Tissue Distribution of the Rous Sarcoma Virus during the Incubation Period

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The Rous chicken sarcoma and its causative virus have been intensively studied since Peyton Rous first reported the transmissibility of the tumor by a filtrable agent (10). It is known that Rous sarcoma virus (RSV) can propagate in tissues other than tumor. Groupe and Rauscher (4) demonstrated progressive propagation in chicken brain after intracerebral inoculation. Groupe et al. (5) found virus in the hemorrhagic lesions in liver which followed serial intracerebral passage of RSV in chicks. Milford and Duran-Reynals (8) demonstrated virus in viscera of chick embryos with hemorrhagic lesions induced by intravenous administration of Rous sarcoma filtrates. Karnofsky et al. (6) found virus in liver and other embryo tissues during growth of Rous sarcoma grafts on the chorioallantoic membrane (CAM). The appearance of diffuse hemorrhagic lesions in young chicks (8) after intravenous inoculation of virus probably also reflects virus propagation in viscera. Rous (11) demonstrated virus in the serum of some fowls with advanced sarcoma, and the observation has been confirmed by several later investigators (see literature review in Ref. 3). There has not, however, been any systematic study of the distribution of the virus during the incubation period.

The present study was designed to determine whether there is a phase of systemic virus distribution during the incubation period, following local inoculation, as frequently occurs with other types of viruses, and, if so, to observe the relationship between virus distribution and the occurrence of tumors. The development of the CAM pock-count technic (7) provided a practicable method for such a study.

MATERIALS AND METHODS
White Leghorn chicks 3-5 days of age (from Sunnybrook Farms, Hudson, New York) were given subcutaneous inoculations, in the wing web, of 0.2 ml. of a 10^{-2} dilution of a partially purified Rous sarcoma virus preparation (1).^1

There were four separate studies involving a total of 80 chickens, including eleven uninoculated controls. Chicks were sacrificed at daily intervals after inoculation, and tissues (blood, brain, lung, heart, liver, spleen, kidney, gonad, small and large intestines, muscle, wing web, tumor, and pancreas) were stored in individual, sealed test tubes in a dry-ice box until tested for virus.

Testing for virus was done by the CAM pock-count technic in 12-day-old chick embryos (from Shamrock Farms, North Brunswick, New Jersey). The tissues were ground in teflon or glass tissue homogenizers and diluted 1:5 with 2 per cent rabbit serum-saline containing penicillin (100 u/ml) and streptomycin (100 μg/ml). The CAM was dropped by a conventional method with the use of pin-hole openings and inoculated with 0.2 ml of an uncentrifuged tissue suspension from a tuberculin syringe and 20-gauge needle. Each specimen was inoculated into five eggs. Five negative control eggs were inoculated with the diluent alone (saline plus serum and antibiotics), and ten positive control eggs were inoculated with a virus preparation of known potency with each test run. The holes were sealed with paraffin. After 6 days of incubation at 100°F. the CAM’s were harvested and examined for the pocks against a black background, under a fluorescent magnifier-illuminator lamp with the light striking the membranes obliquely.

As first described by Keogh (7), pocks were discrete, pearl-gray focal lesions scattered over the membrane. They usually were 1 mm. or less in diameter but, viewed through the magnifying lens, were seen easily. (Histologically, these pocks show proliferation of both epithelial and connective tissue cells, with the former predominating in the smaller lesions.) Confluence often occurred. Because of the survey nature of the study and the large number of specimens, a precise titration was unnecessary and impracticable. A semiquantitative estimation of virus content was used. If a specimen produced over 100 pocks on at least one

^1Supplied by Dr. Ray Bryan (preparation #CT-632 and CI-694).
membrane or at least 50 pocks on each of three or more membranes it was considered a strong positive. If a specimen produced eight or more pocks per membrane, but not enough to meet the criteria for strong positive, it was called weak positive. If a membrane had fewer than eight pocks it was called negative. If at least three eggs survived in the test group and they were all negative, the tissue specimen was considered negative for virus; but, if fewer than three eggs survived, it was called "no test" and was retested. Eight pocks were arbitrarily accepted as true evidence of Rous sarcoma virus, because numerous rechecks never failed to confirm that membranes with as few as six to eight pocks contained transmissible virus.

From the virus-inoculated chicks virus was recovered sporadically from a wide variety of tissues during the first 5 days after virus inoculation (Chart 1). Lung and gonad most frequently contained high concentrations of virus ("strong positives"), but in occasional chicks high virus concentrations were found in all tissues studied except pancreas, brain, and wing web. Virus was never detected in pancreas during the first 3 weeks. In brain and in the inoculated wing web, respectively, it was found in low concentration ("weak positives") in only one or two individual chicks. No tumors were grossly detectable in wing or in other tissues during this period.

From the 6th through the 10th day, virus was always demonstrated in high concentration in the inoculated wing web, but in all other tissues it was rarely demonstrated and almost never in high concentration. This virus distribution pattern is in marked contrast to that of the first 5 days. It was during this same period that tiny lesions first appeared in the wing web and rapidly developed into grossly identifiable tumors. Histologic sections were made of a few of these tumors and confirmed that they were sarcomas consistent in structure with the Rous sarcoma.

After the 10th day virus continued to be demonstrable in high concentrations in the wing tumors and became more widely distributed and more highly concentrated in other tissues. Viscera which contained metastatic tumors were always positive for virus in this study, but most of the virus-positive tissues contained no grossly recognizable tumors.

Two incidental observations deserve mention. One of the few experimental chicks in which a wing tumor had not developed by the 13th day was sacrificed and studied in the usual manner. Virus was found in lung, liver, spleen, and large intestines but not in the wing web. One of the few chickens with regressed tumor during the 4th week after inoculation died suddenly on the 59th day while being chased. At autopsy both lungs were almost completely replaced by gross tumor which, on microscopic examination, was consistent with Rous sarcoma. Virus was found in the lung tumor and in liver, spleen, and pancreas. This was the only time that virus was demonstrated in the pancreas among 69 birds tested.

Several of the wing web tumors were studied by Dr. Mellors by the direct fluorescein-labeled antibody technic. They were found to have Rous virus.
within the cytoplasm of sarcoma cells, as judged by the localization of the labeled antibody and the fact that the reaction was blocked by pretreatment of the tissue sections with unlabeled chicken anti-Rous sarcoma serum. These studies have not yet been extended to other tissues.

DISCUSSION

The demonstration of high virus content in many organs during the first 5 days indicates a greater total amount of infectious virus per bird than can be explained by mere dilution and distribution of the inoculum. This, plus the absence of demonstrable residual virus at the inoculation site during this period, is strong evidence that the virus propagated in many tissues during this systemic phase. In this characteristic, this oncolgetic virus resembles many nononcogenic viruses of man and animals.

The absence of demonstrable virus in the inoculated wing web during the first 5 days, but consistent appearance of tumor (and virus) at the inoculation site thereafter, suggest that virus was probably present but was unrecoverable either because of very low concentration or because it was in some incomplete form. This phenomenon appears to parallel that observed in chicken brain after intracerebral inoculation of Rous virus (4). Similarly, Prince (9) observed a "disappearance" of virus after inoculation onto the chorioallantoic membrane, but in his system the period of nonrecoverability lasted only 24-48 hours.

The pattern of apparent disappearance of virus from tissues other than wing during the second 5-day period in the present study, followed by increasingly frequent virus recoveries after 10 days, suggests two possible explanations. The virus may go through a transient unrecoverable phase, comparable to that seen earlier in the wing web. Alternatively, and perhaps less probably, the reappearance of detectable virus may result from a re dissemination of virus from the wing tumor.

The sporadic appearance of tumors in viscera following peripheral inoculation of virus might occur as a result of either systemic virus distribution or cancer cell metastasis. The fact that lung tumors were observed as early as the 12th day in this study, at which time wing web tumors were still in an early stage of development, favors the former possibility. Rous (11) and Duran-Reynals (3) discussed this problem of cellular vs. viral dissemination of tumors to viscera and concluded that, although there are some data suggestive of a direct viral cause of metastases, cellular dissemination is probably much more common.

SUMMARY

Five-day-old chicks were given inoculations of Rous sarcoma virus into the wing web and sacrificed at daily intervals. Numerous tissues were tested for virus by the chorioallantoic pock-count technic. From days 1 through 5 the virus was distributed widely but sporadically and usually in low concentration in many tissues (most frequently in lung and gonad) but was rarely detected in the inoculated wing web. From days 6 through 10 virus was much less frequently found in viscera but was frequently found in the inoculated wing web. It was toward the end of this period that wing tumors became grossly detectable. After the 10th day viscera again contained virus in increasing frequency and higher concentration, and virus was consistently present in the wing tumors.

The early systemic distribution of virus was highly suggestive of widespread virus multiplication. The pattern of virus distribution in time and in various tissues suggested the possibilities that visceral tumors may result from viral distribution (not only from cellular metastases) and that virus may go through a transient nonrecoverable phase in wing web and in viscera.

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REFERENCES

8. Milford, J. J., and Duran-Reynals, F. Growth of a


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