Cell-to-Substratum Adhesion and Guidance of Axonal Elongation

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The behavior of axonal growth cones on surfaces with patterned variations in substratum was observed. Cells from sensory ganglia of 8-day-old chicken embryos were cultured on plastic petri dishes, plastic tissue culture dishes, and polyornithine-coated tissue culture dishes, all of which contained gridlike patterns of palladium (Pd) deposition.

The results indicated that growth cones elongated on the Pd-shadowed areas vs areas lacking Pd deposits depending on the relative adhesivity of the growth cones to the substrata. In petri dishes, growth cones stay on the Pd; in tissue culture dishes, they cross from one surface to the other; and in polyornithine-coated dishes, they elongate for great distances on the Pd-free areas. Analyses of time-lapse movies showed that, on Pd-shadowed polyornithine dishes, growth cones often approach the Pd-coated areas and microspikes touch the Pd surface. Yet, the axon tip continues to elongate on the Pd-free polyornithine surface.

The conclusion is offered that interactions between microspikes and the substratum adjacent to the growth cone are important determinants of the directions and pathways of axonal elongation.

INTRODUCTION

The assumption is often made that the complexity and specificity which exist in the nervous system result at least partially from an ordered or directed growth of nerve fibers during embryogenesis. The constancy of architecture observed in the nervous systems of some invertebrates also suggests that the embryonic growth of axons is not random (Burrows, 1973; Macagno et al., 1973). However, the basic mechanisms that operate to produce such ordered growth have not yet been elucidated (Jacobson, 1970; Harrison, 1910).

One directive force in axonal growth may be contact guidance (Harrison, 1910). Elongating axons seem to be oriented by extracellular topographic features (Goldberg, 1974; Nornes and Das, 1972; Speidel, 1933), cell surfaces (Das et al., 1974; Lopresti et al., 1973, 1974; Rakic, 1971; Rakic and Sidman, 1973), and other axons and axonal growth cones (Dunn, 1971; Nornes and Das, 1972; Lopresti et al., 1973, 1974; Weiss, 1941). Such contact-mediated interactions may restrict axon elongation to certain pathways, but might not specify the particular direction taken. Growing axons that have been experimentally or naturally disoriented from their normal pathways can reverse their incorrect vector of growth and make appropriate connections (Hibbard, 1965; Sperry and Hibbard, 1968). This suggests that other forces may operate in addition to mere contact guidance.

Chemotaxis is another guiding force in cell movements (Adler, 1973; Bonner, 1947). Attempts to demonstrate chemotaxis of growing axons have produced both positive and negative results (Chamley et al., 1973; Coughlin, 1975; Weiss and Taylor, 1944). Hence, chemotaxis should not be excluded as a possible guiding force of axon elongation (Ramon y Cajal, 1928).

Adhesion to the substratum may influence cell movements. Fibroblastic cells move up a gradient of metal deposition on a surface (Carter, 1965). Harris (1973)
showed that cells will actively accumulate on portions of a patterned substratum to which they adhere most strongly. In these studies elongating axons of embryonic sensory neurons were presented with such patterned substrata. The results show that growth cones prefer to elongate on surfaces to which they adhere most firmly. This suggests a role for cell-to-substratum adhesion in determining the pathways of nerve fiber growth.

MATERIALS AND METHODS

Preparation of substrata. Four types of culture dishes were used: plastic petri dishes (Falcon Plastics, Oxnard, CA), plastic tissue culture dishes (Falcon Plastics), collagen-coated tissue culture dishes, and polyornithine-coated tissue culture dishes. The last two types were prepared as described in Letourneau (1975).

The patterned substrata were made as follows: Several size #2200 Effa, general electron microscope grids (Ernest F. Fullman, Inc., Schenectady, NY) were put into an open dish. Palladium (Pd) wire was shadowed onto the dishes using a vacuum evaporator. The dishes were inverted to remove the copper grids, and a clear pattern of Pd deposition was seen. The patterns consisted of 80-μm squares of Pd deposition with 27-μm-wide lanes of unshadowed Pd-free surface between the squares (see Figs. 1-7). Each shadowed dish was washed overnight with sterile water before use.

Cell culture. Dorsal root ganglia from 8-day-old chicken embryos were dissected and dissociated into single cells as described in Letourneau (1975). To a culture dish cells were added (8 12 x 10^4) in 2.5–3.0 ml of modified F12 (Spooner, 1970) with 10% fetal calf serum (Pacific Biological Supplies) and nerve growth factor (prepared as in Letourneau, 1975). Cultures were incubated overnight in a 37°C, humidified, 5% CO2 incubator before being filmed, photographed, or otherwise manipulated.

Cell-substratum adhesion. The Gail and Boone air-blaster method of assaying cell-to-substratum adhesion was used to compare the adhesion of growth cones to a Pd-shadowed surface and to tissue culture plastic (Gail and Boone, 1972; Letourneau and Wessells, 1974; Letourneau, 1975).

Time-lapse cinematography. Time-lapse movies were made on a Zeiss inverted microscope, using a Bolex 16 mm movie camera, controlled by a Sage cinematographic apparatus. The culture medium was changed to L-15 (Pacific Biological Supplies) supplemented with 10% fetal calf serum and nerve growth factor just before filming was begun. L-15 maintained the medium at pH 7.4 in the absence of 5% CO2 concentrations. During filming, the dish was kept at 37°C with a Sage Aircurtain Incubator.

RESULTS

The adhesion of growth cones to Pd-shadowed tissue culture dishes and to Pd-free tissue culture dishes was compared with the air-blaster method. As seen in Table 1, there is no significant difference in the adhesion of growth cones to Pd or to tissue culture plastic. This equality is further indicated by the fact (Table 1) that the percentage of neurons which form axons and the average length of axon per neuron at 24 hr in vitro on Pd-coated polyornithine or tissue culture plastic is not different from data for neurons on Pd-free tissue culture plastic (Letourneau, 1975). Growth cones are more adhesive to collagen, polyornithine, and the upper surfaces of glial cells than to tissue culture plastic (Table 1; Letourneau, 1975).

Growth cone-substratum adhesion was not assayed in plastic petri dishes because too few neurons form axons in petri dishes to do an adhesion assay (except in serum-free medium; Ludueña, 1973). Many cell types do not adhere well to Falcon petri-dish plastic (Harris, 1973; Martin and Rubin, 1974). We, therefore, assume that
TABLE 1
GROWTH CONE ADHESION, AXON INITIATION, AND MEAN AXON LENGTH AT 24 HR

<table>
<thead>
<tr>
<th>Dish</th>
<th>% Initiation</th>
<th>Axon/neuron (µm)</th>
<th>% Distracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pd-coated tissue culture</td>
<td>27 (n = 137)</td>
<td>158 (n = 20)</td>
<td>48 (n = 54)</td>
</tr>
<tr>
<td>Pd-coated polyornithine</td>
<td>29 (n = 86)</td>
<td>148 (n = 20)</td>
<td>-</td>
</tr>
<tr>
<td>Tissue culture</td>
<td>25 (n = 642)b</td>
<td>154 (n = 60)b</td>
<td>53 (n = 95)</td>
</tr>
</tbody>
</table>

Growth cone–substratum adhesion was assayed at a blasting distance of 2.4 cm and an air flow-rate of 3.0 l/min. Duration of blast was 0.085 sec. The difference in percentage of growth cones distracted on these substrata is not significant. Methods of determining percent of axon initiation and average µm of axon/neuron are described in Letourneau (1975). n, number of neurons counted.

*b Data taken from Letourneau (1975).

growth cone–petri-plastic adhesion is relatively weak.

From these data a hierarchy of substrata for growth cones can be postulated, based on the relative strengths of growth cone–substratum adhesion; Glia ≈ polyornithine > collagen > tissue culture plastic ≈ Pd > petri plastic. Hence, the patterned substrata used in these studies consisted of Pd squares surrounded by lanes of substrata to which growth cones are less, equally, or more adhesive (i.e., petri plastic; tissue culture plastic; collagen or polyornithine, respectively).

Cultures were observed after approximately 16 hr in vitro. On the Pd-shadowed petri and tissue culture dishes axons were seen which extended from Pd onto the unshadowed plastic and vice versa (Figs. 1–5). Growth cones also were seen on both the Pd squares and on the unshadowed lanes.

In marked contrast to these results, in collagen or polyornithine-coated dishes with Pd patterns, axons extended for long distances along the unshadowed lanes between the Pd squares (Figs. 6–11). After 48 hr, axons longer than 1000 µm extended down the collagen or polyornithine lanes. Frequently, axons bent around 90° corners...
or had branches going down several lanes at an intersection (Figs. 6, 7). Some nerve cell bodies were located on Pd squares with axons which had crossed onto and continued along the Pd-free lane.

Although axons did extend along the unshadowed lanes in petri and tissue culture dishes, the frequency of such observations and the lengths of axons were much less than in collagen or polyornithine dishes.

In order to estimate the preference of growth cones for various substrata, the percentage of growth cones that were on (a) Pd, (b) unshadowed lanes, and (c) upper surfaces of flattened glial cells were determined in the petri dishes, tissue culture dishes, and polyornithine-coated dishes. The data are presented in Table 2. If the percentage of growth cones on a surface is a measure of relative preference for that surface, then on petri dishes, growth cones prefer (1) glia, (2) Pd, and (3) petri plastic; in tissue culture dishes the order is (1) glia, (2) Pd, and (3) plastic, but the differences are small; and on polyornithine dishes growth cones prefer (1) polyornithine, (2) glia, and (3) Pd.

These numbers are not absolute indications of the preference of growth cones for a surface because of the geometry of the patterns. The Pd squares comprise about 67% of the surface area, but they are islands surrounded by continuous lanes of unshadowed substratum. Obviously, growth cones can elongate for long distances on the lanes but not for more than 115 μm on the Pd squares. Glia cover little (less than 5%) of the surface, at the cell densities plated here. However, glia do migrate, and opportunities for encounter with an axon or growth cone are greater than suggested by the 5% figure.

From photographs of the cultures, the numbers of glia on the Pd or on the unshadowed lanes were counted. As seen in Table 3, the distribution of glia is similar to that for growth cones. In addition, it conforms with Harris' (1973) results with several cell types, except that he reported that cells strongly prefer tissue culture plastic to Pd.

The 35 mm photographs and the proportions of growth cones situated on various substrata suggested that growth cones tend to remain on substrata to which they are more adhesive. Time-lapse cinematography was used to observe the behavior of growth cones on these dishes.

<p>| TABLE 2 |
| PERCENT OF GROWTH CONES ON SUBSTRATA* |</p>
<table>
<thead>
<tr>
<th>Dish</th>
<th>Substratum</th>
<th>Pd</th>
<th>Unshadowed lane</th>
<th>Glia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petri plastic</td>
<td>35</td>
<td>18</td>
<td>47 (n = 98)</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plastic</td>
<td>32</td>
<td>31</td>
<td>36 (n = 207)</td>
<td></td>
</tr>
<tr>
<td>Polyornithine-coated</td>
<td>4</td>
<td>74</td>
<td>22 (n = 274)</td>
<td></td>
</tr>
</tbody>
</table>

* The distribution of growth cones on Pd, unshadowed lanes, and glia, respectively, was determined by counting the number of growth cones on these substrata in several randomly selected microscope fields. The data for growth cones on glial cells was omitted in calculating the percentages shown in the lower panels. n, number of neurons counted.

<p>| TABLE 3 |
| PERCENT OF GLIA ON SUBSTRATA* |</p>
<table>
<thead>
<tr>
<th>Dish</th>
<th>Substratum</th>
<th>Pd</th>
<th>Unshadowed lane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petri plastic</td>
<td>86</td>
<td>14 (n = 50)</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plastic</td>
<td>59</td>
<td>41 (n = 66)</td>
<td></td>
</tr>
<tr>
<td>Polyornithine-coated</td>
<td>29</td>
<td>71 (n = 55)</td>
<td></td>
</tr>
</tbody>
</table>

* The distribution of glia on Pd and on the unshadowed lanes, respectively, was determined from photographs of cell cultures. Only those glial cells that were wholly on one substratum type or almost wholly, except for marginal areas, were counted. n, number of glia counted.
On Pd-shadowed petri dishes growth cones would move across the Pd to the edge of a square. Several behaviors followed. Often, they would remain active (i.e., intensive microspike activity; Ludueña and Wessells, 1973) but not advance onto the plastic. This was occasionally followed by retraction of the growth cone and axon. Another observation was that the growth cone would move laterally along the border, staying on the Pd. There were cases of the growth cone crossing from Pd onto petri plastic. When this happened, the growth cone usually would not elongate far, although it did continue microspike activity. It seemed as though the growth cones were “slipping back” after accomplishing a short advance.

On Pd-shadowed tissue culture dishes, growth cones frequently crossed from Pd to plastic and from plastic to Pd. One filming sequence of 5.5 hr was a particularly good example. An axon started on Pd and moved onto the plastic, where it elongated about 80 μm in 2 hr. Then, it crossed onto a Pd square and grew 100 μm or more to the other side and moved onto plastic again. Another axon from the same cell body elongated on the plastic for over 4 hr and then crossed onto the Pd.

On Pd-shadowed collagen and polyornithine dishes, growth cones of axons elongating along an unshadowed, Pd-free lane did not stay in the middle of a lane; instead, they meandered from one edge of the lane to the other (Figs. 8–11). Microspikes waved about in close proximity to the Pd surface (Figs. 12–17). It seemed that microspikes protruded from various edges of the growth cone and were contacting the Pd and the unshadowed substrata simultaneously. Yet, the growth cone continued to elongate on the unshadowed surface. In addition to microspikes many thin veilike expansions of microspikes formed on collagen and polyornithine. These veils are reminiscent of the distinctive flattening of growth cones observed in cultures with elevated Ca²⁺ levels (Letourneau, 1975). Such flattened veils may result from a strong adhesion to the substratum. Related to this is the impression that microspikes adhere to the collagen or polyornithine surface for longer time periods than to the Pd.

Growth cones were observed extending around a corner at the intersection of two unshadowed lanes. The growth cone stayed on the collagen or polyornithine as it moved from one lane to the other. In such cases, if the axon was not adherent to the substratum, it straightened out across the corner, thereby extending over the Pd surface. This arrangement could give the impression that the axon had elongated directly across the Pd from one lane to the next. However, the film shows clearly that the growth cone grew around the corner and the axon slipped sideways in a passive fashion (as predicted by Ludueña (1973) and above).

Another interesting series of observations with time-lapse cinematography was the movement of growth cones onto the upper surfaces of glia. The actual adhesion of the growth cone to a glial surface was often not observed, but the result was (Figs. 18–21), the axon rapidly straightened as if pulled tight. The cause of this is unknown, but a possible explanation is that the adhesion of the growth cone, followed by an advance, created tension to pull the axon toward the glial cell.

DISCUSSION

The basic results of these experiments are: (1) A hierarchy of growth cone-substratum adhesion can be postulated; glia ≈ polyornithine > collagen > tissue culture plastic ≈ Pd > petri plastic. (2) On Pd-shadowed petri dishes, growth cones do not move well on petri plastic and often do not cross from Pd to petri plastic. (3) On Pd-shadowed tissue culture dishes, growth cones cross from plastic to Pd and vice versa frequently. (4) On Pd-shadowed collagen or polyornithine dishes, growth cones elongate for hundreds of μm's on the un-
shadowed lanes, even though the growth cones approach the Pd surface very closely. These results may have important implications for neuronal axon formation. One can understand why growth cones will not cross from Pd to petri plastic if they adhere only weakly to petri plastic (adhesion may occur in the absence of serum;
Fig. 18–21. Frames from the same movie of a growth cone as shown in Figs. 12–17. (18) The growth cone (GC) is near the middle of an unshadowed lane (U). A microspike (arrow) has just contacted the upper surface of a glial cell (G). × 880. (19) Taken 296 sec later. The growth cone is beginning to extend towards the glial cell. Also, note the movement of the glial cell’s leading edge towards the growth cone (arrow). × 880. (20) Taken 312 sec after Fig. 19. The growth cone has moved onto the upper glial cell surface. Note the increased ruffling (arrows) of the glial cell margin where it contacts the growth cone. × 880. (21) Taken 288 sec after Fig. 20. The growth cone has elongated across the glial cell surface and microspikes (arrows) are extending over the dish substratum on the far side of the glial cell. Note the extremely long microspike (M), which is touching the dish substratum. × 880.

Ludeña, 1973). Harris (1973) reports that 3T3 cells undergo vigorous ruffling activity on petri dishes, giving the impression of “a frantic, but unsuccessful struggle to spread, which is continually frustrated by repeated de-adhesions at the margin.”

However, what can be offered to explain the response of growth cones to the same substratum, Pd, in different ways? Growth cones do not often cross from collagen or polyornithine surfaces onto Pd. Yet, Pd is a perfectly acceptable substratum for axon elongation, when petri or tissue culture plastic is the alternative substratum! Do growth cones prefer to stay on collagen or polyornithine because they adhere more tightly? Or are they unable to move onto the Pd-coated surface? The fact that neurons do initiate and extend axons on Pd-shadowed, polyornithine-coated dishes which lack patterned deposits eliminates the possibility that Pd-coated polyornithine is an unacceptable surface for growth cone function.

These results suggest a mechanism for regulating the directions in which axons elongate. Microspikes and their veillike expansions often contact the substratum ahead of the growth cone (Wessells et al., 1973). Bray (1973) has postulated that microspikes and other filopodia grow via addition of intracellular vesicles to the surface of the microspike tip. It could be that addition of vesicles and microspike enlargement are enhanced by adhesion of the microspike to a surface (Letourneau, 1975; Rovensky and Slavnaya, 1974; Vasiliev and Gelfand, 1973, p. 326). This may lead to expansion of the microspike and advancement of the growth cone in that
direction. In this manner the growth cone will advance on that substratum adjacent to the growth cone to which microspikes adhere more often or more strongly.

A criticism of these experiments is the artificial nature of the patterned substrata, which are not likely to represent accurately the subtle differences of the microenvironment in an embryo. Nevertheless, the results, however crude, do suggest a way of guiding an axon to its destination. Pathways of relatively high adhesion or adhesion gradients may be crucial elements for both gross axonal routing and for aspects of neuronal connection specificity.

This model does not rule out chemotaxis as a possible directive force. If a chemical concentration gradient could make microspike interactions with the substratum more effective in a particular direction, elongation would be similarly guided. The diffusing molecule could act either on the growth cone surface or on the substratum. Jacobson (1970) has stated well the error of making a hard distinction between chemical and mechanical guidance of nerve fiber growth because “at short range they boil down to the same thing; the physicochemical interaction between the nerve fiber and its environment.”

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