The Effect of Low Temperatures on Serial Transplantability of Mouse Sarcoma 37*

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The recent investigations of Gye and associates (5—7, 11, 12), dealing with the successful transfer of frozen and frozen-dehydrated tumor tissue, have led to a re-evaluation of this technic as a means of demonstrating a virus or virus-like factor in the transmission of neoplasms. Certain aspects of this subject have been dealt with by us in previous reports (3, 4, 13), and several other investigators (1, 8) have also recently considered various other aspects of this problem.

Two observations in our previous experiments (13) appear to be particularly inconsistent with the behavior of a virus: (a) the loss of the growth potential of the tumor following repeated freezing and thawing and (b) the sudden inability of frozen tumor to grow after several serial transplantations without further exposure to low temperature. Additional observations concerning this latter phenomenon are reported here, utilizing the PR8 strain of Influenza A virus for the purpose of comparing the response of a known virus with tumor tissues.

MATERIALS AND METHODS

Sarcoma 37 was used, as in previous investigations (13); it has been shown that this tumor has a low strain specificity and has now been carried in an unfrozen state through many transplant generations in four different strains used in this laboratory, as well as between these various strains; the latter are St. L. U., Rockland, CAF-1, and CFW. Exposures to various low temperatures were carried out in a manner described previously, but storage was for only 4 hours, except in the case of Influenza A virus.

Chorioallantoic fluid infected with Influenza A virus was stored at —70°C. several times during the course of serial transfer, as shown in Table 2. Infectivity titers were determined according to a method previously described (9), in order to note any change in potency of the virus resulting from storage at low temperatures.

RESULTS

The data in Table 1 show a striking cessation of tumor growth following the fourth serial transplantation of this tumor after a single freezing and thawing. The variations in latent period are not significant, since a similar variability has been demonstrated for unfrozen tumor in our previous report. Tiny nodules could be palpated at the site of transplantation for several days after the fourth serial transplantation had been accomplished, but these gradually disappeared completely during a subsequent period of approximately 1 week.

The experiment shown in Table 1 represents the sixth time we were able to demonstrate this phenomena; it is characteristic of the end result in every instance in which serial transplantation was attempted. This includes three experiments in which serial transfer was limited to a single strain, but in each instance a different strain was used; it also includes three experiments in which combinations of strains were utilized. In addition, two other investigators utilized our frozen tumor material for unrelated investigations and observed the same phenomenon.1,2 Of these eight observations, five were with tumor stored at —30°C., and

**TABLE 1**

<table>
<thead>
<tr>
<th>TRANSPLANT No.</th>
<th>GENERATION</th>
<th>STRAIN</th>
<th>+</th>
<th>—</th>
<th>&quot;takes&quot; (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>19</td>
<td>1</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>CAF-1</td>
<td>8</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Rockland</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

1 D. Harris, personal communication.

2 L. P. Laskowski, personal communication.
three with tumor stored at $-70^\circ$ C. Storage periods have varied from 24 hours to approximately 3 months. The longest period of survival was through seven transplant generations; in the latter experiment CAF-1 mice received tumor which had been stored for 7 days at $-30^\circ$ C.

In contrast to the results obtained with tumor tissue, Influenza A virus frozen at $-30^\circ$ C. can apparently be transferred through many generations without significant loss of potency. Nor does the duration of storage or repeated freezing and thawing appear to have any appreciable effect on the infectivity titer. Data illustrating these points are shown in Table 2.

**TABLE 2**

<table>
<thead>
<tr>
<th>Transplant generation</th>
<th>Treatment</th>
<th>Infectivity titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unfrozen</td>
<td>$-8.0$</td>
</tr>
<tr>
<td>2</td>
<td>$-$</td>
<td>$-7.7$</td>
</tr>
<tr>
<td>3</td>
<td>$-$</td>
<td>$-8.4$</td>
</tr>
<tr>
<td>4</td>
<td>$-$</td>
<td>$-8.4$</td>
</tr>
<tr>
<td>5</td>
<td>$-$</td>
<td>$-7.8$</td>
</tr>
<tr>
<td>6</td>
<td>$-30^\circ$ C., 7 days</td>
<td>$-8.0$</td>
</tr>
<tr>
<td>7</td>
<td>$-$ 5 weeks</td>
<td>$-7.2$</td>
</tr>
<tr>
<td>8</td>
<td>$-$ 12</td>
<td>$-7.0$</td>
</tr>
<tr>
<td>9</td>
<td>$-$ 18</td>
<td>$-8.8$</td>
</tr>
<tr>
<td>10</td>
<td>$-$ 6 months</td>
<td>ND t</td>
</tr>
<tr>
<td>11</td>
<td>Unfrozen</td>
<td>ND t</td>
</tr>
<tr>
<td>12</td>
<td>$-$</td>
<td>ND t</td>
</tr>
<tr>
<td>13</td>
<td>$-$</td>
<td>ND t</td>
</tr>
<tr>
<td>14</td>
<td>$-$</td>
<td>ND t</td>
</tr>
<tr>
<td>15</td>
<td>$-$</td>
<td>$-7.8$</td>
</tr>
</tbody>
</table>

* Figures in this column represent only exponents. For example, complete figure would be $10^{+8}$, $10^{-4}$, etc. t ND represents no determination.

In contrast to the results obtained with Sarcoma 37 frozen at $-30^\circ$ C. or $-70^\circ$ C., tumor placed in liquid nitrogen for 15 minutes before storage at $-70^\circ$ C. can be successfully carried through many transplant generations. Data substantiating this conclusion are shown in Table 3. The variation in time between implantation and first appearance of the tumor (latent period) is again within the limits seen following transplantation of unfrozen tumor mince, as are also the variations in percentage "takes." The low percentage of takes in the first transplant generation is probably caused by technical error.

**DISCUSSION**

These observations, then, confirm and extend those noted previously (13). Sarcoma 37 frozen at $-30^\circ$ C. or $-70^\circ$ C., and subsequently serially transplanted, fails to proliferate after three to seven transplant generations. Likewise, as reported previously, multiple freezing and thawing also cause a cessation of tumor growth. In contrast, Influenza A virus suspended in chorioallantoic fluid can be transferred serially for an indefinite period without apparent loss of infectivity, despite the interjection of numerous exposures to freezing at $-30^\circ$ C. and subsequent thawing. The duration of storage at low temperature appears to be without influence on these phenomena. On the other hand, tumor mince frozen relatively rapidly at $-190^\circ$ C. before storage at $-70^\circ$ C. behaves in a manner similar to virus, in that such neoplastic tissue can also be serially transferred indefinitely; multiple exposures of this type have not been tested to date.

Previously, we had pointed out the importance of the rate of freezing in protecting normal and neoplastic cells from the injurious effects of ice crystal formation; reported differences in experiments with tumors on the effects of rapid and slow freezing represented only different rates of slow freezing. In none of the latter investigations did the rate of freezing approach several hundred degrees per second, which Luyet and Gehenio (10) have shown to be necessary to obtain an intracellular vitreous state; the experiment shown in Table 3 in which tumor was first placed in liquid nitrogen probably represents the nearest approach to such a rate of freezing.

The behavior of tumor tissue after freezing-thawing is therefore different from the behavior of a typical infectious virus. It resembles virus in behavior only when conditions of freezing are such as to minimize injury to tumor cells. These experiments further indicate that the injurious effects may not appear immediately, but may become manifest only after several transplant generations. In this respect, the injurious effects seem to be somewhat different from those more immediately produced by various chemical and biological...
agents. However, we wish to point out again the desirability of carrying tumor, apparently unsuccessfully treated with such agents, through several transplant generations before reaching a conclusion as to an injurious or destructive effect.

The mechanism of this delayed manifestation of cell damage is not apparent from these observations, although several possibilities deserve consideration.

1. Relatively slow freezing may produce a somatic mutation incompatible with continued life of the cell. As far as we have been able to determine, such an alteration has not been demonstrated, nor, in the event of development of a lethal factor, is it likely to require a number of transplant generations before becoming manifest. On the contrary, we have observed cells of frozen-thawed thyroid autotransplants in the guinea pig which respond to thyrotropin stimulation with apparently normal mitotic processes (3). The mechanism of injury of cells in freezing-thawing experiments appears to be related to the formation of ice crystals; chromosomal abnormalities have not been reported.

2. The frozen-thawed cell may develop an antigenicity not resident in the unfrozen tumor cell, to which host animals can develop antibodies in a relatively short time. Such antibodies could conceivably enter tumor cells and build up a sufficient concentration to prove lethal only after several transplant generations.

3. There is also the possibility that freezing-thawing causes destruction of certain critical enzyme systems which may be necessary for the removal of injurious metabolites. A sufficient accumulation of such metabolites to prove lethal to tumor cells may necessitate a period longer than that necessary for tumor to kill several of its hosts and therefore would become manifest only after several serial transfers.

4. On the other hand, these observations may also be interpreted as resulting from the destruction of a stimulating or virus-like agent, following which the tumor is able to maintain an autonomous growth for only several subsequent transplant generations. In support of such a thesis are the observations of Jensen (9) on the growth of tumors in beets and mangels induced by Phytophthora tumefaciens. In these plants the tumor persisted and could be successfully transplanted even after the bacteria had apparently disappeared. While such tumor transplants grew effectively for three or four generations, the abnormal proliferative power eventually disappeared. The validity of such an interpretation would rest upon the demonstration of a continuing stimulating agent in tumor cells, and would therefore tend to disprove the concept of tumor as an autonomous growth.

The present experiments are therefore pertinent to a critical evaluation of the concept of tumor as an autonomous growth. In the case of some chemically and hormonally induced tumors it is known that, even when the growth stimulus is removed before the tumor develops, neoplasms subsequently occur, and such data tend to support the autonomy hypothesis. On the other hand, in the case of mammary tumors in mice and possibly in some other tumors, the "milk factor" may constitute a continuing stimulus. In the case of the Rous sarcoma of the chicken and other virus tumors, the virus may represent both the initiating and continuing stimuli. However, a continuing factor has not been demonstrated in either human cancer or in the vast majority of animal tumors.

From our observations, it does not appear likely that a viral agent in the same sense as that used to define a sub-microscopic infectious agent can be implicated as an initiating agent to the development of mouse Sarcoma 37, but some stimulating agent may be required for the apparent autonomous growth of this tumor.

The concept of initiating and continuing factors in neoplastic growth is, of course, not a new one. If the present observations are to be cited in support of such a concept, then it must also be concluded that, unlike the effect on a virus, freezing-thawing destroys rather than preserves such an agent, except probably under conditions which also preserve the integrity of the cell.

**SUMMARY**

The present experiments confirm and extend those reported previously. Mouse Sarcoma 37, frozen at —30° or —70° C. and subsequently serially transplanted, fails to grow after three to seven transplant generations. This phenomenon occurs both when transplantations are carried out in a single strain or between various strains. On the other hand, tumor mince frozen rapidly at —190° C. before storage at —70° C. can be serially transferred through numerous transplant generations without apparent loss of growth potential.

Influenza A virus suspended in chorioallantoic fluid can be transferred serially in the chorioallantoic cavity of the chick embryo for an indefinite period without apparent loss of infectivity, despite the interjection of numerous exposures to freezing at —30° C. and subsequent thawing. This is in contrast to the effect on mouse Sarcoma 37, noted above, as well as to the injurious effect of repeated freezing and thawing on the growth of this tumor.
Possible mechanisms to explain the effect on serial transplantability are discussed, as is also the relationship of this finding to the demonstration of initiating and continuing factors in neoplastic growth.

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The Effect of Low Temperatures on Serial Transplantability of Mouse Sarcoma 37

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