Growth of a Chicken Sarcoma Virus in the Chick Embryo in the Absence of Neoplasia*

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Previous studies from this laboratory (2) showed that chickens injected intravenously with the viruses of the Rous and Fujinami sarcomas responded with notably different types of lesions according to the age of the bird. In the newly hatched chick, a host devoid of virus-suppressing antibody (3), the disease frequently revealed itself by the exclusive development of hemorrhagic lesions in the absence of grossly detectable neoplasia. In pullets neoplasia was observed side by side with hemorrhage. In the adult chicken, a host endowed with virus-suppressing antibody, the lesions when present were mostly neoplastic. The naturally developing virus-suppressing antibody not only conditions the age resistance but also the individual resistance of chickens to tumors (3). Its importance in conditioning the virus activity was further shown (4) by the fact that in chicks infected intravenously with the Rous virus and treated daily with serum from normal adult chickens there was a shifting from hemorrhagic to neoplastic lesions or a complete absence of all lesions.

Histologic study of the early hemorrhagic lesions in chicks failed to disclose neoplasia, and after discussion of the different mechanisms that may lead to hemorrhage the possibility was considered that this hemorrhage may result from direct action of the virus on the vessel wall; however, proofs of a necrotizing effect on its endothelial cells or fibroblasts were lacking. It was then decided to extend the studies to chick embryos, a still more vulnerable host than the newly hatched chick, with the hope, first, that serial sections of the whole embryo would bring irrefutable proof concerning the lack of neoplasia, and second, that the destructive vascular lesions that may lead to hemorrhage would be shown in these hosts. The present paper records the results of inoculating filtered or unfiltered extracts of the Rous sarcoma into the celomic cavity and blood stream of 3 day old chick embryos and outlines a few similar experiments in older embryos.

Murphy and Rous (9) transplanted Rous tumor cells into many chick embryos at the seventh or eighth day of development with the result that tumors in the membranes developed in most instances. By indiscriminate implantation growths were also induced in the embryo along the track of the needle. Of 23 embryos grafted at the second or third day of development in the outer zone of the blastoderm only 5 showed small growths. Tumor filtrates and suspensions of desiccates injected into embryos presumably 7 or 8 days old induced tumors noticeable 3 or 4 days later and having the same location as those produced by living cells. No blood-born metastases occurred. No mention is made of hemorrhagic lesions in the embryo or membranes although it is stated that hemorrhage in the tumor substance was not uncommon.

Other observations on the hemorrhagic lesions occurring in tumor-bearing chickens have been reviewed in a previous publication (2).

MATERIALS AND METHODS

The host animal used routinely in these experiments was the 72 hour white Leghorn chick embryo. Instances in which other stages of development were made use of are indicated in the results. Rous sarcoma extract, 1:5 and 1:10 in normal saline, and Rous filtrate 1:10 (Berkefeld N) were injected in quantities up to 0.05 cc. In operating, a rectangular window was cut in the shell and its membrane and the embryos thus exposed. A manually controlled glass micropipette was then introduced either into a vein or into the celomic cavity, and the inoculum injected under pressure. All injections were intracelomic unless otherwise indicated in the text. The shell was then replaced, and the embryos incubated either until candling indicated death or until an adequate period of development had elapsed.

Tissues sectioned were fixed in formol, cut at 10 microns, and stained in hematoxylin and eosin. The presence of virus in the diseased embryos was investigated by injection of their extracts at 1:10 in saline solution into a pair of normal barred Rock chicks or pullets. Bacteriological examinations were routinely made.
INTRACELOMIC INJECTIONS OF FILTERED TUMOR EXTRACT

Experiment 1.—A preliminary series of ten 72 hour embryos was injected with 0.05 cc. of 1:10 tumor filtrate and examined after 8 days' incubation. One was found to have hemorrhagic blebs in the heart, lung, and liver, and another to have identical lesions in the heart, lung, and proventriculum. The viscera of these embryos were pooled and extracted, and those from two that showed no lesions were similarly extracted. Of these respective extracts 1 cc. was injected subcutaneously into right and left breast areas of pullets. In each case the extracts of embryos with demonstrable lesions induced tumors, while those from embryos with no visible lesions did not.

Experiment 2.—Inoculum, 0.05 cc. tumor filtrate; 16 recipients.

7 days after injection.—Of 5 embryos examined, 1 had a hemorrhagic bleb on the right ventricle.

8 days after injection.—Three of 11 embryos opened showed lesions. Each of these had subepicardial petechiae, and one had blebs within the breast musculature. These lesions, with the immediately surrounding tissues, were extracted and the extract was inoculated into a series of 72 hour embryos, which failed to develop lesions. The remaining viscera of embryos with lesions, and those from embryos that were normal in appearance, were extracted by identical methods and the extract was injected into the breasts of pullets. Again, extracts from embryos with lesions induced tumors, while those from healthy embryos did not.

Experiment 3.—Inoculum, 0.05 cc. tumor filtrate; 14 recipients.

14 days after injection.—Dissection of one dead embryo revealed a single hemorrhagic lesion on the liver. An extract of this embryo was injected into the celomic cavities of 14 chick embryos 3 days old with the result that one embryo, which hatched and died on the 21st day of incubation, developed blebs on the liver and spleen and its extracts induced tumors in pullets; the other embryos of this group died without lesions and their extracts did not induce tumors in pullets.

17 days after injection.—Of 6 embryos opened, one had on the chorioallantoic membrane a small white, soft, nodular tumor that measured 1.5 mm. in diameter. The embryo itself had hemorrhagic blebs on the spleen and heart. The tumor was extracted and the extract injected into seven 3 day chick embryos and two chicks; one embryo developed a typical bleb on the liver after 11 days, and the chicks died of tumors after 1 month. Extracts of the viscera of the tumor-bearing embryo induced neoplasia when injected into pullets. Other embryos of the series died without lesions.

Experiment 4.—Inoculum, 0.05 cc. 1:10 tumor filtrate; recipients, six 6 day embryos.

4 days after injection.—Dissection of 4 embryos revealed no lesions.

17 days after injection.—Two embryos hatched and examination showed small heart blebs. Their viscera were extracted separately, and the respective extracts induced tumors in chicks in one instance, not in the other.

Experiment 5.—Inoculum, 0.5 cc. 1:5 tumor filtrate; recipients, seven 13 day embryos; intravenous inoculation.

6 days after injection.—Two embryos examined showed no lesions.

7 days after injection.—Four embryos opened were found to have subepicardial petechiae, but no other lesions. However, extracts of these embryos induced tumors when injected into chicks.

18 days after injection.—Two animals hatched, of which one had developed myocardial blebs.

In an additional series 1 cc. of 1:5 Rous filtrate introduced upon the chorioallantoic membranes of 13 day embryos elicited no response whatsoever.

Experiment 6.—Small fractions of a cubic centimeter of 1:10 Rous filtrate were injected intravenously into six 3 day chick embryos. After 10 days one embryo had widespread subcutaneous petechiae but no definite blebs. However, extracts of this embryo were effective in inducing tumors in chicks.

INTRACELOMIC INJECTIONS OF UNFILTERED TUMOR EXTRACT

Experiment 7.—Inoculum, 0.05 cc. unfiltered tumor extract; 13 recipients.

5 days after injection.—Five embryos dissected, of which 2 had developed hemorrhagic lesions. Over the next 4 days 8 embryos died and were dissected. Each of them had extensive hemorrhagic disease.

Experiment 8.—In this experiment serial passages of the hemorrhagic disease through 6 groups of 3 day old embryos were accomplished. The embryos of the first passage were injected intracelomically with 0.05 cc. of unfiltered tumor extract 1:10. Subsequent passages were carried out by injection of the same amount of embryo extract 1:5 and proved to be sterile by cultures. The results are summarized in Table I.

Experiment 9.—Inasmuch as the time interval between injection and the development of lesions in the passage series described above was moderately uniform in the second, third, and fourth passages, and since the majority of embryos examined at the time specified were dead, we decided to run a second series, opening the recipient embryos 7 days after injection, thus using live embryos with lesions for the preparation of ex-
tracts. The embryos selected in each case had widespread hemorrhagic lesions, and 1:5 extracts were employed in each instance. The results, given in Table II, showed again that the hemorrhagic disease could be serially transmitted through embryos.

Summarizing the outcome of the entire series of experiments one can state that about 40 per cent of the embryos thus injected would have died of their hemorrhagic lesions. It is clear, however, that the above figures are far from representing exact percentages of susceptibility since many embryos were purposely killed at an early date and others died of incidental causes.

The tumors induced in chicks and pullets by extracts of diseased embryos did not differ in any way from the tumors induced in these hosts by the ordinary tumor virus or cells.

The most important observation made during the experiments was the complete absence of grossly detectable neoplasia in the embryos. In 2 cases tumors developed but the growths were incorporated within the tissues of the chorioallantoic membrane and had not penetrated the embryo proper either by extension or by metastasis, although in one case the embryo itself showed hemorrhagic lesions.

Histological studies.—The nonneoplastic nature of the hemorrhagic lesions of the embryos was fully corroborated by microscopic examination of sections from many lesions and of sections mounted in series at 100 micron intervals of entire embryos (Figs. 1 to 8).

Findings suggesting direct or indirect destructive effects of the virus were largely confined to hemorrhages into the subepicardial tissues, liver (Fig. 9), and spleen (Fig. 10), and to the considerable edema, which was largely limited to the cuts but which was present also in the myocardium and great vessels leaving the heart. Some cell necrosis was present in these areas, but no infiltration. Desquamation and slight swelling of the endothelium was noticed in the hemorrhagic areas, but no undue mitosis. Capillaries were frequently surrounded by blood elements, but in no instance were these of such a type as to suggest that anything more than rupture of capillary walls had preceded their aggregation in these sites. The tumors that in two instances developed in the chorioallantois were loose sarcomas of the ordinary type.

Control injections.—Often during the course of the passages extracts from normal chicken embryos were injected intracelomically into 3 day embryos without lesions resulting in any case.

Attempts to protect the embryo against the virus by means of serum from normal adult chickens.—Several experiments of this sort were carried out in an attempt to duplicate on embryos what had been achieved in chicks (4). In one experiment, adult serum incubated with an equal quantity of tumor extract during 1 hour failed to prevent the appearance of hemorrhagic lesions in nine 3 day embryos. There was, similarly, no protection provided to twelve 3 day old embryos that received adult serum 2 days subsequent to the tumor extract, or to another twelve that received the extract 12 days subsequent to the adult serum. It will be remembered that in chicks, too, protection was

| Table I: First Serial Passage of the Rous Virus through 3 Day Old Chick Embryos |
|---|---|---|---|
| Passage No. | Number of embryos | Time after injection when embryos died or were killed, days | Number showing hemorrhagic lesions | Results of injecting embryo extract into pullets |
| 1 | 12 | 14 | 1 | 1 |
| 2 | 7 | 7 | 5 | Tumors |
| 3 | 12 | 8 | 10 | Tumors |
| 4 | 18 | 9 | 3 | Tumors |
| 5 | 12 | 25 | 1 | (after hatching) |

Passage to the fifth series of embryos was made by the injection of extract of a desiccate prepared from the embryos of the fifth passage. The process of desiccation reduced either the virulence or the relative amount of the virus, because among 12 embryos lesions were observed in the heart and liver of the only individual that hatched and was killed 7 days later.

| Table II: Second Serial Passage of the Rous Virus through 3 Day Old Chick Embryos |
|---|---|---|---|
| Passage No. | Number of embryos | Time after injection when embryos died or were killed, days | Number showing hemorrhagic lesions selected for next passage | Results of injecting embryo extract into chicks |
| 1 | 12 | 7 | 2 | Tumors |
| 2 | 16 | 7 | 2 | Tumors |
| 3 | 8 | 7 | 4 | Tumors |
| 4 | 5 | 7 | 2 | Tumors |
| 5 | 6 | 7 | 2 | Tumors |
| 6* | 5 | 7 | 2 | |
| 7‡ | 6 | 7 | 0 | |

* The embryos were extracted after having been kept frozen for 4 days. Again the preservation of material for subsequent injection resulted in loss of potency of the inoculum.

‡ Some of the embryos of this series were allowed to continue development until the 11th postinjection day. Upon dissection at this time, one animal was found to have a small nodular tumor on the chorioallantoic membrane. This tumor was excised from the living animal and transplanted to the celomic cavities of two 72 hour chick embryos. The tumor tissue grew but the embryos, dissected 5 and 9 days after implantation, had developed hemorrhagic blebs without evidence of tumor extension or metastasis.

119 embryos 3 days old when injected with the virus intracelomically developed hemorrhagic lesions. Embryos 6 and 13 days old, 13 in number, responded to intracelomic or intravenous inoculation in 60 per cent of cases, but the amount of virus injected was larger and the disease apparently less severe, 3 of the injected embryos hatching and living for a few days. In experiment 7 possibly all the thirteen 3 day old embryos injected would have died of their hemorrhagic lesions. It is clear, however, that the above figures are far from representing exact percentages of susceptibility since many embryos were purposely killed at an early date and others died of incidental causes.

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Figs. 1 to 8.—Serial sections showing the heart and adjacent regions of one of the embryos of experiment 9. The deeply staining area is subepicardial hemorrhage. Because no definitive lesion other than hemorrhage and edema could be made out, higher magnifications were not photographed. Mag. X 12.
achieved only when the serum was injected during several successive days, a single high dose being ineffective. Daily successive injections were tried in embryos also but this resulted only in their premature death.

but no lesions occurred. Another 5 embryos received small implants of Rous tumor in the chorioallantois. Two hatched, and these died 3 and 5 days later with hemorrhagic and neoplastic lesions in the heart, lungs, spleen, and liver.

Experiments on duck embryos.—Murphy and Rous (9) grew the chicken tumor in duck embryos, but did not mention the occurrence of hemorrhagic lesions. We injected intravenously 60 duck embryos at the 18th day of incubation with 0.5 cc. of tumor extract

FIG. 9.—Section through an excised liver bleb. The almost indiscernible border between liver tissue and hemorrhage is typical. Mag. X 95.

Fig. 10.—Section through a bleb in the spleen of an embryo. The tissues surrounding appear normal. Mag. X 95.

Fig. 11.—Bleb in breast muscle of an embryo. Hemorrhages occurred not infrequently in skeletal muscle and in subcutaneous connective tissue. Mag. X 40.

DISCUSSION

Since microscopically and in the gross no neoplasia was observed in the numerous lesions studied and in whole embryos serially sectioned it would seem that the only reason for persistence in maintaining that
hemorrhage is the result of tumor growth would be the reluctance to admit that a cancer virus can behave as an ordinary cell-destroying virus under certain conditions.

Yet this conclusion is upheld by the fact that destructive lesions in the capillary endothelium and underlying connective tissue were observed. Although of a much milder sort, they can compare with similar lesions induced in the chick embryo by necrotizing viruses, e.g., herpes virus, as shown by Anderson, and hemorrhage was induced by that virus also (1).

It is easily conceivable that the weakened vessel wall gives way under the blood pressure and "blebs" form that may or may not rupture. Let us point out here that in young birds these typical lesions have been observed with all viruses studied that induce sarcoma in chickens, ducks, turkeys, and guinea fowls (2, 5, 7).

It is quite conceivable also that the destructive lesions are more clearly shown by the early embryo than by chicks injected at the age of 1 day, and dying several days later, because the processes of immunological maturation are detectable very early after hatching. Anderson (1) observed, too, a pronounced resistance to herpes virus in the 18 day chick embryo as compared to the 9 day embryo, and it is important to note that the resistance of the older embryos manifested itself by the development of proliferative lesions in the membranes, whereas the susceptibility of the younger embryos was shown by necrotizing lesions.

Still other reasons for the lack of hemorrhagic lesions in more resistant chicks showing widespread neoplastic involvement of tissues and vessels have been considered in another discussion (2).

There is an apparent contradiction between our results and those of Murphy and Rous (9), who induced tumors in the membranes of embryos and in the embryos themselves with tumor filtrates or desiccates at 7 to 8 days' incubation and reported no hemorrhagic lesions. While the embryos used routinely in the present investigation were 4 or 5 days younger one must point out that 6 and 13 day embryos (experiments 4 and 5) injected intravenously with tumor filtrate and allowed to develop for 7 days showed mild hemorrhagic lesions, but no tumors. Neoplasia in the chorioallantois developed in only 2 cases, and in one of them typical hemorrhagic lesions developed in the embryo but no tumors. The same occurred in another case when tumor tissue was implanted in the membrane.

An important point to keep in mind in trying to explain these discrepancies is that besides injecting

1 The blood serum from 24 hour old chicks occasionally contains enough natural viral antibody for the Rous virus to be demonstrable by available neutralization tests. Serum from 5 hour old ducklings already contains natural neutralizing antibodies for the virus of the duck variant of the Rous sarcoma (8).

older embryos Murphy and Rous worked with a virus from the earliest passages, therefore with one far less active than ours. Hence, the discrepancy seems to indicate that the same "tumor" virus in a state of low activity for adults induces neoplastic lesions in most cases in the highly susceptible embryo, whereas in a state of high activity for adults it induces destructive lesions, followed or not by hemorrhage, in embryos and young birds. That the virus inducing neoplasia or cell destruction is the same is shown by the fact that in the present investigation the injection of the embryo lesions into older hosts consistently resulted in tumors.

The principle outlined above is essentially the same as that previously enunciated (3, 4), and further confirmed in this study; namely, that neoplasia by chicken "tumor" viruses of high activity evolves only when the host is endowed with a certain resistance against these viruses. A higher resistance results in no lesions; a lower resistance in destructive ones.

Other data supporting these views have been brought forward in relation to the virus of rabbit fibroma (6) and will be reported in future publications.

SUMMARY

The virus of the Rous sarcoma of chickens injected intracelomically or intravenously into a total of 132 three, six, and thirteen day chick embryos multiplied in 64 of these hosts without eliciting tumors, but produced hemorrhagic lesions in all 64 analogous to those previously described in chicks.

These lesions were serially transmitted to other embryos in 6 successive passages, again without induction of neoplasia.

The injection of extracts of these lesions, however mild, into chicks and pullets resulted in tumors of the ordinary type, but embryos in which no lesions developed apparently contained no transmissible virus.

Tumors in the chorioallantois developed in only 2 of the 64 embryos that developed lesions. Thus such growths may coexist with typical hemorrhagic alterations in the embryo proper.

Microscopic study of the hemorrhagic lesions disclosed the presence of destructive changes in the vessel wall and adjacent connective tissue and confirmed the absence of neoplasia.

REFERENCES


2 At the time Murphy and Rous carried out their investigation the tumor was still in a phase before the sixth passage, when it succeeded only in Plymouth Rock chickens. Maximum malignancy was attained at the sixth passage and hemorrhagic lesions in highly susceptible chickens were not observed until the eighth passage, 15 months from the time of the original transplantation (10).


7. Duran-Reynals, F. Unpublished observations.

8. King, J., and Duran-Reynals, F. Unpublished observations.


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