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Differential growth of sensory neurons in vitro in presence of dermis and epidermis. A quantitative time-lapse analysis

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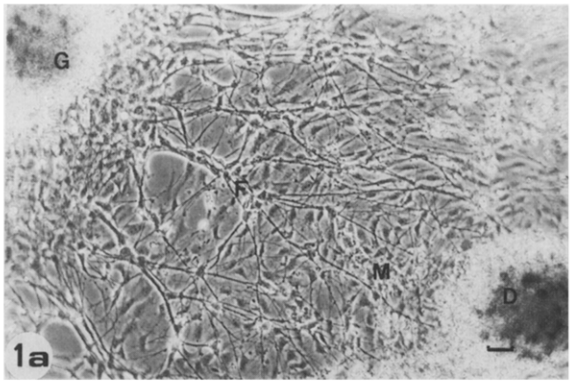
The influence of dermal and epidermal cells on the growth of nerve fibres from chick embryo sensory neurons was investigated in vitro. A previous quantitative analysis showed that the growth of nerve fibres is profoundly modified in the close vicinity of epidermis. This change is mainly characterized by erratic trajectories of nerve fibres resulting from numerous lateral displacements of the growth cones. In contrast, no such behaviour is observed far away from the epidermis or in the presence of dermis. In this latter case, neurites exhibit a straighter direction of extension. These observations suggest that the epidermis exerts some kind of control on the establishment of nerve fibre pattern in the dermis.

culture; directionality; growth; peripheral innervation; skin

Introduction

During the last third of bird embryo development, the histogenesis of sensory corpuscles (in particular, Herbst and Grandry corpuscles) results from morphogenetic interactions between nerve endings and dermal cells (see Saxod, 1978, for a review). However, by the end of the first week of the embryonic life, the axons of the dorsal root ganglia neurons have reached the skin and already formed a complex network of nervous interconnections (Verna and Saxod, 1979). Thus, about one week elapses from the time of the first contacts between the sensory nerve fibres and the skin to the onset of morphogenesis of the sensory corpuscles. It was therefore interesting to study the cellular interactions taking place during this period to determine their role in the subsequent developmental phase of cutaneous innervation.

Due to the dynamic nature of the cellular interactions involved, we studied the behaviour of growing nerve fibres cultivated in vitro with skin cells. A previous study (Verna, 1985) demonstrated that nerve fibres from chick embryo dorsal root ganglia neurons behave differently with respect to dermis or epidermis (Fig. 1). Indeed, the presence of an epidermal sheet causes various modifications in the growth of neurites, resulting in a deflection of their trajectories to avoid it. No such phenomenon occurs in the presence of dermis, and the outgrowth zone as well as the dermal explant itself are readily invaded by the growing neurites. Using quantitative analysis, it was possible to show that in the close vicinity of epidermis, (i) the growth rate is slowed, and (ii) numerous directional changes in the growth cone extension take place. The present study was undertaken to analyse more precisely the respective influences of dermis and epidermis upon the orientation of sensory nerve fibres in vitro. This quantitative



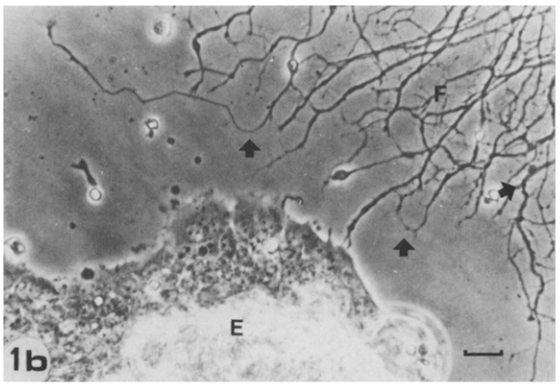


Fig. 1 (a) 3-day culture on polylysine substrate. Nerve fibres (F) extending out of the spinal ganglion explant (G) maintain their directions of elongation when encountering the cells (M) migrating from the dermal explant (D). Phase optics. Scale bar: 50 μm. (b) 2-day culture on collagen substrate. The growth behaviour of nerve fibres (F) is changed over in the close vicinity of epidermis (E); some neurites deviate 'at a distance' (arrows) from the epidermal layer, others are deflected after contact. Phase optics. Scale bar: 25 μm.

study was conducted using an index (straightness index) which defines the deviation of a trajectory from a straight line. Results obtained are consistent with those gathered previously and demonstrate a profound alteration of the direction of neuritic growth in the close vicinity of epidermis. It moreover appears that the dermis has a promoting effect on the direction of nerve fibre extension. The implications of these findings are discussed in relation to the formation of bird cutaneous innervation.

Materials and Methods

Cultures

Co-cultures were prepared as described elsewhere (Verna, 1985). Briefly, the culture dishes were coated either with a thin layer of bovine skin type I collagen (0.5 mg/ml solution) or with a poly-L-lysine solution (10 μg/ml in distilled water). Serum-free supplemented culture medium prepared according to Bottenstein et al. (1980) (N1 medium) with L15 medium as basal medium was complemented with nerve growth factor (NGF) (10 ng/ml) and glucose (6 mg/ml). Back skin and dorsal root ganglia (DRG) from 7-day chick embryos were used for this study. Following the incubation of skin in a 0.5% trypsin solution in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) at 4°C for 15 min, the epidermis was separated from the dermis. DRG were grown alone (control cultures) or with either epidermis or dermis (co-cultures). Co-cultures were carried out by positioning one row of DRG approximately 1 mm away from a row of either dermis or epidermis; each row was made of 5 to 6 explants. Cultures were maintained at 37°C in a humidified atmosphere. Randomly selected areas located between the two rows of explants were used to make time-lapse films. For this purpose, the edge of epidermal (or dermal) explant was placed at one side of the field of view (average size 400 μm), the

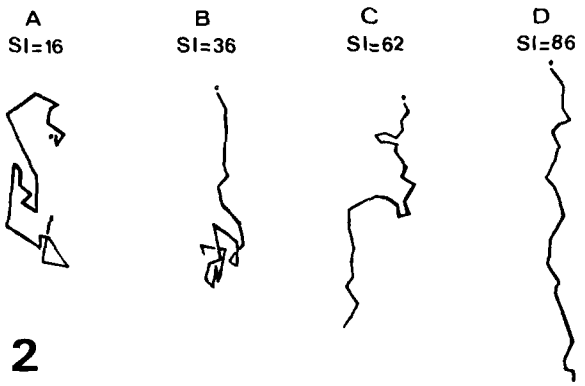


Fig. 2. Typical nerve fibre trajectories taken from each SI class (A–D; see text for details). The value of SI is given for each depicted trajectory.

neurites at the other. Commonly, at the beginning of filming, 5 to 20 neurites were present within the field and their growth cones located at various distances from the associated explant. Filming was done at a rate of one frame every 5–15 min between the first and the fourth day of culture.

Quantitative analysis

Cinematographic records were analysed with an interactive computer system (see Verna, 1985, for further details). The coordinates of successive positions of the growth cone were recorded every 15 min and processed by the computer (growth cone extension was analysed only when no contact was made with other neurites or glial cells). In the present study, we have measured how much the path of a single growth cone deviates from a straight line by using a straightness index (SI). This index is obtained by calculating the ratio of distance to path length (Batschelet, 1981). The distance is the straight line length between the positions of the growth cone recorded in the last and first frames of the sequence. The path length is obtained by summing the distances between the consecutive positions of the growth cone (four successive positions are needed to give a significant SI). To make this index range from 0 to 100, the ratio is multiplied times 100. Nerve fibres are then grouped in four distinct classes as a function of their SI values: A class from 0 to 25 (erratic behaviour); B class from 25 to 50 (wandering trajectory); C class from 50 to 75 (directional tendency); D class from 75 to 100 (strong directional tendency). An illustration of these is given in Fig. 2 showing typical trajectories of fibres taken from each class.

To summarize the results, a multivariate statistical method is used (Lebart et al., 1982). Correspondence analysis (CA) gives us a synthetical view of the relations between experimental groups and different behavioural classes. The interpretation of CA is enhanced using graphical representation of the factorial planes. Such graphs must be read in terms of distances between the different groups weighted by their respective number of elements (growth cones). When the respective distributions of two experimental groups do not differ significantly, their positions within the factorial plane are close to each other. The location of an experimental group in the neighbourhood of a particular behavioural class means that this class is preponderant for the group.

Results

Correspondence analysis is conducted using the four previously defined behavioural classes to build a four-dimensional space. The reduction to the first factorial plane keeps 96% of the total information contained within the four-dimensional space. Accordingly, only the first factorial plane is shown in Fig. 3a. Following the increasing order of SI, the four behavioural classes A, B, C and D are linked in the graph by a continuous line. It appears that the projections of the classes on the first factorial axis (summarizing 66% of the information) are ordered with respect to their SI magnitude. Consequently, this axis which opposes the extreme values of SI can be interpreted as a mean SI axis for the experimental groups. The second factorial axis (summarizing 30% of the information) opposes the medium classes B and C to the extreme classes A and D. The projection of a group on this axis expresses its distribution within the classes. So the location of groups are interpreted as follows: (1) when the group is located in the first or fourth quadrant (upper and lower right), the mean SI is high and the majority of its members belongs to the D class; (2) when located in the second quadrant (upper left), a group has a low mean SI and the A class is preponderant; (3) when located in the third quadrant (lower left), a group has a medium SI and is normally distributed.

(a) Co-cultures with dermal explants

Their projections (groups 3 and 4; Fig. 3a) are located in the first and fourth quadrant of the factorial plane. This expresses a preponderance of the D class and a high mean SI.

In the presence of dermis (Fig. 3b), D class frequency strongly increases to the detriment of other class frequencies, and especially C class

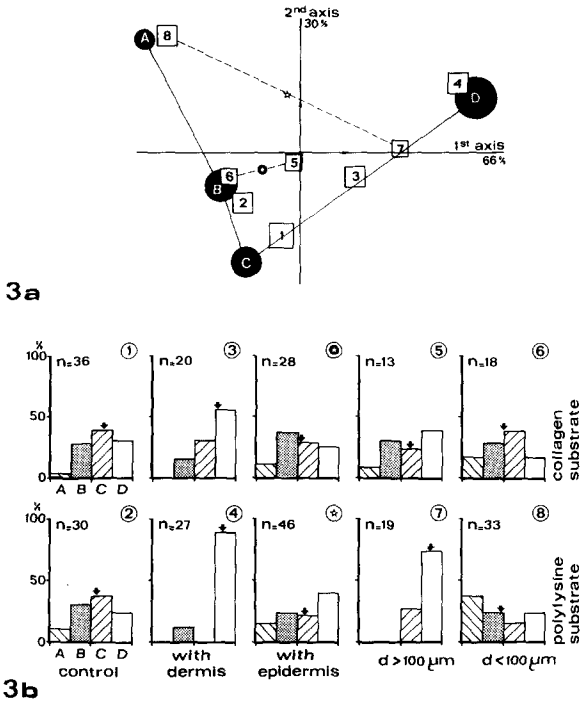


Fig. 3 (a) Correspondence analysis of the SI: graph of the first factorial plane resuming 96% of the total information (see Materials and Methods). Each experimental group is symbolized by a square and each defined class by a circle. The areas of the squares and circles are proportional to the number of neurites involved. (b) Frequency distribution of the SI. n = number of neurites studied in 3 to 5 cultures for each group. d = distance from the epidermal explant. The black arrows indicate the location of the mean SI value in each group.

frequency (resulting in the following mean SI values: polylysine, 84 with dermis, 60 in control; collagen, 76 with dermis, 53 in control). The location of control cultures (groups 1 and 2) in the third quadrant and the clear modification of the distribution (Fig. 3b) occurring in the presence of dermis suggest a promoting effect on the direction of growth due to dermal explants. Furthermore, this effect appears stronger on polylysine substrate than on collagen substrate.

(b) Co-cultures with epidermal explants

The distributions differ from those obtained in the presence of dermis and in control cultures. Moreover, these differences are modulated by the nature of the culture substrate.

On polylysine, the D class is still the greatest, but an increase in the frequency of the other classes takes place (in particular the A class, which is greater than in control, Fig. 3b).

On collagen, the B class is predominant and the D class much weaker. The mean SI values obtained on the two substrates (polylysine, 60; collagen, 53) are similar to those of the control cultures (polylysine, 56; collagen, 63). Thus, no marked promoting effect on the orientation of nerve fibre growth seems to occur in the presence of epidermis when compared to dermis; however, the distribution in the different classes needs a more detailed study. For this purpose, the data used to build the histograms of SI (Fig. 3b, stars) were split as a function of the distance from the epidermis. The SI of a fibre was calculated separately on either side of an arbitrary border located at 100 μm from the epidermis (about 1/4 of the field of view). Because it was not always possible to get a sufficient number of positions (at least four) to calculate a significant SI when data were split, the number of neurites is therefore reduced in these groups (Fig. 3b). The resulting subgroups were used to carry out the CA. The initial groups (open star and black circled star, Fig. 3a) are then projected into the factorial plane as supplementary elements; they are linked to their subgroups with an interrupted line.

On polylysine substrate there is a striking difference between the data collected beyond and within a distance of 100 μm from the epidermal explants. This leads to the opposite locations of groups 7 and 8 in the factorial plane (Fig. 3a). Beyond a distance of 100 μm (group 7), the neurites express a strong directional tendency with a mean SI of 85. In contrast, the A class is preponderant within group 8 and the mean SI falls to 46. The frequency distributions obtained on polylysine substrate as a function of the distance separating the neurite tips from the epidermis, as well as the mean values of SI in both cases, are very different (Fig. 3b). Far from the epidermis, the growth of nerve fibres is about the same as the growth recorded in the presence of dermis. In the close vicinity of epidermis a profound alteration

of the neurite behaviour occurs (i.e, drastic change in the frequency distribution and in the mean value of SI) which results in the appearance of random paths. Thus, the vicinity of epidermal explant strongly disturbs the direction of nerve fibre elongation.

In co-cultures grown on collagen, no such striking results are obtained but similar tendencies are observed (Fig. 3a, groups 5 and 6). Far from epidermis, the growth behaviour of nerve fibres resembles that observed in the presence of dermis (frequency distributions are equivalent, and the mean SI values – 62 with epidermis and 76 with dermis – do not differ significantly, Student t test). But in the vicinity, the frequency distribution is no longer comparable to that observed in the presence of dermis or in the control. Thus, on collagen also, the presence of epidermis modifies the nerve fibre behaviour in a noticeable but less pronounced way than on polylysine.

Discussion

The present study was undertaken with a particular emphasis on the trajectories of the neuritic growth cones when confronted in vitro with chick embryo dermal or epidermal cells.

The use of the SI, which defines how much a trajectory deviates from a straight line, associated with a multivariate data analysis allowed us to quantify the profound alteration of the neuritic walk caused by epidermal cells. The SI provides information about the directional tendency (erratic to linear) of the nerve fibre course, but does not specify the orientation of elongation. Moreover, the value of SI expresses the deviation of the growth cone displacement from a linear trajectory rather than a pure axonal elongation. Therefore, the determination of SI can be a way (less refined, however, than methods such as those designed by Katz et al., 1984 and Gundersen and Park, 1984) to separate proper growth cone movement from broad nerve fibre extension. This is of interest in considering the importance of the growth cone in the guidance of nerve fibre.

From the correspondence analysis (Fig. 3a), the following remarks can be made. (1) In control experiments (plots 1 and 2 in Fig. 3a), the frequency distributions as well as the SI mean values indicate that there is a predominance of intermediate classes. Thus, in agreement with other observations (Katz et al., 1984), this demonstrates the wandering nature of the growth cone movement in vitro. (2) By projecting each experimental group on the first factorial axis (which represents the mean SI axis; see Results), two sets of groups can be separated as a function of their location relative to the control groups; (i) the first set, characterized by a low SI, is exclusively made of groups 6 and 8 (growth cones elongating at less than 100 μm from the epidermis); (ii) all other groups belong to the second set. Thus, taking into account the 'normal oscillatory behaviour' (Katz et al., 1984) of the growth cone, it nevertheless appears that the close vicinity of epidermis leads to a marked modification of the growth cone behaviour. This latter is characterized by the appearance of strong erratic translocations (very low SI) as if the growth cones facing an unfavourable environment have to search for a new path. As a result, nerve fibre routes are deflected around the edge of the epidermal explant. This again demonstrates the respective importance of the growth cone and environmental influences in the process of neuritic guidance (see Letourneau, 1983). The nature of the mechanism involved in the deviation reaction remains unknown, and we are currently testing two hypotheses: (i) the existence of a concentration gradient of a factor(s) released by epidermal cells and affecting neurite translocation; and (ii) the possible implication of cell membrane incompatibility between sensory growth cones and epidermal cells.

Many reports (for reviews see Jacobson et al., 1980; Barde et al., 1983; Berg, 1984) have pointed out the influence of target tissues in stimulating a directed growth of neurites through soluble neurotrophic factors (such as NGF). The significant increase of SI mean values and the net shift of the frequency distribution to high SI classes (Fig. 3b) may be an expression of such an influence of dermal tissue upon sensory neurite growth. Results obtained with nerve fibres elongating at more than 100 μm from the epidermis are not so significant with, however, the exception of cultures grown on polylysine substrate. A more detailed study is

thus needed in order to determine whether (i) such a promoting effect of epidermis occurs, and (ii) the factor(s) involved binds preferentially to polycationic substrates, as reported in similar experiments on nerve fibre growth in the presence of conditioned media (Collins, 1980; Adler et al., 1981; Coughlin et al, 1981; Lander et al., 1982).

The epidermis of chick embryo could thus influence neuritic growth at a distance by attracting nerve fibres and, once the latter are in close vicinity, by repelling the majority of them except the normal intraepidermal fibres. Observing a similar phenomenon of avoidance in sympathetic ganglia–spinal cord explants co-cultures, Ebendal (1982) suggested that "the function of spinal cord in repelling ganglionic fibres may be to prevent sympathetic fibres from entering the central nervous pathways". Such a function may be exerted by the epidermis of the chick embryo during the establishment of cutaneous innervation and it may explain how the sorting out of sensory nerve fibres between dermis and epidermis is realized. The lost necessity of having a dense network of intraepidermal nerve fibres in birds might be a background to the expressed fibre-repelling effect exerted by epidermal tissue (Saxod, 1978). Actually, Lumsden and Davies (1984) recently demonstrated a directed growth of trigeminal ganglion neurites towards epithelium (but not mesenchyme) from the presumptive whisker field of the mouse embryo which is densely innervated in the adult.

Finally, the observation reported here can be related to the finding of Feinberg et al. (1983) demonstrating in chick embryo the control exerted by the ectoderm on the position of the blood vessels in the underlying mesoderm. Therefore, the existence of an epidermal monitoring on the nervous network should be considered in explaining how the pattern of cutaneous innervation is laid down in birds.

Acknowledgements

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