Measurements of Growth Cone Adhesion to Culture Surfaces by Micromanipulation

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Abstract. Neurons were grown on plastic surfaces that were untreated, or treated with polylysine, laminin, or L1 and their growth cones were detached from their culture surface by applying known forces with calibrated glass needles. This detachment force was taken as a measure of the force of adhesion of the growth cone. We find that on all surfaces, lamellipodial growth cones require significantly greater detachment force than filopodial growth cones, but this difference is, in general, due to the greater area of lamellipodial growth cones compared to filopodial growth cones. That is, the stress (force/unit area) required for detachment was similar for growth cones of lamellipodial and filopodial morphology on all surfaces, with the exception of lamellipodial growth cones on L1-treated surfaces, which had a significantly lower stress of detachment than on other surfaces. Surprisingly, the forces required for detachment (760–3,340 μdynes) were three to 15 times greater than the typical resting axonal tension, the force exerted by advancing growth cones, or the forces of retraction previously measured by essentially the same method. Nor did we observe significant differences in detachment force among growth cones of similar morphology on different culture surfaces, with the exception of lamellipodial growth cones on L1-treated surfaces. These data argue against the differential adhesion mechanism for growth cone guidance preferences in culture. Our micromanipulations revealed that the most mechanically resistant regions of growth cone attachment were confined to quite small regions typically located at the ends of filopodia and lamellipodia. Detached growth cones remained connected to the substratum at these regions by highly elastic retraction fibers. The closeness of contact of growth cones to the substratum as revealed by interference reflection microscopy (IRM) did not correlate with our mechanical measurements of adhesion, suggesting that IRM cannot be used as a reliable estimator of growth cone adhesion.

T HE motility of the growth cone plays a major role in elongation of neural axons (Lockerbie, 1987; Letourneau et al., 1991). The growth cone also possesses "sensory" capabilities that are responsible for the recognition of guidance cues of various kinds (Taghert et al., 1982; Bentley and Toroian-Raymond, 1986; Kapfhammer and Raper, 1987; Baier and Bonhoeffer, 1992; Davenport et al., 1993). Both their motile and sensory functions require growth cones to make and break contacts with their environment. Indeed, the molecular composition of the substrate on which neurons grow, in culture or in situ, has significant effects on axonal development; regulating guidance (Letourneau, 1983; Hammarback and Letourneau, 1986; Gunderson, 1987; Bentley and O'Connor, 1992; Clark et al., 1993); axonal elongation rate (Kleitman and Johnson, 1989; Thomas et al., 1990; Buettner and Pittman, 1991; Lamoureux et al., 1992); degree of branching (Bray, 1987; Burmeister and Goldberg, 1988; Buettner and Pittman, 1991);

and growth cone morphology (Caudy and Bentley, 1986; Bovolenta and Mason, 1987; Payne et al., 1992).

The mechanism of these substrate effects is poorly understood, but hypotheses fall into two broad categories that are not mutually exclusive. One general mechanism is that the membrane proteins that mediate cell attachment act as receptors, which in turn stimulate intracellular chemical signals, e.g., production of second messengers and/or activation of protein kinases, that act to alter cellular physiology (Juliano and Haskill, 1993). This report, however, focuses on the other general mechanism to explain substratum effects on axonal development: that neurons respond to the mechanical force of adhesion (Letourneau, 1983; Bastiani and Goodman, 1984; Bray, 1982, 1991). In some cases, for example, growth cones preferentially steer onto culture surfaces that are more adhesive when presented with substratum choices (Letourneau, 1975; Hammarback and Letourneau, 1986). Recent studies, however, have questioned the generality of this latter mechanism. Several studies have shown that adhesion to culture substrata is poorly correlated with guidance preferences (Gunderson, 1987; Calof and Lander, 1991; Lemmon et al., 1992), axonal elongation rate (Buett-

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ner and Pittman, 1991; Lemmon et al., 1992), or fasiculation (Lemmon et al., 1992).

A difficulty in assessing the contribution of adhesive forces to axonal development and growth cone function has been quantifying adhesion by cells and growth cones. Although the relative difficulty of dislodging growth cones or neurons from various culture substrata has been assessed by several workers (Hammarback and Letourneau, 1986; Gunderson, 1987; Calof and Lander, 1991; Lemmon et al., 1992), the actual magnitude of the forces involved in growth cone attachment in culture are unknown. We report here measurements of the force required to dislodge growth cones from culture substrata treated with various physiological and non-physiological "adhesion molecules." Growth cones were detached by micromanipulation with calibrated glass needles. This is a relatively direct method that provides force values for growth cone detachment in absolute units.

Materials and Methods

Materials

Horse serum and N-2 growth supplement (containing 5 μg/ml insulin, 100 μg/ml human transferrin, 0.0063 μg/ml progesterone, 16.11 μg/ml putrescine, 0.0052 μg/ml selenium) were purchased from GIBCO-BRL (Gaithersburg, MD). Laminin, poly-L-lysine, BSA, trypsin, 7S nerve growth factor, L-15 medium was purchased from Sigma Chemical Co. (St. Louis, MO) The fluorescent dye RH-414 was purchased from Molecular Probes (Eugene, OR). L1 was the kind gift of Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH).

Cell Culture

Chick sensory neurons isolated from lumbosacral dorsal root ganglia of 9-12-d-old chicken embryos were cultured essentially as described by Sinclair et al. (1988); however, serum-free medium was used to avoid adding adhesion molecules normally found in serum. Cells were cultured at 37°C in L-15 medium supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, and 136 μg/ml streptomycin, 1% (1×) N2 supplement and 100 ng/ml 7S nerve growth factor.

Cells were grown on four different culture surfaces: (a) 60-mm Corning tissue culture dishes as supplied (plain plastic); (b) The same dishes treated with 10 μg/ml laminin as described by Lamoureux et al. (1992); (c) The same dishes treated with poly-L-lysine. Poly-L-lysine dishes were prepared by incubating dishes in 0.1 mg/ml poly-L-lysine in sterile borate buffer (pH 8.5) for a 2-h period. These dishes were then rinsed five times with sterile distilled water before the addition of cells. Because cells in serum-free medium were extremely sensitive to the toxicity of poly-L-lysine, BSA (5 mg/ml) was added to the culture medium in these dishes (Calof and Lander, 1991); and (d) The same dishes treated with L1 as follows: To conserve L1, 30 μl of L1 solution (230 μg/ml) was applied only to a central region of a dish marked by a circle on the dish's undersurface. The treated dish was allowed to incubate for 30-40 min in a humid chamber at room temperature, and washed with sterile distilled water once before the addition of cells suspended in serum-free medium.

Measurements of Force Required for Growth Cone Detachment

We used glass needles of known bending modulus, fabricated, and calibrated as previously described (Dennerll et al., 1988; Lamoureux et al., 1989), to measure the force required to dislodge the growth cones of the chick sensory neurons. A calibrated needle was mounted in a dual-head micromanipulator so that its tip was a short distance from the tip of a stiffer, uncalibrated reference needle. The uncalibrated needle served as a reference for the bending of the calibrated needle and as a control for any drift in the micromanipulator system. The calibrated needle was brought into position at the distal end of the neurite shaft, i.e., the base of growth cone, and gradually moved perpendicular to the neurite axis applying a force load that eventually caused the growth cone to detach from the substratum. However, as described later (see Fig. 6), some small regions of attachment associated

with retraction fibers typically remained after the bulk of the growth cone region had been dislodged. In some experiments, force was applied to the cell body of neurons lacking neurites by pushing against the soma with a calibrated needle until the cell body detached from the substratum. The whole experimental process was recorded on videotape which was later analyzed for the force of detachment, as described below. The area of the growth cone was determined by tracing around digitized images of growth cones whose area was then integrated by computer (SigmaScan; Jandel Scientific, San Rafael, CA). Throughout the micromanipulation procedure, care was taken to avoid dragging the needle on the dish surface, i.e., we took precautions to ensure that cellular structures were the sole source of resistance to the movement of the calibrated needle (hydrodynamic drag on the needle was not apparent at the speeds at which the needle was moved, which varied between 0.8 and 4 μm/s, with a mean of 2.1 μm/s for six measurements). Those few manipulations in which replay of the videotape suggested dragging of the needle on the culture surface were not included in the data set.

The force required to detach growth cones was calculated from four measurements taken from the videotape of the micromanipulation: the original length of the neurite (d), the lateral deflection of the needle at the moment of detachment (D), the distance along the neurite shaft from the cell body to the needle tip at the same time (c), and the distance along the neurite shaft from the needle to the middle of the growth cone also at the time of detachment (b). The axial force applied to the growth cone along the neurite shaft was calculated from these measurements and the known stiffness of the calibrated needle, which varied between 15 and 36 μdynes/μm of bending, using vector algebra and geometry, essentially as described by Dennerll et al. (1988), according to the following equation:

Force on growth cone = F_{gc}

$$= (D \times \text{calibration}) \frac{\left(\dfrac{d^2 + c^2 - b^2}{2dc} \right)}{\sqrt{1 - \left(\dfrac{b^2 + c^2 - d^2}{2bc} \right)}}$$

Fluorescent Labeling of Neuronal Membrane

Neuronal membranes were fluorescently labeled with a lipid-incorporating dye by a modification of the method of Balice-Gordon and Lichtman (1993). Before some micromanipulations, a stock solution (8.5 mM in DMSO) of RH-414 (Molecular Probes) was added to the culture medium to produce a final concentration of ∼40 μM (10 μl stock to 2 ml medium). After a 5 min incubation, a growth cone was detached as described above. Following growth cone detachment, cells were allowed to incubate for an additional 2 min, and then rinsed twice in warmed, fresh culture medium, as described above, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. Samples were examined using an Odyssey confocal microscope with fluorescein optics (Noran Instrument, Middleton, WI).

Interference Reflection Microscopy

Cells were cultured on glass coverslips with or without the same surface treatments described above for plastic dishes. Cells were allowed to grow for 18-24 h after which the coverslip was removed and inverted onto a glass slide and supported at two sides by thin strips of glass cut from coverslips. The resulting chamber was infused with serum-free medium to bathe the cells. Phase contrast and interference reflection microscope (IRM)[1] images were viewed using a Zeiss 210 Laser Scanning Microscope (Carl Zeiss Inc.). Interference reflection images were obtained with epi-illumination provided by an argon laser (488 nm) using either 40× (NA 1.3) or 100× (NA 1.25) oil immersion lenses. This simple approach has been shown to produce high quality reflection images of diaminobenzidine products in PC12 cells (Whallon et al., 1994) and we directly confirmed interference reflection imaging by observing the "bull's-eye" interference fringes produced by an opaque polystyrene microbead (2.9-μm diam; Polysciences, Warrington, PA) pressed between a glass slide and a coverslip, as described by Davies et al. (1993). In recording IRM images of growth cones, we attempted to equalize background radiance among the various samples, which required only very minor adjustment with the brightness and contrast controls of the microscope. The digitized images from the microscope were

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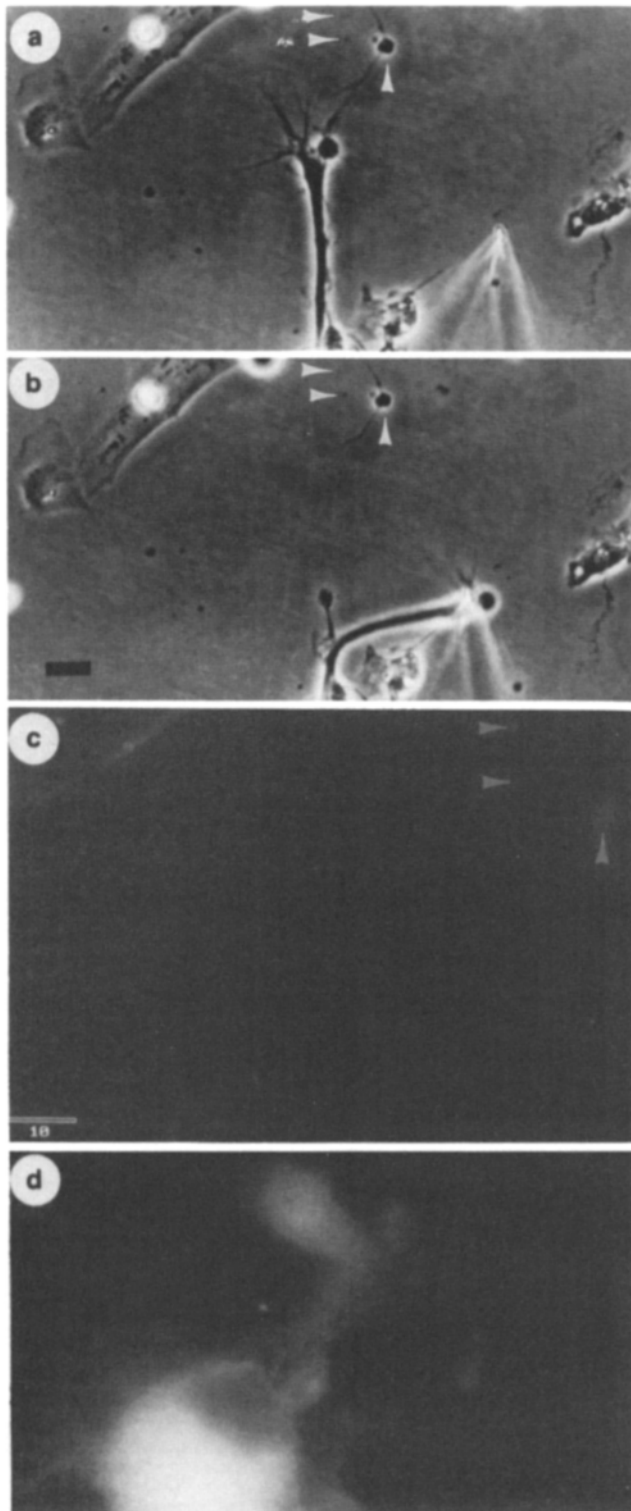1. *Abbreviation used in this paper*: IRM, interference reflection microscope.

Figure 1. Growth cone detachment by micromanipulation in the presence of RH-414. As described in Materials and Methods, cultured chick sensory neurons were incubated in RH-414 before, during, and after growth cone detachment to label the neuronal plasma membrane. The phase images are of a neuron before (*a*) and after (*b*) growth cone detachment. Note the white arrowheads that mark two bits of detritus and a cell fragment used as markers for the original location of the growth cone. Panel *c* shows that after growth cone detachment, the region previously occupied by the growth cone shows no evidence of fluorescently labeled membrane, al-

Results

Detachment of Growth Cones by Micromanipulation

As shown in Fig. 1, micromanipulation by glass needles was used to detach growth cones of chick sensory neurons from the culture substratum. We took growth cone detachment to be the removal of the entire central region of the growth cone. As described later, however, some small regions of attachment associated with retraction fibers often remained after the bulk of the growth cone region had been dislodged (see Fig. 6). In ~80% of the cases, there was no phase-dense residue at the former location of the growth cone, suggesting that detachment left little or no residue from the growth cone. To confirm that no membranous material was left behind following detachment, in five experiments we incubated neurons before, during, and after growth cone detachment in the presence of a styryl dye, RH414, previously used for vital staining of neuronal membrane in situ (Balice-Gordon and Lichtman, 1993). As illustrated by Fig. 1 *c*, no evidence of labeled membrane could be seen at the original location of the growth cone in any of the five experiments, although the detached growth cones, with their axons and cell bodies were clearly fluorescent (Fig. 1 *d*).

Adhesion of Filopodial and Lamellipodial Growth Cones

It has frequently been noted that growth cones vary widely in their morphology both in culture and in situ (Argiro et al., 1984; Kleitman and Johnson, 1989; Bovolenta and Mason, 1987; Payne et al., 1992). Growth cones can be divided rather generally into filopodial and lamellipodial forms, which may play functionally distinct roles (Bovolenta and Mason, 1987; Mason and Godement, 1991; Kleitman and Johnson, 1989). The veil-like form of lamellipodial growth cones typically cover a larger area of substratum than do filopodial growth cones (Fig. 2 and Table I), which might be expected to reflect and/or affect their adhesion to the substratum. For these reasons, we analyzed filopodial and lamellipodial growth cones separately, based on subjective judgements as to form as shown in Fig. 2. An effort was made to analyze both forms of growth cones on all surfaces, although we found different surfaces were associated with characteristic forms and with varying frequencies of the two forms as reported previously by Payne et al. (1992). On laminin for example, lamellipodial growth cones were rare. Conversely, filopodial growth cones were rare on L1-treated surfaces and the characteristic lamellipodial growth cones on this surface had a significantly ($P < 0.05$) larger surface area than lamellipodial growth cones on other surfaces (Table I).

Fig. 3 summarizes our data for the force required for detachment of growth cones on the various surfaces. The average forces required for detachment varied between 760 to 3340 µdynes. These forces are substantially larger than ei-

though the neuron is brightly labeled (*d*). We observed, as did Balice-Gordon and Lichtman (1993), that neuronal cell membranes appeared to label more intensely with RH 414 than other cell types. Bars: (*a* and *b*) 20 µm; (*c* and *d*) 10 µm.

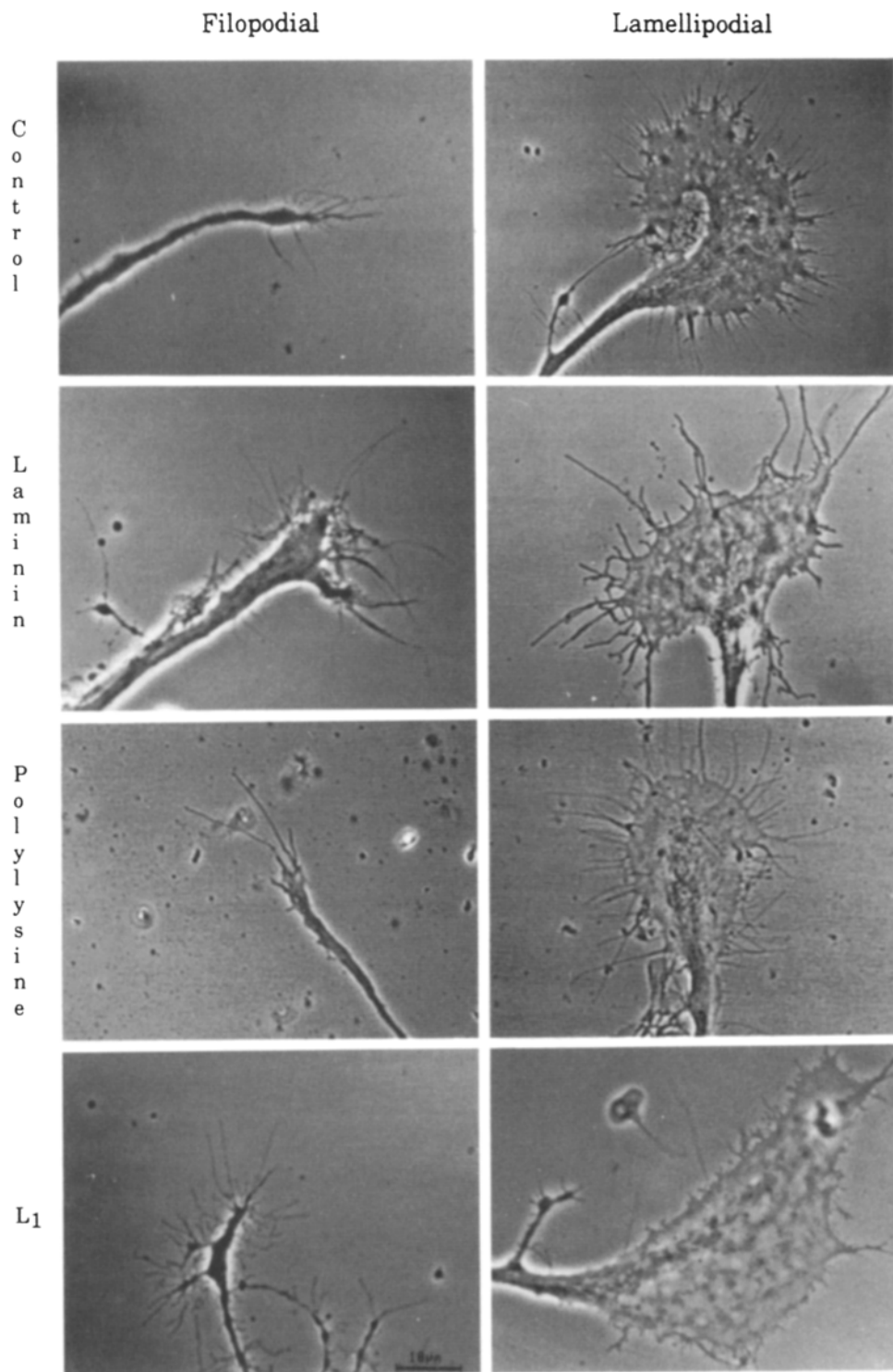Filopodial | Lamellipodial

Control
Laminin
Polylysine
L₁

Figure 2. Phase images of growth cones on the different substrata judged as being filopodial or lamellipodial. (*Left*) Filopodial growth cones on the various surfaces as listed to the extreme left. (*Right*) Lamellipodial growth cones on the same surfaces. Bar, 10 μm.

ther the forces exerted by advancing growth cones in this cell type or the average static tension load of neurites (rest tensions) for this cell type, which typically fall between 100–200 μdynes (Lamoureux et al., 1989, 1992). Fig. 3 shows that on every surface, lamellipodial growth cones required about twice the average force for detachment than filopodial growth cones on the same surface. On all the surfaces, the differences in the forces of detachment between filopodial and lamellipodial forms were significant at the 0.05 level

using a one-tailed t-test (testing that lamellipodial growth cones have a greater force of detachment than filopodial growth cones).

It seemed likely that the larger area of lamellipodial growth cones was one contributor to these differences. To assess whether the differences in adhesion of filopodial and lamellipodial growth cones reflected differences in area or actual differences in the intensity of adhesion, we calculated a force of detachment per unit area, i.e., the stress required

Table I. Area of Growth Cones (sq. μm ± SEM)

	Control	Laminin	Polylysine	L1
Filopodial growth cone	147.79 ± 31.53	63.89 ± 8.9	187.30 ± 45.87	227.37 ± 104.38
Lamellipodial growth cone	280.89 ± 71.11	227.24 ± 66.96	336.99 ± 73.50	758.55 ± 192.99

for detachment, for each growth cone by dividing its detachment force by its measured area. As shown in Fig. 4, when viewed from the perspective of adhesive intensity, filopodial and lamellipodial growth cones are quite similar, typically between 10 and 20 μdynes/μm^2. Except for growth cones on L1-treated dishes, the intensity of adhesion on a given surface did not differ significantly for filopodial and lamellipodial growth cones. That is, normalizing for the differences in growth cone area virtually eliminated the differences in adhesion between filopodial and lamellipodial growth cones on plain plastic, laminin-, and polylysine-treated surfaces. However, because of their large area, lamellipodial growth cones on L1-treated surfaces had a significantly lower (one tailed t-test, $P < 0.05$) average intensity of adhesion than filopodial growth cones on this surface.

Comparison Of Adhesion On Different Surfaces

The force or intensity of adhesion for particular growth cone types, either lamellipodial or filopodial, among the different substrata indicated few significant differences. Pairwise t-tests of filopodial growth cone adhesion on the various surfaces indicated no significant differences in either the force (Fig. 3) or the intensity of adhesion (Fig. 4). The detachment forces on plain, laminin-, and polylysine-treated surfaces averaged between 1,000 and 1,500 μdynes. The few filopodial growth cones found on L1 required a somewhat lower average force of detachment, 760 μdynes, but this was not significant at the 0.05 level of confidence. As explained later

in the presentation of IRM data, it seems possible that filopodial growth cones on L1 occupied regions that did not adsorb the protein, i.e., were actually adhering to plain plastic. The average stress for detachment of filopodial growth cones varied between 9 and 17 μdynes/μm^2 among the four surfaces with no significant differences.

The situation was somewhat more complex for lamellipodial growth cones but, again, adhesion was quite similar. As shown in Fig. 3, the force required for lamellipodial growth cone detachment on plain plastic, laminin-, or polylysine-treated surfaces averaged between 2,300 and 3,300 μdynes with no significant differences. However, the average force of detachment for lamellipodial growth cones on L1 (1,440 μdynes) was significantly less than the average detachment force on plain or polylysine treated plastic at the 0.05 level, but not significantly different than the detachment force on laminin. The average detachment stress for lamellipodial growth cones on plain plastic, polylysine, and laminin varied between 10 and 17 μdynes/μm^2 with no significant differences. However, with the large area of lamellipodial growth cones on L1, the average stress of detachment of neurons on L1 (3 μdynes/μm^2) was significantly less ($P < 0.05$) than the stress of growth cones on any other surface.

We were concerned by the lack of differences in detachment force for growth cones on the various substrata, partic-
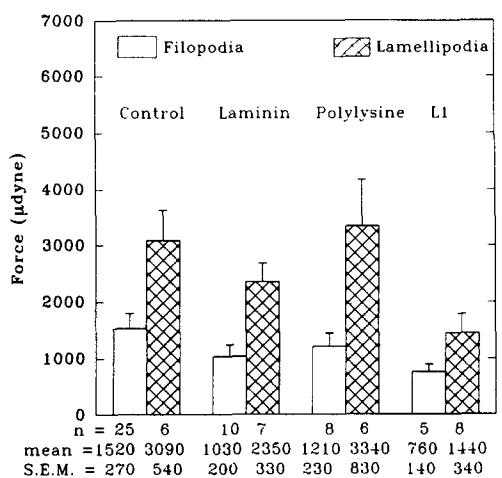

Figure 3. Mean applied forces required to dislodge growth cones from the various culture surfaces. Open bars reflect measurements of filopodial growth cones on the various surfaces and cross-hatched bars reflect measurements of lamellipodial growth cones on the same surfaces. Shown below each bar in the graph are the number of growth cones measured, the mean detachment force in μdynes and its standard error.
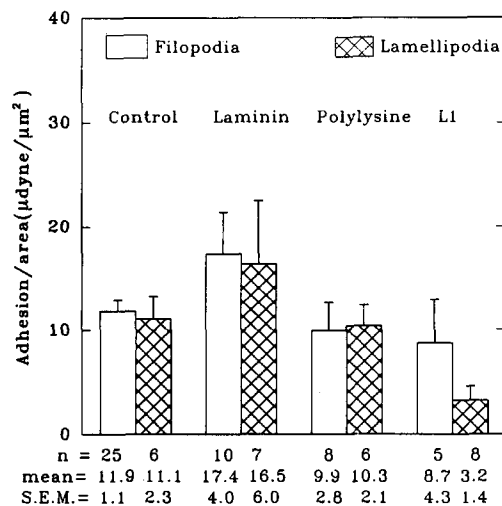

Figure 4. Mean stresses (force/area) required to detach growth cones from the various culture surfaces. The stress of detachment was calculated by dividing the measured force of detachment for each growth cone by the area of the growth cone, as determined by the SigmaScan image analysis computer program. Open bars reflect the mean stresses of filopodial growth cones on the various surfaces and cross-hatched bars reflect the mean stresses of lamellipodial growth cones on the same surfaces. Shown below each bar in the graph are the number of growth cones measured, the mean stress in μdynes/μm^2 and its standard error.
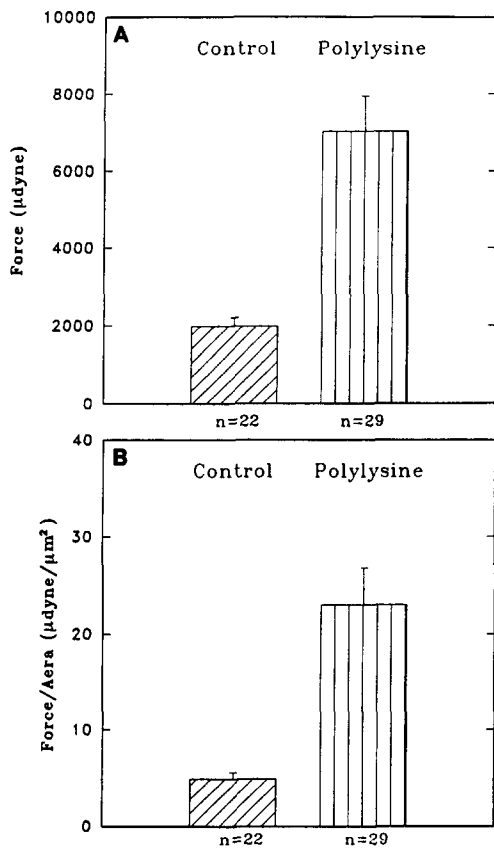
Figure 5. Mean forces and stresses required to dislodge cell bodies on untreated and polylysine-treated plastic. (A) Mean force of detachment of cell bodies on untreated plastic (control, ▨) and polylysine-treated plastic (▥). (B) Mean detachment stress of cell bodies, calculated as in Fig. 4, on untreated and polylysine-treated plastic surfaces.

ularly to polylysine, which has long been known to increase cell adhesion by several measures. Recently, for example, Calof and Lander (1991) found that mouse neurons from olfactory epithelium were substantially more adherent to polylysine-treated plastic than to plain or laminin-treated plastic as measured in an assay that compared the relative centrifugal forces required to dislodge cells from the substratum. However, this and most other assays of cell adhesion focus on the attachment of the cell body. We thus compared the adhesion of cell bodies on plain and polylysine-treated plastic using our micromanipulation method (Fig. 5). The mean detachment force for cell bodies on polylysine-treated plastic was three times greater than that on untreated plastic (Fig. 5 a), a difference that was significant at the 0.05 level of confidence. Unlike growth cones that vary considerably in their area, cell bodies showed no differences in degree of spreading among the various substrata, so there was a statistically significant difference in detachment stress as well (Fig. 5 b).

Mechanically Resistant Points Of Attachment

As mentioned in the Materials and Methods section, small regions of the growth cone that were more resistant to detachment typically remained following the detachment of most of the area of the growth cones (Fig. 6). These regions were most frequently located at the distal ends of filopodia or lamellipodia, but they were occasionally observed along the length of a neurite. These regions of attachment remained connected to the detached regions of the growth cone/neurite by retraction fibers that were highly elastic. That is, upon release of the applied force, the growth cone would rapidly spring back to near its original position within less than a second, as shown in Fig. 6. These retraction fibers were observed to contain actin by standard fluorescent labeling procedures for actin using rhodamine phalloidin (data not shown).
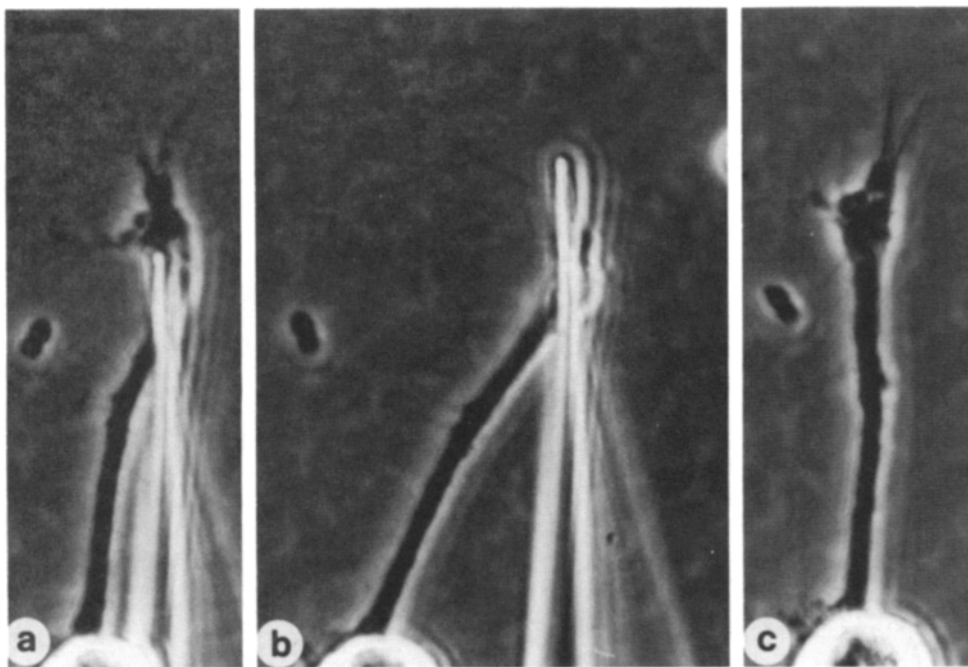

Figure 6. Phase images of retraction fibers observed during growth cone detachment. (a) A growth cone immediately prior to detachment. A needle was placed at the base of growth cone and gradually moved perpendicular to neurite axis applying a force load. (b) Following the detachment of the principal structure of the growth cone, retraction fibers continue to connect the growth cone and, in this case, the neurite shaft, to the substratum. The diameters of the attachment points of these retraction fibers were between 0.6–1.2 μm as determined by the SigmaScan computer program. (c) Micrograph taken immediately upon releasing the neuron from the needle: the growth cone rapidly sprang back to its original position within less than a second. Bar, 10 μm.

We were unable to accurately measure the force of detachment of these retraction fibers, either the neurite shaft or the fiber parted before sufficient force could be applied to cause detachment at or near the dish surface. However, a lower limit can be calculated in nine cases where we were able to measure the force applied before the neurite shaft or retraction fiber parted (e.g., Fig. 6 b). The average force borne by the retraction fibers in these nine cases was 640 ± 110 μdynes. From this we estimate that the adhesion stress was at least 100 μdynes/μm^2 at the attachment site of the retraction fibers, a 6- to 30-fold higher intensity of adhesion than we found for actual detachment of the remaining areas of the growth cone (Fig. 4). This estimate is based on our observations that the regions of attachment were typically 1 μm in diameter (area ~0.8 μm^2) and we observed an average of about eight of these spring-like fibers for each dislodged growth cone.

Interference Reflection Microscopy of Growth Cones on Various Surfaces

IRM images of growth cones produced by zero order reflections between the "ventral" surface of the growth cone and the underlying substratum are a variable pastiche of dark to light grey areas, representative of regions of relatively close contact (Curtis, 1964; Izzard and Lochner, 1976; Verschueren, 1985) interspersed with bright areas, indicative of greater separations from the substratum. Black regions, indicative of focal contacts when seen beneath fibroblasts, were not observed with these neurons except in one instance. In this case, the black region ran along the entire edge of a very thin lamella, as judged from a phase image, which is a well-described artifact produced by reflections from the top surface of these unusually thin cytoplasmic layers (Gingell, 1981; Heath, 1982; Verschueren, 1985).

As previously reported by others (Letourneau, 1979; Gundersen, 1988), the interference pattern produced by growth cones are quite distinctive and characteristic for the different surfaces and our images are in good agreement with the earlier observations (Fig. 7). In general, we attempted to obtain images of both filopodial and lamellipodial growth cones on all surfaces, but we were unsuccessful in obtaining an image of a lamellipodial growth cone on laminin, owing to their relative rarity. On plain glass, small areas of dark to medium grey, representative of regions of relatively close contact are interspersed with white areas. Similarly, filopodia also have relatively extensive white areas, or are completely invisible, suggesting even larger separations from the substratum. Like Gundersen (1988), we observed that growth cones on laminin were generally quite light with extensive regions of white throughout the structure of growth cones. On polylysine, we observed, as did Letourneau (1979), a substantially darker image with large dark zones beneath the central region of the growth cone and dark filopodia. On L1, the IRM images of lamellipodial growth cones, by far the most frequent type on this surface, were particularly distinctive. We observed well-spread, medium to dark grey growth cones interspersed with punctate white regions beneath the broad lamellae. The distinctive appearance of the interference patterns characteristic of the various substrata argues that these substrata do indeed vary systematically as a result of the treatment or lack of it. A possible exception is the few filopodial growth cones

on L1, whose IRM images were not as distinctive, showing a combination of dark and bright regions similar to untreated glass. These filopodial growth cones may possibly have been attached to regions of the coverslip that did not adsorb L1, which is usually applied to surfaces pretreated with nitrocellulose or polylysine (Lemmon et al., 1992; Payne et al., 1992).

The neurons used for IRM imaging were grown on treated, glass cover slips, whereas the detachment force measurements reported above were taken from cells grown on treated plastic. To determine whether growth cones showed substantial differences in their adhesion to these two basic substrata, we compared the forces of detachment of growth cones grown on glass cover slips with those on similarly treated plastic culture dishes. As shown in Fig. 8, the forces of adhesion to treated-glass surfaces were entirely similar to those of treated-plastic surfaces for both filopodial and lamellipodial growth cones.

Discussion

The difficulties inherent in measuring cell adhesion and the limitations of various methods are reviewed by Curtis and Lackie (1991a). We used a micromanipulation method in which force exerted on the neurite shaft by a needle is used to dislodge the distal growth cone. An advantage of this method is that it should mimic what appears to be the principal physiological stress on growth cone adhesions of chick sensory neurons in culture: tension in the neurite shaft (Dennerll et al., 1989; Lamoureux et al., 1992) created by the pulling force of the growth cone (Lamoureux et al., 1989), or, in some circumstances, created by the retraction of the axon (Dennerll et al., 1989). A disadvantage of this method shared with most others is that it is difficult to discern the mechanism of dislodgement; for example, whether we are peeling or shearing the growth cone from the dish. Based on our observations at the moment of dislodgement and the fact that the needle is introduced from above the cell, we postulate that our measurements include a substantial contribution from peeling the growth cone. This would also be the case for dislodgement of growth cones by fluid flows (Hammarback and Letourneau, 1986; Gundersen, 1987; Lemmon et al., 1992) which we expect would combine peeling, shear, and lift. Peeling typically requires less force to separate two surfaces than shearing or direct lifting as dramatically seen in comparing the force required to separate adhesive tape from a surface by peeling, lifting, or sliding. For this reason, we believe our measurements may be conservative estimates of the forces required for dislodgement, and yet the measured forces for detachment are quite high compared to the forces normally borne by the growth cone, as we discuss below. Our evidence suggests that, in general, growth cones apparently detached cleanly at the membrane/substratum interface, also supporting a peeling mechanism. In most experiments, no phase-dense material was observed at the original site of the growth cone, and experiments in which neuronal membrane was labeled with a lipid-incorporating fluorescent dye indicated no membranous residue remaining at the original site of the growth cone following detachment (Fig. 1). Although a few micromanipulation experiments left an obvious "footprint" of cellular material at the original site
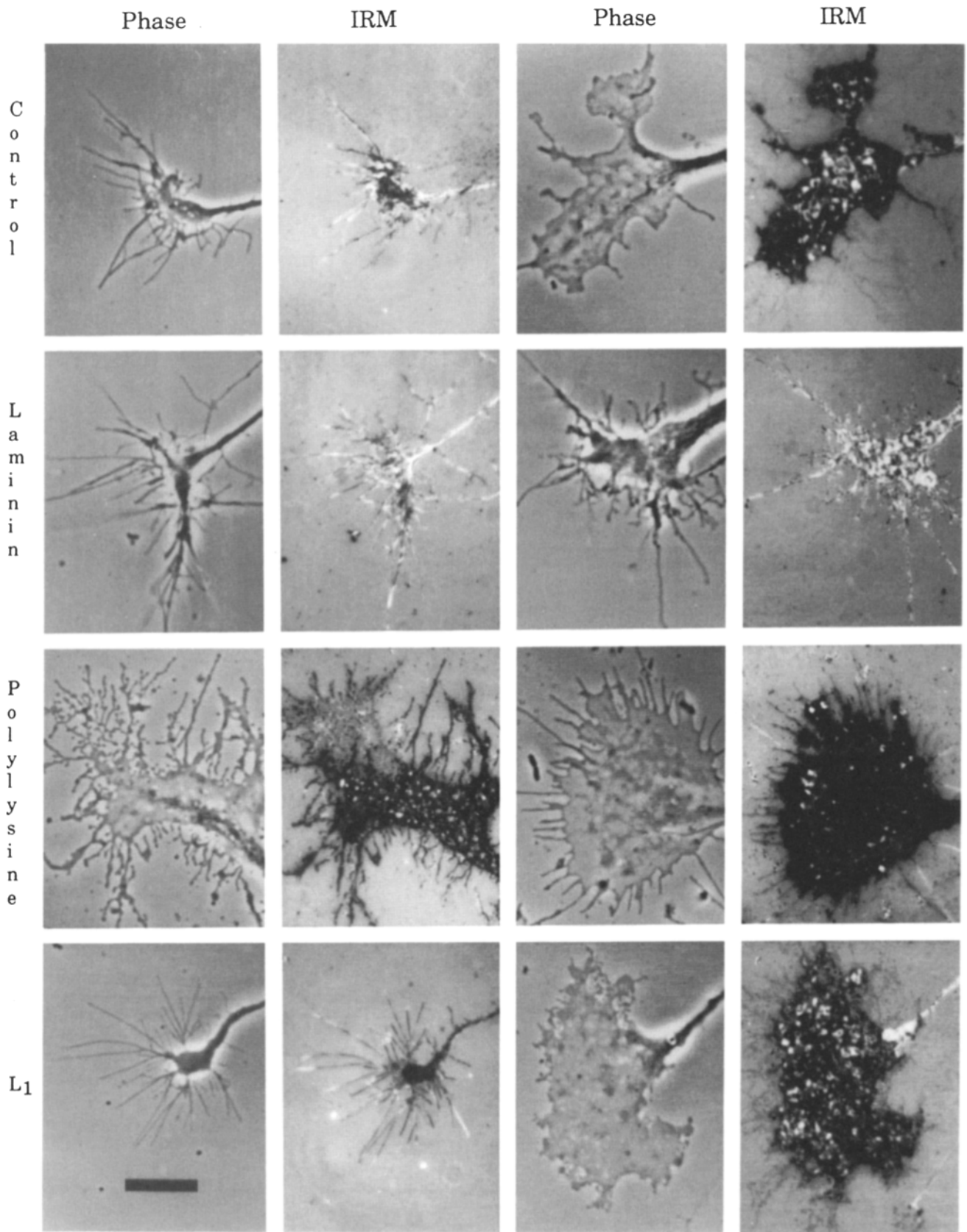
Figure 7. IRM of growth cones on various culture surfaces. Each row shows paired phase and IRM images of two growth cones on a particular growth surface, from top to bottom: plain plastic, laminin, polylysine, and L1. We attempted to provide examples of a filopodial growth cone, pairs at left; and lamellipodial growth cones, pairs at right. However, we were unable to find a lamellipodial growth cone on laminin during these experiments. Bar, 10 μm.
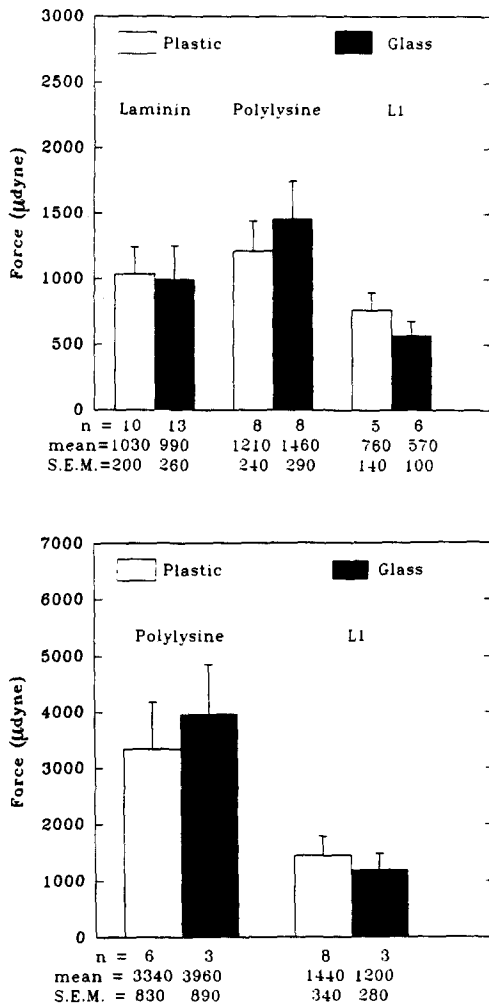
Figure 8. Comparison of mean forces required to dislodge growth cones cultured on glass and plastic substrata. (a) Mean forces required to dislodge filopodial growth cones on plastic (□) and glass (■) culture surfaces treated with laminin, polylysine or L1. For each condition, the number of growth cones detached, the mean and its standard error are given below each bar. (b) Mean forces required to detach lamellipodial growth cones on plastic or glass culture surfaces treated with polylysine or L1. Lamellipodial growth cones were not observed on laminin-treated glass and so no comparison was possible.

of a dislodged growth cone, we do not believe these reflect cellular disruption for several reasons. These detached growth cones continued to show apparently motile behavior, and could occasionally be observed to reattach at a new site. Our experience with micromanipulation is that cellular disruptions are accompanied by obviously pathological cellular responses, which were not observed during any growth cone detachment reported here. Further, the magnitude of force required for detachment of growth cones leaving a footprint did not differ systematically from the majority of cases in which the growth cone apparently came away cleanly. Still, measured detachment forces for those cells that putatively ruptured would again be conservative estimates of the actual force of adhesion of these cells, i.e., the cell membrane ruptured before the membrane–substratum adhesion.

The principal advantage of our method is that it provides an estimate of adhesion forces in absolute units, which, in turn, allows a comparison with the previous measurements of tensions associated with resting neurites (Zheng et al., 1991; Lamoureux et al., 1992) and with growth cone advance (Lamoureux et al., 1989, Heidemann et al., 1991) on the various substrata. Although there is substantial variation in these forces in this cell type, typical values for both these tension measurements are between 100–200 μdynes. This is between three and 15 times less than the forces we have measured for growth cone detachment. In no instance have we measured internally generated forces that are >1,000 μdynes, whereas mean detachment forces were larger than this value on all surfaces except L1. We postulate two possible mechanisms by which the large detachment forces may arise. One is that we are measuring the typical, physiological force of adhesion for the particular growth cone, which is unaffected by the experimental intervention of measurement. Alternatively, the growth cone may respond to our attempts to dislodge it by "digging in," i.e., the growth cone compensates for the intervention by increasing the force of adhesion. Curtis and Lackie (1991b) briefly mention the possibility that cells change their adhesion in response to measurement interventions, but, to our knowledge, experimental reports have tacitly assumed that this does not occur. Yet, we cannot confidently interpret our data to distinguish between the two different mechanisms. One might postulate, for example, that an adhesive compensation to experimental pulling would also involve a change of shape or area, and that an absence of these changes would reflect the absence of an adhesive compensation. However, the immediate response of growth cones to manipulation by the needle is highly variable, changing shape and area in some instances but not others.

Whether or not the growth cone responds to our intervention, our data suggest that growth cones rapidly and markedly alter their adhesion to the growth surface. Observations of growth cone advance and its relationship to elongation (Goldberg and Burmeister, 1986; Aletta and Greene, 1988) indicate that the central region of the growth cone "consolidates" into new neurite shaft, which on laminin and plain plastic is not adherent to the dish. Thus, assuming that our interventions measure typical forces of growth cone adhesion, growth cone advance is apparently accompanied by a change from very firm growth cone attachment in the central region to complete detachment from the surface in the short period of time (10–30 s) required for consolidation. Alternately, if growth cones increase adhesion in response to applied forces, this necessarily requires that growth cones can rapidly alter their adhesion.

We found few adhesive differences of growth cones among the different surface treatments. Only lamellipodial growth cones on L1 showed significant differences when compared with other surfaces and growth cone morphologies. Our results are consistent with reports in which the measurement included or focussed on detachment of cell bodies to the substratum, for example the reports of Hammarback and Letourneau (1986) and of Calof and Lander (1991), because we found that cell bodies adhere differentially to surfaces with different treatments. However, Gundersen (1987), using the same cell type used here, and Lemmon et al. (1992), using chick retinal cells, both found differences in growth cone

adhesion to different surfaces using jets of medium to dislodge the growth cones. The relative adhesions of growth cones in these studies were assessed by measuring the pressure required by a jet of constant duration to dislodge the growth cone (Gundersen, 1987) or by measuring the duration of a jet of constant pressure to cause dislodgement (Lemmon et al., 1992). Both approaches, however, contain a difficult assumption, namely that growth cones do not vary in their hydrodynamic drag. That is, the force exerted on the growth cone by a jet depends not only on the fluid flow, but also on the resistance to the flow provided by the growth cone, which varies very sensitively on its shape and size. For example, standard hydrodynamic equations predict that the force exerted by a given jet will scale with the cube of the height of the growth cone above the surface, and also linearly with the width of the growth cone facing the flow. Further, if the angle of contact between the growth cone and the surface was ramp-like for the flow, the jet would tend to push the growth cone down into the dish; whereas a "lip" formed by the growth cone at the edge of attachment would cause the jet to lift the growth cone, again affecting the apparent force of growth cone attachment. Therefore, differences in the size and shape of growth cones, which are reasonably characteristic for a particular surface, almost certainly contributed to previous results showing differences in forces of adhesion.

Our results argue against an attractive model for growth cone guidance based on postulating that the force of attachment to the substratum limits the growth cone's ability to exert tension for advance (Bray, 1982; Letourneau, 1983). Under these circumstances, growth cones would tend to follow adhesive gradients (Berlot and Goodman, 1984): growth cones preferentially steer onto more adhesive surfaces because growth cones or filopodia exerting more tension than the local adhesion limit pull free and are lost, leaving only those attached to more adhesive surfaces. We believe our findings are inconsistent with this force balance mechanism whether one assumes our data reflects static or compensatory forces of adhesion. That is, we find no evidence that substratum attachment limits growth cone tension as postulated by the model. Assuming that the large forces of detachment we measure are normal resting forces of adhesion, then these are clearly more than sufficient to allow the growth cone to exert additional forces. The force magnitudes exerted by growth cone advance or neurite retraction, as previously documented (Dennerll et al., 1989; Lamoureux et al., 1989), could not cause detachment by simple force imbalances. If our measurements reflect a compensatory mechanism of adhesion by growth cones, then this mechanism would presumably also function during the "tug of war" between regions of a growth cone. That is, additional tension exerted by one region of the growth cone would be met by an increase in the adhesion by those regions "feeling" the increased tension. Our data also suggest that well-described guidance preferences of cultured neurons cannot be explained by differential adhesion. For example, growth cones of cultured chick sensory neurons steer preferentially onto laminin-treated surfaces (Hammarback and Letourneau, 1986; Gundersen, 1987; Clark et al., 1993), yet we found nothing exceptional about adhesion to laminin. Our interpretation is in agreement with results of McKenna and Raper (1988), who showed that despite the preference of growth

cones for laminin treated surfaces, growth cones did not follow a gradient of laminin as would be expected if the steering were due to forces of adhesion per se.

Our data show that the force of adhesion scales with the area of the growth cone because all growth cones (except lamellipodial growth cones on L1), share a similar intensity (stress) of adhesion. Thus, lamellipodial growth cones with their relatively large area have larger forces of attachment than filopodial growth cones on all surfaces. Yet our data on the intensity of adhesion to differently treated surfaces also suggest that the form, particularly the area, of growth cones is not a simple result of differential adhesion. If area of contact reflected differences of adhesive intensity, growth cones of large area would be expected to have high stresses of adhesion. Yet both the force and the stress of adhesion of lamellipodial growth cones on L1 is significantly lower than on the other substrata, although these growth cones have a significantly greater area than on any of the other surfaces.

Previous micromanipulations to study the adhesion of a variety of cell types have noted the presence of firmly attached retraction fibers at the cell periphery following detachment of most of the cellular area (Harris, 1973 and older work cited therein). Similarly, we observed retraction fibers particularly at the tips of filopodia and the distal regions of lamellipodia. These retraction fibers remained attached to the substratum at very small regions at their distal ends after the remainder of the growth cone has been dislodged. These observations and our estimate of the relatively greater intensity of adhesion at these regions are consistent with recent reports suggesting a significant role of filopodial tips in growth cone advance and guidance. We previously remarked on the firm attachments at filopodial tips and their role in bearing the pulling forces generated by contractile filopodia during growth cone motility (Heidemann et al., 1990). O'Connor et al. (1990) have shown that the attachment of a single filopodial tip of a pioneer neuron to a guidepost cell mediates a dramatic steering event and reorientation of axonal elongation within grasshopper limbs. Smith (1994) found that initiation of neurites from cultured chick sympathetic neurons occurred only after filopodial tips contacted either the substrate or some three-dimensional object. In all these cases, it appeared that a single attachment point by a filopodium was sufficient to withstand the forces accompanying a subsequent "engorgement" of cytoplasm (Goldberg and Burmeister, 1986) to advance, reorient, or form a growth cone. Further investigation of the nature of the adhesive interaction at filopodial tips should be of considerable interest.

We imaged growth cones by interference reflection microscopy, which has been applied as an indirect and non-invasive measure of growth cone adhesion (Letourneau, 1979; Gundersen, 1987), in order to compare these results with our direct, mechanical adhesion measurements. At high illuminating apertures, interference reflection microscopy provides information about the closeness of contact of cells to the underlying substratum: with the intensity of interference (level of darkness) scaling with distance from the substratum (Izzard and Lochner, 1976; Gingell, 1981; Verschueren, 1985). IRM imaging has been most successful in contributing to our understanding of cell adhesion via focal contacts or focal adhesions. These are seen as characteristic, small blue-black streaks in IRM that identify regions of min-

imal cell-to-substrate distance (estimated to be 15 nm) that reflect firm cellular attachment between the cytoskeleton and underlying extracellular matrix (Verschueren, 1985 for review). More problematic has been "close contacts," broad regions beneath cells that appear various shades of iron grey by IRM and that are estimated to represent regions of membrane ∿30 nm from the surface. Close contacts are associated with rapidly moving cells (Kolega et al., 1982) and, based on our IRM observations and those of others, also with growth cones. Based in part on the reasonable, but unproven, assumption that closeness of contact correlates with strength of adhesion, close contacts have also been associated with areas of adhesion. However, we found essentially no correlation between the strength of adhesion and the observed area of close contact regions or the apparent closeness of contact as revealed by IRM. Although growth cones on laminin and polylysine show similar forces and stresses of adhesion measured by micromanipulation, IRM images of growth cones on polylysine show quite dark, rather extensive regions of contact while IRM images of growth cones on laminin indicate that much of the growth cone is relatively distant from the substratum. Similarly, the relatively dark and extensive regions of apparent contact revealed by IRM of lamellipodial growth cones on L1-treated surfaces fail to correlate with the significantly lower mechanical adhesion of these growth cones to this surface. Additionally, we were unable to observe regions of close contact between filopodial tips and the substrate by IRM, probably because of their small size. We can only speculate on possible reasons for the lack of correlation between IRM images and our mechanical measurements of adhesion. The assumption that closeness of contact correlates with adhesion may be fundamentally flawed. Alternatively, the growth cone may be a poor structure for IRM microscopy. Gundersen (1988) discusses some possibilities for a lack of correlation between an IRM image of the growth cone and the force of adhesion. Whatever the underlying cause for the lack of correlation between IRM images of the growth cone and mechanical measurements of adhesion, our data indicate that IRM of growth cones cannot be used as a reliable estimator of adhesion despite the distinctive interference patterns characteristic of the various substrata.

Although the growth cone must regulate its adhesion to achieve its motile and sensory functions, our data provide little support for the idea that differences in mechanical adhesion to various surfaces play a major role in this regulation. Our studies are limited to cultured neurons, which may or may not accurately reflect the situation in situ, but cultured neurons have played a major role in the current ideas about the role of adhesion in axonal development. In cultured neurons, at least, simple force balances postulated by differential adhesion mechanisms seem not to play a role in guidance preferences or growth cone morphologies. Nevertheless, our mechanical data focus attention on two aspects of growth cone adhesion that have received relatively little attention: the importance of growth cone detachment and of adhesions at the extreme leading edge of growth cones. To our knowledge, all studies of growth cone adhesion, including the present report, have highlighted the attachment aspect, but our findings of more-than-sufficient attachments of growth cones to all surfaces suggest that the detachment phase may be most significant. In the grasshopper limb, for example, some navigational "decisions" by pioneer growth cones are

achieved in large measure by the apparent detachment of growth cone regions that had been stably attached (O'Connor et al., 1990). More generally, while the attachment of cells to non-living substrata has received intense experimental scrutiny (e.g., Hynes, 1992), crawling motility requires coordinated detachments, phenomena that have not received much experimental attention. Measurements of growth cone adhesion, again including those reported here, have focused on the attachment of the broad region of the growth cone. Yet, as outlined earlier, there is reason to suspect that adhesions at the very tips of filopodia and lamellipodia may be more functionally significant than that of overall adhesion of the growth cone. We follow Harris (1973) in noting that micromanipulation studies of adhesion raise different questions and produce a rather different view of cell adhesion than that derived from other methods. Given that micromanipulation is a relatively direct means of investigating cell adhesion, we believe it will be important to reconcile these differences to achieve an accurate understanding of this important aspect of animal cell function.

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