Claude's Chicken Tumor Virus-10: Local Variability in Virus Titer and Effect of Amputation

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SUMMARY

Infectivity titrations of halves of individual tumors produced in the wing web of chicks with serial dilutions of chicken tumor virus-10 showed marked variability in titratable virus. No pattern of recoverable virus was apparent and virus-neutralizing antibodies were not detected in sera from the birds. Attempts to prevent the development of chicken tumor virus-10 tumors by amputation immediately after infection failed, and tumors developed at the site of amputation. Chicken tumor virus-10 hyperimmune sera injected i.m. protected against subsequent challenge with homologous virus when administered 1 day before, but not 1 day after, infection.

INTRODUCTION

CT-10 is a hard spindle cell sarcoma which is highly invasive but rarely metastasizes. Claude and Murphy (7) and Claude et al. (8) demonstrated that desiccates of filtrates of transplanted CT-10 produced tumors in chickens, and the tumor cells contained virus-like particles measuring 60 to 70 nm in diameter located near the cell membrane. Claude (5, 6) separated an inhibitor in CT-10 extracts by high-speed centrifugation and demonstrated that this inhibitor was similar to and probably represented serum antibody. Immunological studies in this laboratory (17, 18) with cross-neutralization tests revealed an antigenic relationship between Claude's CTV-10 and the Bryan strain of RSV. In addition, CTV-10 produced well-defined red and white pocks on the CAM of embryonated chicken eggs, suggesting the existence of a mixed viral population in stocks of CTV-10 (17, 18). The CAM was found to be the method of choice for the bioassay of CTV-10, and enumeration of pocks, irrespective of their type, was related to the dilution of CTV-10 which was inoculated. Histological studies revealed that the white pocks were carcinosarcomas with numerous epithelial pearls, while the red pocks were composed of ectasia-like lesions and multiple hemorrhagic cysts. The vascular lesions appeared 4 days after infection, followed by the development of carcinosarcoma 3 days later. No evidence of cell destruction was found to be associated with the vascular lesions produced by CTV-10 (17, 18).

The present studies were undertaken to characterize further some virus-host interactions with CTV-10, with the aim of establishing a model avian tumor virus system to complement the existing and intensively studied RSV.

MATERIALS AND METHODS

CTV-10. Methods for preparation of frozen stocks of CTV-10 from chicken tumors were previously described (17, 18). A 10% suspension (w/v) of tumor homogenized for 1 min was allowed to defoam for 15 min. This procedure was repeated twice, and the suspension was centrifuged twice at low speed and then at 105,000 × g for 60 min. The pellet was resuspended to 10% of the original volume in 0.85% NaCl solution containing 10% heat-inactivated calf serum, distributed in sealed glass ampuls and stored at −70°.

Chickens and Tumors. Unsexed White Leghorn chicks (Shamrock Farms, North Brunswick, N. J.), 1 to 3 days old and healthy in appearance, were inoculated s.c. with 0.2 ml of appropriately diluted virus into the left wing web, as previously described (14, 18), and observed daily for periods up to 7 weeks. Whole tumors removed from the wing web after treatment of the skin with 70% ethanol were dissected free of surrounding tissue, cut in half, and stored at −70° in screw-cap vials. In a similar manner, wing webs free of tumor were also collected for control assay. Histological sections of tissues fixed in 10% formalin-0.85% NaCl solution were cut at 6 μ and stained with hematoxylin and eosin.

Bioassay. The average latent period for tumor production was determined by the graphic rankit method, which utilizes probability units (rankits) based on a normal distribution curve for analysis of graded biological responses with experimental groups involving fewer than 30 animals (3). Birds were examined daily for development of tumors, the average rankit values were calculated for the respective dose groups, and the data were plotted on the ordinate versus the reciprocal of days × 100 (100/days) on the abscissa. Days

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4The abbreviations used are: CT-10, chicken tumor-10; CTV-10, chicken tumor virus-10; RVS, Rous sarcoma virus; CAM, chorioallantoic membrane; pfu, pock-forming units, ED50, median effective dose.

Received June 30, 1969; accepted February 12, 1970.
Frosted specimens were thawed, weighed, and ground in a mortar with sterile Alundum. Sufficient diluent (0.85% NaCl solution, 2% heat inactivated horse serum, 100 units of penicillin G, and 100 µg of streptomycin sulfate per ml) was added to make a 10% suspension (w/v), which was centrifuged for 10 min at 500 × g. Inoculums of 0.1 ml of the supernatant fluids were injected into fals air sacs prepared over the CAM in 9-day-incubated eggs, the openings were sealed with colored collodion, and the eggs were incubated upright for 10 days at 38° in a humidified, forced-draft incubator (10). The eggs then chilled at 4° for at least 4 hr, and the pocks were counted at 10 X magnification with a stereoscopic microscope. No attempts were made to differentiate between red and white pocks and all totals reflect a sum of pocks regardless of type (18). The number of pfu calculated from these totals were used in comparing infectivity titers of the various biological materials assayed.

Hyperimmune Sera and Virus Neutralization. Hyperimmune anti-CTV-10 sera were prepared by s.c. inoculation into the wing web of 6 groups of White Leghorn chicks with 0.2 ml volumes of stock CTV-10 diluted 10^4 through 10^6. Birds from each dose group were challenged in the opposite wing web at 4 weeks with either a 10^3 or a 10^3 dilution of standard CTV-10. Twelve weeks after the original virus inoculation, one-half of the birds randomly selected from each group were exsanguinated, and at 16 weeks the remainder were bled. Blood samples taken from chickens by cardiac puncture and allowed to clot individually at room temperature were held overnight at 4°. The clots were cut for 10 mm. The sera were recentrifuged and stored at —20° in 10-fold dilutions of standard virus, and the tubes were incubated for 45 min at 4°. The mixtures in 0.1-ml volumes were inoculated onto the CAM, and the pfu for each group were harvested, cut in half, and stored as described. Each bird was bled before the tumor was removed. Pooled sera of each dose group from either single day or adjacent days of harvest were tested for neutralizing antibodies to CTV-10.

Chart 1 shows that all birds inoculated with a 10^2 dilution (10^2.5 E50/ml) or more CTV-10 developed tumors. With less virus (4) tumor incidence decreased, and only 6% of the 40 birds receiving 10^6 dilution showed growths.

RESULTS

Relation of Infecting Dose to Recovery of Virus from Tumors. Seven groups of 10 to 40 3-day-old birds, each from the same lot of chickens, were inoculated into the wing web with serial 10-fold dilutions of CTV-10. When the tumors, examined daily, reached a size of 2 to 3 g, they were harvested, cut in half, and stored as described. Each bird was bled before the tumor was removed. Pooled sera of each dose group from either single day or adjacent days of harvest were tested for neutralizing antibodies to CTV-10.

Infectivity titers of the halves of the respective tumors are indicated in Chart 2 with the lower titer of each pair plotted 1st as an open bar. The results show great variation in infectivity, both between portions of the same tumor and among growths in the same dose group. The values in Chart 2 summarized in Chart 3 show the average log potency for infectivity, both between portions of the same tumor and among growths in the same dose group. The character of the distribution of virus titers relative to dose was consistent with a linear regression of infectivity levels over the entire initiating dose range. The significance of this 1.5 log difference is uncertain, since it was based on values of considerable variation. In Chart 4, the neutralization indices of pooled sera and infectivity titers are plotted against the day on which the tumor was collected. With this type of analysis, there was again no obvious pattern of virus yield relative to the dose inducing the tumors. The neutralization indices show insignificant levels of antibodies in sera from birds prior to termination of the experiment on the 35th day. Previous experience (17, 18) shows that antibodies appeared much later, e.g., 8 to 12 weeks after infection.

Failure of Amputation to Prevent Tumor Development. A series of 270 chicks inoculated s.c. into the wing web with CTV-10 diluted 10^-1 (10^2.5 E50/ml) was divided into 3 equal groups. The infected wings in the 1st group (amputees) were amputated at 4 hr intervals up to 24 hr after infection. The uninoculated wings of an equal number of chicks were amputated (surgical controls), and the remaining chicks served as infected controls (no amputation). Chicks were
regularly where the infected wing was amputated, but rarely at the amputation wound in the uninfected (opposite) wing. Of particular interest were 2 subgroups of 15 chicks each in which the wings were amputated within 5 mm after infection. Even with this short delay, all of the amputees (infected wing) developed tumors at the stump and died 2 to 3 weeks after infection. Stump tumors did not appear in the surgical controls (opposite wing), but lethal tumors occurred at the site of virus inoculation. Chart 5, summarizing the mortality rates of all groups tested, shows that amputation of the infected wing neither prevented development of the tumor nor prolonged survival of the bird. Each bird was necropsied, and metastases were not observed. The tumors were grossly and histologically identical with other CTV-10 tumors (14, 17, 18).

Attempts to Induce Secondary Tumors by Trauma. The possibility that experimentally induced trauma might enhance metastasis of CTV-10 was also explored. Two groups of 30 chicks, 1 day old, were inoculated in the left wing web with 10\(^{-1}\) and 10\(^{-3}\) dilutions of standard CTV-10, respectively. Immediately after infection, trauma was produced in the right wing web with a mouse ear punch by making 3 holes approximately 2 mm in diameter in the form of a triangle, with 2 holes in the connective tissue of the web and 1 in the bordering muscle. Bleeding was minimal, and all of the tissue was removed from each punch opening. The birds were observed daily for 10 days and every 3 days thereafter for 6 weeks. All birds developed tumors where virus was inoculated and 4 developed small enlargements at the site of trauma which were not large enough for bioassay. Histological examination showed that these proliferative
lesions were only scar tissue formed in response to the wound.

**Growth of CTV-10 in the Wing Web.** Table 1 summarizes data showing the growth curve of CTV-10 in the chick wing web. Two groups of 90 chicks each were inoculated with 0.2 ml of CTV-10 diluted $10^1$ and $10^3$ respectively. Thirty birds were included as controls. On Days 3 through 13, 5 birds of each group were killed and the wing webs were collected. Half of each was fixed in formalin, and the other portions were pooled and frozen at $-70^\circ$ for bioassay. Table 1 shows that, even with the higher virus input, no infective virus was detected until the 9th day after infection, although the latent period with the $10^1$ dilution was 4 days, and all of the birds had palpable tumors by Day 7. With the lower virus dose, infectivity was not detected until Day 12, but the latent period was 11 days. All control samples were negative.

**Table 1**

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Infective titer of wing web injected with CTV-10 diluted:</th>
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<tbody>
<tr>
<td></td>
<td>$10^1$</td>
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<tr>
<td></td>
<td>ED$_{50}$ log</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>9</td>
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<tr>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>2.3</td>
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<tr>
<td>12</td>
<td>2.5</td>
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<tr>
<td>13</td>
<td>Negative</td>
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<tr>
<td>18</td>
<td>3.5</td>
</tr>
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Values shown represent 3 wing webs assayed as a pool.

Sections showed no tumor cells until Day 4 and 11, respectively, corresponding to the appearance of visible macroscopic tumors. It is clear that the development of the tumor preceded the appearance of titratable virus.

**Passive Immunization in Chicks.** Seven groups of 15 chicks each were treated as follows: Groups 1, 2, and 3 were injected i.m. in each leg with 1.5-ml volumes of 0.85% NaCl solution, pooled normal sera, and pooled immune sera, respectively (a total of 3.0 ml/bird). Groups 4, 5, 6, and 7 received no injections at this time. Twenty-four hr later, all 7 groups were inoculated s.c. in the wing web with 0.2 ml of a $10^3$ dilution of standard CTV-10. Twenty-four hr afterward, Groups 4, 5, and 6 were treated with 0.85% NaCl solution, pooled normal sera, and pooled immune sera, respectively. Group 7 served as untreated, infected controls. Chart 6 shows that the latent period for tumor response was delayed when chicks were treated with immune serum 24 hr before infection. No effect occurred when like immune serum was administered 24 hr after infection.

**DISCUSSION**

The enormous variation in infectivity, not only among tumors produced with common virus inocula, but also
between portions (halves) of the same tumor, is of general interest. Unlike RSV-induced tumors in turkeys (9), such variation is independent of circulating, neutralizing antibody. The importance of infecting dose of RSV to recoverable virus is well known (4, 11, 12, 19, 21). The differences in titratable virus between halves of CTV-10 tumors are difficult to explain. It is possible that tumor cells may die and consequently not produce virus. Alternatively, a tumor might have restricted sites of virus synthesizing activity. The data presented here (Charts 2, 3, and 4) do not suggest that the infecting dose of CTV-10 is a critical factor in determining the virus content of the resulting tumor. However, like RSV (1, 2, 13, 22, 23), the latent period for tumor production is inversely related to the infecting of CTV-10 (17, 18). Secondary tumors were not observed with CTV-10 (17, 18) in the lungs or viscera as they are with RSV (15, 23, 24).

Interestingly, amputation of the infected wing immediately after infection resulted in tumor development at the stump, in contrast to the findings in similar studies with RSV (16). These stump tumors are of particular importance because they did not occur in the surgical controls, i.e., virus plus amputation of the uninfected (opposite) wing. Clearly, close proximity of the amputation to the site of infection is necessary for tumor development. This suggests that cellular elements were not involved in the development of tumors at the site of amputation.

REFERENCES

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