Induction of Accessory Limbs and of Sarcoma in the Newt (Triturus viridescens) with Carcinogenic Substances

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Among adult vertebrates, only some of the amphibia are capable of regenerating their limbs. Several years ago experiments were begun in an attempt to induce neoplasia in limb tissues capable of such regeneration. The common newt, Triturus viridescens, was used because of its hardiness and availability. The injection of a variety of supposedly carcinogenic substances into the forelimb of over 500 newts resulted in the development of malignant neoplasms in only two animals. Both neoplasms were sarcomas, and both followed the injection of methylcholanthrene. However, a large number of animals developed non-neoplastic "new growths." These consisted of well organized reduplications of the injected limb. The results of the early experiments have been briefly summarized in preliminary reports (2, 3).

The experiments to be reported here deal with the specificity of the stimuli necessary for the induction of the accessory limbs and of the sarcomas, and with the nature of the accessory limbs.

MATERIALS AND METHODS

Adults of Triturus viridescens, caught in ponds near Philadelphia, were maintained in aquaria. Some were kept at 20°-22° C. in semi-darkness, in aerated water, and were fed on Tubificid worms. Others were kept in well lighted rooms at 25°-30° C. in standing water containing floating plants, and were fed raw muscle or liver. There was no striking difference in response to injections among the two groups; in general, the latter group was better nourished and developed accessory growths earlier.

The substances tested included carcinogenic Pennsylvania coke oven tar, methylcholanthrene, benzpyrene, acetylaminofluorene, scarlet red, vaseline, beryllium hydroxide, and particles of two amphibian neoplasms. These substances were injected directly into the loose subcutaneous and muscular tissues of the proximal region of the forelimb in amounts of 0.005-0.02 cc., with a tuberculin syringe and an 18- to 23-gauge needle. The animals were anesthetized by placing them in ether vapor.

Coal tar, when injected alone, was first heated to 100° C. in order to dissolve its precipitated constituents. It was then drawn into a warm syringe and chilled under the water tap for injection. When pure carcinogens were added to tar, they were dissolved in heated tar at about 180° C., and the mixture chilled in a syringe, as stated above. Details of the preparation of fractions of coal tar for injection are described later. Carcinogens in olive oil or vaseline were dissolved and injected in the same way. Rapid chilling resulted in fine, easily injectable crystals of those carcinogens that tended to crystallize out at room temperature. In one experiment, methylcholanthrene crystals were ground in a mortar with sufficient olive oil to make a paste, which was then injected into the forelimb with an 18-gauge trocar.

Beryllium hydroxide was prepared by adding sodium hydroxide to beryllium nitrate. The flocculent precipitate was purified by washing and by dialysis against distilled water. A 5 per cent suspension was injected in amounts of 0.02 cc. [1 mg. Be(OH)₂] into the forelimb.

Frog kidney carcinoma and sarcoma of the newt were injected as tumor particles, using 18-gauge trocars.

Deep thermal injury to the forelimb was caused by contact with a steel wire heated to about 500° C.

Fracture of the humerus was produced by means of a small rongeur, through a longitudinal incision.

EXPERIMENTAL RESULTS

I. INDUCTION OF ACCESSORY LIMBS AND OF SARCOMA

Experiments with coal tar and pure carcinogen.

—Table 1 shows the incidence of accessory limbs and of sarcoma among newts receiving a single injection into the proximal region of the forelimb of various substances or subjected to severe thermal or mechanical injury of the same region. The variety of accessory limbs or limb parts induced is illustrated by representative photographs in Figures 1-4.

In the first attempts at tumor induction in newts with tar, 4 per cent each of methylcholanthrene, benzpyrene, acetylaminofluorene, and scarlet red were added to it. As seen from Table 1, 40 per cent of animals injected with this mixture developed accessory limbs. In order to determine the active ingredient, injections were made with components of the mixture. Tar alone proved less
active than the mixture, inducing accessory limbs in 26 per cent of animals. Tar containing 4 per cent added methylcholanthrene or benzpyrene or acetylaminofluorene was even less active than tar alone, while tar containing 4 per cent added scarlet red in olive oil was as active as the original mixture. However, 4 per cent scarlet red in olive oil was inactive in ten animals.

Beryllium hydroxide (Table 1) induced accessory limbs in six of eighteen animals injected.

Experiments with tumor tissue.—Particles of spontaneous carcinoma of the kidney of the leopard frog, a tumor probably caused by a virus (14), induced three accessory limbs among 75 animals injected (Table 1). In contrast, none of 274 animals injected with particles of the two strains of transplantable methylcholanthrene-induced sarcoma referred to above developed accessory limbs.

Effect of thermal and mechanical injury.—No accessory growths developed on the forelimbs of 21 animals burned with a hot wire, so as to involve the humerus, and of 23 animals in which the humerus was fractured with a rongeur and allowed to heal.

The incidences listed in Table 1 refer to all types of accessory limb structure that could be identified as such. The simplest growths were single digit-like projections arising from the site of injection (Fig. 4). Others ranged in complexity up to doubly reduplicated limbs, i.e., two accessory forelimbs growing from the injection site. However, it is noteworthy that only those animals receiving coal tar developed the growths early (41–87 days); and only those receiving coal tar, alone or with added carcinogens, or beryllium developed the growths in high incidence. Furthermore, only tar and beryllium produced complex growths. The other accessory growths were relatively simple, having no more than two digits, and appeared late (150–800 days).

Experiments with various fractions of coal tar.—Berendel and Schoental (1) have shown that petroleum ether effectively extracts benzpyrene and at least one other potent carcinogen from tar. Such extracts are clear yellow solutions that, on evaporation, leave a yellow-brown semicrystalline residue.

Pennsylvania coke oven tar (200 ml.) was extracted with petroleum ether (500 ml.) by shaking the two together at intervals over a period of 4 days at 37°C. The extract was evaporated to dryness. Ten grams of the residue were dissolved in 100 ml. of petroleum ether, and the solution was passed through an adsorption column containing 250 gm. of light powdered magnesia. Petroleum ether was continuously added to the top of the column. A first fraction (1-A) of 250 ml. and a second fraction (1-B) of 500 ml. were thus collected. A third fraction (1-C) of 500 ml. was obtained by adding benzene to the column. Each fraction was evaporated on a water bath, the residue dissolved in 2 parts of vaseline at 190°C and chilled in a syringe under the water tap to

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**Table 1**

<table>
<thead>
<tr>
<th>Material injected*</th>
<th>No. ANIMALS INJECTED</th>
<th>Time of appearance of first accessory limb (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coal tar</td>
<td>76</td>
<td>No.</td>
</tr>
<tr>
<td>Coal tar + 4% MCA, BP, AAP, and SR</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Coal tar + 4% MCA</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Coal tar + 4% AAP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Olive oil + 4% MCA</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Olive oil + 4% AAP</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Olive oil + 4% SR</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Vaseline + 4% BP</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Vaseline</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>MCA crystals wet with olive oil</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>Beryllium hydroxide</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>* MCA = 3-0-methylcholanthrene; BP = 5,4-benzpyrene; AAP = 3-ace- tylaminofluorene; SR = scarlet red.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>† Animals surviving 50 or more days.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>‡ Sarcoma developed in 2 animals. One of the tumors arose at the base of the accessory limb.</td>
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</table>

Acetylaminofluorene in olive oil and benzpyrene in vaseline were also inactive, while vaseline alone induced a single accessory digit-like growth after 900 days. Methylcholanthrene in olive oil induced a small accessory limb with two digits in one of four animals after 900 days. Of 34 animals, each receiving approximately 1 mg. of methylcholanthrene crystals wet with olive oil and injected with a trocar, one developed an accessory limb without digits (Fig. 8). However, a sarcoma developed in this limb a year later (Fig. 9). Another animal developed sarcoma 168 days after injection, but an accessory limb did not form. These two animals are described in Part III of "Experimental Results."
form a jelly-like mass for injection. Each fraction thus prepared was injected in amounts of 0.01 cc. into the proximal region of the forelimb of sixteen animals.

The results are shown in Table 2. Fraction 1-A, be very toxic, and more than half of the injected animals died within 2 weeks.

The high activity of all the fractions, as shown in Table 2, makes it difficult to ascribe the accessory limb induction to one or a few compounds present in tar. It is possible that, during industrial distillation, changes such as cracking and recombination of molecules occur, so that large molecules appear in all fractions. At any rate, it is of interest that tar fraction 1-C and fuel pitch both induced the same high incidence of accessory limbs (63 per cent), a result consistent with the hypothesis that at least some heavy molecules in tar show a high degree of activity.

There was some indication that 25 per cent fuel pitch extract in vaseline, when stored in the refrigerator, loses its activity. A sample stored for 5 months was tested in the following concentrations of fuel pitch extract in vaseline: 25 per cent, 12.5 per cent, and 6.25 per cent, which gave, respectively, incidences of 1 in 20, 2 in 19, and 1 in 20 newts with accessory limbs. These incidences are of the order observed with vaseline alone (Table 1).

Effects of injection of sites other than the forelimb.

—The tar mixture containing 4 per cent added methylcholanthrene, benzpyrene, and scarlet red was injected in amounts of 0.1 cc. into the hind limb of twelve newts. One animal lost the injected limb, due to infection and necrosis, and then regenerated a double hind limb, the two members of which were mirror images of each other. No growths of any kind appeared among the remaining eleven animals.

The same mixture was injected into the side of the tail of twelve newts. Six developed flat shelf-like projections that extended laterally from the injection site and had the gross and microscopic appearance of diminutive accessory tails.

Injection of the tar mixture into the back of twelve newts was followed by the development of raised pigmented nodules containing the injected material. No accessory structures or neoplasms developed.

II. Properties of Accessory Limbs

Mode of development and structure of accessory limbs.—Examination of a number of microscopic sections at various periods after the injection of tar into the forelimb showed the following changes: there was at first an apparently nonspecific inflammatory reaction around the injected mass that lasted from a few weeks to several months. During this time there was a concomitant degeneration of striated muscle and resorption of bone. These changes were very similar to the "dedifferentia-
tion” that is said to occur after amputation of a limb and before a regeneration blastema has formed (7). At any rate, the differentiated tissues of the injected region were soon replaced by a tissue resembling regeneration blastema. It was this tissue that eventually gave rise to the muscle and bone of the accessory limb, with accompanying ingrowth of nerve from the host limb and migration and growth of host limb epithelium over the accessory growth. The first readily identifiable limb structure to develop was a cone of cartilage, such as is shown in Figure 6. It was surrounded by muscle cells in various stages of differentiation, together with a few nerve fibrils. Further growth and differentiation might or might not occur. When it did, organization was of such a degree that the resulting accessory limb tissues could not be distinguished from normal limb tissues (Fig. 5).

In some animals the initial degeneration (or “dedifferentiation”) of muscle and bone were very extensive; sometimes fracture of the humerus occurred at the site of injection. However, destruction of tissue was not directly related to accessory limb formation. Injury to tissue was as severe in the methylcholanthrene series, where incidence of accessory limbs was low, as in the tar series, where it was high. The three tar fractions, 1-A, 1-B, and 1-C (Table 2), produced about the same degree of local tissue destruction but were quite different as regards their ability to induce accessory limbs. Furthermore, thermal injury or fracture of the humerus did not result in accessory limb formation (Table 1), nor did any accessory limbs develop among many animals whose limbs were partly destroyed by a chronic fungus-like infection.

It is noteworthy that the accessory limbs or limb parts in no case became larger than their normal counterparts. Also, the induced growths always consisted of reduplications of structures found distal to the point of injection, e.g., digits, carpal bones, and radius and ulna. In these respects, and in their high degree of tissue organization, the growths resembled regenerates such as might be expected following amputation of a limb at the point of injection.

**Fate of accessory limbs after amputation and transplantation.**—The foregoing observations suggested that the accessory “new growths” represented alterations of the normal regeneration mechanism and were not neoplasms. It was possible, however, that neoplastic or preneoplastic cells were present in them, cells somehow held in check by the actively differentiating blastema. For example, the methylcholanthrene-induced papillomas of rabbits, of apparently “benign” nature, extensively investigated by Friedewald and Rous (10) and others, provided a basis for this concept. These tumors may develop into invasive carcinomas when subjected to trauma or other nonspecific injuries. In the present experiments the effects of trauma and of a new tissue environment were tested by amputating the growths and observing their regeneration, as well as by transplanting fragments of the growth to different sites in the same animal or to different animals.

**Amputation of host limbs.**—It was of interest first to determine whether the presence of an accessory limb changed the regenerative qualities of the host limb. The forelimbs of six newts, each bearing an accessory limb, were amputated proximal to the accessory growth. All six animals regenerated normal limbs without accessory growths. Subcutaneous autotransplants of accessory limb tissue to the opposite limb and side failed to grow during 1 year of observation of all six animals.

**Amputation of accessory limbs.**—In the remaining experiments the accessory limb itself was amputated, 0.5–1 mm. from its base. The regenerates appearing after amputation of accessory limbs were varied, but in general they either approximated the structure of the amputated growth or were less complex. These relations are shown in Table 3, where the number of digits on the amputated limb before its amputation is compared to the number on the regenerate arising from the amputation stump. In all animals the regenerates ceased growing at or before the time they reached the size of corresponding normal structures. No changes suggestive of neoplasia were seen in any of them.

In fifteen animals, fragments of the accessory limb were autotransplanted to the opposite limb and the body wall, while the growths removed from two animals were transplanted to a total of ten other newts. Only one of the transplants be-
came established. This was an autotransplant to the opposite limb. A small conical projection slowly grew from the injection site and reached a height of 1 mm. after 3 months. It grew no more during 1 year of observation. None of the other transplants showed evidence of becoming established. These results contrast with those obtained with the two methylcholanthrene-induced sarcomas, both of which could readily be transplanted by the same technic.

### III. Induction of Sarcoma by Methylcholanthrene

Two sarcoma strains arising from tumors in newts injected with methylcholanthrene (cf. Table 1) are being maintained by transplantation in other newts. Their properties will be described in detail in a later publication. The induced tumors are shown in Figures 9 and 10, a transplanted tumor in Figure 11, and the microscopic appearance of the tumors in Figures 12, 13, and 14.

It is of interest that both of the sarcomas occurred in male newts, whereas the incidence of accessory limbs was the same in males and females. Curiously, the sarcomas when transplanted have taken better in females.

As many injected animals as possible are being kept for extended periods in order to determine whether other neoplasms arise, particularly in animals bearing accessory limbs. Over 230 have been observed for 1–2 years.

### DISCUSSION

When newts that have been taken from their natural habitat are examined with care, an occasional animal bearing an accessory or reduplicated limb is found. In this laboratory, only two among some 1,500 T. viridescens have been seen, one an imperfect accessory growth on the forelimb and the other a reduplicated hind limb.

Experimentally, accessory limbs have been produced by severe injuries that apparently involved nerves. Detailed accounts of the induction of such growths in salamanders by the surgical deviation of limb nerves are given by Guyénot and Schotte (12) and by Guyénot et al. (11) and others. If a nerve is shifted from its normal course and made to emerge near the skin, an accessory limb frequently develops near its tip. Fibers growing from the end of the nerve seem to be capable of initiating the formation of blastema.

It is possible that nerve deviation has played a part in the present experiments as a consequence of the destruction and regeneration of nerve. An injured, inflamed, or scarred region may prevent regenerating nerve from following its original course. The new twigs may then be forced far enough out of their original path to emerge near the skin, under conditions similar to those attending surgical deviation. If nerve deviation is involved in the present experiments, it appears necessary to assume that the limb-inducing substances affected in some chemical manner the directional growth of nerves, since other substances incapable of limb induction were just as injurious to tissue, and presumably to nerves, as those known to be active.

Nerve is necessary but not of itself sufficient for the formation of limb regenerates (8, 16, 18). Cells capable of forming a blastema are also required, and these are derived from the limb itself. This fact has been demonstrated by Butler (6) in experiments involving the inactivation of the blastema-forming cells by means of roentgen rays. Similar inactivation was achieved by Thornton, using beryllium (17). However, Brunst (4) and Brunst and Figge (5) have shown that roentgen rays in small doses are capable of stimulating the blastema-forming tissue, and have in this manner induced secondary (accessory) limbs and tails in young axolotls. In their experiments it is improbable that nerve deviation occurred. The results lend support to the hypothesis that in the present experiments a direct stimulation of blastema-forming tissue by the injected substances occurred.

There is little evidence to connect the accessory growths and sarcoma in a causal manner. Only methylcholanthrene proved carcinogenic, yet it induced relatively few accessory limbs. Coal tar and certain fractions of tar, and beryllium, which is known to induce osteogenic sarcoma in rabbits (9), induced a high incidence of accessory limbs but no neoplasms. It seems likely that, of substances carcinogenic in mammals, some may induce only blastema formation in the newt or may be inactive, whereas methylcholanthrene can induce neoplasia as well. It is of interest that beryllium is a powerful inhibitor of alkaline phosphatase (18), an enzyme known to be of importance for the growth of bone, which forms a prominent part of the accessory limbs.

A number of investigators have attempted to induce neoplasia in cold-blooded vertebrates by means of chemical agents. In a review published in 1949 Lucké and Schlumberger (15) state: “No convincing evidence has been produced that true neoplasia has been induced by any of the chemicals used in any cold-blooded vertebrate.”

In the present experiments such induction has been accomplished.
SUMMARY AND CONCLUSIONS

Adults of the common newt, Triturus viridescens, were given injections of various supposedly carcinogenic and noncarcinogenic substances into the proximal region of the forelimb. Other newts were subjected to thermal and mechanical injury of the same region.

Certain fractions of coal tar, or tar either alone or with added methylcholanthrene, benzpyrene, acetylaminofluorene, or scarlet red, induced the formation of accessory limbs in a high incidence (11-63 per cent). The accessory limbs grew from the site of injection and consisted of re-injected tissue, and the animal used it in walking. X4.

Sarcoma developed in two animals that had been injected with methylcholanthrene; in one the sarcoma was at the base of an accessory limb. Both sarcomas were highly invasive and have been serially transplanted in other newts.

It is concluded that some carcinogenic substances are capable of inducing not only neoplasia but highly organized growth as well.

REFERENCES

3. BERENBLUM, C. Transplantable Sarcoma of the Salamander Induced by Methylcholanthrene. Ibid., 11:239, 1951.
FIG. 5.—Section through a well-developed accessory limb, having 4 digits, induced by injection of coal tar 92 days previously. The carpal cartilages are shown at X. A longitudinal section of one digit extends distally from them. Striated muscle and nerve fibers are present at Y. The humerus of the host limb, from which the accessory limb arises, is shown at Z. The normally occurring bone of the humerus has largely been replaced by newly formed cartilage. Holmes' silver impregnation. ×28.

FIG. 6.—Section through a cone-shaped poorly differentiated accessory limb induced by coal tar injected 92 days previously. Its gross appearance was similar to the growth shown in Fig. 4. A central cone of cartilage is to be seen at X. Striated muscle is present at Y, and nerve fibers at Z. The growth is covered by mature skin. Holmes' silver impregnation. ×125.

FIG. 7.—Higher magnification of the region of striated muscle seen in Fig. 6 (Y). The striations are not very prominent, as the muscle is still in the process of differentiation. Holmes' silver impregnation. ×450.
FIG. 8.—Adult T. viridescens. Cone-shaped growth from right forelimb and axillary region 168 days after injection of this site with approximately 1 mg. methylcholanthrene crystals wet with olive oil. This structure is an accessory limb without digits. ×4.

FIG. 9.—Same animal as shown in Fig. 8, but 488 days after injection. The base of the cone has become greatly thickened, and several engorged veins have appeared. There is also swelling of the right forelimb and of the right occipital region. The animal was sacrificed 2 days later. Upon microscopic examination the swelling was found to be an invasive sarcoma (Sarcoma 5) that had arisen at the base of the accessory limb and had extended to the base of the skull. ×4.

FIG. 10.—Adult T. viridescens injected 168 days previously into the proximal region of the right forelimb with approximately 1 mg. methylcholanthrene crystals wet with olive oil. The entire right forelimb shows nodular thickening, the swelling increasing proximally, so that limb and body join in a fusiform manner. The animal was sacrificed 3 weeks later and the tissues examined microscopically. The entire swollen region was found to be invaded by sarcoma (Sarcoma 27). ×4.

FIG. 11.—First passage of sarcoma shown in Fig. 9, 133 days after injection of tumor fragments into the right forelimb. ×4.
FIG. 12.—Section of Sarcoma 27 (see Fig. 10), showing the predominance of spindle-shaped cells and their arrangement in whorls. Hematoxylin and eosin. ×100.

FIG. 13.—First passage of Sarcoma 27. The tumor cells are shown invading muscle. One cell in the lower right-hand corner is in mitosis. Giemsa. ×450.

FIG. 14.—Third passage of Sarcoma 5. The central portion of a large tumor is shown. One cell just below the center is in mitosis. Giemsa. ×450.
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