Viral Oncolyis Studies with a Metastatic Human Tumor in Chicks*

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The fact that many viruses will propagate in, and some will damage, tumor tissue has been demonstrated by many investigators using many experimental systems. Oncotropism and oncolysis have also been studied in clinical experiments. The literature through 1953 has been reviewed by Moore (5). More recent clinical studies include the systemic administration of certain arthropod-borne viruses1 and adenoviruses (8, 9) and local or regional injection of several adenoviruses (7). In these clinical studies virus frequently propagated in tumor tissue. The oncotropism was sometimes selective, and in one study (10) virus was demonstrated within the cancer cells. Tumor regression resulted occasionally but was always transient, incomplete, and seldom of therapeutic significance.

The development of human cancer cell lines which can be maintained in continuous passage has made possible certain studies of human cancer in the laboratory. Tissue culture is not well suited to the evaluation of oncolytic viruses because it differs so markedly from any in vivo situation. The study of viral oncolysis in human tumors growing in conditioned animals has been difficult because the conditioning (x-ray or cortisone) which permits the growth of the heterologous tumor also makes the animals unduly susceptible to virus infections (6, 12).

Dagg and Kornofsky and co-workers (1, 13) demonstrated that the human epidermoid carcinoma #3 (H.Ep. #3) growing on the chorioallantoic membrane (CAM) of the fertile hen’s egg would metastasize into the embryo. A high percentage of these embryos continued to develop, and the resulting chicks bore metastatic human cancer—usually killed within 4 weeks after hatching. Chicks less than a week old can be infected by many of the arthropod-borne viruses with viremia that lasts as long as a week but with no apparent ill effects (11, 13). Thus, the baby chick bearing metastatic human carcinoma seemed uniquely suitable for the study of viral oncolysis.

MATERIALS AND METHODS

A fragment of H.Ep. #3 freshly harvested from previous CAM passage was placed onto the dropped CAM of 8-day fertile hens’ eggs. Incubation was then continued in individual compartments until hatching. After hatching, shells were examined for CAM tumors. If none was found, chicks were discarded from the experimental group; but many were kept for observation. When CAM tumors were found they were measured, and chickens were so distributed into treatment and control groups that CAM tumors of various sizes were proportionately represented. Chicks were inoculated subcutaneously not later than 72 (usually 48) hours after hatching, with 0.1 ml. of the virus preparations. Mouse brain-passaged viruses were used as 20 per cent suspensions, and tissue culture-virus preparations were used as undiluted supernatants. Such inocula contained at least 100 and often over 1,000,000 mouse intracerebral LD50 doses of virus. Untreated controls were included in every experiment in numbers equal to those in the treated group. Treated and control chicks were housed in separate incubators and examined daily for deaths and for development of tumors.

In all but one study, a few of the chicks which did not have tumors on the CAM were also injected with virus and bled at intervals to ascertain infectivity of the virus. In a few studies, groups of chicks which did have tumor on the CAM were set aside to be sacrificed serially for study of virus distribution.

Passage history of the Egypt 101 virus preparations was as follows: mouse brain preparation, seven or eight mouse passages since original isolation from human blood; H.Ep. #2 passage line, seven mouse brain passages followed by 67-142 serial passages in stationary cultures of human epidermoid carcinoma #2 growing on glass; H.Ep. #3 passage preparation, seven mouse brain passages followed by 126 H.Ep. #2 tissue culture passages followed by seventeen to 48 passages in H.Ep. #3 in stationary tissue cultures of H.Ep. #3 cells on glass.

All other viruses were from serial mouse brain passage, but the number of passages is unknown. Statistical methods and x2 tables are from Hill’s textbook (3).

RESULTS

Significance of membrane tumor size.—Although chicks were proportionately distributed into con-
trol and treatment groups according to the size of CAM tumors at hatching, analysis of correlation between diameter of the CAM tumor and subsequent development of metastases in the chicks showed that this precaution was unnecessary.

Comparison of CAM tumor size vs. day of chick death showed no correlation (r = +0.04). Comparison of CAM tumor size vs. day of first detectable metastases in the chicks gave a correlation coefficient of −0.22 (standard deviation of the coefficient ±0.08), indicating that large CAM tumors did tend to cause earlier metastases, but this tendency was so slight as to have little or no effect on experimental results (regression coefficient = −0.2).

In all, 474 chicks hatched from H.Ep. #3-inoculated eggs were observed until day 35 or until death. Of the 356 which had CAM tumors at hatching, 284 (60 per cent) died before day 35, whereas mortality among the 118 which did not have CAM tumors was only 27 per cent. Metastatic tumors were proved in 187 chicks by either external inspection or autopsy. One of treatment with all viruses except West Nile. It should be noted that because of the variability in different experiments each experimental group can only be compared against its own simultaneous control group. When more than one experiment was conducted with a virus, all are combined in these tabulations, because separate analyses of individual experiments gave similar results. Bwamba and Russian viruses showed no effect on chick survival or tumor growth. Bunyamwera, Mengo, and Semliki Forest viruses appeared to hasten death. (Two of the eleven Mengo virus-infected chicks became paralyzed.) Because of the small numbers in these experimental groups the results could easily have resulted by chance, but they do suggest that these viruses may be pathogenic in tumor-bearing chicks even though they cause no apparent disease in normal chicks—possibly owing to decreased host resistance or excessive virus propagation in the tumor tissue (see virus distribution data below). The only virus in Table 1 which appeared to prolong survival of the tumor-bearing chicks was Uganda virus. The differences between Uganda-treated chicks and untreated controls were significant (p < 0.05).

### TABLE 1

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>NO. CHICKS</th>
<th>MEAN* SURVIVAL TIME (DAYS)</th>
<th>MEDIAN</th>
<th>SURVIVORS*</th>
<th>REGRESSIONS</th>
<th>NO. TUMORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>C</td>
<td>Rx</td>
<td>C</td>
<td>Rx</td>
<td>C</td>
<td>Rx</td>
</tr>
<tr>
<td>Bunyamwera</td>
<td>15</td>
<td>15</td>
<td>18</td>
<td>15</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Bwamba</td>
<td>8</td>
<td>10</td>
<td>19</td>
<td>19</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Mengo</td>
<td>11</td>
<td>11</td>
<td>17</td>
<td>12</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Russian SSE</td>
<td>4</td>
<td>4</td>
<td>17</td>
<td>16</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Semliki Forest</td>
<td>8</td>
<td>10</td>
<td>26</td>
<td>16</td>
<td>55</td>
<td>6</td>
</tr>
<tr>
<td>Uganda</td>
<td>29</td>
<td>29</td>
<td>17</td>
<td>22</td>
<td>11</td>
<td>19</td>
</tr>
</tbody>
</table>

* Studies terminated 35 days after virus inoculation. For computation of mean survival times survivors are included as dying on day 35.

† C = controls; Rx = virus-treated.

A hundred and seventy-five of these were in chicks which had tumors on the CAM when hatched—a proved metastasis rate of 49 per cent—whereas metastases were observed in only 10 per cent of the 118 chicks which did not have tumors on CAM when hatched. In addition, many deaths occurred before day 10 and were not investigated for cause. Such early deaths occurred in 30 per cent of the CAM-positive chicks and in only 13 per cent of the CAM-negative chicks. If it be assumed that most of these early deaths were due to tumors, it would follow that nearly 80 per cent of chicks which hatched from eggs with CAM tumors died of metastatic tumor, and less than 25 per cent of chicks from eggs without tumor died of metastases.

**Viral oncolysis data.**—Table 1 presents results of treatment with all viruses except West Nile.
and their parallel controls in mean and median survival time and in number of 35-day survivors could be due to chance in experiments involving such small numbers of chicks. (Analysis of mean survival times and per cent survivors gave P values = >0.05). It may be of significance, however, that during the 2d and 3d week after virus inoculation the cumulative mortality in the virus-treated group lagged behind the controls in a manner similar to that seen in the experiments with West Nile virus, as illustrated in Chart 1 and discussed below.

Table 2 and Chart 1 summarize results of experiments on the Egypt 101 isolate of West Nile virus. The mouse brain and H.Ep. #2-passage virus preparations had no effect upon the H.Ep. #3 tumor in chicks as judged by average survival times or frequency of survivors or tumor regressions. However, with the H.Ep. #3 tissue culture-passaged virus, the average survival time was slightly increased as judged by mean and median values and by cumulative mortality curves. The frequency of survival was also slightly increased, owing to more frequent tumor regressions. There was no difference in survival during the first week after virus inoculation (Chart 1). During the 2d and 3d week very few of the treated chicks died, but the incidence of death in the control group increased progressively. During the 4th week the mortality in the virus-treated group increased sharply, but total mortality remained less than in the control group.

While these differences do not show a therapeutically impressive degree of oncolysis by the H.Ep. #3-passage virus, it is unlikely that they occurred by chance. Analysis of the significance of the difference between mean survival times of chicks treated with the H.Ep. #3-passage Egypt virus and their parallel controls gave a P value of 0.01. χ² analysis of the number of regressions in treated vs. control groups gave a P value of <0.01. Thus, the observed differences between treated and control chicks could be expected on the basis of pure chance only about once in 100 times. Significance of these differences is also suggested by the fact that in each of the six experiments with H. Ep. #3 tissue culture-passaged Egypt virus (from which pooled data are presented in the table) survival was consistently slightly greater in the virus-treated groups. Caution is still necessary, however, in accepting differences of this magnitude as biologically significant when dealing with a system with many and great inherent variables.

These data suggest that infection by the H.Ep. #3 tissue culture-passaged line of Egypt virus culture-passaged virus, the average survival time was slightly increased as judged by mean and median values and by cumulative mortality curves. The frequency of survival was also slightly increased, owing to more frequent tumor regressions. There was no difference in survival during the first week after virus inoculation (Chart 1). During the 2d and 3d week very few of the treated chicks died, but the incidence of death in the control group increased progressively. During the 4th week the mortality in the virus-treated group again increased sharply, but total mortality remained less than in the control group.

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These data suggest that infection by the H.Ep. #3 tissue culture-passaged line of Egypt virus
Viremia studies were usually limited to one chick on each of 2 days during the 1st week after virus inoculation, it is probable that failure to prove viremia in some of the experiments was owing merely to insufficient study.

Virus distribution (planned for detailed presentation elsewhere) was studied in several chickens at various times after inoculation of Egypt 101 virus. During the first 3 days after inoculation virus was widely distributed through the viscera but was rarely found and only in low titer in brain and eye. On days 6 through 8 virus was still widely distributed in viscera and was also found in increasing titers in brain and eye. On days 9–13 virus was found in brain and eye but rarely in viscera. No tumors which could be tested for virus were found in the chicks sacrificed on days 1 through 6. Four of the chickens studied on days 7 through 10 had tumors, and of these tumors two contained virus in high titer. From day 16 through 27, four chicks were studied which had metastatic tumors. In each of these only tumor contained high titers of virus, and the only other tissues containing any detectable virus were brain or eye. Four chicks without detectable tumors were also studied during this same period (day 16 through 27) and had no detectable virus in any of the tested viscera or eye or brain. In normal chicks (from uninoculated eggs) Egypt 101 virus has not been demonstrated in any tissue later than 10 days after inoculation. These findings demonstrate that Egypt 101 virus becomes localized in tumor tissue, and the fact that it was found in tumors in high titers long after it disappeared from other tissues indicates that it was actually propagating in the tumor tissues.

DISCUSSION

Although the oncolytic effect of Egypt 101 virus which has been passaged through H.Ep.#3 cells in tissue culture was not impressive, it is of interest that this passaged virus gave highly suggestive evidence of a slight oncolytic effect against this same cell type in the chick, whereas all other preparations of West Nile and of the other viruses tested had no oncolytic effect in this system. If these data do indicate an actual development of increased oncolysis in the H.Ep. #8-passaged virus, it is of interest because the adaptation was achieved by serial tissue culture passages in the cell type against which an oncolytic effect was being sought. Studies by Huebner and co-workers (4), using Coxsackie and adenoviruses in HeLa cell cultures, failed to demonstrate increased oncolysis after tissue culture passage, although such adaptations were achieved through passage in an in vivo system. The results are of particular interest, because the oncolytic effect was directed against a type of cell (epidermoid carcinoma) which has never shown convincing evidence of regression in clinical trials of this and other viruses. These findings suggest that serial virus passage through tissue cultures of an individual patient’s carcinoma might provide a means of increasing the therapeutic potential of a virus for that individual.

In comparison with other methods now available for the laboratory study of viral oncolysis against human cancers, this technic most closely approaches the biological situation of clinical virus trials, because it utilizes a nonconditioned host bearing metastatic human cancer and susceptible to a nonpathogenic infection by the viruses under study. This method is, however, technically complex and expensive in time and materials.

SUMMARY

The effect of virus infections upon chicks bearing metastatic human epidermoid carcinoma (H. Ep. #3) was investigated. Bunyamwera, Bwamba, Mongo, Russian, and Semliki Forest viruses showed no evidence of antitumor effect in this system. Uganda caused a slight but statistically insignificant increase in survival. Egypt 101 (West Nile) virus, after serial passage through tissue cultures of the H.Ep. #3 cell, caused slight increases in survival and in frequency of tumor regressions. Although statistically significant, this apparent oncolytic effect must be accepted with caution in view of the many variables in this experimental system. Egypt 101 virus, which was propagated in mouse brain or serially passaged through tissue cultures of H.Ep. #8 cells, had no oncolytic effect.

Studies of the distribution of Egypt 101 virus indicated that virus was present in high titer in the tumors longer than in normal tissues of tumor-bearing or normal chicks.

There was no relationship between size of the tumors on the chorioallantoic membrane and time of chick death and only a slight inverse correlation between size of tumor on the membrane and time of appearance of metastases.

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REFERENCES


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