Effect of Alzheimer's Brain Extracts on Dynein Immunoreactivity in PC12 Cells

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Abstract. **The neurodegenerative process in Alzheimer's disease (AD) has been suggested to occur as a consequence of microtubule disruption and subsequent loss of intracellular transport. Structural microtubule-associated proteins (MAPs) have been investigated for their role in the etiology of AD, but dynein, a force-producing MAP which mediates intracellular transport, has not been examined. In this report, dynein (MAP1C) immunoreactivity in AD brain tissue homogenates was observed increased 3.7-fold compared with control brain homogenate preparations. Similarly, NGF-differentiated PC12 cells cultured in the presence of soluble extracts prepared from AD brain tissue homogenates, exhibited an approximate 15-fold increase in dynein immunoreactivity compared to that of control brain tissue extracts. In contrast, AD clarified extracts had little effect upon "kinesin-like" protein immunoreactivity increased (approximately 2-fold); whereas, τ immunoreactivity was observed to be moderately increased (5-fold) over that of control brain extract treated PC12 cells. Chemical dephosphorylation and alkaline phosphatase treatment of AD extract–treated PC12 cell lysate prior to Western blotting resulted in complete loss of immunoreactivity, suggesting the dynein being monitored is a phosphorylated isoform. Furthermore, treatment of clarified brain tissue extracts with trypsin and $(NH_4)_2SO_4$ suggests the endogenous elements giving rise to increased PC12 cell dynein intermediate chain immunoreactivity to be proteinaceous in nature. The observed increase in dynein intermediate-chain dynein immunoreactivity following exposure of neuronal cells to endogenous elements of AD brain may be reflective of dynein-microtubular array differences. Such an approach may be useful in assessing the effect of endogenous biomolecules on retrograde axonal transport in neuronal culture models.**

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The pathogenesis of Alzheimer's disease (AD) involves defective microtubule function resulting in impaired axonal and cellular transport, and, ultimately, in neuronal death (1–3). Loss of intracellular transport may explain many of the observed deficiencies in the AD brain such as decreased neurotransmitter output (4, 5), abnormal membrane turnover (6, 7), accumulation of abnormally phosphorylated cytoskeletal proteins (1, 8, 9), and accumulation of β-amyloid peptide in the neuropil (10, 11).

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τ, a microtubule-associated protein (MAP) responsible for assembly and stability of microtubules, has been shown to be abnormally phosphorylated in AD neurons by mechanisms that are not yet completely understood (12–15). Hyperphosphorylated τ is a major component of paired helical filaments (PHF) found in neurofibrillary tangles (16), which along with senile plaques are the two most common neuropathological findings in AD. It has been reported that abnormally phosphorylated τ exhibits decreased binding affinity for microtubules (17), and is functionally impaired in its ability to promote microtubule assembly (18). Since the precise relationship between accumulation of abnormally phosphorylated τ and loss of microtubule function is currently unresolved, impairment of intracellular transport may occur *via* changes in molecules in addition to τ.

Molecular motors are enzymes that transport a variety of cargo (i.e., organelles and vesicles) about the cell by binding and moving along microtubules. Dynein (MAP1C) is a multisubunit phosphoprotein which couples the energy

of ATP hydrolysis to mechanical movement toward the negative end of microtubules (19–21). It co-localizes with lysosomes (22), is responsible for the perinuclear positioning of the Golgi apparatus (23) and movement of endocytic vesicles from early to late endosomes (24), and is involved in nuclear migration processes (25).

While much understanding of the pathology associated with AD has been gained directly from studies utilizing autopsy tissue, there are many limitations, especially when studying the cytoskeleton. Postmortem delay can result in artifacts in cytoskeletal proteins, while leaving other proteins intact. For example, phosphorylation changes in τ can be detected within 4 hr after death, whereas protein kinase C activity is stable over a 24-hr postmortem period (26, 27). In addition, early developments in AD pathology are difficult to reconstruct from observations made on autopsy tissue. Pheochromocytoma (PC12) cells are neuroendocrine cells derived from rat adrenal medulla which can differentiate to a neuronal-like phenotype after treatment with NGF (28). Differentiated PC12 cells contain dense core and synaptic vesicles, secrete neurotransmitters, and form functional synapses in culture (29). In several respects, NGF-differentiated PC12 cells share similar characteristics to CNS cholinergic neurons (29). This aspect makes PC12 cells an interesting test system in which to study effects of endogenous but unknown elements of AD brain, since it is known that NGF-dependent cholinergic neurons in the basal forebrain are lost in AD.

To our knowledge, dynein has not yet been examined with regard to a possible role in AD despite the fact that other microtubule-associated proteins (3, 30) have been implicated in the disease process which progressively involves disruption of microtubular structure. The interaction of endogenous components of AD brain tissue with viable neurons may provide some insight into specialized long distance axonal transport in AD brain. In this report, we show that (i) dynein intermediate chain immunoreactivity of AD autopsy brain tissue homogenates is increased 3.7-fold when compared with control brain tissue homogenates and (ii) NGF differentiated PC12 cells cultured in the presence of clarified extracts prepared from AD brain tissue homogenates exhibit significantly elevated (15-fold) dynein immunoreactivity.

Materials and Methods

Preparation of PC12 Cell Lysates. Rat pheochromocytoma (PC 12, cf. Ref. 28) cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Opti-MEM (Gibco-BRL, Gaithersburg, MD) supplemented with 4% w/v fetal bovine serum (Sigma Chemical Co., St. Louis, MO), gentamicin, penicillin, and fungazone (Gibco-BRL). Cells were cultured at concentrations between 3200 and 16000 cells/cm^2 in six-well plates (Corning, Corning, NY) precoated with poly-L-lysine (0.1 mg/ml). Differentiation was initiated by addition of 100 ng/ml 2.5S nerve growth factor (NGF) (Harlan Bioproducts for Science, Inc., Indianapolis, IN) for 7–10 days prior to the addition of brain extracts. For all experiments using differentiated PC12 cells, NGF was maintained at a final concentration of 100 ng/ml. PC12 cells were cultured in the presence of brain extracts for the indicated times prior to harvesting and immunoblot analysis. Cells were harvested by gentle scraping from culture dishes, followed by centrifugation at 1000*g* for 5 min. Cell pellets were washed twice in ice-cold phosphate-buffered saline (PBS), resuspended in 0.25 *M* Tris buffer, pH 6.8, containing 2% w/v SDS, and boiled for 5 min.

Preparation of Crude Brain Homogenates. Brain homogenates were prepared from temporal cortex of AD (64–88 years of age) and nondemented, control (65–91 years of age) brain obtained at autopsy from the Tissue Procurement Facility (Birmingham, AL) and the Audie Murphy Veterans Administration Hospital (San Antonio, TX). AD and corresponding control samples were (i) matched as closely as possible with regard to age and postmortem delay (typically less than 24 hr) and (ii) treated in identical fashion. Brain tissue was frozen immediately after removal and stored at −80°C. Frozen tissue (2 g wet weight) was homogenized in four volumes of ice-cold extraction buffer (0.05 *M* PIPES, pH 7.0) containing 2 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* PMSF, phosphoramidon (10 μg/ml), TLCK (50 μg/ml), TPCK (70 μg/ml), pepstatin (10 μg/ml), leupeptin (10 μg/ml), and BAME (10 μg/ml) using a Dounce homogenizer. Homogenates were sonicated for 30 sec while immersed in an ice slurry, analyzed for protein (an average of 6.2 mg/ml/homogenate), aliquoted and stored at −20°C.

Preparation of Clarified Brain Tissue Extracts. Clarified extracts were prepared by subjecting crude homogenate material to centrifugation at 45,000*g* for 45 min. Resulting supernatants were carefully decanted and assayed for protein content followed by dilution to 2 mg/ml with extraction buffer. Supernatants, diluted to a final concentration of 0.25 mg extract protein/ml, were sterilized by filtration through a 0.22-μm filter, and then added to culture media supplemented with 100 ng NGF/ml.

Protein Content Determination. Protein content of PC12 cell lysates, crude brain homogenates, and clarified brain tissue extracts was determined using the BCA reagent (Pierce, Rockford, IL) and bovine serum albumin as standard.

Determination of Viability and Cell Number. Cell number was determined using trypan blue dye (Gibco-BRL) exclusion and cell lysate DNA content. DNA content was determined using the bis-benzimide (Hoechst 33258) reagent (31, 32). Cell lysate material (5–10 μl) was diluted in 10 m*M* Tris buffer, pH 7.4, containing 1 m*M* EDTA, 0.2 *M* NaCl, and 0.19 μ*M* bis-benzimide to a final volume of 2.0 ml. Fluorescent measurements were made (350 nm excitation and 455 nm emission) using a LS-5 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). A standard curve relating cell number and DNA fluorescence was de-

termined to be linear between 5,000 and 100,000 cells/ml (data not shown).

Immunoreagent Characterization. Anti-dynein intermediate chain antibody (mAb 70.1, Sigma) raised against dynein purified from chick brain was characterized by immunoblot and immunocytochemistry in chick embryo fibroblasts (33). mAb 70.1 reacts with a triplet band of approximately 74-kDa molecular weight in preparations of chicken embryo fibroblasts. This antibody also recognizes dynein isolated from chinese hamster ovaries, HeLa cells, and rat tissues. Anti-τ monoclonal antibody (τ-2) and anti-kinesin heavy-chain antibody were obtained from Sigma. Densitometric analysis of anti-τ–reacting species were summed. Anti-kinesin heavy-chain antibodies (1B11) were raised against bovine brain kinesin preparations and reacts with multiple bands in both human brain and PC12 cells. This antibody is referred to as an anti–"kinesin-like" protein antibody to reflect cross-reactivity with several members of the kinesin superfamily.

Enzyme-Linked Immunosorbent Assay. PC12 cell lysates were prepared as previously described in Materials and Methods. Enzyme-linked immunosorbent assays (ELISAs) were carried out according to the method of Uchida and Tomonaga (34).

Western Blot and Densitometric Analysis. PC12 cell lysate proteins were separated on 12% polyacrylamide gels according to the method of Laemmli (35) and transferred to nitrocellulose membranes (36) using a Mini Protean II gel apparatus (Bio-Rad, Richmond, CA). Nitrocellulose membranes were blocked using 5% (w/v) nonfat dry milk (Carnation) dissolved in PBS for 1 hr at room temperature. Subsequent wash steps were performed using 0.5% (w/v) dry milk in PBS containing 0.05% (w/v) Thimerosal (Sigma). Primary antibody was incubated with nitrocellulose membranes overnight at room temperature. Membranes were washed and incubated for 1 hr with horseradish peroxidase goat anti-mouse IgG conjugate and IgM (Boehringer Mannheim, Indianapolis, IN) secondary antibody. All blots were visualized on x-ray film (Kodak, Eastman Chemical Company, Rochester, NY) using a chemiluminescent substrate (Amersham Corp., Arlington Heights, IL) under strict conditions of linearity. Film exposure was linear up to 30 sec for all protein concentrations examined (data not shown). Bands were analyzed by scanning densitometry using a GS 300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). Total protein and molecular weight standards were visualized using Colloidal Gold protein stain (Bio-Rad).

Results

Dynein Immunoreactivity—AD and Control Brain Tissue Homogenates. Shown in Figure 1A is a representative Western blot of AD and control brain tissue homogenates indicating a single band migrating at an approximate molecular weight of 73,000 daltons. Densitometric analysis of Western blots for five AD and control brain

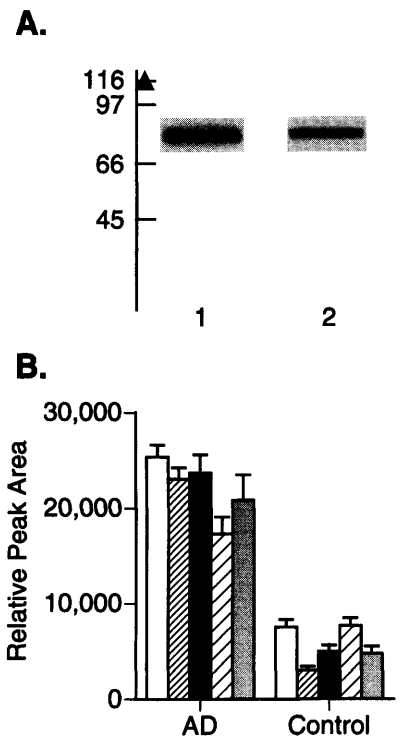

Figure 1. Levels of dynein intermediate-chain immunoreactivity in AD and control brain crude homogenates. Preparation of tissue homogenates and Western blotting were carried out as described in Materials and Methods. (A) Representative Western blot showing dynein immunoreactivity in AD (Lane 1) and control (Lane 2) brain tissue homogenates (25 μg protein/lane). (B) Scanning densitometric analysis of Western blots of AD ($n = 5$) and control ($n = 5$) brain tissue homogenates. Each sample was analyzed in triplicate and results averaged. Error bars represent ±SEM.

tissue homogenates indicate dynein immunoreactivity to be increased an average of 3.7-fold in AD brain (Fig. 1B). Although not the focus of this report, τ immunoreactivity of AD brain homogenates was increased 3.9-fold when compared with control brain homogenates (data not shown).

Effect of Dilution of Clarified Brain Tissue Extracts and Increasing Time of Exposure on PC12 Cell Dynein Immunoreactivity. Shown in Figure 2 is the effect of dilution of AD brain extracts on dynein immunoreactivity indicating little effect at concentrations less than 0.1 mg AD brain extract protein/ml culture media. Although 0.5 mg AD brain extract protein/ml culture media resulted in a higher-fold increase, due to the small amount of tissue available and the requirement for multiple media changes, all subsequent experiments were conducted using 0.25 mg AD and control brain extract protein/ml culture media. Shown in Figure 3 is the effect of increasing time of culture of NGF-differentiated PC12 cells in the presence of AD and control brain tissue extracts on dynein immunoreactivity. Cells grown in the presence of control brain tissue extracts exhibited a slight increase in dynein immunoreactivity over the duration of the experiment. In contrast, cells cultured for up to 10 days in the presence of AD brain tissue extracts exhibited steadily increasing dynein intermediate-chain immunoreactivity (elevated approximately 15-fold af-

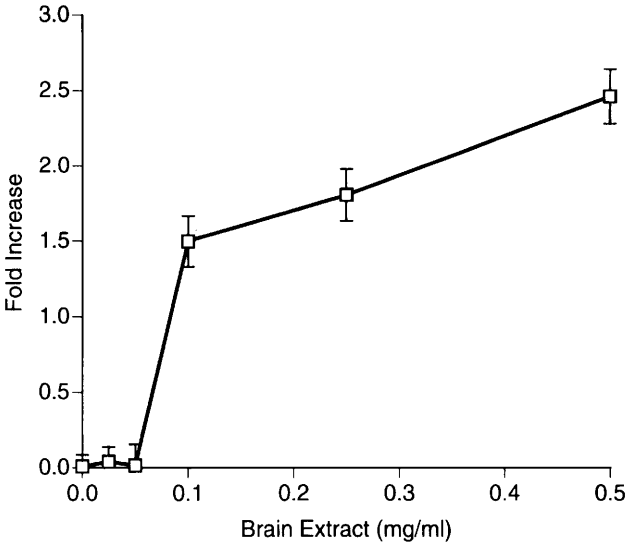

Figure 2. Effect of dilution of clarified brain tissue extracts on dynein intermediate-chain immunoreactivity in PC12 cells. Brain tissue extracts and NGF-differentiated PC12 cells were prepared as described in Materials and Methods. AD and control brain tissue extracts ($n = 3$) were diluted as indicated, applied to PC12 cells, and cultured for 3 days. Dynein immunoreactivity of cell lysate preparations was determined using an established ELISA method as described in Materials and Methods. Fold increase represents the ratio of values (AD/control) observed following exposure of PC12 cells to respective brain tissue extracts.

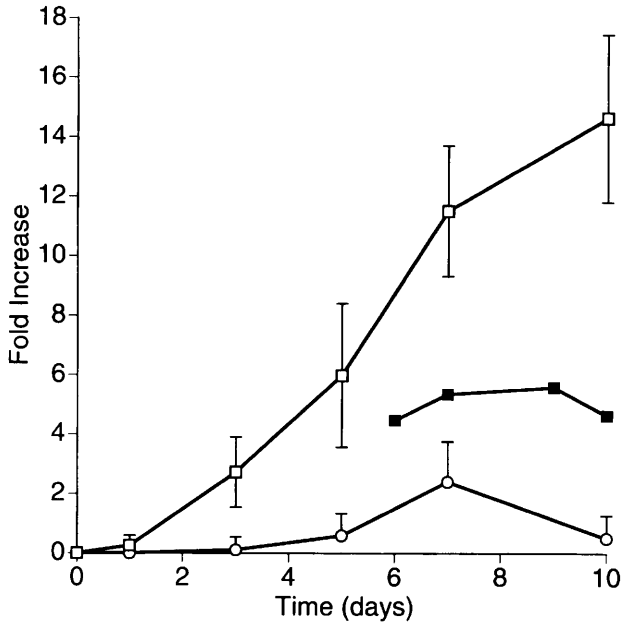

Figure 3. Effect of increasing time of exposure to clarified brain tissue extracts on dynein intermediate-chain immunoreactivity in PC12 cells. Brain tissue extracts and NGF-differentiated PC12 cells were prepared as described in Materials and Methods. AD (□) and Control (○) brain tissue extracts (0.25 mg extract protein/ml media) were applied to PC12 cells and cultured for the indicated times. Cell lysate proteins were separated by SDS PAGE, blotted onto nitrocellulose, and Western blots developed as described in Materials and Methods. PC12 cells previously exposed to a clarified AD brain tissue extract (5 days) were washed once with fresh "extract-free" culture media. Following washing, the incubation was continued for the indicated times in the presence of "extract-free" media containing 100 ng NGF/ml culture media (■). Respective peak areas obtained by densitometric analysis of immunoblots were normalized to cell number derived from lysate DNA content. Fold increase represents the increase in dynein intermediate chain immunoreactivity following culturing of PC12 cells in the presence of clarified AD and control tissue extracts for the indicated times compared with values observed for day 0, respectively. Data shown (□; ○) are averages of three different experiments using extracts prepared from three different AD and control brains. Error bars represent ±SEM. Removal of AD extract material at Day 5 (■) was carried out using one clarified AD and control tissue extract preparation.

ter 10 days). In order to ascertain in preliminary fashion the mode of action of endogenous brain elements upon PC12 cell intermediate chain dynein immunoreactivity, a simple "wash-out" experiment was carried out. Replacement of AD brain tissue extract with extract free media indicated dynein immunoreactivity was maintained at a level equivalent to a 3- to 4-fold increase out to 10 days culture time (Fig. 3).

A Comparison of Dynein, Kinesin, and τ Immunoreactivity following Exposure to Clarified Brain Tissue Extracts. We were interested in determining what effect incubation of PC12 cells in the presence of AD brain tissue extracts had on related cytoskeletal proteins (e.g., kinesin and τ). As shown in Figure 4, kinesin (i.e., "kinesin-like" protein) immunoreactivity was affected considerably less than that of dynein, which was increased approximately 15-fold. τ immunoreactivity was elevated approximately 5-fold.

Effect of Clarified Brain Tissue Extracts on PC12 Cell Morphology, Number, and Mitochondrial Dehydrogenase Activity. Shown in Figure 5 is the effect of clarified AD and control brain tissue extracts on NGF differentiated PC12 cell morphology. PC12 cells appear to respond favorably to control brain tissue extract (Panel A) as evidenced by the appearance of many neurites from a single cell mass. Long neuritic processes with a high degree of branching accompanied by growth cones at the tips of neurites can be observed. In contrast, cells cultured in the presence of AD brain extracts (Panel B) exhibited shorter, finer neurites with little or no branching. In order to characterize further the effect of clarified extracts on cultured PC12 cells, we evaluated cell viability and number as well as mitochondrial dehydrogenase activity (Fig. 6). No significant differences were observed in cell viability and number following culture in the presence of control and AD brain tissue extracts (Panels A and B, respectively). Cells cultured in the presence of control brain extracts exhibited a gradual increase in mitochondrial dehydrogenase activity with maximal activity observed after 10 days in culture (Panel C). In contrast, cells cultured in the presence of AD brain extracts maintained mitochondrial dehydrogenase activity at levels that were not significantly different from starting dehydrogenase activity.

Effect of Dephosphorylation of PC12 Cell Lysate Dynein on Immunoreactivity. Pfister and coworkers (37) have demonstrated that different cell types from brain have different dynein (i.e., phosphorylated intermediate-chain isoforms). We were interested in deter-

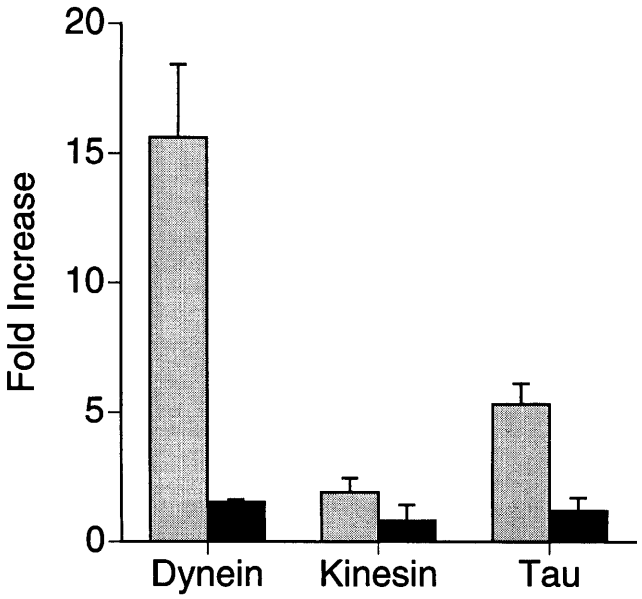

Figure 4. Effect of clarified brain tissue extracts on dynein, "kinesin-like" protein, and τ immunoreactivity in PC12 cells. Brain tissue extracts were prepared from AD (■) and control (▨) brains and applied to PC12 cells for 10 days as described in Materials and Methods. Cell lysate proteins were separated by SDS PAGE, blotted onto nitrocellulose, and Western blots developed as described in Materials and Methods. Dynein and kinesin immunoreactivity were determined in duplicate using three different brains. τ immunoreactivity was determined in duplicate but from only one brain. Fold increase represents the increase in immunoreactivity after culturing PC12 cells in the presence of clarified AD and control brain tissue extracts for 10 days compared with values observed for Day 0, respectively. Error bars represent ±SEM.

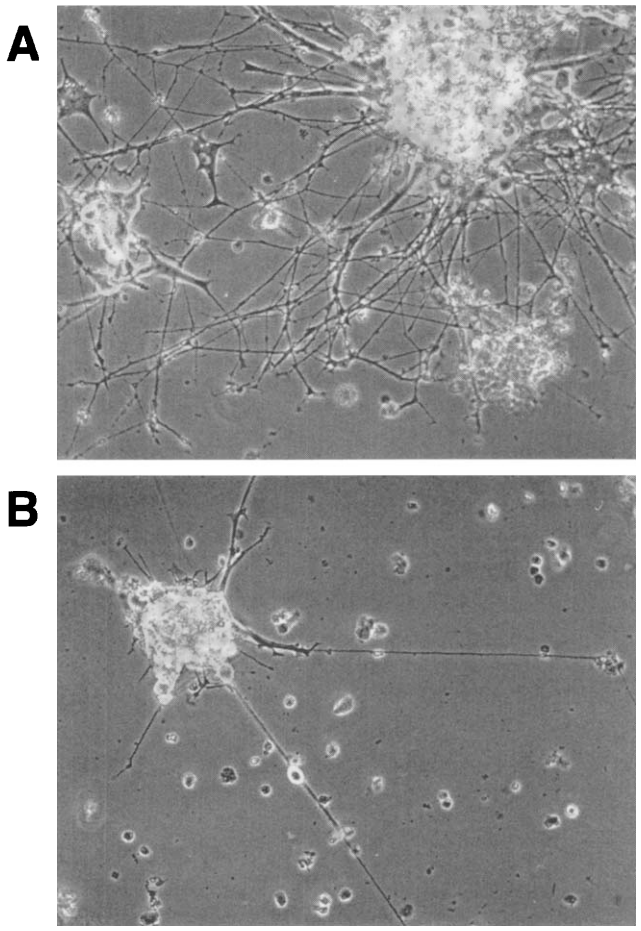

Figure 5. Photomicrographs of PC12 cells cultured in the presence of clarified brain tissue extracts. Cells were incubated for 5 days in the presence of clarified brain tissue extracts (0.25 mg clarified tissue extract protein/ml culture media) and diluted in culture media containing 100 ng/ml NGF. (A) PC12 cells cultured in the presence of control brain tissue extract. (B) PC12 cells cultured in the presence of AD brain tissue extract.

mining if dephosphorylation of PC12 cell lysate dynein would effect dynein immunoreactivity. As shown in Figure 7A, treatment of cell lysate from clarified AD extract–treated PC12 cells with purified, protease-free, alkaline phosphatase resulted in complete loss of immunoreactivity. Chemical dephosphorylation using NaOH treatment also resulted in essentially complete loss of immunoreactivity (Figure 7B). Shown in Figure 7C is a protein-stained gel of lysate protein before and after alkaline phosphatase treatment indicating no detectable differences arising from any contaminating protease activity associated with the alkaline phosphatase preparation.

Effect of Trypsin and $(NH_4)_2SO_4$ Treatment of Clarified Brain Tissue Extracts on PC12 Cell Dynein Immunoreactivity. Shown in Figure 8 is the effect of prior treatment of clarified AD brain tissue extracts with trypsin resulting in abolishment of the previously described increased dynein immunoreactivity. Treatment of AD brain extracts with $(NH_4)_2SO_4$ resulted in partial separation of endogenous components in a 30% and 60% $(NH_4)_2SO_4$ precipitated pellet (Table I).

Discussion

Examination of control and Alzheimer's disease autopsy brain tissue homogenates for dynein intermediate-chain immunoreactivity indicated a 3.7-fold average increase in dynein intermediate-chain immunoreactivity in Alzheimer's disease brain tissue homogenates when compared with that of control preparations. Consistent with the observed increases of dynein in autopsy AD brain, dynein immunoreactivity of AD brain extract–treated NGF-differentiated PC12 cells was also observed increased. NGF-differentiated PC12 cells grown in the presence of Alzheimer's brain tissue extract for 10 days exhibited a 15-fold increase in dynein immunoreactivity when compared with PC12 cells grown in the presence of extracts prepared from control brain tissue. The number of different brain specimens constituting the experimental and control groups is small. Although the standard deviation values reported are small, ideally this study should be expanded to include more samples in the respective groups. Following removal of Alzheimer's brain tissue extract and replacement with extract free growth media, dynein immunoreactivity was observed to be maintained at an elevated level, albeit a level less than that observed prior to removal of extract from the media. Although not conclusive, this is consistent with

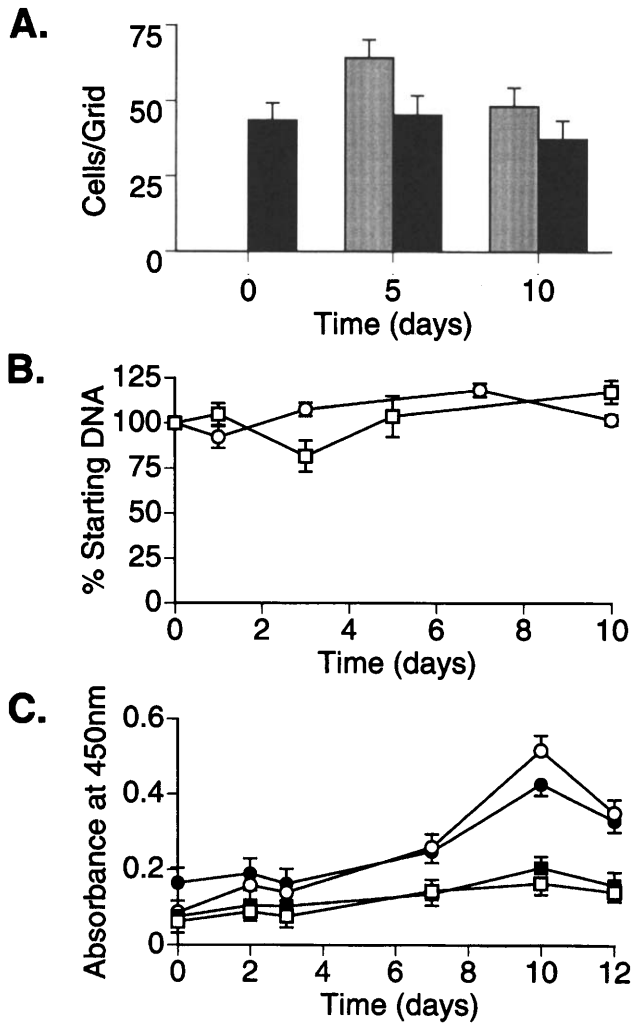

Figure 6. Effect of clarified brain tissue extracts on PC12 cell viability, cell number, and mitochondrial dehydrogenase activity. (A) Cell viability was determined following treatment of NGF-differentiated PC12 cells with AD (▩) and control (■) brain tissue extracts. Cells were harvested, diluted with trypan blue, and counted using a hemocytometer. Eight replicates were counted and averaged. (B) Cell number was estimated from cell lysate DNA content by fluorescent spectrophotometric measurements as described in Materials and Methods. Cell number estimates following treatment with AD (□) and control (○) brain tissue extracts is reported as the percentage of starting DNA content prior to addition of extracts. (C) Mitochondrial dehydrogenase activity was determined (12 replicates) as described in Materials and Methods following culturing in the presence of two different AD (□; ■) and control (○; ●) brain tissue extracts cultured for the indicated periods of time. For all experimentation, NGF was maintained at 100 ng/ml. Error bars represent ±SEM.

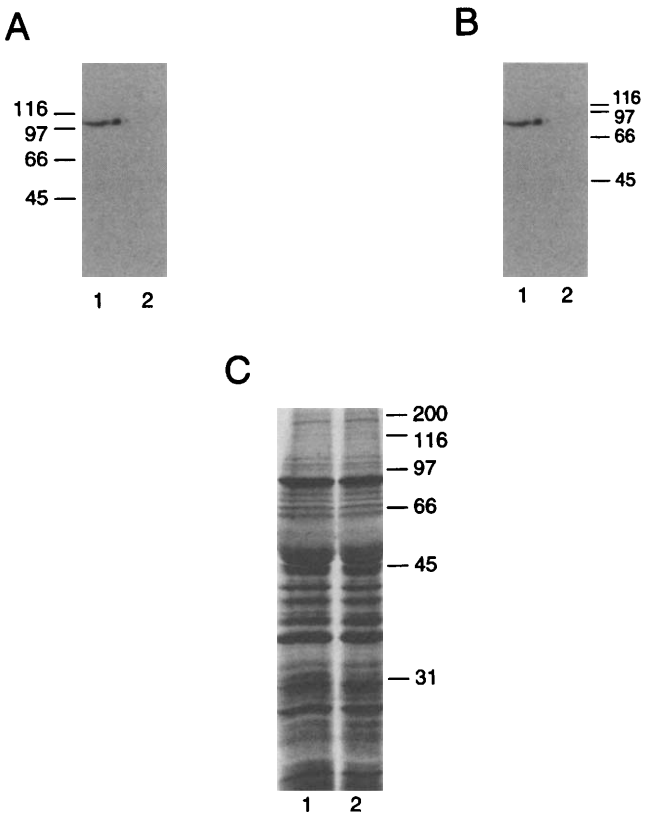

Figure 7. Effect of dephosphorylation of PC12 cell lysate dynein on dynein intermediate chain immunoreactivity. Following exposure of PC12 cells to clarified AD brain tissue extracts, cell lysates were centrifuged at 35,000*g* for 1 hr at 4°C. (A) Alkaline phosphatase treatment. Pelleted material was resuspended in 50 m*M* Tris buffer, pH 8.5, containing 0.1 m*M* EDTA, and incubated for 4 hr at 37°C in the absence (Lane 1) or presence (Lane 2) of alkaline phosphatase (60 U/ml). (B) Chemical dephosphorylation. PC12 cell lysates were boiled in the absence (Lane 1) or presence (Lane 2) of 4 *N* NaOH for 15 min. Samples were neutralized by addition of 2 *N* HCl, diluted in Tris buffer, pH 8.5, and concentrated in Centricon 100 microconcentrators. Concentrated samples (5 µg protein/lane) were subjected to SDS-PAGE and Western blotting as described in Materials and Methods. (C) Protein staining of PC12 cell lysates before (Lane 1) and after (Lane 2) treatment with alkaline phosphatase.

removal of an unknown element that exerts its effect *via* the cell surface.

Although AD brain tissue extract treated PC12 cells were significantly different in appearance than that observed for control brain tissue extract–treated cells, neither extract exerted any apparent adverse effects upon NGF-differentiated PC12 viability. Control extract–treated cells exhibited long neuritic processes with a high degree of branching and the presence of growth cones at the tips of neurites; whereas, cells cultured in the presence of Alzheimer's brain tissue extracts exhibited shorter, finer neurites with little or no branching. Cell viability and number remained relatively constant. Although extracts prepared from Alzheimer's brain tissue are not toxic to NGF-differentiated PC12 cells, they differ significantly with regard to their respective effect on mitochondrial dehydrogenase activity. Mitochondrial dehydrogenase activity of PC12 cells grown in the presence of Alzheimer's brain tissue extract did not parallel that observed for control brain tissue extracts, which exhibited a 2.5-fold increase after 10 days' culture. Cells cultured in the presence of Alzheimer's brain tissue extracts for 10 days exhibited mitochondrial dehydrogenase activity essentially identical to that observed at the beginning of the experiment. One possible explanation for this difference in mitochondrial dehydrogenase activity could be a preferential effect of excess deposition of β-amyloid in the AD brain. Several lines of evidence suggest a role for oxidative abnormalities, specifically in mitochondrial enzymes, in the pathophysiology of AD (38–41).

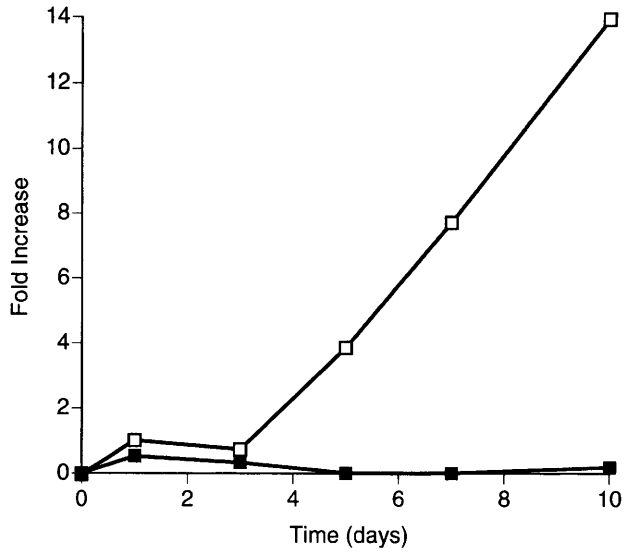

Figure 8. Effect of prior trypsin treatment of clarified brain tissue extracts on dynein intermediate-chain immunoreactivity. AD and control brain tissue extracts (n = 2) were prepared as previously described under Materials and Methods. Brain extracts were denatured in 6 *M* guanidine hydrochloride (Sigma) for 30 min at 60°C. Dithiothreitol (0.01 *M* final concentration) was added to reaction mixtures and incubated for an additional 30 min in order to reduce all disulfide bridges. Alkylation of sulfhydryl groups was accomplished by addition of iodoacetamide (0.05 *M* final concentration). Following denaturation, extracts were dialyzed extensively against PBS and digested with 2% (w/v) trypsin for 24 hr at 37°C. Trypsin was removed by incubation of extracts with immobilized trypsin inhibitor (covalently attached to agarose beads; Sigma) for 30 min at room temperature with gentle mixing followed by centrifugation for 1 min at 500*g*. The extent of digestion was monitored by SDS-PAGE. Trypsin-digested extracts were diluted to 0.5 mg protein/ml, and filtered through a 0.2-μm filter. NGF was added to the filtrate (100 ng/ml final concentration). Cells were cultured for 7 days in the presence of extracts (0.25 mg clarified tissue extract protein/ml culture media). Cell lysates were prepared and dynein intermediate-chain immunoreactivity determined by Western blotting and scanning densitometry as described in Materials and Methods. Trypsin-treated (■); untreated (□). Fold increase represents the ratio of values (AD/control) observed following exposure of PC12 cells to the respective clarified brain tissue extracts.

Recently, Scott and co-workers have observed widespread increases in cortical and subcortical NGF (42). Although these increases are small (approximately 550 pg/g wet weight AD temporal cortex versus 450 pg/g wet weight control temporal cortex), could the AD clarified brain extract provide significant additional trophic support resulting in increased dynein immunoreactivity? Calculations based upon wet weight of AD temporal cortex, i.e., approximately 550 pg NGF/g tissue and dilutions (25-fold of clarified homogenate preparations presented to NGF-differentiated PC12 cells in this study) indicate the contribution of endogenous homogenate NGF to total NGF (300 ng total, 3.8 n*M*) is approximately 40–50 pg corresponding to 0.02% of the total NGF present. Slot blot analysis further supports the conclusion that neither extract contributes significantly to the level of NGF (data not shown). Furthermore, Jette and co-workers (43) have concluded that levels of mRNA in general, and of NGF mRNA in particular, are unchanged in the frontal cortex of individuals affected by AD.

Table I. Partial Fractionation of Clarified Brain Tissue Extracts by $(NH_4)_2SO_4$ Precipitation

$(NH_4)_2SO_4$ fraction	Fold increase
30% supernatant	0
30% pellet	2.5
60% supernatant	−2.0
60% pellet	3.0

Clarified AD and control brain tissue extracts (n = 2) prepared as described in Materials and Methods were subjected to $(NH_4)_2SO_4$ precipitation by addition of solid $(NH_4)_2SO_4$ required to produce a desired change in the percent saturation at 25°C (45). Proteins that precipitated in the presence of $(NH_4)_2SO_4$ (i.e., pellets) or remained soluble in the presence of $(NH_4)_2SO_4$ (i.e., supernatants) were dialyzed against PBS, added to culture media containing NGF, and added to PC12 cells for 7 days. Final concentration of brain extracts added to PC12 cells was 70 μg/ml. PC12 cell lysates were prepared and dynein intermediate-chain immunoreactivity determined by Western blotting and scanning densitometry as described in Materials and Methods. Fold increase represents the averaged ratio (AD/control) observed following exposure of PC12 cells to the respective $(NH_4)_2SO_4$ fractions. The "negative" value depicts a 2-fold decrease (i.e., inhibitory effect of this fraction).

The removal of phosphate from PC12 cell lysate dynein after exposure to AD extract, but prior to Western blot analysis, resulted in complete loss of immunoreactivity. This would indicate the increase in dynein immunoreactivity reported here to represent only that of a phosphorylated isoform. If total dynein increases, dynein mRNA in treated PC12 cells should similarly increase; if the only change is in phosphorylation, dynein mRNA would not be expected to increase. This issue is currently being investigated. Sodium hydroxide treatment removes phosphate from serine and threonine residues leaving phosphotyrosine residues intact (44). Thus, it appears that the presence of phosphoserine and phosphothreonine on the dynein intermediate chain is required for antibody binding.

Although PC12 cells treated with control brain extracts exhibited more neurites than that of AD brain extract–treated cells, we did not observe dynein immunoreactivity to correlate with neurite growth. It is possible that identification of the AD brain extract endogenous factor(s) may shed some light on this discrepancy. Presently, we can only speculate that the observed increased levels of PC12 cell dynein immunoreactivity following exposure to AD brain tissue extracts may involve receptors coupled to specific kinases. Preliminary characterization suggests the endogenous elements responsible for increasing dynein immunoreactivity may be proteinaceous in nature as evidenced by sensitivity to trypsin as well as precipitation by $(NH_4)_2SO_4$. However, the possible involvement of lipid as well as carbohydrate free and/or bound cannot be ruled out. It is apparent that prior trypsin digestion of the clarified AD tissue extract significantly reduces dynein intermediate chain immunoreactivity. $(NH_4)_2SO_4$ precipitated, clarified AD tissue extract protein when added to PC12 cell cultures does increase dynein intermediate-chain immunoreactivity but not to the same extent as observed for native, untreated tissue extracts. On the basis of the fold increase which should be

observed for untreated clarified AD brain tissue extract (i.e., approximately 10- to 12-fold), total recovery following $(NH_4)_2SO_4$ precipitation at maximum is approximately 40%–50%.

Importantly, there are many limitations when working with human brain 24 hr postmortem, especially when phosphorylation changes are suspected. However, considering all parameters being equal and controlled for, dynein intermediate-chain immunoreactivity was increased in neuronal cells cultured in the presence of endogenous components of AD brain tissue. These data indicate that differences in the dynein-microtubular array occur which may contribute to altered intracellular transport processes.

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