EFFECTS OF MOUSE TUMOR TRANSPLANTATION ON THE NERVOUS SYSTEM

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Mammalian tumors were first grown successfully on the chorio-allantoic membrane of the chick embryo by Murphy.[9] The recent development of this technique has been surveyed by Karnofsky, *et al.*[7] The first intra-embryonic transplants of normal mouse tissue were performed by Rawles.[10] Bueker[2] has combined both techniques: he implanted mouse tumor in the body wall of three-day embryos. This experiment was performed in connection with certain neuro-embryological problems. Previous experiments had shown that the differentiation of primary sensory and motor systems in amphibia and in chick embryos is to a large extent under the control of extrinsic factors.[4] The effect exerted by the periphery is not a species-specific; transplantations in Amphibia (Harrison,[6] Detwiler[3]) had proved the capacity of nerve fibers to invade heteroplastic structures. The same compatibility was demonstrated by Bueker[1] to exist between nerve fibers of the chick embryo and the peripheral field provided by the guinea hen embryo.

By transplanting mouse tumors intra-embryonically, Bueker wanted to test the capacity of nerve fibers to invade tissue of a different class. In addition, it was his intention to confront the nerve fibers with a homogeneous tissue, rather than with a set of heterogeneous and complex structures, as represented by a limb. He performed transplantations of three different tumors: mouse adeno-carcinoma which completely failed to grow, fowl Rous sarcoma which caused extensive hemorrhage and had no effect on the nerve structures, and mouse sarcoma 180, which grew vigorously and was invaded by nerve fibers of the host. The attention was then focused on sarcoma 180.

The characteristics of tumor growth and the response of the spinal nerve centers were investigated in embryos between six and nine days of incubation, that is, between three and six days after the implantation of the tumor. In this short lapse of time, the tumor had infiltrated the somatopleure of the body wall of the embryo and bulged as a conspicuous mass in the coelomic cavity. The mesonephros was partly invaded and destroyed. In eight- to nine-day embryos, nerve bundles were found inside the tumor. The sensory ganglia on the side and at the level of the tumor were considerably enlarged in comparison with the contralateral ganglia. The size increase was 33 per cent on the average. The motor column at the same level was, on the contrary, hypoplastic. The increase in size of the sensory ganglia and the coincident reduction of the motor column were taken as evidence for the sensory nature of the nerve fibers in the tumor. The author concluded that histochemical properties of the tumor favored the branching of sensory nerve fibers, whereas somatic motor fibers were refractory to these agents.

Three important facts were thus established: (1) the sensory ganglia are responsive not only to tissues of the same class, but also to structures of a

different class; (2) the effect of sarcoma tissue on nerve fibers is selective, as proved by the admission of sensory fibers and the lack of response of somatic motor fibers; and (3) the nerve fibers act as mediators of this effect on the respective nerve centers, the spinal ganglia.

We have repeated the experiment on a large scale, using mouse sarcomas 180 and 37.[8] The investigation was extended to the entire developmental period, and the silver impregnation technique of Cajal-De Castro was employed. The use of this technique permitted a more penetrating analysis of the relations of nerve fibers and neoplastic cells and it gave more precise information on the pattern of nerve growth inside the tumor at different developmental stages. The results of Bueker were of particular interest to us in connection with our previous study of the development of the spinal ganglia. This investigation had demonstrated in the spinal ganglia two classes of cells, which differ from each other in their developmental pattern, their affinity for silver and their topographic position.[5] It was of interest to determine whether both classes or only one would supply nerves to the tumor.

Our investigation confirmed the findings of Bueker of a selective invasion of the tumor by sensory but not by motor fibers. A careful study of the hyperplastic spinal ganglia led to the conclusion that only the late differentiating medio-dorsal sensory neurons were involved, whereas the early differentiating ventro-lateral cells did not contribute nerves to the tumor. The overall hyperplasia in volume reached a maximum of 250 per cent in the second week of development. Another source of nerve supply to the tumor was identified in the sympathetic ganglia; their contribution of nerves to the tumor seems to be even larger than that of the sensory ganglia if one uses the degree of hyperplasia as a criterion. Sympathetic ganglia adjacent to the tumor underwent a hyperplasia which reached a maximum of 600 per cent. In both sympathetic and spinal ganglia, the overall increase in volume is the result of at least three different factors: increase in cell number, increase in the size of individual cells, and acceleration in the processes of cell differentiation.

The ingrowth of nerve fibers into the tumor begins on the sixth day of development. In the preceding stages (four to six days), nerve fibers grow out from the somato-motor column and from the early differentiating sensory neurons. They fail to invade the tumor, although the latter is readily accessible to both.[18] From the sixth day on, nerves enter the tumor in increasing numbers. Sensory and sympathetic components can be easily traced to their origin (FIGURE 1); in the tumor, the fibers are intermingled and not distinguishable from each other (FIGURE 7). In the majority of the cases, the nerve bundles end abruptly without establishing contact with individual tumor cells. They branch in an irregular fashion and give origin to an extremely dense net of nerve fibers. No embryonic or adult tissue shows a nerve distribution of comparable density.

As a result of this investigation, it was concluded that sarcomas 180 and 37 release a growth promoting agent which stimulates selectively the growth of late-differentiating sensory cells and of sympathetic ganglia. Since the

publication of our first results, a large number of additional intra-embryonic transplants were performed, including 95 transplants of sarcoma 180, and 225 of sarcoma 37. An effort was made to raise the embryos to advanced stages. However, only a few embryos older than 14 days were recovered. The high rate of mortality of embryos in later developmental

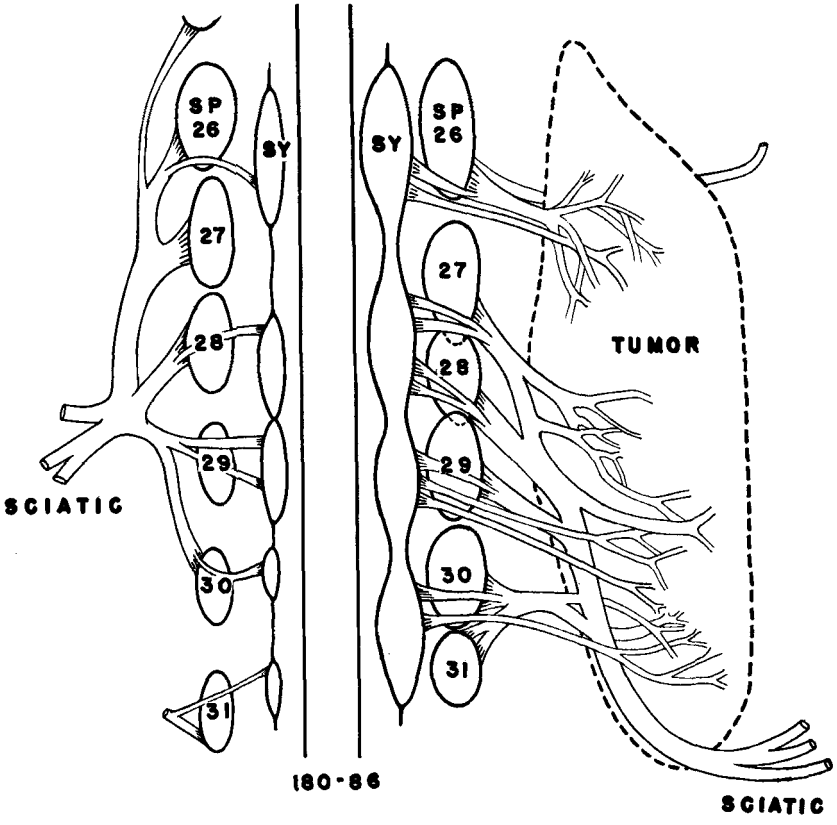


FIGURE 1. Fifteen-day embryo. Semi-diagrammatic reconstruction of the distribution of nerve fibers in the tumor (sarcoma 180). SP, spinal ganglia; SY, sympathetic chain ganglia. (From Levi-Montalcini and Hamburger, 1951, J. Exp. Zool. 116: FIGURE 3.)

stages seems to be correlated with toxic effects of the tumors, which reached a very large size in the third week of incubation.

It was found that, in addition to nerve centers which are directly connected with the tumor by nerve fibers, paravertebral and prevertebral ganglia which had no fiber connections with the tumor were also hyperplastic. This effect is particularly striking in the thoracic segments of the prevertebral sympathetic chain of 10- to 16-day embryos. Where one finds normally a pair of ganglia at the two sides of the aorta, and two slender strands of nerve fibers connecting these ganglia with the adrenal and hypogastric plexuses, one finds now large ganglionic complexes bulging laterally to the

aorta (FIGURES 4, 9, and 10). Thick nerve bundles emerge from their caudal ends and give origin to the prevertebral chains. They are many times larger than those in control embryos. The celiac plexus which, under normal conditions, is rather rudimentary until the tenth day of incubation, is already conspicuous in experimental animals on the tenth day of incubation. It forms a dense meshwork of fibers around the celiac artery, which increases in density during the following days. The celiac plexus is the main source of nerve supply to the abdominal viscera. The pattern of nerve ingrowth into the viscera of normal embryos and embryos carrying tumor transplants will be described below.

In addition to the ganglia adjacent to the aorta, one finds in normal embryos, at the level of the adrenal primordia, groups of nerve cells which are scattered among the strands of cortical cells. Under the impact of the tumor, these clusters of cells are now transformed into large ganglionic masses. An equally striking enlargement was found to occur in the hypogastric ganglia. As a result, the entire prevertebral thoracic and abdominal chain is transformed in some cases into a massive cellular column. Moreover, one occasionally finds ganglionic masses stemming from this column which project into the fringe of the tumor (FIGURE 8); they have no equivalent in normal embryos.

The intrinsic ganglia of the stomach and intestine (Meissner and Auerbach plexuses) were entirely unaffected.

The overgrowth of the ganglia which had no fiber connections with the tumor suggested that this effect on the sympathetic system was mediated through the blood circulation. To test this hypothesis, the tumor was transplanted to the extra-embryonic coelomic cavity, near the presumptive yolk stalk of three-day chick embryos. When it was realized that some sympathetic nerve fibers could still reach the periphery of the tumor, the grafts were made to the allantoic vesicle of four-day chick embryos. A total of 260 transplants were performed: 138 in the yolk stalk and 122 on the allantoic vesicle. The embryos were fixed between seven and 17 days of incubation. They were impregnated with silver and sectioned serially. The tumor grafts were recovered in every case and studied histologically.

Extra-embryonic Transplants

1. *Transplants Near the Presumptive Yolk Stalk.* The tumor became incapsulated in the yolk stalk and bulged laterally in the extra-embryonic coelomic cavity (FIGURE 2). Toward the tenth day of incubation, it had, in some cases, reached the size of a pea. Generally, however, it was of a smaller size. All embryos of this series were fixed between seven and ten days. Sections showed that a few nerves emerging from the extrinsic intestinal ganglia (Remak nerve) had reached the periphery of the tumor and branched in its peripheral parts.

2. *Transplants onto the Allantoic Vesicle.* At four days, the allantois bulges into the extra-embryonic coelomic cavity as a small pear-shaped vesicle. It has not yet come in contact with the chorion membrane. One or two fragments of sarcoma 37 or 180 were grafted onto the allantois

after perforation of the chorion. A considerable number of transplants became established and grew vigorously during the days following the transplantation. At ten days, the size of the grafts was about the same as in the

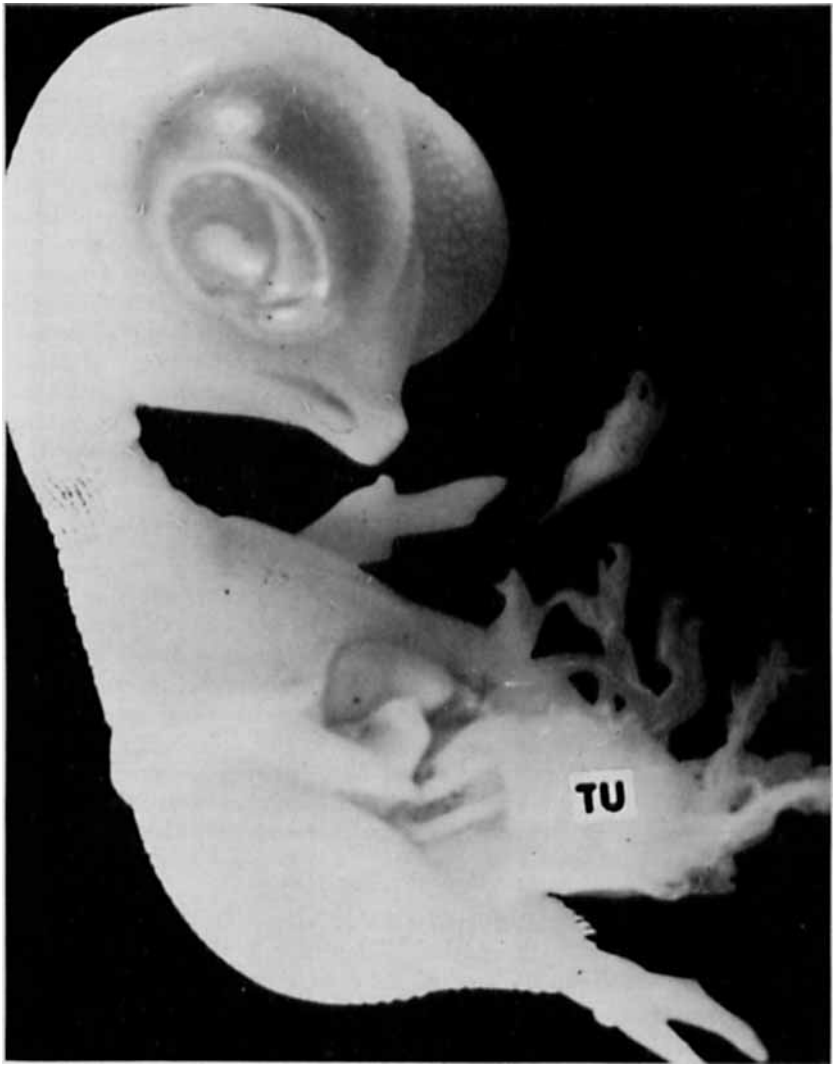


FIGURE 2. Ten-day embryo. Tu, tumor (sarcoma 37) embedded in the yolk stalk.

previous experiments. In the following days, they reached the size of a bean (FIGURE 3) and they were even larger in those embryos which survived until the 16th day of incubation. Up to 12 days, the embryos showed no sign of impairment. From then on, they appeared to be significantly smaller than control embryos: the spleen was enlarged and the liver deeply suffused

with bile. Similar and other noxic effects had been described as a result of serial transplantation of mouse adeno-carcinoma to the yolk sac by Taylor and Carmichael.[11] The rate of mortality was highly increased after the 14th

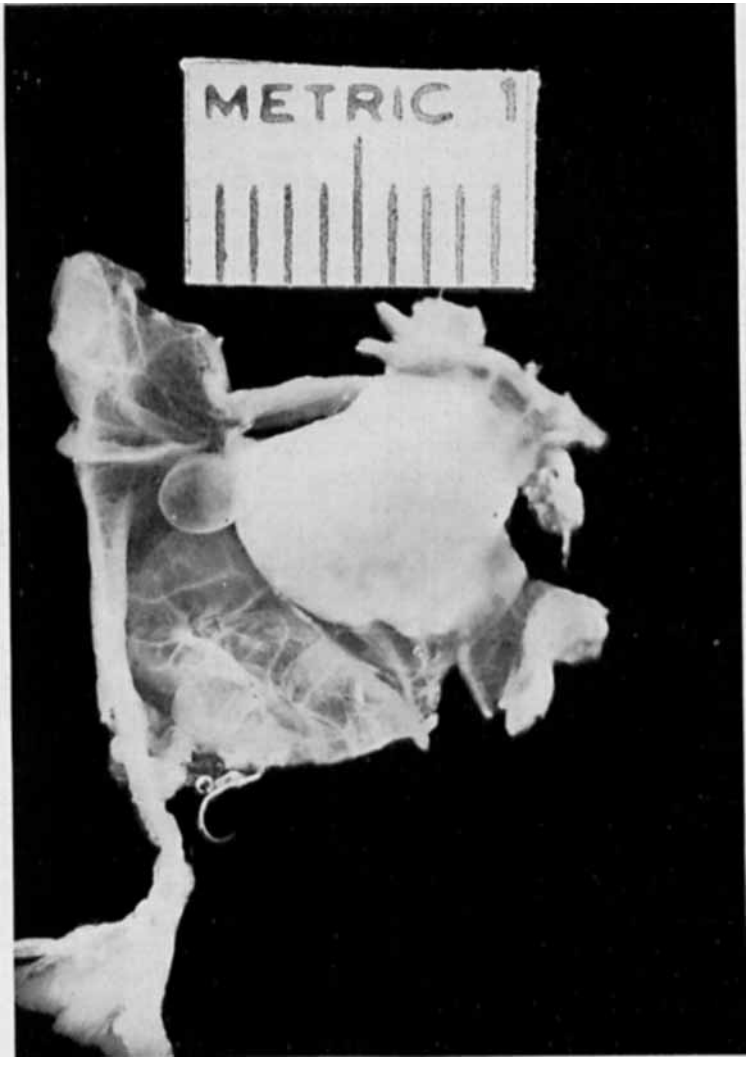


FIGURE 3. Tumor (sarcoma 37) growing on the allantoic membrane of a 14-day embryo.

day, as in the intra-embryonic transplants, and no embryo survived the 17th day of incubation. In many cases, the tumor infiltrated the blood vessels of the chorio-allantoic membrane, resulting in lethal hemorrhages.

In no cases of transplants to the allantoic membrane were nerve fibers

found in the tumor. In the majority of extra-embryonic transplants, metastatic tumor cells were found in the host. Small neoplastic nodules were scattered in the mesenteries or became established at the fringe of the thoracic or abdominal organs. The mesonephroi were the preferential site of their formation. In a few cases, metastases completely failed to occur. If the metastatic nodules happened to be within reach of visceral nerve fibers, they were invaded by them. There was no correlation, however, between the hyperplastic condition of the visceral ganglia, as described below, and the nerve connections with tumor metastases. In cases in which metastases did not occur, the effect of the extra-embryonic tumor was the same as in the cases with multiple metastases. Therefore, the occasional ingrowth of nerves into the metastatic nodules was not instrumental in the overall tumor effect. The same reasoning holds for the nerve ingrowth into the peripheral areas of yolk-sac transplants. The yolk transplants and the allantoic transplants, therefore, will be considered together in the following discussion.

The effects of extra-embryonic transplants on the sympathetic paravertebral and prevertebral chain ganglia were qualitatively identical with intra-embryonic transplants of the tumor (FIGURE 4). In all cases, the whole sympathetic system was highly hyperplastic. A total of 35 embryos were investigated in detail. Area measurements were made of the prevertebral sympathetic chain ganglia of the thoracic and abdominal level between the caudal end of the brachial spinal cord and the rostral end of the lumbo-sacral cord. Camera lucida drawings of the contours of the ganglia were made in every other section, and the areas measured with the planimeter; altogether, nine experimental cases and six control embryos were used. The results are represented in FIGURE 5. A hyperplasia of the sympathetic system was already detectable at the end of the seventh day of incubation, whereas the intra-embryonic transplants had no effect in corresponding stages. In the following stages, between eight and 12 days, the effects of intra-embryonic and extra-embryonic transplants are similar in every respect. After the 12th day, the intra-embryonic transplants have a more conspicuous effect than the extra-embryonic transplants. In these late developmental stages, the intra-embryonic tumors have generally reached a larger size than the extra-embryonic transplants, and a relation between the size of the tumor and the amount of the hyperplasia of the sympathetic system suggests itself. No quantitative measurements were made, however.

FIGURE 5 shows that the effect of both intra- and extra-embryonic tumors are progressive and that the effect of the former reaches a maximum of over 800 per cent in 16-day embryos.

Peripheral Distribution of Visceral Nerve Fibers

The next question to be discussed is the distribution of the nerve fibers which emerge from these hyperplastic ganglia.

A complete series of silver-impregnated normal embryos was available for comparison. It may suffice to mention here that, in normal embryos, from the ninth day on, a very small number of visceral nerve fibers can be traced along the main blood vessels into the spleen, ovary, testis, liver, meta-

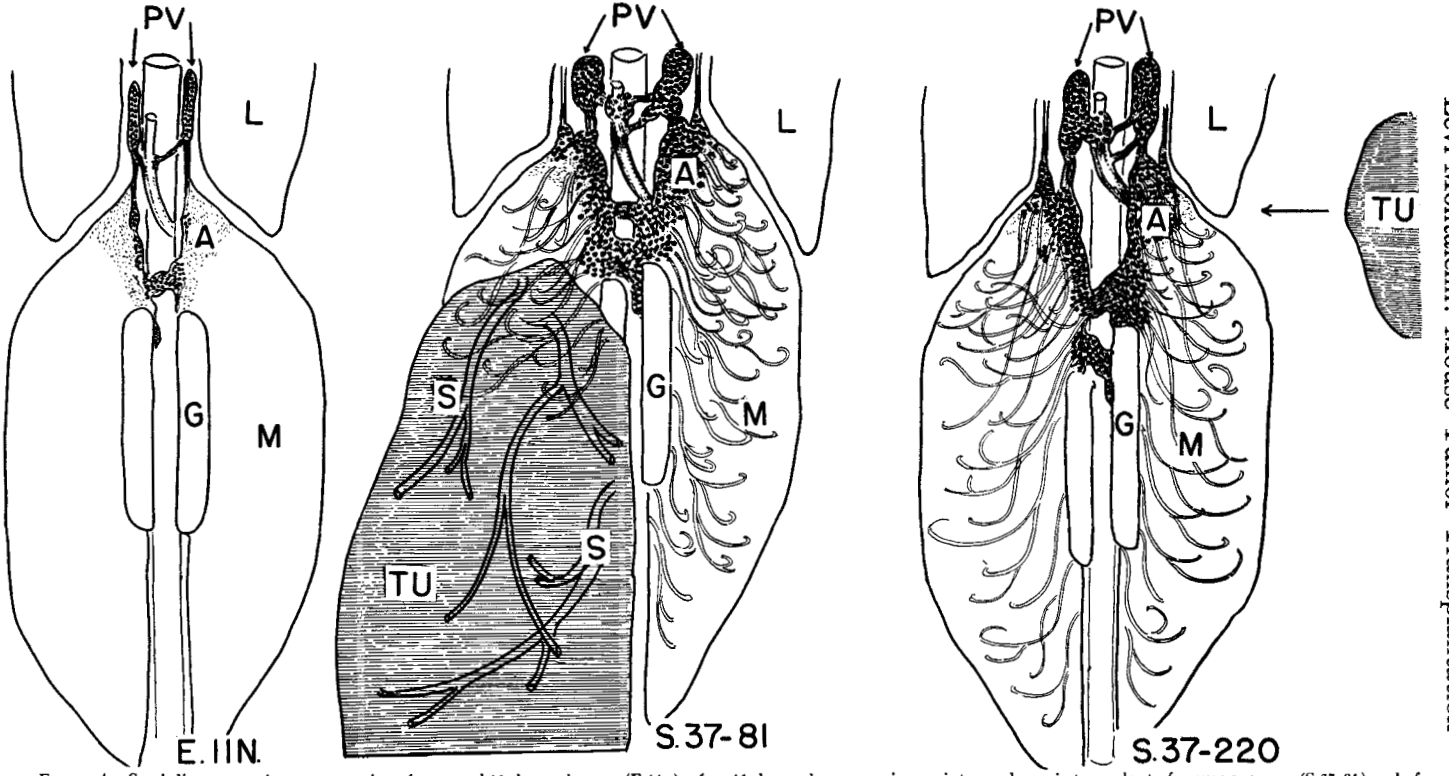


FIGURE 4. Semi-diagrammatic reconstruction of a normal 11-day embryo. (E 11n), of an 11-day embryo carrying an intra-embryonic transplant of mouse sarcoma (S 37-81) and of an 11-day embryo with transplant of sarcoma 37 on the allantoic vesicle (S 37-220). Notice the hyperplastic growth of the prevertebral chain ganglia in embryos carrying tumor transplants. Visceral nerve fibers from these ganglia invade the nearby mesonephroi. A, adrenal; G, gonad; L, lung; M, mesonephros; PV, prevertebral ganglia; S, sensory nerves; Tu, tumor.

nephros, thyroid gland and pancreas. In the mesonephros, a visceral innervation was not observed. In embryos with tumor grafts, all these organs were swamped with nerve fibers from the very beginning of their differentiation. Nerves from the nearby paravertebral and prevertebral ganglia enter the rostral edge of the mesonephros at the end of the seventh day of incubation and grow along the mesonephric tubules, following a rostrocaudal course. In the following days, the number of these nerve bundles

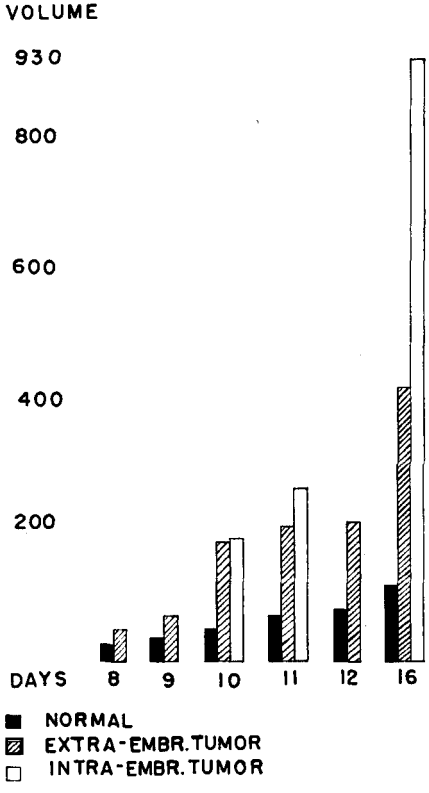


FIGURE 5. Volume increase (in arbitrary units) of the prevertebral thoraco-abdominal chain ganglia in control and experimental embryos, between 8 and 16 days of incubation.

increases rapidly and the entire organ is literally flooded with visceral nerve fibers which can be traced easily to the enlarged ganglia (FIGURE 4). A maximal density of nerve fibers was observed in those cases in which the area measurements had shown a maximal hyperplasia of the sympathetic ganglia. The nerves do not establish connections with the mesonephric tubules; they follow the blood vessels and wrap themselves around them (FIGURES 15, 16).

A similar pattern of nerve distribution was observed in the other organs mentioned above. The spleen, liver, ovary (FIGURES 13, 14), testis, metanephros, pancreas, ultimo-branchial bodies, and thyroid gland (FIGURES 17,

18) are all invaded by nerve fibers as soon as they start to differentiate. In every organ, the nerves enter with the blood vessels. Whereas the pattern of invasion is consistently the same in all cases, the density of nerve fibers

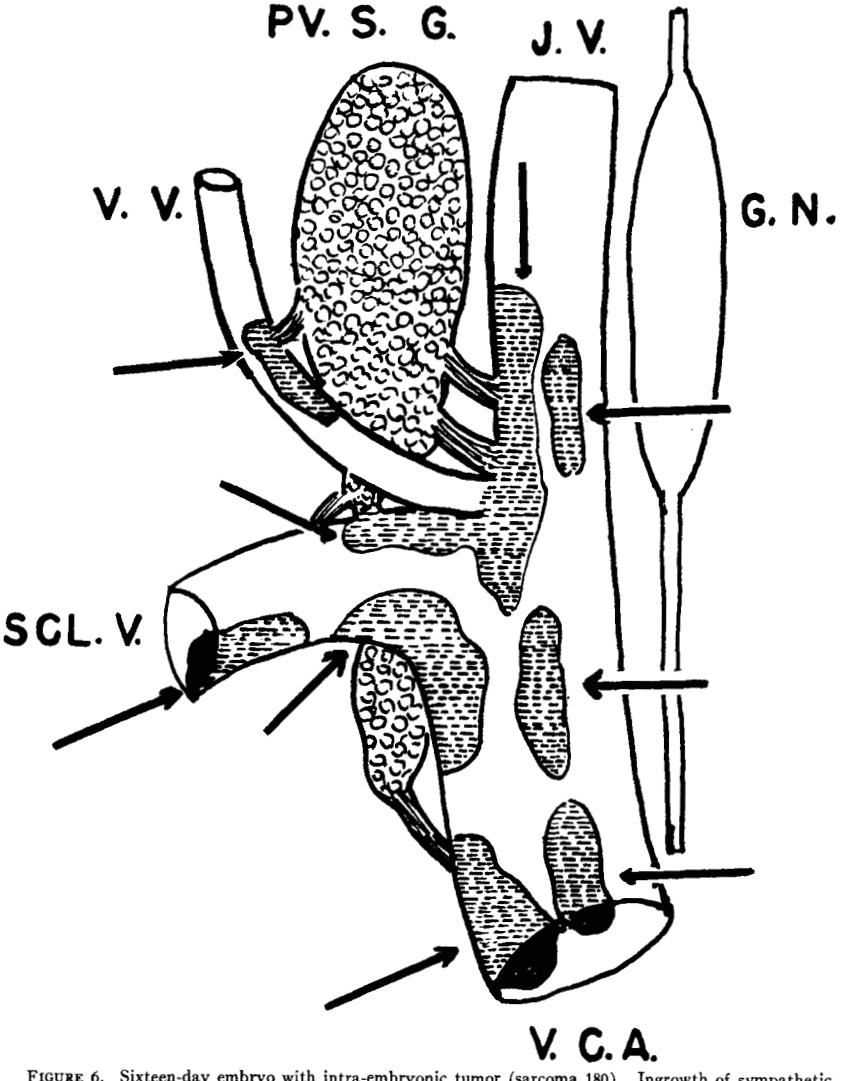
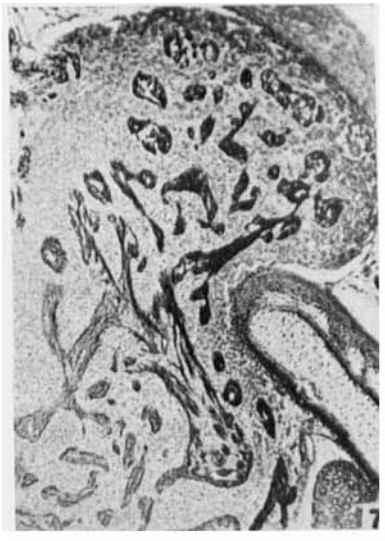
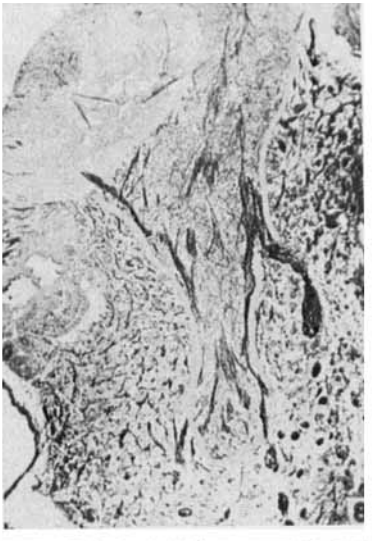
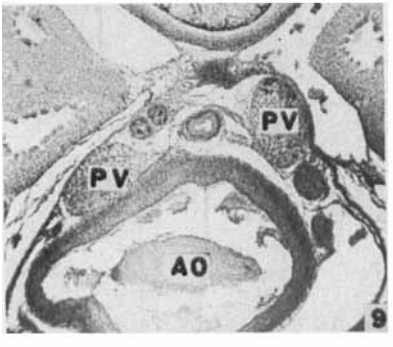


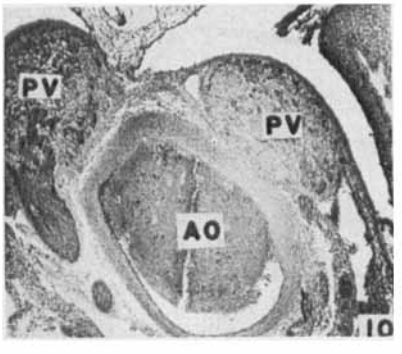
FIGURE 6. Sixteen-day embryo with intra-embryonic tumor (sarcoma 180). Ingrowth of sympathetic nerve fibers into the Jugular, Vertebral, Subclavian, Anterior Caval Veins. GN, Ganglium Nodosum; JV, Jugular Vein; Pv.SG, Paravertebral Sympathetic Ganglion; SCL.V, Subclavian Vein; VCA, Anterior Caval Vein; VV, Vertebral Vein. Arrows point to nerve agglomerations.

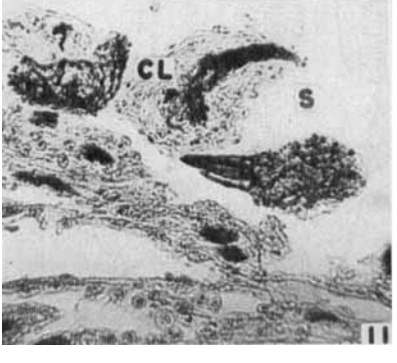
shows a high degree of variation in the different embryos. The amount of nerve fibers in the viscera is proportional to the degree of hyperplasia of the visceral ganglia. This, in turn, is dependent on the size of the tumor, as mentioned above.











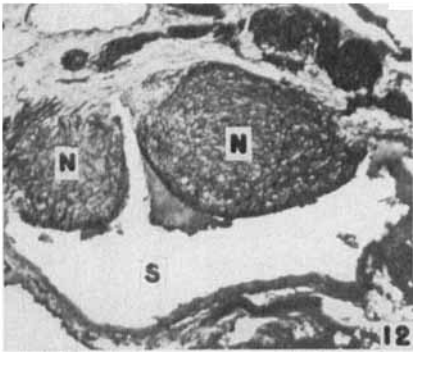


PLATE I*

FIGURE 7. Intra-embryonic tumor (sarcoma 180) in 11½-day embryo. Section through part of the tumor to show density and irregular branching of nerve bundles. (From Levi-Montalcini and Hamburger, 1951, J. Exp. Zool. **116:** FIGURE 14.)

FIGURE 8. Neoformation of sympathetic ganglion projecting into the fringe of the tumor (sarcoma 180) of an 11-day embryo. Nerve fibers from the ganglionic mass invade the tumor.

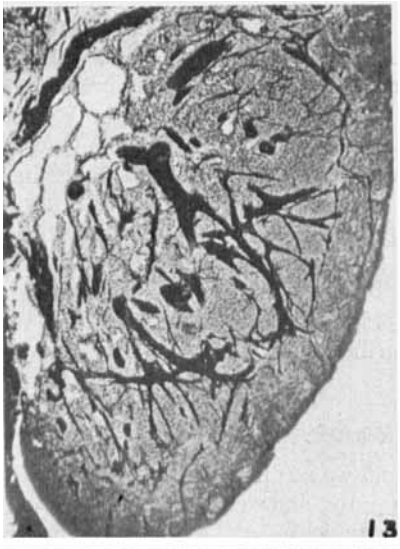
FIGURE 9. Prevertebral sympathetic ganglia (PV) on both sides of the aorta (AO) in a normal 16-day embryo.

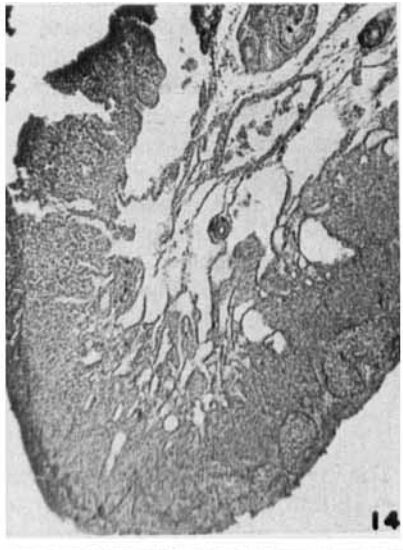
FIGURE 10. The same ganglia in a 16-day embryo with an intra-embryonic tumor (sarcoma 180). AO; Aorta; PV, Prevertebral sympathetic ganglia. Same magnification as FIGURE 9.

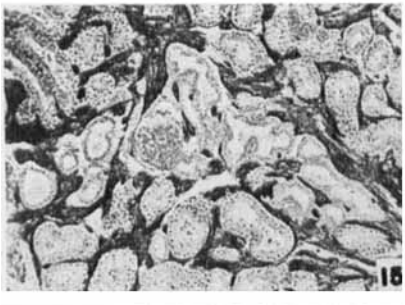
FIGURE 11. Twelve-day embryo with graft of sarcoma 180 on the allantoic membrane. Visceral nerve bundles have perforated the intima and end freely inside the subcardinal vein (S). Blood clots surround the nerve fibers (CL)).

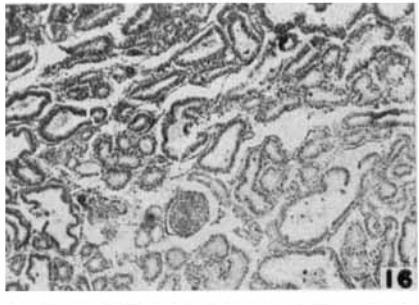
FIGURE 12. Sixteen-day embryo with intra-embryonic tumor (sarcoma 180). Two large nerve agglomerations of visceral nerve fibers (N) bulge into the subclavian vein (S).

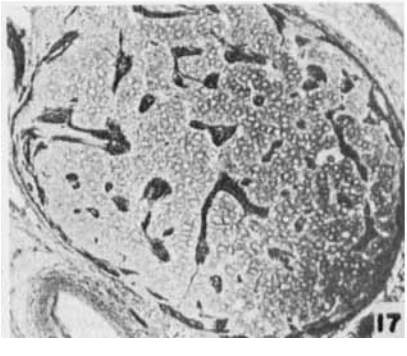
* All photographs are of silver-impregnated material (De Castro's modification of Cajal's method); all are unretouched; white demarcation line added to FIGURE 8.











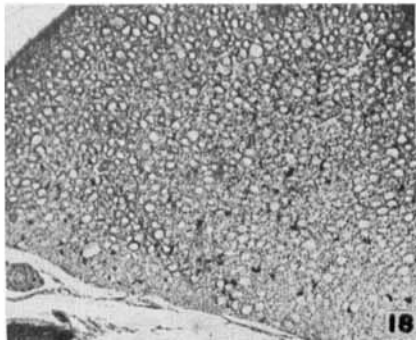


PLATE II

FIGURE 13. Branching of visceral nerve fibers in the ovary of a 16-day embryo with an intra-embryonic tumor (sarcoma 180).

FIGURE 14. Ovary of a control embryo of 16 days.

FIGURE 15. Visceral nerve fibers in the mesonephros of a 16-day embryo with an intra-embryonic tumor. The nerve fibers wrap themselves around the blood vessels.

FIGURE 16. Mesonephros of a control embryo of 16 days.

FIGURE 17. Thyroid gland of a 16-day embryo with an intra-embryonic tumor (sarcoma 180). The nerves enter the gland with the blood vessels.

FIGURE 18. Thyroid gland of a control embryo of 16 days.

A very unusual relation was found between sympathetic nerve fibers and the large veins of the thoracic and abdominal level. Whereas one normally finds small numbers, at best, of fine fibers in the media, but none in the intima, the experimental cases show at 11 days the penetration of numerous visceral nerve bundles into the intima of the large veins, such as the jugular, pre-caval, subclavian, and the subcardinal (FIGURES 6, 11, and 12). At 16 days, these nerves have enlarged to a very conspicuous size and bulge into the lumen of these veins in the form of thick nerve agglomerations (FIGURE 12). In some instances, they grow to such a size that the lumen of the blood vessels becomes obliterated. Clusters of blood cells or large blood clots surround the protruding nerve fibers. The arteries and the capillaries are spared from invasion. The death of practically all embryos toward the 17th day of incubation prevented a further analysis of the fate of these aberrant fibers.

Concluding Remarks

The investigation is still in progress and we refrain from a theoretical interpretation of these results. We can state, however, that the humoral transmission of a tumor-produced agent, which affects profoundly the development of the sympathetic system, is definitely established by the extra-embryonic transplants. These results are not in conflict with the previously-advanced hypothesis[2, 18] that nerve fibers mediate the tumor effect whenever they penetrate the tumor. We are not yet in a position to decide if we are dealing with two different modes of action or whether the same mechanism operates in both instances.

We should like to emphasize the exceptional character of the response of the visceral nervous system to the tumor. The results reveal a morphogenetic effect for which there is no parallel. The selective susceptibility of some neurons to the effect of the tumor and the absence of species specificity may suggest an analogy between this and hormonal effects. One aspect of the effect of the tumor does not fit in this picture, however. The tumor promotes an excessive growth of the sympathetic system with total disregard of the requirements of the whole organism. In fact, it upsets its harmonious development to such an extent that it causes profound changes in the sequence of developmental processes. Whereas, under normal conditions, proliferation and differentiation of the sympathetic ganglia are held within limits and kept in pace with the development of other structures, we find that under the impact of the tumor the sympathetic system overtakes other systems. The increase in the size of the sympathetic chain ganglia which, in one particular case, amounts to more than eight times the normal size, is even more impressive if one realizes that the embryos were considerably smaller than normal embryos, due to toxic tumor effects. The latest stages investigated are 16- and 17-day embryos, and there is no indication that the hyperplastic process has come to an end at that time.

The accommodation of an enormous number of nerve fibers in the viscera is equally intriguing. It indicates another severe infraction of the laws which govern the mechanics of development. Under normal conditions,

nerve fibers are admitted to organs according to a rigid time schedule, and the quantity of the entering fibers is strictly limited; each organ has a characteristic density, and regulative mechanisms are in operation to prevent hyper-neurotization. In the present instance, all barriers seem to have broken down, and the organs surrender to the invading fibers. It remains to be seen whether some properties of the nerve fibers are changed, such as their invasiveness or growth potential, or whether the organs themselves are directly affected by the tumor. An even more striking instance of the abnormal behavior of visceral nerve fibers is represented by their perforation of the intima of the veins, and the formation of nerve agglomerations in the lumen of these vessels. Nothing can be said concerning the nature of the agent and our research is now heading in this direction.

Two other questions arise in connection with the present investigation:

1. Are the nerve cell bodies or the nerve fibers the immediate target of the tumor agent?

2. What is the source of origin of the greatly enlarged and the newly-formed sympathetic ganglia? In view of their enormous volume and relatively late formation of additional ganglia (after the seventh day), it seems difficult to accept their derivation from the neural crest. In this connection, the contention of Tello[12-14] that the sympathetic cells are mesodermal derivatives and have a common origin with the endothelial cells of thoracic blood vessels deserves serious consideration.

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Discussion of the Paper

DR. DAVID A. KARNOFSKY (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): Dr. Levi-Montalcini has found that the embryos transplanted with Sarcoma 180 die about the 16th day of development, presumably due to the growth of the tumor. We have noted that the chick embryo is relatively resistant to the nitrogen mustards. The injection of a dose of methyl-*bis* (B-chloroethyl) amine hydrochloride, about $\frac{1}{5}$ of that lethal to the chick embryo, is destructive to the sarcoma 180 previously transplanted to the chorio-allantoic membrane. (Ref.: Approaches to Tumor Chemotherapy, A. A. A. S., pp. 293, 1947). After the sarcoma 180 has induced the enlargement of the ganglia in the chick embryo, it may be possible to save the embryo by destroying the tumor with nitrogen mustard, and then see if the overgrowth of nervous tissue persists or regresses.