Frozen Storage of 34 Various Solid and Ascites Tumors*

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SUMMARY

A variety of transplantable mouse, rat, hamster, and ehieken tumors have been successfully stored at \(-76^\circ\) C. (dry-ice chest) for a period of 1 year. These tumors are seven sarcomas, a mammary carcinoma, a skin carcinoma, a bladder carcinoma, a lung carcinoma, two lymphosarcomas, a glioma, a leukemia, a virus leukemia, a pancreatic tumor, and a small intestine tumor. Transplantability of a majority of mammary-type tumors was greatly damaged by frozen storage at \(-76^\circ\) C. Transplantability of very slowly growing tumors—namely, Harding-Passey melanoma, Andervont hepatoma, Iglesias functional ovarian tumor, and Iglesias functional adrenal tumor—was either completely or almost completely abolished by frozen storage at \(-76^\circ\) C. for 3 months.

The viability of Ridgway osteogenic sarcoma was completely destroyed by frozen storage for 1 month at \(-76^\circ\) C. Transplantability of Wagner osteogenic sarcoma was markedly reduced following storage at \(-76^\circ\) C. The viability of Ridgway osteogenic sarcoma was almost completely destroyed by frozen storage for 1 day at \(-76^\circ\) C. or \(-25^\circ\) C. Maintenance of this tumor for 1 day at \(4^\circ\-5^\circ\) C. did not alter its transplantability. Hauschka's slow freezing technic (from \(0^\circ\) C. to \(-25^\circ\) C.) of neoplastic tissues appears to be less harmful to the viability of frozen tumor (Ridgway osteogenic sarcoma) than the technic of freezing described in the present report.

During the past several years we have used a spectrum of 40 transplantable animal tumors for chemotherapy studies. We have found that some of these tumors either are very resistant to the action of compounds and antibiotics or possess identical sensitivity toward various chemical agents. Therefore, it was thought best to discontinue a number of such tumors and replace them by new tumors having different characteristics. A decided disadvantage in discarding some of our presently carried tumors would be that a number of them are not available elsewhere and might be desired in the future by other investigators. Therefore, we decided to store our solid and ascites tumors in a dry-ice chest at \(-76^\circ\) C. Different methods for frozen storage of solid and ascites tumors have been described by Breedis and Furth (1), Snell and Cloudman (5), Craigie (2), Hauschka (4), and by many other investigators. This paper presents the results of such a study with a spectrum of tumors.

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MATERIALS AND METHODS

We used the following technic for frozen storage of solid and ascites tumors. Small pieces of approximately 1.5 millimeters cube, weighing about 6 mg., and suitable for insertion into trocars, were dissected from non-necrotic portions of solid tumors. A dozen such cubes were dropped into 1.25 cc. of mammalian Ringer solution,\(^1\) a modified Locke-Ringer solution, recommended by Hauschka plus 10 per cent glycerol in 2.5-cc. vials which were then sealed with a propane torch. The sealed ampules were kept in a refrigerator for about \(\frac{1}{2}\) hour at \(4^\circ\-5^\circ\) C. and in the freezing chamber at \(-25^\circ\) C. for another half an hour; they were then placed into a dry-ice chest at \(-76^\circ\) C. for frozen storage.\(^2\)

The ascites tumor cells were withdrawn from the host with a 2- or 5-cc. syringe through a 20-gauge needle and placed in a 2.5-cc. glass vial; 0.1 cc.

\(^1\) Concentration of salts in mammalian Ringer solution is: 0.83 per cent NaCl, 0.02 per cent KCl, 0.02 per cent CaCl\(_2\). The mammalian Ringer solution was adjusted to pH 7.2 with 0.1 N NaOH or 0.1 N HCl.

\(^2\) We did not immerse the sealed ampules into dry ice-alcohol slush (\(-76^\circ\) C.). Such a cabinet was not available at the initial stage of the present study.
of glycerol was added to 1.0 cc. of tumor ascites, and the vials were then heat-sealed for freezing. As a control, animals were given inoculations of untreated tumor tissues or ascites tumors (before frozen storage) immediately after their removal from the tumor-bearing host.

Following various intervals of time, solid tumor tissues were thawed and injected by trocar into healthy young animals subcutaneously in the region of the right axilla. Frozen ascites tumors (10) were diluted 1:4 with mammalian Ringer solution and injected intraperitoneally into young mice. In the case of Friend virus leukemia (12), the leukemic spleens were prepared as a 10 per cent homogenate in mammalian Ringer, and 0.2 cc. was injected intraperitoneally. The progress of the transplanted tumors was recorded graphically by measuring them every week by means of calipers for 8 weeks. The animals were maintained on a standard pellet diet (Purina Laboratory Chow) and water ad libitum.

The tumors used in the present study are listed in Tables 1 and 2. The history, biological properties, and cytological description of most of these tumors have been presented previously (13, 17–19). These tumors are: Sarcoma 180, Ehrlich carcinoma, Nelson ascites tumor, Bashford carcinoma 63, Harding-Passey melanoma, and Friend virus leukemia in Swiss albino mice; Sarcoma T241, Adenocarcinoma E 0771, Lewis bladder carcinoma, Lewis lung carcinoma and Gliona 26 in C57BL mice; Sarcoma MA 387, Miyono adenocarcinoma, Carcinoma 1025, Wagner osteogenic sarcoma, Ridgway osteogenic sarcoma, Meeca lymphosarcoma, and leukemia L 4946 in AKR mice; Andervont hepatoma in C mice; Goldfeder spindle-cell mammary tumor DBAG and Goldfeder mammary adenocarcinoma DBAH in DBA mice; Flexner-Jobling carcinoma and Walker carcinosarcoma 256 in Sherman rats; Jensen sarcoma in Sprague-Dawley rats; Murphy-Sturm lymphosarcoma in Wistar rats; Iglesias functional ovarian tumor, Iglesias sarcoma (the product of a spontaneous transformation of Iglesias functional ovarian tumor), and Iglesias functional adrenal tumor in A X C rats; Crabb sarcoma, Fortner adenocarcinoma of pancreas No. 1, and Fortner adenocarcinoma of small intestine No. 1 in golden Syrian hamsters; and Rous chicken sarcoma in white leghorn chicks.

**RESULTS**

The results obtained with nineteen solid mouse tumors, seven solid rat tumors, three hamster tumors, one mouse leukemia, one mouse virus leukemia, three mouse ascites tumors, and one chicken tumor after they were frozen stored for 3 months ± 15 days, 6 months ± 15 days, and 12 months ± 1
month at $-76^\circ$ C. are summarized in Tables 1 and 2. Each frozen tumor was tested with ten animals, and each experiment was repeated; the data presented are averages of the results of multiple experiments. Evaluation of tumor growth has been based on results observed 6 weeks after tumor inoculation, except for the Andervont hepatoma and the Iglesias functional ovarian and adrenal tumors. Because of the very slow growth of these tumors in animals, observations were made after 12 weeks.

From the tabulated data in Table 1 it is evident that transplantability of mouse sarcomas was not altered by prolonged frozen storage. Thus, the percentage of growth of Sarcoma 180, Sarcoma T241, and Sarcoma MA 387 which had been frozen for from 3 to 12 months at $-76^\circ$ C. was essentially the same as that of transplants of untreated tumor tissues immediately after their removal from the tumor-bearing animals; however, the appearance of the palpable tumors was delayed for approximately 1 week except in the case of Sarcoma T241, which was not delayed. In contrast, transplantability of all five mammary carcinomas, namely, Bashford carcinoma 65, Adenocarcinoma E 0771, Miyono adenocarcinoma, Goldfeder spine-cell mammary tumor DBAG (3), and Goldfeder mammary carcinoma DBAH, was greatly damaged by frozen storage for as short a period as 3 months. Ehrlich carcinoma which arose 50 years ago as a mammary carcinoma withstood the deleterious action of the frozen state (cold-treated transplants grew normally).

Carcinoma 1025, a skin tumor, Lewis bladder carcinoma, and Lewis lung carcinoma were highly resistant to cold treatment. The growth of these tumors approximates that of normal transplants.

It is interesting that immersion of both Wagner osteogenic sarcoma and Ridgway osteogenic sarcoma in a mammalian Ringer solution at pH 7.2 and being kept frozen at $-76^\circ$ C. for 3, 6, and 12 months resulted in complete inhibition of tumor growth. Mecca lymphosarcoma withstood prolonged frozen storage relatively well, but Harding-Passey melanoma was greatly damaged by very low temperature. Glioma 26 (a brain tumor) showed satisfactory results, having more than 50 per cent growth. Almost no inhibitory effect was observable with Friend virus leukemia and Leukemia L 4946 even after frozen storage of 12 months, the growths being nearly the same as that of untreated control. The transplantability of Andervont hepatoma was completely destroyed by storage at $-76^\circ$ C. for 3 months or more.

In the case of ascites tumors, Sarcoma 180 was most resistant to the freezing treatment, followed by Ehrlich carcinoma and Nelson carcinoma.

Similar to mouse tumors, the transplantability of rat sarcomas was not greatly altered, but that of rat carcinomas was greatly damaged by frozen storage at $-76^\circ$ C. (Table 2). All three hamster tumors—namely, Crabb sarcoma, Fortner pancreatic tumor No. 1, and Fortner intestinal tumor No. 1—were not altered in their transplantability by frozen storage for 12 months except for a short delay in the appearance of palpable tumors.

Transplants of Iglesias functional ovarian tumor and Iglesias functional adrenal tumor which had been kept at $-76^\circ$ C. for 3-12 months completely failed to grow following passage to A X C rats.

In summary, the appearance of palpable tumors was delayed for 7 days. The only exceptions included: Sarcoma T241, Ehrlich carcinoma, Lewis lung carcinoma, and Iglesias rat sarcoma.

The Rous agent was unaffected by prolonged frozen storage at $-76^\circ$ C. of either tumor fragments or cell-free filtrate of the chicken sarcoma.

The histological examinations of a number of the tumor fragments after immersion in a mammalian Ringer solution, pH 7.2, and frozen storage at $-76^\circ$ C. for 12 months showed varying degrees of decreased size of cells with smaller, hyperchromatic nuclei. These tumors (Ridgway osteogenic sarcoma, Wagner osteogenic sarcoma, Bashford carcinoma 65, Adenocarcinoma E 0771, Iglesias functional ovarian tumor, and Iglesias functional adrenal tumor), however, either completely failed to grow or grew in a small percentage of mice or rats. Apparently no relationships can be established between the cellular alterations resulting from freezing and viability, since a number of frozen tumors grew in 50-100 per cent of transplanted animals.

As part of the present study, we determined the effect of long frozen storage (at $-76^\circ$ C.) on the chemical sensitivity of tumors. Since mitomycin C completely destroyed Jensen rat sarcoma, this tumor was chosen. Sarcoma 180 and Mecca lymphosarcoma were selected, since the former is sensitive whereas the latter is resistant to 6-mercaptopurine. The tumors were transplanted one passage prior to examining their responsiveness to various drugs. Results of these experiments indicate that daily intraperitoneal injections of 0.5-1.0 mg/kg of mitomycin C for 7 days caused complete destruction of 1-day-old Jensen Sarcoma (Table 3). Similarly, the intraperitoneal injections of 30 mg/kg of 6-mercaptopurine had a destructive effect on 1-day-old Sarcoma 180 (Table 3) but no effect on Mecca lymphosarcoma. These findings demonstrate that tumor tissues after 1 year of storage in the frozen state show no detectable change in their sensitivity to the chemotherapy agents tested.
DISCUSSION

It is evident from the data in Tables 1 and 2 that the transplantability of mouse, rat, hamster, and chicken sarcomas was not altered appreciably by prolonged storage of from 3 to 12 months at -76°C. However, the transplantability of all five mammary carcinomas of the mouse and all rat carcinomas was greatly damaged by frozen storage for even 3 months. Previous studies in our laboratory with Flexner-Jobling rat carcinoma (16), Rous chicken sarcoma (15), Sugiura rat sarcoma (6), Sarcoma 180 (7), Bashford carcinoma osteogenic sarcomas completely lost transplantability during 3 months’ frozen storage at -76°C.

Transplantability of all the very slowly growing tumors—namely, Harding-Passey melanoma, Andervont hepatoma, Iglesias functional ovarian tumor, and Iglesias functional adrenal tumor—was completely or nearly abolished by the frozen storage at -76°C for 3 months or longer. This fact is in agreement with the observation of Snell and Cloudman (5), who pointed out that some of the slowly growing tumors do not survive any method of freezing.

TABLE 3

**Comparison of Drug Sensitivity of Frozen and Nonfrozen Tumors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tumor*</th>
<th>Dose (mg/kg/d)</th>
<th>No. Deaths</th>
<th>AV. WT. CHANGE TESTED/CONTROL 1st wk.</th>
<th>RESULTS OF TREATMENT AT 8ND WEEK</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>JRS (normal)</td>
<td>Controls 0/10</td>
<td>0/10</td>
<td>-15/12</td>
<td>0</td>
<td>+++ at 3 wk.</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>JRS (normal)</td>
<td>Controls 0.5</td>
<td>0/10</td>
<td>+20/20</td>
<td>0.05</td>
<td>+++ at 3 wk.</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>JRS (frozen)</td>
<td>Controls 0/10</td>
<td>0/10</td>
<td>-10/10</td>
<td>0</td>
<td>+++ at 3 wk.</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>JRS (frozen)</td>
<td>Controls 0.5</td>
<td>0/10</td>
<td>+12/10</td>
<td>0</td>
<td>+++ at 3 wk.</td>
</tr>
<tr>
<td>6-MP</td>
<td>S-180 (normal)</td>
<td>Controls 0/10</td>
<td>0/10</td>
<td>-1.5/10</td>
<td>0.37</td>
<td>+</td>
</tr>
<tr>
<td>6-MP</td>
<td>S-180 (frozen)</td>
<td>Controls 0/10</td>
<td>0/10</td>
<td>-1.0/10</td>
<td>0.25</td>
<td>+</td>
</tr>
<tr>
<td>6-MP</td>
<td>MLS (normal)</td>
<td>Controls 0/10</td>
<td>3/10</td>
<td>0/+1.5</td>
<td>0.96</td>
<td>Rapid growth</td>
</tr>
<tr>
<td>6-MP</td>
<td>MLS (frozen)</td>
<td>Controls 0/10</td>
<td>3/10</td>
<td>0/+1.5</td>
<td>0.96</td>
<td>Rapid growth</td>
</tr>
</tbody>
</table>

* JRS = Jensen rat sarcoma; S-180 = Sarcoma 180; MLS = Mecca lymphosarcoma.

† Solid tumors: Diameters of treated tumors/diameters of control tumors.

- = No effect; + = moderate inhibition; ++ = marked inhibition; +++ = complete inhibition or destruction of tumors.

63 (8), and Harding-Passey melanoma (9) indicated that these tumors showed distinct differences in their reaction to varying hydrogen ion concentrations. Since no tumor inhibition was noted at pH 7.2 in our previous studies, the pH of our mammalian Ringer solution was always adjusted to 7.2 and checked after immersion of tumor fragments. Thus, the variability of transplantability following this method of freezing cannot be ascribed to pH factors.

It is interesting to note that the Wagner osteogenic sarcoma, which is relatively resistant to chemotherapeutic agents, and the Ridgway osteogenic sarcoma, a relatively sensitive tumor, are both equally susceptible to low temperature. Both All three hamster tumors—namely, Crabb sarcoma, Fortner pancreatic tumor No. 1, and Fortner small intestinal tumor No. 1—did not alter their transplantability by storage at -76°C for 12 months. Crabb hamster sarcoma is relatively drug-sensitive, while Fortner pancreatic tumor No. 1 and Fortner small intestinal tumor No. 1 are very resistant to the action of compounds and antibiotics (13, 14). Among 300 selected compounds tested, only 5-fluorouracil and 5-fluorouridine have a marked inhibitory effect on Fortner intestinal tumor No. 1. On the other hand, Crabb hamster sarcoma is sensitive to many compounds and antibiotics. Therefore, it would appear that no correlation can be established between drug
susceptibility and susceptibility to maintenance in a frozen state. Furthermore, the “relatively chemical responsive” ascites tumors, Carcinoma 1025, Jensen rat sarcoma, and Iglesias rat sarcoma, are resistant to freezing treatment. Our earlier study on the relative sensitivity of the solid and ascites forms of Sarcoma 180 and Ehrlich carcinoma to inhibitory compounds (182 selected compounds) showed that in almost all cases the ascites tumors were more sensitive to the carcinostatic action of agents than were their respective solid tumors (11).

Most probably other factors are involved, including the method of freezing and thawing. To investigate these possibilities, the following experiments were undertaken. Fragments of Adenocarcinoma E 0771, Bashford carcinoma 63, Wagner osteogenic sarcoma, and Ridgway osteogenic sarcoma were dropped into a mammalian Ringer solution at pH 7.3 plus 10 per cent glycerol and kept at 4°-5° C. for 2 minutes immediately prior to transplantation into normal AKR mice to compare the effect of the slow freezing technic (Hauschka [4]) with our technic on the transplantability of Ridgway osteogenic sarcoma. Ampules containing fragments of Ridgway osteogenic sarcoma were placed in a dry ice-alcohol slush and subjected to slow freezing at a cooling rate of about 0.5° C. per minute from 0° to -25° C., then a rapid descent to -76° C. Ampules were stored for either 1 day or 5 days. Results showed that these tumor fragments grew from 40 to 50 per cent, although there was delayed appearance of tumors for 3-6 weeks. On the other hand, the tumor fragments kept 30 minutes at 4°-5° C. and 30 minutes at -25° C., then at -76° C. for 1 and 5 days, grew from 10 to 30 per cent; thus, Hauschka’s slow freezing technic of neoplastic tissues is slightly less harmful to the viability of frozen tumor. However, fragments of Ridgway osteogenic sarcoma and Wagner osteogenic sarcoma subjected to the slow freezing technic of Hauschka from 0° C. to -25° C., and then kept at -76° C. for 3 months, failed to grow when implanted into normal mice.

In an earlier paper (17) it was shown that both Wagner and Ridgway osteogenic sarcomas contained high alkaline phosphatase; the former had about 50 units alkaline glycerophosphatase per gram tissue, whereas the latter about 25 units/gram. This high enzyme activity of osteogenic sarcomas appeared to be retained after storage of tumor tissues at -76° C. for 5 days. Frozen-treated and frozen-untreated osteogenic sarcoma tissues showed essentially similar quantities of alkaline phosphatase by histochemical technic (Gomori) for this enzyme.

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