Eur J Cancer Clin Oncol, Vol. 21, No. 8, pp. 957-964, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

Sympathetic Ganglia Augment Growth of Neuroblastoma *in Vitro**

EWA CHELMICKA-SCHORR,†‡§ RILEY C. YU,† MICHAEL G. SPORTIELLO† and BARRY G. W. ARNASON†

The University of Chicago, Departments of †Neurology and ‡Pediatrics, and the Brain Research Institute, 5841 South Maryland Avenue, Chicago, IL 60637, U.S.A.

Abstract—*The sympathetic nervous system modulates the growth of C-1300 mouse neuroblastoma (C-1300 NB)* in vivo. *We now report that the presence of sympathetic cervical ganglia in cultures containing C-1300 NB or dispersed S-20 neuroblastoma (S-20 NB) cells augments growth of these tumors* in vitro. *Sympathetic ganglia-conditioned medium increases proliferation and survival of S-20 NB cells, indicating that nervous system-derived growth factors can exert an effect on neoplastic cells.*

INTRODUCTION

WE HAVE shown previously that the sympathetic nervous system (SNS) modulates growth of C-1300 NB *in vivo*. In mice treated with 6-hydroxydopamine either as newborns (sympathectomy) or as adults (axotomy) and then transplanted with C-1300 NB, growth of tumor was retarded significantly [1, 2]. To the contrary, when newborn mice were pretreated with nerve growth factor (NGF) prior to tumor inoculation (this treatment produces hypertrophy of the SNS), C-1300 NB tumor growth was augmented significantly [2]. Prior SNS axotomy failed to influence growth of the A-10 mouse breast adenocarcinoma, demonstrating relative specificity for the effects observed. We also have shown that administration of chlorisondamine, a nicotinic blocking agent that prevents afferent input into adrenergic neurons by competing with acetylcholine for receptor sites and in newborn mice arrests maturation of the SNS, slows growth of C-1300 NB in mice pretreated as newborns [3]. Our experiments indicate that an intact SNS is essential for NB growth. Whether this relates to the common embryologic origin of NB and SNS is not established. We now report on augmented growth of cells from C-1300 NB explants and dispersed S-20 NB cells in the presence of sympathetic ganglia or sympathetic ganglia-conditioned medium.

MATERIALS AND METHODS

C-1300 mouse neuroblastoma (C-1300 NB) was obtained from Mason Research Institute (Worcester, MA) and carried subcutaneously throughout in A/J mice. S-20 cells, a cholinergic clonal cell line derived from C-1300 NB, was a gift from Dr. R. Rosenberg and was maintained in culture in Dulbecco's modified Eagle's medium (DME; Gibco Lab, Grand Island, NY, Cat. No. 430-2100) containing 10% fetal bovine serum (FBS). Passages 8-16 were used in our experiments. A-10 mouse breast adenocarcinoma (A-10) was obtained from Jackson Labs (Bar Harbor, ME) and grown subcutaneously in AHe mice. Superior cervical sympathetic ganglia (SCG) were obtained from 1-day old Wistar-Furth rats. Each sympathetic ganglion was cut into eight pieces and 3-4 explants were seeded onto collagen-coated Petri dishes (surface area 9 cm^2). SCG explants were cultured in DME enriched with 10% FBS and NGF 84 ng/ml at 37°C in humidified 5% CO_2 balanced with air. Outgrowth of fibers from SCG explants was visible after 24 hr. Skin fibroblasts were obtained from newborn Wistar-Furth rats and grown as explants (3-4 explants/dish) or as dispersed cells (5×10^3/dish) in DME enriched with 10% FBS. SCG-conditioned medium was obtained from cultured SCG that had extended multiple processes. Culture medium containing 1% FBS and NGF was substituted for regular

Accepted 22 February 1985.
*This work was supported in part by grants from the National Institutes of Health No. 5 RO1 NS-18413-02, Sarah D. Mayer Memorial Grant for Cancer Research of the American Cancer Society PDT No. 253, and the Brain Research Foundation.
§To whom requests for reprints should be addressed.

medium for the 24-hr period prior to supernatant removal. Fibroblast-conditioned medium (FCM) was obtained from cultured skin fibroblast explants or dispersed fibroblasts using the method described above for SCG-conditioned medium.

In experiments in which tumor explants or cells were grown in the presence of SCG that had extended multiple processes, C-1300 NB explants, S-20 cells or A-10 tumor were added. Each dish contained 3–4 C1300 NB or A-10 explants or 10^6 S-20 cells and 2–3 SCG explants. Control cultures consisted of 3–4 C-1300 NB or A-10 explants or 10^6 S-20 cells in the absence of SCG. The medium was changed twice a week in cultures containing C-1300 NB explants and every other day in cultures containing S-20 NB cells.

For experiments with SCG-conditioned medium, 5×10^4 (4-day experiment) or 10^5 (9-day experiment with photographs) S-20 cells were seeded onto Petri dishes and grown for 24 hr in DME containing 10% FBS. After 24 hr in experimental dishes, SCG-conditioned medium containing 1% FBS and NGF replaced DME medium containing 10% FBS. In control dishes DME with 10% FBS was replaced by DME containing 1% FBS and NGF. Medium was changed every 3 days. Cells were counted on the fourth and ninth days in culture and photographs were taken after 14 days in culture. For experiments with FCM, 5×10^4 S-20 cells were seeded on the Petri dishes and cultured in FCM containing 1% FBS. Cells in control dishes were cultured in DME containing 1% FBS. Medium was changed once. Cells were counted after 4 days in culture.

RESULTS

C-1300 NB explants grown in the presence of SCG had abundant growth of NB cells from the explants (Fig. 1). C-1300 NB explants grown in the absence of SCG had only sparse growth of the NB cells at the margins of the explants (Fig. 2). These findings were highly reproducible in each of four experiments. S-20 NB cells grown in the presence of SCG covered the entire area in proximity to the SCG (Fig. 3). When S-20 NB cells were grown in the absence of SCG, only small aggregates formed (Fig. 4). S-20 cells grown in SCG-conditioned DME enriched with 1% FBS and NGF covered the entire Petri dish after 14 days in culture (Fig. 5), whereas after this same time only scattered S-20 cells had survived in control dishes fed with DME containing 1% FBS and NGF (Fig. 6). This result was reproducible in each of five experiments. In additional experiments S-20 cells grown in the presence or absence of conditioned medium were counted. After 4 days in culture the number of cells grown in conditioned medium was $2.2 \pm 0.29 \times 10^5$/dish; the number of cells in control medium was $1.2 \pm 0.31 \times 10^5$/dish (eight experiments). This difference was statistically significant at $P < 0.01$. After 9 days in culture the number of cells grown in conditioned medium was $9.6 \pm 0.8 \times 10^5$ and the number of cells in control medium was $3.5 \pm 0.3 \times 10^5$ (three experiments). This difference was again statistically significant at $P < 0.01$. The numbers of S-20 cells grown in FCM did not differ from controls. The number of S-20 cells grown in control medium was 1.7×10^5 dish, the number of cells grown in skin explant conditioned medium was 1.6×10^5/dish and the number of cells grown in dispersed fibroblast conditioned medium was 1.8×10^5/dish (each number is a mean from three dishes). Growth of A-10 breast adenocarcinoma tumor in culture was not influenced by the presence of SCG in culture (three experiments).

DISCUSSION

We have shown that the presence of SCG in cultures containing C-1300 NB explants or dispersed S-20 NB cells augments growth of mouse neuroblastoma *in vitro*. SCG-conditioned medium increases proliferation and survival of S-20 cells in medium containing 1% FBS. We have also shown that non-neuronal cell fibroblasts which are present in SCG cultures do not have any trophic effect on S-20 NB cells and that growth of a non-neuronal tumor, A-10 breast adenocarcinoma, is not influenced by the presence of SCG in culture. These findings are in agreement with those of our previous experiments in which we showed that growth of neuroblastoma in mice with either a totally (sympathectomy) or partially (axotomy) ablated SNS was slowed while growth of neuroblastoma in mice with an hypertrophied SNS was augmented. The data point to a nervous system directed control over growth of certain tumor cells.

That the nervous system can exert trophic influences on normal tissues is well documented. The neurotrophic phenomenon was initially demonstrated in studies of regeneration of the limbs of larval and adult newts and salamanders [4–6]. Denervation of the stump immediately or shortly after amputation prevents limb regeneration in these animals. Denervation during early regeneration leads to a decreased incorporation of specific radioactive precursors into DNA, RNA and protein. In additional experiments, protein synthesis was shown to be partially or totally restored by infusion of nerve homogenate, or of newt or frog brain homogenate into denervated regenerating limb buds [7]. It was also shown that recovery of DNA synthesis could be

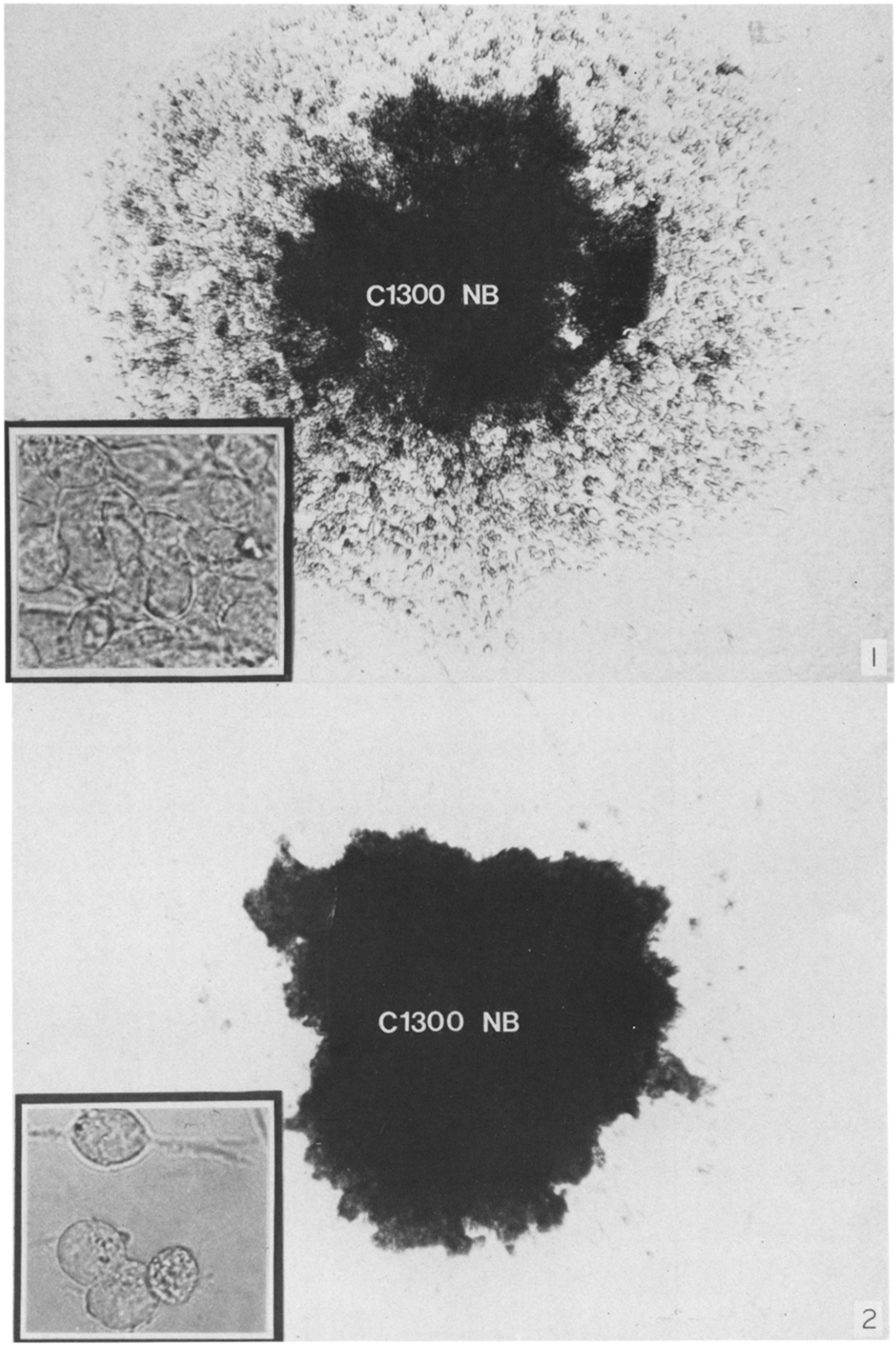
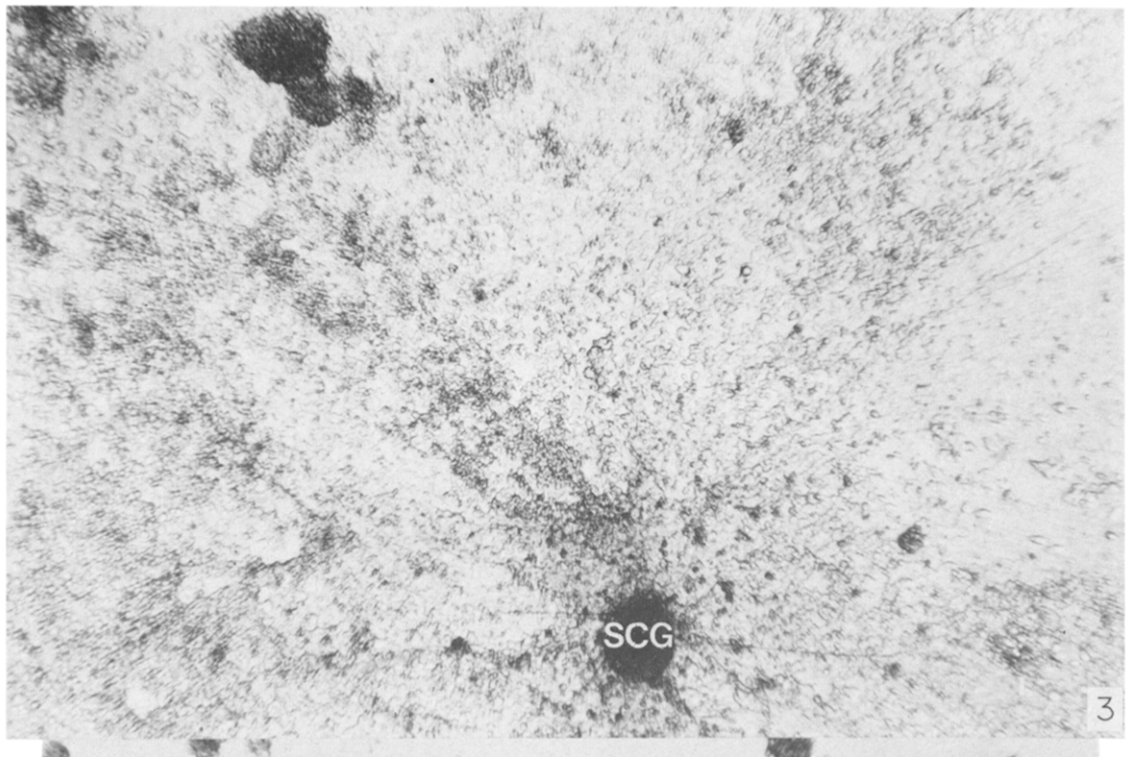


Fig. 1. A representative C-1300 NB explant grown in DME enriched with 10% FBS and NGF in the presence of SCG (× 130). *Cell growth at and beyond the margin of the C-1300 NB explant is abundant* (9 days *in culture*). *The insert is a high-power view of tumor cells in the outgrowth zone* (× 680).

Fig. 2. A representative C-1300 NB explant grown in DME enriched with 10% FBS and NGF in the absence of SCG (× 130). *Cell growth at and beyond the margin of the C-1300 NB explant is sparse* (9 days *in culture*). *The insert is a high-power view of tumor cells in the outgrowth zone* (× 680).



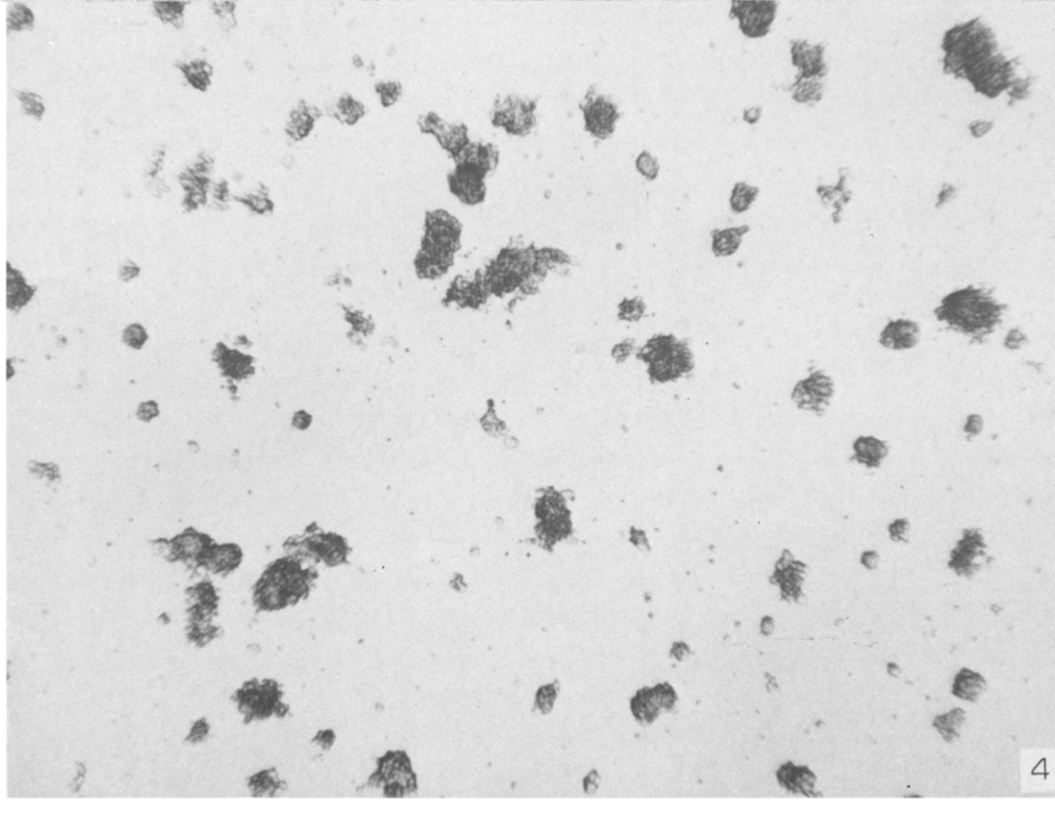


Fig. 3. Dispersed S-20 cells grown in DME enriched with 10% FBS and NGF in the presence of SCG (× 130). S-20 cells cover the entire area in proximity to the SCG (9 days in culture).

Fig. 4. Dispersed S-20 cells grown in DME enriched with 10% FBS and NGF in the absence of SCG (× 130). The S-20 cells have formed small aggregates (9 days in culture).

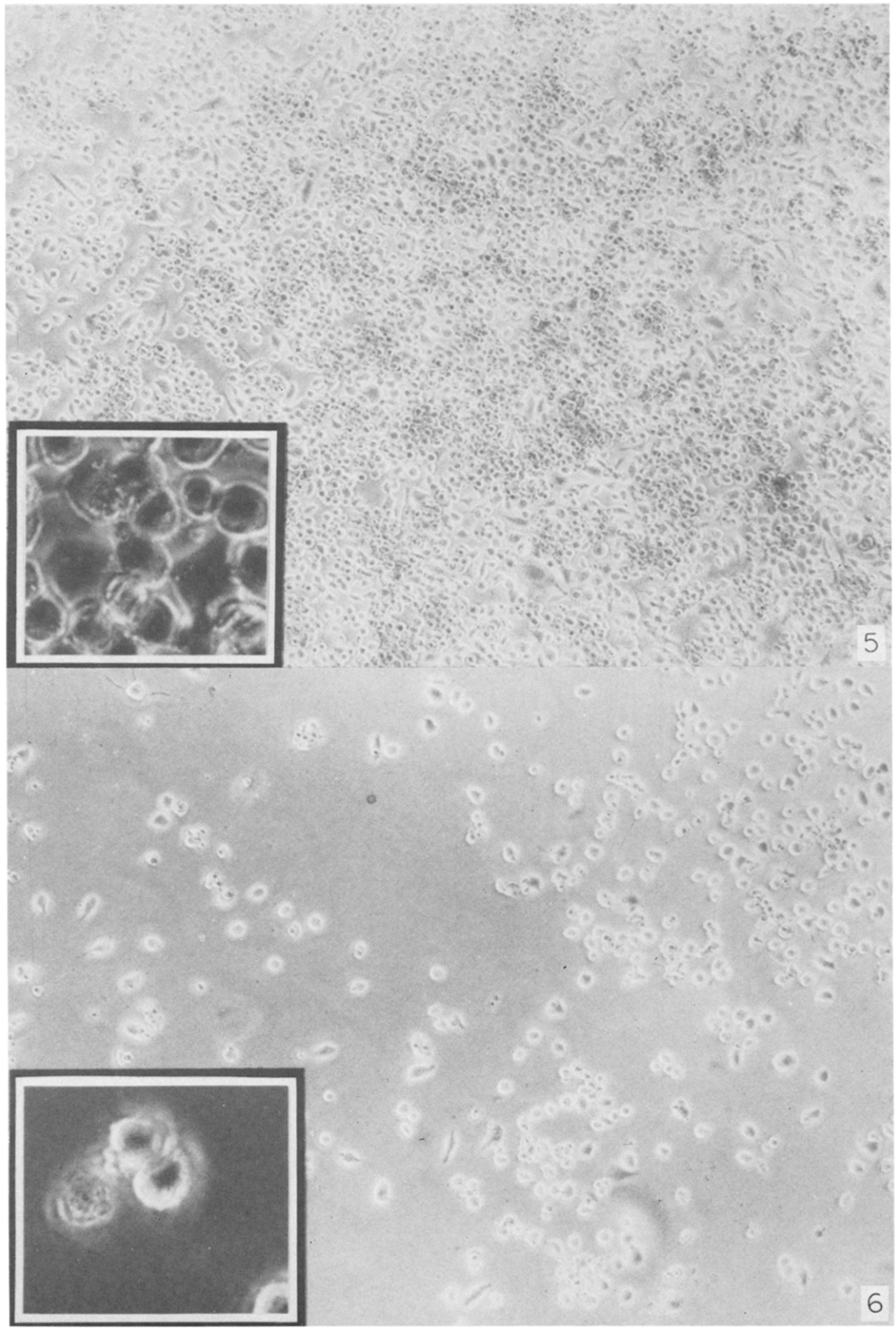


Fig. 5. Dispersed S-20 cells grown in SCG-conditioned medium enriched with 1% FBS and with NGF for 14 days (× 130). *Cells cover the entire Petri dish. The insert shows a representative high-power field* (× 680).

Fig. 6. Dispersed S-20 cells grown in DME enriched with 1% FBS and with NGF (× 130). *After* 14 days *in culture few S-20 cells have survived. The insert shows a representative high-power field* (× 680).

induced in a denervated regenerating stump with newt brain extract [8]. It was postulated that some neurotrophic factor controls the cell cycle during amphibian limb regeneration since when the cell cycle in denervated limbs was studied, it was shown that there were fewer cycling cells in denervated than in control limbs. One explanation for this finding would be that some cells are completely blocked and removed from cycling, another that cells in the denervated regenerate were arrested in the G_1 stage of the cell cycle [9, 10].

Additional evidence for the existence of neurotrophic factors is provided by the discovery that sensory neurons provide a mitogenic stimulus for Schwann cells. The neural-derived mitogen is located on the neurite surface. Direct contact with the neurite surface or a neurite membrane fraction is necessary for Schwann cell stimulation. Conditioned media or the soluble fraction of neurite homogenate failed to provide a mitogenic signal in these experiments [11-14].

Embryonic sympathetic neurons have been shown to increase ganglionic non-neuronal cell proliferation. Co-culturing of neuronal cells with non-neuronal cells resulted in an increased [^{3}H]thymidine incorporation by the latter. The effect was obtained only if cells were grown in direct physical contact and was neuronal cell-dose dependent. Exposure to neuronal sonicates was also followed by an increased non-neuronal ganglionic cell proliferation and this effect again was dose-dependent. Norepinephrine was ruled out as a mitogenic factor [15, 16].

That nerve exerts a neurotrophic effect on muscle is well recognized but the chemical nature of the factor involved remains unknown [17]. It has been shown that chick brain extract stimulates proliferation and protein synthesis and accelerates maturation of chick embryonic muscle cells in culture [18]. A trophic effect of sympathetic ganglia has also been shown on normal and dystrophic chicken skeletal muscle. Co-culture of muscle with sympathetic ganglia prolonged the survival of normal and dystrophic muscle *in vitro* [19].

While neurotrophic effects on growth and regeneration are well documented, little is known about neurotrophic effects on tumor growth. However, altered tumor growth (either suppression or augmentation) has been noticed by several investigators following manipulation of the nervous system [20-23]. To our knowledge neuroblastoma growth in denervated tissue has been studied by only one group of investigators in addition to ourselves. The experimental results reported by them are in agreement with ours. They showed that growth of C-1300 NB in denervated muscle in the mouse is retarded as compared to growth in innervated normal muscle [24]. In addition C-1300 NB grown in denervated tissue had a significantly lower mitotic rate than control tumors [25].

Our earlier *in vivo* experiments have shown that augmentation of the function of the SNS augments growth of C-1300 NB while suppression of SNS function results in suppression of take and growth of this tumor. The *in vitro* experiments reported here extend our *in vivo* findings. Growth of C-1300 NB and S-20 NB cells in culture is augmented in the presence of SCG. SCG-conditioned medium increases proliferation and survival of S-20 cells in culture. We postulate that the SNS is secreting a trophic factor which supports growth of C-1300 NB and that this factor is at least relatively specific since it does not influence the growth of A-10 breast adenocarcinoma. The findings may have implications for cancer therapy.

REFERENCES

1. Chelmicka-Szorc E, Arnason BGW. Effect of 6-hydroxydopamine on tumor growth. *Cancer Res* 1976, **36**, 2382-2384.
2. Chelmicka-Schorr E, Arnason BGW. Modulatory effect of the sympathetic nervous system on neuroblastoma tumor growth. *Cancer Res* 1978, **38**, 1374-1375.
3. Chelmicka-Schorr, Arnason BGW. Suppression of growth of mouse neuroblastoma and A10 adenocarcinoma in newborn mice treated with the ganglionic blocking agent chlorisondamine. *Eur J Cancer* 1979, **15**, 533-535.
4. Singer M. Neurotrophic control of limb regeneration in the newt. *Ann NY Acad Sci* 1974, **228**, 308-322.
5. Singer M. The influence of the nerve in regeneration of the amphibian extremity. *Q Rev Biol* 1952, **27**, 169-200.
6. Singer M. The nervous system and regeneration of the forelimb of adult triturus. IV. The stimulating action of a regenerated motor supply. *J Exp Zool* 1946, **101**, 221-240.
7. Singer M. Neurotrophic activity of brain extracts in forelimb regeneration of the urodele, *Triturus*. *J Exp Zool* 1976, **196**, 131-150.
8. Jabaily JA, Singer M. Neurotrophic stimulation of DNA synthesis in the regenerating forelimb of the newt, *Triturus*. *J Exp Zool* 1977, **199**, 251-256.

9. Tassava RA, McCullough WD. Neural control of cell cycle events in regenerating salamander limbs. *Am Zool* 1978, **18**, 843-854.
10. Maden M. Neurotrophic control of the cell cycle during amphibian limb regeneration. *J Embryol Exp Morphol* 1978, **48**, 169-175.
11. Salzer JL, Bunge RP. Studies on Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development of Wallerian degeneration and direct injury. *J Cell Biol* 1980, **84**, 739-752.
12. Salzer JL, Bunge RP, Glaser L. Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *J Cell Biol* 1980, **84**, 767-778.
13. Salzer JL, Williams AK, Glaser L, Bunge RP. Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction. *J Cell Biol* 1980, **84**, 753-766.
14. Wood PM, Bunge RP. Evidence that sensory axons are mitogenic for Schwann cells. *Nature* 1975, **256**, 662-664.
15. Hanson GR, Partlow LM. Stimulation of non-neuronal cell proliferation *in vitro* by mitogenic factors present in highly purified sympathetic neurons. *Brain Res* 1978, **159**, 195-210.
16. McCarthy KD, Partlow LM. Neuronal stimulation of [^{3}H]thymidine incorporation by primary cultures of highly purified non-neuronal cells. *Brain Res* 1976, **114**, 415-426.
17. Guth L. "Trophic" influences of nerve on muscle. *Physiol Rev* 1968, **48**, 645-687.
18. Oh TH. Neurotrophic effects: characterization of the nerve extract that stimulates muscle development in culture. *Exp Neurol* 1975, **46**, 432-438.
19. Kobayashi T, Tsukagoshi H, Shimizu Y. Trophic effects of sympathetic ganglia on normal and dystrophic chicken skeletal muscles in tissue culture. *Exp Neurol* 1982, **77**, 241-253.
20. Kavetsky RE, Turkevich NM, Balitsky KP. On the psychophysiological mechanism of the organisms' resistance to tumor growth. *Ann NY Acad Sci* 1966, **125**, 933-945.
21. Nayar KK, Arthur E, Balls M. The transmission of tumors induced in cockroaches by nerve severance. *Experimentia* 1971, **27**, 183-184.
22. Scharrer B. Gastric cancer experimentally induced in insects by nerve severance. *JNCI* 1949, **10**, 375-376.
23. Stein-Werblowsky R. The sympathetic nervous system and cancer. *Exp Neurol* 1974, **42**, 97-100.
24. Batkin S, Piette LH, Wildman E. Effect of muscle denervation on growth of transplanted tumor in mice. *Proc Natl Acad Sci USA* 1970, **67**, 1521-1527.
25. Batkin S, Rayner MD. Mitotic changes in neuroblastoma transplanted into denervated host tissue. *Res Commun Chem Pathol Pharmacol* 1976, **13**, 145-148.