Friend and Haddad\textsuperscript{4) tried to titrate virus content of spleens and subcutaneous tumor tissues comparatively until the 21st generation using their own transplantable tumor induced from Friend's viral leukemia. Their results
revealed no difference in virus content between spleen and tumor tissue.

On the other hand, the present study using Friend virus ascites sarcoma showed that virus content in tumor tissues or tumor ascites was far smaller than that in the spleen. Electron microscopic examinations also revealed numerous virus particles in intercellular spaces of immature mononuclear cells and in intracellular vacuoles of macrophages in spleen, but no virus particle in tumor cells of both solid and ascites form at the same level of precision. These findings suggest the possibility that an appearance of generalized virus-induced leukemia following transplantation of tumor ascites may be due to macrophages included in ascites in the approximate ratio of 1 to 70 tumor cells and not to tumor cells themselves. As was expected, numerous virus particles were found in intracellular vacuoles of macrophages in tumor ascites by electron microscopic examination. If viruses were transmitted by tumor cells themselves, virus content in tumor cells would be extremely small. It seems of first importance to determine whether viruses are always transmitted in association with tumor cells themselves or not. In further studies, it must be clarified whether the virus is merely contaminant or truly requisite for the proliferation of tumor cells.

The authors are indebted to Drs. T. Kasuga and K. Oota, Cancer Institute, for original solid tumor strain. They also express their thanks to Dr. M. Yamada, National Institute of Health, Tokyo, and to Dr. K. Maruyama, National Tama Institute for Leprosy, for their advice and cooperation throughout this study.

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7) Kasuga, T., Oota, K., Gan-no-Rinsho, 8, 251 (1962).
EXPLANATION OF PLATES VII～IX

Photo 1. ddN mouse of 28th generation sacrificed 21 days after intraperitoneal transplantation of tumor cells.

Photo 2. Smear preparation of tumor ascites. One lymphocyte is seen at lower left besides tumor cells.

Photo 3-8. Materials obtained from ddN mice which died of tumor.

Photo 3. Solid tumor tissue in omentum.

Photo 4. Portion of the same. Silver impregnation.

Photo 5. Spleen showing Friend virus-induced proliferation of mononuclear cells and erythroblasts.

Photo 6. Liver showing tumor metastasis in portal lymphatics in lower part and Friend virus-induced proliferation of mononuclear cells and erythroblasts in sinusoidal space in upper part.

Photos 7-9. Materials obtained from ddN mouse 20 days after intraperitoneal transplantation of tumor cells.

Photo 7. Solid tumor tissue in omentum. Note marked pleomorphism and well-developed rough-surfaced endoplasmic reticulum of tumor cells. ×3,920

Photo 8. Ascites tumor cell. ×9,945

Photo 9. Macrophage in spleen, showing numerous virus particles in intracytoplasmic vacuoles and extracellular spaces. ×40,035