Cultivation of 4-Dimethylaminoazobenzene-induced Rat Liver Tumors in Yolk Sacs of Chick Embryos*

M. I. ARMSTRONG, A. E. GRAY,† AND A. W. HAM

(Departments of Anatomy and Physics, University of Toronto, Toronto, Canada)

INTRODUCTION

This communication deals with our experiences in attempting to grow a number of 4-dimethylaminoazobenzene-induced liver tumors of rats in the yolk sacs of chick embryos and in maintaining three of these tumors in serial cultivation in this medium for a period of 2½ years.

MATERIALS AND METHODS

Hybrid white rats of from 90 to 200 gm. were maintained on the synthetic diet containing 0.06 per cent 4-dimethylaminoazobenzene recommended by Geise, Miller, and Baumann (8). After 3-12 months the livers became irregularly enlarged. When, on palpation, they were found to occupy half or more of the abdomen, tumorous masses were removed from them to glass plates. After a piece of each mass was fixed for histological study the remainder was finely chopped with curved scissors, placed in the barrel of a syringe, and forced through a No. 18 or No. 20 needle into a vial where they were diluted with a mixture of amniotic and allantoic fluids and yolk of the harvested egg. The degree to which they were diluted before being injected into further eggs depended on how lethal the tumors were as is explained in the section headed “On the lethality of tumors 91, 108, and 124.”

Since there is always a danger of contamination, several batches of eggs were injected at each transfer, each batch being injected with a different lot of tumor tissue and diluting fluids.

RESULTS

PROPORTION OF RAT LIVER TUMORS THAT GREW IN EGGS

Thirty-five tumors, proved by gross and microscopic examination, were each injected into a batch of eggs. The results follow:

- Fourteen failed to grow on their initial injection but survived for two transfers and were kept for later study.
- One grew for fifteen transfers but was discarded because of a mold contamination that was picked up as it was transferred.
- Nine produced very small growths on their first transfer, but failed to survive either the second, third, fourth, or fifth transfer.
- Fourteen failed to grow on their initial injection into eggs.

In summary: Of 28 uncontaminated tumors injected into yolk sacs, five lent themselves to successful serial cultivation.

RELATION BETWEEN THE PRESENCE OF METASTASES IN RATS WITH LIVER TUMORS AND THE SUCCESSFUL SERIAL CULTIVATION OF THE PRIMARY TUMORS IN YOLK SAC

The 28 rats from which the uncontaminated tumors were obtained were examined at autopsy for metastases, and these were found in the lung,
mesentery, spleen, abdominal lymph nodes, para-adrenal fat, or para-ovarian fat in seven of the 28 rats.

Tumors from three of the seven rats with metastatic growths have been serially cultivated in eggs for 94, 93, and 64 generations, respectively. Another grew to a large size for two transfers but was lost accidentally. The liver tumors from the other three rats grew slightly on their first transfer but did not survive more than four transfers.

Twenty-one of the rats did not have metastases. The primary tumor from one of these was cultivated for fifteen transfers in eggs before it was discarded with a mold contaminant. The liver tumors from twenty of the 21 rats without metastases either failed to grow when injected into eggs or grew only slightly from one to four transfers.

In summary: The primary tumors from four of the seven rats with metastases lent themselves to successful serial cultivation in eggs. Of the 21 rats which revealed no metastases the primary tumors were successfully serially cultivated in only one instance.

SOME GENERAL FEATURES OF THE THREE RAT LIVER TUMORS THAT HAVE BEEN CULTIVATED IN EGGS FOR 94, 93, AND 64 TRANSFERS, RESPECTIVELY

Percentages of takes.—After excluding the eggs in which the embryos died before the tenth day of incubation (from injury, contamination, or unknown reasons), about 99 per cent of the yolk sacs injected with suitable amounts of tumor tissue on the fourth day of incubation contained tumors when they were harvested.

Site and volume of tumors grown in eggs.—Tumors grew along the margin of the yolk sac. The average volume of tumor tissue obtained from each egg was about 0.4 cc., although larger tumors with a volume of 1 cc. or more were not unusual.

Constancy of growth pattern.—Sections taken from these three egg-grown tumors at different transfers showed a constant histological pattern. Two of the tumors that were transferred at their thirteenth and 71st egg transfer, respectively, from eggs to rats grew in the rats and exhibited the same histological pattern that they had in the eggs. Both tumors, when transferred from the rats back into yolk sacs, grew and again exhibited the same microscopic structure.

Stroma and blood vessels.—The chick-embryo-derived stroma of all three tumors was very delicate and not obvious in most areas in sections stained with eosin and hematoxylin. It was seen to best advantage in the cores of the papillary projections of liver tumor 124 (Fig. 5).

The blood vessels in all three tumors were lined with endothelium supported by a small amount of delicate connective tissue. They were much larger than capillaries and resembled thin-walled non-muscular veins (Figs. 2–6).

Necrosis.—The amount of necrosis in yolk sac-grown tumors varied from being almost negligible to moderate in amount. In general, its percentage increased with the time the tumor-bearing egg was incubated. Necrotic tissue was generally red because of hemorrhage into it; in some instances, however, it was an opaque white. Its different color permitted it to be dissected away from the healthy tissue.

SOME DETAILS REGARDING THE THREE RAT LIVER TUMORS THAT HAVE BEEN TRANSFERRED 94, 93, AND 64 TIMES, RESPECTIVELY, IN YOLK SAC

Liver tumor 91.—The primary tumor was an adenocarcinoma, the cells of which were growing both in solid masses and in glandular formations (Fig. 1). Goblet cells were sometimes seen among the glandular cells. An extension of the tumor into the mesentery revealed the same type of histological picture.

Figures 2 and 3 illustrate the tumor as it grew in yolk sac. Figure 3 shows that the cells of the tumor alternated, in different areas, between being packed closely together and being loosely arranged. In some instances the tumor cells were packed closely in a radial arrangement around the blood vessels (Fig. 3). In other areas the cells showed some differentiation into glandular structures (Fig. 2).

The tumor cells varied considerably in shape according to whether they were arranged in glandular formations or in tightly packed or loosely packed formations. The nuclei, however, were consistently large and ovoid with large prominent nucleoli. Mitotic figures were very numerous. No goblet cells were seen in the egg-grown tumors.

Liver tumor 108.—The primary tumor was an adenocarcinoma with cells growing in fairly solid masses but showing some tendency to form glandular structures. In some areas there were papillary formations. Secondary growths in the lung and mesentery exhibited the same histological structure.

This tumor in eggs showed glandlike structures in the midst of irregular masses of tumor cells. The cells varied in shape according to their position, but their nuclei were similar in being of moderate to large size and in containing well defined nucleoli (Fig. 4).

Although some goblet cells were seen among the tumor cells that formed glandular structures in both the primary and in some of the secondary
growths in the rat, they were never observed in the tumor in the yolk sac.

Liver tumor 124.—The primary tumor was an adenocarcinoma, the cells of which formed fairly solid masses in some sites but glandular structures in others. Papillary projections sometimes extended into the glandular spaces. Metastatic nodules in the para-adrenal fat and near the ovary showed this same pattern of growth.

In eggs the tumor cells in some areas formed papillary projections (Fig. 5) and in other areas tightly or loosely packed masses of cells with glandlike structures in their midst (Fig. 6).

Liver tumor 124—The primary tumor was an adenocarcinoma, the cells of which formed fairly solid masses in some sites but glandular structures in others. Papillary projections sometimes extended into the glandular spaces. Metastatic nodules in the para-adrenal fat and near the ovary showed this same pattern of growth.

In eggs the tumor cells in some areas formed papillary projections (Fig. 5) and in other areas tightly or loosely packed masses of cells with glandlike structures in their midst (Fig. 6).

ON THE LETHALITY OF TUMORS 91, 103, AND 124

As noted previously, deaths before the tenth day of incubation are not caused by tumor growth; they occur even when physiological saline alone is injected and are probably the aftermath of the trauma of the injection procedure. Deaths after the tenth day of incubation, however, are generally the result of tumor growth. Chart 1 shows the percentages of a given number of embryos inoculated with liver tumor 103 that were dead on each day from the ninth to the seventeenth days of incubation and after different numbers of transfers. It can be seen that the tumor was lethal for chick embryos. It should be noted that eggs were injected with more dilute suspensions of tumor tissue in the later than in the earlier transfers of the tumor; increasing the dilution decreases the lethality (1). In spite of increasing its dilution, the tumor became more lethal as it was serially cultivated. Liver tumors 124 and 91 acted similarly.

The large number of chick embryo deaths that occur when tumors are grown in yolk sacs makes the method somewhat laborious, for the number of eggs that are inoculated must always greatly exceed the number that are needed on the day of harvesting.

The way in which the lethality of the carcinogen-induced rat liver tumors increased as the tumors were serially cultivated in eggs is very similar to the way in which the lethality of mouse mammary tumors and the Walker 256 rat tumor increased with serial egg passage (1, 5, 7). The increase in lethality of mouse mammary tumors, on serial cultivation in yolk sacs, could possibly be explained by the milk agent's increasing in virulence. (The milk agent has been shown still to be present in yolk sac-cultivated mouse mammary tumors after 31 transfers [2].) But it is difficult—though not impossible—to reason that the increase in lethality that occurs in a carcinogen-induced tumor on serial transfer in yolk sacs could be caused by an increase in the virulence of a tumor-inciting virus. The possibility of the increased lethality being due to the introduction of a new contaminant or the increased growth of a pre-existing one must always be borne in mind. As yet we have been unable to demonstrate any contaminant that could account for the lethality, but the possibility of there being one continues to give us concern.

Armstrong and Ham (1) found that, when mouse mammary tumors became lethal on continued serial egg passage, the chick embryos developed a severe anemia; whereas before they became lethal they produced only a slight or no anemia. Unfortunately, the liver tumors became lethal so quickly that we did not obtain hemoglobin estimations on chick embryos with non-lethal liver tumors. Hemoglobin levels were obtained, however, on a group of 33 14-day-old chick embryos bearing liver tumor 103 between its tenth and twelfth transfers. The average was 4.6 ± 1.3 gm., the lowest 2.2 gm., and the highest 6.4 gm. (The average hemoglobin level of 45 nontumor-bearing chick embryos of the same age was 7.7 ± 0.7 gm.; the highest was 9.6 gm., and the lowest was 5.8 gm.)

Armstrong and Ham (1) found enlarged flabby hearts and enlarged livers with widely dilated
Fig. 1.—A section of primary rat liver tumor 91. Mag. X200.

Fig. 2.—A section of liver tumor 91 growing in yolk sac showing an attempt by the tumor cells to form a glandlike structure. Mag. X200.

Fig. 3.—A section of liver tumor 91 growing in yolk sac showing the usual arrangement of its cells; that is, clumps of closely packed cells separated by more loosely arranged cells. Note the tendency of the tumor cells to pack closely where they surround blood vessels. Mag. X75.

Fig. 4.—A section of liver tumor 103 growing in yolk sac. Mag. X600.

Fig. 5.—A section of liver tumor 124 growing in yolk sac. Note the papillary projections with chick stroma and blood vessels in their cores. Mag. X150.

Fig. 6.—A section of liver tumor 124 growing in yolk sac showing a glandlike formation in the midst of a mass of tumor cells. Mag. X200.
sinusoids in anemic chick embryos that had lethal mouse mammary tumors growing in their yolk sacs. They attributed these lesions to circulatory failure caused by the severe anemia. It is of interest that these lesions did not occur in anemic chick embryos with lethal carcinogen-induced rat liver tumors growing in their yolk sacs.

**DISCUSSION**

This work was undertaken in the hope that it would enable us to have a steady supply of carcinogen-induced malignant tumor tissue that would be relatively pure, of a consistent quality, and of a kind that would permit us to obtain embryonic, normal functioning, and regenerating cells of the same origin as the tumor cells for purposes of comparison. In this quest we think we were reasonably successful, for we have shown that some carcinogen-induced malignant rat liver tumors can be serially cultivated in yolk sacs to produce a steady supply of relatively pure tumor tissue of a consistent type.

Primary tumors produced in rats with carcinogens are of somewhat dubious value for exploring, e.g., the biochemical processes of malignant cells. The types of primary tumors that develop vary. Furthermore, a slice, cut from what is presumed to be a tumor, commonly contains much that is not tumor tissue; for example, areas of fatty liver, hyperplastic parenchyma, proliferating bile ducts, fibrous tissue, and necrotic material. Finally, as Opie (6) has pointed out, there is no guarantee, unless metastases are found, that the tumor is malignant; and even then it is difficult to be certain that the malignant change responsible for the metastases had occurred in any substantial portion of the piece of tissue selected from the primary tumor.

In contrast to primary tumors, the yolk sac-grown ones are much more of the nature of a pure culture. They consist almost entirely of tumor cells with only a few chick blood vessels and stroma; in addition, a few strands of yolk sac and a little yolk, albumen, and allantoic fluid may still adhere to them even after careful dissection. They are of a standard quality from one transfer to the next. Necrosis is not usually extensive and, when present, may be recognized grossly and dissected out. Finally, our finding that a high proportion of the tumors that metastasized could be cultivated serially in yolk sacs, while those that had not metastasized could not usually be so cultivated, suggests that malignancy is a requirement for serial cultivation in yolk sacs. In this respect, our findings are similar to those of Greene (4), who found that tumors that had metastasized are most likely to grow on transplantation to the anterior chamber of the eye of a heterologous host.

Accordingly, we think that liver tumor tissue cultivated serially in yolk sacs is about as close to a pure tissue in which the properties of malignancy are embodied as it is possible to obtain provided more substantial amounts of tissue are required than can be procured by tissue culture methods. While not particularly costly, the method requires close attention, for to maintain adequate supplies of tumor tissue large numbers of eggs must be injected every 8–14 days, and all the injected eggs must be candled daily.

**SUMMARY**

Of 35 4-dimethylaminoazobenzene-induced rat liver tumors introduced into the yolk sacs of fertile eggs, seven infected the eggs. Of the remaining 28, four of the seven that had metastasized lent themselves to successful serial cultivation, while only one of the 21 that had not metastasized could be serially grown.

Three of these tumors have now been transferred in yolk sacs 94, 93, and 64 times, respectively, and have shown no change in their histological pattern. All three tumors, however, on serial transfer, have become increasingly lethal for chick embryos.

Rat liver tumors cultivated in yolk sacs have many advantages over primary tumors obtained from livers; they are of a consistent pattern, have only a little stroma, and contain no other parenchyma than tumor cells. Moreover, since only liver tumors that have metastasized generally grow in yolk sacs, their malignancy is probably assured.

**REFERENCES**

2. ———. Demonstration of Milk Factor in a CSH Mouse Mammary Tumor after the Tumor Had Been Transferred 51 Times in Fertile Eggs. Ibid., 10:901, 1950.
Cancer Research

Cultivation of 4-Dimethylaminoazobenzene-induced Rat Liver Tumors in Yolk Sacs of Chick Embryos

M. I. Armstrong, A. E. Gray and A. W. Ham