CYTOSKELETAL CHANGES IN SY5Y NEUROBLASTOMA CELLS EXPOSED TO ACRYLAMIDE: AN IMMUNOCYTOCHEMICAL STUDY

by

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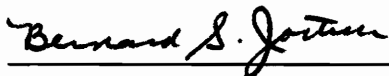

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(ABSTRACT)

The neuronal cytoskeleton is vital for normal growth and differentiation of the nervous system, as well as for maintenance of the normal intracellular environment. Without it , major functional deficits occur due to interference with cellular transport of membrane components, proteins and neurotransmitter substances and as a result, inadequate maintenance of the distal axon occurs. Through the study of both nervous tissue and primary neuronal culture, specific cytoskeletal markers have been found to predominate in axonal or dendritic processes, as well as in different stages of neuronal development. In vitro study of neuroblastoma cell lines has also been utilized to develop hypotheses of neuronal development. These hypotheses attempt to explain the appearance of certain cytoskeletal elements, such as phosphorylated neurofilament proteins, in relationship to functional maturity of the neuron. We used SY5Y human neuroblastoma cells as an in vitro model of neurotoxicity to investigate cytoskeletal changes that may occur from the exposure of the nervous system to a known neurotoxicant. Cells were differentiated with either retinoic acid (RA) or dibutyryl cyclic adenosine monophosphate (dbcAMP) and 3-isobutyl-1-methyl-xanthine (IBMX). Differentiation was based morphologically on the appearance of neuritic processes in a majority (>50%) of the cells. Using the peroxidase-antiperoxidase technique, cells were labeled with monoclonal antibodies to cytoskeletal proteins (phosphorylated neurofilament, microtubule associated protein 2, vimentin and low molecular weight neurofilament protein)

either specific for axonal markers or linked to stages in neuronal development. Staining patterns were compared to undifferentiated cells using the same protocol. There were no differences in staining patterns found between methods of differentiation or between differentiated cells and undifferentiated controls. Axonal markers of differentiation, defined as phosphorylated neurofilament immunopositivity, were only detected in cells exposed to retinoic acid for 9 days. Once these studies were completed, both differentiated and undifferentiated cultures were exposed to acrylamide as an example of a neurotoxicant with known cytoskeletal effects. Cells were fixed and stained after the observation of cellular swelling 24 hours post acrylamide treatment. In spite of obvious alterations in morphology in unstained cells in culture, the cytoskeletal staining pattern was unchanged after acrylamide treatment. We conclude that there is no difference in the cytoskeletal immunoreactivity of SY5Y neuroblastoma cells when differentiated with RA or dbcAMP/IBMX after three days. Retinoic acid differentiated cells, however, do develop immunoreactivity to axonal markers of differentiation after nine days of treatment. We also conclude that acrylamide does not affect the cytoskeletal structure of SY5Y neuroblastoma cells in undifferentiated or in RA or dbc AMP differentiated cells at the time and concentration tested.

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And without faith it is impossible to please God, because anyone who comes to him must believe that he exists and that he rewards those who earnestly seek him.

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ABBREVIATIONS

CO_2	carbon dioxide
CRABP	cellular retinoic acid binding protein
cm^2	centimeter squared
°C	degrees Centigrade
DAB	3,3'diaminobenzidine
dbcAMP	N_6, O_{12}-dibutyryladenosine 3':5'- cyclic monophosphate
EtOH	ethanol
FBS	fetal bovine serum
F12	Ham's F12 medium
g	grams
IBMX	isobutylmethylxanthine
KSP	lysine-serine-proline amino acids
l	liter
µ	micron
µl	microliter
µM	micromolar
MAP2	microtubule associated protein 2
MAP2c	microtubule associated protein 2, subtype c
ml	milliliter
mm^2	millimeter squared
mM	millimolar
MEM	minimal essential medium
M	molar
NF-H	two hundred kilodalton molecular weight neurofilament protein
200 kD NFP	two hundred kilodalton molecular weight neurofilament protein
NF-L	seventy kilodalton molecular weight neurofilament protein
70 kD NFP	seventy kilodalton molecular weight neurofilament protein
NF-M	one-hundred fifty kilodalton molecular weight neurofilament protein
150 kD NFP	one-hundred fifty kilodalton molecular weight neurofilament protein

ABBREVIATIONS (continued)

NGS/TBS	normal goat serum in Tris buffered saline
PAP	peroxidase/anti-peroxidase
RA	all-trans-retinoic acid
SCa	slow component a
SCb	slow component b
TBS	Tris buffered saline

I. INTRODUCTION

The neuronal cytoskeleton is vital to the normal growth and differentiation of the mammalian central and peripheral nervous systems. It is composed of a variety of proteins, many of which have been identified and partially characterized as to their role in neuronal structure and function. One cytoskeletal protein is vimentin, an intermediate filament more commonly recognized in other non-epithelial cell types. Vimentin is expressed in most dividing neuroepithelial cells [1], but it is gradually replaced by neurofilament proteins in neurons as they approach maturity [2-4]. Neurofilament proteins, defined here as the triplet proteins of molecular weights 70kD, 150kD and 200kD or NF-L, NF-M and NF-H respectively, are also classified as intermediate filaments in the assembled state. They are characteristically present only in the nervous system and act to stabilize the neuron during maturation as well as to provide a framework for axonal transport. Phosphorylation of neurofilament proteins is thought to occur late in neuronal maturation as axonal processes mature [5-7] and the phosphorylation process provides a physical expansion of the axonal diameter, thus providing a greater surface area for conduction of electrical impulses [3]. Microtubule associated protein 2 (MAP 2) is a component of the interconnecting fibrils between microtubules and neurofilaments in the neuronal cytoskeleton [8], and is generally accepted as cell body and dendrite specific in its location in the cell [9]. Some authors believe that due to the early appearance and persistence of MAP2 during development, its presence may label neuritic processes destined to become dendrites [10].

Neuronal development has been studied in both primary neuronal cell culture and various neuroblastoma cell lines, including the SY5Y human neuroblastoma cell line. This line has been well characterized and used extensively in the *in vitro* study of neuronal development [11-19]. Several compounds have been used to induce the formation of neurites, defined as elongated extensions of the cell body, with the goal of promoting differentiation toward a neuronal phenotype [13, 14, 16, 20-22]. Retinoic acid, a vitamin A derivative, and dibutyryl cyclic adenosine monophosphate (dbcAMP), an

analog of intracellular cAMP, have been used successfully to this end [12, 13, 23, 24]. In one mouse neuroblastoma cell line (NB2a/d1), application of these compounds resulted in both the formation of a dendritic phenotype with retinoic acid, and an axonal phenotype with cAMP [25]. Both compounds have been used in the SY5Y line, but comparison of their effects by immunocytochemical labeling of cytoskeletal elements has not been performed. The goals of this work were to differentiate the cells with both compounds (after modification of the cAMP protocol to incorporate isobutyl-methyl-xanthine [IBMX], a phosphodiesterase inhibitor), to characterize the cytoskeleton by immunocytochemistry using the peroxidase-antiperoxidase technique, and to classify the cells with respect to their extent of development according to a proposed hypothesis [5], noting any differences in the two protocols. After characterization of the cytoskeletal changes, the cells were additionally exposed to acrylamide, a neurotoxicant with suspected cytoskeletal effects [26-28]. Immunocytochemical staining was repeated with acrylamide treated cells, once again noting any cytoskeletal changes in the staining pattern that could be attributed to exposure to the toxicant. This information, therefore, provides further evaluation of SY5Y neuroblastoma cells as an *in vitro* model of cytoskeletal neurotoxicity and neuronal development.

II. REVIEW OF THE LITERATURE

II. A. CYTOSKELETAL PROTEINS AND NEURONAL DEVELOPMENT AND FUNCTION

The neuronal cytoskeleton, with its associated proteins, is vital to the normal growth and differentiation of the mammalian central and peripheral nervous systems. Of no less importance is the role of the cytoskeleton in the function of the neuron. It acts both as a structural framework as well as a dynamic organelle that allows adaptation of the neuron to its microenvironment. Some of these modifications include spindle formation associated with cell division in developing neurons, movement of various structural proteins and neurotransmitter substances through bidirectional axonal transport, and neuronal "plasticity," or, the ability of the neuron to modify its structure and function in association with learning and other complex mental tasks [29]. Study of the individual proteins within the cytoskeleton has revealed an intricate structural and functional working relationship as the nerve cell matures from a primitive neuroblast to a fully functioning neuron. Among the cytoskeletal proteins are vimentin, the neurofilament proteins and microtubule associated protein 2 (MAP2) (Figure 1).

II. A. 1. VIMENTIN

Vimentin, an intermediate filament commonly found in mesenchymal cells, is expressed in most dividing neuroepithelial cells [1], but is gradually replaced by neurofilament proteins in neurons as they become postmitotic [2, 4, 5]. Vimentin subunits can coexist transiently with neurofilament subunits within the same neurite *in vivo* [2], but they assemble into separate systems as differentiation occurs. In adult mammals, vimentin is found in non-neuronal cells such as astrocytes and ependymal cells.[30].

NB2a/d1 mouse neuroblastoma cells and their cytoskeletal proteins have been studied by Shea and colleagues extensively as a model of neuronal development. In order to study the transformation of a primitive or

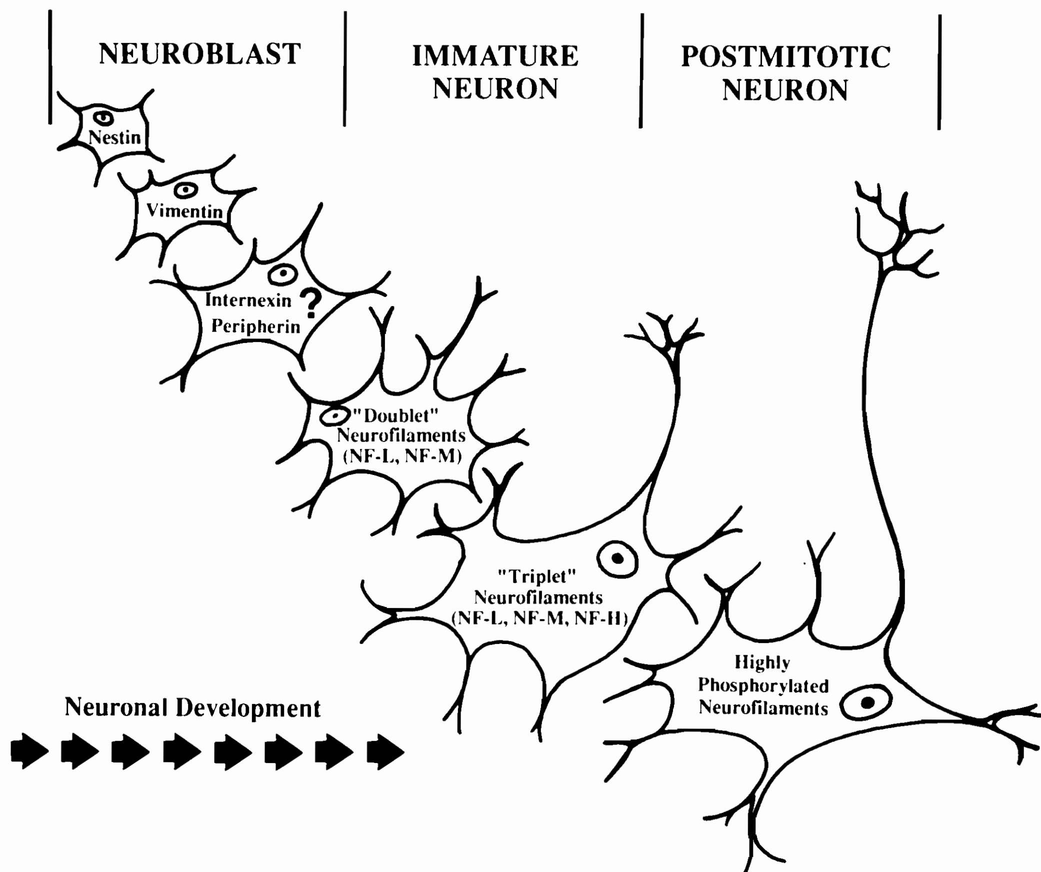

Figure 1. Diagram illustrating the estimated order of appearance of intermediate filament proteins in the neuronal cytoskeleton during development. Nestin first appears in the neuroblast and aids in neuroblast migration. Nestin is replaced by vimentin, which has a vital role in cell division and is believed to aid in initial neurite outgrowth. Internexin and peripherin first appear in transitional stages between the neuroblast and immature neuron. Both play a role in maintaining neuronal plasticity, but it is not known how long they persist in mature neurons. Neurofilament proteins NF-L and NF-M establish the neuronal phenotype, first in singlet and doublet forms, and the addition of NF-H further stabilizes the neurofilament structure. Phosphorylation of neurofilaments occurs as they are transported into maturing axonal structures. Not all filament types shown are found in all neurons (internexin and peripherin) and the intervals as illustrated are approximate. MAP2 is not represented, due to a lack of information about the timing of subtype appearance in immature neurons. MAP2c is present in immature brain and MAP2a and b are present in adult brain.

undifferentiated cell to a differentiated cell that is closer phenotypically to a neuron, neuroblastoma cells were treated with any one of a number of chemical compounds. These compounds induced the formation of neurites, as well as the synthesis of neuron specific enzymes and neurotransmitters. Differentiation of NB2a/d1 cells with cAMP resulted in the development of an axonal morphology based on the appearance of phosphorylated high molecular weight neurofilament proteins, microtubule associated protein 1, and tau protein within the neurite [6, 31]. These same cytoskeletal proteins are found in axonal processes present in nervous tissue as well as axons in primary culture [1, 2, 10, 32, 33]. Research with this model revealed that vimentin plays a role in initial neurite outgrowth, but it is gradually replaced by neurofilament proteins as the neurite becomes stable and develops its axonal morphology. This conclusion was reached when neurite outgrowth was prevented in transiently permeabilized cells loaded with anti-vimentin antisera, but similar treatment with antineurofilament-L did not prevent the elaboration of neurites. Vimentin expression was also prevented by treatment of the cells with antisense oligonucleotides and subsequently, initial neurite outgrowth did not occur. In both instances, pre-existing neurites were unaffected, but new neurite outgrowth was prevented [34]. From these studies it can be concluded that the presence of vimentin alone would likely identify an undifferentiated or partially differentiated cell or a cell without specific cytoskeletal markers of neuronal phenotype.

II. A. 2. NEUROFILAMENT PROTEINS

Neurofilament proteins, defined here as the triplet subunit proteins of molecular weights 70kD, 150kD and 200kD or NF-L, NF-M and NF-H respectively, are also classified as intermediate filaments when assembled into neurofilaments and are characteristically present only in the nervous system. Neurofilaments were first identified by electron microscopy and were determined to be approximately 10nm in diameter and to contain unique processes that appear to form bridges between adjacent filaments. These processes are thought to be localized to the carboxyterminal "tail" of the neurofilament structure. Biochemical studies and protein sequencing of the three neurofilament proteins show that NF-H and NF-M both have unusually

large carboxyl termini which may correspond to the site of these bridges. Also present on the carboxyl end of NF-M and NF-H are multiple repeats of lysine-serine-proline amino acids, known as KSP repeats by the single amino acid code [3]. Although not unique to these two proteins, (similar structures are present on MAP 2 and tau), they are powerfully immunogenic and represent the site against which antibodies are made [35]. The KSP sequence is also thought to be the major site of phosphorylation (see below).

Studies in chick neural tube and mouse neuroblastoma cell culture have established that the neurofilament proteins typically first appear during postmitotic development. They can in some instances co-exist with vimentin, even within the same filament [3, 4, 6]. Most investigators agree that vimentin is replaced by neurofilament proteins that assemble into neurofilaments as the neuron matures [3, 5, 36]. NF-L and NF-M are thought to appear as singlet or doublet forms before the appearance of NF-H and subsequent incorporation of the triplet form into the neurofilament backbone [35, 37] (See also Figure 1). These immature forms are detected by immunocytochemistry before the recognition of neurofilaments by transmission electron microscopy, and they are thought to be structurally unstable [38].

Neurofilament protein function includes the mechanical stabilization of the cytoskeleton. These filaments seem to have a largely passive role in axonal transport and phosphorylation of neurofilaments is thought to be responsible for physical widening of the axonal cytoskeleton. This would allow greater surface area for conduction of action potentials [3]. Other functions include stabilization of the axon during maturation, which is associated with phosphorylation of the KSP sequences (see below), and possible cross-linking of neurofilaments with microtubules and other structures via other cytoskeletal proteins [5].

II. A. 3. PHOSPHORYLATED NEUROFILAMENTS

As mentioned previously, the KSP sequences present on NF-M and NF-H are highly immunogenic and are considered the primary sites of phosphorylation. The correlation between phosphorylation and localization of immunoreactivity to axonal structures has been determined on immunoblots and tissue sections of rat brain and labeled with antibodies derived from mice immunized with hypothalamic material. This material was subjected to

phosphatase treatment after antibody labeling, and localization and degree of immunoreactivity were assessed and compared to controls. The data indicated that neuronal cell bodies, proximal axons and dendrites contain nonphosphorylated neurofilaments and distal axons and terminal segments contain largely phosphorylated neurofilaments [7]. Further studies in rabbit retinal explants and other systems indicated that phosphorylation occurred after neurofilaments entered the axon, providing further evidence of their axonal specificity [39-41]. This correlates with slowing of neurofilament transport [42] and stabilization of the axonal cytoskeleton , as studied in retinal cells and feline corpus callosum [43, 44, 45]. Phosphorylation also leads to a physical expansion of the axonal diameter due to the presence of multiple phosphate groups on the already complicated carboxyterminus of NF-M and NF-H. The axonal expansion provides a wider surface area for conduction of electrical impulses, and corresponds to the observation that axons are longer and usually of greater diameter than dendrites [3].

Labeling of phosphorylated and nonphosphorylated neurofilaments has also been performed in neuroblastoma cell culture. Mouse NB2a/d1 neuroblastoma cells were differentiated with dbcAMP and Triton extracted to reduce non-cytoskeletal immunoreactivity and insure penetration of the antibodies across the cell membrane. NF-H was identified by immunocytochemistry in neurites and occasionally in perikaryal regions. NF-M was present throughout the neurites and cell body, and was occasionally detected in the perikarya of undifferentiated cells. Phosphorylated neurofilament was predominantly detected in the neurites of differentiated cells and in the perikarya of both undifferentiated and differentiated cells [6]. Further study with the same model revealed the perikaryal staining to be due to a soluble protein fraction not associated with the cytoskeleton, but with the same molecular weight, electophoretic pattern and ability to incorporate radiolabelled phosphate groups as NF-H[46]. The finding of phosphorylated neurofilament proteins in neuritic processes of these cells corresponds to the location of the same proteins in axons of normal mouse brain [7].

II. A. 4. MICROTUBULE ASSOCIATED PROTEIN (MAP2)

Microtubule associated protein 2 (MAP2) is a component of the interconnecting bridges between microtubules and neurofilaments in the neuronal cytoskeleton [8]. It is a major component of brain microtubules and generally considered neuronal specific. MAP 2 functions include stimulation of microtubule assembly and stabilization of assembled microtubules as well as crosslinking of microtubules to each other, to organelles and to other cytoskeletal elements, including NF-L [9]. Like NF-M and NF-H, MAP2 has multiple sites available for phosphorylation and these are thought to play a role in the assembly and disassembly of microtubules [47]. In the adult central nervous system, MAP2 is localized in dendrites and cell bodies and thus absent from most axons [10, 48-51]. One explanation for this finding is based on the fact that ribosomes are found in dendrites but not in axons, therefore, local synthesis of MAP 2 may occur within the dendrite [52]. In the developing brain, there are multiple subforms of MAP2 (a, b, and c) and they vary in the time of their appearance and disappearance during the neuronal maturation process. One of these forms, MAP2c, is most abundant in immature brain, while MAP2a is present only in adult brain and MAP2b can be in either, depending somewhat on the species [43][49, 53-55]. Some authors believe that, due to the early appearance and persistence of MAP2 during development, its presence may label neuritic processes destined to be dendrites [10], while others claim that specific subforms are also present in axons [43].

MAP2 has also been studied in cell culture. Previous studies of primary cultures of hippocampal cells have established the development of axons beginning 48 hours after culture. In one study, neurite outgrowth in hippocampal neurons was categorized into three stages using a polyclonal antibody to MAP2 and a novel monoclonal antibody to phosphorylated NF-H. The majority of neurites were MAP2 positive alone in the first 24 hours, followed by coexpression of MAP2 and NF-H within 48 hours. Segregation of neurites based on positivity for one or the other occurred between 48 hours and 4 days [32]. These data support the theory that phosphorylated neurofilament is predominantly present in axons, but also suggests there is an early form

present in neurites that can be coexpressed with MAP2 before segregation of neuritic processes into axons and dendrites.

II. B. USE OF SY5Y NEUROBLASTOMA CELLS IN RESEARCH

The neuroblastoma is a neoplasm of the autonomic nervous system that is commonly found in children and is estimated to account for 7% of the cases of childhood cancer diagnosed annually [56]. It was first described by Wright, who noted the characteristic "blast" or immature cell with neuronal like processes [57] Although these tumors are commonly attributed to sympathetic ganglia of the adrenal medulla, they are also known to originate from other dividing nerve cells [16]. Cushing and Wolbach [58] were the first to describe the phenomenon of spontaneous differentiation of the neuroblastoma into its benign counterpart, the ganglioneuroma. Subsequently, this spontaneous regression of the malignant phenotype has been classified into a subgroup of neuroblastomas in infants. The biological behavior of tumors belonging to this subgroup results in one of two possible clinical outcomes. In the first, the tumor undergoes differentiation with remission of the disease. In the second, the tumor undergoes partial differentiation with proliferation of undifferentiated subpopulations of the neoplastic cells and mortality [20, 59]. The malignant behavior of neuroblastoma is thought to be due to abnormalities in the regulation of the differentiation process, resulting in immature cells that continue to divide and proliferate [16], but the precise mechanisms remain unknown. Since the identification of this tumor subtype, interest in differentiation of neuroblastoma cells toward a more mature phenotype has incresed becuase of the possibility of developing therapeutic strategies and also as a means to study the maturation of so-called "neuroblasts" into neurons [20].

Numerous neuroblastoma cell lines have been developed for the purpose of studying neuronal differentiation. Ideally, neural precursors could be cultured from embryonal tissue and studied as they matured, but this has been difficult to achieve due to the inability to maintain dividing neuroblasts in continuous culture. As a result, cloned cells from clinically derived neoplastic

tissue have served as a useful alternative [20, 60]. These cloned cells retain many of the characteristics of normal differentiated neurons. These include generation of action potentials, formation of neurites, and the presence of neurotransmitters and neuron specific enzymes [60]. Some of these characteristics can be enhanced by the addition of a number of substances to the culture medium as will be discussed later. Both human and rodent (rat and mouse) neuroblastoma lines have been studied extensively [16, 15, 60], but there are distinct advantages to using human lines instead of rodent derived cell lines. Mouse neuroblastoma lines all originate from a single tumor (C1300) and typically have an aneuploid or tetraploid chromosome number, implying severe abnormalities in the nuclear regulation of cellular events. Injection of mice with cells derived from the original neoplasm does not consistently result in tumors histologically classifiable as neuroblastoma, suggesting that the biological behavior of the tumor *in vivo* is questionable [15]. Human lines, on the other hand, are derived from a variety of tumors and are nearly diploid and therefore closer genotypically to primitive neuroblasts [60, 15].

The SY5Y clone of human neuroblastoma is derived from the parent cell line SK-N-SH, which was cultured from a bone marrow metastatic site of a four year old child with neuroblastoma. SY5Y cells are often referred to as the "thrice cloned" line from SK-N-SH because they were subcloned from the parent line, then again from the SHSY line, and lastly, from the SH-SY5 line to become SY5Y on the third cloning [11, 17]. The cloning was an attempt to separate the neuroblast phenotype from an epithelial or "flat cell" population, but it has since been established that there is morphological interconversion between these two cell types in all SY5Y lines [17, 18, 61]. The epithelial cells, when studied by electron microscopy, also reveal two populations of cells. One cell type contains variable numbers of melanosomes and neurosecretory granules within the cytoplasm, identifying it as a probable melanocytic precursor. The other contains a discontinuous basal lamina with prominent intercellular attachments, characteristic of Schwann cells. These conclusions were further substantiated by the presence of S-100 protein in both epithelial cell types but not in the neuroblast-like cell. Extracellular matrix proteins laminin and type IV collagen were also detected within cells with Schwann cell phenotypic characteristics but not in neuroblast-like cells [18]. SY5Y cells have

a stable, nearly diploid karyotype and contain high levels of dopamine ß hydroxlase, a catecholaminergic neurotransmitter synthesizing enzyme, moderate levels of tyrosine hydroxylase, which is involved in melanin and catecholamine synthesis, and low levels of choline acetyltransferase, an enzyme involved in the synthesis of acetylcholine [14, 17]. Based on the presence of these enzymes, they are generally classified as adrenergic in origin [13]. The neuroblast phenotype, which usually predominates in the SY5Y cultures (95% of cells), is a small cell with short to medium length processes called neurites that extend radially from the cell soma. There are several karyotypic abnormalities that distinguish cells originating from the SK-N-SH parental line, and a unique chromosomal abnormality is present in cells derived from SH-SY cells (of which SY5Y is a subclone)[17]. As a result, SY5Y cells have 47 chromosomes, including 7 marker chromosomes. They also are known to have an abnormal N-ras oncogene, which would imply there is an inherent dysfunction in the regulation of normal growth and differentiation regulated at the nuclear level [13].

Characterization of intermediate filament types in cultured neuroblastoma cells has been determined in a number of cell lines. Western blot procedures performed on cells of the SK-N-SH lineage were undertaken to attempt to correlate cellular morphology with the presence of intermediate filament types. SY5Y cells were classified in this study based on their morphologic appearance as having an N-type or neuronal morphology as opposed to an S type (large, flat epithelial like cell), or I type (intermediate between the two). All N type lines were reported to have none or only trace amounts of vimentin, while the majority of them contained NF-L and NF-M neurofilament proteins. SY5Y cells specifically were reported as variably vimentin positive, but containing both NF-L and NF-M, not NF-H [70]. Other neuroblastoma cell lines are reported as vimentin positive and when compared to neuronal cell explants are considered less differentiated based on the absence of neurofilament protein immunoreactivity [8]. In summary, the current literature, including Nixon and Shea's model of neuronal differentiation [5], would categorize SY5Y cells as partially differentiated, with cytoskeletal markers typical of mesenchymal cells (vimentin) and neuronal cells (NF-L, NF-M).

II. C. DIFFERENTIATION OF SY5Y NEUROBLASTOMA CELLS _IN VITRO_

The term differentiation in neuroblastoma cell culture refers to the measurement or detection of characteristics present in chemically modified cells with promotion of the cell phenotype toward that of a mature neuron, both morphologically and biochemically. These characteristics range from the extension of cell processes (neurites) and the inhibition of cell proliferation as morphological measures of differentiation, to the synthesis of neurotransmitters and enzymes characteristic of adrenergic or cholinergic neurons. Other physiological measures of differentiation include spontaneous generation and propagation of action potentials and the presence of ultrastructural characteristics considered typical of mature neurons, such as the cytoskeletal neurofilament proteins [29, 12]. Immunocytochemical staining of both biochemical and cytoskeletal markers of differentiation have also been used in conjunction with gel electrophoresis to establish the presence of other markers of maturity in both normal nervous tissue and in neuroblastoma and other cell lines [4, 6, 7, 31, 33, 34, 39, 46, 54, 62-67]. Many compounds have been used to induce characteristics of differentiated cells, including retinoic acid and cyclic adenosine monophosphate (cAMP).

II. C. 1. RETINOIC ACID

Retinoic acid and retinoid derivatives have been used in a variety of cell types to induce changes consistent with cellular differentiation and growth. These include cultured cells of carcinoma, melanoma, and lymphoma origin and epithelial and fibroblastic cells [68]. Retinoids, which include retinoic acid, are synthetic derivatives of vitamin A. Their effects on cultured cells include growth inhibition and alterations in gene expression and cell membrane structure and function [25]. The lesions associated with vitamin A deficiency are well documented in animals and are characterized by squamous metaplasia of epithelial tissues, an effect which is readily reversible when nutrient levels are restored to normal [13]. The earliest report of this effect was documented in

1925 by Wollbach and Howe [69], in rats deprived of vitamin A. They observed that stem cells failed to differentiate into epithelial cells in rats with Vitamin A deficient diets.

The mechanism of action of retinoids on cells both *in vitro* and *in vivo* is largely unknown, although there are comparisons to the effects of steroid hormones due to the fat soluble nature of vitamin A derivatives [68]. One theory on the mechanism by which retinoic acid causes cells to differentiate relates the binding of retinoic acid to cellular retinoic acid binding proteins (CRABP and CRBP). These two proteins are believed to transfer retinol to nuclear chromatin, where it induces effects at the nuclear level [20]. Several studies have reported positive correlations between retinoid and ara-retinoid compound affinity for CRABP and the amount of differentiation observed in embryonal carcinoma cells treated with those compounds [70, 71]. This theory is controversial, however, as differentiation resistant variants have been isolated in several cell types, both with and without CRABP. Treatment of such cells with retinoid compounds has failed to confirm this mechanism [72, 73, 74, 20].

An alternate theory relates increases in protein kinase activity, specifically cAMP-dependent protein kinase and the calcium dependent protein kinase C, to the mechanism of retinoic acid differentiation. These enzymes are involved in phosphorylation mechanisms and linked to control of signal transduction and differentiation of tumors in mechanisms of carcinogenesis [20]. Treatment of embryonal carcinoma cells with retinoic acid has been reported to cause an increase in both the cytosolic and membrane bound cAMP dependent kinases [75] by one group of investigators. Protein kinase activity has also been increased in both melanoma and HL-60 lymphoma cells with a direct correlation between increased cAMP-dependent protein kinase and phosphorylation of substrates [20]. Still another theory of retinoic acid differentiation relates to ion channel control. Cation transport has been implicated in regulation of cell growth and differentiation [20]. Retinoic acid has been shown to block potassium channels in human lymphocytes and this blockage has been demonstrated to alter cellular responses to immune stimulation [76, 77], therefore some authors believe there could be a link to differentiation in other cell types [20].

The capability of retinoic acid to differentiate neuroblastoma cells is well documented [13, 16, 19, 20, 22, 61, 78-80]. Human neuroblastoma cells are considered sensitive to the effects of retinoic acid and both biochemical and morphological effects have been reported for a variety of cell lines [80, 61, 79]. SY5Y cells are reported to increase in choline acetyltransferase activity and to contain increased amounts of neuron specific enolase, both considered biochemical markers of a mature neuronal phenotype [21, 81]. The increase in choline acetyltransferase may indicate differentiation toward a cholinergic phenotype [22]. SY5Y cells also demonstrate a mild increase in noradrenalin production after differentiation with retinoic acid, but this is mild compared to phorbol ester induced increases in the same cells [22].

Biochemical markers of differentiation are often correlated with morphologic evidence of differentiation. Most authors use neurite outgrowth as a measure of differentiation and correlate this change with a decrease in growth rate, which would be considered characteristic of a neuronal population approaching maturity [60]. Cell division is inhibited by many compounds used to differentiate neuroblastoma cells, but neurite extension and cell division have been established as independent phenomena [82, 83]. Therefore, neurite extension alone may be used to evaluate differentiation independent of a decrease in growth rate. Markers of morphological differentiation in SY5Y cells treated with retinoic acid include neurite extension, reduced growth rate, and the formation of neurite bundles and cellular aggregates [24, 13]

II. C. 2. CYCLIC ADENOSINE MONOPHOSPHATE

Cyclic adenine monophosphate or cAMP is a cellular second messenger involved in many cell functions, including normal growth and differentiation. Its effects were first reported in 1971, when increases were associated with morphological differentiation of fibroblasts and Chinese hamster ovary cells in culture [84, 85]. Since that time, cAMP has been implicated in many of the regulatory roles associated with cellular differentiation, including transmission of cellular signals that increase or decrease protein phosphorylation [23, 20, 86]. One mechanism of action of cAMP involves alterations in cell function through the activation of the cAMP dependent protein kinases, leading to

protein phosphorylation and/or modulation of oncogene expression [87, 20, 88]. Study of these mechanisms in cell culture has been performed with the use of cAMP derivatives, such as dibutyrl cAMP, either alone or in combination with phosphodiesterase inhibitors, which act to decrease the turnover of endogenous cAMP within the cell. The use of cAMP derivatives in human neuroblastoma cell lines with or without phosphodiesterase inhibitors results in neurite outgrowth and a reduction in growth rate similar to retinoic acid, although some authors maintain that morphological differences exist between the two [14, 20]. Dibutyrl cAMP treatment of SY5Y neuroblastoma cells also cuases a decreased rate of cell-substrate adhesion, resulting in clumping of cells with a refractile appearance, but enhanced neurite outgrowth, and generation of action potentials when cells are stimulated with electrical current [23, 12]. Interestingly, SY5Y cells have been shown to exhibit a dramatic enhancement of intracellular cAMP levels in cells differentiated with retinoic acid and stimulated with prostaglandin E_1 [19].

II. D. IMMUNOCYTOCHEMISTRY USING THE PEROXIDASE-ANTIPEROXIDASE METHOD

The term immunohistochemistry refers to the specific recognition of fixed antigen in tissue section by antibody, followed by visual localization of the antibody by one of several labeling methods [89]. Extrapolation of this methodology to cells in tissue culture, as well as impression smears and other diagnostic specimens, has resulted in coining of the term immunocytochemistry, reflecting the difference in the original specimen. There are several systems utilized for the detection of the antigen-antibody complex. Some of these systems involve labeling of either the primary antibody bound to the antigenic site, or a secondary antibody specific for the primary antibody. Others systems involve an additional step and use a complex of molecules that bind to the secondary antibody already in place on the antigen-antibody complex. The goal in all these detection systems is specific and accurate detection of small

amounts of antigen, while keeping nonspecific background staining to a minimum [89].

The peroxidase-antiperoxidase (PAP) method has been employed in a variety of immunocytochemical studies, both in primary neuronal culture and in neuroblastoma cell culture [4, 6, 25, 31, 33, 39, 46, 62, 63, 65]. A detailed description of the technique is as follows: (see also Figure 2)

1. After adequate fixation of the specimen, cells are incubated with antibody specific for the cellular antigen in question. This antibody is referred to as the primary antibody or antisera.

2. A second or secondary antibody is then applied that is specific for a binding site on the primary antibody. The primary and secondary antibodies are derived from different species to increase binding specificity, as the secondary is specific for the immunoglobulin class of the primary antibody species. For example, if the sample to be stained was of human origin, a primary antibody could be mouse anti-human. The secondary antibody would be raised against a specific mouse immunoglobulin G (IgG) in a different species (e. g., goat anti-mouse IgG). The specificity of the secondary antibody can be further enhanced by incubation of the cells with species specific normal serum before application of the primary antibody. The normal serum binds to nonspecific antigens that may cross react with the secondary antibody purely due to species differences, freeing the antibody to react with its intended target. This results in decreased background staining of the sample after application of the chromogen.

3. The third step involves incubation of the sample with a tertiary enzyme-antienzyme complex, in this case, peroxidase-antiperoxidase. Each complex contains at least three peroxidase molecules bound to two peroxidase antibodies and the complex as a whole binds to a site on the secondary antibody. In the past, the sensitivity of the available peroxidase antibodies has been less than desirable but recent improvements in antibody quality and

conjugation primarily attributed to the work of the Sternbergers have made this technique one of the most specific available for immunocytochemistry [89]. The complex is bound to a site on the secondary antibody and the peroxidase molecules are unbound and available for binding to an appropriate substrate.

4. The last step involves application of the combination of a suitable chromagen such as 3,3'diaminobenzidine (DAB) and hydrogen peroxide. The peroxidase on the PAP molecule reacts with this combination to form a dark brown reaction product that is readily visible. After the completion of all four steps, the site of the tissue antigen is thus "labeled".

Immunocytochemistry using the PAP technique has been used in cell culture and tissue sections to label cytoskeletal proteins both alone or in conjunction with immunoblotting procedures. The availability of primary antibodies to vimentin and neurofilament proteins has enabled researchers to formulate theories of neuronal development and to suggest pathologic mechanisms in diseases such as Alzheimer's disease and aluminum and 2.5-hexanedione intoxication as well as a variety of spontaneous motor neuron diseases in animals [33, 67, 90, 91, 5]. The PAP technique is readily reproducible, and due to the availability of commercially produced high quality antibodies and reagents, is considered by some to be an excellent detection system with perhaps the highest sensitivity of all immunohistochemical methods [89, 92].

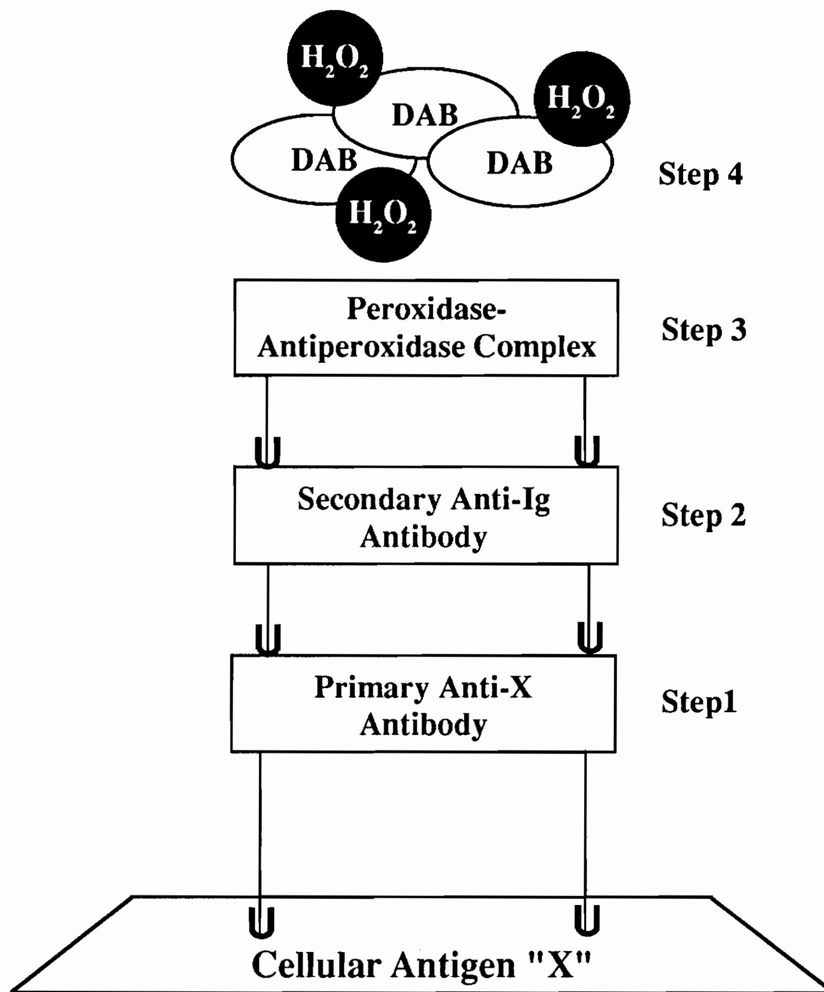

Figure 2. Diagram illustrating the peroxidase-antiperoxidase method of immunocytochemical staining. The cellular antigen is first incubated with a primary antibody, then a secondary antibody is added to the antigen-antibody complex. The peroxidase-antiperoxidase complex is added in the third step. The reaction of DAB in the presence of hydrogen peroxide with the peroxidase results in a detectable color change visible by light microscopy. See text for a more detailed description.

II. E. PATHOLOGY OF THE CYTOSKELETON

The study of the cytoskeletal proteins has been the source of recent research efforts directed toward understanding the mechanisms of nervous system diseases in both humans and animals. One landmark study involved the identification of phosphorylated neurofilament proteins in the perikarya of neurofibrillary tangles characteristic of Alzheimer disease. As reviewed earlier with the section on neurofilament proteins, phosphorylated neurofilaments are characteristically restricted in location to axons and the phosphorylation process occurs distal to the initial segment, and during transport within the axon. The presence of phosphorylated neurofilament proteins within perikaryal Alzheimer tangles led to the conclusion that this lesion may be due to an imbalance in specific protein kinases responsible for normal phosphorylation of neurofilament proteins [67]. Since the publication of this study, it has been determined that perikaryal accumulations of phosphorylated neurofilament protein are also present in other diseases, such as amyotrophic lateral sclerosis and Parkinson's disease [93, 94]. Similar findings have been documented for a variety of animal diseases of varying etiologies, including those associated with hereditary and nutritional causes [90].

Other pathologic processes associated with abnormalities in neurofilament distribution include toxicologic mechanisms. One study of 2,5-hexanedione toxicity in rats demonstrated decreased amounts of neurofilament proteins, especially NF-L, as detected by immunoblotting of nervous tissue in exposed animals [91]. Another study of mouse spinal cord and dorsal root ganglion in primary culture revealed axonal swellings composed of neurofilaments that were detected by immunocytochemical labeling after animals were exposed to 2,5-hexanedione [63]. The same group of researchers documented the aggregation of vimentin intermediate filaments into perinuclear accumulations in cultured human fibroblasts exposed to 2,5-hexanedione or acrylamide [95]. Finally, mouse neuroblastoma cells have been used as a model for aluminum salt toxicosis, with immunocytochemical

detection of phosphorylated neurofilamentous accumulations in the perikarya of treated cells [66].

In summary, it appears likely that cytoskeletal alterations are an important feature of the pathologic mechanisms responsible for nervous system disease in both humans and animals and cell culture can be used to study these processes at the cellular level.

II. F. ACRYLAMIDE AND ITS EFFECTS ON THE CYTOSKELETON

Acrylamide, also known as acrylic amide and propenamide, is a water soluble compound used in industry, primarily in its polymeric form. Its primary uses are as a flocculant in sewage and wastewater treatment plants, in the paper industry for improved quality of paperboard products, and in the petroleum industry as an additive to enhance oil recovery. In the biomedical sciences, polyacrylamide gels are used in chromatography and electrophoresis for research and diagnostic purposes. Acrylamide monomer is also used for the production of grout and soil stabilizers in the highway and construction industries [96, 97]. Acrylamide is readily water soluble and contains an electrophilic vinyl group which can be easily manipulated to produce the polymeric form. This same reactivity makes acrylamide a likely candidate for interaction with biological macromolecules. One example of such an interaction is the preferential binding of acrylamide to sulfhydryl groups of hemoglobin in erythrocytes [98, 99], although this has not been established as a singular mechanism of toxicity [100]. Acrylamide is widely distributed throughout the body after absorption with largely equal distribution to all tissues except erythrocytes. Tissue distribution is only slightly affected by the route of administration and there is no preferential localization to nervous tissue, even after repeated dosing [98].

Acrylamide is metabolized by one of two mechanisms. The major metabolic route is through conjugation with glutathione, which results in detoxification and excretion of metabolites [100, 96]. This is supported by the increased neurotoxicity of acrylamide when non-protein sulfhydryl content is

depleted experimentally [101]. Biotransformation, however, may occur when acrylamide interacts with the microsomal cytochrome P-450 system. Manipulation of the P-450 system with phenobarbital has resulted in conflicting reports of the delay in expression of acrylamide induced neuropathy in rats and the disappearance of free acrylamide detected in liver homogenates from phenobarbital treated animals [102, 103]. Therefore, it is not known whether metabolites of acrylamide are neurotoxic in addition to the parent compound [96]. Evaluation of the neurotoxic effects of several structural analogs of acrylamide in animal studies has led to the conclusion that elimination of the double bond of acrylamide or deletion of the nitrogen atom prevents neuropathy [103]. Based on these studies, it is believed that the acrylyl moiety (CH_2CHCO) is essential for the development of neurotoxic effects [100].

The first reported instances of acrylamide neurotoxicity were documented in the late 1950s and in 1960 the first detailed clinical descriptions were published in Japan [104]. The Japanese documented illness in production workers who presented with clinical signs ranging from nervousness and irritability to paraesthesia and numbness in hands and feet with abnormal perspiration. Specific nonneurologic signs of acrylamide poisoning considered characteristic for the disease include redness and peeling of the hands due to skin exposure to the compound and excessive perspiration, the pathogenesis of which is unknown. The main clinical features of acrylamide toxicity in humans are listed in Table 1 [105].

TABLE 1. CLINICAL FEATURES OF ACRYLAMIDE NEUROPATHY

Red, peeling hands
Weight loss
Excessive perspiration
Urinary incontinence
Distal limb weakness
Distal limb sensory loss
In severe, acute intoxication:
    confusion and hallucinations
In moderate, subacute intoxication:
    drowsiness, loss of concentration and ataxia

In 1966, Fullerton and Barnes [106] were the first to describe changes in peripheral nerves of rats that were consistent with nerve fiber degeneration. Soon after, Prineas [107] described the ultrastructural lesions of abnormal neurofilament accumulations present primarily in distal axons and postulated that this lesion could be consistent with the "dying back" hypothesis of Cavanagh as it related to organophosphate toxicity. Briefly, this hypothesis suggests that there is initial involvement of long and large diameter axons, with degeneration beginning in distal regions and continuing proximally with increasing exposure time. The dying back neuropathy resembles Wallerian degeneration following axotomy in that the segment distal to the transection degenerates first, but then proceeds in a stepwise fashion proximally [108]. Prineas speculated that these accumulations of neurofilaments could be due to a lack of substrates necessary for enzymatic mechanisms of axonal transport or a failure of energy production necessary for proper neurofilament transport [107]. Further study in rats revealed the earliest ultrastructural lesions due to repeated dosing with acrylamide are accumulations of neurofilaments and membranous materials in terminals and preterminal nodes of Ranvier [109, 110]. Thus, most authors believe at least part of the pathogenesis of acrylamide neuropathy involves some abnormality in axonal transport mechanisms [100,

111, 108]. Other lesions of acrylamide neuropathy are chromatolytic changes in the neuronal cell body and the formation of structures resembling microtubule organizing centers in cerebellar Purkinje cells [112, 108]. In general, sensory nerves are also considered more sensitive to the effects of acrylamide than motor nerves, but this selectivity is unexplained [111].

As stated previously, most authors believe that the underlying mechanism of acrylamide toxicity is related to its effect on axonal transport. Axonal transport systems are classified as to their rate and/or direction of flow down the axon. There are at least three anterograde transport mechanisms and two retrograde transport systems. Anterograde systems are made up of slow anterograde transport (at a rate of 0.1-4.0 mm per day) which supplies most of the cytoskeletal proteins to the axon as well as most of the components that maintain glycolytic function [113]. The slow anterograde systems are further classified into slow component a (SCa) which is responsible for the transport of neurofilament triplet proteins and tubulin and slow component b (SCb) which maintains other structural proteins, such as actin, and most of the glycolytic enzymes [114]. Fast anterograde transports systems (at rates of up to 400mm per day) move mostly membrane associated proteins and glycoproteins for maintenance of the axonal membrane, as well as neurotransmitter peptides for use in synaptic communication [100]. The retrograde transport system is responsible for the movement of a variety of substances, including acetylcholinesterase, adrenergic granules, and lysosomal proteins. In addition, retrograde transport is utilized by the neuron to move substances taken up at axon terminals for movement to the cell soma. Such substances include nerve growth factor [100]. A slow retrograde transport system has been identified and so far it is only known to transport a single protein, thought to be albumin [115].

Studies of the effect of acrylamide on axonal transport have been somewhat conflicting. Experiments on treated animals with clinical signs and morphologic signs of axonal degeneration have indicated either no changes or slight decreases in the rate of fast anterograde axonal transport and no change in the rate of slow anterograde transport [116, 117, 118]. Other investigators, however, have suggested that fast anterograde transport of protein is indeed affected by acrylamide [119, 120]. A recent review by Miller and Spencer [100], suggests that caution is warranted in interpretation of this data. Alterations may

have been present due to the direct effects of acrylamide or as a sequela to axonal damage after the primary lesion was no longer detectable. Also, multiple doses of acrylamide were used in these studies to induce neuropathy, further complicating interpretation of the data. Single dose studies in animals indicated that retrograde transport is dramatically affected by acrylamide due to a reduction in the quantity of material being transported. This alteration in transport quantity preceded the development of functional signs of neuropathy and behaved in a dose dependent manner, thus supporting its role as a primary mechanism of action.

Recent studies using single high doses of acrylamide support a direct effect on slow anterograde axoplasmic transport. Radiolabelling and morphometric studies of sciatic nerve complexes of rats compared the effects of single high dose administration of acrylamide with continuous administration. Two different abnormalities in slow axonal transport were found. In single high dose animals, the leading edge of the slow component was reduced in rate and coincided with the appearance of neurofilamentous accumulations in proximal axons and increased axonal diameter. Chronically dosed animals had lesions consistent with distal axonal degeneration and the proportion of neurofilaments in the slow component was greatly reduced with a decrease in axonal diameter when compared to neural lesions in single dosed animals. The authors suggested that the toxicity of acrylamide after a single high dose is the result of a primary effect on slow axonal transport with retarded movement of neurofilaments down the axon. No other cytoskeletal proteins were affected by acrylamide in this study [121].

Other effects of acrylamide on the cytoskeleton have been reported, including the depletion of microtubule associated proteins 1 and 2 (MAP1, MAP2) in the brains of treated rats. Rats were given continuous doses in water for two weeks and brain tissue examined for immunoreactivity to MAP1 and MAP2, using the peroxidase-antiperoxidase technique described earlier [7]. The immunoreactivity of MAP1 and MAP2 was substantially decreased in hippocampus, cerebellum, and cerebral cortex in preliminary studies. Further study of the extrapyrimidal system was undertaken to determine the relationship between toxicant induced neuronal damage and MAP reactivity. The caudate-putamen area was more severely affected (based on loss of immunoreactivity)

than other areas of the extrapyrimidal tract, compared to controls, illustrating a differential effect of acrylamide in this area of the rat brain. A significant decrease in MAP2 immunoreactivity was also observed in dendrites in affected areas. The authors concluded that the depletion of MAP immunoreactivity was probably an early biochemical event preceding peripheral neuropathy. Additionally, they concluded that the loss of MAP2 reactivity in dendrites was indicative of dendritic damage in addition to axonal damage in affected neurons [28, 27, 122].

In vitro studies of the effects of acrylamide have been undertaken with a variety of cell culture systems and explants, including rat dorsal root ganglion and sciatic nerve, chick spinal ganglia, Chinese hamster ovary cells, and proximal kidney tubule cells [123-127]. Several studies have also utilized neuroblastoma cell lines. Hoosima, et. al. [123], compared the sensitivity of mouse C1300 neuroblastoma cells with other cell culture systems using morphological and electrophysiological parameters to evaluate the cytotoxicity of acrylamide. One parameter evaluated in C1300 cells exposed to acrylamide was plating efficiency, and even though a decrease in plating efficiency was observed, it was not considered statistically significant compared to controls [123]. Another cytotoxicity study involved the coculture of liver cells with neuroblastoma cells. This *in vitro* system was developed to evaluate the metabolic activation of toxic compounds by exposing them to hepatocyte metabolism concurrently with a target cell type, in this case, N1E.115 mouse neuroblastoma cells. The cytotoxic effects of acrylamide increased significantly when hepatocytes cocultured with N1E.115 cells were stimulated by phenobarbital, bringing into question the effect of metabolism of acrylamide on its toxicity [124].

Acrylamide's effects on neurite outgrowth and neuritic transport of organelles in differentiated cells were also evaluated in N1E.115 mouse neuroblastoma cells. In an extensive study using acrylamide and several structural analogs to acrylamide, organelle transport rate within neurites was measured by videomicroscopy of serum deprived cells (for the induction of neurite outgrowth) in culture. Cells were exposed to cytotoxic levels (1mM) of acrylamide and glycidamide for 48 hours and the bidirectional rate of organelle transport within neurites measured. Organelle transport rate was unaffected by

treatment with these compounds at a concentration equivalent to a 50% effective concentration (EC_{50}), determined by radioactive labeling with ^{51}Cr. Neurite outgrowth was also impaired in approximately 50% of the cells by this concentration. The authors concluded that the neurotoxicity of acrylamide is probably due to a mechanism other than a direct effect on either rapid anterograde or retrograde axonal transport [26].

Acrylamide studies done in other cells in culture include PtK_1 and PtK_2 lines which are proximal tubule cells of renal origin. Eckert [125], using PtK_1 cells found juxtanuclear accumulations of vimentin and keratin intermediate filaments in acrylamide treated cells. These accumulations, first identified with immunofluorescent antibody labeling, were immunoreactive for both vimentin and keratin and were also identified by transmission electron microscopy. Microtubules and microfilaments were unaffected by the same treatment. Unlike the intermediate filament disruption that occurred from injection of cells with antibody to these filaments, washing of the cells caused the juxtanuclear aggregate to disperse and treated cells return to normal. No cytotoxic effects were found at the dosage used (5mM). Treatment of PtK_2 cells with 0.5 - 10mM acrylamide resulted in perinuclear accumulations of vimentin intermediate filaments. At the higher concentrations, microtubule distribution was similarly affected, but separate from the vimentin bundles. These findings were detected by immunofluorescent antibody labeling and confirmed as an altered migration pattern of vimentin-antibody complexes in immunoblotting studies. These authors suggested a parallel between the accumulation of vimentin intermediate filaments in epithelial cells and neurofilaments in similarly treated neurons and related their findings to a hypothesis of altered electrostatic charge in filament proteins that causes them to aggregate into focal accumulations [126].

In summary, the mechanism of acrylamide neuropathy is far from completely understood. Effects on all axonal transport systems have been documented and alternately disputed. Most researchers, however, still believe that the altered transport of neurofilaments in neurons and intermediate filaments in other cell types is at least part of the toxic mechanism, but it has been difficult to isolate the exact sequence of events either *in vitro* or *in vivo.*

III. GENERAL MATERIALS AND METHODS

III. A. CELL CULTURE

SY5Y human neuroblastoma cells, passage 19, were a generous gift of Dr. June Biedler, Memorial Sloan-Kettering Cancer Center, New York. These cells were maintained in a 1:1 mixture of Eagle's modified essential media (MEM), obtained from Gibco BRL (Grand Island, NY) and Ham's Nutrient Mixture F12 (F12) obtained from Sigma Chemical Company, (St. Louis, MO). Cells were further supplemented with 15% fetal bovine serum (FBS) also obtained from Sigma Chemical Company. Cells were maintained at 37°C in an incubator suitable for cell culture with a humidified atmosphere composed of 95% air and 5%CO_2. All research was conducted with cell passages from 19 - 29. Cells were grown as monolayers in 75-cm^2 tissue culture flasks (Corning, Cambridge, MA) with media changes at approximately 48-72 hour intervals. Upon confluency, cells were then harvested by trypsinization and diluted at a 1:2 or 1:3 ratio, cells to media. Cultures were monitored periodically by observation with a Nikon UFX-II phase contrast microscope.

In order to maintain SY5Y cells within 10 passages, cells were periodically frozen in liquid nitrogen by the following protocol: Confluent cultures in 75cm^2 flasks were harvested by trypsinization, centrifuged at 1000 rpm for 10 minutes and the pellet resuspended in a mixture of 10% dimethylsulfoxide (DMSO) and media. One microliter (ml) aliquots were dispensed into cryovials suitable for freezing and suspended in liquid nitrogen. Cells were thawed by suspending a vial in a 37° water bath until the aliquot was visibly liquidified, and diluted into a 75cm^2 culture dish in 9 ml of media. The media was changed 24 hours later to remove the DMSO and the culture maintained until confluency.

III. B. DIFFERENTIATION OF CELLS IN CULTURE

All-trans-retinoic acid (RA) was obtained from Sigma Chemical Company (St. Louis, MO.), dissolved in absolute ethanol and diluted into 50 millimolar (mM) stock solution. This stock solution was dispensed into 1 ml aliquots and maintained frozen in liquid nitrogen at -196°C until use. Dibutyrl cyclic adenosine monophosphate (dbcAMP) and isobutylmethylxanthine (IBMX) were also obtained from Sigma Chemical Company. Using a protocol kindly provided by Dr. Marga Oortgiesen [128], dbcAMP was dissolved in double distilled water, diluted into a 25 mM stock solution and maintained frozen at -20°C until use. IBMX was dissolved in a 50% ethanol solution, diluted into a 50 mM stock solution and maintained frozen at 0°C until use.

Preliminary studies of cell differentiation with RA were conducted with SY5Y cells in 35mm^2 tissue culture dishes (Corning). Cells were seeded at 3 x 10^5 cells/dish and allowed 24 hours to adhere to the dish surface. RA media was applied to the cells after 24 hours at a final concentration of 20μM RA per 2.5 ml of media and refreshed every 48 hours [25]. Cells were photographed at 24 hour intervals for up to 96 hours with a Nikon UFX-II phase contrast microscope with attached camera body. Differentiation was defined as the presence of neuritic processes on a majority (>50%) of the cells. Neurites were defined as processes extending from the cell body at least two times the cell diameter in length. Neurites were present on approximately 50% of the cells at 48 hours. This percentage seemed to increase to 60-75% at 72 hours, but cells were seeded too heavily to evaluate accurately. A subsequent study using different seeding densities was performed to determine the optimum seeding rate for visualization of neurites under the stimulation of RA. The optimum seeding density for neurite outgrowth was determined to be 5 x 10^4 cells/35mm^2 dish.

Preliminary studies with dbcAMP were performed in a similar fashion, with cells seeded at 5 x 10^{4}cells/35mm^2 dish. Cells were again allowed 24 hours to attach to the surface. Dibutyrl cAMP was diluted into serum free media, passed through a 0.2μ filter, and serum added to the filtrate for a final

concentration of 1mM dbcAMP in sterile media [19, 23]. Previous studies had become contaminated due to the possibilitiy of bacterial growth in the powdered dbcAMP, therefore, preparation of media was altered to include filtration for bacterial organisms. Photographs taken at 24 hour intervals for 96 hours were inconclusive, with no apparent increase in neuritic outgrowth. Additional studies incorporated the use of isomethylbutylxanthine (IBMX), a phosphodiesterase inhibitor, as per the protocol of Dr. Oortgiesen [128]. IBMX, by virtue of its action, further increases endogenous cAMP within the cell and delays the breakdown of endogenous or exogenous cAMP, therefore increasing the effect of treatment [20, 129]. Cells were seeded on 60 mm^2 dishes and treated after 24 hours, this time with filtered media containing 1mM dbc AMP and 1mM IBMX together. Cells were monitored at 24 hour intervals. Neurite outgrowth was present at 24 and 48 hours with an apparent reduction in cell proliferation rate based on visual comparison to control cells. Cells appeared to migrate into small clusters of two to five cells with neurite extension present on several cells. By 48 hours, cells appeared to detach and round up within a group, leaving one or two cells attached with neurite extension unaffected. This process continued so that by 72 hours, few cells remained attached with neurites intact.

In preparation for immunocytochemical studies, cells were grown on eight well chamber slides (Lab-Tek chamber slides, Nunc, Inc., Naperville, IL). Preliminary studies revealed that the SY5Y cells would not attach to glass slides, therefore, plastic Permanox chamber slides were obtained from Nunc, Inc. (Naperville, IL). The optimum seeding density for cell growth and differentiation with RA or cAMP/IBMX was determined to be 2 x 10^3cells per well.

III. C. TOXICANT AND DOSING

Acrylamide was obtained from Sigma Chemical Company, (St. Louis, MO) as a crystalline material stored in a sealed and labelled container at room temperature. Because of its highly water soluble nature, stock solution was made up in distilled water at 0.5 molar (M) and kept under refrigeration at +4°C.

Initial acrylamide treatment of cells was conducted with three concentrations of media containing 0.25 mM, 0.5 mM and 1.0 mM acrylamide. Undifferentiated cells in 35 mm^2 dishes were treated with supplemented media for 48 hours, and photographs were taken at 12, 24 and 48 hours. From this study, an optimum time and concentration of acrylamide was chosen to use with cells differentiated with RA. For immunohistochemical studies, cells were grown in eight well Permanox chamber slides (Lab-Tek chamber slides, Nunc, Inc., Naperville, IL), and treated, fixed and stained *in situ.*

III. D. IMMUNOCYTOCHEMISTRY

The type of detection system used was the peroxidase-antiperoxidase (PAP) system developed by Sternberger and Sternberger [7] based on its rather widespread and successful use in cell culture [4, 6, 31, 33, 34, 39, 65, 130]. The monoclonal antibodies for phosphorylated neurofilament and MAP2 came from Sternberger Monoclonals, Inc. (Baltimore, MD). The product used to label phosphorylated neurofilaments consists of a cocktail of monoclonal immunoglobulin G antibodies to hypo and hyperphosphorylated neurofilaments and is called panaxonal marker due to its specificity for axons in tissue sections and cultures [131]. The product chosen to label MAP2 is specific for MAP2c, a subclass of MAP2 detected in immature brain [9]. Both of these products are manufactured in mice and produced for use in human tissue. Both products were diluted in a 1% normal goat serum/tris buffered saline solution (1% NGS/TBS) to a concentration of 1:1000 before use.

The monoclonal antibodies to vimentin and to NF-L were obtained from Boehringer Mannheim Corporation (Indianapolis, IN). Both products were manufactured in mice and produced for use in human tissue. Anti-vimentin was obtained as a lyophilized product, which was resuspended in 1.0 ml of double distilled water and applied at a dilution of 1:5 in a 1.0% NGS/TBS solution. Anti-neurofilament-L was also lyophilized and resuspended in 2.0 ml of double distilled water, and applied at a dilution of 1:4 in a 1.0% NGS/TBS solution.

The secondary antibody was obtained from Sternberger Monoclonals, Inc. (Baltimore, MD). This antibody binds to mouse monoclonal antibody-antigen complexes via one Fab binding site and is manufactured in goats. It was diluted in 1.0%NGS/TBS and used at a dilution of 1:1000. The peroxidase-antiperoxidase reagent is Sternberger's ClonoPAP®, a product containing high affinity antiperoxidase immunoglobulin bound to peroxidase molecules. This product binds to the second Fab site on the secondary antibody. It was also diluted in 1.0%NGS/TBS and used at a dilution of 1:100.

The diaminobenzidine (DAB) was obtained in tablet form from Sigma Chemical Company (St. Louis, MO). Tablets were dissolved in Tris buffered saline and kept as a stock solution for 7 days. A 5.0 ml aliquot was used for staining and 50 µl of hydrogen peroxide added just before use.

Cells were grown in eight well chamber slides as previously described. Fixation of cells prior to immunocytochemical staining was performed by immersion of cells in a 1:1 acetone/methanol mixture for 2.0 minutes, followed by replenishment of the wells with fresh solution and further incubation for 8.0 minutes. Cells were rinsed once in TBS (pH 7.6) and allowed to air dry for 30 minutes. Cells were rinsed again in TBS and a solution of 3.0% NGS/TBS was applied with incubation for 30 minutes at room temperature.

After aspiration of the 3.0% NGS, cells were incubated with the appropriate dilution of primary antibody (anti-MAP2, anti-panaxonal marker, anti-NF-L, anti-vimentin) for the incubation time and temperature recommended by the manufacturer. Two wells of each slide were used as controls for the staining procedure and remained in 3.0% NGS until they were incorporated into the protocol. One control well was processed without primary antibody, and one well was processed without primary or secondary antibodies. After the appropriate incubation time, wells were aspirated, filled with TBS and gently agitated on a stir plate for 5.0 minutes. This washing procedure was repeated twice, for a total of three washes. Secondary antibody was applied to the appropriate wells and incubated at room temperature for 30 minutes in a humidified atmosphere. Triplicate washes in TBS were repeated at 5.0 minutes each and then the ClonoPAP® was applied to all wells with room temperature incubation for 30 minutes. Aspiration of the wells was followed by a single 5.0 minute wash in TBS, then wells were removed from the slides and washed in a

Copeland jar containing TBS for 7.0 minutes. Slide surfaces were flooded with DAB solution and incubated at room temperature for 3.0 minutes, then immersed in distilled water. Slides were covered with a water soluble mounting media (Supermount®; BioGenex Laboratories, San Ramon, CA) and stored at room temperature in a slide box.

Cells were evaluated as positive or negative for each antibody based on the presence or absence of a brown precipitate within the cytoplasm visualized by light microscopy. Nonspecific (background) staining was evaluated by observation of the controls for each slide. The control well containing no primary or secondary antibody was free of any stain precipitate and the control well containing no primary antibody contained little or no stain precipitate. In order to consider a slide positive for a particular antibody, a marked difference in the degree of staining was present between control wells and sample wells.

IV. MANUSCRIPT: THE EFFECTS OF ACRYLAMIDE ON THE CYTOSKELETON OF SY5Y NEUROBLASTOMA CELLS: AN IMMUNOCYTOCHEMICAL STUDY

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Abstract: SY5Y human neuroblastoma cells were used as an in vitro model of neurotoxicity to investigate cytoskeletal changes that may occur from the exposure of the developing nervous system to acrylamide. Cells were differentiated with either retinoic acid or dibutyryl cyclic adenosine monophosphate (dbcAMP) and a phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX). Differentiation was based morphologically on the appearance of neuritic processes in a majority (>50%) of the cells. Using the peroxidase-antiperoxidase technique, cells were labeled with monoclonal antibodies to cytoskeletal proteins (phosphorylated neurofilament, microtubule associated protein 2c, vimentin and low molecular weight neurofilament protein) either specific for axonal markers or linked to stages in neuronal development. Staining patterns were compared to undifferentiated cells using the same protocol. There were no differences in staining patterns found between RA or dbcAMP/IBMX treated cells or between differentiated cells and undifferentiated controls. Both RA differentiated and undifferentiated cultures were additionally exposed to acrylamide-treated media. Cells were fixed and stained after the observation of cellular swelling 24 hours post acrylamide treatment. In spite of obvious alterations in morphology in unstained cells in culture, the cytoskeletal staining pattern was unchanged after acrylamide treatment. We conclude that there is no difference in the cytoskeletal immunoreactivity of SY5Y neuroblastoma cells when differentiated with retinoic acid or dcAMP/IBMX after three days. In addition, it appears that acrylamide

does not affect the cytoskeletal structure of SY5Y neuroblastoma cells in undifferentiated or in RA differentiated cells.

KEY WORDS: acrylamide, neuroblastoma, SY5Y, cytoskeleton, peroxidase-antiperoxidase

INTRODUCTION

The neurotoxic effects of exposure to acrylamide have been recognized since the late 1950's (LeQuesne 1985), however, the mechanism of action of acrylamide has never been fully elucidated, despite extensive research efforts (Miller and Spencer 1985). Most investigators believe that a defect in axonal transport is primarily responsible for the focal accumulation of neurofilaments that accompany distal axonal degeneration in acrylamide neuropathy. The specific transport mechanism or mechanisms affected in acrylamide neuropathy, however, are unclear (Edwards, Sporel-Ozakat et al. 1991; Griffin 1989; Miller, Carter et al. 1982). Radioactive labelling studies in young rats exposed to single high doses of acrylamide demonstrated a direct toxic effect on slow axonal transport of neurofilaments in conjunction with proximal neurofilamentous accumulations (Gold, Griffin et al. 1985). Immunohistochemical studies with rat nervous tissue have implicated acrylamide as a cause of altered immunoreactivity to cytoskeletal proteins, leading to speculation about its effects on slow anterograde transport in the nervous system (Chauhan, Sabri et al. 1991; Gold, Griffin et al. 1985). *In vitro* studies in non-neuronal cell types have also demonstrated alterations in vimentin immunoreactivity upon exposure to acrylamide (Eckert 1985; Sager and Matheson 1988). Together, these studies suggest a possible neurotoxic role for acrylamide in the developing cytoskeleton of the mammalian nervous system.

Neuronal development has been studied in both primary neuronal cell culture and various neuroblastoma cell lines, including the SY5Y human neuroblastoma cell line. This line has been well characterized and used extensively in the *in vitro* study of neuronal development (Biedler, Helson et al. 1973; Kuramoto, Werrbach-Perez et al. 1981; Pahlman, Ruusala et al. 1984;

Perez-Polo 1985; Perez-Polo, Werrbach-Perez et al. 1979; Prasad 1974; Ross, Splenger et al. 1983; Tsokos, Scarpa et al. 1987; Yu, Hochhaus et al. 1988). Several compounds have been used to induce the formation of neurites, defined as process-like extensions of the cell body, with the goal of promoting differentiation toward a neuronal phenotype (Abemayor and Sidell 1989; Adem, Mattsson et al. 1987; Pahlman, Mamaeva et al. 1990; Pahlman, Ruusala et al. 1984; Perez-Polo, Werrbach-Perez et al. 1979; Prasad 1974). Retinoic acid, a vitamin A derivative, and dibutyryl cyclic adenosine monophosphate (dbcAMP), an analog of intracellular cAMP, have been used successfully to this end (Kuramoto, Werrbach-Perez et al. 1981; Pahlman, Ruusala et al. 1984; Schulze and Perez-Polo 1982; Sidell, Sarafian et al. 1986). We proposed to differentiate SY5Y cells with both compounds (after modification of the cAMP protocol to incorporate isobutyl-methyl-xanthine [IBMX], a phosphodiesterase inhibitor), and expose the cells to a neurotoxic dose of acrylamide. Characterization of cellular immunoreactivity was performed using monoclonal antibodies for cytoskeletal proteins, noting any staining differences in the two protocols or any changes in the staining pattern that could be attributed to exposure to the toxicant. This information, therefore, provides further evaluation of SY5Y neuroblastoma cells as an _in vitro_ model of cytoskeletal neurotoxicity.

MATERIALS AND METHODS

CHEMICALS AND IMMUNOCHEMICALS

All-trans retinoic acid (RA), N_6, O_{12}-dibutyryladenosine 3':5'- cyclic monophosphate (dbc AMP), 3-isobutyl-1-methyl-xanthine (IBMX), and 3, 3'-diaminobenzidine (DAB), were all obtained from Sigma Chemical Company, (St. Louis, MO). Retinoic acid was dissolved in absolute ethanol and maintained frozen at -196°C in liquid nitrogen until diluted into media. Using a protocol kindly provided by Dr. Marga Oortgiesen (Oortgiesen 1994), dbc AMP was dissolved in double distilled water, diluted into a 25 mM stock solution and maintained frozen at -20°C until use. IBMX was dissolved in a 50% ethanol solution, diluted into a 50 mM stock solution and maintained frozen at 0°C until use. Monoclonal antibodies for vimentin and low molecular weight

neurofilament protein (NF-L) were obtained from Boehringer Mannheim Corporation, (Indianapolis, IN). Monoclonal antibodies for hypo and hyper-phosphorylated neurofilament (panaxonal marker), microtubule associated protein 2c (MAP2c), normal goat serum, and other antibody conjugates (goat anti-mouse IgG, peroxidase-antiperoxidase complex) were obtained from Sternberger Monoclonals Incorporated, (Baltimore, MD). Acrylamide was obtained from Sigma Chemical Company (St. Louis, MO), dissolved in distilled water as a 0.5M solution and kept under refrigeration at +4°C until use.

CELLS AND CULTURE CONDITIONS

SY5Y human neuroblastoma cells, passages 19-29, were a generous gift of Dr. June Biedler, Sloan-Kettering Memorial Cancer Center, New York. Cells were grown and maintained in a 1:1 mixture of Eagle's Minimal Essential Media (Gibco BRL, Grand Island, NY) and Ham's F12 Nutrient Mixture (Sigma Chemical Company, St. Louis, MO) supplemented with 15% heat inactivated fetal calf serum in a 37°C humidifed atmosphere consisting of 95% air and 5% CO_2. For differentiation studies, cells were plated at 2×10^3 cells per well in eight well chamber slides (Lab-Tek Chamber Slides, Nunc, Inc., Naperville, IL), given 24 hours to attach to the slide surface and treated with either 20μM RA media or 1mM dbc AMP/IBMX media. Control slides were seeded simultaneously and either treated with media containing 0.2% absolute ethanol or plain media as controls. Media was changed at 48 hour intervals until >50% of the cells extended neurites greater than two somal diameters in length. Media containing acrylamide (1mM) was applied after differentiation and cells were maintained for 24 hours before staining. Undifferentiated cells and cells containing 0.2% ethanol in media were given fresh media changes in an identical manner and stained accordingly.

IMMUNOCYTOCHEMICAL STAINING

Cells in 8 well chamber slides were fixed in a 1:1 acetone:methanol solution and allowed to air dry for 30 minutes. Two control wells were designated for each slide, one processed without the application of primary antibody and one processed without the application of primary or secondary antibody. Slides were then rinsed with Tris buffered saline, pH 7.6. All wells

were covered with 3% normal goat serum in Tris buffered saline, (3.0% NGS/TBS) and incubated for 30 minutes at room temperature. Slides were then incubated with their respective primary antibodies, at dilutions of 1:4 for NF-L, 1:5 for vimentin, and 1:1000 for panaxonal marker and MAP2c, respectively. After rinsing with TBS, slides were incubated with the appropriate secondary antibody for 30 minutes at room temperature. Rinsing was repeated and slides were incubated with a peroxidase/antiperoxidase conjugate (ClonoPAP®, Sternberger Monoclonals, Inc., Baltimore, MD) for 30 minutes, rinsed and reacted with DAB in the presence of hydrogen peroxide for 3 minutes. Cells were rinsed, covered with a water soluble mounting media (Supermount®, BioGenex Laboratories, San Ramon, CA) and observed by light microscopy. Cells were graded positive or negative based on the presence of a brown granular reaction product within the cytoplasm of stained cells. Cells were also evaluated by comparison to control wells on each slide and to undifferentated controls.

RESULTS

RETINOIC ACID DIFFERENTIATION

Cells treated with retinoic acid extended fine, occasionally branched neurites that were present in approximately 50% of treated cells by 48 hours post-treatment. At 72 hours post-treatment, approximately 75% of the cells were considered differentiated based on the presence of neurites at least two cell somal diameters in length (Figure 1M). Cells differentiated with retinoic acid were processed simultaneously with undifferentiated cells and cells treated with .2% ethanol as controls. All groups of cells were positive for vimentin and MAP2c and negative for panaxonal marker at 72 hours. Cell staining appeared uniform, with no predilection for either a perikaryal or neuritic distribution (Figures 1M, 2M and 3M). Cells stained for NF-L, however, contained focal areas of reaction product in perinuclear regions, again, in all three groups examined (Figure 4M). Each slide was negative for immunoreactivity when primary antibodies were eliminated from the staining protocol.

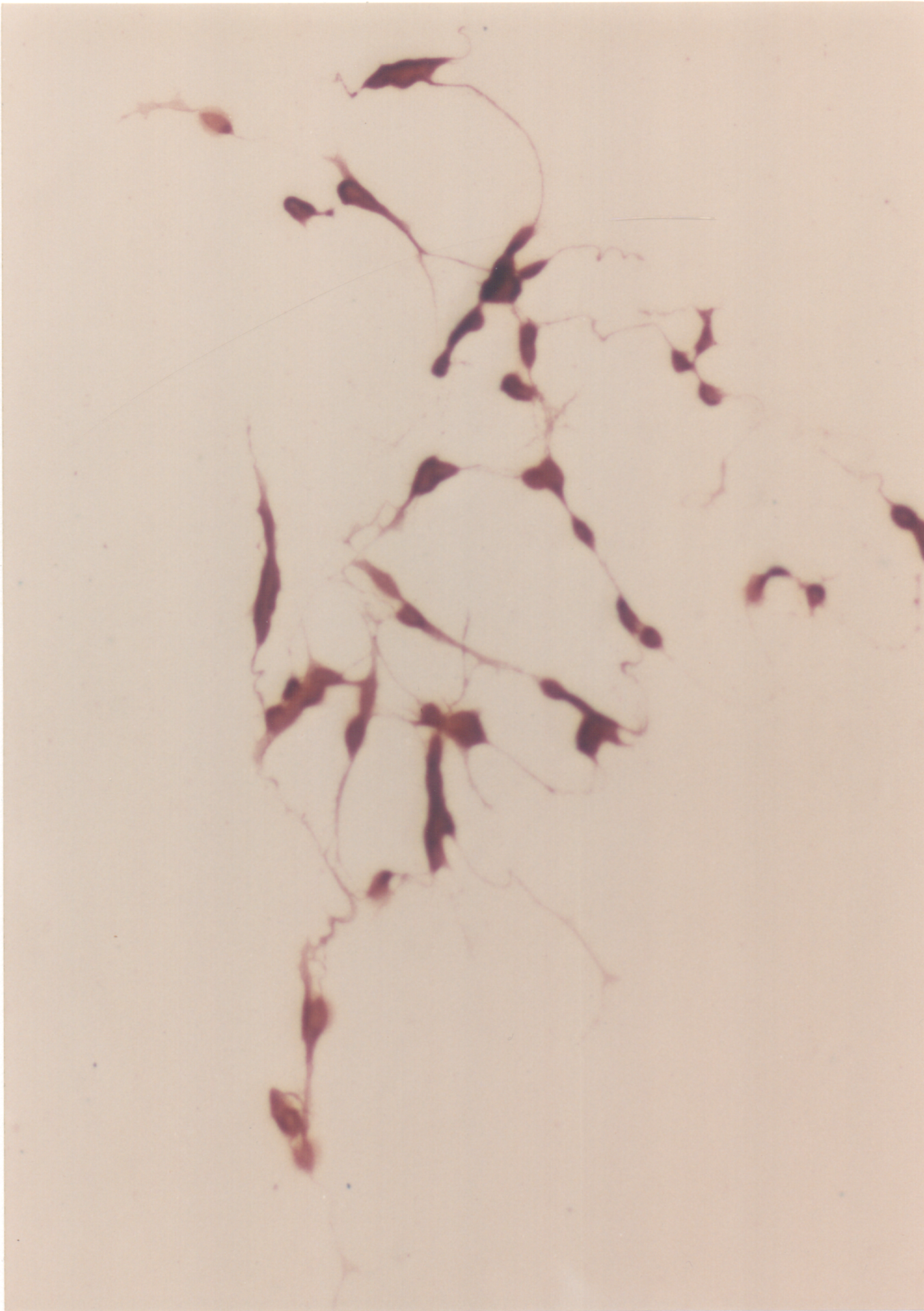

FIGURE 1M-SY5Y neuroblastoma cells at 72 hours post-treatment with retinoic acid (RA). 75% of the cells contain neurites that are at least 2 times the cell body diameter in length. These cells are also immunopositive for MAP2. Vimentin immunopositive cells displayed an identical staining pattern. Light microscopy, 20X.

FIGURE 2M-SY5Y neuroblastoma cells at 72 hours post-treatment with RA. These cells are immunonegative for panaxonal marker antibody. Light microscopy, 20X.

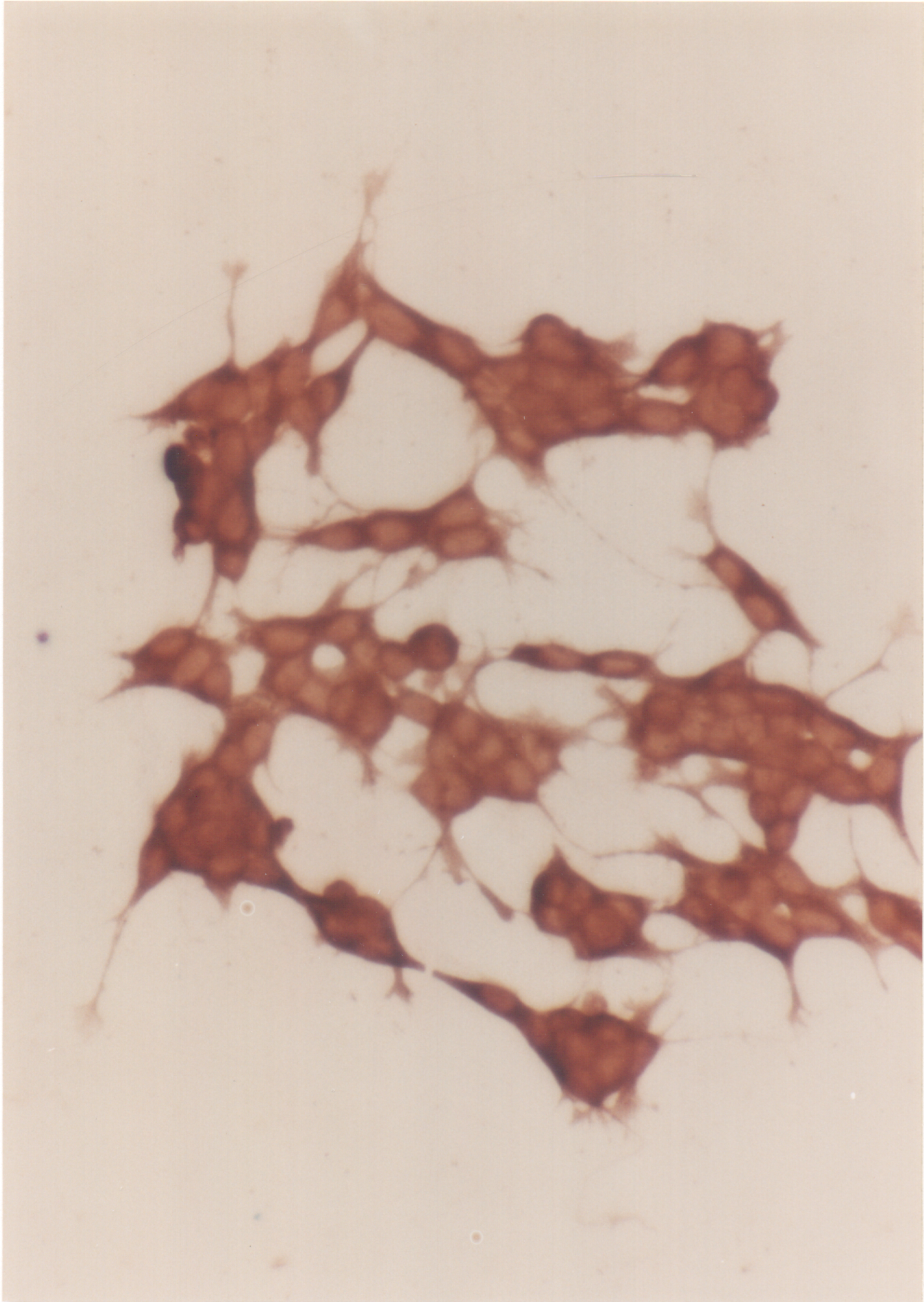

FIGURE 3M-SY5Y neuroblastoma cells at 72 hours post-treatment with 0.2% ethanol as a control. Cells are diffusely immunopositive for vimentin, both within the cytoplasm and neuritic processes. Light microscopy, 20X.

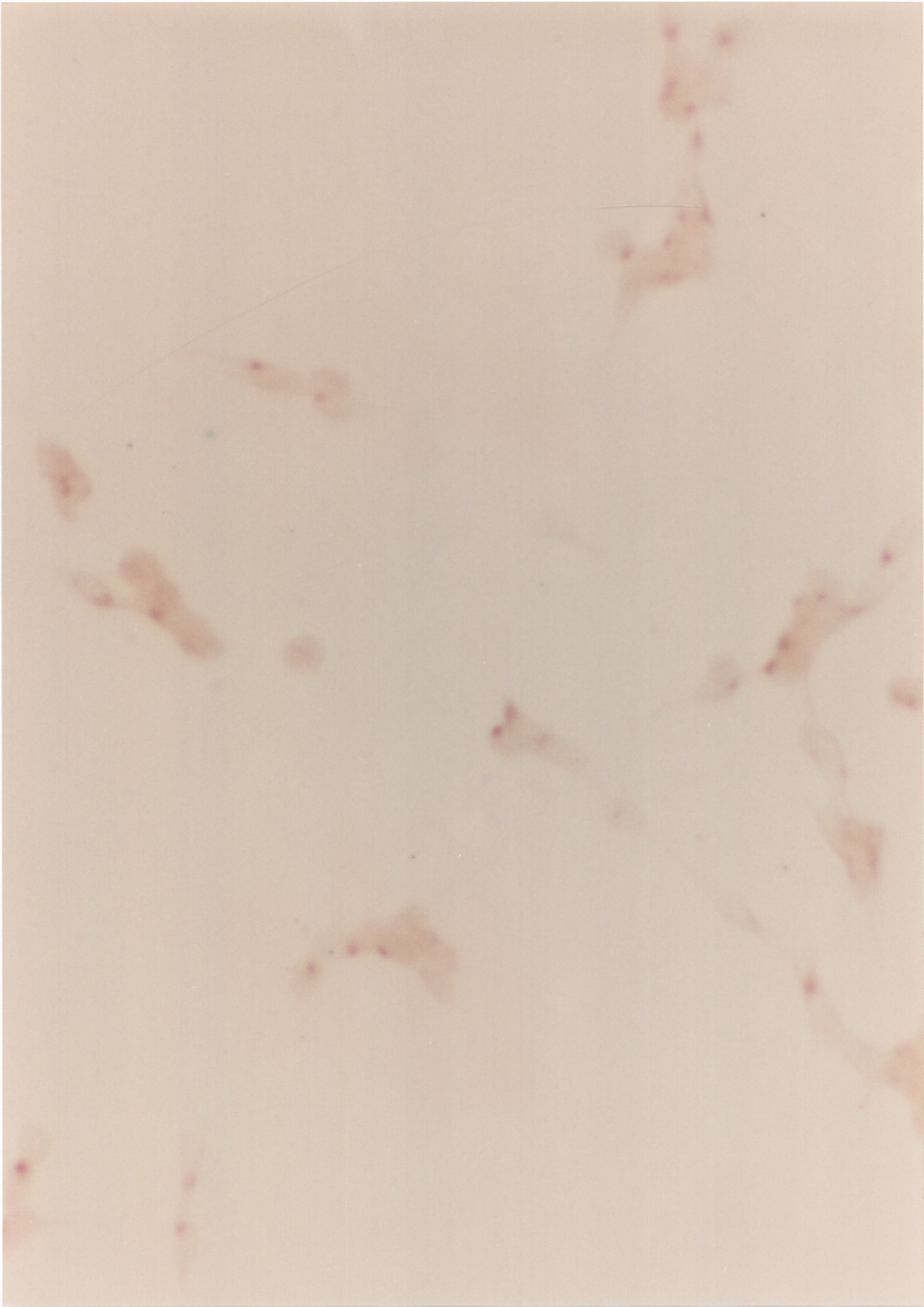

FIGURE 4M-SY5Y neuroblastoma cells at 72 hours post-treatment with RA. Cells contain focal areas of immunopositive staining for NF-L in perinuclear areas. Light microscopy, 20X.

dbcAMP/IBMX DIFFERENTIATION

Cells treated with dbcAMP/IBMX at 24 hours demonstrated migration of cells into small clusters with neurite extension in some cells (<50%). At 48 hours, cell clusters contained several shrunken, rounded cells within a group and increased somal diameter among the remaining cells. Several cell fragments were present and floating in the media. At 72 hours, fewer cells were present on the culture surface and there was an increase in shrunken cells within a group (Figure 5M). Remaining cells (>50%) contained vacuolated cytoplasm and with neurites many times the cell soma in length. Staining patterns were identical to RA differentiation, with cells positive for vimentin and MAP2c, and negative for panaxonal marker. dbcAMP/IBMX cells also contained perikaryal areas that were immunoreactive for NF-L (Figure 6M).

SY5Y IMMUNOREACTIVITY AFTER ACRYLAMIDE EXPOSURE

A preliminary study revealed diffuse cellular swelling in undifferentiated cells treated with 1mM acrylamide at 12, 24 and 48 hours illustrated by a progressive increase in somal diameter of individual cells (Figure 7M). Cells were differentiated with RA for 72 hours to allow a majority of the cells to extend neurites. Media was replaced with acrylamide media and cells were allowed to incubate for 24 hours. Acrylamide-treated RA differentiated cells showed swelling similar to that noted in undifferentiated cells (above). They were unchanged in their cytoskeletal immunoreactivity being positive for vimentin and MAP2c, and negative for panaxonal marker (Figures 8M, 9M and 10M)..

DISCUSSION

Neurite outgrowth has been used extensively as a morphologic indicator of differentiation in neuroblastoma. It is the most commonly measured parameter of differentiation in neuronal cell lines (Bottenstein 1981). Some investigators measure growth rate in association with neurite extension, but neurite extension and cell division are independent events (Bottenstein and Sato 1979; Sato 1973). Neurite extension has also been measured in

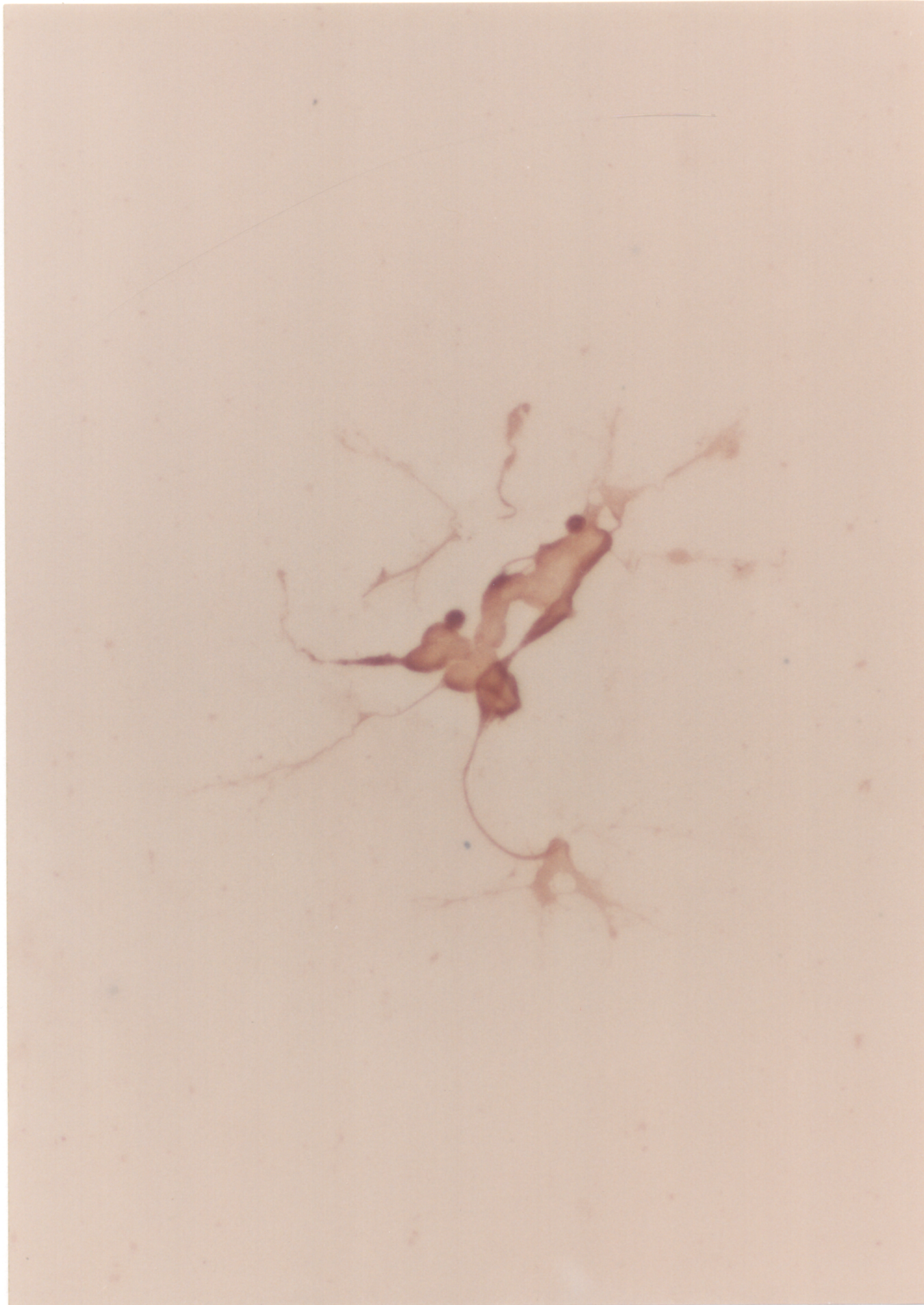

FIGURE 5M-SY5Y neuroblastoma cells treated with dbcAMP/IBMX for 72 hours. Cells are clumped with well developed neurites and some cells are no longer present on the culture surface. These cells are immunopositive for MAP2c. Light microscopy, 20X.

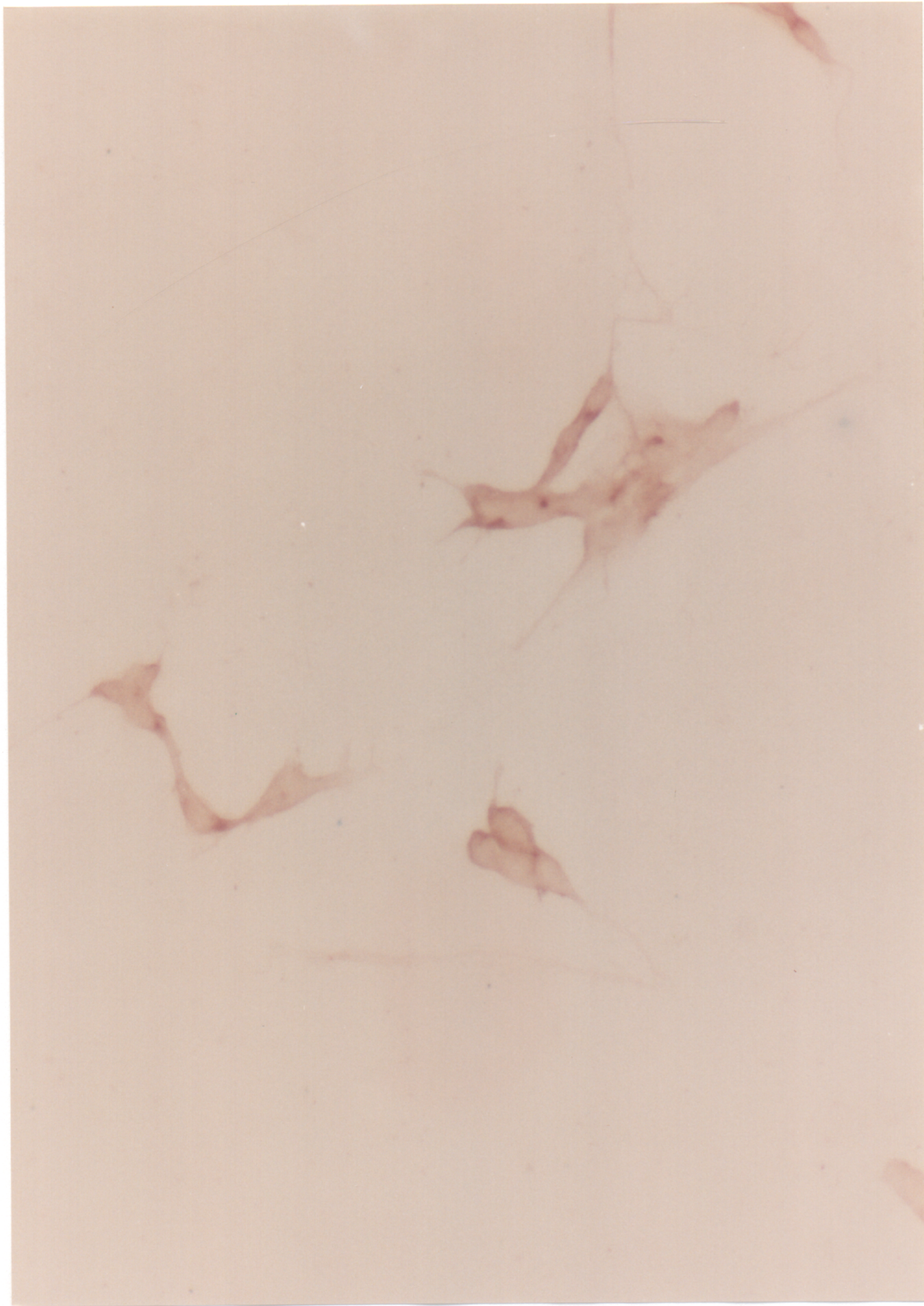

FIGURE 6M-SY5Y neuroblastoma cells treated with dbcAMP/IBMX for 72 hours. Several cells contain perikaryal areas of immunopositivity for NF-L. Light microscopy, 20X.

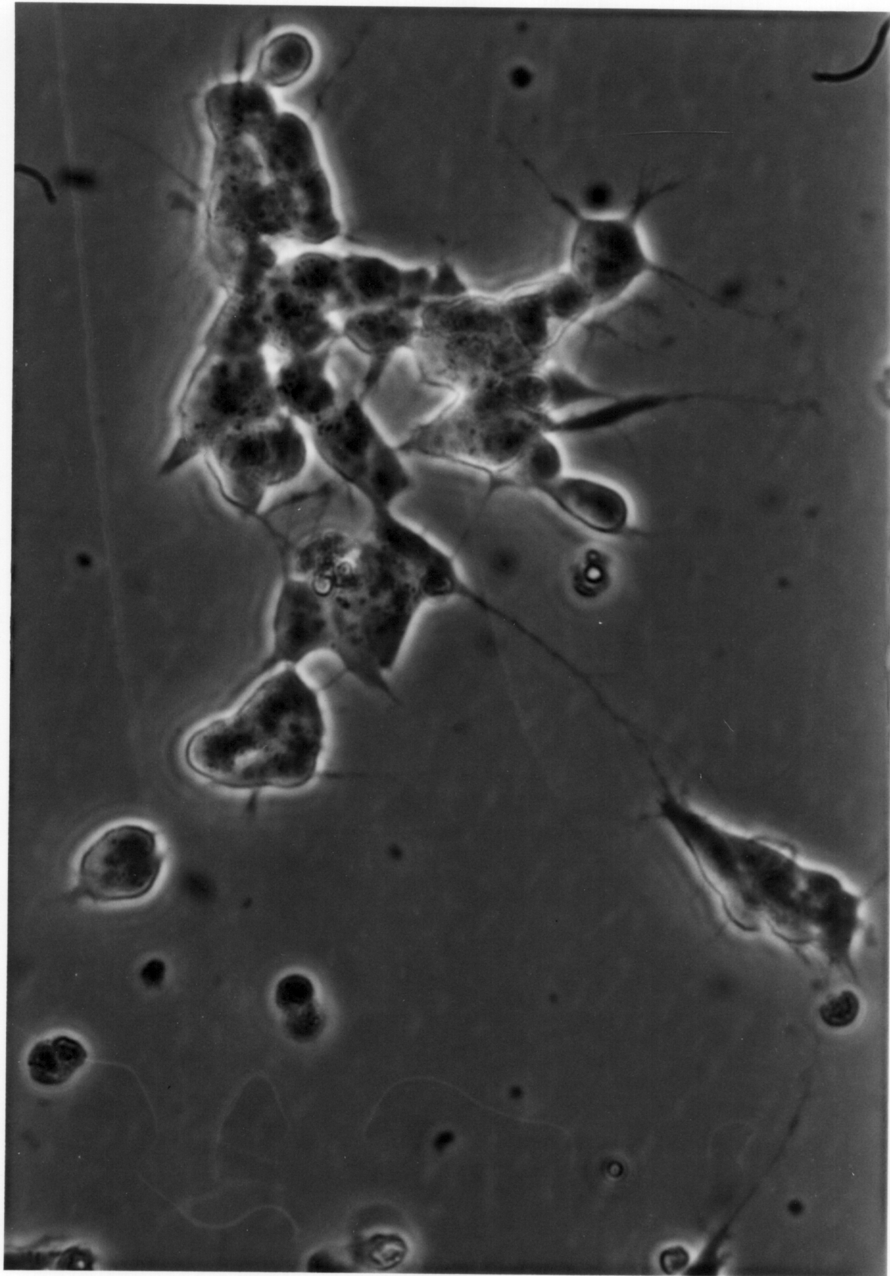

FIGURE 7M-SY5Y neuroblastoma cells (undifferentiated) after 24 hours exposure to 1.0mM acrylamide. Cells are diffusely swollen and rounded but remained attached to the culture surface. Phase contrast microscopy, 20x.

FIGURE 8M-SY5Y neuroblastoma cells differentiated with RA and exposed to 1.0mM acrylamide for 24 hours. Cells are somewhat swollen, but remain diffusely immunoreactive for vimentin. Light microscopy, 20X.

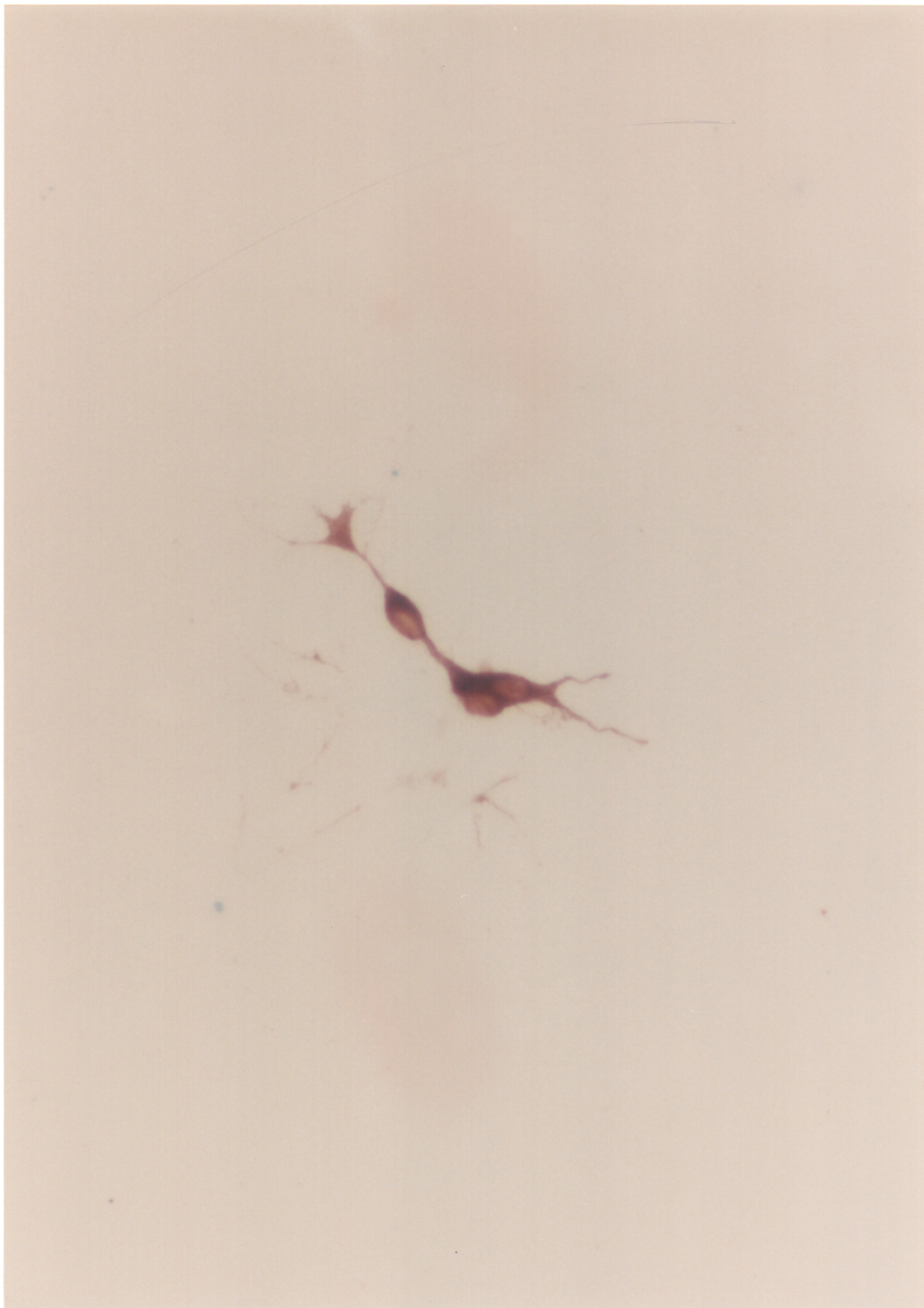

FIGURE 9M-SY5Y neuroblastoma cells differentiated with RA and exposed to 1.0mM acrylamide for 24 hours. Cells remain diffusely immunopositive for MAP2c with well developed neurites. Light microscopy, 20X.

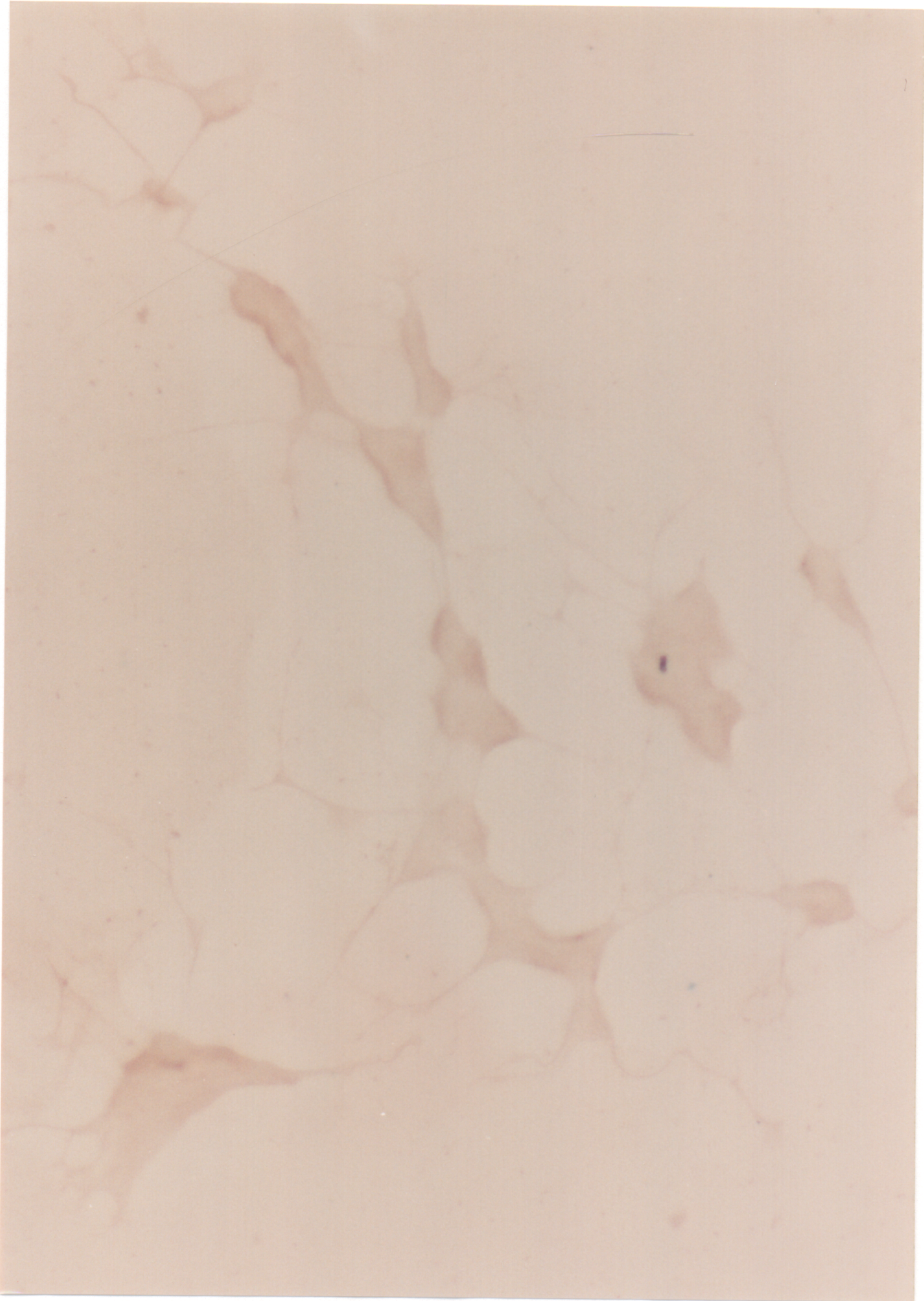

FIGURE 10M-SY5Y neuroblastoma cells differentiated with RA and exposed to 1.0mM acrylamide for 24 hours. Cells remain immunonegative for panaxonal marker. Light microscopy, 20X.

conjunction with biochemical and physiological markers of differentiation in SY5Y neuroblastoma cells. Examples of such markers associated with RA or dbcAMP differentiated SY5Y cells include the spontaneous generation of action potentials (Kuramoto, Werrbach-Perez et al. 1981), increased neuron specific enolase activity, and increased norepinephrine levels (Pahlman, Ruusala et al. 1984). In this study, the presence of neurite outgrowth in >50% of the RA or dbcAMP/IBMX treated cells is consistent with the parameters used for morphologic differentiation in SY5Y neuroblastoma cells in previous studies (Pahlman, Ruusala et al. 1984; Perez-Polo 1985; Prasad 1974).

The mechanism of retinoic acid differentiation is unknown. One theory relates the effect of retinoic acid to increases in protein kinase activity, specifically cAMP-dependent protein kinase and the calcium dependent protein kinase C (Abemayor and Sidell 1989). These enzymes are involved in phosphorylation mechanisms and linked to the control of signal transduction and the differentiation of tumors in mechanisms of carcinogenesis (Kumar, Cotran et al. 1992). Cyclic AMP and its derivatives are also thought to act by stimulating phosphorylation of proteins through kinase activation. IBMX, a phosphodiesterase inhibitor, acts to decrease the metabolism of endogenous cAMP, therefore adding to its stimulatory effects (Abemayor and Sidell 1989). Both RA and dbcAMP/IBMX cells had the same immunostaining patterns after treatment, as would be expected if both compounds elicit cell differentiation along the same pathways. However, control cells with no exposure to either compound had identical immunoreactivity. Therefore, it seems that cytoskeletal differentiation does not, in fact, occur in SY5Y neuroblastoma cells when exposed to these compounds, even though biochemical differentiation reportedly does (Adem 1987; Tsokos, et. al. 1987; Yu et. al 1988).

Nixon and Shea (Nixon and Shea 1992) have established a hypothesis of neuronal development based on the presence or absence of certain cytoskeletal proteins in studies with primary neuronal cultures and mouse neuroblastoma cells. According to this hypothesis, vimentin is present early in neuronal development, but is eventually replaced by the neurofilament triplet proteins, NF-L, NF-M and NF-H. NF-L and NF-M are thought to establish the neuronal phenotype and NF-H appears later in development as neurofilaments

are assembled into the cytoskeleton. As axonal processes are established, the neurofilaments become phosphorylated and provide stability to the axonal cytoskeleton. The presence of phosphorylated neurofilaments has subsequently been used a marker of axonal development as well as evidence suggesting differentiation toward a neuronal phenotype (Nixon and Shea 1992; Shea, Majocha et al. 1988; Shea, Sihag et al. 1988). In our study we observed neurite extension in a majority of cells in the absence of staining for panaxonal marker. Such an absence of cytoskeletal markers of neuronal differentiation suggests that neurite outgrowth alone should not be used as evidence of morphologic differentiation in RA or dbcAMP/IBMX treated SY5Y cells.

Vimentin is an intermediate filament commonly found in mesenchymal cells, but it is also expressed in most dividing neuroepithelial cells (Cambray-Deakin 1991). As previously mentioned, vimentin is gradually replaced by neurofilament proteins in neurons as they become postmitotic (Cochard and Paulin 1984; Nixon and Shea 1992; Shea 1990). Vimentin subunits can coexist transiently with neurofilament subunits within the same neurite *in vivo* (Cochard and Paulin 1984), but they assemble into separate systems as differentiation occurs. Research with mouse neuroblastoma cells has demonstrated that vimentin plays an initial role in neurite outgrowth, but it is gradually replaced by neurofilament proteins as the neurite becomes stable and develops its axonal morphology (Shea and Nixon 1988). The immunoreactivity of SY5Y cells for vimentin regardless of their treatment with RA or dbcAMP/IBMX suggests that these cells are not maturing towards a neuronal phenotype after 72 hours exposure to these differentiating agents.

NF-L is a 70 kilodalton protein and one of the neurofilament triplet proteins. SY5Y cells have been reported to contain NF-L, based on Western blots performed on the cytoskeletal fractions of undifferentiated cells (Ross, Ciccarone et al. 1988). If the hypothesis proposed by Nixon and Shea is accurate, then perhaps these cells (regardless of treatment with RA or dbcAMP/IBMX) would be classified as early in the differentiating process, that is, at the point where vimentin and neurofilaments can co-exist, but before the appearance of phosphorylated neurofilaments or the disappearance of vimentin from the cytoskeleton. We have no explanation for the apparent focal perikaryal location of the NF-L staining. Shea, et. al. (Shea, Sihag et al. 1988), have

reported labeling of the nuclear envelope by neurofilament antibodies and discussed the possibility of the cross-reaction of antibodies with nuclear laminins. The nuclear laminins are nuclear envelope proteins that share antigenic determinants with neurofilament proteins (Fisher, Chaudhary et al. 1986). However, this cross reactivity should manifest itself in a circumferential pattern, rather than a single focus. It is possible that this area is a local area of NF-L production (as in the Golgi apparatus) and the protein is localized in such a manner until the time of assembly into neurofilaments.

Microtubule associated protein 2 (MAP2) is a component of the interconnecting bridges between microtubules and neurofilaments in the neuronal cytoskeleton (Hirokawa 1991). It is a major component of brain microtubules and generally considered neuronal specific. MAP2 functions include stimulation of microtubule assembly and stabilization of assembled microtubules as well as crosslinking of microtubules to each other, to organelles and to other cytoskeletal elements, including NF-L (Burgoyne 1991). In the adult central nervous system, MAP2 is localized to dendrites and cell bodies and thus absent from most axons (Bernhardt and Matus 1982; Burgoyne and Cumming 1983; Burgoyne and Cumming 1984; Caceres, Banker et al. 1984; De Camilli, Miller et al. 1984). In the developing brain, there are multiple subforms of MAP2 (a, b, and c) and they vary in the time of their appearance and disappearance during the neuronal maturation process. One of these forms, MAP2c, is most abundant in immature brain, while MAP2a is present only in adult brain and MAP2b can be in either, depending somewhat on the species (Riederer 1992; Binder, Frankfurter et al. 1984; Burgoyne and Cumming 1984; Couchie and Nunez 1985; Garner, Brugg et al. 1988). Some authors believe that due to the early appearance and persistence of MAP2 during development, its presence may label neuritic processes destined to be dendrites (Bernhardt and Matus 1982). The presence of MAP2c immunoreactivity during neurite outgrowth in SY5Y cells is not unexpected, given that other markers of neuronal maturation do not appear to be present, i.e., phosphorylated neurofilament (panaxonal marker). It is tempting to speculate that the neuritic processes are dendritic in nature, but the antibody that we used is specific for MAP2c (Sternberger 1994) and we are therefore unable to rule out positive staining due to the immaturity of the cells. MAP2c has also been reported to be

abundant in axons in developing brain (Burgoyne 1991), therefore no conclusions can be drawn as to the specificity of the staining in these cells other than they are probably still immature.

NB2a/d1 mouse neuroblastoma cells and their cytoskeletal proteins have been studied by Shea and colleagues extensively as a model of neuronal development. Differentiation of NB2a/d1 cells with cAMP results in the development of an axonal morphology based on the appearance of phosphorylated high molecular weight neurofilament proteins, microtubule associated protein 1, and tau protein within the neurite (Shea, Beerman et al. 1989; Shea, Sihag et al. 1988). Other morphologic findings include the formation of long, unbranching, bipolar neurites with ultrastructural characteristics of axons (no ribosomes, bundles of filament, long microtubules) (Shea, Fischer et al. 1985). Differentiation of NB2a/d1 cells with RA leads to the development of a dendritic morphology based on the formation of highly branched neurites and ultrastructural features consistent with dendrites (numerous polysomes, no intermediate filaments, no junctional densities at contact points with other cells) (Shea, Fischer et al. 1985). The SY5Y cells did not respond to RA and dbcAMP in the manner of NB2a/d1 cells, even though the exposure times and final concentrations were similar to that used by Shea and colleagues (Shea, Fischer et al. 1985) In fact, there was no response to dbcAMP when used alone in our study and a phosphodiesterase inhibitor (IBMX) was necessary for initial neurite outgrowth (unpublished observation). This difference could be due to species differences, as SY5Y neuroblastoma cells are of human origin, or differences in the nature of the neoplastic cells themselves, i. e., differences in the neoplastic mechanism of individual neuroblastomas. SY5Y cells elaborated long, occasionally branched neurites with either protocol. Based on the data thus far, it appears that SY5Y cells react in a similar fashion with either of our differentiation protocols based on their immunoreactivity to cytoskeletal proteins.

Cytoskeletal elements in neurons are thought to be transported by the slow anterograde transport system. Acrylamide has been reported to decrease slow axonal transport rate, resulting in the accumulation of neurofilaments in proximal axons of sciatic nerve complexes of treated rats (Gold, Griffin et al. 1985). In our study, SY5Y cells stained for NF-L in a perikaryal location and

treatment with acrylamide did not appear to alter that distribution. Proximal kidney tubule cell lines and cultured fibroblasts have demonstrated perikaryal accumulations of vimentin intermediate filaments upon exposure to acrylamide (Eckert 1985; Durham, Pena et al. 1983; Sager and Matheson 1988). SY5Y cells, however, both in undifferentiated and RA differentiated cells, were positive for vimentin, and the distribution was not altered by treatment with acrylamide. Therefore, SY5Y cells do not appear to react in the same way as mesenchymal cells in culture to acrylamide. Acrylamide is metabolized primarily by conjugation with glutathione in the liver, but action by the microsomal P450 system also occurs. Whether acrylamide is bioactivated by P450 metabolism is a source of controversy among investigators, but all agree that the parent compound alone is neurotoxic (Miller and Spencer 1985). Therefore, if the effect of decreased slow antergrade transport is mediated by a metabolite, our data is inconclusive. However, based on the data thus far, it appears that acrylamide does not affect the cytoskeletal structure of SY5Y neuroblastoma cells after 24 hour exposure to a neurotoxic dose.

In summary, SY5Y human neuroblastoma cells appear to have a stable cytoskeletal structure composed of vimentin, MAP2c and NF-L that does not progress to that of a mature neuron despite neurite outgrowth after exposure to RA or dbcAMP/IBMX for 72 hours. Exposure of RA differentiated cells to acrylamide for 24 hours does not appear to alter the distribution of cytoskeletal components despite visible cellular swelling. We are confident of the specificity of the antibodies used for this study, however specificity could be confirmed with immunoblotting studies of the cytoskeletal fractions of these cells in future studies.

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V. ADDITIONAL RESULTS AND DISCUSSION

V. A. ADDITIONAL RESULTS

After concluding there were no differences between cells treated with RA and cells treated with dbcAMP/IBMX after 72 hours, further experimentation was pursued with RA differentiation alone. Cells were seeded as previously described for RA differentiation and incubated for 9 days in RA, with media changes every 72 hours. Cell proliferation appeared to slow down dramatically and remaining cells proceeded to migrate into small clusters with neurite extension. After 9 days, individual cells within the cluster proceeded to appear shrunken and somewhat detached from the culture surface, while underlying cells remained and continued to extend neurites. This appearance was identical to that of dbcAMP/IBMX cells after 72 hours. Cells were again fixed and stained by the same protocol. The staining pattern differed from previous experiments in that cells became markedly panaxonal marker immunopositive (Figures 3 and 4).

Further experimentation with the 9 day differentiation period was conducted. Cells were again seeded in eight well chamber slides and differentiated with retinoic acid. A slide containing ethanol control cells (0.2% ethanol) was also incubated for 9 days. These cells also demonstrated neuritic outgrowth with migration into clusters, but cells continued to proliferate. After 9 days, 1.0mM acrylamide media was applied to the RA differentiated cells. Ethanol controls were untreated. After 24 hours, all cells were fixed and stained as described previously. Acrylamide treated cells were again visibly swollen after 24 hours as visualized by phase contrast microscopy. Staining patterns in RA/acrylamide cells again illustrated immunoreactivity for vimentin, MAP2 and panaxonal marker (Figure 5). Ethanol control cells were also positive for panaxonal marker (Figure 6).

A preliminary experiment was performed with undifferentiated cells in 60mm^2 Permanox (Nunc, Inc., Naperville, IL) Petri dishes. Cells were cultured for 3 days, fixed and stained for vimentin. Further processing for electron microscopy was performed [134] and cells thin sectioned and examined for the presence and location of DAB immunoreactivity. Fixation of the cells in

acetone:methanol 1:1 for electron microscopy provided less than ideal quality, but DAB complexes were located intracellulary.

Preliminary experiments with fixation protocols were performed prior to selecting acetone:methanol as the appropriate fixation for SY5Y cells on Permanox slides. Cells were seeded in eight well chamber slides as previously described and fixed by the following 5 protocols: 1) Immersion in 1:1 acetone:methanol for 10 minutes, followed by 30 minutes of air drying [135]; 2) Immersion in 1:1 acetone:methanol for 10 minutes, followed by immersion in Tris buffered saline [135]; 3)Immersion in 4.0% paraformaldehyde in .1M phosphate buffer, followed by immersion in Tris buffered saline [136]; 4) Immersion in 95% ethanol for 5 minutes followed by 80% for 3 minutes, then 70% for 3 minutes and then immersion in water [137], and 5) Air drying cells to 90% dry, followed by immersion in 100% acetone for 2 minutes, then addition of fresh acetone with 5 minute incubation [137]. Following fixation, all slides were stained with Meyer's hematoxylin for 3 minutes and examined with light microscopy. The protocol that provided the best preservation of cellular morphology was 1:1 acetone:methanol with 30 minutes air drying and this protocol was used for the remainder of the study.

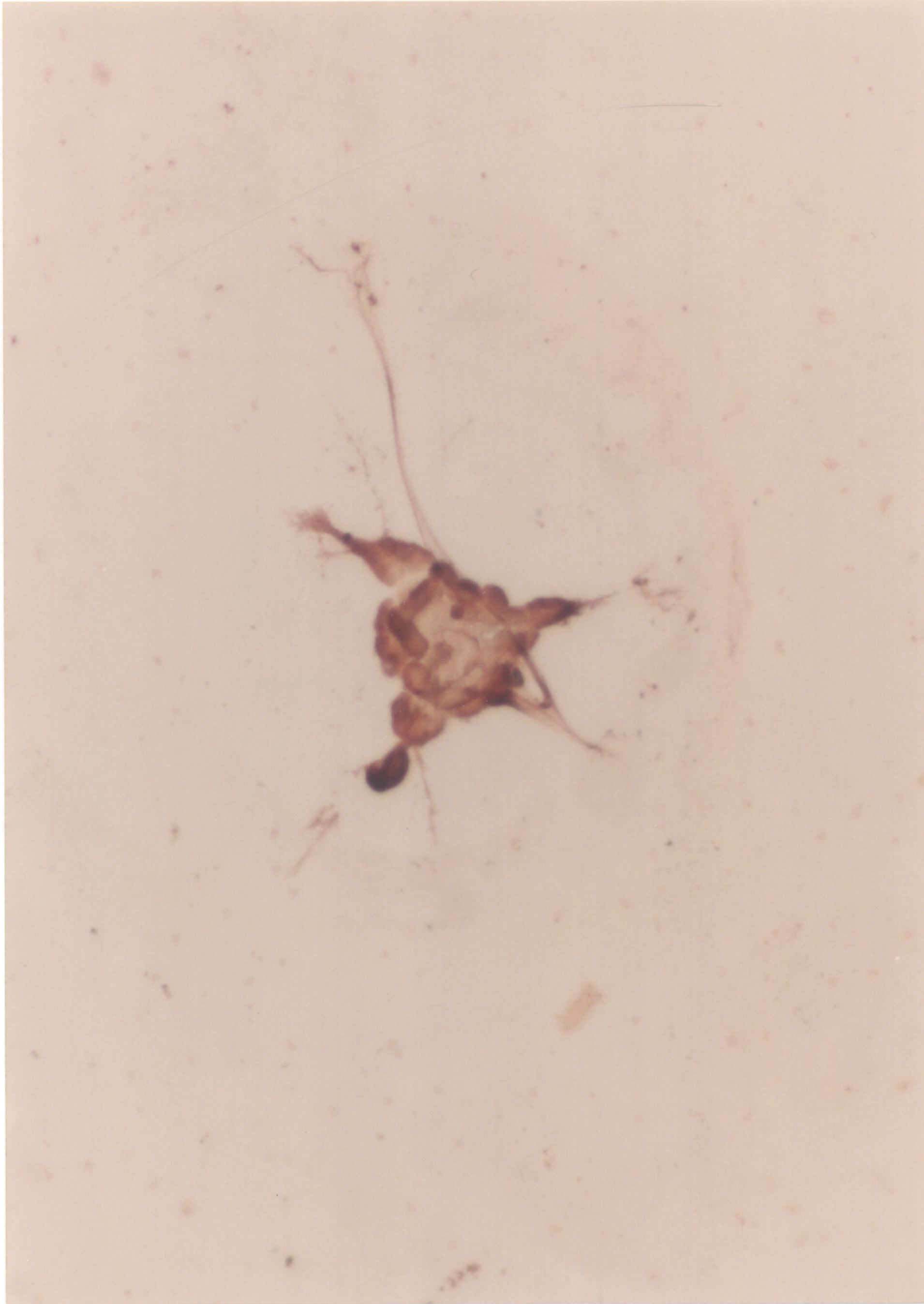

FIGURE 3-SY5Y neuroblastoma cells treated with RA for 9 days. Cells are clumped with well developed neurites. Cells are now diffusely immunopositive for panaxonal marker. Light microscopy, 20X.

FIGURE 4-SY5Y neuroblastoma cells treated with RA for 9 days. These cells were processed without primary antibody for panaxonal marker and are immunonegative when compared to figure 3 cells.

FIGURE 5-SY5Y neuroblastoma cells differentiated with RA for 9 days and exposed to 1.0mM acrylamide for 24 hours. Cells are clumped as with RA treatment alone, but are swollen. All cells and neuritic processes remain diffusely panaxonal marker immunopositive. Light microscopy, 20X.

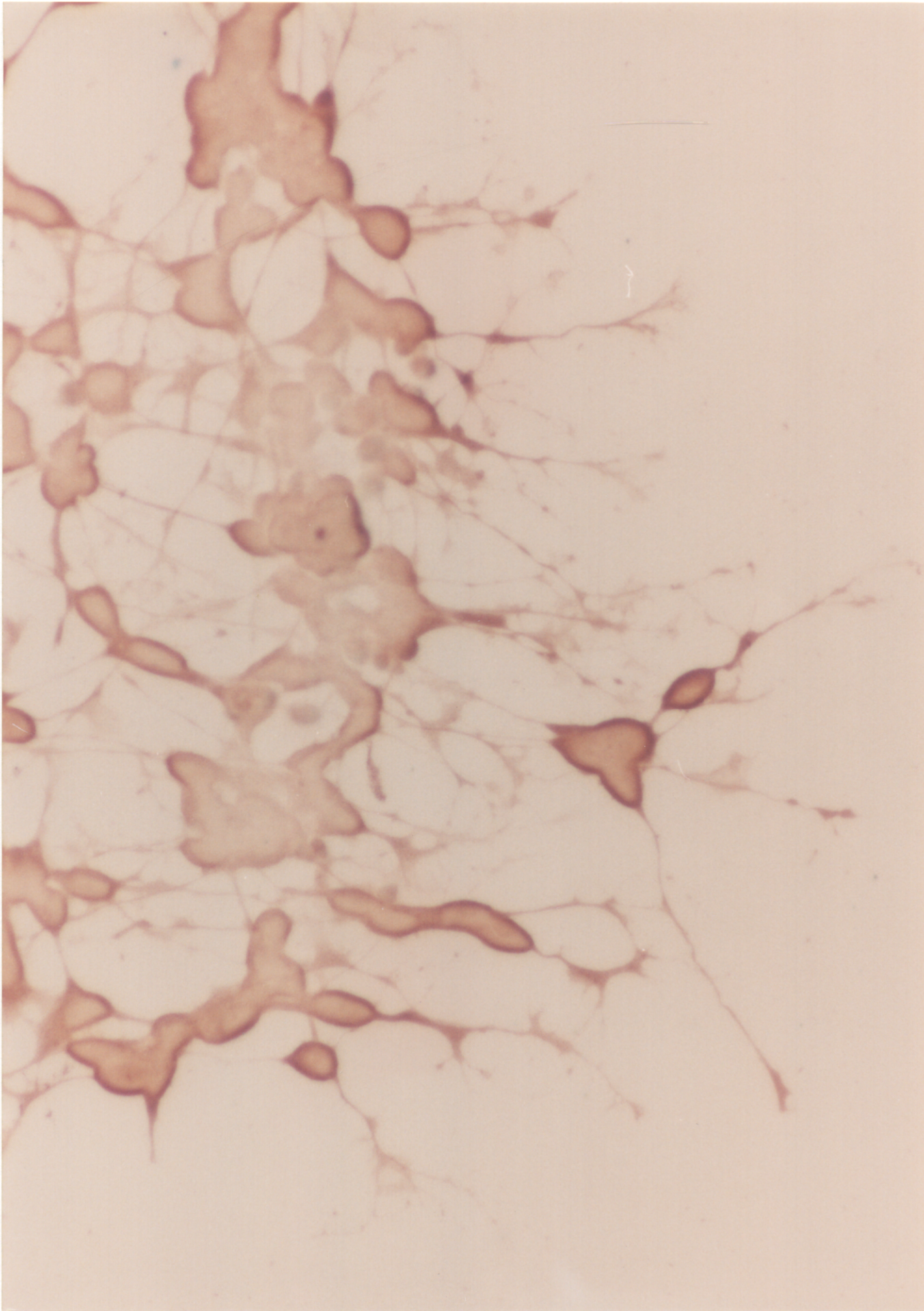

FIGURE 6-SY5Y neuroblastoma cells treated with 0.2% ethanol as controls for 9 days. Cells are diffusely immunopositive for panaxonal marker with extensive neuritic outgrowth and an apparent increase in cell number. Light microscopy, 20X.

V. B. ADDITIONAL DISCUSSION

Both ethanol control cells and RA treated cells demonstrated neurite outgrowth and cell migration into ganglion-like clusters. Neurite outgrowth was present in ethanol control cells maintained for 9 days, therefore, morphological differentiation did occur despite the lack of exposure to retinoic acid. It is possible that ethanol has some effect on SY5Y cells when they are exposed for an extended time period, but to the author's knowledge, this phenomenon has not been reported. The percentage of ethanol used (0.2%) is comparable or less than that reported in other studies using RA for differentiation of SY5Y cells. In these studies, controls were reported as unaffected [13, 25]. Ethanol-treated cells also appeared to increase in number compared to RA treated cells, but this was a subjective evaluation and growth curves were not performed. In order to further investigate this finding, control cells without ethanol should also be evaluated at the same seeding density and after the same 9 day incubation. Cells exposed to ethanol for 72 hours in earlier studies did not demonstrate this behavior.

The finding that both ethanol control cells and RA treated cells were both positive for panaxonal marker was unexpected. Panaxonal marker is composed of antibodies to both hypophosphorylated and hyperphosphorylated neurofilaments, the latter of which is most common in the axonal cytoskeleton [5, 7]. The monoclonal antibodies used for panaxonal marker and MAP2c in this procedure are of high manufacturing quality and very specific for their substrates [131]. Therefore, it may be presumed that SY5Y cells contain either hypo or hyperphosphorylated neurofilaments after 9 days, regardless of whether or not they are exposed to RA. Slides were compared to 72 hour ethanol controls and RA treated cells (Figure 2M). Nixon and Shea's hypothesis of neuronal development states that NF-L is probably manufactured before NF-H and indeed, triplet subunits of nonphosphorylated neurofilaments are presumed to be present before phosphorylation occurs [5]. NF-L was not evaluated in ethanol control cells after nine days and the RA treated cells stained for NF-L contained fixation artifacts that made evaluation difficult.

Comparison of undifferentiated cells, ethanol control cells and RA treated cells stained for NF-L after 9 days would be interesting, and would likely complicate interpretation of the results, due to the presence of vimentin immunoreactivity. Vimentin subunits can coexist transiently with neurofilament subunits within the same neurite *in vivo* [2], but they are thought to assemble into separate systems as differentiation occurs. The presence of all the components of the neuronal cytoskeleton simultaneously would indicate that in SY5Y cells, Nixon and Shea's hypothesis is not applicable.

Nine day RA-treated SY5Y cells were also positive for vimentin and MAP2c. A study using western immunoblots conducted on SY5Y cells states that these cells are variably positive for vimentin, and this finding was correlated with their neuroblastic morphology [132]. In contrast, we find that SY5Y cells are uniformly positive for vimentin immunocytochemistry. The source of the antibody used in the immunoblotting study is the same as the one used in our study (Boehringer Mannheim, Indianapolis, IN); however, during the processing procedure for immunoblots, proteins are denatured with detergents and some compromise in configuration can be expected. In order to compensate for this effect, higher concentrations of antibody are used in immunoblot labeling [138]. The anti-vimentin antibody in our study was applied at a dilution of 1:5, which is very concentrated compared to the other antibodies we used (1:1000 for MAP2c and panaxonal marker). This dilution was suggested by the manufacturer for use in cell suspensions, frozen tissue sections and paraffin embedded tissue sections. Therefore, it is possible that the immunoblotting study was done with an inappropriately diluted antibody and thus vimentin was not labeled accurately.

Cells remained MAP2c positive throughout the study. To the author's and the manufacturer's knowledge [131], this antibody has not been used in cell culture previously. MAP2c is abundant in immature brain in both axons and dendrites. Higher molecular weight MAP2 subunits (a and b) are not abundant in immature brain and they are located in dendrites and cell bodies of neurons in adult brain [9]. Whether or not the antibody we used (to MAP2c) would label MAP2 in adult brain is not known, therefore conclusions about the role of MAP2c in differentiation of SY5Y cells cannot be made based on their immunoreactivity. It is interesting to note that cells staining for panaxonal

marker, considered an indicator of neuronal maturation, maintained their immunopositivity for MAP2c, an indicator of immaturity.

The osmium labeling procedure we attempted was originally used to confirm that the antibodies were localized intracellularly. Some immunolabeling studies use detergents to remove noncytoskeletal components or microinjection techniques to insure intracellular placement of antibodies, but other studies do not [33, 66, 25]. Osmium combines with DAB to form complexes that can be localized by electon microscopy [134]. We attempted this procedure using the same fixation protocol used throughout the study (acetone:methanol), but found it inadequate for electron microscopic use, although the complexes were identifiable and located intracellularly. Further refinement of this procedure and incorporation of immunoblotting studies would confirm the presence and location of the antigen antibody complexes within the cell. Phosphorylation of neurofilaments increases the size and width of axons due to the physical expansion of individual filaments by multiple phosphate groups [3] and such filaments should be detectable by electron microscopy. Immunoblotting studies would confirm the molecular weight of the proteins and their migratory pattern as compared to normal brain. These studies would prove invaluable in confirming the findings reported here.

VI. SUMMARY AND CONCLUSIONS

In summary, SY5Y human neuroblastoma cells were shown to be immunopositive for MAP2c, vimentin and NF-L after 72 hours exposure regardless of their treatment with RA or dbcAMP/IBMX, with the exception of RA treatment for 9 days. RA differentation induces neuritic outgrowth within 24 hours, with neurite extension in a majority of the cells at 72 hours. Subsequent differentiation of SY5Y cells with RA for 9 days results in an apparent reduction in cell proliferation, with the migration of cells into ganglion-like clusters and continued neurite extension. The presence of phosphorylated neurofilaments, considered an indication of cytoskeletal differentiation, is detected after 9 days, but is also present in ethanol treated control cells incubated for the same length

of time. This indicates some capacity for SY5Y cells to morphologically differentiate without exposure to known differentiating compounds.

Both RA and dbcAMP/IBMX induce neuritic outgrowth at 72 hours, but there are no differences in cytoskeletal staining for the markers used in this study. Both protocols result in positive staining for MAP2c, vimentin and NF-L. NF-L is focal and perinuclear in its distribution, but the significance of this finding is unclear and may indicate some early localization of the protein in the cell soma. The presence of MAP2c in SY5Y cells regardless of treatment with RA or dbcAMP/IBMX may indicate a lack of maturity compared to adult nervous tissue, but its role in differentiation of neuroblastoma is unknown. Uniform vimentin immunopositivity contrasts with previous immunoblotting studies using SY5Y cells, but is consistent with dividing neuroepithelial cells and studies in other neuroblastoma lines.

Acrylamide, a neurotoxicant hypothesized to interfere with the axonal transport of neurofilaments, does not appear to alter the distribution or intensity of cytoskeletal immunostaining of RA differentiated SY5Y cells after 24 hours of exposure. Cells are morphologicaly altered by cellular swelling at 24 hours, but the pattern of immunoreactivity is unchanged after 72 hours or 9 days of RA differentation. Phosphorylated neurofilament was immunolabeled after 9 days and remained consistent in its distribution and staining intensity compared to ethanol controls in spite of 24 hours of acrylamide exposure. Acrylamide does not appear to acutely alter the distribution of the cytoskeleton of SY5Y neuroblastoma cells in undifferentiated or RA differentiated cells.

In conclusion, it appears that SY5Y neuroblastoma cells are useful in the study of neuronal differentiation, but only to a limited degree. Two of the most commonly used differentiating agents, RA and dbcAMP (with IBMX), produced morphologic evidence of differentiation (neurite outgrowth), but our findings did not correspond to a theory of neuronal cytoskeletal differentiation based on extensive *in vivo* and *in vitro* evidence. In contrast, the evaluation of these cells as an *in vitro* model of neurotoxicology is incomplete without the use of more potent cytoskeletal disrupting agents other than acrylamide in this system. The uncertainty of acrylamide's effects on the cytoskeleton made it an imperfect choice for this type of study, but its water soluble nature made it an excellent choice for testing in cell culture. Further evaluation of the system with other

neurotoxicants and the addition of other techniques to the system would confirm its usefulness.

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VITA

Delana Dawn Taylor was born to Barney and Linda Taylor on July 19, 1960 in Nowata, Oklahoma. Delana graduated from Eastern Oklahoma State College with an Associate of Science degree in 1980. She received her Bachelor of Science degree as a first year veterinary student at Oklahoma State University, where she completed her Doctor of Veterinary Medicine degree in 1985. After two and one-half years of private mixed practice in Bedford, Virginia, Delana became a Veterinary Medical Officer with the United States Department of Agriculture. In 1988, she accepted a position as Laboratory Director/Diagnostician for the Virginia Department of Agriculture and Consumer Services at the Lynchburg Regional Laboratory. After one and one-half years of pathology-related work as a non-pathologist, Delana began a pathology residency at the Virginia Maryland Regional College of Veterinary Medicine to further her training. Delana's pathology residency was completed under the direction of Dr. Geoff Saunders in August of 1993 and she became a Diplomate of the American College of Veterinary Pathologists in September of the same year. Her graduate research has been directed by Dr. Bernard S. Jortner. She will be starting her pathology career as a surgical pathologist in Dallas, Texas in 1994. Delana's interests include horseback riding, baseball and hiking. She resides with her dog, Hobo, her parrot, Quincy, her iguana, Waldo, and three Spanish Mustangs.

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RECOGNITIONS AND MEMBERSHIPS

1993	Diplomate, American College of Veterinary Pathologists
1993	Member, C. L. Davis Foundation
1992	Member, Society of Toxicology
1992	Member, Veterinary Cancer Society
1984	Member, Phi Zeta, National Veterinary Honor Fraternity
1993	Pauline Willson-Gunn Scholarship

PUBLICATIONS

Taylor D., Ehrich M., Jortner B., Dunnington E., and Siegel P. "Organophosphorus ester-induced delayed neuropathy (OPIDN) in chickens; the effect of genotype on lesion severity" American College of Veterinary Pathologists Meeting, San Diego, CA, November 1992. Veterinary Pathology, Vol. 29, No. 5, Page 448, No. 108, 1992.

Taylor D., Rowles T. K., Nostrandt A. C., and Ehrich M. "Early morphologic changes in SH-SY5Y neuroblastoma cells after exposure to a neuropathy-inducing organophosphorus compound, mipafox." In vitro toxicology, 10 year symposium of the Center for Alternatives to Animal Testing, Baltimore, MD., April 14-16, 1992, in Alternative Methods in Toxicology, Vol. 9, 1993, Mary Ann Liebert, Inc. New York, NY.