*IN VITRO AND IN VIVO EFFECTS OF A NERVE GROWTH-STIMULATING AGENT ISOLATED FROM SNAKE VENOM**

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Communicated by V. Hamburger, July 19, 1956

INTRODUCTION

Previous reports have dealt with a nerve growth-stimulating effect of mouse sarcomas 180 and 37 on sensory[1] and sympathetic ganglia of the chick embryo.[2] The effect consisted in a striking increase in size and cell number of sensory and sympathetic ganglia adjacent to the tumor and of an extensive invasion of their fibers into the tumor. Transplantation of the tumor onto the allantoic membrane had a growth-promoting effect similar to the intra-embryonic transplantations.[3, 4] In the allantoic transplants the tumor was not accessible to nerve fibers; the effect was therefore mediated through the circulation which the tumor shared with its host. Embryos bearing mouse tumors in the abdominal wall or on the allantoic membrane differed from controls also in another respect: the viscera were overloaded with sympathetic nerve fibers, whereas control embryos showed practically no visceral innervation at corresponding stages of development. Large nerve bundles were also found in blood vessels, where they formed agglomerates projecting into the lumen of the vessel. The agent released by sarcomas 180 and 37 has therefore a dual effect: it stimulates the overgrowth of sensory and sympathetic ganglia and is responsible for the aberrant distribution of sympathetic nerves in the viscera and blood vessels. Two alternative hypotheses were offered: either the agent affects primarily the growth and differentiation processes of nerve cells, or changes of the periphery are the primary effect and the overgrowth of the ganglia is an indirect effect.[4] The following experimental result favored the first hypothesis.

Sensory and sympathetic ganglia were explanted in vitro in proximity to a fragment of mouse tumor. Under these conditions the ganglia are directly exposed to the tumor, and influences of the organism are excluded. Ganglia explanted together with normal embryonic tissues of chick or mouse served as controls. Whereas very few nerve fibers emerged from the control ganglia in the first 24 hours, a dense fibrillar "halo" surrounded the ganglia exposed to the tumor at the end of the first day.[5] These experiments indicated a direct action of the neoplastic agent on nerve cells. The tissue-culture method was then used to test the effect of a cell-free homogenate of the tumor. When it was found to possess the same activity as the growing tumor in vitro, the investigation was directed toward the purification and identification of the agent.[6, 7]

In the course of this work another growth-stimulating agent was discovered in snake venoms. The properties of this agent will be dealt with in another communication.[8] It is the object of the present report to describe its biological effects on sensory and sympathetic ganglia in vitro and in vivo and to compare them with those of the tumor factor.

EXPERIMENTS IN VITRO

The investigation was centered on sensory ganglia of 7-day chick embryos. The standard hanging-drop technique was used. The medium consisted of one part plasma, one part synthetic medium, and one part of the snake venom to be assayed. We used crude venom from *Crotalus adamanteus* (rattlesnake) and *Agkistrodon piscivorus* (moccasin) and a protein fraction (DEAE fraction) derived from the moccasin venom.[8] In each culture three to four ganglia were explanted. A total of several hundred ganglia were tested. A series of controls was run in which one part of an isotonic saline solution was added to the medium instead of the snake-venom fraction. The cultures were incubated at 37° and examined after 6, 12, and 24 hours.

Results.—The addition to the medium of 7 μg/ml of the crude snake venom or of 1 μg/ml of the DEAE fraction had identical effects. The following description therefore applies to the experiments performed with both. The results were in fact remarkably consistent, and only minor fluctuations were observed.

Six hours after the beginning of the experiment, a dense "halo" of short nerve fibers surrounded the ganglia. The fibers increased rapidly in length and density in the following hours. At 24 hours the fibrillar halo had reached its maximal density (Fig. 1). The characteristics of this most unusual fiber outgrowth were identical with those described in previous investigations dealing with mouse tumor;[5, 6] they will therefore not be recounted here (Figs. 3 and 4). It may only be mentioned that the cultures differed from controls also in another respect. Whereas in control cultures the fiber outgrowth was very scanty in the first 24 hours and the nerve fibers grew in a wavy, irregular fashion (Fig. 2), in the present experiments the fibers grew straight, radially, and showed extensive branching, forming a brushlike border at their tips. In most cases, a second, shorter halo formed in the clot between the twelfth and the twenty-fourth hour. As in the previous experiments with tumor, very few or no spindle cells were found among the nerve fibers in the first 24 hours. In the following days, however, a large number of these cells migrated from the explant between the fibers. In the present experiments a considerable degree of liquefaction of the plasma occurred. As a result, a disintegration of the fibrillar halo was observed in many of the cultures after 48–72 hours. The cultures were then discontinued.

A more limited number of experiments were performed with sympathetic ganglia explanted from 8- to 9-day chick embryos. The results were in all respect similar to the ones described above.

EXPERIMENTS IN VIVO

It had been suggested previously that the in vitro and in vivo effects of tumors were produced by the same agent. However, preliminary experiments of injections of active tumor fractions into the yolk of 7-day embryos were negative. We attribute this failure to the fact that the pure fraction was available only in very small amounts and that probably the quantities injected were considerably smaller than those produced continually by living tumor. The striking similarity between the in vitro effects of the tumor and of the snake venom, and the fact that the activity of the purest fraction of snake venom is 1,000 times that of the purest tumor fraction (on a dry-weight basis), suggested a repetition of the injection experiments with snake venom.

The effect of the venom was assayed by injecting it into the yolk of embryos ranging between 6 and 8 days of development. A small window was opened in the shell, and the fraction DEAE of the venom (containing 150 μg protein/ml together with 100 μg/ml of terramycin) was injected into the yolk with a 1-ml. syringe.

In these preliminary experiments the total amount of venom injected, the stage of the embryos, and the length of time of the experiments were varied, in order to determine (*a*) the tolerance of the embryo, (*b*) the stages of development which are sensitive to the effect of the venom, and (*c*) the length of time required to secure an effect.

The injections were repeated two or three times a day. A total of 120 embryos were injected; 17 were recovered and impregnated with silver following the Cajal–De Castro technique. They were sectioned serially and used for the present investigation.

Results.—The toxicity of the venom was reflected in the severe mortality and in the diffuse edema which affected the surviving embryos. The results proved beyond doubt that the venom possesses a growth-stimulating effect on sensory and sympathetic ganglia of the embryo similar in every respect to the effect of mouse sarcomas. The increase in size of the sympathetic system and, in particular, of its prevertebral chain was so striking that it overshadowed the reaction of the sensory ganglia. The same results were obtained in embryos bearing mouse sarcomas. The analysis was then focused on the sympathetic system.

Of the 17 embryos, 11 had been injected over a period of between 36 hours and 4 days, and 6 over a period of only 26 hours. Ten of the 11 embryos injected for a longer period showed a considerable increase in size of the sympathetic ganglia and an atypical distribution of nerve fibers in the viscera. The intensity of the effect varied in different embryos: it increased parallel to the length of the time the embryos had been exposed to the venom. A definite increase in the prevertebral sympathetic complex was observed in one embryo injected over a period of 36 hours, from 6 to $7^1/_2$ days, with a total amount of 0.5 ml. Nerve bundles emerging from the prevertebral complex entered the adjacent mesonephroi (Figs. 9 and 10). No nerves are found in the mesonephroi at corresponding stages in control embryos. The nine other embryos which showed a positive effect were fixed 2, 3, and 4 days after the beginning of the experiment. The total amounts of venom injected were: 0.6 ml. in one, 1.3 ml. in four, 1.8 ml. in two, and 3.0 ml. in two cases. A maximal effect was observed in the two embryos injected over a period of 4 days with 3.0 ml. and recovered at 10 days. The embryos injected for only 26 hours showed no effect.

In one of the embryos injected over a period of 4 days, one ganglion of the prevertebral sympathetic complex at the level of the adrenal was measured. Each section was projected with the help of the camera lucida, and the contour of the ganglion was drawn on cardboard. The total number of sections was then weighed, and the weight was compared to that of the same prevertebral ganglion of a control embryo.

The results gave an increase in weight of 3.2 times the control. Since the weight is proportional to the volume, the figure indicates a similar increase in volume. Estimates of cell numbers were obtained by inserting a micrometer disk in the ocular and counting the number of cells in 200 squares each in comparable regions of ganglia from normal and injected embryos, using oil immersion. The results are

1,066 cells in 200 squares of the injected embryo and 2,158 in the control. The ratio of cell number per unit area was therefore 1:2. The volume ratios were then divided by the cell-count ratios to obtain an estimate of the relative increase in cell numbers. The latter amounts to 1.6. Hence the over-all increase in size is due to a greater extent to cellular hypertrophy and to a lesser extent to an increase in cell numbers. Comparable results were obtained in embryos bearing tumors.[2]

A comparison of hyperplastic and normal ganglia (Figs. 5 and 6) shows that the cells of the injected embryos are not only larger than the controls but also in a more advanced stage of differentiation. This is attested by the presence of neurofibrils in the cytoplasm of almost all the sympathetic neurons in the experimental cases, but only in very few of the controls (Figs. 7 and 8). Furthermore, the nerve bundles which emerge from the hyperplastic and hypertrophic ganglia are much larger than those in the controls.

COMPARISON OF THE IN VIVO EFFECTS OF MOUSE SARCOMAS AND SNAKE VENOM

The results of the preliminary experiments with snake venoms reported here point to a striking similarity between the two phenomena.

In both groups of experiments not only the mesonephroi (Figs. 9 and 10) but also other viscera—thyroid, metanephroi, spleen, and liver—are invaded by sympathetic fibers. In the experiments with snake venom as in those with tumor, nerve bundles perforated the wall of thoracic veins and projected inside the blood vessels (Figs. 11 and 12). Since the experiments with venom were not performed in as late developmental stages as those with tumor, the neuromas were of small size and comparable only to neuromas found in 9–10-day embryos bearing a mouse tumor. The quantitative data reported above are also in line with those given for tumor effects.[2, 4]

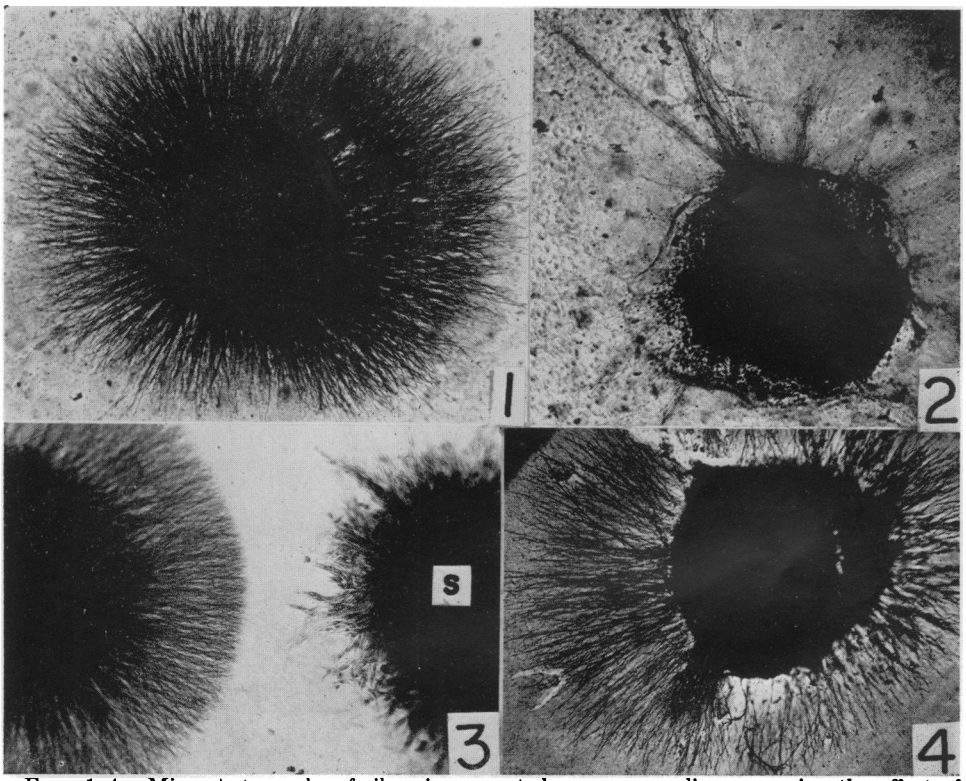
In both groups of experiments no morphological changes were detected in any part of the nervous system other than spinal and sympathetic ganglia.

CONCLUDING REMARKS AND SUMMARY

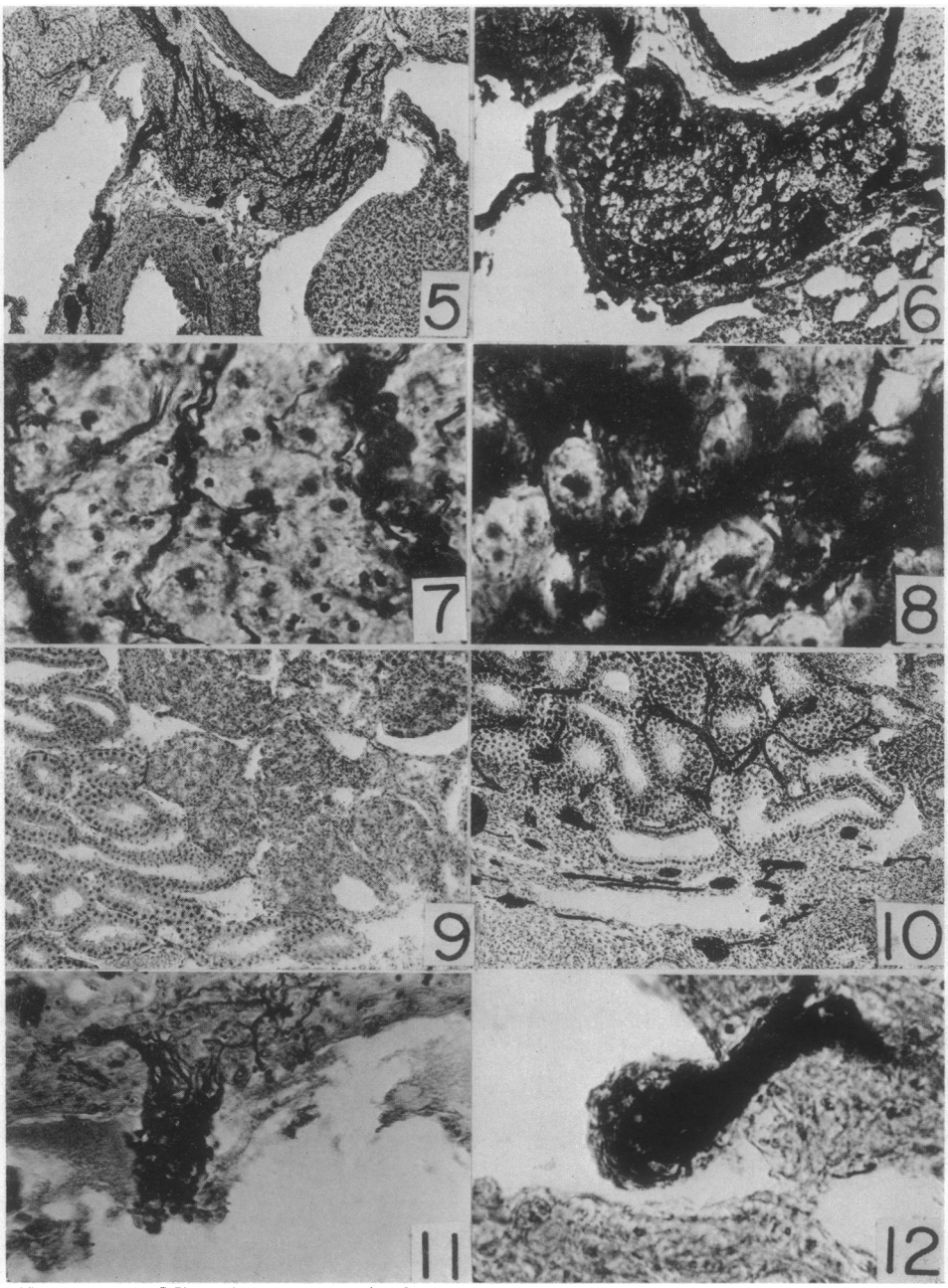
A nerve growth-promoting agent was discovered in snake venoms, and its in vitro and n vivo effects were investigated.

In both sets of experiments the nerve fiber outgrowth from sensory and sympathetic ganglia was found to be far beyond the normal range. Furthermore, the experiments in vivo gave evidence of a remarkable increase in cell size and cell numbers in sympathetic ganglia and of an acceleration of their differentiative processes. The nerve fibers produced in excess entered into the adjacent viscera.

These observations show that the snake venoms duplicate the effects of a "protein fraction" of mouse sarcomas in vitro and the effect of living sarcomas in vivo. The similarity of the two agents is further emphasized by the data which were obtained in attempts to purify them. In both instances, we are apparently dealing with a protein or a substance bound to protein.[7, 8] The purest fraction of snake venom obtained so far is approximately 1,000 times as effective as our purest fraction of mouse tumor (on a dry-weight basis).[8] This fact as well as the ready availability of different snake venoms will facilitate the further analysis of the phenomena reported above.



FIGS. 1–4.—Microphotographs of silver-impregnated sensory ganglia, comparing the effect of the snake venom, of the intact tumor, of the cell-free extract of the same tumor, and a control ganglion. All ganglia were isolated from 7-day embryos and cultured for 24 hours. Fig. 1, sensory ganglion growing in a medium to which the fraction DEAE of moccasin venom had been added. Fig. 2, control ganglion. Fig. 3, ganglion combined with a fragment of sarcoma 37 (*S*). Fig. 4, ganglion growing in a medium to which cell-free extract of the tumor has been added.



FIGS. 5–12.—Microphotographs of silver-impregnated control and experimental embryos. Fig. 5, prevertebral sympathetic ganglion of a 10-day embryo. Fig. 6, prevertebral sympathetic ganglion of a 10-day embryo injected with moccasin snake venom (fraction DEAE) for 4 days. Figs. 7 and 8, enlarged sectors of Figs. 5 and 6, respectively. Figs. 9 and 10, mesonephros of a control and of an injected 10-day embryo. Notice the sympathetic nerve fibers between the mesonephric tubules in the injected embryo. Fig. 11, neuroma projecting into the lumen of a vein of a 9-day embryo with an extra-embryonic sarcoma 37. Fig. 12, same, in a 10-day embryo, injected with moccasin venom, fraction DEAE (see text).

Our failure, so far, to obtain positive effects after in vivo injection of mouse tumor fractions was attributed to the relative weakness of the tumor fraction rather than to the presence of two different agents in the tumor—one that would be responsible for the in vivo effects and one responsible for the in vitro effects. The present finding that the much stronger venom factor reproduces both effects in every detail gives further strong support to our contention.

* This work has been supported by Grant B-463 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service, by a grant of the American Cancer Society, recommended by the Committee on Growth, National Research Council, and by a contribution from an institutional grant of the American Cancer Society to Washington University. The able assistance of Miss Barbara Bankhead is gratefully acknowledged.

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ON TIME LAGS IN EQUATIONS OF GROWTH

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Communicated by G. E. Hutchinson, July 27, 1956

An earlier paper by one of the authors[1] analyzed the equation

$$\frac{dN_{(t)}}{dt} = bN_{(t)}\left[\frac{K - N_{(t-\tau)}}{K}\right], \qquad (1)$$

which has been suggested[2] as a mathematical description of a population growing at a constant reproductive rate (b) toward a saturation value (K), with a self-regulatory system represented by $(K - N)/K$ which takes a finite time (τ) to react to changes in the environment caused by the approach of the population to the saturation level.

When this equation is used to describe real populations, a second time lag, although not expressed, is clearly implied. In the species of animals normally used for laboratory experiments in population dynamics a finite amount of time is needed for the animal to respond to changes in its environment. The lag introduced into the self-regulatory system in equation (1) becomes of great importance as (N) approaches (K). While the population is still in the exponential stage of its growth, and (N) is quite small in comparison to (K), another lag, the time needed for the reproductive process to take place, is more important.

The equation as written states that the change in the number of animals in the time interval (dt) is some function of the number of animals present at time (t).