Multiplication of Influenza Virus in the Ehrlich Ascites Carcinoma*

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The effect of viruses on malignant tumors has been examined by a number of investigators (9). With the conversion of solid tumors into ascites form (8), however, it has become possible to carry out a more definitive study of the growth of viruses in cancer cells. The present communication deals with the normal growth characteristics of a hyperdiploid line of the Ehrlich ascites carcinoma of the mouse and the effects of influenza virus on this tumor. The development of changes in the volume of the tumor, the number of tumor cells, the mitotic index, and the pH of the tumor were analyzed. The tumors were also examined for the presence of virus inhibitors. The results of injecting influenza virus into tumors of different ages were observed in terms of the growth of the virus in the tumor, the destruction of tumor cells, the distribution of the virus in other tissues, the lethal effect on mice, and the pH of the tumor.

MATERIALS AND METHODS

**Virus.**—WS (neurotropic) type A influenza virus had been transplanted for 55 passages (1) in a near-tetraploid line of the Ehrlich ascites tumor,1 and was able to proliferate in the tumor used in the present study.

**Tumor.**—This was the Lettré, hyperdiploid line, having a modal count of 42 chromosomes per cell. It was originally procured from Dr. T. S. Hauschka.

**Mice.**—Of several strains of mice examined, the National Institutes of Health strain of albino mouse was found to be the most expedient for carrying the tumor. Female mice weighing 23-26 gm. were employed in all studies.

**Virus assay.**—Dilutions of the virus were prepared in 10 per cent horse serum in 0.85 per cent NaCl. The 50 per cent egg-infective dose (EID$_{50}$) was determined by employing six 10-day embryos per tenfold dilution and inoculating each with 0.05 ml. The inoculated eggs were incubated at 37°C. for 3 days before being screened for the presence of hemagglutinin.

**Normal characteristics of the tumor.**—Studies on the growth pattern of the tumor were carried out on mice which had been inoculated with 0.2 ml. of a 5-day tumor (approximately 40 million tumor cells). Six mice were sacrificed per day, and studies were executed on a pool of an equal quantity of tumor from each mouse. Tumor volumes were estimated by aspiration procedures, after which the cells were enumerated by standard blood cell counting technics. The mitotic indices were determined at the same time each day (10 A.M.) by making squash preparations in aceto-orcein, as suggested by Dr. Hauschka. To measure the pH, the tumor fluid was removed from the mouse with a needle and syringe. It was then injected into a Beckman hypodermic-type electrode (#290-80) which had just been flushed with pure nitrogen.

**Virus distribution.**—Tumor-bearing mice used to ascertain the distribution of virus in the lungs and brain bore 5-day tumors at the time of virus inoculation. Each of 25 tumor-bearing mice and 25 normal mice was given an intraperitoneal inoculation of 0.1 ml. of a 1:100 dilution of a virus preparation having an EID$_{50}$ titer of log 7.5. Three mice per day were sacrificed from each group, and the tumor, brains, and lungs were appropriately pooled. The last two, after being ground in a mortar with alundum, were prepared as 20 per cent suspensions by weight in 10 per cent horse serum.

**Cell destruction by the virus.**—An analysis of the extent of tumor cell destruction in 24 hours, under circumstances in which the cells were present in excess of the inoculated infective virus particles, was carried out after inoculating 4- to 10-day tumors with 0.2 ml. of a 1:10 dilution of a virus suspension having an EID$_{50}$ titer of log 7.1. Concomitant volumes from six tumors of each age group were pooled and examined. In this same study, the multiplication of virus in 4-, 7-, and 10-day tumors was analyzed over a 5-day period. Six mice were
Inhibitor determinations.—In the analysis of the tumor for inhibitors of hemagglutinin or infectivity, dilutions of the cell-free tumor fluid and the serum controls were made in a solution containing 0.85 per cent NaCl and 0.1 per cent CaCl₂ · 2H₂O. These were prepared in twofold steps in a volume of 0.3 ml., starting at 1:5. The serum and tumor fluid were not heated prior to use. The titers given refer to the initial dilutions, i.e., before 0.3-ml. volumes containing five agglutinating doses of virus and the 1 per cent suspension of chicken erythrocytes were added. Fifteen minutes elapsed between the addition of the virus and the erythrocytes. The agglutination pattern was read in about 30 minutes. A mouse line of the Seerey A prime strain of influenza virus was employed as the alpha indicator. The allantoic fluid containing this virus was first held at 37° C. for 2 hours to destroy any alpha inhibitor that might be present. It was then converted to the indicator state by being heated at 56° C. for 30 minutes. The WS virus could not be converted to an indicator. An unheated egg line of the Seerey virus was used as the beta indicator. For details see the following references: Anderson (2), Brans et al. (3), Briody et al. (4), Francis (6), and Stone (11). In neutralization studies in the chick embryo, with the Seerey egg-line virus used as the beta indicator, 0.5 ml. of cell-free tumor fluid from 10-day tumors was inoculated into the allantoic cavity of each egg. A suspension of the virus was assayed in eggs containing tumor fluid and in eggs without tumor fluid. These were incubated at 35° C. and tested for the presence of hemagglutinin in 48 and 72 hours. The titers were then compared to determine the degree of neutralization by the tumor fluid. Similar tests were carried out with the mouse line of Seerey virus and the WS virus.

Viability staining.—Eosin was employed in a manner described elsewhere (5). Trypan blue was prepared by saturating 100 ml. of Ringer’s solution with 2 gm. of dye and centrifuging out any undissolved material. The solution was diluted 1:100 for use, and to 1.2 ml. of this was added 0.05 ml. of the tumor. In additional respects, it was handled the same as eosin.

RESULTS

Normal characteristics of the tumor.—Following the injection of 40 million tumor cells, the increase in the tumor volume and in the total number of tumor cells (Chart 1) proceeded in a more or less parallel manner. A marked rise began at the 9th day and continued to the 11th day. Thereafter, the volume continued to increase, while the number of tumor cells declined. The animals invariably died on the 14th day. Looking at the combined effects in terms of tumor cells per unit of volume (Chart 1), it is evident that the changes in volume and cells, although somewhat parallel, were not proportional. The maximum concentration of cells was reached on the 5th day.

The percentage of nontumor cells, excluding erythrocytes, was found to be about 15 per cent on the 1st and 2d days of tumor development. It then dropped to about 6 per cent and stayed at this level until the 14th day, when it rose to about 15 per cent. The precise number of erythrocytes was not determined, but it may be stated that it has been unusual to detect any red coloration in the tumor. The mitotic index of the tumor cells was highest on the 1st day, being 6.9 per cent. After this, it remained in the neighborhood of 4 per cent until the 11th day, at which time it fell to 2 per cent and continued at this level. It should be noted that the 11th day is the point at which the total tumor cell count dropped off.

The pH of the tumor up to the 14th day was found to fluctuate between 6.85 and 7.15, usually being close to 7.00. On the 14th day, or when the animals became moribund, the pH went as high as 7.47 before the animals died. Following infection of 5-day tumors with 0.2 ml. of a 1:10 dilution of virus preparations having EID₆₀ titers in the vicinity of log 7.0, the pH went to 7.40 in 48 hours. Generally, these animals died 3-4 days after infection.

Stability of the virus.—When the WS virus was assayed by methods involving dilution in saline, incubation of the inoculated eggs at 35° C., or incubation for only 2 days, the titers were 2-3 logs lower than those resulting when the virus was assayed in tumor-bearing mice. The 50 per cent tumor-infective dose was determined following the intraperitoneal inoculation of six mice bearing 5-day tumors per dilution of the virus. Further studies revealed that egg titers which were comparable to the tumor titers could be obtained by diluting the WS virus in 10 per cent horse serum and incubating the inoculated eggs at 37° C. for 3 days. The egg assay is preferred because of the short time required. When tumor-bearing mice are employed, it is necessary to wait 10 days to obtain an answer.

Virus proliferation and cell destruction.—The WS (neurotropic) virus is not easily adapted to the tumor. After a number of attempts, Ackermann and Kurtz (1) succeeded in adapting this virus to a tetraploid line of the tumor on only one occasion. This involved somewhat circuitous procedures.
Numerous attempts at adaptation were unsuccessful in our laboratory. It is of interest that the virus which Ackermann and Kurtz adapted to the tetraploid tumor was able to proliferate in the hyperdiploid line employed in the present study.

After infecting the tumor, the virus migrated to the brain and lungs (Table 1). This migration also occurred in normal mice which had been inoculated intraperitoneally with virus. In view of the high concentration of virus produced in the tumor, it is not surprising that it appeared more quickly in the brain and lungs of the tumor-bearing mice. All the tumorous mice were either sacrificed or had died in 5 days. Of the ten normal mice which had not been sacrificed, all were alive 3 weeks after inoculation.

When equivalent amounts of virus were inoculated into tumors 4–11 days old and when tumor cells were present in excess of the infective virus particles at the time of inoculation, the tumors were equally capable of supporting virus growth for a certain length of time. This is inferred from the fact that the number of cells destroyed in 24 hours was nearly the same regardless of the age of the tumor. The actual values for 4-, 5-, 7-, 9-, 10-, and 11-day tumors, as may be calculated from the data in Table 2, were, respectively, 2.3, 2.5, 2.5, 3.0, 3.0, and 2.0 × 10⁶ tumor cells. Projected studies up to the 4th day, relating the age of the tumor to the percentage of cells destroyed, are also presented in Table 2. With the increase in the number of cells, the time required to destroy almost all the tumor cells became protracted. The growth of virus in the 4-, 7-, and 10-day tumors disclosed extreme degeneration and partial disintegration in many. In view of this, the results could not be interpreted with any confidence.

Inhibitors.—There was a small amount of alpha inhibitor in the early tumor, and as the tumor developed there was a moderate increase (Table 3). It is of some interest to note that, while the ratio of alpha-inhibitor to beta-inhibitor was 1:8 in mouse serum, it was reversed in the tumor. No alpha-inhibitor could be detected in tumor fluids at 1, 2, and 3 days after infection of the tumors with virus. Presumably, the virus had destroyed...
all the inhibitor. In this analysis the virus was first removed from the tumor by repeated centrifugation, and the remaining tumor fluid was extracted with ether. This was necessary to remove a substance, possibly lipide from the disintegrated tumor cells, which caused the virus-free tumor fluid to give an atypical hemagglutination to a low titer. In control tests, ether extraction was found to have no effect on either the alpha or beta inhibitor titers of normal tumor fluids.

What role any inhibitor may play in conditioning the multiplication of influenza virus in the tumor is only speculative. The neutralization of the virus, as found in this study, may be a phenomenon seen only in the chick embryo to which cell-free tumor fluid has been added. This is suggested by the observation that, when the 50 per cent in-

**TABLE 2**

<table>
<thead>
<tr>
<th>TUMOR AGE:</th>
<th>4 DAYS</th>
<th>5 DAYS</th>
<th>7 DAYS</th>
<th>9 DAYS</th>
<th>10 DAYS</th>
<th>11 DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells</td>
<td>$\times 10^6$</td>
<td>$\times 10^6$</td>
<td>$\times 10^6$</td>
<td>$\times 10^6$</td>
<td>$\times 10^6$</td>
<td>$\times 10^6$</td>
</tr>
<tr>
<td>Time after inoc. with virus†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Hours</td>
<td>29.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Day</td>
<td>96.0</td>
<td>48.1</td>
<td>32.0</td>
<td>32.6</td>
<td>12.6</td>
<td>12.6</td>
</tr>
<tr>
<td>2 Days</td>
<td>95.6</td>
<td>93.8</td>
<td>83.4</td>
<td>83.0</td>
<td>88.3</td>
<td>44.6</td>
</tr>
<tr>
<td>3 Days</td>
<td>95.3</td>
<td>96.5</td>
<td>94.0</td>
<td>88.5</td>
<td>88.3</td>
<td>59.0</td>
</tr>
<tr>
<td>4 Days</td>
<td>95.5</td>
<td>96.5</td>
<td>93.4</td>
<td>94.9</td>
<td>93.0</td>
<td></td>
</tr>
</tbody>
</table>

† The tumors were given inoculations of 0.2 ml. of a 1:10 dilution of a virus preparation having an EID₅₀ titer of log 7.1.

‡ The calculated percentages are based on the total number of cells present at the time the virus was inoculated into the tumor.

**TABLE 3**

<table>
<thead>
<tr>
<th>INHIBITOR TITERS*</th>
<th>TUMOR FLUIDS</th>
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<tbody>
<tr>
<td>Alpha</td>
<td>Beta</td>
</tr>
<tr>
<td>4-Day</td>
<td>30</td>
</tr>
<tr>
<td>5-Day</td>
<td>30</td>
</tr>
<tr>
<td>6-Day</td>
<td>30</td>
</tr>
<tr>
<td>7-Day</td>
<td>60</td>
</tr>
<tr>
<td>8-Day</td>
<td>120</td>
</tr>
<tr>
<td>9-Day</td>
<td>160</td>
</tr>
<tr>
<td>10-Day</td>
<td>80</td>
</tr>
<tr>
<td>11-Day</td>
<td>160</td>
</tr>
<tr>
<td>12-Day</td>
<td>120</td>
</tr>
</tbody>
</table>

* A heated mouse line of the Seerey A prime strain of influenza virus was employed as the alpha-indicator. The above fluids all gave titers of <10 in control tests with unheated mouse-line Seerey virus. An unheated egg line of the Seerey virus served as the beta-indicator.

The low hemagglutinin inhibition titers against the egg-line virus (Table 3) suggest the presence of beta-inhibitor in the tumor. In the more sensitive neutralization test (Table 4), however, mouse-line virus was inhibited to the same degree as the egg-line virus, thus indicating the action of a neutralizing substance other than beta-inhibitor. This could be properdin, since it is found in mouse serum in moderate concentration (10). No beta-inhibitor could be detected in virus-infected tumor fluids.
the cells more quickly than in the chick embryo, thereby allowing less time for a neutralizing substance to exert its effect. Although there is approximately the same number of cells in a 5-day tumor and in the chorioallantoic membrane of a 10-day chick embryo, the freely suspended tumor cells provide much more surface area and adsorbing potential.

**SUMMARY**

When 40 million tumor cells were injected intraperitoneally into susceptible mice, a uniformly fatal outcome ensued in 14 days. The progress of the tumor was accompanied by an increase in the number of tumor cells, which reached a peak of 16 \times 10^6 cells on the 11th day, and by a maximal volume on the 14th day of 19 ml. The number of cells/cu mm reached a peak of 25 \times 10^4 cells on the 5th day and declined thereafter. The mitotic index was highest on the 1st day at 0.2 per cent. The concentration of nontumor cells approached 15 per cent the first 2 days, dropped to 6 per cent, and stayed at this level until the 14th day, when it returned to 15 per cent. The pH of the tumor fluid remained close to 7.0, but just before the animals died it rose to 7.5. The concentration of alpha-inhibitor increased moderately as the tumor developed, reaching a peak on the 9th day.

Concomitant with multiplication of the virus in the tumor there was a marked increase in the number of tumor cells destroyed and a rise in the pH of the tumor. Low levels of virus were found in the lungs and brains of normal as well as tumor-bearing mice, and the normal mice survived the infection. Death of the tumor-bearing mice ensued in 2-5 days after injection of virus. The cause of death was not investigated, but it may be related to the toxicity observed in connection with high concentrations of influenza virus. Alpha-inhibitor, although present in normal tumors, was not detectable in virus-infected tumors.

**ACKNOWLEDGMENTS**

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