

Interactions between neurites and somite cells: inhibition and stimulation of nerve growth in the chick embryo

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SUMMARY

After neural processes emerge from the neural tube in the chick embryo, their growth is restricted to the cranial halves of the neighbouring somites. In this study we have developed an *in vitro* system to model the interactions between these tissue types. Pioneer neurites display a hierarchy of preferences in terms of the substrates they can grow on. As expected, tissue culture plastic does not support neural outgrowth, but this can be overcome by coating the plastic substrate with either collagen or poly-L-lysine. Neural crest, cranial half somite, and a number of other tissues support growth well, while caudal half somite and tail bud mesenchyme do so to a much smaller extent.

The binding pattern of a variety of lectins was assessed in cryostat sections of embryos and in cultured cells of the above tissues. It was found that peanut agglutinin can discriminate between cranial and caudal sclerotome both *in vitro* and in the embryo, since it binds preferentially to caudal sclerotome in both cases. This difference is expressed as soon as the sclerotome forms.

The significance of these findings is twofold: first, they show that the interactions that take place during peripheral neural segmentation can be modelled *in vitro*; second, they represent the first instance of a molecular difference between the cranial and caudal halves of the sclerotome, detectable both in culture and in the embryo.

INTRODUCTION

Segmentation of the body in developing higher vertebrate embryos arises in craniocaudal sequence by the formation of two rows of epithelial spheres, or *somites*, which flank the developing embryonic axis. In all vertebrates, with the exception of some anuran amphibia, the somites are formed by sequential epithelialization of two rods of mesoderm (the segmental plates), which lie on either side of the regressing primitive streak. Each somite then disperses as it splits into three distinct components: the *myotome* (which will give rise to the skeletal muscles), the *dermatome* (which will give rise to the dermis) and the *sclerotome* (from which will arise the vertebral column). The sclerotome is found most medially, adjacent to the developing spinal cord (neural tube).

Key words: chick embryo, somite, segmentation, neural outgrowth, pathfinding, pattern formation.

When motor and sensory axons emerge from the neural tube region of the chick embryo, their growth is restricted to the cranial half of each sclerotome (Keynes & Stern, 1984, 1985). Craniocaudal rotation of a portion of the neural tube by 180° does not affect the pattern of axonal growth, whilst a similar rotation of the segmental plate results in axons traversing the new caudal halves of the rotated sclerotomes (Keynes & Stern, 1984). These findings show that there must be intrinsic differences between the cranial and caudal halves of each sclerotome that determine the position of axon growth: cranial sclerotome permits axonal growth and/or caudal sclerotome inhibits it. These differences between the cranial and caudal halves of the sclerotome are determined early, prior to the segmentation of each somite (Bellairs, Veini, Stern & Keynes, in preparation). At the time of segmentation, cells destined to become cranial or caudal half sclerotome already occupy their appropriate positions in the epithelial somite (Stern & Keynes, in preparation).

At present, nothing is known about the nature of the differences between cranial and caudal cells at a molecular level. In this study we have developed an *in vitro* model which may be used in future experiments designed to identify such molecular differences. In the course of the work, we have also succeeded in identifying the first molecular marker which can discriminate between the two cell populations.

MATERIALS AND METHODS

Cultures

Fertile hens' (Light Sussex or Rhode Island Red) eggs were incubated for 48–72 h, until the embryos had reached stages 9–19 of Hamburger & Hamilton (1951). Embryos were then explanted into a 0.1 % solution of trypsin (1:250, DIFCO) in Ca^{2+} -/ Mg^{2+} -free Tyrode's saline at room temperature, pinned out in Sylgard dishes and dissected. In this way neural tube segments, either whole or halved along the sagittal plane, could be cleaned of adherent cells. Somites were also removed from various craniocaudal levels, either whole or as halves. For older somites that had already differentiated into dermomyotome and sclerotome, sclerotome halves or whole dermomyotome were dissected. Endoderm, lateral plate mesoderm and 2-day tailbud mesenchyme were also used for comparison.

Medium

Medium 199 with Earle's salts (Flow) was used (Bellairs, Sanders & Portch, 1978). The culture medium consisted of 9 ml medium 199: 1 ml foetal calf serum (Gibco), to which was added 0.1 ml of antibiotic solution (Flow) giving final concentrations of 50 i.u. ml^{-1} penicillin and 50 $\mu\text{g ml}^{-1}$ streptomycin.

Cultures were incubated at 37–38°C for periods between 1 and 7 days in gas-tight plastic boxes. Each box contained two or more small vials, with distilled water and one soda tablet in each vial to provide humidification and a source of CO_2 . Under these conditions the pH of the culture medium remained constant at around 7.3 for at least 24 h, after which time the boxes were opened for examination of the cultures and new tablets placed in the vials.

Growth vessels

30 mm diameter Sterilin tissue culture dishes were used. When explants were to be confronted, or somites grown alone, glass rings (about 8 mm I.D., 3 mm high, cut from 1 mm-thick

glass tubing) were placed in the dishes, held by a small amount of autoclaved silicone grease, to decrease both the effective volume of the dish and convection; this modification was found to improve the growth of most of the tissues cultured. Cooke 'U'-microtitre plates (Sterilin) were also used for this purpose.

In some experiments, the dishes or wells were coated with different materials as follows: Collagen dishes were prepared by pouring 1 ml of Vitrogen 100 (rat tail collagen, Flow) onto the dishes; gelling was aided by addition of a few drops of medium and/or a few seconds' exposure to ammonia vapour at 4°C. Poly-L-lysine (PLys)-coated dishes or wells were prepared by wetting the plastic substrate with a 1 mg ml⁻¹ solution of PLys (Sigma poly-L-lysine hydrobromide, $M_r > 500 \times 10^3$) followed by air drying in a laminar flow hood for 2–24 h. To prevent any free PLys from attaching to the cells in the explant, PLys-coated dishes were washed with serum-free medium 199 prior to addition of the explant in full culture medium.

Fixation and lectin staining of cultures and embryos

After various periods of incubation, cultures were washed with serum-free medium and fixed in absolute ethanol. They were then either rehydrated and stained with haematoxylin or processed for lectin binding by the following procedure. Cultures were rehydrated with PBS and then incubated with 200–250 μ l of a 0.01 mg ml⁻¹ solution of horseradish-peroxidase-labelled *Arachis hypogaea* agglutinin (Peanut lectin, Sigma) for 1–1.5 h at 22°C or 37°C. They were then washed with PBS, and placed in a saturated solution of diaminobenzidine (DAB, Sigma) at room temperature for 5 min, after which H₂O₂ was added to a final concentration of 0.3–0.5%. After a further 5 min the cultures were washed again with PBS, intensified by exposure to 0.1% OsO₄ for a few seconds, air dried and mounted in Permount.

The binding of four horseradish-peroxidase-labelled lectins was also assessed in coronal (horizontal) cryostat sections of embryos. The four lectins used were concanavalin-A, wheat germ agglutinin, soybean agglutinin and peanut lectin (all obtained from Sigma). Embryos to be stained were transferred to dishes containing Tyrode's saline, fixed for 48 h in absolute ethanol at 4°C, washed in PBS, transferred to 5% sucrose solution in PBS for 2–4 h, and then placed in 15% sucrose overnight at 4°C. They were then infiltrated with two changes of a mixture of 7.5% gelatine (Sigma, 300 Bloom) and 15% sucrose/PBS for at least 3 h at 37°C. After this they were transferred to plastic embedding moulds and allowed to solidify in blocks for a few minutes at room temperature. They were then stored at 4°C for up to one week prior to sectioning. The blocks were frozen by immersion in isopentane (BDH) cooled with liquid nitrogen and sectioned at 10 μ m in a Bright cryostat at -22°C. Sections were collected onto gelatine-subbed slides, air dried and stored with silica gel in air-tight boxes at 4°C. By this method, excellent preservation of tissue structure and lectin binding were achieved. Lectin staining of the sections was carried out as described above for cultures, but no osmium intensification was necessary.

Control sections to assess the specificity of lectin binding were incubated in a 5% solution of one of the following sugars (Sigma) in PBS: D+-mannose as a control for Concanavalin-A, N-acetyl D-galactosamine for Soybean agglutinin, N-acetyl D-glucosamine for Wheat Germ agglutinin, and 1-0-methyl- α -D-galactopyranoside or β -D-galactose for peanut lectin. In all cases, the presence of the sugar reduced the lectin binding to background levels. Some sections were preincubated with 1 μ g ml⁻¹ neuraminidase or 0.05% trypsin for 5–7 min at room temperature prior to lectin staining.

Immunological methods

The monoclonal antibody HNK-1 (anti-human Leu-7, Becton Dickinson) has an affinity for embryonic chick trunk neural crest cells (Tucker *et al.* 1984). Cultures to be stained with HNK-1 were first fixed either with absolute ethanol for 48 h at 4°C or with 1.5% glutaraldehyde in PBS for 20 min at room temperature. After fixation, the cultures were washed several times with PBS, incubated in 1% bovine serum albumen (BSA, BDH) in PBS, and then washed again several times in PBS. They were then incubated in a 1:10 dilution of the monoclonal antibody, washed again, and incubated in a 1:20 dilution of horseradish peroxidase-conjugated rabbit anti-mouse total Ig (Nordic). After a final series of washes, the peroxidase

reaction was carried out with DAB as described for lectin staining. Each of the above incubations was carried out at 37°C for 1 h.

Living cultures to be treated with HNK-1 and complement were incubated for 15–30 min at room temperature with a mixture of the monoclonal antibody (final dilution 1:10) and pooled guinea pig complement (Miles, final dilution 1:1) in medium 199 without serum. They were then gently washed several times with medium 199, covered with normal culture medium (199+serum+antibiotics) and returned to the incubator for further growth. In order to assess the lysis of HNK-1 positive cells, cultures were fixed and stained with HNK-1, either with or without previous incubation with HNK-1 and complement. It was found that all HNK-1 positivity was lost from treated cultures, and that the cells that had not lysed during treatment (the HNK-1 negative cells) survived the period of subsequent culture. In cranial half sclerotome cultures, treatment lysed 10–30 % of the cells; in cultures of caudal sclerotome no cells were lysed, and in cultures of cells which had emigrated from a neural tube younger than stage 17 all the cells were lysed. This is therefore a convenient method for eliminating HNK-1 positive cells selectively from mixed cultures.

Examination of results

Cultures were examined after various periods of incubation before and after fixation under a Prior phase-contrast inverted microscope. They were drawn using a *camera-lucida* attachment fitted to a Leitz Laborlux microscope or photographed on a Zeiss photomicroscope with either PanF or Agfa 50L 35 mm film.

RESULTS

(A) Culture experiments

A total of about 1000 cultures was set up, at least 10 for each separate experiment.

Growth of single explants

(1) Neural tube

(a) *Plastic substrates.* Neural tube explants were grown on plastic tissue culture dishes. Cells with a stellate appearance emigrated from one edge of the tube, and by 1–2 days' culture they surrounded it. These cells were seen leaving neural tube explanted at stages 9–16 and were found to stain with HNK-1 antibody (Fig. 1) (see 'Immunological experiments' below). However, neural tube taken from wing bud to occipital levels of embryos older than stage 16 did not produce them, and remained unchanged after attachment for up to 6 days on tissue culture plastic.

2–3 days after the emigration of cells from the neural tube, neurites were seen to grow out from the neural tube explant. Their growth cones contacted the emigrated cells and were never seen to be free on the plastic substrate (Figs 2, 3). It was clear that the emigration of cells from explants of neural tube younger than stage 17 always preceded the elongation of neurites in culture by about 24–48 h.

(b) *Collagen substrates.* When neural tube younger than stage 17 was grown on collagen, an identical pattern was observed. Growth cones were almost always associated with cells, although occasionally one could be seen extending off the cell sheet (Fig. 4). Emigrated cells formed a more cohesive, confluent sheet on the

collagen substrate than on plastic. Neural tube from stage-17 or older embryos did not produce cells or extend neurites on collagen.

(c) *Poly-lysine-coated plastic substrates*. When stage-17 neural tube was grown on PLys coated dishes, neurites emerged from the tube in the complete absence of other cells (Figs 5, 6). Considerable fasciculation was seen in these cultures, suggesting that axons preferred adhering to each other to adhering to the substrate.

(2) *Somite and sclerotome*

Whole or half somites from the two or three most recently segmented somites of stage-10 to -13 embryos, or whole or half sclerotomes from stage-12 to -18 embryos were cultured on tissue culture plastic. Reducing the volume of medium markedly improved the growth of both somite and sclerotome explants. This was achieved in two ways: explants were grown either within glass rings in the dishes or in small wells which were either uncoated or PLys coated. The morphologies of cells from the cranial and caudal halves of the most recently segmented somites were identical (Figs 7–10), as were those from cranial and caudal half sclerotomes.

Growth of confronted explants

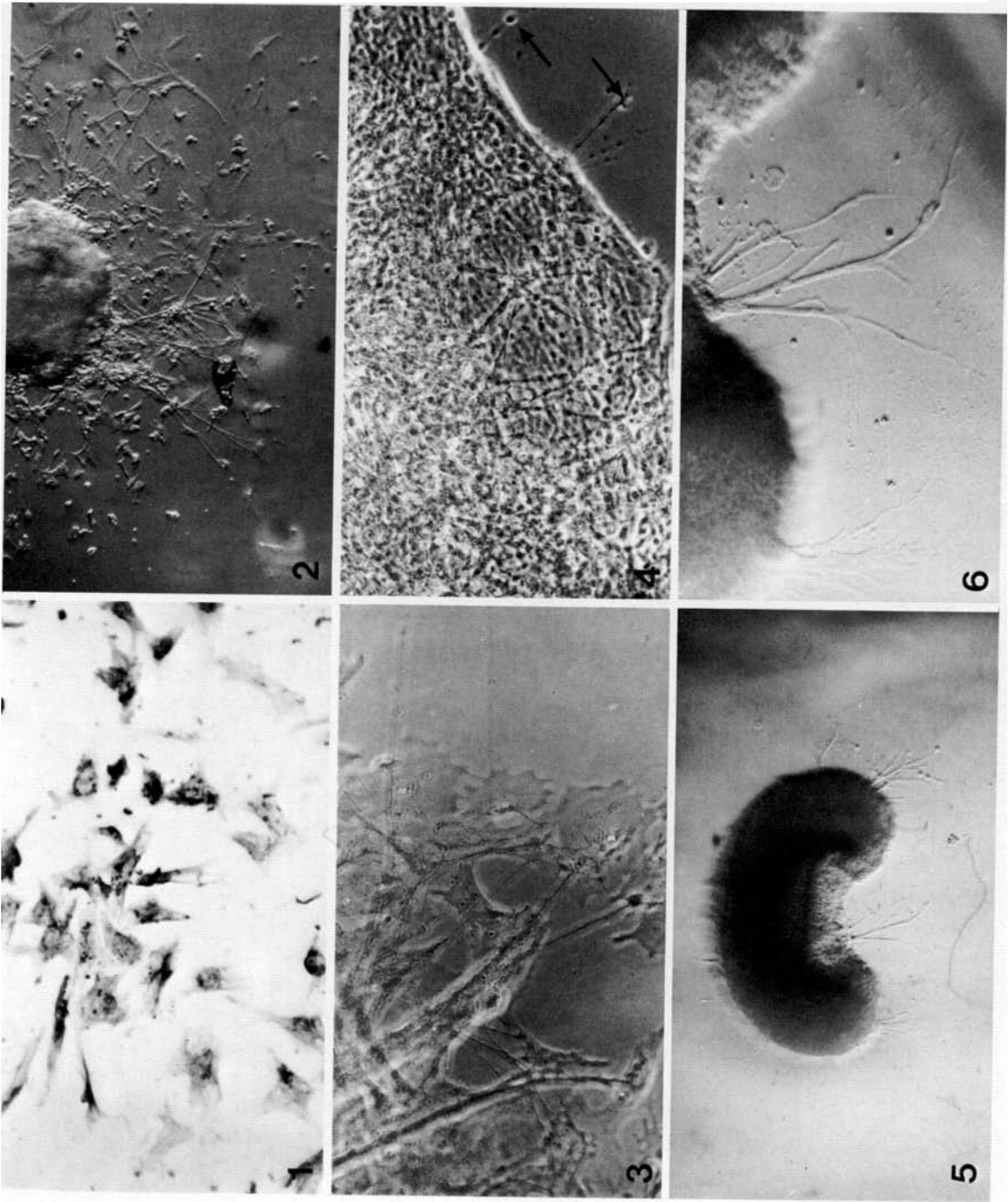
(1) *Cocultures of neural tubes of different stages on plastic*

To determine whether cranial neural tube from stage-17 embryos was capable of producing neurites at all on plastic, explants of stage-17 neural tube were placed onto a spread culture of emigrated cells from a stage-9 to -14 neural tube whose unspread centre had been removed with tungsten needles. Both neural tubes had been in culture for equal periods of time (24 h) and the stage-17 neural tube had not produced cells or neurites. It was found that the stage-17 neural tube attached and developed neurites. They were seen *only* in association with spread cells derived from the first explant, and appeared after 18 h coculture.

(2) *Cocultures of neural tube and newly segmented somite*

One somite length of one half of the neural tube was used as the experimental explant. The other half was used as a control to ensure that neurite growth was not associated with cells which had emigrated from the neural tube explant. In these cultures, neural tube halves were explanted with either the cranial or caudal halves of the three most recently segmented somites, the somite halves being placed in contact with each tube in a well. In all cases, neurite outgrowth was seen within 24 h, while the controls had not developed beyond attachment of the explant to the substrate. Both cranial and caudal halves of the somite stimulated nerve growth.

To enable a more direct comparison between somite halves, a series of experiments was undertaken in which one half of the neural tube was cultured with two to three cranial half somites, and the other half of the neural tube with the caudal halves of the same somites. After 22–24 h in culture, the cultures were examined and counts made of the number of exit points of neurites from each half-neural



tube. The outgrowth pattern was also drawn with a *camera lucida* and the cultures were then fixed in absolute ethanol. It was found that both cranial and caudal halves stimulated nerve outgrowth, but to differing degrees: there were more points of outgrowth in cranial half-somite cocultures (c.f. Figs 11, 12. $P < 0.05$ in both two-tailed paired Student's t-test and Wilcoxon. The results are shown in Table 1). All the growth cones were associated with cells.

(3) Cocultures of neural tube and sclerotome

Cocultures of neural tube (st. 17) and sclerotome halves, obtained from wing-bud-level somites of stage-16 to -18 embryos were also set up. It was found that the growth of neurites was more extensive than in the cocultures with somite halves from the most recently segmented somites described above. As a result of fasciculation and branching, satisfactory quantitation of neurite growth proved impossible. Instead, an estimate of the degree of fasciculation was made, being conducted as a series of 'blind' trials. The sclerotome cells from cranial and caudal halves showed a similar degree of spread. In all cases with caudal half sclerotome, fasciculation dominated the pattern of neurite outgrowth; neurites tended to bundle together into thick trunks. With cranial half sclerotome, on the other hand, the pattern of growth consisted of a fine meshwork of neurites spreading over most of the surface of the sclerotome explant (c.f. Figs 13, 14).

Cocultures of cranial or caudal sclerotome halves and stage-17 neural tube were also carried out on PLys-coated substrates. In both cases, growth cones were found to be associated only with sclerotome cells and were never free on the PLys substrate.

When comparing the growth of neurites on cells derived from younger neural tube with that on cells of the somite or sclerotome, it was found that outgrowth occurred a few hours earlier in neural tube-neural tube confronts than in neural tube-sclerotome or neural tube-somite confronts.

Fig. 1. Neural crest cells from a 24 h explant of a stage-12 neural tube after glutaraldehyde fixation and staining by indirect immunoperoxidase with the monoclonal antibody HNK-1. $\times 135$.

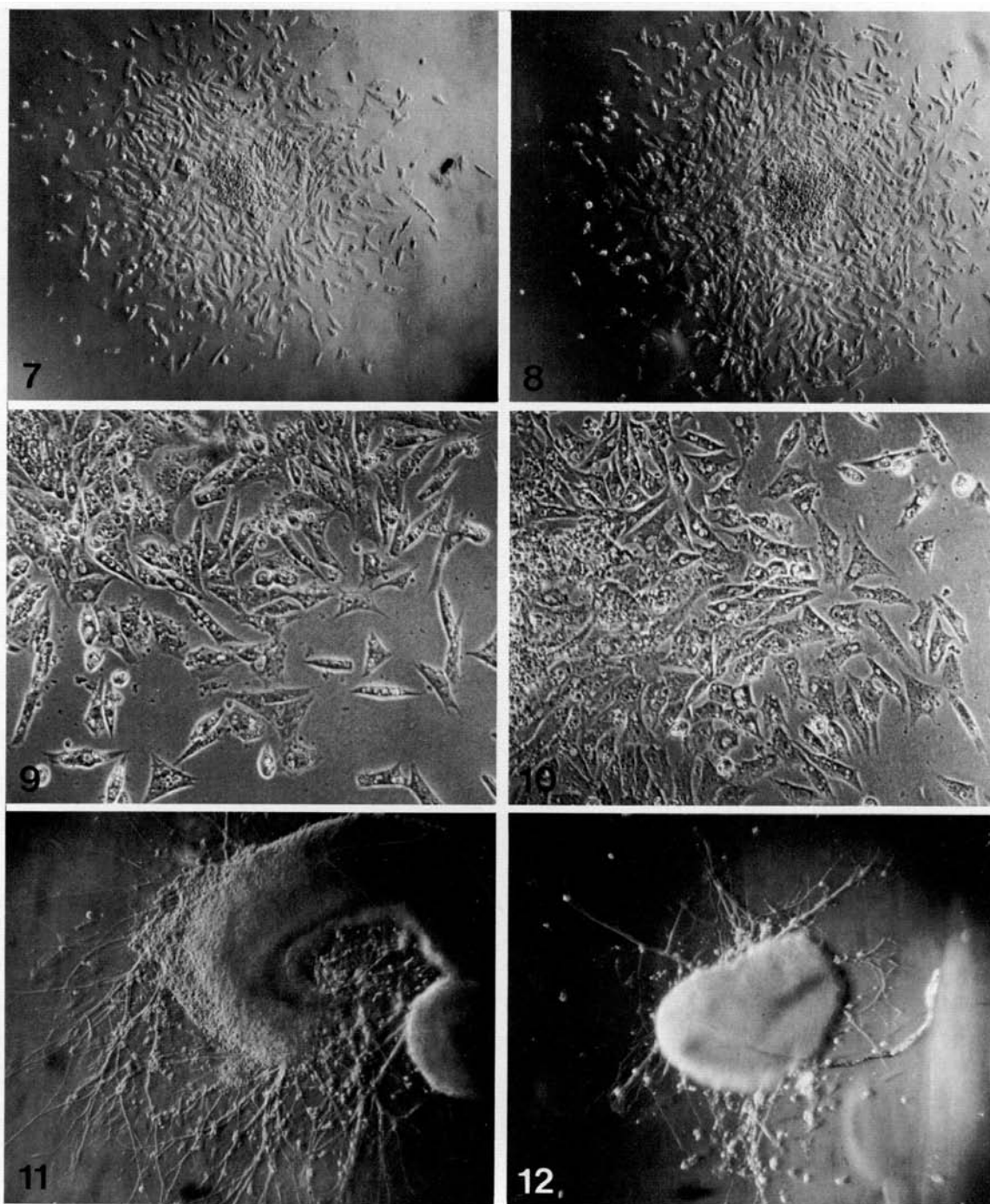
Fig. 2. Neurites emerging from a neural tube explant (top) after crest cell emigration. Note how all the neurites end on crest cells and reach the periphery of the spread explant. Nomarski DIC, $\times 50$.

Fig. 3. A higher magnification micrograph showing the association of the nerve endings with crest cells. Phase contrast, $\times 150$.

Fig. 4. Explant of a stage-10 neural tube on collagen. Note how the crest cells are confluent (cf. Fig. 2). Though most of the growth cones are still associated with the crest cells, a few growth cones (arrows) are seen on the collagen substrate. Phase contrast, $\times 160$.

Fig. 5. A stage-17 cranial neural tube explant, after 2 days on a PLys-coated dish. Neurites can be seen in the absence of crest cells. Nomarski DIC, $\times 45$.

Fig. 6. A higher magnification picture of the centre of the explant in Fig. 5. Numerous processes are seen lying free on the PLys-coated dish. Note the high degree of fasciculation present between the outgrowing axons. Nomarski DIC, $\times 140$.



Figs 7–10. Epithelial somite halves in culture, viewed with Nomarski DIC optics. The cranial (Fig. 7, $\times 35$) and caudal (Fig. 8, $\times 35$) halves look identical after 24 h in culture. Under higher magnification ($\times 200$, phase contrast) the cranial (Fig. 9) and caudal (Fig. 10) cells still look indistinguishable.

Figs 11, 12. Neural tube halves grown with either cranial (Fig. 11, $\times 55$) or caudal (Fig. 12, $\times 45$) halves of epithelial somites after 3 days in culture. Note the difference in the extent of neurite outgrowth.

Table 1.

Dish	No. of exit points from the neural tube explant with:	
	cranial half somite	caudal half somite
1	12	6
2	13	2
3	16	14
4	0	9
5	14	9
6	1	0
7	8	12
8	15	15
9	12	9
10	2	9
11	8	7
12	18	20
13	13	12
14	13	7
15	14	0
16	13	2
17	18	0
18	14	1
19	13	15
20	6	0
Mean diff. = 3.7	Student's $t = 2.3276$	
s.d. (diff.) = 7.1	D.F. = 19	
Variance (diff.) = 50.5	P (2-tailed) = 0.0311	
No. of zero diff. = 1	Sum +ve ranks = 145	Wilcoxon's $T = 45$
No. untied pairs = 19	Sum -ve ranks = 45	P (2-tailed) = 0.0446

(4) *Other cocultures*

Stage-17 neural tube was cultured with cranial pieces of segmental plate, pieces of 2-day tailbud mesenchyme, and endoderm. Neurite outgrowth occurred with segmental plate and endoderm, but not with 2-day tailbud mesenchyme.

Neural tube halves were also cocultured with caudal half sclerotome or dermomyotome. Neurite outgrowth was more extensive, more highly branched and denser with caudal sclerotome than with dermomyotome.

(5) *Conditioned medium test*

Conditioned medium tests were conducted in an attempt to stimulate neurite extension in the absence of direct contact between neurites and somite cells. First, three cranial or caudal sclerotome halves were grown for two days in a well; the medium from the culture was then transferred using a siliconized glass pipette to a fresh well. Stage-17 neural tube halves were then placed in wells with this conditioned medium, and control halves placed in fresh medium. No neurite outgrowth was seen within 2–3 days in either the experimental or control halves, whether the medium came from cranial or from caudal half sclerotome cultures.

In a second type of conditioned medium test, plastic dish/glass ring cultures were used. A line of silicone grease was used to divide the ring into two semicircles; in one half, several somite or sclerotome halves were placed; in the

other, half of a neural tube. In these experiments, although the neural tube halves attached, and the somite or sclerotome halves attached and spread, no neurite outgrowth was seen within 2–3 days. As the cultures aged, the somite cells died, and longer term cultures could not be established.

Since it is possible that neurites cannot attach directly to plastic under any conditions, the same experiments were conducted using PLys-coated dishes or wells. Again, no significant differences were found between media conditioned by cranial or caudal half somite or half sclerotome.

(B) *Immunological experiments*

In order to determine the nature of the emigrated cells, 24 h cultures of cells from stage-9 to -16 tubes, fixed with glutaraldehyde for 20 min, were stained by indirect immunoperoxidase using the monoclonal antibody HNK-1, which recognizes neural crest cells (Tucker *et al.* 1984). It was found that all the emigrated cells stained with the antibody (Fig. 1). Caudal half-sclerotome cultures (Fig. 15), or cultures of newly segmented somite, did not stain with the antibody. In cranial half-sclerotome cultures, however, 10–30 % of the cells stained with the antibody. There was a predominance of these cells near the periphery of the explant (Fig. 16).

When living, 24 h neural tube cultures were incubated at room temperature with a mixture of this antibody and guinea-pig complement, all spread cells lysed within 15–20 min. In contrast, no cell lysis was seen in 24 h cultures of somites or caudal halves of sclerotomes treated in the same way. In cultures of cranial half sclerotome, on the other hand, again about 10–30 % of cells lysed. After treatment with antibody and complement, washing several times with fresh medium and re-incubation, no HNK-1 positive cells remained. When cultures of cranial half sclerotome that had been treated with HNK-1 and complement were washed and confronted with stage-17 neural tube explants, neurites extended from the neural explant as if on untreated cranial sclerotome. Therefore, the differences between cranial and caudal half sclerotome in terms of their ability to support neurite growth or fasciculation are not due to the presence of HNK-1 positive cells.

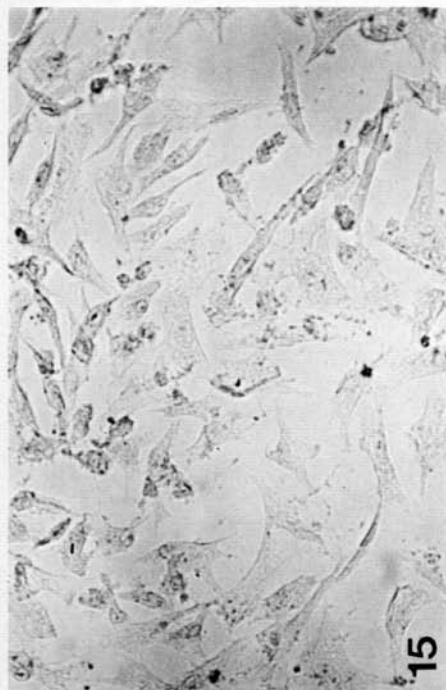
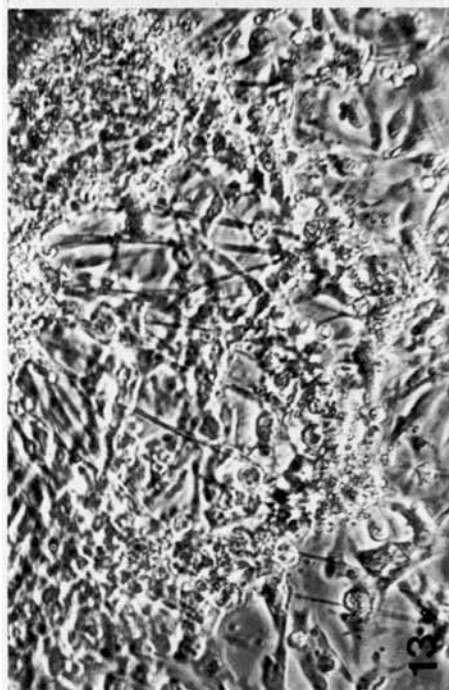
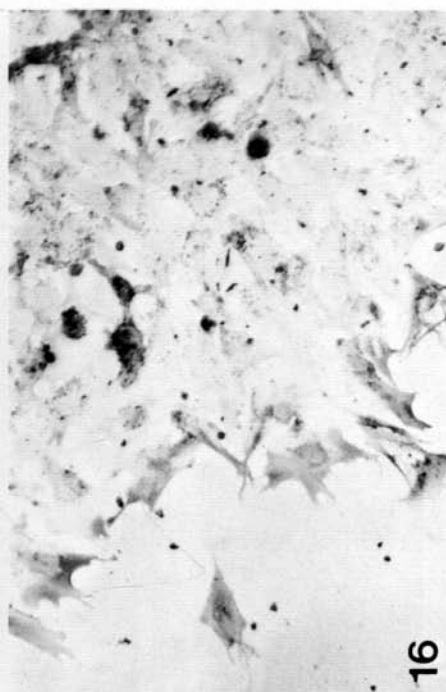
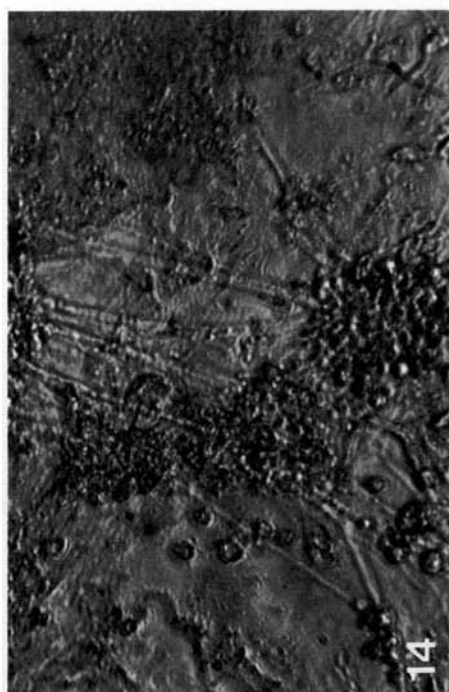
(C) *Lectin staining*

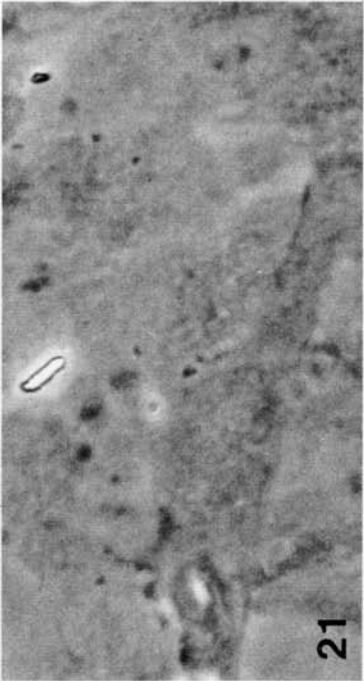
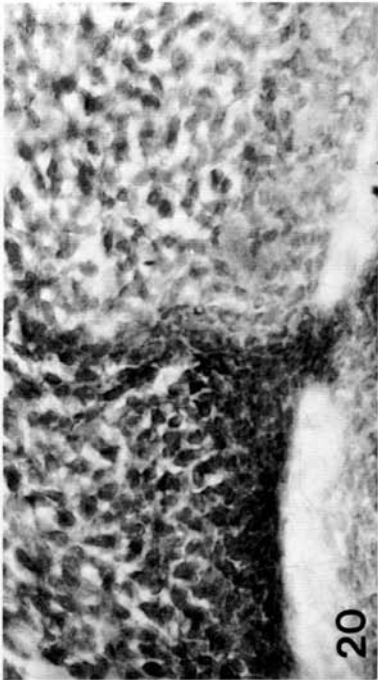
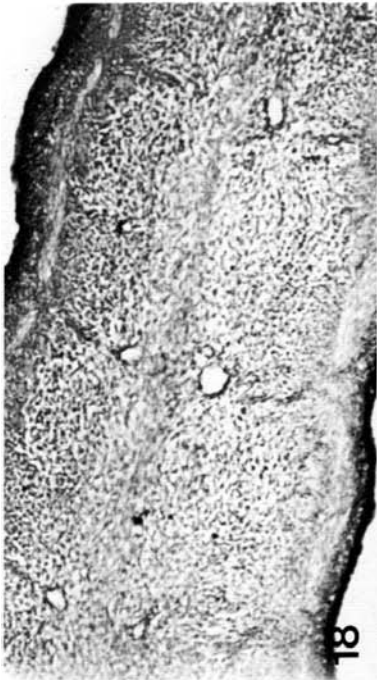
Coronal (horizontal) cryostat sections of gelatine-embedded stage-11 to -18 embryos were stained with a variety of horseradish-peroxidase-conjugated lectins:

Figs 13, 14. Cocultures of stage-17 neural tube halves with cranial half sclerotome (Fig. 13) or the corresponding caudal half (Fig. 14) after 24 h in culture. Neurites form a fine meshwork of fibres on the cranial half-sclerotome cells but only thicker, fasciculated processes are seen with caudal half sclerotome. Phase contrast, $\times 140$.

Fig. 15. Edge of the explant in a 24 h culture of three caudal half sclerotomes from a stage-16 embryo, stained by indirect immunoperoxidase with HNK-1. No staining is seen. $\times 135$.

Fig. 16. Edge of the explant in a 24 h culture of three cranial half sclerotomes from a stage-16 embryo, stained with HNK-1. A proportion of the cells, particularly at the periphery of the explants, stain with the antibody. $\times 135$.





concanavalin-A, soybean agglutinin, wheat germ agglutinin and peanut (*Arachis hypogaea*) agglutinin.

The following binding patterns were observed: concanavalin-A bound to all regions, especially epithelial tissues (e.g. ectoderm, neural tube) and their basal laminae. Soybean agglutinin also bound to most regions, but especially to material in the intersclerotome spaces and to the dermatome. In addition, it stained the caudal halves of the sclerotomes slightly more than the cranial halves (Fig. 17). Wheat-germ agglutinin staining was less intense than that obtained with the other lectins, but it was also widely distributed. Unlike any of the other lectins, it stained the myotome (Fig. 18). Peanut agglutinin bound to many regions, but a striking pattern of binding was found in the somites. The myotome was completely free of reaction product. The dermatome and the *caudal* half of the sclerotome were both heavily labelled, but there was much less label in the cranial half of the sclerotome (Figs 19, 20). This distribution of binding generated a clear banded pattern along the rostrocaudal axis of the embryo. Comparison of the cranial and caudal halves of the sclerotome under higher power ($\times 40$ and $\times 100$ objectives) revealed that this pattern reflected different degrees of binding to individual cells and not differences in cell density (Fig. 20). At the resolution of these objectives, the binding formed a uniform coat over the entire area of the labelled cells in the caudal half sclerotome. The difference in lectin binding between the two halves of the sclerotome was seen in all those somites which had differentiated into sclerotome and dermomyotome. However, it could not be seen in the most recently segmented, epithelial somites.

Neither neuraminidase nor trypsin preincubation led to loss of the craniocaudal difference in peanut agglutinin binding, although trypsin pretreatment attenuated peanut lectin staining throughout the embryo.

Peanut lectin binding was also assessed in ethanol- or glutaraldehyde-fixed 24 h cultures of somite and sclerotome halves. It was found that caudal half sclerotome stained with the lectin, in contrast with cultures of cranial half sclerotome, or

Fig. 17. Coronal cryostat section of a stage-13 chick embryo stained with peroxidase-labelled soybean agglutinin. There is little localization of binding. $\times 165$.

Fig. 18. Coronal cryostat section of a stage-13 chick embryo stained with peroxidase-labelled wheat germ agglutinin. There is very little labelling and the binding is generalized. $\times 280$.

Figs 19, 20. Coronal cryostat sections of a chick embryo stained with peroxidase-labelled peanut lectin. In Fig. 19 ($\times 225$) note the banding generated by alternate darkly stained caudal halves of the sclerotomes (caudal end towards left of picture) and the lightly stained cranial halves. The myotome is unstained. At higher power (Fig. 20, $\times 750$) it can be seen that the staining is associated with the cells, that the caudal (left) cells are stained more darkly than the cranial (right) cells and that the staining is uniform over the surface of the cells.

Figs 21, 22. Peanut lectin staining in culture. High-magnification views of explants of cranial (Fig. 21) and caudal (Fig. 22) half sclerotome after 24 h in culture, fixed with glutaraldehyde and stained with peroxidase-labelled peanut lectin. Note the meshwork of lectin-positive fibrils over the surface of the caudal half-sclerotome cells. Both $\times 1000$.

cultures of either half of newly segmented somite, none of which showed lectin binding. Because binding of the lectin was seen after fixation with glutaraldehyde, which does not permeabilize cells, as well as after ethanol fixation, which does, we can conclude that the lectin probably recognizes a molecule on the cell surface. In contrast with the pattern of lectin binding to caudal sclerotome in embryo sections, where binding was uniform over the area of each cell, cultured caudal sclerotome cells showed a reticular distribution of binding (Figs 21, 22).

DISCUSSION

(A) *Summary of results*

Our results can be summarized as follows: cultured explants of pre-stage-17 neural tube gave rise to HNK-1 positive cells that emigrated from the explant and spread on the substrate. Neurites were extended by post-stage-17 explants on PLys and on HNK-1 positive cells, but not on untreated tissue culture plastic or collagen. Cranial and caudal halves of recently segmented somites also allowed nerve growth, the cranial halves being better in this respect than the caudal halves. In cocultures of neural tube and sclerotome, neurite growth was still more extensive than in cultures with recently segmented somites. There was more fasciculation of neurites with caudal half sclerotome than with cranial half sclerotome. 2-day tailbud mesenchyme and dermomyotome did not support neurite extension, whereas segmental plate and embryonic endoderm did. In each case growth cones were always associated with cells.

Coronal (horizontal) cryostat sections of embryos showed that the binding of peanut lectin to the sclerotome reflects the division of the sclerotome into cranial and caudal halves: the horseradish-peroxidase-labelled lectin stained the caudal sclerotome cells intensely and the cranial sclerotome cells much less so. Caudal sclerotome cells *in vitro* also stained with lectin much more intensely than did cranial sclerotome cells. While in sections the staining pattern formed a uniform coat over the caudal half cells, caudal sclerotome cells *in vitro* bound the lectin in a reticulated pattern, so that some areas of the cell were not stained.

(B) *Growth of neural tube explants*

By comparison with published accounts, the cells that emigrate from younger neural tube explants are neural crest cells (see Cohen & Konisberg, 1975; Le Douarin, 1982; Weston, 1982; Kahn & Sieber-Blum, 1983; Rovasio *et al.* 1983). This conclusion is confirmed by the results of our immunocytochemical studies using the monoclonal antibody, HNK-1, which recognizes this population of cells in the embryo (Tucker *et al.* 1984).

What neuronal types are extended by the neural tube explants? Since the neurites emerge from the ventral edge of the neural tube explant, the majority of outgrowths are likely to be from motor nerves. However, it is also possible that the outgrowth includes others, for example from interneurons.

In Letourneau's (1975a,b) experiments a correlation was obtained between adhesion of growth cones to the substrate and elongation of neurites. On this basis, and following similar concepts introduced earlier by Steinberg (1963, 1970) and Carter (1965), Letourneau proposed that nerves may be guided by the ability of filopodia to test the adhesivity of the proximal environment of the growth cones, the filopodia directing the growth cones towards areas of increased adhesivity.

Because in our experiments even non-innervated tissues (endoderm, segmental plate, epithelial somite and caudal half sclerotome) support neurite outgrowth *in vitro*, it is not possible to invoke solely target- or pathway-specific mechanisms to explain neural growth. A general mechanism, such as differential adhesion, is required. Neurites were not extended at all by stage-17 neural tube on tissue culture plastic or collagen, but were on PLys and cellular substrates. Moreover, given the choice, they preferred sclerotome cells to PLys, suggesting that, for these tissues at least, the hypothesis could be applicable.

(C) *Do neurites grow on neural crest or on somite cells?*

In a recent study using HNK-1, Rickmann *et al.* (1985) showed that trunk neural crest cells migrate through the cranial half of each segment. They begin to do so as soon as each epithelial somite disperses, that is, some six to eight somites rostral to that that has most recently segmented.

As a result of this observation, a problem in interpreting the present experiments arises: since the cranial half sclerotome contains neural crest cells, in confrontation experiments we could be seeing growth of neurites on crest cells rather than on cranial sclerotome cells. This could then explain the differences between the behaviour of confronts with cranial and with caudal half sclerotomes. We therefore treated cranial and caudal sclerotome cultures with HNK-1 antibody and guinea pig complement prior to confrontation. In cranial half cultures, some of the cells lysed, whereas in caudal half cultures no cells lysed. Subsequent confrontation experiments with post-stage-17 neural tube produced the same results as those in which the neural crest cells had not been removed, arguing that neurites can respond differently to cranial and caudal sclerotome regardless of the presence or absence of neural crest cells. In support of this conclusion, Rickmann *et al.* (1985) have found that if the neural crest is ablated *in ovo*, motor nerves still pass through the cranial half of each sclerotome, as in the normal embryo (Keynes & Stern, 1984). These results show that motor nerves and neural crest cells can respond independently to the craniocaudal differences in the sclerotome.

Another possible problem with our experiments is that the differences in lectin binding within the sclerotome could reflect differences between crest and sclerotome and not between cranial and caudal sclerotome cells. Two observations argue against this: first, in the embryo, *all* the cells of the cranial halves of the sclerotomes stain less intensely with peanut lectin than do all the cells of the caudal halves of the same sclerotomes; second, *in vitro*, half-sclerotome cultures in which crest cells were lysed with HNK-1 and complement, followed by lectin staining, still displayed the same difference in lectin binding as untreated cultures.

From the above results we can conclude that the presence of neural crest cells in the cranial halves of the sclerotomes is not responsible for either the differences in lectin binding or the differences in the behaviour of neurites in confrontation cultures.

(D) *Interactions between neurites and somite cells, and the molecular basis of neural segmentation*

In vivo, the caudal half sclerotome never supports motor nerve growth (Keynes & Stern, 1984, 1985). Lewis, Chevallier, Kieny & Wolpert (1981) showed that when the somitic mesoderm is destroyed, nerves emerge from the entire length of the neural tube and segmentation of the outgrowth is lost. These results suggest that the caudal half sclerotome in some way inhibits nerve growth *in vivo*. We have found that, *in vitro*, both cranial and caudal half somite and sclerotome support neurite growth, but to different extents: cranial half somite supports growth better than caudal half somite, while cranial sclerotome produces more arborescent outgrowth than caudal sclerotome, which leads to fasciculation. An experiment that would have modelled the *in vivo* interactions more accurately would have been to culture neural tube explants together with a mixture of cranial and caudal cells, to allow outgrowing neurites to choose between them. However, such an experiment proved difficult to carry out, because of the problem of recognizing individual cells in the mixture as cranial or caudal.

How can these *in vivo* and *in vitro* results be reconciled? One clue may be provided by the peanut lectin staining pattern. *In vivo* the labelling is uniform around the cells of the caudal half sclerotome, while *in vitro* the labelling adopts a reticulated pattern. A similar reticular pattern of binding of peanut lectin to other cultured cells has been reported recently (Trejdosiewicz, Southgate, Hodges & Goodman, 1985). Peanut lectin binds to the disaccharide (D-galactose, N-acetyl-D-galactosamine), which may be part of a glycoprotein or glycolipid. The observed difference in staining may therefore be due to a capping-like redistribution of plasma-membrane-associated components, possibly as a result of adhesion of the cells to the substrate in the culture environment (Grinnell, 1977; Trejdosiewicz *et al.* 1985). This redistribution *in vitro* may expose areas on the caudal half-sclerotome cell surface that allow adhesion of growth cones. However, the inhibitory properties of the cell surfaces are not entirely lost, so that later outgrowing axons tend to stick to the 'pioneer' axons rather than the sclerotome cell surfaces. That is, growth cone-axon adhesion is greater than growth cone-cell adhesion for some parts of the caudal half-sclerotome cell, resulting in fasciculation.

From the data available, we cannot decide whether the peanut lectin receptor is itself an inhibitor of neurite attachment. The change in the distribution of lectin binding *in vitro* with respect to its distribution in the embryo could reflect similar changes in the distribution of other surface molecules during culture, and one or more of these molecules might be inhibitory to nerve growth.

Our results do not exclude the simultaneous existence of a stimulant in the cranial half. It is known from heat-shock experiments that the craniocaudal difference within each somite is determined approximately 12 h before the somite separates from the segmental plate (Bellairs, Veini, Stern & Keynes, in preparation). The present experiments show that the newly formed somite already expresses a craniocaudal difference to which nerves are able to respond, before any differences in lectin binding are detectable. Cranial halves of newly segmented somites produce more points of neurite outgrowth than both the corresponding caudal halves and several other embryonic tissues.

How might the postulated inhibitor and stimulant work? The conditioned medium experiments failed to provide evidence either for or against the existence of diffusible stimulators or inhibitors of nerve growth. This may be because the molecules produced are labile. More experiments to test this possibility are in progress in our laboratory.

(E) *Conclusions*

The *in vivo* phenomenon of segmentation in the vertebrate peripheral nervous system can be modelled *in vitro*. One or more inhibitors of nerve growth probably exist on the surface of caudal half-sclerotome cells, which may also be present on cells of the caudal half of the newly segmented somite. In addition, a stimulant may be associated with the cranial half of the epithelial somite. No evidence was found for or against the existence of diffusible stimulators or inhibitors.

The specific molecules involved remain to be identified, and the disaccharide (D-galactose, N-acetyl-D-galactosamine) may be associated with an important molecule.

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