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GROWTH CONE ADVANCE: AN IMMUNOCHEMICAL AND ULTRASTRUCTURAL STUDY OF ACTIN, MYOSIN, TROPOMYOSIN, AND FIBRONECTIN IN NEUROBLASTOMA CELLS

City University of New York PH.D. 1987

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GROWTH CONE ADVANCE: AN IMMUNOCHEMICAL AND ULTRASTRUCTURAL STUDY OF ACTIN, MYOSIN, TROPOMYOSIN, AND FIBRONECTIN IN NEUROBLASTOMA CELLS

by

DAVID MUIR

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

GROWTH CONE ADVANCE: AN IMMUNOCHEMICAL AND ULTRASTRUCTURAL STUDY OF ACTIN, MYOSIN, TROPOMYOSIN, AND FIBRONECTIN IN NEUROBLASTOMA CELLS

by

David Muir

Adviser: Professor Soll Berl

The constitutive neurite-producing human neuroblastoma (Nb) cell line SH-SY5Y was used as a model for developing autonomic neurons in vitro. Neuronal and non-neuronal characteristics of these cells were observed and aspects of cellular differentiation were examined in response to morphology-altering agents, particularly the tumor promoter 4 beta-phorbol 12 beta-myristate 13 alpha-acetate (PMA). SH-SY5Y Nb cells extended neurites with growing tips which had arrays of filament bundles that coursed into filopodial protrusions. These structures were examined in detail in growth cones prepared as whole mounts for light and transmission electron microscopy and immunolabeled using specific antibodies against actin, myosin, tropomyosin and fibronectin (FN). The potential role of these proteins in the process of filopodia-mediated growth cone advance was elucidated by the localization of actin and myosin to filopodia and filament bundles associated with sites of substratum attachment containing FN.

SH-SY5Y Nb cells secreted FN into defined medium and formed

substratum-attachment sites that contained FN. Cells immunolabeled for FN with colloidal gold probes demonstrated densely labeled substratum-attachment plaques on filopodia (contact pads) and on the underside of distal growth cones (focal plaques) that appeared in close association with microfilament bundles coursing into filopodia. Immunolabeling live cells with a pulse of anti-FN antibody provided evidence that filopodial contact pads may be the precursor form of focal attachment plaques later to be found under the body of the advancing growth cone. Both sites were more abundant on the distal tips of cells grown in defined medium supplemented with serum FN and correlated with neurite production.

In contrast, neurite sprouting in serum-free medium was substantially retarded in the presence of PMA; the alteration in neurite elongation was partially antagonized by the addition of FN to the medium or substratum. PMA-treated SH-SY5Y cells produced neurites with growth cones lacking filopodia and microfilament bundles. Actin filaments were rearranged into a dense meshwork and the association with myosin and tropomyosin was diffuse. In addition, FN-containing sites were scant and greatly diminished in FN-immunolabel. Substratum-attachment plaques were not evident on PMA-induced growth cones nor were arrays of cytoskeletal filament bundles.

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ABBREVIATIONS

CNBR -cyanogen bromide

EDTA -ethylenediamine tetraacetate

EGTA -ethylene glycol bis(amino-ethyl ether) tetraacetate

ELISA -enzyme-linked immunosorbant assay

EM -electron microscope, microscpoy

FBS -fetal bovine serum

HEPES -hydroxyethylpiperazine ethanesulfoic acid

IgG -immunoglobulins

kD -kilodaltons

Mr -molecular weight (relative)

Nb -neuroblastoma cell line

NGF -nerve growth factor

OD -optical density

PBS -phosphate buffered saline

PHEM - buffer containing PIPES, HEPES, EGTA, $MgCl_2$

PIPES -piperazine bis(ethane sulfonic acid)

PMA -4 beta-phorbol 12 beta-myristate 13 alpha-acetate

PPi -pyrophosphate (inorganic)

RA -retinoic acid

SDS-PAGE-sodium dodecylic sulfate-polyacrylamide gel electrophoresis

WMTEM -whole mount transmission electron microscopy

INTRODUCTION

Neuroblastoma Cells

Establishment of continuous cell lines of Nb has made it possible to initiate studies on induced differentiation in a well-defined and well-controlled environment. Peripheral Nb is an embryonic tumor derived from neural crest cells which can be compared to its normal counterpart, developing autonomic neurons. Nb has one one of the highest rates of spontaneous regression amoung human tumors (Everson, 1964), yet at specific stages can be maintained and propagated in culture while retaining the capacity for morphologic maturation (Goldstein et al., 1964). Many continuous Nb lines express neuronal features under standard culture conditions. The near-diploid karyotype of many human Nb lines makes them a useful model for studying the control of differentiation. Certain lines have extrachromosomal features which have been a focus for exploring the molecular biology of cellular differentiation; particularly, a long, nonbanded homogeneously staining region and small, paired chromatin bodies known as double-minutes are noteworthy features of many Nb lines (Spengler and Biedler, 1979).

The adrenergic, human Nb line SH-SY5Y is a clone of SK-N-SH established from an aspiration biopsy of a bone marrow metastasis eight months after the excision of a large thoracic primary found in a 4-year-old female with continuing elevated levels of urinary catecholamines and vanillyl-mandelic acid (Biedler et al., 1973). Barnes and coworkers (1981) have found that, for the most part, in culture these cells do not display the cytoplasmic fine structure characteristic of differentiated neurons. Considered together with other features, such as neurotransmitter synthesizing enzyme expression, these cells are comparable to normal autonomic neurons at early stages of their differentiation. In addition, neuronal characteristics are expressed to varying

degrees depending on culture conditions. Nb cells have been induced to differentiate, but there have been conflicting results regarding drug-induced terminal differentiation in culture (Littauer, 1980).

Nb lines, subclones and variants have been established with various neurite-producing potential. SH-SY5Y cells constitutively produce neurites in the presence of serum, and more so in serum-free medium. In addition, SH-SY5Y and other human Nb lines demonstrate altered morphology, neurite outgrowth and neurochemical expression in response to a variety of agents including nerve growth factor (NGF), retinoic acid (RA) and tumor-promoting phorbol esters such as 4 beta-phorbol 12 beta-myristate 13 alpha-acetate (PMA).

Culture Treatments

1. The Tumor Promoter PMA

Because of the substantial overlap in the activities of tumor promoters and oncogenic viruses, it is hoped that elucidation of the mechanism of action of tumor promoters may also provide insight into the process of malignant transformation. The pleiotropic effects of phorbol ester tumor promoters on cultured mammalian cells have been extensively reported (See Vandenbark and Niedel, 1984). One of the main features of phorbol esters is their ability to change the differentiation stage of various cell types, and many phenotypic alterations are reminiscent of neoplastic transformation (Blumberg et al., 1976; Kellie et al., 1985; Vandenbark and Niedel, 1984). This includes both dedifferentiation of normal cells (Feuerstein et al., 1984; Ishii et al., 1978; Rovera et al., 1977) and differentiation or maturation of cultured tumor cells (Feuerstein et al., 1984; Ishii, 1978; Lotem and Sach, 1979; Rovera et al., 1979).

In medium supplemented with serum, the adrenergic SH-SY5Y line responded to the phorbol ester PMA by formation of cell processes and a

decrease in the proliferation rate. Concomitantly there was a 30-fold increase in the catecholamine content and the number of neurosecretory granules seen by electron microscopy was increased (Pahlman et al., 1981). Furthermore, PMA-treated Nb cells had a higher proportion of neuron specific enolase, an induction known to occur in maturing neurons (Schemchel et al., 1980). Taken together, the phenotypic alterations induced by PMA indicate that SH-SY5Y cells develop towards more mature or differentiated cells.

The presence of phorbol ester receptors on mammalian cells has been established (Shoyab et al., 1979) and more specifically a PMA receptor was reported (Driedger and Blumberg, 1979). A specific phorbol ester aporeceptor was found in mouse brain which copurifies with protein kinase C, the Ca^{++}-activated, phospholipid-dependent protein kinase (Lehto and Virtanen, 1983). In accordance with this observation, changes in the pattern of protein phosphorylations after treatment with PMA have been reported (Kikkawa et al., 1983; Nishizuka, 1984; Werth and Pastan, 1984). For example, smooth muscle myosin is phosphorylated by protein kinase C, which results in changes in myosin conformation (Umekawa et al., 1985). Myosin light-chain phosphorylation was suggested to serve as a signal which might be preferentially involved in the PMA effect in the acceleration of growth of non-transformed cells and growth inhibition of certain neoplastic cells (Feuerstein et al., 1984). Morphologic and ultrastructural studies have indicated that PMA alters cell shape by inducing a redistribution of actin microfilaments (Kellie et al., 1985; Schliwa et al., 1984) Although PMA and protein kinase C have not been shown to interact directly with actin, the ultrastructural changes induced by PMA may be mediated by a phosphorylated product which regulates actin function and location. In this context it is of interest that unphosphorylated gelsolin (a protein which gels F-actin) has been detected in areas of ruffling membranes of transformed

fibroblasts (Wang et al., 1984). In addition, the adhesion plaque protein vinculin is a phosphoprotein and has been shown in vitro to be a substrate for protein kinase C during PMA activation (Kikkawa et al., 1983). Accordingly, the level of phosphorylation is a possible regulatory event; an idea strengthened by the observation that in the transformed cells, vinculin is "over phosphorylated" (Werth and Pastan, 1984). This biochemical change is accompanied by loss of adhesion plaques and a redistribution of the cytoskeleton in growing cell margins (David-Pfeuty and Singer, 1980). Furthermore, it has been reported that changes in vinculin plaques might preceed changes in the organization of actin-containing filament bundles following PMA treatment (Kellie et al., 1985; Schliwa et al., 1984). It is interesting that these changes may be related to similar alterations seen after viral transformation. In this regard, PMA-induced alterations may affect the expression of mRNAs for actin and vimentin in a leukemic line (Siebert and Fukuda, 1985).

2. Nerve Growth Factor

Nerve growth factor (NGF) is a chemically characterized protein that has been explored as a paradigm for tropic action in the nervous system. NGF is required for the survival and development of sympathetic neurons and acts to promote neurite outgrowth, both in vivo and in vitro (Greene and Shooter, 1980; Varon and Bunge, 1978). Studies of the NGF response in clonal lines have indicated that NGF-promoted neurite outgrowth involves separable pathways, one which is transcription dependent and another which is transcription independent (Burnstein and Greene, 1978). Modulation of neurite elongation by NGF has been shown to result from local actions on the neurite and its growth cone which alter regional shape and motility within minutes (Seeley and Greene, 1983). Receptors for NGF are present and active in the distal neurite and

growing tip (Johnson et al., 1978) and NGF is locally required for the maintenance and extension of rat sympathetic nerve endings (Camponet, 1979). In addition, substratum adsorbed NGF can support neurite formation and elongation as well as guide the direction of neurite elongation (Gundersen, 1985).

The capacity of NGF to enhance survival in embryonic neurons (Mobley et al., 1977) and in fetal adrenal chromaffin cells (Aloe and Levi-Montalcini, 1979) and both to enhance survival and arrest growth in the closely related rat pheochromocytoma cells (Greene, 1978) is apparently lost in human Nb cells (Sonnenfeld and Ishii, 1982). Thus, human Nb shares with other cell types the loss of response to normal effectors that follows malignant transformation. However, an NGF receptor subtype is present in many Nb lines, including SH-SY5Y, in which NGF-treatment increases neurite production (Sonnenfeld and Ishii, 1982). Apparently, the effect of NGF on growth and neurite outgrowth is largely uncoupled in SH-SY5Y cells. Consequently, the response to NGF treatment in the form of enhanced neurite outgrowth, haptotaxis and chemotaxis can be examined under conditions that might threaten the survival of NGF-dependent primary cultured neurons.

3. Retinoic Acid

Vitamin A and its metabolite, retinoic acid (RA), have attracted attention as antipromoter agents in experimental carcinogenesis. In culture, retinoic acid can induce morphologic differentiation in certain neoplastic cell types including human melanoma cells (Meyskins and Fuller, 1980). Like melanoma, Nb is a neural crest derivative; this similarity prompted the studies of Sidell who reported that RA can inhibit mitosis and promote morphologic differentiation of the malignant phenotype of the Nb cell line LA-N-1 (Sidell, 1982). Also, a detailed examination of RA induced neurite outgrowth has documented the

increased appearance of growth cones, outgrowth and biochemical differentiation in Nb (Robson and Sidell, 1985). These effects are accompanied by a reduction in DNA synthesis and a decrease in the ability of these cells to grow in soft agar cultures, and it has been suggested that RA reduces the tumorigenic potential of Nb cells (Sidell et al., 1983). Recently, it has been found that Nb cells contain specific intracellular binding proteins (Haussler et al., 1983) which are implicated in the functioning of RA in other cell types (Lotan, 1980).

4. Fibronectin

The developmentally regulated synthesis of the extracellular matrix protein fibronectin (FN) during embryogenesis and its affinity for both cells and components of the extracellular matrix suggest a role of FN in morphogenetic events such as cell migration and differentiation. Accordingly, FN has been shown to promote adrenergic differentiation of neural crest cells in vitro (Sieber-Blum et al., 1981). The appearance of FN in the nervous system and the expression of the FN-receptor by neurons has received recent attention (Boucaut et at., 1984; Duband and Thiery, 1982). In the developing embryo, cell interactions with extracellular matrix are important in regulating cell behavior (Hynes, 1981) and the presence of the extracellular protein FN has been correlated with the migration of neural crest cells (Duband et al., 1986). The interaction of FN with the cell surface is a requirement for cell movement, since blocking the FN-binding site inhibits cellular migration (Boucaut et al., 1984). Neuronal cells are dependent on adhesive contacts with their surrounding surface in order to form neurites (Letourneau, 1975). In vitro, FN promotes cell adhesion, spreading and formation of specialized adhesion sites (Duband and Thiery, 1982). Sympathetic ganglia attach to and extend neurites on FN-coated

surfaces (Greensberg et al., 1981; Rogers et al., 1983). Peripheral Nb cells show enhanced sprouting on FN-coated surfaces and have been used as a convenient model system to study adhesion and neurite outgrowth (Bottenstein and Sato, 1980; Rauvala, 1984). They produce cellular attachment sites and remnant substratum-attached material which contain FN (Culp et al., 1980). The composition of this material may vary according to culture conditions and the neurite-producing potential of certain Nb variants (Chernoff et al., 1983; Domen and Culp, 1981).

Bottenstein and Sato (1979) demonstrated that a rat Nb line was able to proliferate in the absence of serum in a hormone supplemented medium provided the cells were preincubated in serum prior to plating. Subsequently, they documented that the addition of plasma FN to serum-free medium and precoating the tissue culture dish with polylysine at each subculture permitted cell division to occur without serum preincubation (Bottenstein and Sato, 1980). It is interesting that the effect of FN on the proliferation and morphology of Nb cells might be related to findings that FN partially restores normal morphology to transformed fibroblasts (Ali et al., 1977; Hynes, 1981; Mautner and Hynes, 1977). Alteration of the cellular response to FN may be an important aspect of the malignant transformation associated with a pleiotropic change in cellular characteristics including, the loss of surface FN (Chen et al., 1984; Mautner and Hynes, 1977; Vaheri and Ruoslahti, 1975), reduced adhesion (Ali et al., 1977; Chen et al., 1984), rounded morphology (Ali et al., 1977; Hynes, 1981; Mautner and Hynes, 1977), loss of specific cytoskeletal organization (Ali et al., 1977; Boucaut et al., 1984; Mautner and Hynes, 1977), and an increased production of proteases that degrade FN at cell-substratum contact sites that were correlated with phenotypic changes (Chen et al., 1984). Because all of these changes can be reverted to some extent by the addition of FN, loss of FN may be important for

producing the transformed phenotype and be associated with detachment and subsequent invasion by malignant cells in vivo. Possible explanations for the loss of FN in transformed cells include a) reduced synthesis, b) reduced binding, or c) increased rate of degradation.

Neuronal Motility

Virtually all eukaryotic cells have the ability to maintain a specific shape and to change shape and form. Cells also have contractile properties providing for motility and the transport of materials and organelles throughout their cytoplasm. Neurons are motile and locomotory when first seen at the ventricular zone, where they translocate the nucleus and, following cell division, begin to migrate into outer layers of the developing embryo. Once the neuron is properly located and activated it sends out processes, while the cell body remains essentially stationary.

As a requirement for neuronal development and differentiation, the establishment of an axon can be described as a sequence involving neurite sprouting and elongation, and growth cone motility and target seeking. Neurons have a highly anisotropic morphology and the growing axon is a specialization which meets a greatly exaggerated structural demand. The major ultrastructural feature of the axon is its cytoskeleton, which can be viewed as a labile assemblage of filamentous proteins continually moving toward its distal tip, the growth cone. The axon has a cytoskeleton core of neurofilaments and microtubules oriented longitudinally, which extend to but do not invade the growing tip (Lasek and Hoffman, 1976). Neurofilaments and microtubules only splay-out into the broaden growth cone, whereas the axonal cortex is filled with an actin filament network, without any conspicuous orientation, which contacts the neurolemma and penetrates inward among the thicker cytoskeletal

filaments. Interestingly, the advance of the growing tip of the axon approximates the rate of the slow axonal transport component containing actin (Lasek, 1981). The rates of axonal transport for actin and myosin are considerably greater than that of the microtubule and neurofilament proteins. Consequently the growing tip can easily accumulate contractile proteins which may provide for motility requirements for sustained development and survival of the axon as it elongates through the extracellular microenvironment.

Evidence supports the idea that the growing tip is readily responsive to the milieu within its reach. The growth cone is guided by tactic responses which may involve extracellular gradients; these include a chemotaxic mechanism (Letourneau, 1978) utilizing gradients of suspended or soluble factors and haptotaxic guidance by the extracellular matrix and by substrate-bound molecules involving a gradient of process adhesions (Gundersen, 1985; Letourneau, 1975). Nerve growth factor has been the paradigm for both initiation and guidance of neurite outgrowth (Greene and Shooter, 1980). Evidence suggests that many of the NGF-induced alterations of growth cone form are the result of transcription-independent processes (Burnstein and Greene, 1978). Growth cone morphology changes within seconds upon the addition of NGF to the neuronal microenvironment (Gundersen and Barrett, 1980). It follows that the growth cone must be capable of the independent management of its cytoskeletal constituents; this capacity suggests the cytoskeleton assemblies required for motility are contained and reorganized within the distal neurite and growing tip. Interestingly, when the distal neurite is severed from the axon proper, the growing tip retains (for a period) its ability to migrate by tactic guidance (Seeley and Greene, 1983). One possible explanation for the local regulation of growth cone motility is the intracellular transduction of signals by factors which bind to specific receptors on the cell

surface. Occupied receptors form membrane-bound complexes which result in the alignment of microfilament bundles (Albertini and Herman, 1984). The elongation of a neuronal process involves actin containing microfilaments associated with the plasmalemma. A concerted conversion of the cellular subplasmalemmal actin meshwork into polar microfilaments and bundles may initiate process extension and provide for the directionality in continued elongation (Condeelis, 1981). It seems likely that the sensory-effector system that directs neurite outgrowth involves actin rearrangement into tension-generating arrays (Bray, 1982).

Transition from a smooth surface to a protrusion is, in a sense, a maximizing of filament-membrane contacts, at the expense of internal-filament interactions. These transitions may require alterations in the interactions between actin and myosin-like proteins (Pollard, 1976) or alterations in the gel-sol condition of the cytoplasm (Condeelis and Taylor, 1977). Another model suggests that the material and the force required for protrusion are provided by the polymerization of new elements. Observations in support of this hypothesis include studies on the polarity of actin at the leading edge of growing cells (Small, et. al., 1978) and reports of amorphous or finely granular material, which may represent polymerizing filaments, within newly spread veils (lamellipodia) in neurons (Wessels and Nuttall, 1978).

Transmembrane induction of microfilament binding to the plasmalemma appears to be a common way of transducing signals into cells. Two apparently interrelated systems are involved: the transmembrane interaction of ligand-clustered surface receptors with actin; and the formation of a family of cell contacts known as adherens junctions. Surveys of a large variety of biological systems suggested that the induction of surface specialization due to interaction between cytoskeleton and the inner surfaces of the plasmalemma can play

multiple roles in processes such as cell motility, attachment, division, and recognition (Geiger, 1985). Adherens junctions and transmembrane linkage to actin filaments may be of two major classes: contacts with non-cellular surfaces such as basal lamina or tissue culture substrates and direct intercellular attachments between neighboring cells. The distinctive property of all adherens junctions is their association with the microfilament system through a cytoplasmic, membrane-bound plaque that contains vinculin; often FN and alpha-actinin are enriched in its vicinity (Geiger, 1985). Experimental manipulation of adherens junctions suggested that besides adhesive extracellular surface molecules (e.g. FN) they contain three major molecular domains: 1) integral 'contact receptors' in the membrane, (e.g. the FN receptor complex); 2) a peripheral, membrane-bound plaque at the endofacial surfaces of the contact area; and 3) a bundle of actin-containing microfilaments (Singer, 1982).

Many cells have specialized microprojections on their surface which are extended and sometimes make contact and attach to surrounding surfaces (Trelstad et al., 1967). In fibroblasts, bundles of microfilaments are not found in suspended cells and the process of attachment leads to formation of microspikes and then the appearance of stress bundles (Bragina et al., 1976). Intermittent cell contacts with the substrate often appear electron dense and are termed plaques or attachement sites, and are closely associated with microfilament fibers that appear to emanate from these specializations (Byers et al., 1984). Stereo high voltage electron microscopy confirms that the ends of microfilament bundles are coincident with linear focal contacts with the substrate. The pattern of linear contacts within living cells corresponds closely with alpha-actinin distribution and are subjacent to the actin staining stress fibers (Wehland et al., 1979). Recent studies have investigated the relationship of actomyosin (Letourneau, 1981; Pollard, 1981), alpha-actinin (Geiger et al., 1981; Lazarides

and Burridge, 1975), tropomyosin (Geiger et al., 1981; Lazarides, 1976, Lazarides, 1976), FN (Hynes and Destree, 1978; Wylie et al., 1979), and vinculin (David-Pfeuty and Singer, 1980; Geiger et al., 1981; Singer, 1982) to adhesion sites and stress fibers in nonmuscle cells using immunocytochemical microscopy.

Two kinds of anchorage structures probably involved in the attachment of cells to their substratum are focal contacts and close contacts. Focal contacts represent the closest substratum approach (10-15 nm) and have always been found at the distal ends of microfilament bundles or stress fibers (Heath and Dunn, 1978). Focal contacts are stationary during locomotion and become most numerous in completely spread cells. Motile cells tend not to form focal contact sites. The bulk of the evidence indicates that focal contacts are mainly responsible for process adhesion. FN is a constituent of focal contacts (Culp and Bruniel, 1976; Culp et al., 1980; Hynes, 1981) and is coincident with actin microfilament bundles on the ventral cell surface (Hynes and Destree, 1978). FN is strongly implicated in processes mediating the adhesion of cells to substrates. In addition, a very close transmembrane relationship between FN and actin microfilaments termed the fibronexus was demonstrated in fibroblasts by electron microscopic examination (Singer, 1979). The fibronexus is often associated with dense submembranous plaques, which correspond to focal contacts (Singer, 1982).

In neurons, the activities of protrusion and retraction are spatially restricted to the growth cone. The soma and sides of the neurite have a latent capacity to produce protrusions, but unlike the temporarily inactive sides of moving fibroblasts, they normally remain quiescent (Bray et al., 1978; Wessells and Nuttall, 1978). The neuronal growth cone margin and its surface protrusions has been described as an actin network anchored to a membrane lanced by filamentous arrays extending into filopodia and parts of lamellipodia (Tosney and

Wessells, 1983). Neurons do not have stress fibers as seen in fibroblasts, but all neuronal microspikes (filopodia) contain aligned arrangements of microfilaments, similar to but smaller than stress fibers (Kuczmarsky and Rosenbaum, 1979). Microfilament bundles, in neurons have been located in the basal cortex in regions of cell-substrate contacts in vitro (Goldman et al., 1981). Unattached filopodial extensions and lamellipodial excrescences have an open, criss-cross array of filaments, whereas substratum-attached filopodia contain more aligned filaments which extend as a small bundle into the growth cone. Compact bundles of filaments are probably generated only in association with firm adhesion of a filopodium to the substratum (Tosney and Wessells, 1983). Immunocytochemical staining has confirmed that actin and myosin are concentrated in these arrays (Letourneau, 1978). An added significance of this kind of network with actomyosin filamentous arrays is that they are seen at the sites where cells have made linear adhesions to the substrate (Hynes and Destree, 1978). Thus, the motile margin of a growth cone resembles the locomotory portions of other cells because it contains actin and myosin complexes anchored within the cell to sites of cell-substrate adhesion.

In the newly forming axon, the coupling between growth cone locomotion and axon elongation might simply be mechanical tension. By pulling on the axon, the growth cone may activate stretch-sensitive mechanisms that, in turn, stimulate growth (Bray, 1984). Accordingly, a mechanism by which a neuron responds to tension applied along the longitudinal axis of its axon by making more axon: that is, by increasing the length and number of microtubules and neurofilaments by assembling more components. In the initial formation of an axon, tension would be produced by the growth cone as a result of locomotory (contractile) activity. In the short term, tension-induced neurite growth probably involves the assembly of neurite components from a pool of

precursors. In the longer term, the loss of this material would be balanced by synthesis, and the limiting rate of axonal transport (Johnston and Wessells, 1980).

Examinations of Growth Cone Ultrastructure

Recent advances in preserving and resolving cytoplasmic ultrastructure have provided the means to examine cellular substructure at a molecular level. Particularly the filamentous proteins which compose the cytoskeleton have received considerable attention. Cytoplasmic filaments can appear in characteristic arrays, which, when studied by EM can readily be identified by estimating filament diameter. This characterization by apparent diameter extends to a molecular level in that many filamentous structures are formed by the polymerization of single (or singular sets of) protein. For example, 5-7 nm diameter microfilaments coursing in bundles to protrusions in the cell surface have been characterized as actin polymers. In addition, microtubules and 10 nm intermediate filaments assemble into filaments by polymerization. The intermediate filaments is a class of heterogenous proteins and a variety of cell types can be characterized by the type of intermediate filament protein they contain (Lazarides, 1980). The neurofilaments are a unique case in that they are composed of three subunit proteins (the neurofilament triplet), the relative amounts of which can change during development, creating heteropolymers with variable composition (Willard, 1983). Ultrastructural studies that attempt to detail cytoskeleton composition by ascribing to assumptions of molecular form must often attempt to confirm their premises by analogy from correlated studies. This approach has become increasingly problematic as the levels of resolving ultrastructure have improved. The extensive network of proteinaceous material which closely associates with the cytoskeleton presents such problems. To this network has been ascribed such names as microtrabecula (Ellisman and

Porter, 1980) and cytomusculature (Goldman et al., 1981). Due to the rigorous fixation necessary for preservation of specimens prepared for electron microscopy, which fuses the cytosolic and filamentous phases of the cytoplasm, a heterogeneous composition may be imposed. Identification of the proteins composing this ultrastructural network is necessary for conceptualizing its molecular nature and potential functions.

Immunochemical analysis of proteins has been a great advancement in the attempts to correlate cell biochemistry with intracellular ultrastructure. An antibody probe can be used for both quantitative and qualitative biochemistry, and as well for localizing intracellular antigens and identifying the composition of ultrastructural features. Major difficulties in the immunocytochemical demonstration of intracellular proteins arise when attempting to obtain good morphologic preservation while retaining antigenicity and allowing adequate penetration of immunochemical reagents. Non-ionic detergents are often used to permeabilize fixed cell membranes improving antibody penetration to intracellular antigen-containing sites. Additional emphasis on accessibility is required when immunolabeling is performed for the electron microscope which necessitates the use of bulky electronopaque probes. In this regard, methods which extract the non-ionic detergent soluble cellular material have been used prior to or during fixation. Polyacrylamide gel electrophoresis of such extracted cells demonstrates the retention of large amounts of actin, together with many of the known microfilament associated proteins (Osborn and Weber, 1977; Schliwa and van Blerkom, 1981) including myosin (Zigmond et al., 1979), tropomyosin (Lazarides, 1976), and the intermediate filaments (Henderson and Weber, 1980). In preparing neurons for high resolution immunocytochemistry, much of the cytoplasm can be retained in a three-dimensional organization which in many ways approaches that of the intact cell (Kuczmarsky and Rosenbaum,

1979). Whole mount preparations of intact growth cones can be refined for maximal resolution of the filamentous structures by extracting the non-ionic detergent soluble cytosol. When Triton-extraction and aldehyde fixation are performed simultaneously in the presence of buffer which enhances preservation of the cytoskeleton (Letoureau, 1983; Schliwa and van Blerkom, 1981), a specimen remains which is highly permeant, retains three-dimensionality and allows clear resolution of cytoskeletal elements by standard transmission electron microscopy. The growth cone and distal neurite in vitro provide an advantageous feature in that the cytoskeleton is splayed-out providing a high contrast specimen when stained and viewed by standard transmission EM. Retention of the integrity of whole mount specimens is of paramount importance for reliable interpretation of the complex network within the growth cone. Thorough fixation with glutaraldehyde and tannic acid enhances the preservation of fine microfilament arrays, while limited concentrations of osmium tetroxide may fortify these arrays against the electron beam while incurring minimal fixation artefact (Small and Langanger, 1981). Dehydrating the specimen by critical point drying minimalizes disruption by the transitional stresses created during dehydration, thus protecting its inherent dimensionality (Buckley and Porter, 1975). The whole mount growth cone is a well-preserved specimen that can be observed intact and unsectioned, and provides the additional relief from embedding artefact and loss of contrast due to interference by epoxy resins.

When maximal resolution of ultrastructural elements is a requirement, additional difficulties arise where there is a great importance in the exact localization of the antibody probe. Sensitive labeling methods such as the immunoperoxidase stain often rely on the formation of a precipitate which tends to disperse from the site of antibody binding. To overcome this problem in antigen localization for electron microscopy, antibodies conjugated with

complexes such as ferritin or imposil are useful but hampered by limited size and contrast. Recently, the use of antibody probes adsorbed to colloidal gold particles has overcome these problems when immunolabeling permeabilized or partially extracted specimens. Examination of Triton-extracted, aldehyde-fixed cytoskeletal whole mounts of neurons growing directly on coated EM grids provides an excellent opportunity for reliable localization of contractile and structural proteins by immunogold probes, while adequately preserving specific cellular morphology. Although intact, unextracted cell preparations do demonstrate greater retention of cytoskeletal proteins (especially monomers) and of cytoskeleton-associated proteins, well immunolabeled whole mounts of extracted specimens offer substantial insights which, in combination with intact cell whole mounts and embedded, sectioned material, constitutes an advanced approach to the examination of the roles and interactions of the suspected contractile assemblies and ultrastructural features which provide for growth cone motility and neuronal development.

MATERIALS AND METHODS

Antigen Purifications

The production of monospecific antibodies against proteins of the neuronal cytoskeleton necessitates purification of each antigen for innoculating rabbits, affinity purification of antisera, and performance of the appropriate characterizations and control experiments.

1. Brain Actin

The procedures used for isolating brain actin were described by Maekawa and coworkers (1984). Briefly, bovine brain was cleaned, stripped of menninges and homogenized. Following centrifugation (5000g, 10 min.), the supernatant was made 1 mM in each of $MgCl_2$ and $CaCl_2$, then mixed with DNase 1 -Affigel (Pharmacia) for affinity chromatography. The column was washed and actin-binding proteins were eluted by the addition of 0.6 M NaCl. Actin itself was eluted by a solution containing 2mM EGTA and 6M Urea. Further purification was achieved by Sephadex G-100 column chromatography. The actin content of eluate fractions was assayed by the inhibition of DNase I activity and by SDS-PAGE using smooth muscle actin as a standard.

2. Brain Myosin

The procedures used for isolating brain myosin were described by Pollard and coworkers (1974) and Kuczmarsky and Rosenbaum (1979). Briefly, bovine brain was cleaned, stripped of menninges and homogenized in an extraction buffer containing KCl (0.9 M), PPi, $MgCl_2$ and beta-mercaptoethanol. Following centrifugation (5000g, 10 min.) the supernatant was diluted by dialysis to produce a precipitate which was solubilized in buffer containing KI (0.6 mM), ATP, $MgCl_2$

and beta-mercaptoethanol. This solution was precipitated by ammonium sulfate and the 25-55% saturation precipitate was collected and resuspended in KI buffer. The soluble material was applied to a Biogel A-15M, 200-400 mesh column. Myosin was collected in the void volume, as detected by K^+-ATPase activity. Solubilization, precipitation, and column chromatography were repeated. The purity of the myosin containing fractions was determined by SDS-PAGE using smooth muscle myosin as a standard.

3. Brain Tropomyosin

The method of Fine and coworkers (1973) was applied to the bovine brain crude regulatory protein prep as described by Mahendran and Berl (1977) for the isolation of brain tropomyosin. The preparation of crude regulatory proteins was solubilized in the presence of 1M KCl. The solution was boiled and clarified. The supernatant was brought to pH 4.1 then centrifuged. The precipitated pellet was suspended in 0.2 M KCl buffer. Ammoniun sulfate precipitation was carried out and the 40-55% saturation precipitate was collected, then solubilized, dialyzed, and lyophilized. Purification was qualified by SDS-PAGE.

4. Neurofilament Triplet Protein, 68 kD

The procedures used for the isolation of the neurofilament triplet proteins were described by Schlaepfer (1977). Briefly rat sciatic nerves were cleaned, desheathed and minced in hypotonic Tris buffer, then homogenized. Following centrifugation the supernatant was collected and made 0.1 M in NaCl. The precipitated material was collected by ultracentrifugation and suspended in H_2O. This solution was dialyzed against 8M urea. The soluble material was further reduced and denatured in Laemmli's sample buffer (Laemmli, 1970) and electrophoresed on 7.5% acrylamide preparative gels. Bands were visualized in a

test strip of gel by soaking it in cold 2 M KCl, which served as a guide for cutting out the 68 kD band which was a major band in this preparation. The gel pieces were minced and the protein electroeluted. Following extensive dialysis, the eluted protein solution was electrodialyzed in the presence of beta-mercaptoethanol and urea to quantitatively remove bound SDS. The resulting solution was concentrated, and purity was determined by SDS-PAGE.

5. Vimentin

Vimentin intermediate filaments were collected from cultured fibroblasts according to procedures established by Franke and coworkers (1979). Human fibroblast cultures were established from biopsy tissues in 150 cm^2 harvest flasks. Confluent cultures were washed with PBS and extracted with a sequence of buffered, high (1.5 M) then low (0.05 M) salt solutions containing Triton (0.5%). The residual Triton-insoluble cellular material attached to the culture flask was scaped off and collected by centrifugation. The pellet was washed and resuspended in buffer containing 8 M urea and 10 mM beta-mercaptoethanol. The solution was applied to 10% acrylamide gels in Laemmli's buffer and electrophoresed. The major band at approximately 57 kD was excised after being visualized by soaking gels in cold 2 M KCl. The excised gel pieces from many runs were pooled, minced and electroeluted, electrodialyzed, then analyzed by gel electrophoresis as described above.

The purification of fibroblast vimentin was performed under the auspices of Dr. J. Madri at the Yale University Medical School. Polyclonal antibody production and tests to determine its specificity were documented by J. Madri. Anti-vimentin was received as a gift for the purposes of this study.

6. Fibronectin

Purified FN was isolated from serum by affinity chromatography using the methods described by Engvall and Rouslahti (1977). A 15 ml column (1 cm i.d.) was packed with 4 gm of CNBr-activated Sepharose 4B (Pharmacia) coupled with gelatin (40 mg). The column was equilibrated with PBS then 40 ml of FBS was applied (0.5 ml/min). Following extensive washing with PBS, elution was performed with 8 M urea in 50 mM Tris-HCl, pH 7.5. The eluate was precipitated by making the solution 75% in ethanol and stirring overnight at 4°C. The precipitate was collected by centrifugation, washed, resuspended and dialyzed in PBS. In addition, by repeated application to the gelatin-affinity column, FBS was stripped of FN for use in culture (see below). In this procedure only the peak fractions of the void volume (not diluted by PBS equilibration or washing) were collected and pooled. Purified FN and stripped FBS were assayed by ELISA and immunoblotting (see below) using anti-FN antibody (Dako-Accurate) and purified bovine serum FN (Sigma) as a standard.

Production of Monospecific Antibodies

Polyclonal antiserum was produced against each antigen in New Zealand white rabbits by the following method. Antigen (0.1-0.5 mg) was suspended in complete Freund's adjuvant and injected at multiple subdermal and intramuscular sites. After two weeks, the antigen was similarly injected suspended in incomplete Freund's adjuvant. Two weeks following the second innoculation, antigen was suspended in PBS and injected intravenously. Five days later animals were bled from the ear and tested for specific antibodies by ELISA (Voller et al., 1979 and see below). Blood was collected from responsive animals, which were finally exsanguinated by cardiac puncture. The pooled blood was clotted and serum was collected following centrifugation. The serum was cut by

33% ammonium sulfate and the precipitate was pelleted by centrifugation, then resuspended in PBS.

Affinity purification of each antiserum was performed essentially as described by Warr (1982). Antigen (5 mg) was bound to CNBr-Sepharose (1 g) and packed in a 4 ml column. Following clarification, the serum-IgG preparation was applied until the column was saturated. The specificly bound antibody was eluted by 0.1 M glycine-HCl, pH 2.5 and OD_{280nm}-absorbance peak fractions were analyzed by ELISA in microtiter plates coated with antigen. The specific antibody fractions were pooled, dialyzed against PBS, NaN_3 and then stored at -70°C. Determination of protein for antigens and antibodies was preformed by the Lowry method (1951).

Qualification of Monospecific Antibodies

1. ELISA

Each antiserum was characterized by an enzyme-linked immunosorbant assay (ELISA) (Voller et al., 1979) using the purified antigens detailed above. First, 96-well microtiter plates were coated with 500 ng/well of antigen in 0.1 ml bicarbonate buffer (pH 9.6). Antibody titer was determined by serial dilutions in PBS containing 0.06% Triton X100. Antibody bound to the solid-phase was detected by peroxidase-conjugated anti-rabbit IgG (1:400, Dako-Accurate) or by biotinylated anti-rabbit IgG and the ABC reagent coupled to peroxidase (Vectastain). The soluble colorimetric reagent O-phenylene diamine / H_2O_2 (0.05% / 0.02%) dissolved in buffer made with dibasic sodium phosphate and citric acid (pH 5.0) was used and the immunoperoxidase reaction rate (delta OD_{492nm}) of each well was read by a multiscan spectrophotometer. Specific antibody activity was determined by an inhibition ELISA by the methods of Madri and Barwick (1983) using purified antigen to block specific binding to an antigen-

coated microtiter plate. An empirically determined antibody concentration was incubated with serially diluted amounts of antigen. Following centrifugation, unabsorbed antibody which bound to the antigen-coated microtiter plate was assayed by an immunoperoxidase method described above. An inhibition curve and the point of maximal inhibition were measured. Inhibition assays were also performed to detect any cross-reactivity with non-specific cytoskeletal proteins.

2. Immunoblotting

Antibody specificity was also determined by immunochemistry performed on SDS-PAGE profiles of brain ether powder or neuronal culture extracts which had been transferred onto nitrocellulose paper. First, samples of neural tissues were chromatographed by SDS-PAGE. Next, the unfixed protein profiles were electroblotted onto nitrocellulose paper (Towbin et al., 1979). Following transfer, the proteins were fixed to nitrocellulose paper then immunostained (see below). The polypeptides recognized by the primary antibody was stained by peroxidase-bound anti-rabbit IgG using diaminobenzidine-$(HCl)_4$ / H_2O_2 (0.05% / 0.02%) as chromogen. Control experiments included the use of pre-immune sera and absorption of specific antibody activity with purified antigen. In parallel with immunoblots: a) gels were fixed with 10% trichloroacetic acid, stained with 0.25% commassie blue dissolved in 8% acetic acid / 5% methanol, then destained in the same solvent; b) electroblots on nitrocellulose sheets were fixed with 25% isopropanol / 10% acetic acid, stained with 0.1% amido black dissoved in 45% methanol / 10% acetic acid, then destained with 90% methanol / 2% acetic acid.

Cell Culture

The human neuroblastoma cell line SH-SY5Y (Biedler et al., 1973) was maintained in Roswell Park Memorial Institute 1640 medium (RPMI) containing 15% fetal bovine serum, 50 U/ml penicillin, and 25 µg/ml streptomycin. Cells were dissociated by brief exposure to 10 ug/ml trypsin and 0.1 mM EDTA in Hank's balanced salt solution. After the addition of 20 µg/ml soybean trypsin inhibitor the cell suspension was collected by centrifugation and resuspended in modified serum-free supplemented N2 medium (Bottenstein and Sato, 1979) using RPMI as a base medium. N2 medium contains a supplement consisting of 5 µg/ml insulin, 5 µg/ml transferrin, 100 uM putresciné, 20 nM progesterone and 45 nM selenate. For some experiments SH-SY5Y cultures were treated with beta-NGF (200 ng/ml) prepared from mouse saliva by the methods of Burton, Wilson and Shooter (1978). 4 beta-phorbol 12 beta-myristate 13 alpha-acetate (PMA) (Sigma) and retinoic acid (RA) (Sigma) were first dissolved in ethanol then used in medium at concentrations of 17 nM and 1 µM, respectively. All culture treatments and incubations were performed at 37°C in 5% CO_2 / humidified air.

Protein Profiles of Treated Nb Cells

Cell cultures were established and treated with NGF, RA or PMA as described above. Cultures were washed, detached and dissociated with PBS containing 1 mM EDTA. Total cell protein was determined for each condition by the Lowry method and cell count was estimated with a hemocytometer. SDS-PAGE profiles of each treatment were obtained by loading samples with equal amounts of total cell protein. Secondarily, Triton-insoluble cytoskeletal proteins were analyzed by gel electrophoresis as described by Lehto and Virtanen (1983), with modifications. Briefly, cells were extracted with 0.5% Triton in PHEM buffer containing the protease inhibitor phenylmethyl sulfonyl fluoride.

Cytoskeletons were collected and solubilized in urea prior to SDS-PAGE. Following chromatography, protein profiles were stained by commassie blue or transfered to nitrocellulose paper and immunostained with antibodies directed against cytoskeleton antigens. For immunoblotting with antibodies raised against brain actin, myosin, tropomyosin and the intermediate filaments, immunoperoxidase staining was performed using modifications of the methods described by Goldstein and coworkers (1983). Fixed immunoblots were treated to suppress non-specific antibody binding with a diluent for antibodies consisting of 1.5% NaCl and 0.1% Triton X100 in 0.05 M Tris-HCl (pH 7.6) containing 2% BSA and 1% normal serum. Affinity purified primary antibodies (1 µg/ml) were applied for 2 hrs with shaking at 20°C. Bound antibody was detected by peroxidase-conjugated secondaries applied for 1 hr. Immunoreactive bands were visualized with diaminobenzidine-$(HCl)_4$ / H_2O_2 in Tris-HCl buffer containing 1.5% NaCl (without Triton).

Immunocytochemical Studies of the Cytoskeleton

1. ELISA with Nb cells as a Solid-Phase: Retention of Antigens and Antigenicity

Cytoskeleton proteins of SH-SY5Y cells grown in 96-well microtiter dishes established in serum-free medium were measured by an ELISA using a fixed cell layer as a solid-phase for immunoassay. Measurements using anti-actin, anti-myosin, anti-tropomyosin, anti-vimentin, and anti-68 kD were made by the immunoperoxidase ELISA described above, with the inclusion of steps to quench unreacted aldehydes, block non-specific binding and suppress low-affinity IgG activity as described for other immunocytochemical procedures applied to cell cultures. This method also provided a measure of the antigenicity of specimens which were variably fixed and Triton-extracted as specimens for immunocytochemical microscopy. The effects of fixation on antigenicity and

the relative extraction of cytoskeletal proteins during extractions were measured and expressed as the relative immunoperoxidase activity normalized to total protein. Cell protein was assayed by the Lowry method on 8 random wells from each 96-well plate and all conditions compared were assayed on the same dish, with separate dishes for the assay of each antigen.

2. Immunoperoxidase and Immunofluorescent Cytochemistry

Cell cultures were established on glass coverslips (pretreated with FBS, overnight) and treated with NGF, RA, and PMA as described above. Cytoskeletal proteins were labeled with antibody probes and staining performed by an indirect method and by the peroxidase and fluorescence methods described by Sternberger (1979) with several modifications. The cultures were fixed with paraformaldehyde (4%) followed by Triton-permeabilization (0.1%) in PBS. Specimens were treated with diluent supplemented with: 0.1 M glycine, to quench unreactive aldehyde groups; 1% BSA to reduce background staining and; 2% normal serum to suppress low affinity IgG activity. Optimal dilution testing was routinely carried-out which varied somewhat with fixation and extraction parameters. Primary antibody was applied in diluent for 2 hours with gentle shaking. For immunoenzyme staining bound primary antibody was detected by peroxidase-conjugated anti-rabbit IgG (Dako-Accurate) (1:50) and a precipitant chromogenic reaction with diaminobenzidine-$(HCl)_4$ / H_2O_2 (0.05% / 0.02%) dissolved in PBS. Coverslips were dehydrated through a graded series of ethanol and cleared with xylenes then mounted on slides with Permount. Micrographs were taken on a Zeiss Universal Microscope using Kodak Technical pan 2415 and HC-110 developer. Immunofluorescent staining was performed as described above except that bound primary antibody was detected by rhodamine-conjugated anti-rabbit IgG (Boerhinger-Mannheim) (1:50). After thorough washing

with PBS, coverslips were mounted on glass slides with glycerol / PBS and examined and photographed on a Nikon Diaphot inverted microscope equipped for epifluorescent illumunation. Micrographs of rhodamine fluorescence were taken with equal exposure times using Kodak Tri-X film.

Ultrastructural Examinations

1. Whole Mount Transmission Electron Microscopy (WMTEM)

For WMTEM, cells were grown directly on 75-mesh gold grids (Pelco) coated with formvar strengthened by sublimed carbon. Following UV-sterilization, the grids were soaked in N2 medium containing 15% FBS. Cultures were grown for 1-6 days in N2 medium or N2 containing either NGF (200 ng/ml), RA (1 μM), or PMA (17 nM). Morphologic examination of growth cones was performed on cultures fixed with 2% glutaraldehyde in buffer containing 60 mM PIPES, 25 mM HEPES, 1 mM EGTA and 2 mM $MgCl_2$ at pH 6.9 (PHEM buffer) (Schliwa and Nakamura, 1984) for 15 min at 37°C, followed by 0.4% aqueous tannic acid (Letoureau, 1983) for 15 min. Otherwise, for resolution of cytoskeletal features, specimens were simultaneously fixed and extracted with PHEM buffer containing 2% glutaraldehyde and 0.1% Triton X100 (proportions were varied), followed by tannic acid mordanting. The specimens were made electron-dense by staining with 2% aqueous uranyl acetate for 30 min at 4°C. Dehydration through a graded series of ethanol preceded critical point drying with CO_2 (see Hayat, 1979).

2. WMTEM and Immunogold Labeling of Cytoskeletal Features

Immunogold labeling (see Horrisberger and von Lanthen, 1978) with antibodies against actin, myosin, tropomyosin, or the intermediate filaments was performed on cultures grown directly on gold grids then simultaneously fixed and

Triton-extracted in PHEM buffer and on specimens that were first fixed then Triton-permeabilized. Grids were pre-incubated in PBS containing 0.1 M glycine and PBS diluent with 1% BSA and 2% normal goat serum. Primary antibody (5 µg/ml) was added to the diluent for 60 min and bound antibody was labeled by goat anti-rabbit IgG adsorbed to colloidal gold particles (5 or 20 nm) (1:50) (Boerhinger-Mannheim). After thorough rinsing with PBS, the specimens were post-fixed with 1% glutaraldehyde in PBS then 0.4% aqueous tannic acid, for 15 min each. Once equilibrated with H_2O, counterstaining with aqueous 2% uranyl acetate was performed at $4^{o}C$ for 10-30 min. The grids were dehydrated through a graded series of ethanol then critical point dried. The specimens were examined by standard transmission electron microscopy on an Hitachi HU12A at accelerating voltages between 75-125 kV and photographed on Kodak EM film 4489 developed for varied contrast with dilutions of D-19 developer.

FN STUDIES

Metabolic Labeling of FN in SH-SY5Y-Conditoned Medium

Trypsin dissociated SH-SY5Y cells were plated and incubated in RPMI / 15% FBS or medium containing NGF (200 ng/ml), RA (1 μM) or PMA (17 nM). After 4 days the media were replaced, with fresh medium containing 5 μCi / ml (^{14}C)amino acid mixture (20 Ci/mM, New England Nuclear). Conditoned media samples were removed from each culture after 4 days of incubation and centrifuged (1000g, 10 min) to remove cellular debris. Phenylmethyl sulfonyl fluoride (1 mM) was added to the samples to inhibit protease activity. To assure that FN was precipitated, gelatin (5 μg/ml) was added to the samples and proteins were concentrated by ammonium sulfate (281 mg/ml) precipitation. The precipitates were collected, resuspended and dialyzed against PBS. The samples were electrophoresed on 5% polyacrylamide gels under reducing conditions. The SDS-PAGE profiles were electroblotted onto nitrocellulose sheets and immunostained with anti-FN antibody (see below). The nitrocellulose strips were cut into pieces, dissolved in acetone and counted by liquid scintillation spectrometry. In addition, a portion of each of the media concentrates was used for immunoprecipitation with anti-FN antibody. Control immunoprecipitate sample were prepared using rabbit IgG. The precipitates were counted by liquid scintillation and electrophoresed to determine purity.

Conditioned Media Assay for FN

SH-SY5Y cultures were seeded in 75 cm^2 flasks in N2 medium and treated with either NGF, RA, or PMA as described above. Cultures were treated for either 0, 2, or 4 days before media was collected for assay. After each period of treatment, the treatment media were replaced and left to be conditioned by the pretreated cells for the next 2 days. The conditioned media samples from each

treatment and interval were collected and made 1 mM with phenylmethyl sulfonyl fluoride and concentrated in dialysis tubing with Aquacide (Calbiochem). In parallel, the cell layers were harvested for cell count and protein assay. Both media and culture (cell) protein were determined by the Lowry method. Conditioned media samples were quantitatively assayed for de novo FN by an inhibition ELISA using the methods described by Madri and Barwick (1983). Gelatin-affinity purified bovine serum FN prepared by the method of Engvall and Rouslahti (1977) and commercially acquired human plasma FN (Sigma) were used as inhibition standards and as the solid-phase in the ELISA. The titer of anti-FN antiserum was inhibited with soluble antigen in the media samples and standard solutions. Unadsorbed anti-FN which bound to FN-coated microtiter plates (Linbro, Flow Laboratories) was detected by biotinylated goat anti-rabbit IgG and the ABC reagent (Vectastain). O-phenylene diamine HCl / H_2O_2 was used as the soluble colorimetric reagent; the reaction was monitored on a microtiter plate spectrophotometer at 492 nm. FN quantitation was expressed as a fraction of the total cell protein in the culture from which the conditioned media sample was rendered.

For immunoblotting, media concentrates were normalized to total protein and then applied in Laemmli's buffer to 5-15% polyacrylamide gradient gels and electrophoresed. The SDS-PAGE profiles were electroblotted onto nitrocellulose sheets (Towbin et al., 1979) then fixed with 25% isopropanol / 10% acetic acid for 30 min. The blots were immunostained following a 2-hour incubation in diluent consisting of 50 mM Tris-HCl (pH 7.6) containing 1.5% NaCl, 0.1% Triton X100, and 2% BSA. Rabbit anti-human plasma FN antibody (1:4000) (Dako-Accurate) was applied to the nitrocellulose strips for 2 hours with shaking. Specifically bound anti-FN was detected by goat anti-rabbit IgG conjugated to peroxidase (1:400) (Dako-Accurate). Diaminobenedine-$(HCl)_4$ / H_2O_2 was used

as the chromagen to visualized the immunoreactive bands.

Immunoassay of Cell Surface FN Binding

Cultures of SH-SY5Y cells maintained in serum-free medium for 2 days were detached by treatment with 10 μg/ml trypsin and 0.1 mM EDTA in Hank's for 5 min. Soybean trypsin inhibitor (20 μg/ml) was added to the cell suspension which was then centrifuged (600g, 5 min). The cell pellet was washed and resuspended to $2X10^5$ cells/ml in N2 medium or N2 containing 17 nM PMA. After pretreatment for 2 hours at 37°C, assays were performed on 0.5 ml samples of the cell suspensions immediately, and 2 then 4 hours later. Purified serum FN (50 μg/ml) was added to each sample and binding was carried out while shaking at 37°C for 20 min. The samples were collected by centrifugation and rinsed several times to remove unbound FN. Controls were incubated in the media without FN. The samples were briefly fixed with 4% paraformaldehyde then thoroughly washed in PBS. Prior to immunoassay, unreacted aldehydes were quenched with 0.1 M glycine and non-specific antibody binding was suppressed by 2% BSA in PBS. Anti-FN antibody was added in saturating dilution to the suspended cell samples, which were then incubated for 30 min while shaking. Bound antibody was detected by goat anti-rabbit IgG conjugated to peroxidase. A colorimetric reaction was carried out by suspending the immunoperoxidase labeled cells in O-phenylene diamine HCl / H_2O_2. The cells were pelleted and the supernatant reaction product was measured spectrophotometrically. The immunoperoxidase activity (delta OD_{492nm}) was expressed per sample, which were equal in cell count (and also had equivalent total cell protein).

Substrata Attachment Assay

Substratum pretreatment was accomplished by adding 20 μg/ml FN to 24-

well tissue culture dishes (Linbro) in bicarbonate buffer (pH 9.6), 0.1 ml/cm^2 for 2 hours at 37^{o}C. Control dishes were pretreated with bicarbonate buffer only. SH-SY5Y cultures were grown for 20 hours in the presence of 0.5 uCi/ml (^{3}H)thymidine (20 uCi/umol; New England Nuclear). Cells were detached with 10 ug/ml trypsin and triturated. Soybean trypsin inhibitor (20 µg/ml) was added to the suspension which was then centrifuged. The cell pellet was washed and resuspended in N2 media or N2 containing 17 nM PMA. After incubating while shaking for 2 hours at 37^{o}C, 10^5 cells were seeded per well. Four hours following seeding, effective attachment was determined by suspending poorly attached cells with orbital shaking (100 rpm) for 10 min. The suspensions were drawn and pooled with an additional rinse then solubilized by 1% SDS in 0.1 M NaOH. Substrata-attached cells were solubilized by the addition of SDS / NaOH. Radioactivity was counted on a liquid scintillation counter.

Cell Sprouting Determination

Cultures were detached and established in 24-well plates as described above. After 4, 8, and 24 hours of incubation, the cultures were rinsed and the outgrowth assay was stopped by the addition of 4% paraformaldehyde in PBS. The percentage of cells sprouting neurites was determined with phase-microscopy and photomicroscopy by scoring 100 cells from randomly choosen areas in each well. Cells sprouting neurites were defined as those bearing processes which were longer than one diameter of a rounded cell body. Substratum pretreatments were performed as stated above.

Immunofluorescence Microscopy

Immunofluorescence studies were carried out with cells grown on coverslips which had been coated with 20 µg/ml FN in bicarbonate buffer (pH

9.6), 0.1 ml/cm^2; this produced a fine homogeneous carpet of substrata-bound FN which was clearly detected by immunofluorescent staining. Cultures were grown for 4-6 days in N2 media with or without PMA and washed twice with PBS. Cultures were fixed for 20 min with 4% paraformaldehyde in PBS, followed by washes containing 0.1 M glycine in PBS. Triton-permeabilization was routinely used prior to antibody incubations except when otherwise indicated for exclusive extracellular FN staining. Non-specific staining was suppressed by incubating the specimens in a diluent consisting of PBS and 2% BSA. Coverslips were incubated with monospecific anti-FN antibody (1:200) for 2 hours. Bound antibody was detected by rhodamine conjugated goat anti-rabbit IgG (Boehringer-Mannheim) diluted 1:50 in the same diluent. Specimens were mounted in glycerin / PBS then examined on a Nikon Diaphot inverted microscope equipped for epifluorescent illumination. Micrographs of rhodamine fluorescence were taken with equal exposure times using Kodak Tri-X film.

Whole Mount Preparation and Immunogold Labeling for FN

Immunogold staining was performed on fixed specimens, with or without Triton-permeabilization, and on specimens simultaneously glutaraldehyde fixed and Triton-extracted in PHEM buffer. The grids were washed several times with PBS then with PBS containing 0.1 M glycine as described above. For FN labeling specimens were incubated in diluent (2% BSA / PBS) for 30 min, anti-FN antibody (1:500) was applied for 2 hours. Bound antibody was localized by goat anti-rabbit IgG adsorbed to 20-nm colloidal gold particles (Boehringer-Mannheim). The results reported concerning adhesion site immunoaccessibility by 5-nm and 20-nm probes involved the consecutive application of the two secondary antibody probes, with the 20-nm probe applied first. Following extensive washing with PBS, immunogold WMTEM specimens were post-fixed

with 1% glutaraldehyde in PBS for 30 min, followed by tannic acid and counterstained by uranyl acetate as described above. All WMTEM specimens were dehydrated by a graded series of ethanol then critical point dried in CO_2 and examined on a Hitachi Hu12A transmission electron microscope at accelerating voltages between 75 and 125 kV.

In order to immunolabel the FN accumulated by filopodial contact pads of living cells, cultures were established on coated grids as described above. After 24 hours in defined medium, purified FN (20 µg/ml) was added for 60 min. The cultures were then washed several times with medium. The living cells were immunolabeled by adding anti-FN antibody to N2 medium containing 1% BSA for 30 min at 37°C. After thorough washing with medium, the cultures were incubated in N2 medium for continued survival up to 2 hours before being fixed. Next, the specimens were simultaneously fixed and extracted and prepared for immunogold labeling as above, but omitting the (re)application of anti-FN antibody. Secondary antibody adsorbed to gold particles was applied to the fixed and extracted specimens and the immunogold labeled cultures were then processed for WMTEM.

RESULTS

Antigens and Antibodies

1. Brain Actin

Two dimensional gel electrophoresis has discriminated three main forms of actin; alpha, beta and gamma actins exist having separable isoelectric points (Kuczmarsky and Rosenbaum, 1979). Whereas the alpha form are found only in muscle, the other two forms were present in non-muscle including neural cells. The character of brain actin may differ from that isolated from other sources. For this reason, brain actin was purified for the production of antisera in this study of neuronal ultrastructure. Actin was isolated from homogenates of bovine brain, having an apparent subunit M_r = 43 kD by SDS-PAGE (Fig. 1). Polyclonal antisera produced against bovine brain actin were affinity purified using highly purified actin bound to Sepharose. The affinity-purified brain actin antibody was shown by immunoblotting techniques to specifically immunolabel a polypeptide with apparent M_r = 43 kD found in a brain ether powder SDS-PAGE profile (Fig. 1). The titer and inhibition of antibody activity by purified brain actin were determined for the eluted antibody fractions by ELISAs (Fig. 2 and 3). Whereas antibody binding to actin was inhibited by prior actin absorption, other cytoskeletal proteins did not effect antibody activity. These results indicate that this antibody probe against bovine brain actin is monospecific to actin.

2. Brain Myosin

SDS-PAGE of purified brain myosin revealed a heavy chain dimer (M_r = 210 - 200 kD) and a light chain (M_r = 23 kD) when stained with commassie blue (Fig. 4). Brain myosin has been reported to have three light chains (17, 20, 23 kD) (Kuczmarsky and Rosenbaum, 1979). With successive precipitations during

Figure 1. SDS-PAGE and actin immunoblots. All samples were electrophoresed on 5-20% polyacrylamide gels under reducing conditions. (Lane 1) Purified bovine brain actin (2 μg) and (Lane 3) bovine brain ether powder (75 μg) profiles stained with commassie blue. (Lane 2) A cocktail of purified brain actin, myosin and tropomyosin (2 μg each) and (Lane 4) brain powder (75 μg) were electroblotted onto nitrocellulose paper and immunoperoxidase stained using anti-actin antibody.

Figure 2. ELISA titration curve for monospecific brain actin antibody. Anti-actin antibody was diluted 1/100 and serial dilutions were made to 1/51,000 in 96-well microtiter plates coated with purified brain actin (500 ng/well). Bound antibody was detected with anti-rabbit IgG conjugated to peroxidase and immunoperoxidase activity was measured using O-phenylene diamine / H_2O_2. The colorimetric reaction product was measured spectrophotometrically (delta OD_{492nm}).

Figure 3. ELISA inhibition plot using brain actin to inhibit the binding activity of anti-brain actin antibody. Actin antibody was used at 1/1000 and inhibitions were carried-out with concentrations of actin ranging from 1 μg/well serially diluted to 2 ng/well.

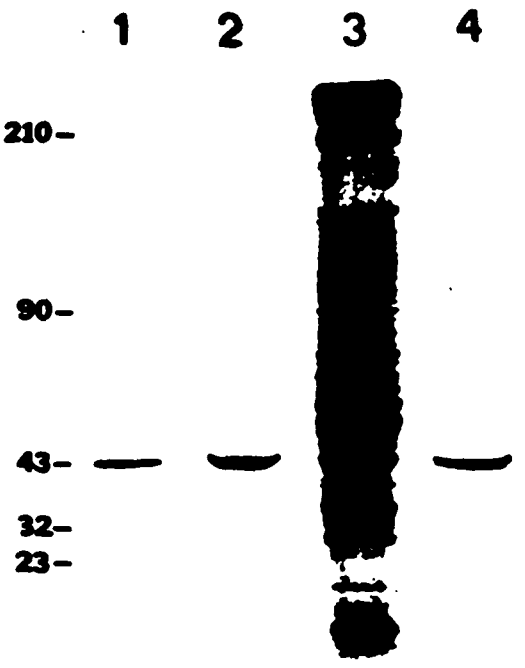

Figure 1.

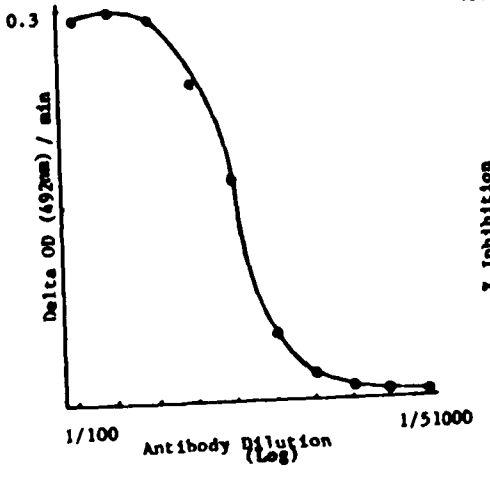

Figure 2.

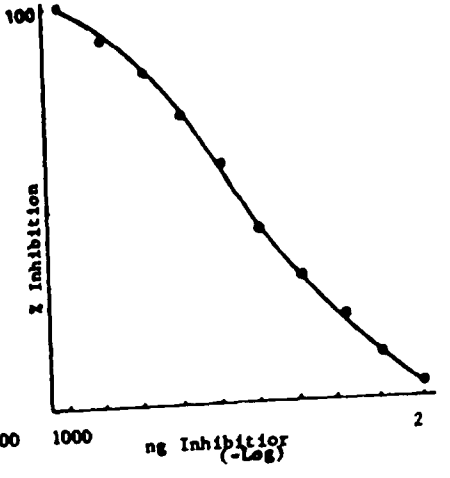

Figure 3.

purification procedures, the 17 and 20 kD light chains were gradually diminished. Another feature of myosin purification was the gradual degradation of the heavy chain profile into a pair of bands (210-200 kD), along with the loss of the light chains. Specific K^+-ATPase activity also diminished. Using this highly enriched brain myosin as antigen, polyclonal antisera were produced and purified. On western blots of the SDS-PAGE profile of brain ether powder, immunostaining with anti-myosin antibody demonstrated a single immunoreactive polypeptide corresponding to the myosin heavy chain with a degradation tail (Fig. 4). Immunoblots of the purified myosin preparation showed the antibody recognized the heavy chain band-pair and the 23 kD light chain. No cross-reactivity with actin or tropomyosin was shown by immunoblotting. The titer and inhibition characteristics of this antibody are shown in figures 5 and 6. Antibody binding was inhibited by myosin absorption as indicated by immunoassay.

3. Brain Tropomyosin

Purified brain tropomyosin, when resolved by SDS-PAGE, showed virtual purity appearing as a band-pair with apparent M_r = 32 kD (Fig. 7). Antiserum raised against brain tropomyosin was characterized by an ELISA (Fig. 8). Although its titer was relatively low this antiserum showed virtually linear inhibition by brain tropomyosin (Fig. 9). Immunoblotting brain ether powder revealed that this antiserum labeled a single 32 kD band most heavily, but a 64 kD band was also detected, perhaps representing the associated band-pair (dimer) proteins. This antibody did not cross-react with actin, myosin or other cytoskeleton preparations.

Figure 4. SDS-PAGE and myosin immunoblots. All samples were electrophoresed on 5-20% polyacrylamide gels under reducing conditions. (Lane 1) Purified bovine brain myosin (2 µg) and (Lane 3) bovine brain ether powder (75 µg) profiles stained with commassie blue. (Lane 2) A cocktail of purified brain actin, myosin and tropomyosin (2 µg each) and (Lane 4) brain powder (75 µg) were electroblotted onto nitrocellulose paper and immunoperoxidase stained using anti-myosin antibody.

Figure 5. ELISA titration curve for monospecific brain myosin antibody. Anti-myosin antibody was diluted 1/100 and serial dilutions were made to 1/51,000 in 96-well microtiter plates coated with purified brain myosin (500 ng/well). Bound antibody was detected with anti-rabbit IgG conjugated to peroxidase and immunoperoxidase activity was measured using O-phenylene diamine / H_2O_2. The colorimetric reaction product was measured spectrophotometrically (delta OD_{492nm}).

Figure 6. ELISA inhibition plot using brain myosin to inhibit the binding activity of anti-brain myosin antibody. Myosin antibody was used at 1/1000 and inhibitions were carried-out with concentrations of myosin ranging from 2 µg/well serially diluted to 4 ng/well.

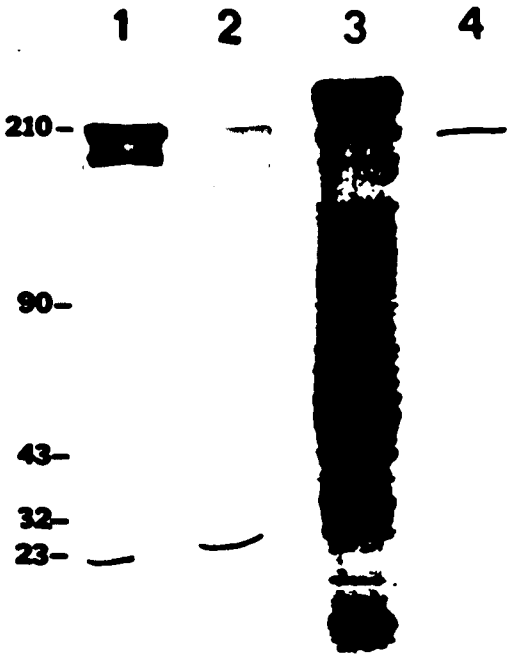

Figure 4.

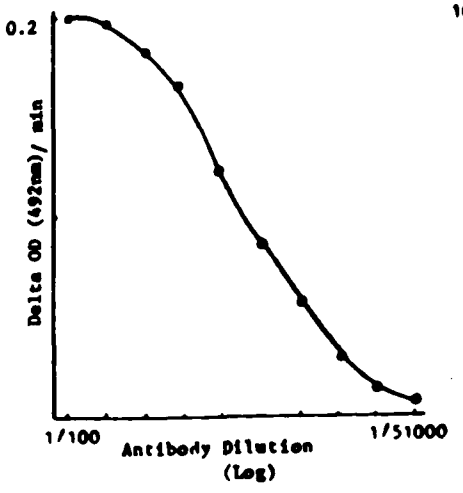

Figure 5.

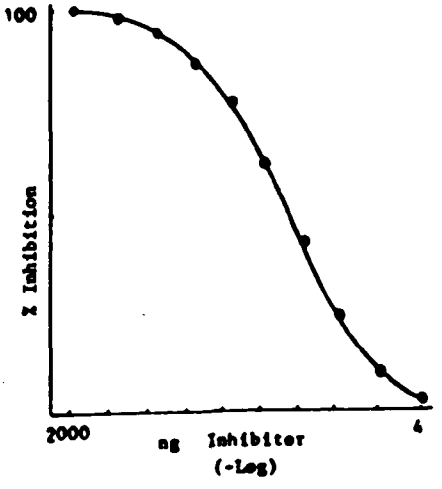

Figure 6.

Figure 7. SDS-PAGE and tropomyosin immunoblots. All samples were electrophoresed on 5-20% polyacrylamide gels under reducing conditions. (Lane 1) Purified bovine brain tropomyosin (2 µg) and (Lane 3) bovine brain ether powder (75 µg) profiles stained with commassie blue. (Lane 2) A cocktail of purified brain actin, myosin and tropomyosin (2 µg each) and (Lane 4) brain powder (75 µg) were electroblotted onto nitrocellulose paper and immunoperoxidase stained using anti-tropomyosin antibody.

Figure 8. ELISA titration curve for brain tropomyosin antibody. Anti-tropomyosin antibody was diluted 1/100 and serial dilutions were made to 1/51,000 in 96-well microtiter plates coated with purified brain tropomyosin (500 ng / well). Bound antibody was detected by anti-rabbit IgG conjugated to peroxidase and immunoperoxidase activity was measured using O-phenylene diamine / H_2O_2. The colorimetric reaction product was measured spectrophotometrically (delta OD_{492nm}).

Figure 9. ELISA inhibition plot using brain actin to inhibit the binding activity of anti-brain tropomyosin antibody. Tropomyosin antibody was used at 1/1000 and inhibitions were carried-out with concentrations of tropomyosin ranging from 2 µg/well serially diluted to 4 ng/well.

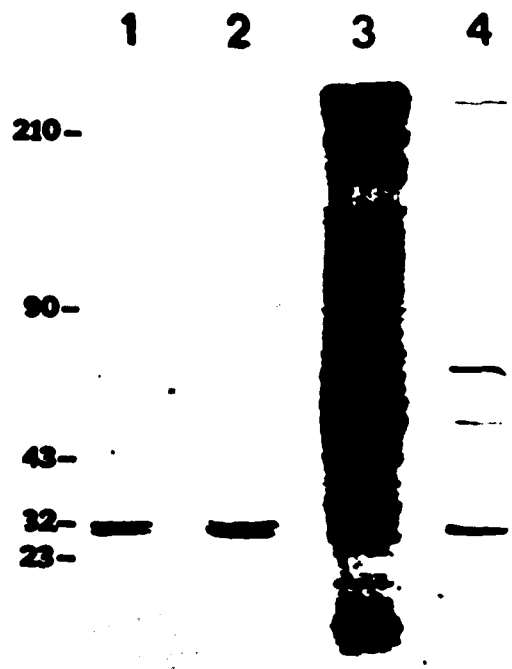

Figure 7.

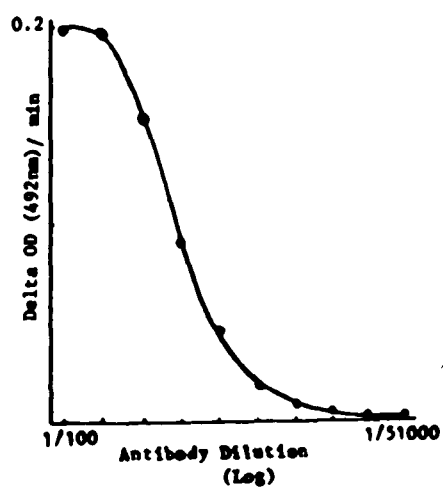

Figure 8.

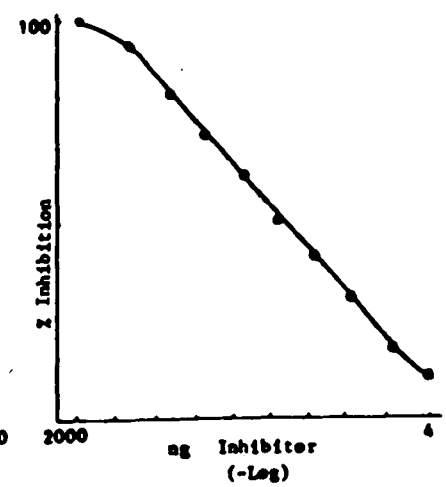

Figure 9.

4. 68 kD Neurofilament Protein.

The neurofilaments are a unique class of intermediate filament consisting of three distinct subunits. Polyclonal antisera produced against the 68 kD subunit generally cross-react with both the 160 kD and 210 kD subunits, yet monoclonal antibodies are available which distinguish the three as being immunologically separable. The 68 kD protein is the major constituent of neurofilaments, and a major band in SDS-PAGE profiles of rat sciatic nerve (Fig. 10). From a precipitate of sciatic nerve homogenate, an electrophoretically pure 68 kD protein was obtained from preparative SDS-PAGE. This procedure required the protein to be electrophoresed, electroeluted and electrodialyzed to obtain it pure and free of SDS for antibody production and purification. Anti-68 kD antibody stained only the 68 kD neurofilament triplet protein on immunoblots of brain ether powder. The 160 and 210 kD components, which were probably trace proteins in total brain ether powder, were not evident. Anti-68 kD did not cross-react with vimentin (M_r = 57 kD) on immunoblots of fibroblast total cell protein. This antibody stained only neurons in brain sections and cultures. In many studies of the intermediate filament content of Nb cells, a commercially acquired (Boehringer-Mannheim) monoclonal antibody produced specifically against the 68 kD neurofilament protein (showing no cross-reactivity to any other intermediate filament protein) was also used.

Intermediate Filament Content of SH-SY5Y Cells

SH-SY5Y cells were found to express vimentin and not neurofilament proteins. Immunoperoxidase staining with anti-vimentin antibody was intense in the cell body and processes, but was absent in the splayed regions of cell margins (Fig. 18). The polyclonal and monoclonal antibodies against the 68 kD

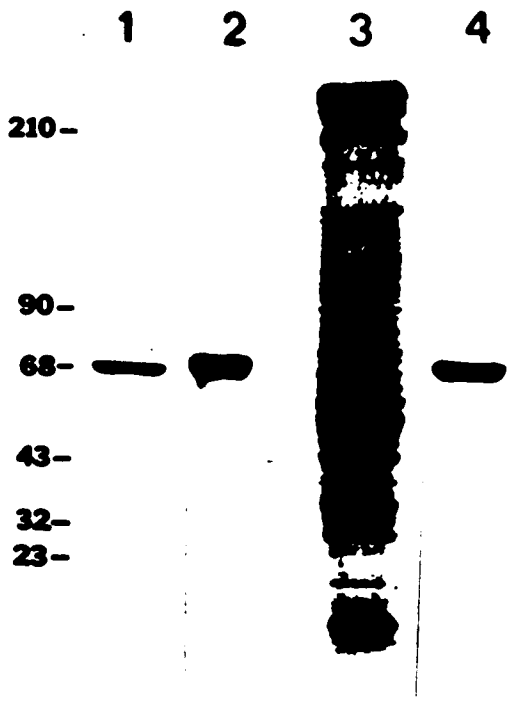

Figure 10. SDS-PAGE and 68 kD neurofilament protein immunoblots. All samples were electrophoresed on 5-20% polyacrylamide gels under reducing conditions. (Lane 1) Purified sciatic nerve, 68 kD neurofilament protein (2 μg) and (Lane 3) bovine brain ether powder (75 μg) profiles stained with commassie blue. (Lane 2) A cocktail of purified brain actin, myosin, tropomyosin and 68 kD protein (2 μg each) and (Lane 4) brain powder (75 μg) were electroblotted onto nitrocellulose paper and immunoperoxidase stained using anti-68 kD antibody.

neurofilament protein did not stain SH-SY5Y cells. Using cells as the solid-phase in an ELISA and as the samples for immunoblotting, vimentin immunoreactivity and the lack of neurofilament protein were confirmed (Fig. 11). In addition, SH-SY5Y cells were treated with NGF, RA, or PMA then assayed for the 68 kD protein. Neither immunoblots nor ELISA detected the neurofilament protein under any conditions tested. Vimentin was consistently found.

Antigen and Antigenicity Retention Experiments

Preliminarily, a major concern was to establish parameters for immunogold labeling of whole mount growth cones which have been variably fixed and extracted. While the appearance of ultrastructural detail was relatively easy to monitor by WMTEM, the parameters for optimal immunocytochemical labeling required substantial investigation. Of particular concern was the retention of cytomuscular components and antigenicity in aldehyde fixed specimen preparations. First, it was necessary to establish whether actin, myosin, and tropomyosin were extracted by Triton, which removes a cytosolic fraction and renders a greatly enriched cytoskeleton or Triton-insoluble preparation. Triton applied in isotonic buffer to a monolayer of SH-SY5Y cells extracted approximately 90% of the total cell protein. While actin, myosin and tropomyosin were detected in the Triton-soluble material, the Triton-insoluble fraction retained much of these proteins and, for instance, actin was due to the extraction of cytosolic proteins (Table I). A more expedient approach to establishing immunocytochemical parameters was to use Nb cells grown in microtiter plates. Here Nb cells served as the solid-phase for antigen detection by ELISA. Wells of adherent cells were fixed and extracted under various conditions; fixatives, non-ionic detergents, and cytoskeleton stabilizing buffers were varied. Following treatment, wells were assayed using antibodies

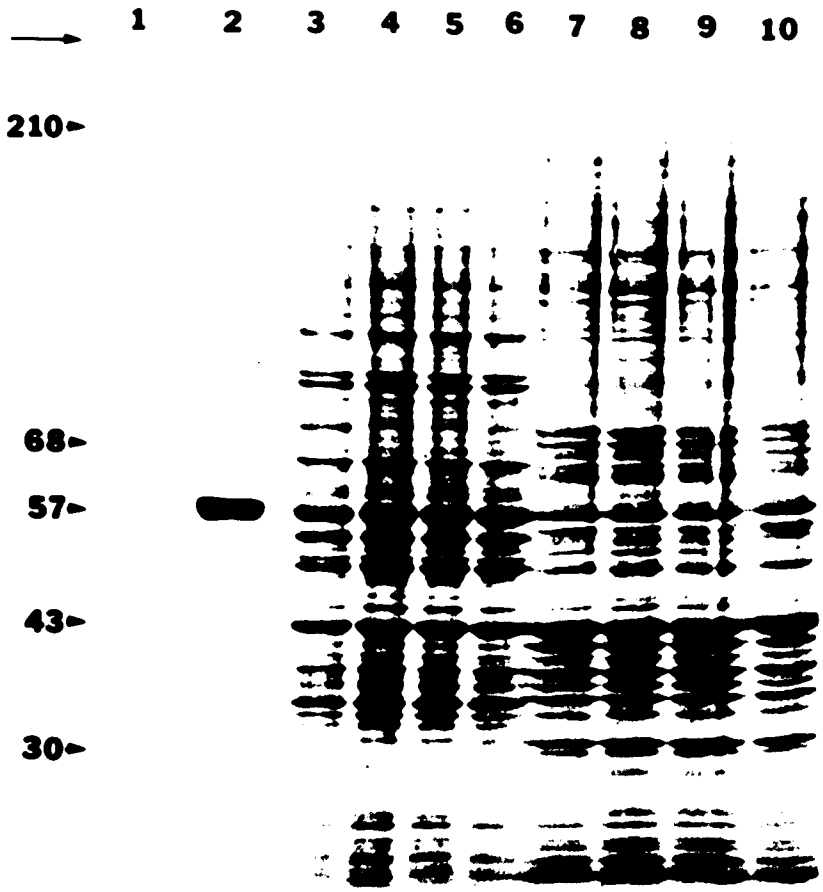

Figure 11. SDS-PAGE of SH-SY5Y cells and Triton-insoluble fractions and intermediate filament immunoblots. (Lane 1) Immunoblot of SH-SY5Y total cell protein using anti-68 kD neurofilament antibody (1:100) and immunoperoxidase staining. (Lane 2) Immunoblot of total cell protein using anti-vimentin antibody (1:500) and immunoperoxidase staining. (Lanes 3-6) SH-SY5Y total cell protein (50 µg) electrophoresed on a 5-15% acrylamide gel under reducing conditions then stained with commassie blue. (Lanes 7-10) SH-SY5Y Triton-insoluble pellet (50 µg) stained with commassie blue. Samples were obtained from cells grown in serum-free N2 medium (Lanes 3 and 7), and N2 containing NGF (Lanes 4 and 8), PMA (Lanes 5 and 9), and RA (Lanes 6 and 10). Standards: brain myosin (210 kD), neurofilament 68 kD protein, fibroblast vimentin (57 kD), brain actin (43 kD) and tropomyosin (30 kD).

against actin, myosin and tropomyosin (Fig. 12). First, it was found that the chelator EGTA and $MgCl_2$, when added to buffers containing Triton and aldehyde fixatives improved retention of actin, while mild hypotonic solutions offered enhanced myosin and tropomyosin detection without diminishing actin. The results shown in figure 12 indicated that paraformaldehyde fixation afforded the greatest relative immunoreactivity for fixed specimens. Glutaraldehyde and Triton applied simultaneously resulted in variable retention of antigens as detected by immunoperoxidase methods. Particularly, the binding activity of anti-myosin was reduced by glutaraldehyde fixatives, and anti-tropomyosin and anti-actin activities were somewhat reduced. However, this fixation and extraction combination provided the preferred level of ultrastructural preservation when performed at 37°C by immediately submerging grids in cytoskeleton stabilizing buffer (PHEM) containing 2% glutaradehyde and 0.1% Triton. The problem of glutaradehyde reduced antigenicity (or antigen retention) was circumvented in certain experiments by initially fixing specimens with paraformaldehyde (in PHEM buffer containing Triton) then post-fixing with glutaraldehyde and tannic acid following immunolabeling. This procedure was effective for improving the immunocytochemical detection of myosin and tropomyosin which otherwise was variable. With this sequence of fixations the preservation of filamentous structures was dependent apon relatively short incubations and gentle immunocytochemical processing. For most immunoelectron microscopy, post-fixations were a definite requirement, especially when initial aldehyde fixation was necessarily brief. Even subtle changes in fixation and extraction were often discernible when specimens were examined as whole mounts by electron microscopy. These effects were closely scrutinized in specimens preparations shown to best retain antibody activities by ELISA.

Figure 12. The effect of buffers, fixatives and Triton-extraction on actin, myosin and tropomyosin immunoreactivity. SH-SY5Y Nb cells were grown to confluency in 96-well microtiter plates. Following fixation and/or extraction by various solutions, indirect immunoperoxidase staining was carried out as described for standard ELISA (here using cells as the solid-phase for antigen detection). Either anti-actin, anti-myosin, or anti-tropomyosin primary antibody were applied to all wells on a given plate and bound antibody was detected by anti-rabbit IgG conjugated to peroxidase. Colorimetric assay using O-phenylene diamine / H_2O_2 was performed and the reaction product measured spectrophotometrically (delta OD_{492nm}). The relative immunoreactivities of cells in each treatment for actin, myosin, and tropomyosin were determined independently. Twelve wells per plate were committed to each of the six treatments and assays for actin, myosin and tropomyosin were performed on separate plates. Each assay was performed in duplicate and the entire experiment was repeated.

A. All fixation solutions contained 1% glutaraldehyde and 0.1% Triton plus:
   1. 0.12 M phosphate buffer, pH 7.2 (P-buffer).
   2. 25 mM HEPES, 65 mM PIPES, pH 6.9 (HP-buffer).
   3. HP-buffer + 2 mM $MgCl_2$.
   4. HP-buffer + 2 mM $CaCl_2$.
   5. HP-buffer + 2 mM EGTA.
   6. HP-buffer + 2 mM $MgCl_2$ + 2mM EGTA (PHEM buffer).

B. Fixations and extractions were carried-out with PHEM buffer containing:
   1. 5% glutaraldehyde + 5% Triton.
   2. 5% glutaraldehyde + 0.1% Triton.
   3. 1% glutaraldehyde + 0.5% Triton.
   4. 1% glutaraldehyde + 0.1% Triton.
   5. 0.2% glutaraldehyde + 0.5% Triton.
   6. 0.2% glutaraldehyde + 0.1% Triton.

C. Fixations and Triton treatments were applied in various sequences.
   1. 0.1% Triton-extraction preceeding 1% paraformaldehyde fixation.
   2. 0.1% Triton-extraction and 1% paraformaldehyde simultaneously.
   3. 1% paraformaldehyde fixation followed by 0.2% Triton.
   4. 0.1% Triton-extraction preceeding 1% glutaraldehyde fixation.
   5. 0.1% Triton-extraction and 1% glutaraldehyde fixation simultaneously.
   6. Triton-extraction and paraformaldehyde/glutaraldehyde fixation.

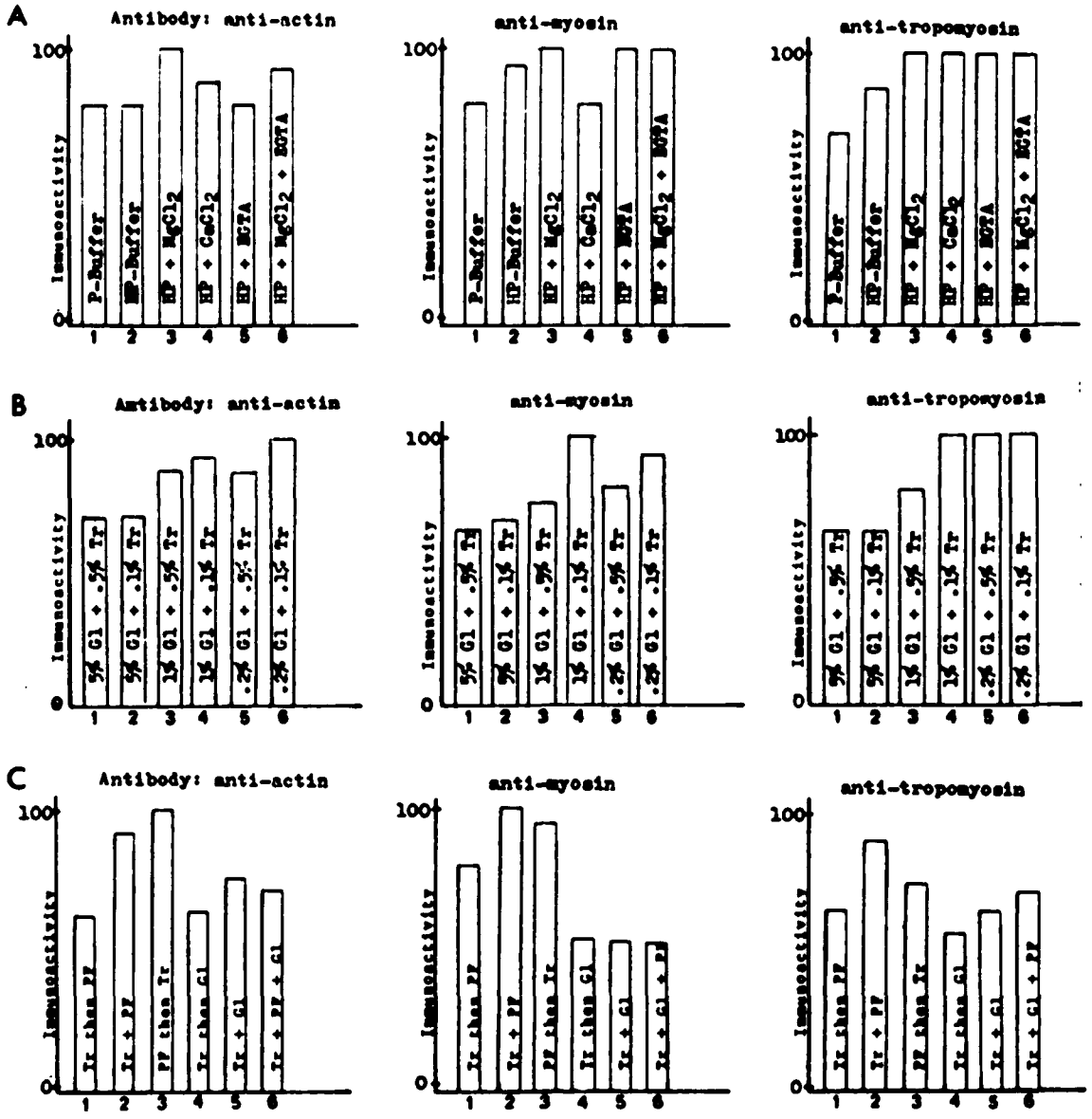
A
Antibody: anti-actin
anti-myosin
anti-tropomyosin
Immunoactivity
100
0
1 2 3 4 5 6
P-Buffer
HP-Buffer
HP + MgCl2
HP + CaCl2
HP + EGTA
HP + MgCl2 + EGTA
B
Antibody: anti-actin
anti-myosin
anti-tropomyosin
.5% Gl + .5% Tr
.5% Gl + .1% Tr
.1% Gl + .5% Tr
.1% Gl + .1% Tr
.2% Gl + .5% Tr
.2% Gl + .1% Tr
C
Antibody: anti-actin
anti-myosin
anti-tropomyosin
Tr than PF
Tr + PF
PF than Tr
Tr than Gl
Tr + Gl
Tr + PF + Gl

Figure 12.

Alterations of Actin, Myosin and Tropomyosin in PMA-Treated Cells

SH-SY5Y cultures are not a morphologically homogeneous cell population. One evident feature is the variabilty of neurite outgrowth and in the predominance of long, thin or splayed, lamellar processes. The effect of PMA on Nb morphology (detailed in following sections) was found to coincide with changes in the proportion of actin, myosin and tropomyosin, and the relative retention of antibody activity by cell preparations extracted by Triton (Table I). Triton extracted approximately 90% (145 pg/cell) of the total protein of SH-SY5Y cells and 87% (184 pg/cell) in cells exposed to PMA. In untreated cultures actin comprised 8.8% of the total cell protein and 43% of this actin was removed from the cell when cytoskeleton preparations were rendered by Triton-extraction. PMA treatment resulted in cells having 5.5% actin, of which only 22% was removed by Triton. The myosin and tropomyosin content of untreated cells was approximately 1%, PMA treatment reduced these to less than 0.3% of the total cell protein.

Morphology of SH-SY5Y Nb

SH-SY5Y cultures proliferate under a variety of culture conditions and exhibit little contact inhibition. In serum-free medium, cells usually remained in contact; at low plating density, cell bodies were clustered in a fashion reminiscent of ganglia formation. In addition, neurites often apposed other neurites or cell bodies and frequently extended onto vacant substratum. Nb cultures were pleiomorphic, neurite dimensions and number varied between cells. The asynchronous mitotic activity, coupled with neurite extension and retraction, resulted in a pleiomorphic population of growing Nb cells. In defined medium, cells were especially sensitive to the nature of the substratum or medium supplements. Pre-coating tissue culture plastic with serum enhanced

Table I. Actin, Myosin and Tropomyosin Content of SH-SY5Y Nb Cells.

Cell Fraction	Culture Treatment N2 medium	N2 + PMA
Total protein (T.C.P.) (pg)	160 (2.3)	210 (3.0)
Triton-soluble T.C.P. (%)	90.4 (4.2)	87.5 (3.7)
Total actin (% T.C.P.)	8.8 (0.7)	5.5 (0.4)
Triton-soluble actin (%)	43	22
Total myosin (% T.C.P.)	0.9 (0.08)	0.3 (0.02)
Total tropomyosin(% T.C.P.)	1.2 (0.13)	0.2 (0.02)

SH-SY5Y Nb were grown in serum-free medium with and without PMA (17 nM). Sub-confluent cultures were harvested; total cell protein (T.C.P.) was determined by the Lowry method and homogenate samples were assayed for actin, myosin, and tropomyosin by an inhibition ELISA (see methods). Also, samples were obtained by Triton extraction, the Triton-soluble supernant fractions were assayed for total protein and for actin content as described above. Each value represents the average of 20 determinations from two experiments. (Standard Deviations).

proliferation and lead to a more dispersed cell population. In contrast, cultures established on FN-coated dishes clustered much like those growing on uncoated plastic but showed enhanced neurite elongation (Fig. 26).

The morphology of Nb cultures grown in serum-free medium was greatly altered by the addition of PMA (17 nM). Initially PMA slowed mitosis (Pahlman et al., 1981) and perhaps allowed neurite elongation to continue longer before neurites were retracted in the mitotic cycle. After PMA treatment, cell bodies appeared larger and more rounded, neurites were compact, longer, staighter and resembled axons, and the total cell protein was increased. Whereas untreated cultures contained cells bearing many large, spread processes lanced by filopodia, PMA-treated cells had fewer neurites which appeared to be more uniform and had growing margins with a common shape. PMA resulted in the formation of large, rounded margins with a lamellar configuration either forming a hood or crown-shaped process continuous with the cell body, or a similarly shaped margin continuous with the distal tip of neurites. These margins were filled with a meshwork of filaments without any conspicuous orientation or bundling and lacked filopdial protrusions. (see below)

The Morphology and Ultrastructure of PMA-treated Neuroblastoma Cells

SH-SY5Y cells grown in serum-free N2 medium displayed elaborate growth cones with many filopodia (13a-b). After cells were allowed to extend neurites, the morphology and ultrastructure of the growth cones were drastically altered upon the addition of PMA. Within 30 minutes the growing tips had begun to take on a rounded appearance and filopodia were partially retracted (Fig. 13c). After continued exposure to PMA, few filopodia remained extended and lamellar growth cone margins appeared that lacked cytoskeletal features such as coursing microtubules and intermediate filaments (Fig. 13d). This was confirmed

Figure 13. Low magnification WMTEM of neuroblastoma cells stained with tannic acid and uranyl acetate. In (a) the substratum was pretreated with serum; (b-f) the substratum was pretreated with serum that had been stripped of FN. Growth cones after 3 days in N2 medium are exemplified in (a,b). Retraction of filopodia by cells first established in N2 medium for 2 days then treated with PMA for 30 min. (c) and 2 hrs. (d). Lamellar growth cones lack filopodia when cells were established and maintained in medium containing PMA for 24 (e) and 48 hrs. (f). Bar 3 μm.

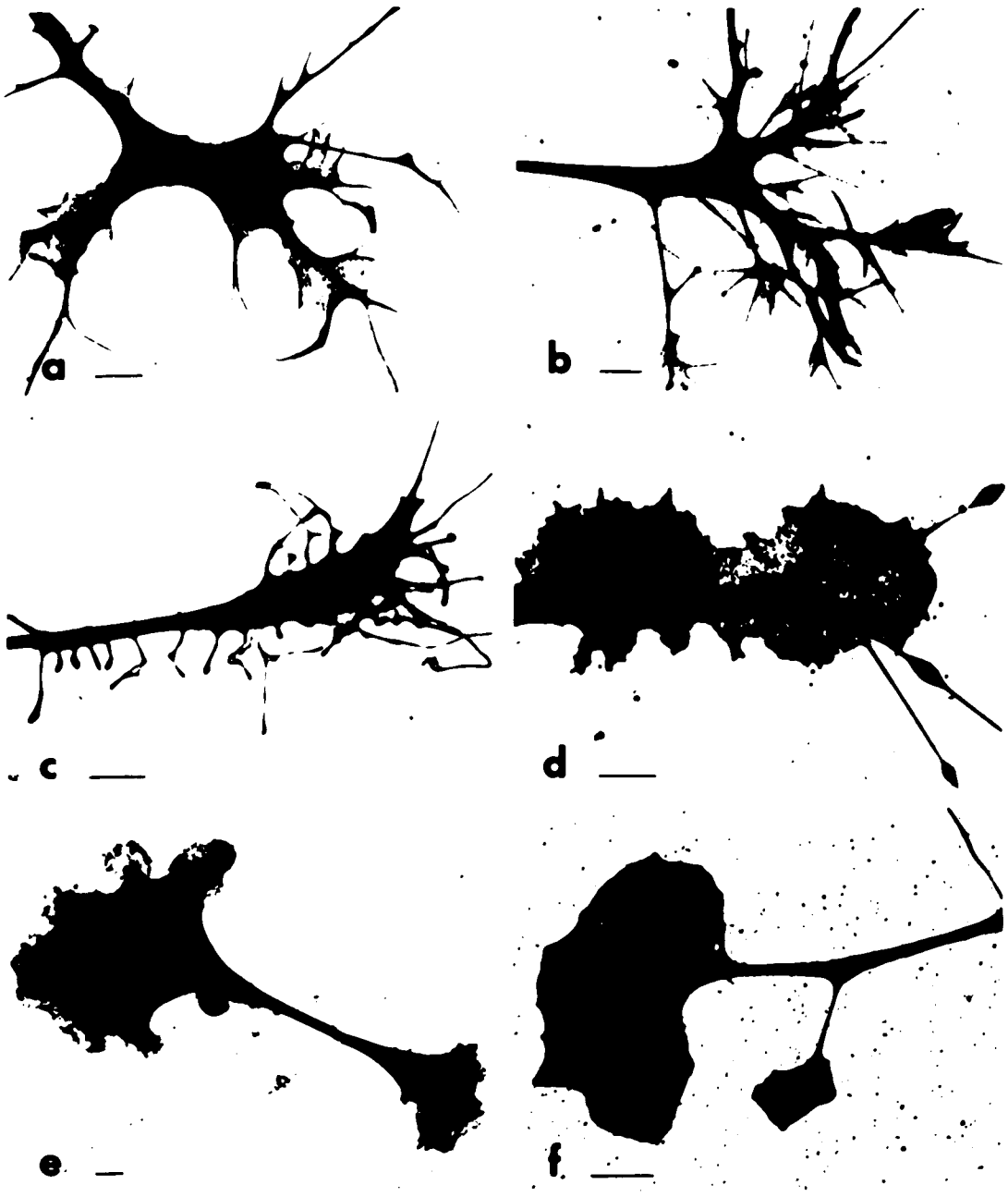

Figure 13.

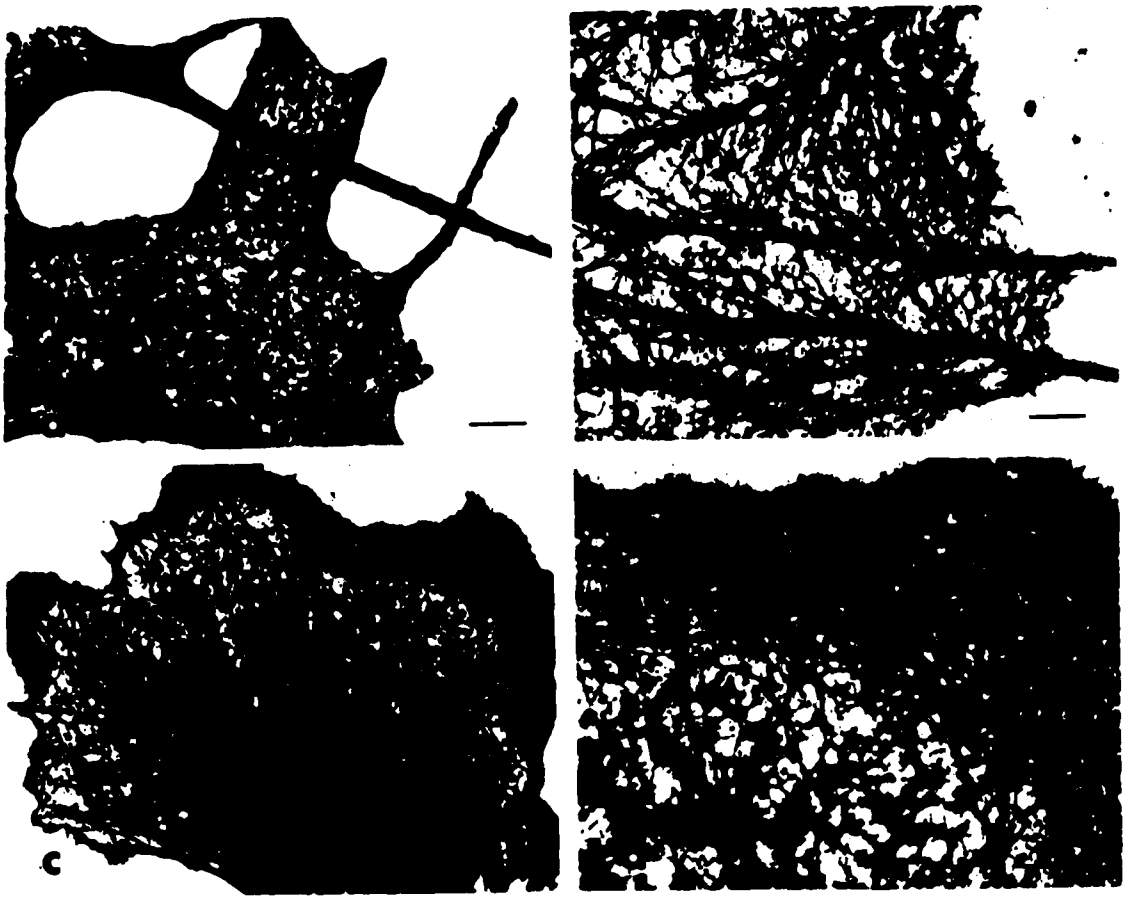

Figure 14. The ultrastructure of neuroblastoma growth cones. WMTEM of cells grown in N2 medium; (a,b) growing margins contain microfilament bundles which project into filopodia. The lamellar margins of cells treated with PMA lack filopodia and contain a microfilament meshwork devoid of microfilament bundles (c,d). Specimens were stained with tannic acid and uranyl acetate. Bar, (a,b,) 0.5 μm; (c,d) 1 μm.

by immunocytochemistry using antibodies against tubulin and intermediate filaments (see below).

The effect elicited by PMA on growth cone morphology was evident at the onset of neurite outgrowth by cells initially plated in the presence of PMA. During the considerable lag in neurite sprouting (shown above), widely spread margins were often seen splayed in many directions as extensions of the cell body (Fig. 13e). Shortly thereafter, neurites began to elongate which retained a splayed distal tip with lamellar configuration. These processes continued to lengthen apparently without ever expressing filopodia (Fig. 13f). It appeared that the cytoskeleton rearrangements induced by PMA interfered with the formation of filopodia by the growth cone, without precluding neurite elongation. The bundles of microfilaments which, prior ot PMA treatment usually extended throughout the distal margin and protruded into filopodia, were greatly diminished and replaced by a microfilament meshwork (Fig. 14). This suggested that the PMA-induced cytoskeletal rearrangements, characterized by a shift from cross-linked microfilament bundles to a filament meshwork, mediated the loss of normal growth cone morphology.

Cytoskeleton Immunocytochemistry of SH-SY5Y Cells

1. Light Microscopy.

Figures 15-18 show immunocytochemical staining for actin, myosin, tropomyosin or vimentin in SH-SY5Y cells grown in N2 medium and N2 containing PMA. Anti-actin immunofluorescence was intense throughout the cytoplasm and all cell processes (Fig. 15). Neurites and growing margins were intensely stained, although due to their flattened configuration, splayed margins at the distal tips of neurites appeared less fluorescent. Often, in untreated cells, small bundles of filaments and filopodia intensely stained for actin were seen.

The spread margins of PMA-treated cells were more densely stained in comparasion to the margins of untreated cells, indicating the concentration of actin. Immunofluorescence cytochemistry also demonstrated that myosin was present throughout the cell and growing processes (Fig. 16). Myosin staining often appeared punctate in the perikaryon with patches of fluorenscence extending down neurites into very diffusely stained growing tips. In untreated cells, the intensity of staining within the growing margins was often coincident with the appearance of phase-dense bundles and filopodia. In contrast, PMA-treated cells showed only diffuse myosin labeling which appeared throughout the growing tip including the leading edge. Tropomyosin was found in many regions of the cytoplasm and cell processes (Fig. 17). Tropomyosin immunofluorescence was very diffuse in lamellar regions of growing margins and was absent from their leading edge. While PMA-treated cells had only diffuse tropomyosin immunofluorescence in growing margins, immunolabel was sometimes coincident with phase-dense bundles in the margins of untreated cells.

SH-SY5Y cells contain vimentin intermediate filaments. Immunocytochemical localization indicated that the core of cell processes was packed with vimentin filaments (Fig. 18). In untreated cells, intensely labeled bundles coursed the extent of cell processes as a core structure from which growing margins seemed to emanate. The vimentin-labeled core complex often branched and entered each cell margin including the distal tip. As a margin spread peripherally, diffuse immunostaining became increasingly faint. In contrast, the growing margins of PMA-treated cells displayed a graded pattern of staining for vimentin. The thin, compact neurites characteristic of exposure to PMA were densely stained; as the neurite spread-out into the growing distal tip, the pattern of staining quickly became diffuse and was absent from the lamellar cortex. Apparently, the growing tip of a PMA-induced neurite

Figure 15. Actin immunofluorescence of SH-SY5Y Nb cells. Cultures were grown in serum-free N2 medium with or without the addition of PMA (17 nM). Cells were fixed with paraformaldehyde and immunofluorescent cytochemistry was performed using anti-brain actin antibody (1:500) and rhodamine conjugated anti-rabbit IgG (1:50). Epifluorescence micrographs were taken with equal exposure times. (a) Actin immunofluorescence in the distal margins of PMA-treated cells and (b) in untreated cells was intense throughout cells and the thin margins at the leading edge, and in untreated cells, in filopodia (arrows). Bar = 10 μm.

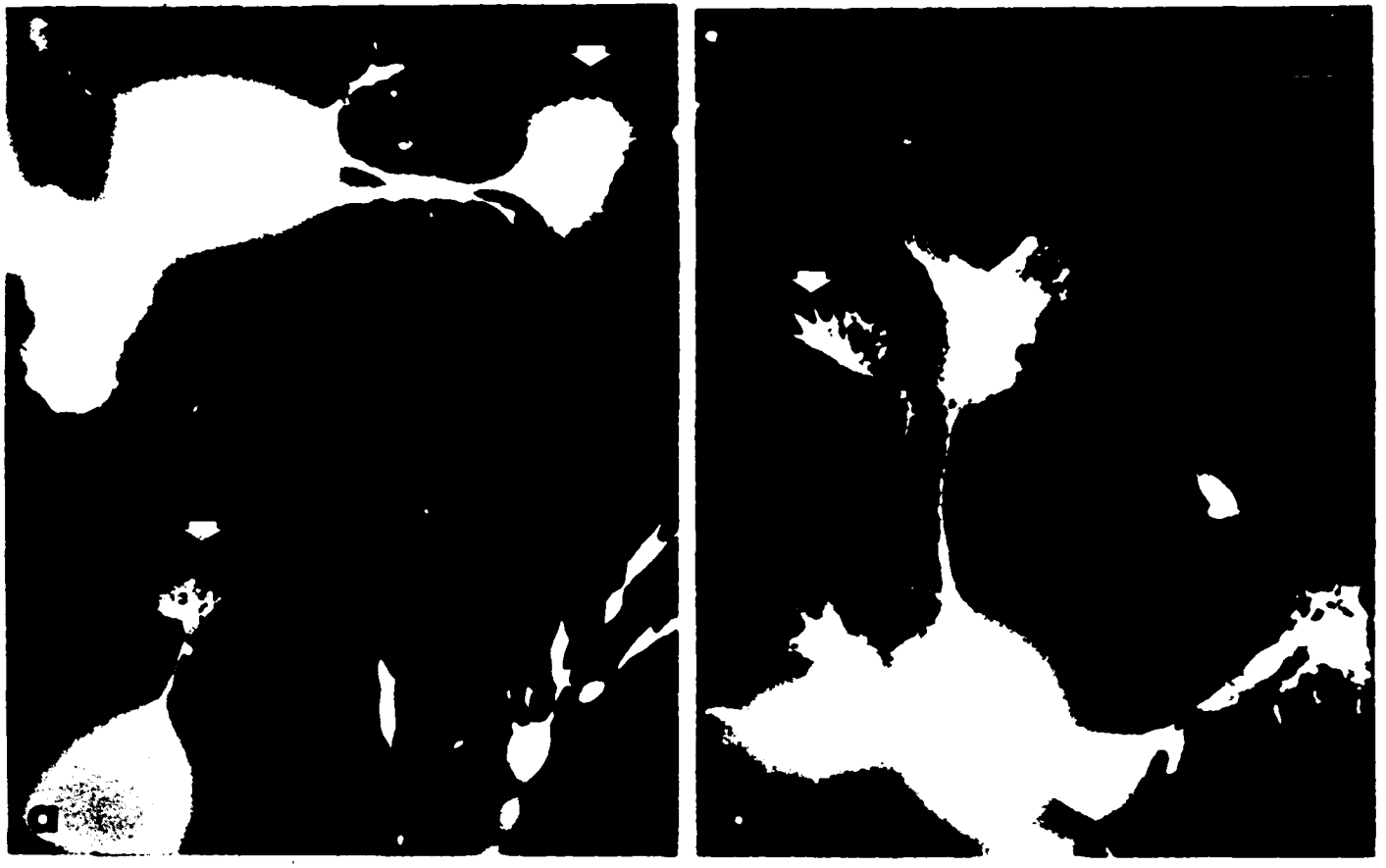

Figure 15.

Figure 16. Myosin immunofluorescent cytochemistry of SH-SY5Y Nb cells. Cells were grown in serum-free N2 medium (b) and in N2 containing PMA (a). Myosin immunofluorescence is seen in cell processes and concentrated at the leading edge of growth cones (arrowheads), and in the filopodia of untreated cells. Bar = 10 μm.

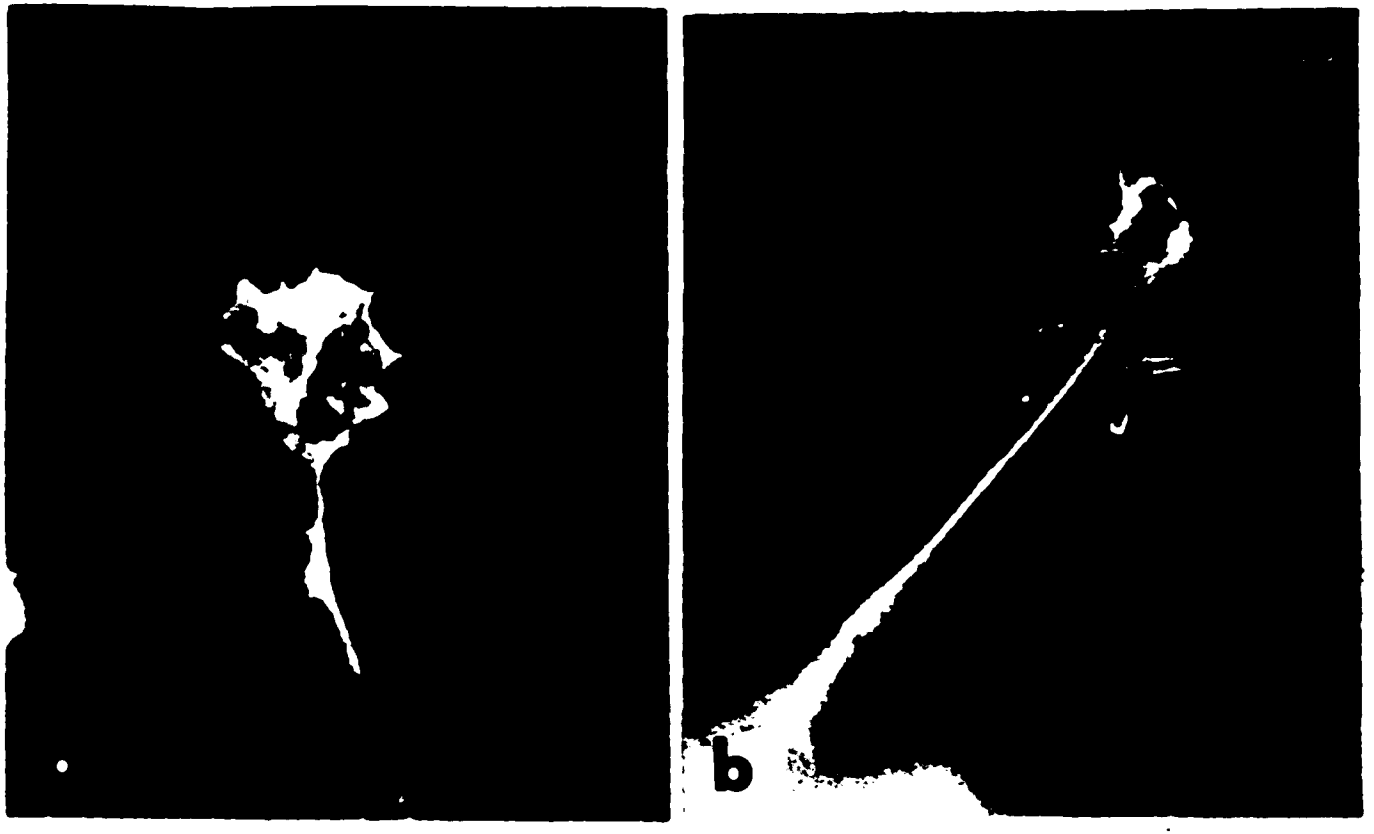

Figure 16.

Figure 17. Tropomyosin immunofluorescent cytochemistry of SH-SY5Y Nb cells with and without exposure to PMA. Immunofluorescence with anti-tropomyosin antibody was absent from splayed margins (arrowheads) of PMA-treated cells (a) and sometimes was seen in a fibrillar pattern (arrows) in the body of growth cones of untreated cells (b). Bar = 10 μm.

Figure 17.

Figure 18. Vimentin intermediate filament immunoperoxidase cytochemistry of SH-SY5Y Nb cells. In (a), Nb cells treated with PMA show an absence of vimentin staining in the cortical regions of the distal tip of neurites (arrowheads) which appeared as the terminus for intermediate filament transport. Untreated SH-SY5Y cells (b) show vimentin staining in branched neurites from which unstained margins emanate (arrowheads). Micrographs were taken using light field illumination. Bar = 10 μm.

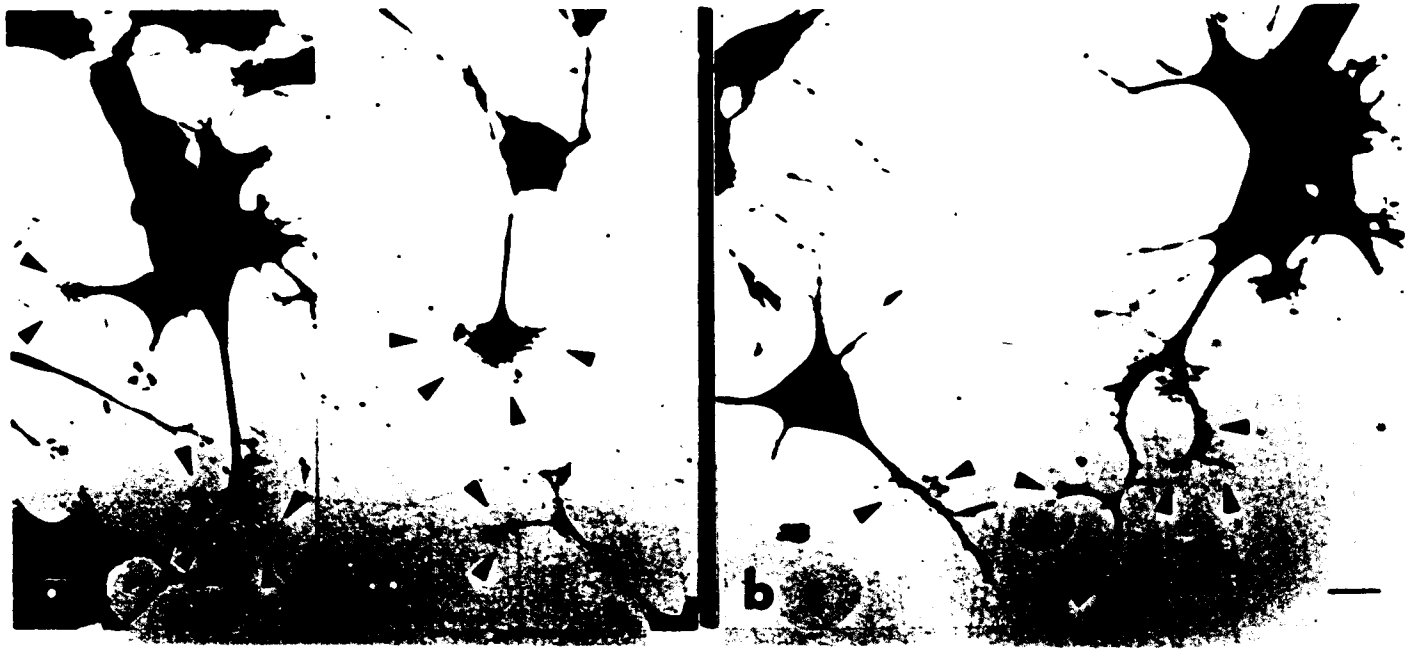

Figure 18.

represented more of a terminal for intermediate filament advance, while in untreated cells the growing margins appeared with intermediate filaments en passant.

2. Immunoelectron Microscopy

SH-SY5Y cells were immunolabeled with colloidal gold probes and examined as whole mounts by transmission electron microscopy. Generally, the fixation and extraction methods required to obtain well immunolabeled specimens did not provide optimal ultrastructural preservation when compared to specimens thoroughly fixed with glutaraldehyde. However, many details were resolved in these specimens after immunolabeling, post-fixation with glutaraldehyde, mordanting with tannic acid, and uranyl acetate counterstaining. In particular, filopodia protruding from the growing tip of Nb cells were clearly resolved by WMTEM. While filopodial staining was difficult to discern by immunofluorescent microscopy, immunogold label on filopodia was very informative when examined by electron microscopy. Filopodia were found to contain several closely associated but separable structural features. Microfilaments within the growth cone coalesced into the roots of filopodia. These microfilament bundles in the cytoplasm were immunogold labeled uniformly with actin antibody (Figs. 19-20) and in a somewhat punctate fashion with myosin (Fig. 23) antibody. Within the cytoplasm, a fine trabecula of interconnected, short filaments was conspicuously unlabeled by antibodies for actin and myosin (Figs. 20 and 23). The nature of this ultrastructural feature was not revealed in this study. Both actin and myosin were found along the length of the filopodium (Fig. 21 and 24). Many filopodia demonstrated a core of filaments and a helical superstructure. The helix was intensely labeled by actin antibody, and actin was probably a major constituent of this filopodial

superstructure. The very distal end of some filopodia had an interesting pattern of actin label that appeared as an actin cap, suggesting that this was a dynamic site of actin organization (Fig. 22). Immunogold label for myosin was found along the filopodium at sites where actin was localized, but appeared scattered or in small patches. The actin-containing helix surrounded a core of long, staight filaments which were loosely bundled and ran the length of a filopodium. The composition of these filaments was not clearly indicated by immunochemistry although their appearance was similar to actin-containing filaments in the cytoplasm. Tropomyosin was not found in filopodia nor in other protrusions from the growing tips of Nb cells.

Another feature of filopodia was a dense plaque which formed a relatively large (0.2-2 μm) protrusion. The filopodium was linked to the plaque by small trabecular bridges made with the helical superstructure that were labeled for actin and were contiguous with the FN-labeling of the plaque structure. The formation and appearance of FN-containing sites of attachment between a filopodium and the substratum was the impetus for a battery of assays and ultrastructural examinations which are described in detail below.

Figure 19. WMTEM of SH-SY5Y cell processes immunogold labeled using anti-actin antibody and anti-rabbit IgG adsorbed to 20-nm colloidal gold particles. Immunogold labeling for actin in specimens initially fixed with paraformaldehyde was intense throughout cell processes and margins which were clearly visible by electron microscopy without heavy metal counterstaining. Magnified 4,000X.

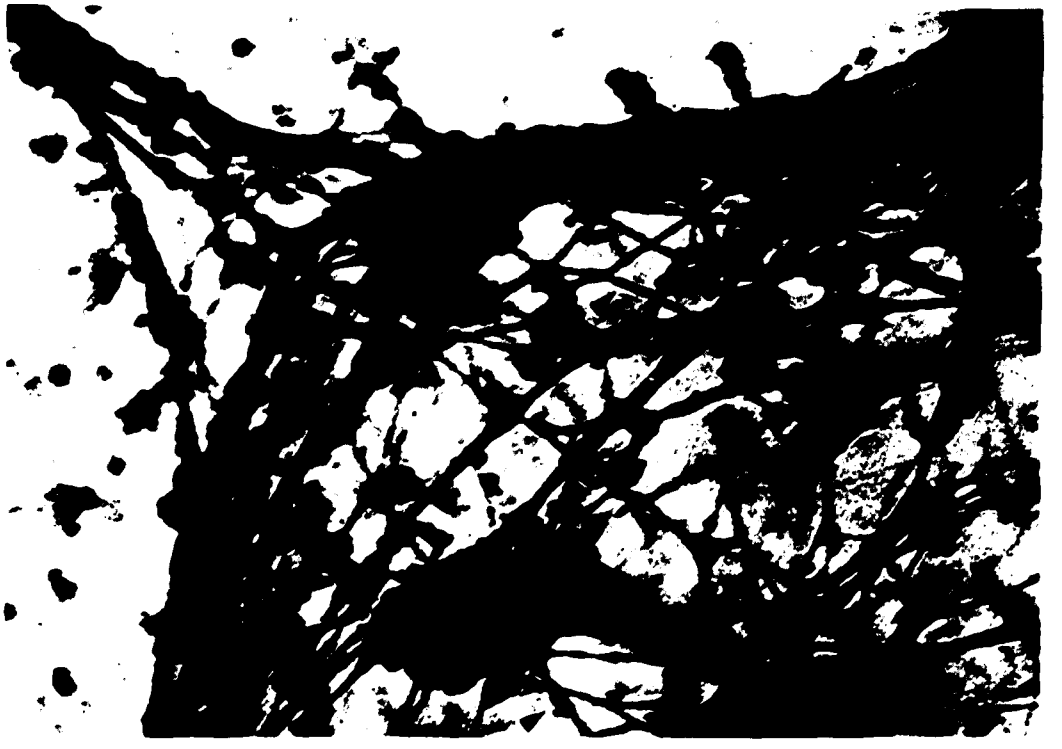

Figure 20. WMTEM of a SH-SY5Y growth cone labeled with anti-actin antibody and secondary antibody adsorbed to 5-nm gold particles. The specimen was simultaneously fixed and Triton-extracted prior to immunolabeling. Counterstaining was acheived with uranyl acetate which showed a fine trabecula that was not labeled for actin (arrowheads). Magnified 300,000X.

Figure 21. WMTEM of SH-SY5Y filopodia immunogold labeled for actin using 5-nm probes demonstrated the actin content of the helical superstructure of filopodia. Magnified 200,000X.

Figure 22. WMTEM of the distal end of a filopodium immunolabeled for actin using secondary antibody adsorbed to 20-nm gold particles. The actin-containing helix and a filopodial cap at the very end of the filopodium are demonstrated. Magnified 100,000X.

Figure 23. WMTEM of a Triton-extracted SH-SY5Y margin immunolabeled for myosin using secondary antibody adsorbed to 5-nm gold particles and counterstained with uranyl acetate. While filament bundles and globular domains (appearing as cross-links) of the trabecular network were well labeled, the trabecular filaments were not labeled by myosin antibody. Magnified 200,000X.

Figure 24. WMTEM of a SH-SY5Y filopodium immunolabeled for myosin with 5-nm gold probes. Magnified 200,000X.

Substrata Attachment

The goal of studying the attachment of SH-SY5Y cells to tissue culture plastic and coated plastic surfaces was to ensure that a subsequent examination of neurite outgrowth, cytoskeleton ultrastructure and FN-containing plaque formation under serum-free conditions was not confounded by poor cell-substrata attachment. On uncoated culture dishes, 93% of the SH-SY5Y cells seeded in defined medium attached effectively (i.e. resistant to mild perturbation) (Table II). Attachment was similar (85%) when cells were pretreated with and seeded in the presence of PMA. In both cases, attachment was virtually unaltered on dishes moderately coated with FN (20 μg/ml, 0.1 ml/cm^2) or by the addition of soluble FN (20 μg/ml) to defined media. However, cell attachment was reduced by about 25% in medium containing 15% serum. SH-SY5Y cells attach effectively to plastic culture dishes and do not require the addition of FN to the media or substrata. These findings confirm observations made in rodent neuroblastoma lines (Bottenstein and Sato, 1979; Rauvula, 1984). Untreated and PMA-treated cells remained adherent and grew neurites in serum-free N2 media. Proliferation was prolonged by FN. We observed that the amount of FN secreted by the neuroblastoma cultures was sufficient for proliferation, provided that FN was not removed during medium replenishment.

Neurite Sprouting and Outgrowth

SH-SY5Y cells constitutively extend neurites in the presence of serum and more so in serum-free N2 medium. For prolonged proliferation and neurite outgrowth both substrata pretreated with FN-containing solutions (including FBS) or FBS stripped of FN were permissive. Uncoated tissue culture dishes provided an adequate substratum for neurite outgrowth in N2 medium and approximately 88% of control cells sprouted neurites within 4 hours of replating (Figs. 25 and

Table II. Substratum attachment.

Treatment	Substratum		Medium	
	culture dish	FN-coating	+ FN	+serum
	% cells attached (standard error)			
N2 medium	93 (1.4)	91 (0.9)	90 (1.6)	67 (2.0)
N2 + PMA	85 (1.6)	84 (1.3)	80 (1.7)	65 (1.8)

SH-SY5Y neuroblastoma cells that had been labeled with (^{3}H) thymidine were detached with trypsin / EDTA and pretreated in suspension for 2 hrs. with N2 medium or N2 containing PMA (17 nm). Cells were seeded ($2x10^5$ / well) on tissue culture plastic or plastic coated with FN (20 µg/ml/0.1 cm^2), and on tissue culture plastic with serum FN (20 µg/ml) or FBS (15%) added to the media. After 4 hrs., poorly attached cells were detached with rotary shaking (100 rpm, 10 min), removed and dissolved in 1% SDS in 0.1 M NaOH for radioactivity counting. Substrata-attached cells were similarly dissolved and counted by liquid scintillation. The values given are averages of four means obtained from determinations with 24 replicates.

26). While a FN-coated surface apparently increased the length of neurites, it did not increase the already high sprouting percentage. In contrast, the sprouting of neurites within 4 hours was nearly eliminated when cells were pretreated with and plated in the presence of PMA. Only 5% of the cells exposed to PMA had sprouted neurites after 4 hours on uncoated plastic, by 24 hours the percentage had only increased to 38%. Coating the culture dishes with FN hastened sprouting of PMA-treated cells; cultures showed 31% sprouting after 4 hours and 77% sprouting after 24 hours. Similar results were obtained when soluble FN was added to the media upon replating. Results showed PMA treatment retarded neurite sprouting and elongation. Also, FN added to the medium or substratum partially antagonized the PMA-induced alteration of neurite outgrowth. Under all conditions tested, neurite elongation was enhanced on FN-coated surfacs (Fig. 26). The addition of anti-FN antibodies to the media greatly disrupted SH-SY5Y cultures and precluded neurite elongation.

FN Content of SH-SY5Y Conditioned Media

The secretion of FN by SH-SY5Y cells was demonstrated by immunoblotting and ELISA using anti-FN antibody (Fig. 27 and Table III). Additionally, metabolic labeling experiments showed that SH-SY5Y cells produced radiolabeled FN from radioactive amino acids which had been added to the media. When SH-SY5Y-conditioned media samples were immunoprecipitated with anti-FN antibody, compared to control antibody precipitations, scintillation counting demonstrated radioactivity was found to be concentrated in the FN-containing precipitate. To test the possibility that certain morphology-altering agents might affect the secretion of FN by SH-SY5Y cells, an inhibition ELISA was used to compare the FN content of defined media that had been conditioned by cultures grown in the presence of NGF, RA, and PMA. When the media

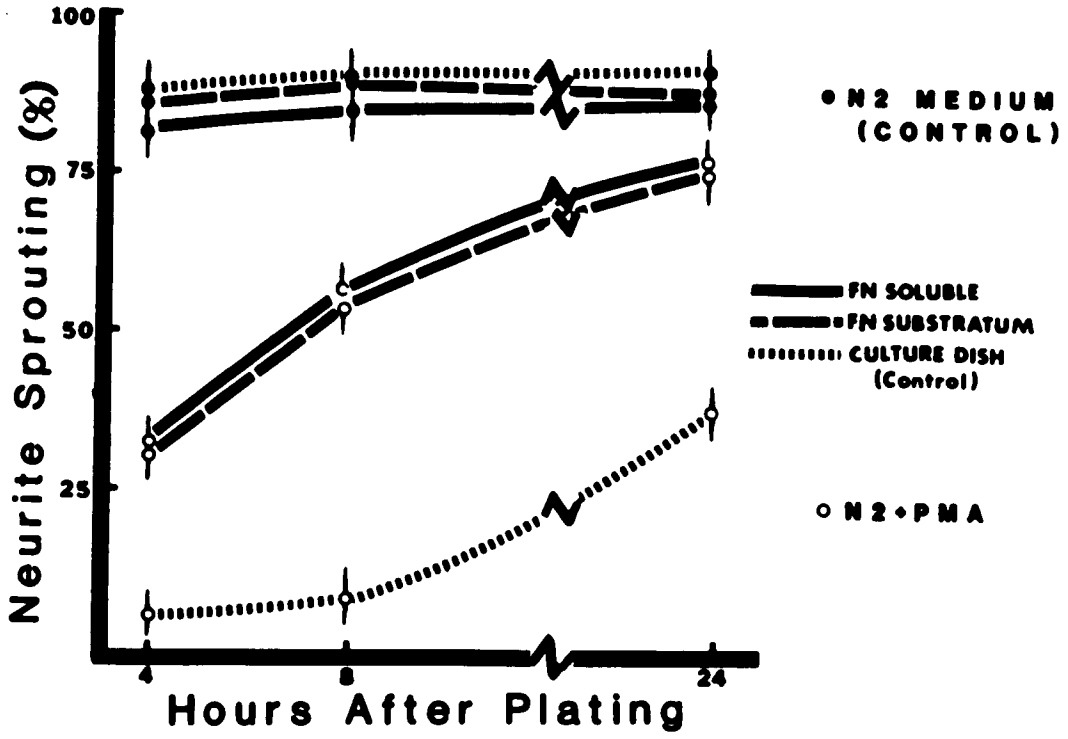

Figure 25. Neurite sprouting. SH-SY5Y cultures were established and treated as described for the attachment assay (see Table 2). After 4, 8, and 24 hrs. neurite outgrowth was stopped by fixation and the percentage of the cells bearing neurites greater than one cell body diameter was determined. Each data point represents the mean of eight determinations from two experiments. Each determination measured 100 cells.

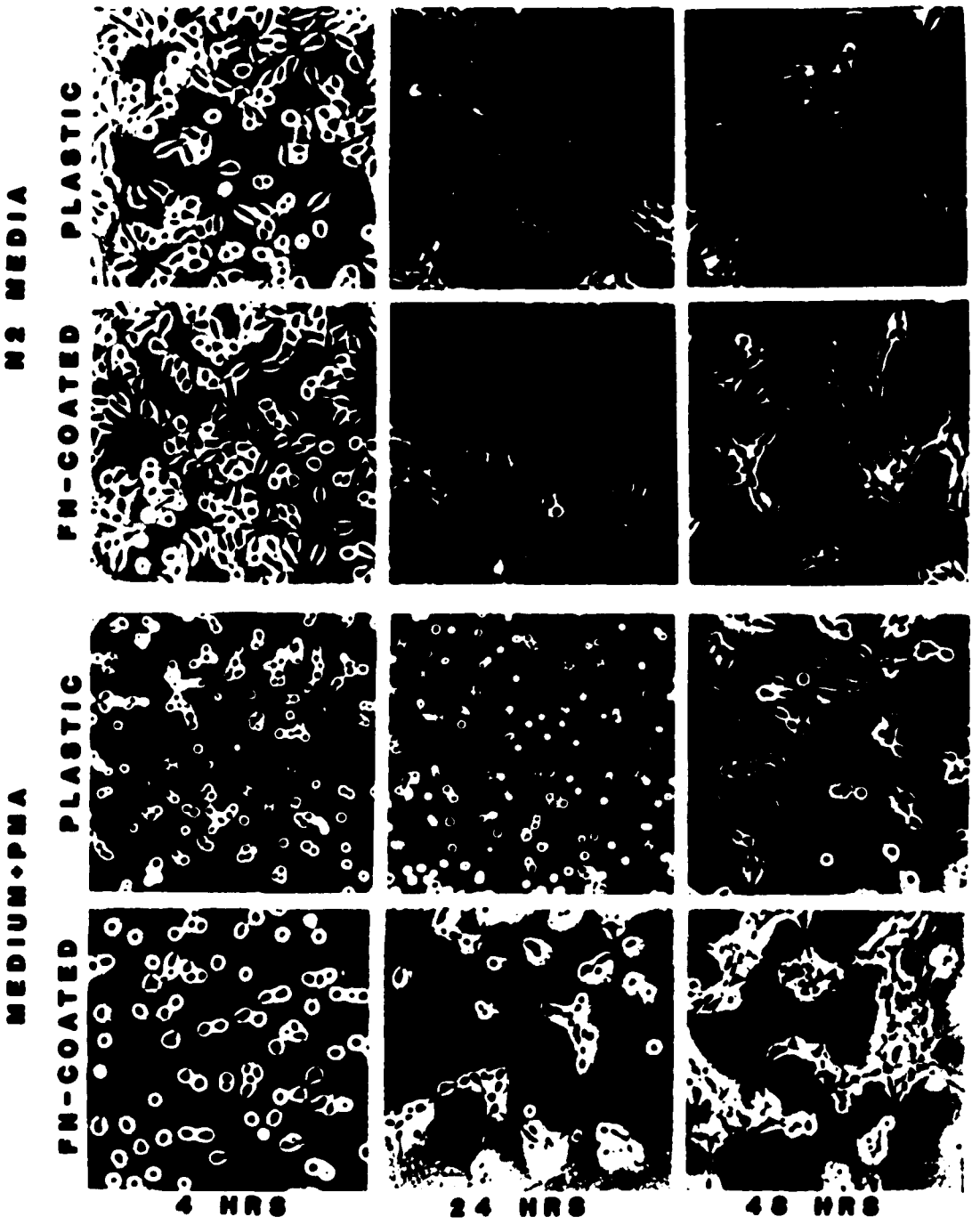

Figure 26. Neurite sprouting and elongation of SH-SY5Y neuroblastoma cultures seeded on culture plastic and FN-coated plastic in N2 medium and medium containing PMA. Phase-contrast photomicrographs were taken after 4, 24, and 48 hrs. of incubation. (Compare Figure 3). Bar 100 µm.

Figure 27. Immunoblots: FN-immunoreactive polypeptides in conditioned media from treated SH-SY5Y cultures. Cultures were grown in defined medium alone or containing either PMA (17 nM), NGF (4 nM), or RA (1 μM) for 6 days. Media samples were concentrated, reduced in Laemmli's buffer, and electrophoresed on 5-15% polyacrylamide gradient gels. The SDS-PAGE profiles were transferred onto nitrocellulose sheets which were then fixed and immunostained with anti-FN antibody (1:4000) followed by peroxidase-conjugated anti-rabbit IgG (1:400). Sites of antibody binding were visualized using diaminobenzedine-$(HCl)_4$ / H_2O_2. (Lane 1) Immunoblot of FBS (10 μg), showing that antibody binds to a band-pair with an apparent polypeptide chain molecular weight 240-220 kD. (Lane 2) Immunoreactivity of FN-polypeptides present in conditioned media was blocked by preincubating anti-FN antibody with purified FN. Immunoblots of conditioned media from SH-SY5Y cultures grown in (Lane 3) N2 medium and in N2 containing (Lane 4) PMA, (Lane 5) RA, and (Lane 6) NGF show similar immunoreactive polypeptides (loads of 10X concentrates were normalized to total culture protein). (Lane 7) Defined N2 medium (75 μl of a 10X concentrate) has no inherent FN-immunoreactivity. (Top To bottom) Myosin heavy chain (200 kD), phosphorylase B (94 kD), BSA (68 kD), and ovalbumin (44 kD) served as molecular weight markers.

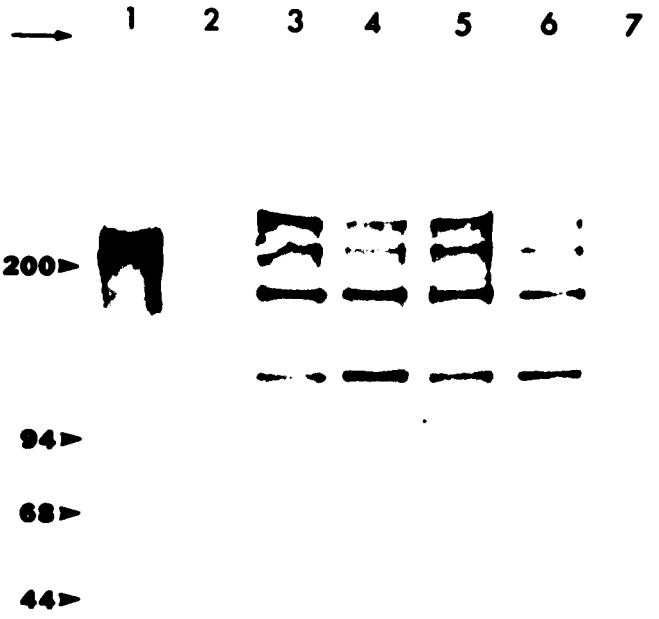

Figure 27.

Table III. FN content of media conditioned by SH-SY5Y cultures treated with morphology-altering agents.

Treatment	Media FN (ng / mg t.c.p.)		
(days)	1-2	3-4	5-6
N2 medium	190	190	200
N2 + PMA	180	190	200
N2 + NGF	170	180	190
N2 + RA	190	200	210

An enzyme-linked immunosorbant assay (ELISA) was used to quantify FN secreted into a defined media by neuroblastoma cultures treated with PMA, NGF, or RA. Cells were grown and treated in 75 cm^2 flasks. Three sets of cultures were established by pretreating cells for 0, 2, or 4 days with N2 medium alone or N2 containing PMA (17 nM), NGF (4 nM), or RA (1 µM) before media assays commenced. The FN (ng) content of the media conditioned for the next 2 days was measured and expressed as a fraction of the total cell protein of the culture (mg t.c.p.) from which the media sample was rendered. The values given are averages from two determinations. (SD less than 5%).

samples were normalized to the total cellular protein of the culture from which they were rendered, the conditioned media samples for each 2-day interval contained approimately 200 ng of FN per mg cell protein. The results of immunoassays (Table III) indicated that FN secretion was virtually uneffected by the morphology-altering treatments. These treatments changed neurite outgrowth and the mitotic activity of SH-SY5Y cells to varying degrees; these changes apparently were not dependent on alterations in FN secretion. SDS-PAGE immunoblots of media samples from each treatment showed nearly identical profiles when immunostained with anti-FN (Fig. 27). In addition to the 240-220 kD FN subunits, several lower molecular weight, FN-immunoreactive species were stained. The latter FN-polypeptides were probably the result of normal cellular protease activity and were demonstrated consistently across treatments. These assays did not account for the full amount of FN secretion since de novo FN was also found on the cell surface and adsorption to the culture flask occurred.

FN Accumulation on the Cell Surface

An immunoassay was used to measure cell surface FN. After trypsin / EDTA detachment, SH-SY5Y cells had little surface FN. Cells maintained in suspension, however, showed the capacity to accumulate soluble FN on their surface. This capacity increased steadily during 4 hours while in suspension following trypsin / EDTA treatment. While suspended in medium containing PMA, FN-immunactivity was substantially reduced relative to untreated cells (Fig. 28). The reduced capacity to bind soluble FN resulting from PMA treatment persisted throughout the duration of the assays. Compared to the binding activities of PMA-treated cells, untreated cells in defined medium showed FN-binding approximately 2-fold greater after two hours and this

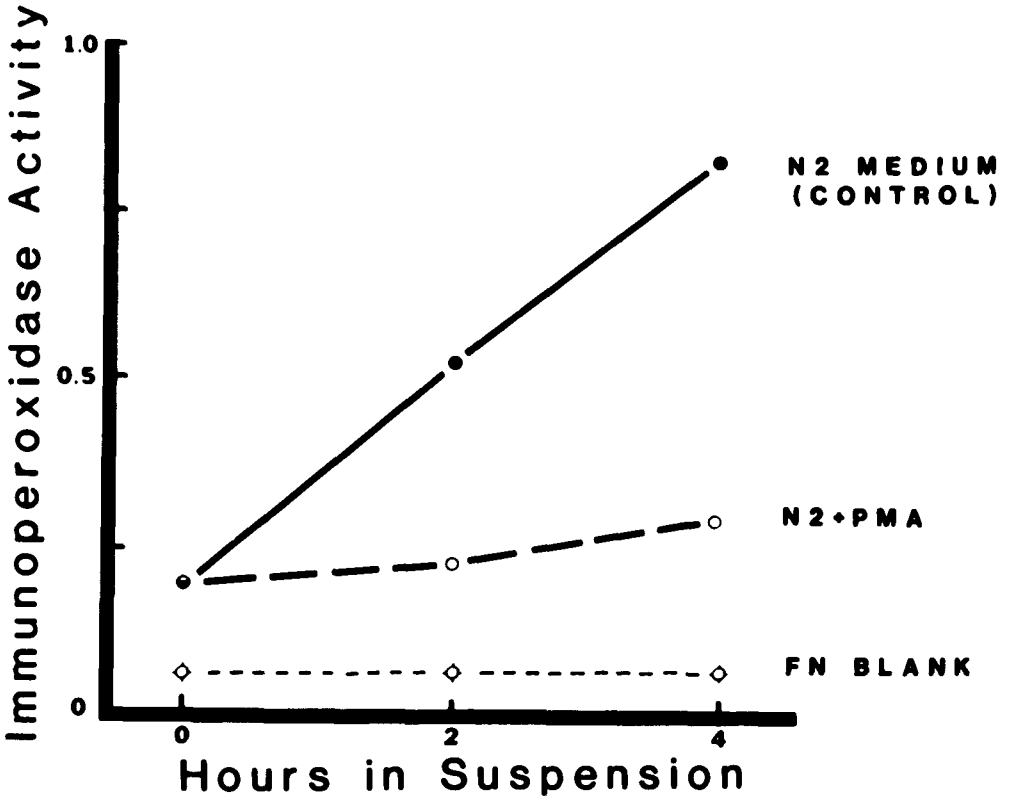

Figure 28. ELISA: FN accumulation on the surface of suspended neuroblastoma cells. Trypsin / EDTA detached cells were treated in suspension with N2 medium or N2 containing PMA. After 0, 2, and 4 hrs., soluble FN (50 μg/ml) was added to the suspensions for 15 min. FN bound on the cell surface was measured by ELISA using FN-antibody and a peroxidase-conjugated secondary antibody. The immunoperoxidase activity per 10^5 immunolabeled cells was measured as a change in absorbance, delta OD_{492nm} following the addition of chromogen. The FN Blank plot represents the FN bound to cells at 0, 2, and 4 hrs. without the addition of exogenous FN, representing the FN secreted by the cells which might have accumulated on the cells during the treatment periods. Each data point represents the mean of nine determinations from two experiments. (SD less than 10%).

increased to 3-fold by four hours. These findings indicate that most of the extracellular FN was dissociated from the cell surface after trypsin / EDTA detachment. Relative to untreated cells, PMA-treated cells showed a significantly reduced rate of recovery in FN-binding capacity following detachment.

Intracellular FN Immunofluorescence

Without permeabilizing pretreatment, immunofluorescence cytochemistry of SH-SY5Y cells grown under serum-free conditions demonstrated only faint, sometimes spotty FN-staining at the periphery of growing margins. After Triton-permeabilization, intracellular FN-immunofluorescence was concentrated in the cell body and in the core of many neurites (Fig. 29). On a FN-coated coverslip (which carpeted the substratum with specific immunofluorescent staining) SH-SY5Y cells produced neurites and cell margins which were clearly outlined against the substratum by their lack of intracellular FN-labeling. Clearly, immunoreagents were unable to gain access to the substratum-bound FN underneath cell pocesses. This allowed simultaneous examination of the intracellular distribution of FN and the extent of lamellar cell processes which did not show substantial immunofluorescence. Some untreated cells had splayed neurites with many lamellar margins and FN-staining appeared only in the cytoskeleton core of major processes (Fig. 29a-c). In contrast, most PMA treated cells had neuron-like morphology with thin, cylindrical neurites which labeled intensely, and large, lamellar distal tips (growth cones) which did not show staining (Fig. 29d-f). Intracellular localizations indicated that FN was, to some extent, transported within neurites but did not enter the growing tips of neurites or spread margins.

Figure 29. FN immunofluorescence staining of intracellular FN. The substratum was coated with FN (20µg/ml/0.1cm^2) which produced a homogeneous carpet of FN-immunofluorescence. SH-SY5Y cultures were grown for 4 days then fixed and permeabilized prior to immunocytochemistry to demonstrate the distribution of cytoplasmic FN. (a-c) Epifluorescence micrographs showing cells maintained in serum-free N2 medium. (d-f) Cells grown in the presence of PMA. Unstained regions are dark compared to intensely stained cell bodies and well differentiated neurites and stand out by contrast against the moderately stained FN-coated substratum. Cytoplasmic FN was often seen in the core of undifferentiated neurites (arrowheads). Bar 25 µm.

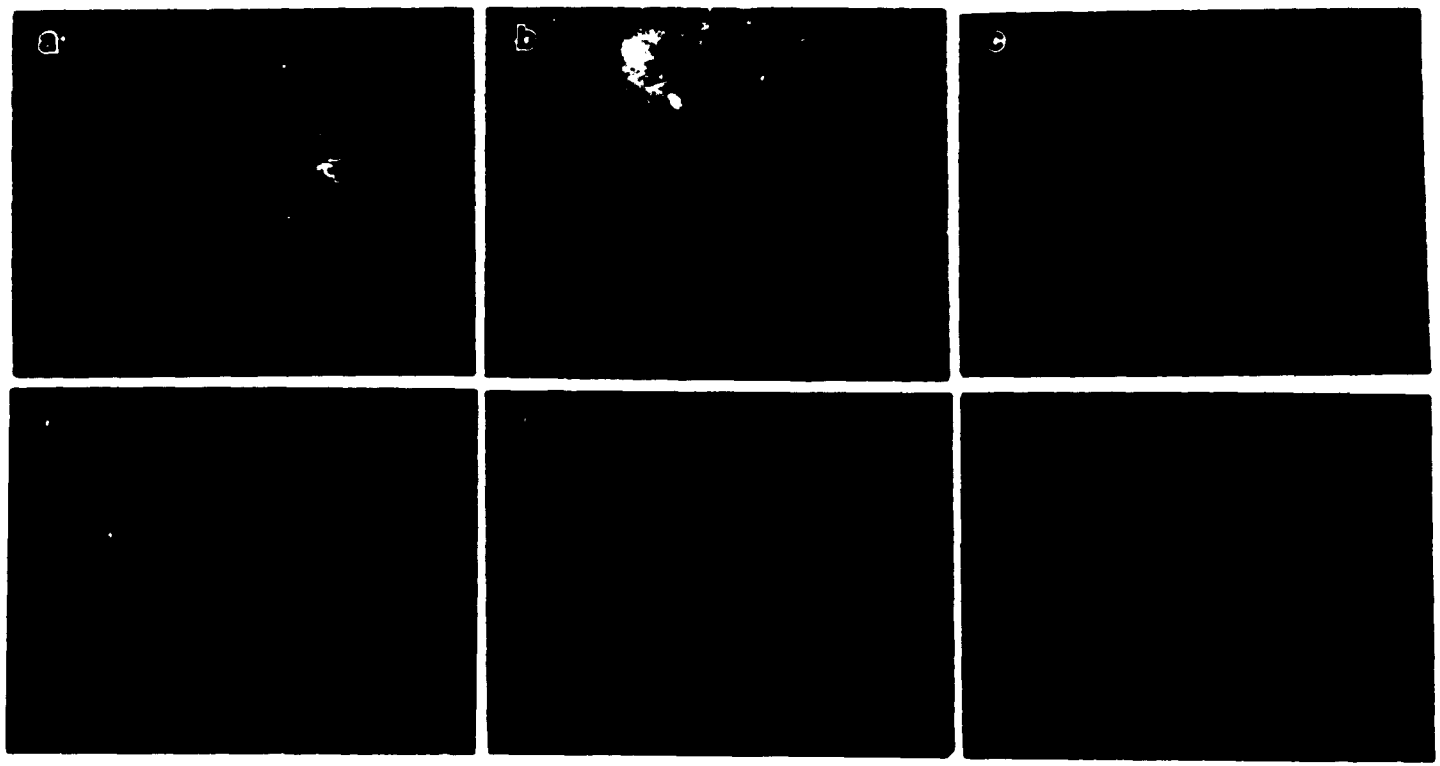

Figure 29.

Whole Mount Specimen Preparations and FN-Immunoaccessibility

Cells grown directly on coated EM grids and processed for WMTEM without Triton-extraction (during fixation) or permeabilization (following fixation) were not penetrated by antibody probes. Indirect immunogold labeling with anti-FN antibody demonstrated that FN was not found diffusely on the cell surface. Only the FN associated with filopodial contact pads was immunolabeled without the aid of membrane permeabilizing agents. Even after Triton-permeabilization, antibody probes did not label FN-containing sites on the ventral aspect of cell margins (also indicated by immunofluorescence, see above). In addition to filopodial contact pads, FN-containing attachment plaques on the underside of cell margins were labeled with immunogold probes when specimens were Triton-extracted during aldehyde fixation. 20-nm gold probes apparently penetrated the specimen to sites of substratum contact, but a pool of FN was better accessed with 5-nm probes (Fig. 30). When applied in sequence to the same specimen, the 5-nm probes always labeled some FN antibody at the attachment site which had not been first bound by 20-nm probes (Fig. 30a-d). The restricted immunoaccessibility of gold probes was a property of the plaque structure or close apposition to the substratum since similar results with 5-nm and 20-nm probes were also demonstrated in the remnant substratum-attached material detached from cells which had been sheared-off during processing (Fig. 30b). Remnant plaques were easily distinguished by their morphology and FN content, and additionally by immunolabeling for actin.

The Effect of Exogenous FN on the Formation of Attachment Plaques

Under serum-free conditions, SH-SY5Y cells secreted FN into N2 medium and produced small attachment sites that labeled moderately for FN using immunogold probes (Fig. 31a-d). The appearance of plaques and the density of

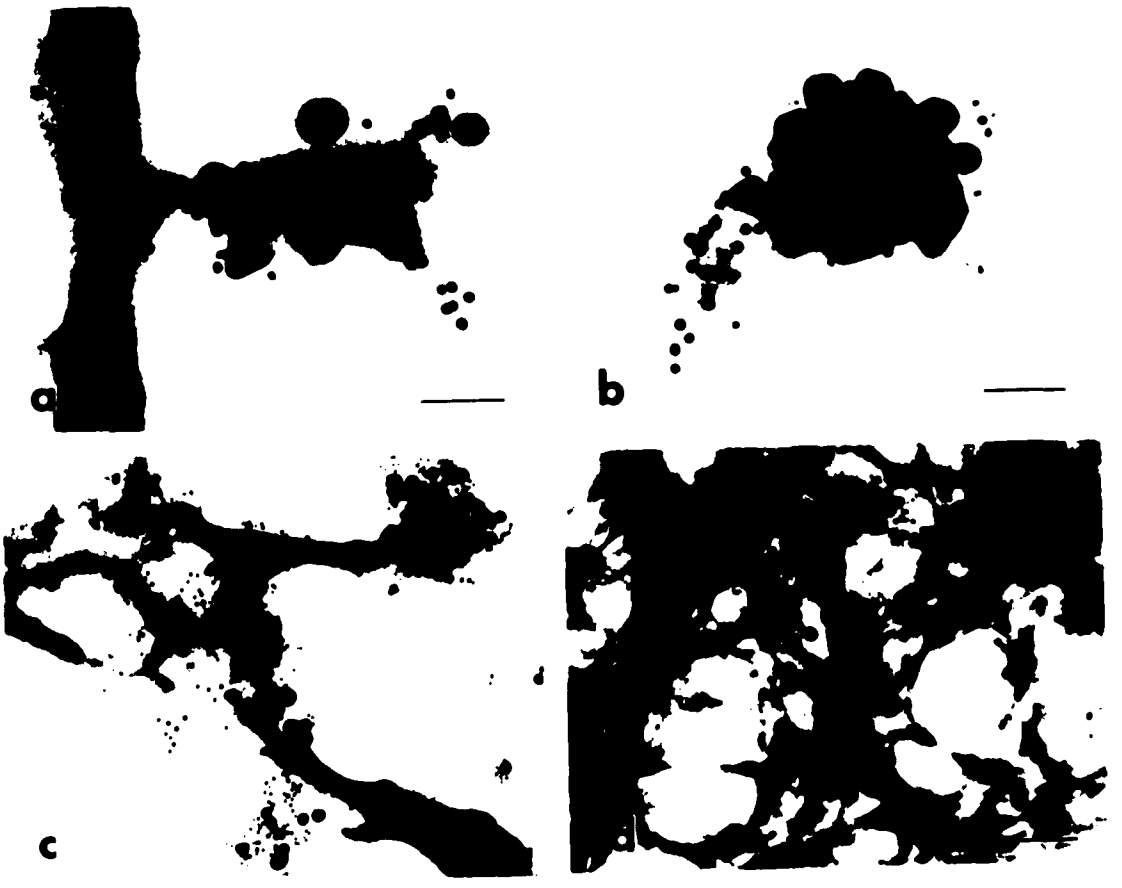

Figure 30. The immunoaccessibility to specimen-bound anti-FN antibody by secondary antibody adsorbed to two different size gold particles. WMTEM of filopodia immunogold labeled for FN demonstrate FN-containing filopodial footpads (a), remnant footpads found free on the substratum (b), and FN-containing plaques under variably Triton-extracted distal margins (c,d). Following application of primary antibody against FN, specimens were labeled with a 20-nm gold particle-bound secondary antibody then additionally with a 5-nm secondary probe. Bar, (a,b) 50 nm; (c,d) 100 nm.

their FN-immunolabel increased on cells maintained in SH-SY5Y -conditioned medium. This occurrence was more evident following the addition of purified serum FN to the medium (Fig. 31e-g). In the presence of soluble FN, filopodia showed numerous FN-containing contact pads within 60 minutes and attachment sites on the ventral surface of the distal growth cone were abundant thereafter. Furthermore, with the addition of exogenous FN, relatively large plaques resulted and the FN immunolabeling of many plaques was conspicuously more intense. These effects were only prevalent in control cells; PMA-treated cells did not aggregate substantial amounts of the exogenous FN and produce plaques (Fig. 32).

Immunogold Labeling with FN-Antibody Applied to Living Cells

As stated above, the formation of FN-containing attachment sites on filopodia was enhanced in SH-SY5Y cells after the level of FN in the media was increased for 60 minutes. Also, once accumulated on the contact pads of filopodia, this pool of FN was accessible to FN-antibody without permeabilizing the cell membrane. In other words, the FN content of contact pads on the filopodia of unfixed, living cells was accessible to FN-antibody. After cells were maintained in culture for up to 2 hours the bound FN-antibody was successfully detected, following fixation and extraction, throughout the specimen with secondary antibody adsorbed to gold particles. Figure 33 shows filopodia and distal margins of cells prepared for WMTEM after immunolabeling for FN as living specimens. First shown is the presence of FN in a filopodial contact pad distal to the leading edge of the microfilament meshwork. It apeared, that in a progressive fashion, contact pads were often covered over by the advancing distal margin as it proceeded to fill the space between two adjacent filopodia. Eventually, these FN-containing sites coud be found under the cell margin

closely associated with a bundle of microfilaments which constituted the once distal filopodium. This association often persisted as the lamellipodia continued to advance, but in some cases was seen to become diffuse as the filamentous bundles in the cytoplasm became less organized (Fig. 34).

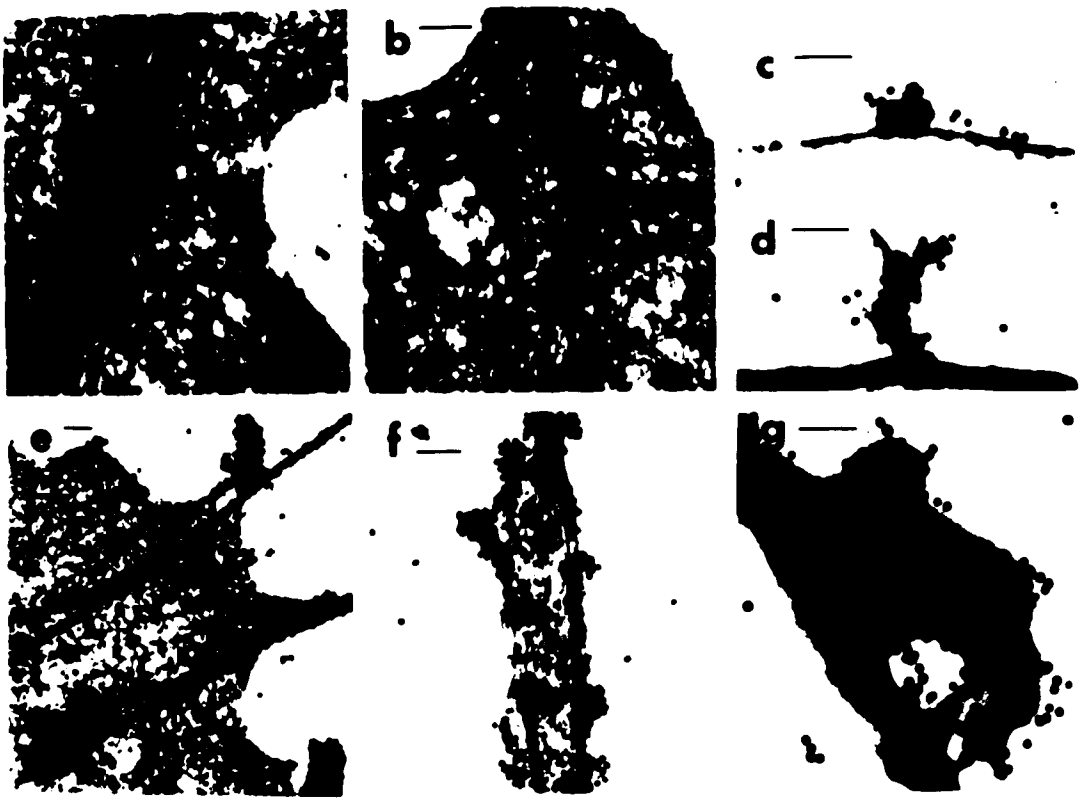

Figure 31. Immunogold WMTEM of FN-containing attachment plaques on SH-SY5Y neuroblastoma neurite tips and filopodia. Without the addition of exogenous FN to the culture, modest FN-labeling of relatively small attachment plaques appeared under neurites (a,b) and on filopodia (c,d). The FN-containing plaques on neuroblastoma cells grown in N2 medium supplemented with purified FN were more abundant, often very large and heavily labeled for FN (e-g) with 20-nm gold probes. The filopodial structures in c, d, and g are equally magnified. Bar 0.2 μm.

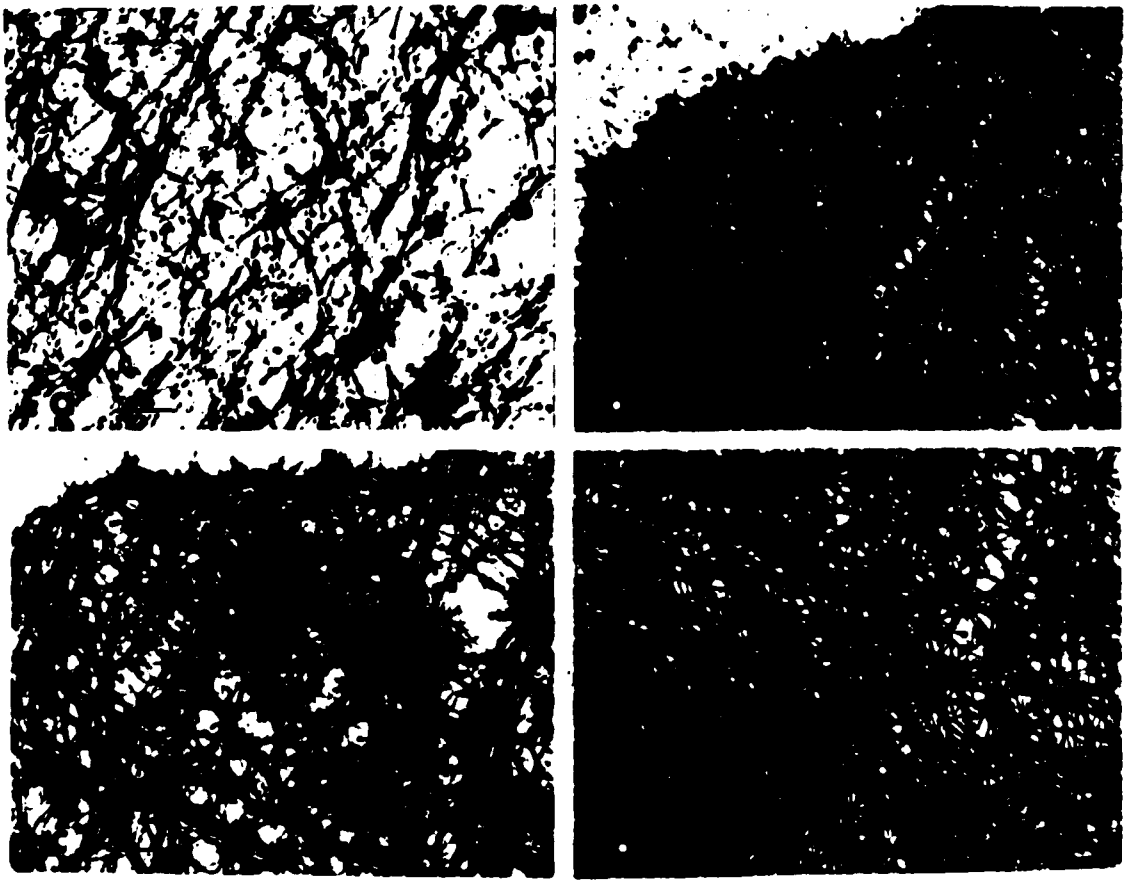

Figure 32. WMTEM of FN-immunogold labeled cell margins of SH-SY5Y cells grown in the presence of PMA. Few accumulations of FN are immunolabeled (arrowheads). Print quality was altered to highlight scant immunogold labeling (a only). Bar, (a) 0.1 μm; (c) 0.2 μm; (b,d) 0.4 μm.

Figure 33. Immunogold WMTEM of FN-containing filopodial footpads, labeling with primary antibody was performed on living cells in culture. Once bound on living cells, the primary antibody followed the FN-containing footpad and appeared to be incorporated into the advancing cell margin. A distal footpad (a) was the only form of substratum-attachment site that was immunoaccessible to primary antibody when applied to living cells. (Figs. a-d) After a 2 hr. growth period the cultures were simultaneously fixed and Triton-extracted (to allow for penetration of secondary antibody). On some filopodia a helical superstructure was evident (small arrowheads) which appeared to link the filopodium to FN-containing contact pads (large arrowheads) Bar 0.2 μm.

Figure 33.

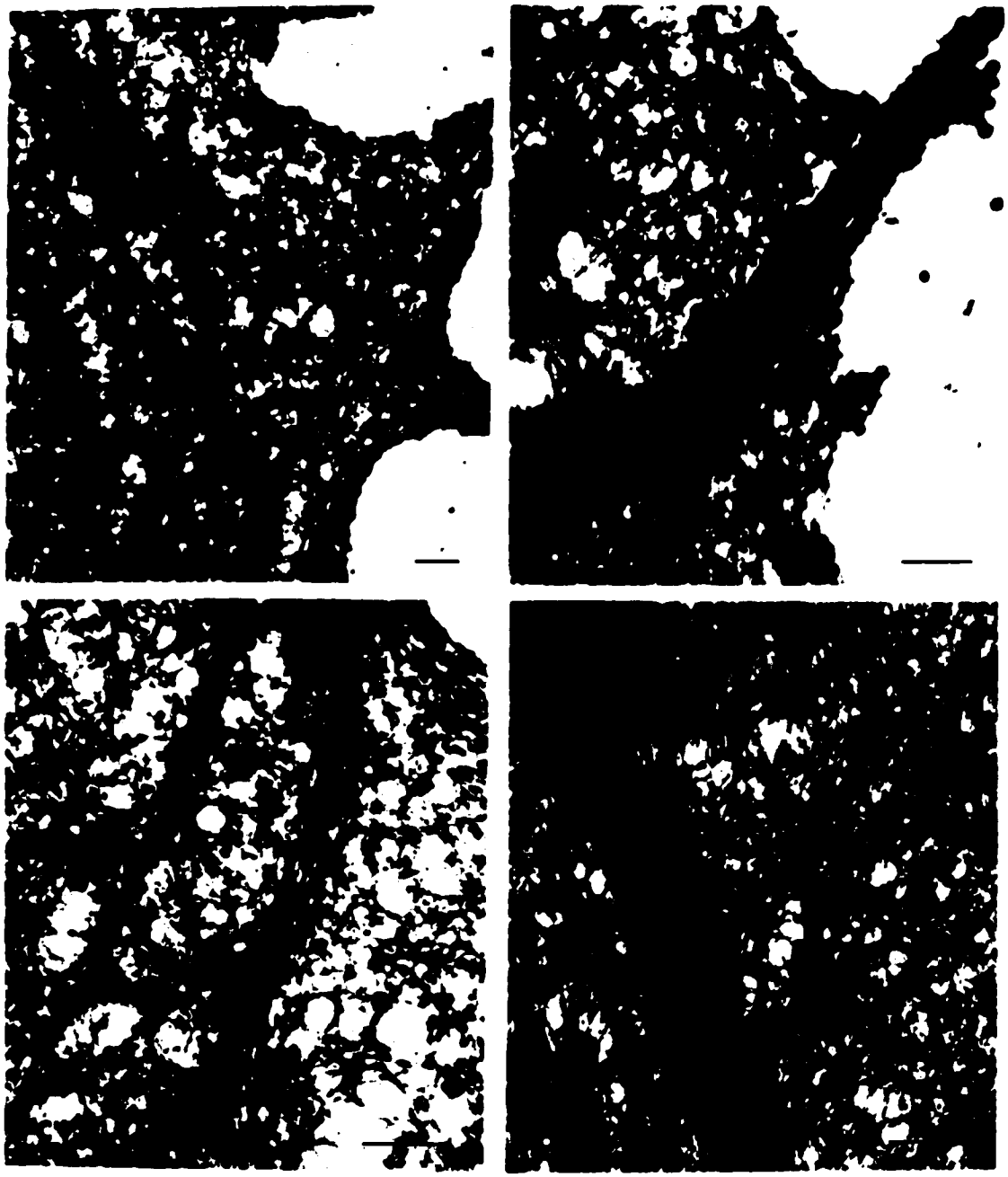

Figure 34. WMTEM of cell margins immunolabeled for FN and counterstained with tannic acid and uranyl acetate illustrate FN-containing attachment sites associated with microfilament bundles which form the roots of filopodia. Bar 0.2 μm.

DISCUSSION

Neuroblastoma cell lines represent a convenient model for studying certain aspects of neuronal development and differentiation. SH-SY5Y Nb cells have many characteristics in common with peripheral neurons in culture. They generate action potentials, and synthesize, store and release catecholamine (Biedler et al., 1973; Biedler et al., 1978). In the course of this study, SH-SY5Y cells were shown to express several embryonic features. Like undifferentiated neurons, they contain vimentin intermediate filaments; under none of the conditions tested were neurofilament proteins detected. For instance, it was previously reported that treatment with PMA resulted in anisotropic morphology and long, straight neurites with varicosities containing vesicles, increased the catecholamine content 30-fold and induced neuron-specific enolase (Pahlman et al., 1981). Our studies confirmed morphologic and neurochemical differentiation of these cells in response to PMA, however, the induction of neurofilament proteins did not accompany the appearance of other neuronal characteristics.

The intermediate filaments is a class of heterogenous proteins, yet a variety of cell types can be characterized by the type of intermediate filament protein they contain (Lazarides, 1980). The neurofilaments are a unique case in that they are composed of three subunit proteins (the neurofilament triplet), the relative amounts of which can change during development, creating heteropolymers with variable composition (Willard, 1983). Early in development, neurons contain variable amounts of vimentin and neurofilament proteins, later to be dominated by neurofilaments. Several reports have been made concerning the intermediate filament content of Nb cell lines. Early studies using preparations of bovine white matter and whole brain to produce antibodies were hampered by a confusion concerning the molecular weight of neurofilament

polypeptides. Conclusions about the neurofilament immunoreactivity of mouse Nb lines (clones of C1300) (Dahl and Bignami, 1978) were later questioned due to the use of antibodies raised against a mistaken protein. More recently, studies using antibodies which specifically recognize the 68 kD neurofilament protein have shown that the C1300 lines do not contain neurofilament proteins and were only stained by intermediate filament antibody raised against vimentin (Virtanen et al., 1981). In the closely related rat PC12 cells both vimentin and neurofilament proteins were found and following treatment with NGF, which induces neuronal differentiation (Greene, 1978), a 3-fold increase in the proportion of 68 kD protein resulted (Lee and Page, 1985).

Consistent with their embryonic nature, SH-SY5Y cells were found to express vimentin. Throughout this study a variety of morphology-altering or neurochemical differentiating treatments were applied to SH-SY5Y cultures. No condition tested induced amounts of neurofilament proteins that were detected by ELISA or immunoblotting assays.

Cell-Substrata Attachment and Neurite Sprouting

Neuronal cells are dependent on adhesive contacts with their surrounding surface in order to form neurites (Letourneau, 1975; Rauvala, 1984; Rogers et al., 1983). Letourneau (1975) has shown that axonal outgrowth is facilitated on a surface with increased adhesiveness . The adhesiveness of a surface per se may not be a sufficient condition for initiation of neurite outgrowth, although it is a necessary one. My results indicate cell-substratum attachment and neurite sprouting are separable processes. This inference is supported by the finding that many substratum coatings, to which cells effectively adhere, fail to enhance sprouting (Rauvala, 1984). For example, SH-SY5Y Nb cells attached to a collagen surface but have attenuated neurite outgrowth (Spinelli et al., 1982). In

addition, cell attachment was generally reduced in the presence of serum, yet in response to PMA an overall increase in neurite production occurred (Pahlman et al., 1981; Spinelli et al., 1982). By comparison, under serum-free conditions, SH-SY5Y cells attached better to tissue culture dishes and PMA treatment retarded neurite sprouting and partially inhibited neurite elongation. In contrast, without PMA treatment Nb cells respond to serum deprivation by producing long neurites. Clearly, PMA alters the cellular response to the neurite-promoting and/or the haptotaxic properties of serum.

FN Binding

Following detachment from the culture dish with trypsin, SH-SY5Y cells in suspension showed an ability to bind soluble FN which increased over four hours; PMA retarded this increase. These results suggested that PMA treatment affected the FN-binding capacity of suspended cells. In suspension, rounded cells normally project numerous filopodia and my results indicated that filopodia are an important site for the clustering of bound FN. Perhaps, as more filopodia are produced, the cells show a greater FN-binding capacity. In this regard, PMA was shown to disrupt cytoskeleton organization and inhibit the formation of filopodial projections as well as reduce the amount of FN which bound to the surface of Nb cells in suspension.

FN Synthesis and Distribution

FNs are high molecular weight adhesive glycoproteins present in soluble form in plasma and other body fluids, and in insoluble form in the extracellular matrix and basal lamina. The FN molecules contain a cell-binding region (the FN-receptor complex) and binding sites for collagen, heparin, ganglioside and fibrin (see Hynes, 1981). Recently it has been shown that FN polymorphism may

occur. In addition, tumor-derived or transformed human cells release into the culture media different FN variants than do normal cells, suggesting a specifically programmed splicing mechanism in different cell types and that in transformed or tumor-derived cells these mechanisms are altered (Castellani et al., 1986).

In this study, SH-SY5Y Nb cells were plated and maintained in serum-free supplemented N2 medium originally described by Bottenstein and Sato (1979) for maintaining the rat Nb line B104 in defined medium. Additionally they reported that the addition of FN to N2 medium and coating tissue culture dishes with polylysine were required to permit cell division to occur (Bottenstein and Sato, 1980). For SH-SY5Y cultures these additions were not necessary for proliferation, which was prolonged by the use of SH-SY5Y-conditioned N2 medium. In light of previous findings that the mouse Nb line C1300 produced FN (Alitalo et al., 1980), SH-SY5Y-conditioned medium was assayed and de novo FN was found. Since it has been reported that normal neuronal cells may not produce FN (Paetau et al., 1980; Schachner et al., 1978), it was thought that certain agents which differentiate Nb cells might alter the secretion of FN and indicate an important aspect in Nb differentiation. In the C1300 Nb line, dibutyryl cyclic AMP did not alter FN secretion (Alitalo et al., 1980). Agents such as NGF (Spinelli et al., 1982), RA (Sidell et al., 1983), and PMA (Pahlman et al., 1981; Spinelli et al., 1982) variably differentated and altered the morphology of SH-SY5Y cells, yet immunoassay of conditioned N2 medium rendered from SH-SY5Y cultures treated with NGF, RA, or PMA showed that FN secretion was unaffected. The relative amounts of secreted FN in SH-SY5Y-conditioned media was consistent and probably not an important variable in the effects of these agents on SH-SY5Y morphology. In addition, FN-immunoblots of the conditioned media showed FN polypeptides and extracellular enzymatic degradation products

were similar for cells under all treatments.

The intracellular distribution of FN was examined by immunofluorescence in Nb cells after treatment with morphology-altering agents. In all conditions, FN was concentrated in the perikaryon and staining was usually evident in the core of major cell processes but not in splayed margins. Well differentiated (axon-like) neurites were densely stained by FN antibody; these processes had splayed growth cones which did not demonstrate cytoplasmic FN. Immunocytochemical localization in Nb cells suggested that FN is synthesized in the cell body and at least some is transported distally within neurites, then possibly externalized at sites proximal to growing margins and growth cones.

The Formation of FN-containing Sites on Filopodia

FN released into the medium did not diffusely accumulate on the cell surface or form fibers along the ventral surface. Immunofluorescence of intact (unpermeabilized) cells showed that FN was localized only in proximity to areas of cell-substratum apposition and was most evident at the distal tip of growing neurites. Electron microscopy of cells prepared as cytoskeleton whole mounts demonstrated immunogold labeling for FN in dense clusters associated with filopodia and the underside of the distal growth cone. FN-containing sites were securely attached to the culture surface. This was supported by the identification of morpohologically and immunologically similar profiles that appeared as remnant plaques attached to the substratum, probably the result of shearing cells from the substratum during specimen processing. Clusters of FN-label were not randomly scattered on the substratum but were found only on morphologically distinct plaques that also labeled for actin and were probably similar to the substratum-attached material of Nb cells described by Domen and Culp (1981).

FN immunogold labeling scarcely appeared on the filopodium itself. FN was found in clusters associated with contact pads that usually appeared as small filopodial appendages. In some cases, clusters of label appeared only at the end of a filopodium with little evidence of FN being transported along the filopodium. This suggested that filopodia sequester FN from the media proximal to FN-containing substratum-attachment sites. The observation that filopodia formed more abundant FN-containing contact pads after the addition of purified serum FN to defined medium supported this view and that either the FN-binding sites were present in excess on the filopodial surface or an increased supply of receptors was made accessible in response to exogenous FN. It seemed likely that once FN was bound to the filopodial surface, FN-binding domains were aggregated during formation of a dense plaque. Heavy metal staining of specimens for electron microscopy showed that plaques appeared and became more electron-dense as the amount of FN in the medium was increased. This was not the case when cells were grown on a coated surface with high concentrations of FN securely bound. Concerning the possibility that attachment sites contained FN that had been removed from the substratum, in a recent report describing substratum-bound FN it was concluded that fibroblasts can remove FN from the substratum and reorganize this material into patches which co-localize with FN-receptors. However, FN was not removed from the substratum when fibroblasts were grown in serum-free medium (Grinnell, 1986).

Recent studies have shown the FN and its 140 kD membrane receptor complex are spatially associated with microfilaments to form cell surface linkage complexes which are thought to mediate adhesive interactions between cultured cells and their substratum. Results suggest that FN interaction with cells regulates the organization of FN-receptor complexes and cytoskeletal components at the cell surface (Chen et al., 1986). Interestingly, while

transformation disrupts the organization of cell surface linkage complexes, transformation had little effect on the total expression of the FN-receptor although large proportions of the total were released into the culture medium by transformed cells (Chen et al., 1986). In addition, arrays of FN and FN-receptor were reconstituted on transformed fibroblasts by the exogenous addition of purified FN. In the present study on Nb cells it was indicated that FN regulated the distribution of its binding sites on growth cone filopodia, and that clustered binding sites were more abundant in the presence of exogenous FN. These sites were stable over short periods of time and were continually formed, again suggesting a regulatory role for FN and FN-receptor interactions in regulating the localization of FN at adhesive contacts throughout the process of growth cone advance. This type of externally controlled receptor system could provide a particularly sensitive mechanism for locally regulating the organization of cytoskeletal elements such as microfilament bundles that may associate directly with the FN-receptor. Such a system could rapidly coordinate microfilament systems and growth cone advance in response to the local extracellular microenvironment.

The association of microfilaments with FN and FN-binding domain has been well documented (see Singer, 1979). In addition, an association between intermediate filaments and the cell membrane complex has been described in epithelial cells where keratin-containing filaments interact in some way with desmosomal junctions with neighboring cells. In other cell types, where cell surface attachment structures are not so apparent, the nature of intermediate filament-cell membrane complex interaction is difficult to approach experimentally. From ultrastructural observations it was concluded that intermediate filaments interact with a cell membrane complex which probably associates with extracellular FN in cultured fibroblasts (Green and Goldman,

1986). Using high voltage electron microscopy, intermediate filaments have been found to link structures of the surface membrane of neuronal axons and the surface of Schwann cells at specialized sites of cell-cell apposition (Deernick and Ellisman, 1985). In addition, intermediate filament proteins and FN were shown to be a constituent of the substratum-attached material of Nb cells detached from culture dishes by calcium chelation. The existence of such an interaction between FN, the FN-receptor complex and intracellular intermediate filaments could have far-reaching implications for many aspects of cell biology such as transduction of signals from the substratum to cytoplasmic constituents, the determination and maintenance of cell shape, the distribution of cell-substrate adhesion points, and intracellular transport. It is interesting that Nb cells treated with PMA lack intermediate filaments in the distal margins of growth cones and coincidentally do not retain the capacity to form FN-containing attachment plaques.

The Role of Filopodia and FN-Containing Sites in Growth Cone Advance

The morphological events in the formation of focal adhesion sites of fibroblasts to serum-coated tissue culture substrata have been elucidated by studies using light and interference reflection microscopy and by transmission and scanning electron microscopy (Avnur and Geiger, 1981; Heath and Dunn, 1978; Rogers et al., 1983; Singer, 1979, 1982; Thom et al., 1979; Wehland et al., 1979). Much more limited information is known about the characteristics of neuronal cell interactions with a substratum involving FN. Neurite outgrowth appears to involve only contact of the growth cone with the substratum and not the neurite (axon) itself (Boucaut et al., 1984; Letourneau, 1975). Studies of Nb cells have reported the formation of a ruffled surface membrane at the end of the neurite during elongation with long prominent filopodia, probably for

substratum-exploratory function (Domen and Culp, 1981). SH-SY5Y cells produce growth cones with numerous filopodia. Immunogold labeling of whole mount cells showed FN-containing contact pads on certain filopodia and not others even after cells were maintained in medium supplemented with FN. In many cases a particular filopodium (or set of proximal filopodia) had several contact pads and FN-containing plaques were found under the distal growth cone in association with microfilament bundles in the cytoplasm which formed the root of the filopodium. Apparently, certain filopodia were associated with multiple sites of substratum attachment involving FN and were continuous with specific bundles of filaments in the distal growth cone. On the basis of cinematographic and high-voltage electron microscopic study, Tosney and Wessells (1983) reported that bundles of microfilaments were found only in attached filopodia while unattached filopodia were filled with a meshwork of filamentous actin. The filopodia of SH-SY5Y cells attached to FN-containing contact pads had dense filament bundles that appeared to be the core for an outer helical structure which spanned its length. The substance of the helix connected with the contact pad and linked the filopodium to the contact pad at multiple sites on the helix. In some cases, two filopodia were linked in this fashion to opposite sides of the same contact pad (Fig. 33). FN was strongly implicated in mediating the association between filopodia and contact pads and, although further study is required to elucidate the nature of this junction, a fine ultrastructural feature of filopodial superstructure immunolabeled by actin antibody was documented (Figs. 21, 22 and 33). The co-localization of actin and FN at these sites is reminiscent of sites of substratum-contact described for other anchorage-dependent cells in vitro.

Despite substantial contributions from the study of motility in other cell systems (Goldman et al., 1976) and recent studies on neurons in tissue culture

(Argiro et al., 1984; Bray, 1982; Johnston and Wessells, 1980) no definitive description of the process of neurite assembly and elongation at the growth cone has emerged. Filopodial extension is an integral element of growth cone behavior and probably participates in neurite growth control and guidance and in determining cell shape (Bray, 1982). Argiro and coworkers (1985) observed neurite outgrowth in embryonic and adult ganglia by time-lapse cinematography. They found that filopodia were straight and rigid throughout their extension, and landmarks along the shaft of a filopodium, such as slight protrusions, remained the same distance from the point of origin as the filopodium reached greater length. Once extended, many filopodia were quickly retracted and absorbed into the growth cone. Some filopodia were more stable, possibly because they adhered to the substratum, until they were expanded from base toward top by the addition of cytoplasm and membrane and became part of a lamellipodium or the body of the advancing growth cone. Examinations of whole mount proparations of cells immunolabeled for FN while actively growing in culture provided evidence that filopodia attached to contact pads served as structural support for growth cone advance (Fig. 33). Filopodia appeared stabilized by their association with FN-containing contact pads, which remained attached to the substratum and demarcated the path of elongation. My results indicate that cytoplasmic advancement does occur between a pair of adjacent filopodia. This interpretation is in accordance with high resolution video-enhanced imaging of regenerative axon growth which showed lamellipodia almost always formed with filopodial borders and protrude between either fully extended or growing filopodia (Goldberg and Burmeister, 1986). The contact pads and filopodia of SH-SY5Y cells continued to demarcate and support growth cone elongation as they appeared to be incorporated into the body of the growth cone and took on the characteristics of focal attachment sites and cytoplasmic

microfilament bundles which retained a close association throughout. It follows that the dimensions of attachment plaques and microfilament bundles are directly attributed to the processes of contact pad formation and the extension of filopodia.

PMA-Induced Alterations

The adhesion plaque protein vinculin is a phosphoprotein and has been shown in vitro to be a substrate for protein kinase C during PMA activation (Leach et al., 1983). Accordingly, the level of phosphorylation is a possible regulatory event, one strengthened by the observation that transformed cells, vinculin is "over phosphorylated" (Werth and Pastan, 1984). This biochemical change is accompanied by loss of adhesion plaques (David-Pfeuty and Singer, 1980). Furthermore, it has been reported that changes in vinculin plaques might preceded changes in the organization of actin-containing filament bundles (Kellie et al., 1985; Schliwa et al., 1984) following PMA treatment.

Tumor promoter-induced changes of the neuronal cytoskeleton have not been the subject of much study. PMA-induced alterations in kidney cells associated with the cytoskeleton were described as a rapidly occurring reorganization of filamentous actin and the adhesion plaque protein vinculin (which often co-localize with cell surface FN). The reorganization occurred in a highly coordinated fashion and involved two seemingly independent but overlapping processes: (a) dismantling of stress fibers and associated adhesion plaques; and (b) development of actin networks with newly formed vinculin sites that are punctate, dotted or diffuse (Schliwa et al., 1984). Therefore, contact to the substratum was largely uncoupled from the distribution of actin and vinculin after PMA treatment. Similarly, PMA-induced microfilament redistribution was paralleled by a rearrangement of alpha-actinin and a reduction in the number of

vinculin-containing adhesion plaques (Kellie et al., 1985). Actin, alpha-actinin, vinculin and FN have all been shown to associate strongly with the plasma membrane and their organization can be profoundly influenced by changes in the distribution of certain membrane receptors or in the extracellular matrix composition (Geiger, 1985; Singer, 1982). In SH-SY5Y cells, the distributions of FN binding and microfilaments was drastically altered by PMA treatment, resulting in scant and diffuse sites of FN aggregation. Microfilament bundles seen in untreated cells were rearranged into a dense meshwork without any obvious orientation (Fig. 32). This reorganization was coincident with a two-fold reduction in the fraction of actin which was extracted from the cell by Triton solubilization (Table I).

Trophic factors can influence neurite outgrowth by regulating growth cone activity. However, the process of neurite elongation is not strictly dependent upon intact growth cone (Marsh and Letourneau, 1984). Growth cones can exist and move at times without filopodia (that is, when they are predominantly lamellipodial in form). The predominance of lamellipodial or filopodial conformations has shown a correlation with the rate of growth cone advance (Argiro et al., 1984). The work of Albrecht-Buehler (1976) suggests that filopodia of spreading fibroblasts serve as organs which explore the environment and react to a certain quality of the substrate. Subsequently they mediate the extension of lamellipodia in the direction of that quality. The described phenomena were reversibly inhibited by treating fibroblasts with the actin-binding protein cytochalasin B. However, neurite elongation can occur in the presence of cytochalasin B although the appearance of filopodia at the growing tip was inhibited (Marsh and Letournau, 1984). In this study PMA was found to inhibit the formation of microfilament bundles, filopodia, and FN-containing attachment plaques by SH-SY5Y growth cones. PMA-induced growth cones had

the shape of large lamellipodia and, while PMA treatment retarded neurite elongation, neurites did slowly extend over several days without the presence of characteristic filopodia.

This examination of Nb cells has provided evidence that filopodia mediate growth cone advance and guidance involving the formation of FN-containing plaques. Filopodia appear to support the advance of lamellipodial excresences. An understanding of whether the attachment of filopodia to FN-containing substratum-attachment plaques constitutes a precise signal for lamellipodial spreading is important for studying growth cone guidance and neuronal development and regeneration.

A critical feature of the role of cell-substrate adhesions is their action on local actin-myosin movements to promote the advance of organelles in the vicinity of firmly adherent protrusions. In vitro observations of neuronal growth cones find numerous long spike-like filopodia which radiate in many directions, transiently exploring their environment and then retracted in a contractile cycle and become indistinguishable within minutes (Argiro et al., 1985; Bray and Chapman, 1985). Some filopodia adhere strongly to specific surfaces through noncovalent adhesive bonds that might involve selective molecular recognition (such as between a receptor and a ligand). If the adhesion is strong enough to withstand the tension of the growth cone contraction, a stress vector is created and polymerization of filamentous proteins increases proximally (Bray, 1982). Lamellar extension is not initiated if the retraction force on the filopodium leads to an actual shortening of the whole filopodium due to inadequate adhesion; that is, lamellar advance requires an isometric retraction of the filopodium (Tosney and Wessells, 1983). Apparently, a cell tests the firmness of a filopodial contact with the substratum before it extends a lamellipodium, by testing whether the retraction force will break contact and retract the filopodium. The proposal,

supported by immunoelectron microscopic localizations, is that those structures linked to myosin, such as sacs of membrane precursors, neurofilaments and microtubules, will be pulled toward the adhesive contacts. This forward movement promotes neurite growth by drawing new membrane to the front of the neurite tip, as well as pulling cytoskeletal fibers forward from the base of the growth cone to positions where they may be further lengthened by terminal additions of monomers (Lasek and Hoffman, 1976).

In the growth cone margins and filopodia of SH-SY5Y cells, actin and myosin are consistently found. Filopodia that were attached to the substratum by FN-containing plaques contain the molecular components which are implicated in contractile activity and the anchorage required for the formation of a stress vector. The filopodia of Nb cells and normal neurons are probably specializations which subserve this exact function. The proposal supported by WMTEM examination of the growing tips of SH-SY5Y neurites is that filopodia represent the ultrastructural apparatus for the formation of a contractile scaffold which supports lamellar excrescence of the growth cone as it advances over the substratum by a mechanism involving haptotaxic guidance. The FN-containing sites and microfilament bundles formed in the process of growth cone advance are the direct result of the management of FN, actin, and myosin by filopodia, and the form and shape of these ultrastructural features of the growth cone are the result of filopodial activity.

REFERENCES

Albrecht-Buehler, G. 1976. Filopodia of spreading 3T3 cells. Do they have a substrate-exploring function. J. Cell Biol. 69: 275-286.

Albertini, D.F. and B. Herman. 1984. Cell shape and membrane receptor dynamics. In: Cell and Motility, Vol. 5: The Cytoskeleton, J.W. Shay (ed); Plenum Press, New York, pp. 235-253.

Ali, I.U., V. Mautner, R. Lanza and R.O. Hynes. 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by the addition of a transformation-sensitive surface protein. Cell 11: 115-126.

Alitalo, K., M. Kurkinen, A. Vaheri, I. Virtanen, H. Rhode and R. Timpl. 1980. Basal lamina glycoproteins are produced by neuroblastoma cells. Nature (London) 287: 465-466.

Aloe, L. and R. Levi-Montalcini. 1979. Nerve growth factor-induced transformation of immature chromaffin cells in vitro into sympathetic neurons. Effect of antiserum to nerve growth factor. Proc. Natl. Acad. Sci. (USA) 76: 1246-1250.

Argiro, V., M.B. Bunge, M.I. Johnson. 1985. A quantitative study of growth cone filopodial extension. J. Neurosc. Res. 13: 149-162.

Argiro, V., M.B. Bunge, M.I. Johnson. 1984. Correlation between growth form and movement and their dependence on neuronal age. J. Neurosci. 4(12): 3051-2062.

Avnur, Z. and B. Geiger. 1981. The removal of extracellular fibronectin from areas of cell-substratum-contact. Cell 25: 121-132.

Baetscher, M., D.W. Pumplin, and R.J. Bloch. 1986. Vitronectin at sites of cell-substrate contact. J. Cell Biol. 103: 369-378.

Barnes, E.N., J.L. Biedler, B.A. Spengler and K.M. Lyser. 1981 The fine structure of continuous human neuroblastoma lines SK-N-SH, SK-N-BE and SK-N-MC. In Vitro 17(7): 619-631.

Beckerle, M.C. 1986. Identification of a new protein localized at sites of cell-substrate adhesion. J. Cell Biol. 103: 1679-1687.

Biedler, J.L., L. Helson, B.A. Spengler. 1973. Morphology and growth, tumorgenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res. 33: 2643-2652.

Biedler, J.L., S. Roffler-Tarlov, M. Schachner, L. Freedman. 1978. Multiple transmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 38: 3751-3757.

Blumberg, P.M., P.E. Driedger, and P.W. Rossow. 1976. Effect of a phorbol ester on a transformation-sensitive surface protein of chick fibroblasts. Nature (London) 264: 446-447.

Bottenstein, J.E. and G.H. Sato. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented media. Proc. Natl. Acad. Sci. (USA) 76(1): 514-517.

Bottenstein, J.E. and G.H. Sato. 1980. Fibronectin and polylysine requirement for proliferation of neuroblastoma cells in defined medium. Exp. Cell Res. 129: 361-366.

Boucaut, J.C., T. Darribere, T.T. Poole, H. Aoyama, K.M. Yamada and J.P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitve peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryo. J. Cell Biol. 99: 1822-1830.

Bragina, E.E., J.M. Vasiliev, I.M. Gelfand. 1976. Formation of bundles of microfilaments during spreading of fibroblasts on the substrate. Exp. Cell Res. 97: 241-253.

Bray, D., C. Thomas and G. Shaw. 1978. Growth cone formation in cultures of sensory neurons. Proc. Natl. Aced. Sci. (USA) 75: 5226-5229.

Bray, D. 1982. Filopodial contraction and growth cone guidance. In: Cell Behaviot, R. Bellairs, A. Curtis and G. Dunn (eds), Cambridge University Press, New York, p. 299.

Bray, D. 1984. Axonal growth in response to experimentally applied mechanical tension. Devel. Biol. 102: 379-389.

Bray, D. and K. Chapman. 1985. Analysis of microspike movements on the neuronal growth cone. J. Neurosc. 5(12): 3204-3213.

Buckley, I.K. and K.R. Porter. 1975. Electron microscopy of critical point dried whole cultured cells. J. Microscopy (Oxford) 104: 107-120.

Buckley, K.M. and S.C. Landis. 1983. Morphological studies of synapses and varicosities in dissociated cell cultures of sympathetic neurons. J. Neurocytol. 12: 67-92.

Burnstein, D.E.and L.A. Greene. 1978. Evidence for both RNA-synthesis-dependent and -independent pathways in stimulation of nerve outgrowth by nerve growth factor. Proc. Natl. Aced. Sci. (USA) 75: 6059-6063.

Burton, L.E., W.H. Wilson, and E.M. Shooter. 1978. Nerve growth factor in mouse saliva: Rapid isolation and characterization of 7S nerve growth factor. J. Biol. Chem. 253: 7807-7812.

Byers, H.R., G.E. White and K. Fujiwara. 1984. Organization and function of stress fibers in cells in vitro and in situ. In: Cell and Muscle Motility, Vol 5: The Cytoskeleton: J.J. Shay (ed), Plenum Press, New York, p. 83.

Camponet, R.B. 1979. Development of sympathetic neurons in compartmentized cultures: I. Local control of neurite growth by nerve growth factor. Dev. Biol. 93: 1-12.

Castellani, P., A. Siri, C. Rosellini, E. Infusini, L. Borsi, and L. Zardi. 1986. Transformed human cells release different fibronectin variants than do normal cells. J. Cell Biol. 103: 1671-1677.

Chen, W-T., K. Olden, B.A. Bernard and F-F. Chu. 1984. Expression of transformation-associated protease(s) that degrade fibronectin at contact sites. J. Cell Biol. 98: 1546-1555.

Chen, W-T., J. Wang, T. Hasegawa, S.S. Yamada, and K.M. Yamada. 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. J. Cell Biol. 103: 1649-1661.

Cheresh, D.A., M.D. Pierschbacher, M.A. Herzig, and K. Mujoo. 1986. Disialogangliosides GD2 and GD3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. J. Cell Biol. 102: 688-696.

Chernoff, E.A.G., G.A. Maresh and L.A. Culp. 1983. Isolation and charcterization of a large, neurite-associated glycoconjugate from neuroblastoma cells. J. Cell Biol. 96: 661-668.

Condeelis, J.S. and D.L. Taylor. 1977. The contractile basis of ameboid movement. V. The control of gelation, solation, and contraction in extracts from Dictyostelium disoidem. J. Cell Biol. 74: 901-927.

Condeelis, J.S. 1981. Reciprocal interactions between the actin lattice and cell membrane. Neurosc. Res. Prog. Bull. 19(1): 83-98.

Culp, L.A. and J.F. Bruniel. 1976. Substrate-attached serum and cell proteins in adhesion of mouse fibroblasts. J. Cell. Physiol. 88: 89-106.

Culp, L.A., R. Ansbacher and C. Domen. 1980. Adhesion sites of neural tumor cells: Biochemical composition. Biochem. 192(5): 5899-5907.

Dahl, D. and A. Bignami. 1978. Neurofilament protein in clonal lines of mouse neuroblastoma. Dev. Neurosc. 1: 142-152.

David-Pfeuty, T. and S.J. Singer. 1980. Altered distributions of the cytoskeletal proteins vinculin and alpha-actinin in cultured fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. (USA) 77:6687-6691.

DeMey, J.R. 1983. The preparation of immunoglobulin gold conjugates (IGS reagents) and their use as markers for light and electron microscopic immunocytochemistry. In: Immunohistochemistry, A.C. Cuello (ed), IBRO, pp. 347-372.

Deerinck, T.J. and M.H. Ellisman. 1984. Transcellualar filaments at the node of Ranvier. Soc. Neurosc. Abstr. 10: 163.

Domen, C. and L.A. Culp. 1981. Adhesion sites of neural tumor cells: Morphogenesis of substratum-attached material. Exp. Cell Res. 134: 329-338.

Driedger, P.E. and P.M. Blumberg. 1979. Specific binding of phorbol ester tumor promoters. Proc. Natl. Acad. Sci. (USA) 76: 5158-5162.

Duband, J-L., S. Rocher, W-T. Chen, K.M. Yamada, J.P. Thiery. 1986. Cell adhesion and migration in the early vertebrate embryo: The putative fibronectin receptor complex. J. Cell Biol. 102: 160-178.

Duband, J-L. and J.P. Thiery. 1982. Appearance and distribution of fibronectin during chick embryo gastrulation and neuralation. Dev. Biol. 94: 337-350.

Ellisman, M.H. and K.R. Porter. 1980. The microtrabecular structure of the axoplasmic matrix: Visualization of cross-linking structures and their distribution. J. Cell Biol. 87: 464-479.

Engvall, E., and E. Rouslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer 20:1-5.

Everson, T.C. 1964. Spontaneous regression of cancer. Ann. N.Y. Acad. Sci. 114: 721-735.

Feuerstein, N., A. Sahai, W.B. Andersen, D.S. Salomon, and H.L. Cooper. 1984. Differential phosphorylation events associated with phorbol ester effects on acceleration versus inhibition of cell growth. Cancer Res. 44: 5227-5233.

Fine, R.E., A.L. Blitz, S.E. Hitchcock and B. Kammer. 1973. Tropomyosin in brain and growing neurons. Nature New Biol. 245: 182-185.

Fogh, J., H. Fogh. 1969. Procedures for control of mycoplasma contamination of tissue cultures. Ann. N.Y. Acad. Sci. 172: 15-30.

Franke, W.W., E. Schmid, S. Winters, M. Osborn and K. Weber. 1979. Widespread occurence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp. Cell Res. 123: 25-46.

Franke, W.W., E. Schmid, M. Osborn, K. Weber. 1979. Intermediate-sized filaments of human endothelial cells. J. Cell Biol. 81: 570-580.

Garrigues, H.J., M.W. Lark, S. Lara, I. Hellstrom, K.E. Hellstrom, and T.N. Wight. 1986. The melanoma proteoglycan: Restricted expression on microspikes, a specific microdomain of the cell surface. J. Cell. Biol. 103: 1699-1710.

Geiger, B. 1985. Microfilament-membrane interaction. Trends Biochem. Sci. 10:456-461.

Geiger, B., A.H. Dutton, K.T. Tokuyasa, S.J. Singer. 1981. Immunoelectron microscopy studies of membrane-microfilament interactions: Distributions of actinin, tropomyosin, and vinculin in intestinal epithelial brush border and chicken gizzard smooth muscle cells. J. Cell Biol. 91: 614-628.

Goldberg, D.G. and D.W. Burmeister. 1986. Stages in axon formation: observations of growth of Aplysia axons in culture using video-enhanced contrast-differential interference contrast microscopy. J. Cell Biol. 103: 1921-1931.

Goldman, R., Pollard, T., Rosenbaum, J. (eds). 1976. Cell Motility, Cold Spring Harbor conference on cell proliferation, Vol. 3, Cold Spring Harbor, New York: Cold Spring Harbor Laboratories.

Goldman, R.D., R.V. Zackroff, J.M. Starger and M. Whitman.1979. Intermediate filaments: assembly, disassembly, reorganization and relation to centrioles. J. Cell Biol. 83: 343a.

Goldman, R.D., B. Chojnacki, A.E. Goldman, J. Starger, P. Steinart, J. Talian, M. Whitman and R. Zackroff. 1981. Aspects of the cytoskeleton and cytomusculature of nonmuscle cells. Neurosc. Res. Prog. Bull. 19(1): 59-82.

Goldstein, M.N., J.A. Burdman, L.J. Journey. 1964. Long-term tissue culture of neuroblastoma. II. Morphologic evidence for differentiation and maturation. J. Natl. Cancer Inst. 32: 165-199.

Goldstein, M.E., L.A. Sternberger, N.H. Sternberger. 1983. Microheterogeneity ("neurotypy") of neurofilament proteins. Proc. Natl. Acad. Sci. (USA) 80: 3101-3105.

Green, K.J. and R.D. Goldman. 1986. Evidence for an interaction between the cell surface and intermediate filaments in cultured fibroblasts. Cell Motility and the Cytoskeleton 6: 389-405.

Greene, L.A. 1978. Nerve growth factor prevents death and stimulates the neuronal differentiation of PC12 pheochromocytoma cells in serum-free medium. J. Cell Biol. 78: 747-755.

Greene, L.A. and E.M. Shooter. 1980. The nerve growth factor: Biochemistry, synthesis and mode of action. Ann. Rev. Neurosc. 3, 353-402.

Greensberg, J.H., S. Seppa, H. Seppa, A.T. Hewitt. 1981. Role of collagen and fibronectin in neural crest cell adhesion and migration. Devel. Biol. 87: 259-266.

Grinnell, F. 1986. Focal adhesion sites and the removal of substratum-bound fibronectin. J. Cell Biol. 103(6): 2697-2706.

Gundersen, R.W. and J.W. Barrett. 1980. Characterization of the turning response of dorsal root neurites toward nerve growth factor. J. Cell Biol. 87: 546-554.

Gundersen, R.W. and J.W. Barrett. 1984. Neurite growth cone-substratum adherence increases in vitro. Dev. Brain Res. 12: 21-26.

Gundersen, R.W. 1985. Sensory neurite growth cone guidance by substrate adsorbed nerve growth factor. J. Neurosc. Res. 13: 199-212.

Haussler, M., N. Sidell, M. Kelly, C. Donaldson, A. Altman, D. Mangelsdorf. 1983. Specific high affinity binding and biologic action of retinoic acid in human neuroblastoma cells. Proc. Natl. Acad. Sci. (USA) 80: 5525-5529.

Hayat, M.A. 1979. Introduction to Biological Scanning Electron Microscopy. University Park Press, Baltimore, pp. 131-166.

Haydon, P.G., C.S. Cohan, D.P. McCobb, H.R. Miller, and S.B. Kater. 1985. Neuron-specific growth cone properties as seen in identified neurons of Helisoma. J. Neurosc. Res. 13: 135-148.

Hayman, E.G., M.D. Pierschbacker, S. Suzuki, and E. Ruoslahti. 1985. Vitronectin - A major cell attachment-promoting protein in fetal bovine serum. Exp. Cell Res. 160: 245-258.

Heath, J.P. and G.A. Dunn. 1978. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflection and high voltage electron microscope study. J. Cell Sci. 29: 197-212.

Henderson, D. and K. Weber. 1980. Immunoelectron microscopic studies of intermediate filaments in cultured cells. Exp. Cell Res. 129: 441-453.

Horrisberger, M. and M. vonLanthen. 1978. Simultaneous localization of a hepatic binding protein specific for galactose and of galactose-containing receptors on rat hepatocytes. J. Histochem. Cytochem. 26: 960-966.

Hynes, R.O. and A.T. Destree. 1978. Relationships between fibronectin (LETS proteins) and actin. Cell 15: 875-881.

Hynes, R.O. 1981. Fibronectin and its relation to cellular structure and behavior. In: Hay, E.D. (ed). Cell Biology of Extracellular Matrix, New York: Plenum Publishing Corp., pp. 295-327.

Ishii, D.N. 1978. Effect of tumor promoters on the response of cultured embryonic chick ganglia to nerve growth factor. Cancer Res. 38: 3886-3893.

Ishii, D.N., E. Fibach, H. Yamaski and I.B. Weinstein. 1978. Tumor promoters inhibit morphological differentiation in cultured mouse neuroblastoma cells. Science 200: 556-559.

Johnson, E.M., R.Y. Andres and R.A. Bradshaw. 1978. Characterization of the retrograde transport of nerve growth factor using highly specific (^{125}I) NGF. Brain Res. 150: 319-331.

Johnston, R.N. and N.K. Wessels. 1980. Regulation of the elongating nerve fiber. In: Current Topics in Developmental Biology, Vol. 16, Academic Press, New York, pp. 165-205.

Kellie, S., T.C. Holme and M.J. Bissell. 1985. Interaction of tumor promoters with epithelial cells in culture: an immunofluorescence study. Exp. Cell Res. 160: 259-274.

Kikkawa, U., Y. Takai, Y. Tanaka, R. Miyake and Y. Nishizuka. 1983. Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. J. Biol. Chem. 258: 11442-11445.

Kuczmarsky, E.R. and J.L. Rosenbaum. 1979. Studies on the organization and localization of actin and myosin in neurons. J. Cell Biol. 80: 356-371.

Kuczmarsky, E.R. and J.L. Rosenbaum. 1979. Chick brain actin and myosin isolation and characterization. J. Cell Biol. 80: 341-355.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriphage T4. Nature 277: 680-685.

Lasek, R.J. and P.N. Hoffman. 1976. The neuronal cytoskleton, axonal transport and axonal growth. In: Cell Motility, R. Goldman, T. Pollard and J. Rosenbaum (eds.), Cold Spring Harbor Labs, p. 1021.

Lasek, R.J. 1981 The dynamic ordering of neuronal cytoskeletons. Neurosc. Res. Prog. Bull. 19(1): 7-31.

Laszlo, A., K. Raedke, S. Chin and M.J. Bissell. 1981. Tumor promoters alter gene expression and phosphorylation in avian cells in culture. Proc. Natl. Acad. Sci. (USA) 78: 6241-6244.

Lazarides, E. and K. Burridge. 1975. Alpha-actinin immunofluorescent localization of a muscle structural protein in non-muscle cells. Cell 6: 289-300.

Lazarides, E. 1976. Two general classes of cytoplasmic actin filaments in tissue culture cells: The role of tropomyosin. J. Supramol. Struc. 5: 511-563.

Lazarides, E. 1976. Actin, actinin and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. J. Cell Biol. 70: 202-219.

Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. Nature 283: 249-256.

Leach, K.L., M.L. James and P.M. Blumberg. 1983. Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. Proc. Natl. Acad. Sci. (USA) 80: 4208-4212.

Lee, V.M. and C. Page. 1984. The dynamics of nerve growth factor-induced neurofilament and vimentin filament expression and organization in PC12 cells. J. Neurosc. 4(7): 1705-1714.

Lehto, V.P. and M. Virtanen. 1983. Immunolocalization of a novel, cytoskeleton-associated polypeptide of Mr 230,000 dalton (p 230). J. Cell Biol. 96: 703-716.

Leibovitch, M.P., S.A. Leibovitch, M. Raymondjean and J. Kruh. 1984. Effects of sodium butyrate on gene expression in a rat myogenic cell line. Bioch. Biophys. Res. Comm. 125(3): 1129-1136.

Letourneau, P.C. 1975. Cell-to-substratum adhesion and guidance of axonal elongation. Dev. Biol. 44: 92-101.

Letourneau, P.C. 1978 Chemotactic response of nerve fiber elongation to nerve growth factor. Dev. Biol. 66: 183-196.

Letourneau, P.C. 1981. Immunocytochemical evidence for colocalization in neurite growth cones of actin and myosin and their relationship to cell-substratum adhesions. Dev. Biol. 85: 113-123.

Letoureau, P.C. 1983. Differences in the organization of actin in the growth cones compared with the neurites of cultured neurons from chick embryos. J. Cell Biol. 97: 963-973.

Littauer, U.Z. 1980. Discussion: cell differentiation. In Evans, E.A. (ed). Advances in Neuroblastoma Research. New York: Raven Press, pp. 195-196.

Lotan, R. 1980. Effects of vitamin A and its analogues (retinoids) on normal and neoplastic cells. Biochem. Biophys. Acta. 605: 33-91.

Lotem, J. and L. Sachs. 1979. Regulation of normal differentiation in mouse and human myeloid leukemic cells by phorbol esters and the mechanism of tumor promotion. Proc. Natl. Acad. Sci. (USA) 76: 5158-5162.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1983: 265-275.

Madri, J.A. and K.W. Barwick. 1983. Use of avidin-biotin complex in an ELISA system: A quantitative comparison with two immunoperoxidase detection systems using keratin antisera. Lab. Invest. 48: 98-107.

Maekawa, S., O. Yasutaka, E. Nishida, H. Sakai. 1984. Isolation of low molecular weight actin-binding proteins. J. Biochem. 95: 377-385.

Mahendran, C. and S. Berl. 1977. Isolation of troponin-like complex from bovine brain cortex. Proc. Natl. Acad. Sci. (USA) 74(6): 2273-2277.

Marsh, L. and P.C. Letourneau. 1984. Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. J. Cell Biol. 99: 2041-2047.

Mautner, V. and R.O. Hynes. 1977. Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells. J. Cell Biol. 75: 743-768.

Meyskins, F.L. and B.B. Fuller. 1980. Characteristics of the effects of different retinoids on the growth and differentiation of a human melanoma cell line and selected subclones. Cancer Res. 40: 2194-2196.

Miller, C. and W.M. Kuehl. 1976. Isolation of myosin from cloned rat glioma and mouse neuroblastoma cells. Brain Res. 108: 115-124.

Mobley, W.C., A.C. Server, D.N. Ishii, R.J. Riopelle and E.M. Shooter. 1977. Nerve growth factor. New Engl. J. Med. 297: 1096-1104.

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature (London) 308: 693-698.

Osborn, M. and K. Weber. 1977. The detergent-resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. Exp. Cell Res. 106: 339-349.

Paetau, A., K. Hellstrom, B. Westermark, D. Dahl, M. Halfin and A. Vaheri. 1980. Mutually eclusive expression of fibronectin and glial fibrillary acid protein in cultured brain cells. Exp. Cell Res. 129: 337-344.

Pahlman, S., L. Odelstad, E. Larsson, G. Grotte and K. Nilson. 1981. Phenotypic changes of human neuroblastoma cells in culture incuded by 12-O-tetradecanoyl-phorbol-13-acetate. Intl. J. Cancer 28: 583-589.

Pahlman, S., A. Ruusala, L. Abrahamsson, L. Odelstad and K. Nilsson. 1983. Kinetics and concentration effects of TPA-induced differentiation of cultured human neuroblastoma cells. Cell Differ. 12: 165-170.

Pollard, T.D., S.M. Thomas and R. Niederman. 1974. Human platelet myosin: Purification by a rapid method applicable to other nonmuscle cells. Annals Biochem. 60, 258-273.

Pollard, T.D. 1976. The role of actin in the temperature dependent gelation and contraction of extracts of Acanthameba. J. Cell Biol. 68: 579-601.

Pollard, T.D. 1981. Cytoplasmic contractile proteins. J. Cell Biol. 91: 1565-1579.

Rauvala, H. 1984. Neurite outgrowth of neuroblastoma cells: dependence on adhesion surface-cell surface interactions. J. Cell Biol. 98: 1010-1016.

Rein, D., E. Gruenstein, and J. Lessard. 1980. Actin and myosin synthesis during differentiation of neuroblastoma cells. J. Neurochem. 34(6): 1459-1469.

Robinson, J.M. and M.J. Karnovsky. 1980. Specialization in filopodial membranes at points of attachment to the substrate. J. Cell Biol. 87: 562-568.

Robson, J.A. and N. Sidell. 1985. Ultrastructural features of a human neuroblastoma cell line treated with retinoic acid. Neurosc. 14(4): 1149-1162.

Rogers, S.L., P.C. Letourneau, S.L. Palm, J. McCarthy, and L.T. Furcht. 1983. Neurite extension by peripheral and CNS neurons in response to substratum-bound fibronectin and laminin. Devel. Biol.: 212-220.

Rosen, J.J. and L.A. Culp. 1977. Morphology and cellular origins of substrate-attached material from mouse fibroblasts. Exp. Cell Res. 107: 139-149.

Rovera, G., T.G.O. O'Brien and L. Diamond. 1977. Tumor promoters inhibit spontaneous differentiation of Friend erythroleukemic cells in culture. Proc. Natl. Acad. Sci. (USA) 74: 2894-2898.

Rovera, G., D. Santon and C. Damsky. 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol ester. Proc. Natl. Acad. Sci. (USA) 76: 2779-2783.

Schachner, M., G. Schoonmaker, R.O. Hynes. 1978. Cellular and subcellular localization of LETS protein in the nervous system. Brain Res. 158: 149-158.

Schlaepfer, W.M. 1977. Immunological and ultrastructural studies of neurofilaments isolated from rat peripheral nerve. J. Cell Biol. 74: 226-240.

Schemchel, D.E., M.W. Brightman and P.J. Marrangos. 1980. Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. Brain Res. 190: 195-214.

Schliwa, M. and J. van Blerkom. 1981. Structural interaction of cytoskeletal components. J. Cell Biol. 90: 222-235.

Schliwa, M., T. Nakamura, K.R. Porter and U. Euteneuer. 1984. A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. J. Cell Biol. 99: 1045-1059.

Seeley, P.J. and L.A. Greene. 1983. Short latency local actions of nerve growth factor at the growth cone. Proc. Natl. Acad. Sci. (USA) 80: 2789-2793.

Shoyab, M., J.E. DeLarco, and G.J. Todaro. 1979. Biologically active phorbol esters specifically alter affinity of epidermal growth factor receptors. Nature (London) 279: 387-391.

Sidell, N. 1982. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. J. Natl. Canc. Inst. 68(4): 589-597.

Sidell, N., A. Altman, M.R. Haussler and R.L. Seeger. 1983. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cells. Exp. Cell Res. 148: 21-30.

Sieber-Blum, M., F. Sieber, K.M. Yamada. 1981. Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells in vitro. Exp. Cell Res. 133: 285-295.

Siebert, P.D. and M. Fukuda. 1985. Induction of cytoskeletal vimentin and actin gene expression by a tumor-promoting phorbol ester in human leukemic cell line K562. J. Biol. Chem. 260(6): 3868-3874.

Singer, I. 1979. The fibronexus: a transmembrane association of fibronectin-containing fibers and bundles of 5-nm microfilaments in hamster and human fibroblasts. Cell 16: 675-685.

Singer, I. 1982. Association of fibronectin and vinculin with focal contacts and stress fibers in stationary hamster fibroblasts. J. Cell Biol. 92: 398-408.

Small, J.V., G. Isenberg and J.E. Celis. 1978. Polarity of actin at the leading edge of cultured cells. Nature (London) 272: 638-639.

Small, J.V. and G. Langanger. 1981. Organization of actin in the leading edge of cultured cells: Influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. J. Cell Biol. 91: 695-705.

Sonnenfeld, K.H. and D.N. Ishii. 1982. Nerve growth factor effects and receptors in cultured human neuroblastoma cell lines. J. Neurosci. Res. 8: 375-391.

Spengler, B.A. and J.L. Biedler. 1979. Prevalence of homogeneously staining regions (HSRs) and double-minute (DM) chromosomes of a human neuroblastoma cell line. J. Cell Biol. 83: 159a.

Spinelli, W., K.H. Sonnenfeld and D.N. Ishii. 1982. Effects of phorbol ester tumor promoters and nerve growth factor on neurite outgrowth in cultured human neuroblastoma cells. Cancer Res. 42: 5067-5073.

Sternberger, L.A. 1979. Immunocytochemistry. Wiley, New York, 2nd Edition.

Thom, D., A.J. Powell and D.A. Rees. 1979. Mechanisms of cellular adhesion: IV. Role of serum glycoproteins in fibroblast spreading on glass. J. Cell Sci. 35: 281-305.

Tomaselli, K.J., L.F. Reichardt, and J.L. Bixby. 1986. Distinct molecular interaction mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. J. Cell Biol. 103: 2659-2672.

Tosney, K.W. and N.K. Wessells 1983. Neuronal motility. The ultrastructure of veils and microspikes correlates with their motile activities. J. Cell Sci. 61: 389-411.

Towbin, H., T. Staehelin, J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gells to nitrocellulose sheets: procedures and applications. Proc. Natl. Acad. Sci. (USA) 76: 4350-4354.

Trelstad, R.L., E.D. Hay and J.P. Revel. 1967. Cell contact during morphogenesis in the chick embryo. Dev. Biol. 16: 78-106.

Umekawa, H., M. Naka, M. Inagaki, H. Onishi, T. Wakabayashi, and H. Hidaka. 1985. Conformational studies of myosin phosphorylated by protein kinase C. J. Biol. Chem. 260(17): 9833-9837.

Vaheri, A. and E. Ruoslahti. 1975. Fibroblast surface antigen produced but not retained by virus transformed human cells. J. Exp. Med. 142: 530-538.

Vandenbark, G.R. and J.E. Niedel. 1984. Phorbol diesters and cellular differentiation. J.N.C.I. 73(5): 1013-1019.

Varon, S.S. and R.P. Bunge. 1978. Tropic mechanims in the perpheral nervous system. Ann. Rev. Neurosc. 1: 327-361.

Virtanen, I., V-P. Lehto, E. Lehtonen, T. Vartio, and S. Stenman. 1981. Expression of intermediate filaments in cultured cells. J. Cell Sci. 50:45-63.

Voller, A., D.E. Bidwell, A. Bartlett (eds). 1979. The Enzyme Linked Immunosorbent Assay (ELISA): A guide with abstracts of microplate applications. The Authors, Dynatech Labs. Inc.

Wang, E., H.L. Yin, J.G. Kreuger, L.A. Caliquiri and I. Tamm. 1984. Unphosphorylated gelsolin is located in regions of cell-substratum contact or attachment in Rous sarcoma virus-transformed rat cells. J. Cell. Biol. 98: 761-771.

Warr, G.W.: Purification of antibodies. 1982. In: Antibody as a Tool. J.J. Marchalonis and G.G. Warr (eds), John Wiley and Sons, Ltd., pp. 59-96.

Wehland, J., M. Osborn, K. Weber. 1979. Cell to substratum contacts in living cells, a direct correlation between interference reflexion and indirect immunofluorescence microscopy using anitobides against actin and actinin. J. Cell Sci. 37: 257-273.

Werth, D.K. and I. Pastan. 1984. Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. J. Biol. Chem. 259: 5264-5267.

Wessells, N.K. and R.P., Nuttall. 1978. Normal branching, induced branching, and steering of cultured parasympathetic motor neurons. Exp. Cell Res. 115: 111-122.

Willard, M. 1983. Nerurofilaments and axonal transport. In: Neurofilaments, C.A. Marotta (ed), University of Minnesota Press, Minneapolis, p. 106.

Wylie, D.E., C.H. Camsky, C.A. Buck. 1979. Use of antisera to surface membranes in the identification of membranes components relevant to cell-substrate adhesion. J. Cell Biol. 80: 386-398.

Yamada, K.M., S.S. Yamada, and I. Pastan. 1976. Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed fibroblasts. Proc. Natl. Acad. Sci. (USA) 73: 1217-1221.

Zigmond, S.H., J.J. Otto, and J. Bryan. 1979. Organization of myosin in a submembranous sheath in well spread human fibroblasts. Exp. Cell Res. 119: 205-219.