Growth of Normal and Rous Sarcoma Virus-infected Chick Embryo Cells in Rat Brains*

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SUMMARY

A comparison was made of the growth capacity in vivo of normal chick embryo cells and identical cells infected with Rous sarcoma virus. The latter cells grew more efficiently and developed into typical Rous sarcomas in the rat brain. This effect was noted even when the cells were allowed to remain in contact with virus for a short period of 15-90 minutes. More abundant growths were often obtained when the cells were exposed to virus for a longer period of time (2 days) prior to transplantation. The infected cells did not give any morphologic evidence of transformation by virus at the time they were implanted. Hemorrhage in many of the Rous tumors was a striking feature.

Excessive, uncoordinated growth constitutes a major aspect of cancer. Yet, under conditions of in vitro growth, there is no consistent pattern separating malignant cells from normal ones. Cells derived from normal tissues often demonstrate a growth capacity in tissue cultures similar to that of malignant cells. On the other hand, in vivo studies, such as those of Foley et al. (2), reveal that tissue culture cells originating from normal tissues differ from cells derived from malignant neoplasms in their growth efficiency when transplanted to an animal host. These authors were able to produce growths in cheek pouches of hamsters, in unconditioned and conditioned animals, by implanting fewer tissue culture cells derived from normal embryos than those of adult non-neoplastic origin. Cells derived from normal embryonal tissues were variable in their capacity to grow: some resembled those of adult normal tissue origin, others exhibited a growth potential similar to cells derived from malignant neoplasms. In our own experiments with the rat brain used as the site of transplantation it has been found that malignant tissue culture cells, such as HeLa and KB, multiplied with much greater efficiency in vivo in conditioned animals than did normal cells whether the latter were obtained from an animal de novo or were first cultivated in vitro.

In the present studies a comparison is made of the growth capacity, in a heterologous host, of normal chick embryo cells with that of similar cells which were infected with Rous sarcoma virus (RSV) but in which there was no visible evidence of transformation to malignancy at the time of transplantation. In a previous report (6) it was shown that minced chick embryo tissue introduced into the brains of conditioned rats was able to survive and develop into a variety of embryonal structures, and that similar tissue exposed to RSV for about 30 minutes prior to transplantation gave rise to growths of Rous sarcoma. More recently it has been noted that trypsin-dispersed chick embryo cells could be used in place of the minced embryos, thus providing a better basis for considering quantitative aspects of the problem. Similarly it has been possible to employ as heterologous transplants normal and RSV-infected chick embryo cells previously grown in vitro.

MATERIALS AND METHODS

Virus.—Rous sarcoma virus, lot number CT 895, was obtained from the laboratory of Dr. W. Ray Bryan at the National Institutes of Health. This virus which had an infectivity titer of $10^{-4.5}$/0.1 ml was adjusted to a final dilution of $10^{-2}$ for

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the subsequent experiments. Infectivity assays were made on the chorioallantoic membrane of the chick embryo.

**Trypsin-dispersed chick embryo cell suspensions.**—Minced 7- to 9-day-old embryos were treated with 0.25 per cent trypsin. The trypsinization process was carried out in vessels placed on a magnetic stirrer. Two to four trypsinizations were performed for periods of 15 minutes. It has been our experience that early stoppage of trypsin action by means of serum protein was beneficial to the cells. For this reason the trypsinized cells were added to a medium containing 10–25 per cent calf serum. The pool of cells was centrifuged at low speed (approximately 1000 r.p.m.) for 2–10 minutes and resuspended in appropriate volumes of Eagle's medium containing 5 per cent calf serum. The pool of cells was centrifuged and resuspended in the manner described above. One group of animals was given tissue culture suspensions containing the desired concentration of cells mixed with Hanks BSS, and another group of animals received cell suspensions to which was added RSV 15–30 minutes prior to transplantation.

In another type of experiment Hanks BSS or RSV was added to primary and secondary cultures of the chick embryo cells on the 3rd–5th day of cultivation. The Hanks BSS-treated and the RSV-infected cultures were allowed to incubate 2 additional days. Then the cells were removed from the glass with the aid of trypsin, centrifuged, and resuspended as in the previous experiments, and were inoculated into the rats.

**Transplantation.**—The method of transplantation was published previously (5). All rats in the present experiment were conditioned by means of x-radiation and cortisone. The desired number of cells injected intracerebrally into each animal was contained in 0.1 ml. of suspension. The animals were sacrificed at 13 days after transplantation. The brains were removed, fixed in 10 per cent formalin, sectioned at five different levels, and prepared for histologic examination.

**RESULTS**

**Growth capacity of normal chick embryo cells.**—In general, normal chick embryo cells did not exhibit much of a tendency to survive and grow in the rat brain. When trypsin-dispersed chick embryo cells without RSV (Table 1) were inoculated into the rat brains, something of significance was observed only when the largest number of cells (10⁶) was employed. This consisted of small collections of round, ovoid, and elongated cells either in the brain substance or in the meninges. Slight glial reaction was identified usually in the vicinity of the cellular areas when they occurred in the brain substance. It was apparent that some fibroblasts were present in these foci because collagen formation was evident, but it was not always possible to differentiate the fibroblasts from glial cells. The groups of cells in the meninges were readily recognized as fibroblasts with collagenous fibers. In previous experiments in this laboratory, such intracerebral and meningeal foci did not develop in rat brains following the inoculation of nonfibroblast-containing cell cultures (5) or the injection of RSV alone (6). Thus it was felt that the collections of cells did not represent merely a reaction to the trauma of injection and that the collagen was produced principally, if not completely, by transplanted cells which persisted and possibly proliferated to some extent. This positive finding was noted in slightly more than half of the animals in the affected group. Similar foci were not seen in the brains of rats receiving 10⁵ or 10⁶ chick embryo cells.

In the brains of animals receiving tissue culture cells without RSV (Tables 2 and 3) similar positive findings were observed in a minority of animals when 10⁴ or more cells were inoculated. No such

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<td><strong>GROWTH OF TRYPSIN-DISPERSED CHICK EMBRYO CELLS WITH AND WITHOUT RSV IN RAT BRAINS</strong></td>
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<tr>
<td>No. cells per rat</td>
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* Numerator is number of animals with Rous sarcoma. Denominator is number of animals inoculated.

† Numerator is number of animals with small groups of cells admixed with collagen fibers. Denominator is number of animals inoculated.

Trypan blue was used to determine the number of nonviable cells. The cell suspensions were inoculated into the brains of rats 15–90 minutes after being mixed with either RSV or Hanks balanced salt solution (BSS) without RSV.

**Tissue culture suspensions.**—Cells from 7- to 9-day-old chick embryos were grown in 8-oz. glass prescription bottles containing Eagle's basal medium supplemented with 10 per cent calf serum. In one type of experiment primary culture cells, after 3–5 days of cultivation, were removed from the glass by using 0.5 per cent trypsin. The cells were centrifuged and resuspended in the manner described above. One group of animals was given inoculations of suspensions containing the desired concentration of cells mixed with Hanks BSS, and another group of animals received cell suspensions to which was added RSV 15–30 minutes prior to transplantation.

In another type of experiment Hanks BSS or RSV was added to primary and secondary cultures of the chick embryo cells on the 3rd–5th day of cultivation. The Hanks BSS-treated and the RSV-infected cultures were allowed to incubate 2 additional days. Then the cells were removed from the glass with the aid of trypsin, centrifuged, and resuspended as in the previous experiments, and were inoculated into the rats.
change appeared when less than $10^8$ cells were employed.

**Growth capacity of RSV-infected chick embryo cells.**—Trypsin-dispersed chick embryo cells exposed to RSV shortly before transplantation (Table 1) grew well and developed into Rous sarcomas in most of the animals. These neoplastic areas were much more obvious than the foci produced after injection of the noninfected cells. Rous tumors occurred when only $10^6$ cells were inoculated. As noted previously, identical cells not infected with RSV did not survive in the rat brain unless $10^7$ cells were employed. Even with this high concentration of cells the frequency of any change suggesting persistence and possible growth of transplanted cells in the noninfected series (56 per cent) was significantly less than the frequency of growths in the corresponding RSV-infected series (89 per cent).

Morphologically, the Rous sarcomas were typical, appearing as foci of bundles of varying sized spindle cells together with prominent round and polygonal cells consisting of distinct nuclei and nucleoli and often vacuolated cytoplasm. Mitotic figures were conspicuous. Myxomatous change was seen in the growths frequently. The tumors were usually multiple, discrete, but sometimes confluent, and found in different areas of the brain. The degree of host reaction in the vicinity of the growths was minimal or slight when present. Relatively recent hemorrhage was observed within and adjacent to many of the foci of Rous sarcoma which was not attributed to the trauma of injection. The individual tumors tended to be somewhat larger in the animals receiving $10^7$ cells. In the group of animals receiving $10^8$ the foci of Rous sarcoma were fewer and smaller.

Tissue culture cells infected with RSV (Tables 2 and 3) were able to initiate growths of Rous sarcoma when the number transplanted was as low as $10^4$ per rat, in contrast to the uninfected cells which exhibited very little tendency to survive and only when injected in the number of $10^6$ or more per rat. In the group of animals receiving primary tissue culture cells exposed to RSV shortly before transplantation (Table 2) the morphologic appearance and distribution of the Rous sarcomas were comparable to those of the tumors initiated by the infected trypsin-dispersed chick embryo cells. Microscopically, the growths associated with tissue culture cells exposed to RSV for 2 days prior to inoculation (Table 3) were similar to those arising from cells that were in contact with the virus only a short time before transplantation, but the individual tumors often were more abundant. In some of the largest masses ischemic necrosis was evident in the centers. An exception to this prolific growth was observed in two rats receiving $10^4$ cells. The tumors in these animals were minute.

**DISCUSSION**

It is apparent in the present studies that cells exposed to an oncogenic virus develop as heterologous transplants more efficiently than do identical cells not exposed to the virus. Under the same conditions of cell type, host specificity, and conditioning, chick embryo cells mixed with RSV exhibited greater growth capacity than did cells which had not contact with the virus. The development of more abundant growths from those cells exposed to RSV for a longer period of time (2 days) prior to transplantation may be owing to the fact that more cells became infected by the virus.

It is to be noted that what is being considered in this paper is the comparison of the growth potential of normal cells with that of RSV-treated but not obviously malignant cells, since the infected cells which eventually developed Rous sarcomas in the rat brains gave no morphological evidence of malignant transformation prior to transplantation. This study differs from that of Morgan and Andrese, who produced Rous tumors in the cheek pouch of hamsters and in the eye of guinea pigs by the inoculation of RSV-treated chick em-
hryo fibroblasts, because they employed cells in which there was visible evidence of transformation by the virus in vitro (3).

It is significant that in many instances tumors developed from cells which were in contact with the virus for less than 90 minutes before transplantation—too short a time to expect any visible changes in the cells. In fact, at the time of transplantation they did not differ morphologically from the normal, uninfected cells. Likewise, the cells mixed with virus for 2 days before injection did not appear to be altered in vitro, although morphologic changes have been identified by others in some tissue culture cells after this period of exposure to RSV (8).

It can be assumed that the changes which usually occur within RSV-infected cells in vitro during the process of transformation by virus were not interrupted by the cells' residence in the rat brain. Indeed, the infected cells took on the properties of malignancy as evidenced by their growth behavior as heterologous transplants. That virus released from the implanted cells did not participate in the production of the Rous tumors is suggested by earlier studies (6) in which it was shown that no pathologic changes occurred in rat brains following the intracerebral injection of RSV alone.

The hemorrhagic feature in many of the Rous sarcomas was striking. Although in the previously published investigation (6) hemorrhage was not recognized in the tumors arising from RSV-infected minced chick embryo tissue implanted in rat brains, this finding has been observed commonly in tumors produced by the same technic in more recent unpublished experiments in this laboratory. The absence of hemorrhage in the earlier studies might have been due to the use of a different lot of RSV. Hemorrhagic Rous sarcomas have been described in the work of Rous and Murphy dealing with a series of transplantations of the neoplasm in fowls (4). Also, hemorrhagic lesions not associated with Rous sarcomas but attributed to RSV have been produced in animals (1, 7).

ACKNOWLEDGMENTS

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