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Expression in Cultured Human Neuroblastoma Cells of Epitopes Associated with Affected Neurons in Alzheimer's Disease

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Of three human neuroblastoma lines tested, IMR32K (and IMR32 parental line) was the only cell line that, after its exposure to a differentiation medium, consistently developed materials recognized immunocytochemically by a panel of antibodies against paired helical filaments (PHF). Ultrastructurally, these cells accumulated, at their perikarya and neuritic extensions, spatially discrete arrays of fibrils, which occasionally occurred in twisted pairs. When these fibrillar structures appeared as paired helices, they exhibited dimensions and configurations reminiscent of PHF found in affected Alzheimer neurons, although less compact. Immunoelectron microscopic examinations of the fibrillar structures in these neuroblastoma cells with one of these anti-PHF immunoprobes revealed that only subsets of fibrillar structures that appeared thickened or aggregated to form bundles were selectively immunolabeled. Cultures of these immortal neuroblastoma lines may provide a convenient model for studying aspects of PHF formation that are hard to examine in Alzheimer brain obtained at autopsy. (Am J Pathol 1990, 136: 867–879)

Alzheimer's disease (AD) is characterized by two key neuropathologic hallmarks, ie, neurofibrillary tangles (NFT) and senile plaques (SP).[1,2] Even though the precise relationship between the frequency of NFT as well as SP and certain phenotypic manifestations of the disease, including severity of dementia, deficit in cortical cholinergic activity, and degree of neuronal loss in hippocampus, remains to be established, it is widely believed that a better understanding of how these markers develop may provide crucial clues about the pathogenesis of this devastating disease.[3] Ultrastructurally, NFT consist mainly of paired helical filaments (PHF)—tightly adherent pairs of 10-nm filaments wound into helices with an 80-nm internode periodicity.[4,5] Immunoprobes have provided valuable insights into the molecular nature of intraneuronal cytoskeletal aberrations in this disease.[6–8] Cumulative evidence to date has indicated the presence of tau[9–13] (probably abnormally phosphorylated),[14,15] neurofilament peptides,[16,17] gangliosides,[18] and ubiquitin[19] in PHF. These specific immunoprobes may also provide useful tools for delineating the mechanisms by which these distinct epitopes accumulate at cellular level and for correlating the sequence of these events with the cytoarchitectural alterations.

Despite impressive progress in recent understanding of the chemical nature and molecular biology of this neuronal aberration,[20–22] detailed mechanistic descriptions of the processes that lead to PHF formation still remain elusive. Specific aspects of this mechanism may well become more amenable to experimental scrutiny in appropriate model systems than in autopsy brain. The use of primary neuronal cell cultures as a model system to study the mechanism of PHF formation has recently been attempted by several laboratories[23,24] with varying degrees of success. Since the intraneuronal accumulation of PHF has been found primarily in humans,[1,25] we have therefore attempted to use cultured human neuroblastoma cells to develop an experimental model for studying the sequence of events that leads to PHF formation.

We report here that one of the three cultured human neuroblastoma cell lines of distinct neurotransmitter types tested, namely IMR32,[26] developed epitopes immunoreactive with polyclonal and monoclonal antibodies against PHF when incubated in a chemically defined, 'differentia-

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Table 1. *Culture Media for Neuroblastoma Cells**

Ingredient	Maintenance medium	Serum-free medium†	Differentiation medium‡
Basal medium	DMEM	DMEM/F12	DMEM/F12
HEPES	15 mmol/l	15 mmol/l	15 mmol/l
L-Glutamine	2 mmol/l	2 mmol/l	2 mmol/l
Fetal bovine serum§	10%	—	—
Insulin	—	5 μg/ml	5 μg/ml
Transferrin	—	100 μg/ml	100 μg/ml
Progesterone	—	20 nmol/l	20 nmol/l
Putrescine	—	100 μmol/l	100 μmol/l
Na selenite	—	30 nmol/l	30 nmol/l
DBcAMP	—	—	1 mmol/l
7S NGF	—	—	0.1 μg/ml
Gangliosides	—	—	10 μg/ml
Na butyrate	—	—	0.5 mmol/l

* DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium; HEPES, N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid; DBcAMP, N^6,$O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphate; 7S NGF, 7S nerve growth factor.
† Bottenstein et al.[29]
‡ Blass et al.[30]
§ Heat inactivated (56 °C, 30 minutes).

tion' condition (1 of the 10 culture conditions tested). They also accumulated fibrillar ultrastructures reminiscent of PHF. The potential application of this cell culture model is discussed. Preliminary results of this study have been presented in abstract.[27]

Materials and Methods

Unless otherwise described, ingredients of culture media and chemical reagents were obtained from GIBCO Laboratories (Grand Island, NY) and Sigma Chemical Company (St. Louis, MO), respectively.

Human Neuroblastoma Cell Cultures

Cultured human neuroblastoma IMR32 (mixed cholinergic-adrenergic) cells[26] were originally obtained from the American Type Culture Collection (Rockville, MD). A subpopulation of cultured cells derived from this line was developed in this laboratory (designated as IMR32K). This subline appeared to provide a homogeneous cell population with regard to their growth characteristics, morphology, and response to culture manipulations and was used throughout this study (except for two preliminary experiments done with the parental IMR32 line). Two additional human neuroblastoma cell lines, SK-N-MC (cholinergic)[28] and SK-N-SH (adrenergic)[28] were kindly provided by Dr. J. Biedler, Memorial Sloan-Kettering Cancer Center, New York, NY.

All these neuroblastoma cells were maintained in T75 flasks (Corning Glass Works, Corning, NY) in the maintenance medium (Table 1)[29,30] in a humidified 37°C incubator supplied with 5% CO_2/95% air. Regular screening indicated that these cultured cells were free of mycoplasma contamination.[31] When needed for experiments, these cells were harvested from flasks after a brief trypsinization and their viability was determined with the trypan blue dye exclusion test.[31] The cells thus collected in suspension, with cell viability of better than 98%, were plated in appropriate culture vessels in the maintenance medium for 24 hours. On the following day, the culture medium was replenished with the aforementioned maintenance medium, or serum-free medium,[29] or 'differentiation medium'[30] (Table 1) for 7 days before various analyses described below. The culture media were replenished on days 3 and 6.

Immunocytochemical and Histochemical Staining

The human neuroblastoma cells were seeded on 9 × 22-mm glass coverslips (Bellco Glass, Inc., Vineland, NJ) at 1.25×10^4 viable cells/sq cm in 60 × 15-mm petri dishes (Corning). After 7 days of cultivation under different conditions as described above, the coverslips were rinsed thoroughly with three changes of Hanks' balanced salt solution (HBSS) and fixed with 4% paraformaldehyde in 0.1 mol/l (molar) phosphate buffer, pH 7.4, at room temperature for 1 hour. The fixed cellular preparations were washed three times with 0.1 mol/l TRIS saline, pH 7.6 (TS).

For immunocytochemical screening for the expression of epitopes reactive with polyclonal and monoclonal antibodies against PHF and the Alz-50 monoclonal antibody, the fixed cultures were processed for immunostaining with the streptavidin biotin-conjugated peroxidase complex (SABC) technique modified from Hsu et al.[32] In brief, the fixed cellular preparations were treated with 3% H_2O_2 in methanol to quench the endogenous peroxidase activity. Then the cells were permeabilized with 0.3% Triton X-100 in TS and exposed to 3% normal goat serum in TS to block any nonspecific binding. All the immunoprobes, except monoclonal antibodies, which were derived from supernatants of respective hybridoma culture media and employed directly without further dilution, used in this study for immunocytochemical staining were diluted immediately before use with the antibody diluent, TS with 1% normal goat serum. This was followed by successive incubations with a primary antibody (anti-PHF [ICN] [1:500 dilution], a rabbit antiserum purchased from ICN Biochemicals, Inc., Lisle, IL; or anti-PHF (SY), a mouse monoclonal antibody [MAb 39][8] provided by Dr. S.-H. Yen, Albert Einstein College of Medicine, Bronx, NY; or Alz-50,[33] a mouse monoclonal antibody provided by Dr. P. Davies, Albert Einstein College of Medicine, Bronx, NY) at 4°C overnight and with its corresponding biotinylated second-

ary antibody (biotinylated, affinity-purified goat anti-rabbit [or anti-mouse] immunoglobulin G [or M] [1:500 dilution] obtained from Zymed Laboratories Inc., South San Francisco, CA) at room temperature for 30 minutes. The immunoreactivity in these cultured cells was detected with freshly prepared SABC, and this immunoperoxidase activity was subsequently visualized with the chromogen diaminobenzidine. The specimens were then dehydrated through an ascending ethanol series, cleared in xylene, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). The cellular preparations then were examined with an Olympus BH-2 research photomicroscope (Olympus Optical Co., Ltd., Tokyo, Japan). A minimum of 100 cells from at least three randomly selected fields were evaluated and their immunostaining intensities were scored independently by three investigators blind to the experimental conditions.

These immunostained cells were evaluated for their immunocytochemical staining intensity, and their immunostaining scores were obtained semiquantitatively by grading against a standardized color photomicrographic template (Figure 1), according to the following criteria to obtain the numbers of immunostained cells for each of the five immunostaining score categories (X_i, i = 0, 1, 2, 3, and 4): 0, no staining of any subcellular structures; 1, perikaryal cytoplasm stained in faint brown but no staining in both nuclei and processes; 2, perikaryal cytoplasm and processes stained in yellowish brown and no nuclear staining; 3, the cell body stained in brown so that the nucleus was obscured and the neuritic extensions were stained in faint brown; and 4, the cell body stained in dark brown so that it was impossible to discern the nucleus and the processes were stained in brown.

The total number of cells evaluated by each observer was represented as T. And, the percentage of cells for each of the five immunostaining score categories (N_i, i = 0, 1, 2, 3, and 4) were thus estimated separately by:

$$Ni = \frac{Xi}{T} \times 100$$

Immunostaining index (ISI) of different culture preparations were then calculated according to:

$$ISI = \frac{N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4}{100}$$

The means were subsequently calculated for each culture condition from the immunostaining indices independently obtained by three investigators blind to the experimental conditions and were subjected to one-way analysis of variance for statistical significance.

If the cultured neuroblastoma cells demonstrated immunocytochemical staining with anti-PHF antibodies in this screening, a subsequent evaluation was performed with an enhanced SABC technique, as will be described below, to further differentiate the immunoreactivities of these cells under various culture conditions. In addition to the aforementioned PHF immunoprobes, another rabbit anti-PHF serum[6] (1:500 dilution) (provided by Dr. D. J. Selkoe, Harvard Medical School, Boston, MA) was also included to evaluate the expression of PHF-associated epitopes in these cultured cells. The fixed cell culture specimens were treated similarly through the blocking step with normal goat serum exactly as in the SABC procedure described above. This was followed by successive incubations with primary antibodies and their corresponding biotinylated secondary antibodies, which were carried out exactly as in the SABC procedure described above, except that these incubations were repeated twice and the treatment with primary antibodies in the second cycle was reduced to 1 hour at room temperature. To efficiently amplify the signals generated by this immunodetection mechanism, complexes with biotin-conjugated peroxidase in excess were prepared in addition to SABC so that there were biotin molecules on these complexes freely accessible to bind streptavidin (referred to hereafter as BSAC). The cellular preparations, previously probed with immunoreagents, were subsequently exposed to three cycles of alternate incubations with SABC and BSAC, 20 minutes each at room temperature (except the first incubation with SABC, which was performed for 1 hour at room temperature), to further enhance the sensitivity of the signal-generating mechanism. The peroxidase activity was visualized with 0.5 mg/ml diaminobenzidine in 50 mmol/l TRIS buffer, pH 7.5, containing 0.3% H_2O_2. The stained culture preparations were subsequently dehydrated, mounted, and evaluated under a microscope for immunostaining scores as described above.

For immunocytochemical controls, replicate neuroblastoma cultures were seeded on glass coverslips and exposed to various culture media (the differentiation medium in particular) exactly as described above. This was followed by the immunocytochemical staining procedure, which was carried out exactly according to the method described above except that similarly diluted nonimmune serum of the animal species (rabbit or mouse) that corresponds with that of the primary antibody employed, or appropriately diluted anti-PHF serum preabsorbed with PHF from the cerebral cortex of a neuropathologically verified AD patient,[34] or 1% normal goat serum in TS were tested as substitutes for the respective primary antibodies used.

For histochemical staining, the fixed culture specimens were processed for silver impregnation with Bielschowsky's staining technique modified by Yamamoto and Hirano,[35] or stained with thioflavin S followed by the examination with fluorescence microscopy,[36] or stained with

Anti-PHF Ab
Maintenance Medium
Differentiation Medium
ICN
DS
SY
A
B
C
D
E
F
Immunostaining Score
0
1
2
3
4

Figure 1. *Photomicrographic template for scoring the immunocytochemical staining intensity of cultured neuroblastoma cells. Photomicrographs of cultured neuroblastoma cell preparations, arranged according to the color intensities of their immunocytochemical staining, which cover the spectrum of immunostaining scores of 0 to 4. This was used as a reference for scoring the immunocytochemical staining of cultured human neuroblastoma cells under different culture conditions. The immunostaining scores were then used to estimate the immunostaining indices of respective cultures, as described in Materials and Methods for semiquantitative immunocytochemical analysis.* **Figure 2.** *Photomicrographs of replicate IMR32 neuroblastoma cultures stained immunocytochemically for their expression of PHF-associated epitopes. The human neuroblastoma cells were cultured in the maintenance or differentiation medium for 7 days before the immunocytochemical staining for PHF. Note the augmented expression of PHF-associated epitopes, demonstrated in parallel by the panel of anti-PHF antibodies used, in these cultured cells when grown in the differentiation medium as compared to those in the maintenance medium. Anti-PHF (ICN), a rabbit anti-PHF serum, was obtained from ICN Biochemicals, Inc., Lisle, IL; anti-PHF (DS), a rabbit anti-PHF serum, was provided by Dr. D. J. Selkoe; anti-PHF (SY), a mouse monoclonal anti-PHF antibody (MAb 39), was provided by Dr. S.-H. Yen. (×350)*

1% Congo red and Mayer's hematoxylin and then examined for green birefringence with polarization microscopy.

Ultrastructural Examinations

The neuroblastoma cells were seeded directly in 60 × 15-mm polystyrene petri dishes and subsequently treated as described above. On the seventh day of exposure to different media, these cultured cells were thoroughly rinsed with three changes of HBSS and processed for *in situ* transmission electron microscopic analysis according to Ko et al.[31] In brief, the rinsed cultures were fixed with 3% glutaraldehyde in 0.1 mol/l sodium cacodylate, pH 7.4, postfixed with 1% osmium tetroxide, stained *en bloc* with 0.1% uranyl acetate, dehydrated through an ascending ethanol series, infiltrated with hydroxypropyl methacrylate, and finally embedded *in situ* with Poly-Bed 812 (Polysciences Inc., Warrington, PA). Ultrathin sections of the *in situ* embedded cultures thus obtained with a Sorvall MT-6000 ultramicrotome were stained with lead citrate and examined at 80 kV in a JEOL JEM-100S or JEM-100CX2 electron microscope (Jeol Ltd., Tokyo, Japan).

For immunoelectron microscopic studies, cells were seeded directly in polystyrene petri dishes (Corning) and incubated under exactly the conditions described above. After brief rinses with three changes of HBSS and PHEM buffer (60 mmol/l PIPES, 25 mmol/l HEPES, 10 mmol/l EDTA, and 2 mmol/l $MgCl_2$, pH 6.9),[37] the cultured cells were permeabilized with 0.5% Triton X-100 in PHEM buffer for 1.5 minutes followed by a thorough rinse with two changes of PHEM buffer. The treated cellular preparations then were fixed with 0.1% glutaraldehyde in PHEM buffer and subsequently exposed to 100 mmol/l each of glycine and sodium cyanoborohydride in PHEM buffer for 20 minutes to block excessive aldehyde before the rinses with PHEM buffer and TS. After blocking with 3% normal goat serum in TS for 2 hours, the fixed preparations were incubated for 30 minutes each with the anti-PHF antibody (ICN) (1:500 dilution) and the biotinylated affinity-purified, goat anti-rabbit IgG (1:500 dilution) at 37°C, and then washed 5 minutes each with TS and TBS (20 mmol/l Tris, 150 mmol/l NaCl, 10 mmol/l NaN_3, pH 8.2). Immediately before use, the streptavidin-labeled immunogold probe, streptavidin absorbed with 15-nm gold colloid (AuroProbe EM streptavidin G15; Janssen Life Science Products, Piscataway, NJ), was rinsed in TBS by centrifugation at 11,500*g* for 5 minutes followed by resuspension with TBS containing 0.5% cold-water fish gelatin[38] to achieve a 1:10 dilution. Incubation of the cellular specimens with this immunogold probe was carried out for 60 minutes at 37°C. The immunolabeled samples were further processed as follows: a 15-minute wash each in TBS and 0.1 mol/l sodium cacodylate (Polysciences, Inc., Warrington, PA), pH 7.4, followed by a 15-minute fixation in 2% glutaraldehyde in 0.1 mol/l sodium cacodylate, pH 7.4, and another rinse in 0.1 mol/l sodium cacodylate, pH 7.4, for 15 minutes. These specimens were subsequently washed in distilled water, postfixed with 1% osmium tetroxide, stained *en bloc* with 0.1% uranyl acetate, then dehydrated through an ascending ethanol series, embedded, and processed *in situ* for transmission electron microscopy as described above.

Results

Among the three cultured human neuroblastoma cell lines of distinct neurotransmitter varieties screened for their expression of epitopes associated with NFT of AD, only IMR32K (and IMR32 parental line) cells demonstrated augmented immunocytochemical staining with the polyclonal and monoclonal antibodies against PHF (Table 2). It is interesting to note that, like SK-N-MC and SK-N-SH cells, cultures of IMR32, grown in the maintenance medium or serum-free medium, exhibited extremely low or no immunostaining with both polyclonal and monoclonal anti-PHF antibodies. In contrast, when exposed to the differentiation medium, cultured cells derived from IMR32 (or its variant IMR32K), but not from SK-N-MC or SK-N-SH, became significantly stained with the polyclonal anti-PHF serum (Table 2). The medium varieties described herein represent only 3 of the 10 different culture conditions screened to date.

Table 2. *Semiquantitative Analysis of Immunocytochemical Staining of Cultured Human Neuroblastoma Cell Lines for Their Expression of Alzheimer Antigens**

	Maintenance medium	Serum-free medium	Differentiation medium
IMR32 or IMR32K (mixed cholinergic-adrenergic)			
Anti-PHF (ICN)‖	0.02 ± 0.02	0.04 ± 0.01	1.00 ± 0.11†‡§
Anti-PHF (SY)¶	0.01 ± 0.01	0.01 ± 0.01	0.18 ± 0.10†‡§
Alz-50#	1.03 ± 0.03†	0.98 ± 0.08†	1.06 ± 0.08†
Negative Controls			
Anti-PHF (ICN) abs**	0.01 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
Normal mouse serum††	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Antibody diluent‡‡	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
SK-N-MC (cholinergic)			
Anti-PHF (ICN)	0.03 ± 0.02	0.05 ± 0.03	0.07 ± 0.03
Anti-PHF (SY)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Alz-50	0.96 ± 0.04†	1.02 ± 0.01†	1.06 ± 0.03†
Negative Controls			
Anti-PHF (ICN) abs	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
Normal mouse serum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Antibody diluent	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SK-N-SH (adrenergic)			
Anti-PHF (ICN)	0.03 ± 0.02	0.05 ± 0.03	0.05 ± 0.03
Anti-PHF (SY)	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
Alz-50	0.94 ± 0.03†	0.92 ± 0.04†	0.94 ± 0.03†
Negative Controls			
Anti-PHF (ICN) abs	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
Normal mouse serum	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
Antibody diluent	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00

* Immunostaining indices of cultured cell specimens of a typical experiment were determined separately by three investigators blind to the experimental conditions on the basis of their immunocytochemical staining intensities with the SABC technique as described in the Materials and Methods. The results are represented as means ± standard error. All values derived from the same immunoprobes were compared with one another and with those of their corresponding negative controls and were subsequently analyzed for statistical significance by one way analysis of variance. The comparisons that are statistically significant ($P < 0.01$) are denoted as follows: †, compared with that of cultures incubated with maintenance medium and probed with antibody diluent; ‡, compared with that of cultured cells incubated with maintenance medium and probed with the same antibody; and, §, compared with that of cultures exposed to serum-free medium and probed with the same immunoprobe.

‖ ICN Biochemicals, Inc., Lisle, IL.

¶ Yen et al.[8]

Wolozin et al.[33]

** The Anti-PHF (ICN) antibody absorbed with PHF prepared from the cerebral cortex of a neuropathologically verified AD patient according to Selkoe and Abraham (1986).[34]

†† Organon Teknika-Cappel, West Chester, PA.

‡‡ 1% normal goat serum in 0.1 mmol/l TRIS saline, pH 7.6.

Because the rabbit anti-PHF serum (ICN) used in this analysis has been reported to react with both epitope(s) unique to PHF as well as those in microtubule-associated tau proteins,[39] questions arise as to whether the elicited immunoreactivity for PHF in these cells reflects the accumulation of epitopes uniquely associated with PHF or the expression of tau proteins, or both. Even if tau proteins were responsible for the immunoreactivities, the demonstrated immunostaining in these neuroblastoma cells most likely resulted from the tau proteins incorporated into the detergent-insoluble cytoskeleton, because the immunocytochemical procedure employed in this study detects only detergent-resistant structures. Furthermore, since the monoclonal antibody MAb 39 is highly specific for PHF,[8] the independent confirmation of immunostaining for PHF with this monoclonal antibody indicates that the expression of PHF-associated epitopes may be elicited in these neuroblastoma cells by the differentiation condition used.

Monoclonal antibody Alz-50, despite its immunoreactivity with tau and its derivatives,[40,41] is thought to recognize neurons vulnerable to the development of NFT before the actual detection of NFT structures,[15,42] was also included to evaluate the expression of its immunoreactivity in these cultured neuroblastoma cells (Table 2). It appears that the Alz-50 immunoreactivity was constitutively expressed in all three human neuroblastoma cell lines tested. Furthermore, the immunocytochemical reactivity in these cells demonstrated with this monoclonal antibody remained virtually unaltered regardless of the different culture conditions used.

Negative controls for the corresponding primary antibodies used in the immunocytochemical staining procedure were always appropriately negative. It is readily evident in Tables 2 and 3 that the immunostaining indices of these negative controls for each of the three cell lines evaluated on the basis of their immunocytochemical reactivity were negligible irrespective of the evaluations independently made by different observers or of the differences in the variety of culture condition used or the type of negative control tested.

Table 3. *Immunocytochemical Analysis of Cultured IMR32K Cells for Their Expression of PHF-associated Epitopes**

Immunoprobe	Maintenance medium	Serum-free medium	Differentiation medium
Anti-PHF (ICN)‖	1.20 ± 0.11†	1.21 ± 0.09†	2.96 ± 0.02†‡§
Anti-PHF (DS)¶	1.93 ± 0.01†	1.98 ± 0.06†	3.95 ± 0.05†‡§
Anti-PHF (SY)#	0.58 ± 0.08†	0.78 ± 0.12†‡	2.08 ± 0.17†‡§
Negative Controls			
Anti-PHF (ICN) abs**	—	—	0.14 ± 0.02
Normal mouse serum††	—	—	0.04 ± 0.01
Antibody diluent‡‡	—	—	0.01 ± 0.01

* Immunostaining indices were determined from a typical experiment independently by three investigators blind to the experimental conditions following the immunocytochemical staining of neuroblastoma culture specimens with the enhanced SABC technique as described in the Materials and Methods. The results are represented as mean ± standard error. All values derived from the same immunoprobes were compared with one another and with those of their corresponding negative controls. The comparisons were analyzed for statistical significance by one way analysis of variance. The comparisons that are statistically significant ($P < 0.01$) are denoted as follows: †, compared with that of cultures exposed to differentiation medium and probed with antibody diluent; ‡, compared with that of cultured cells incubated with maintenance medium and probed with the same antibody; and §, compared with that of cultures incubated with serum-free medium and probed with the same immunoprobe.

‖ ICN Biochemicals, Inc., Lisle, IL.

¶ Ihara et al.[6]

Yen et al.[8]

** The Anti-PHF (ICN) antibody absorbed with PHF prepared from the cerebral cortex of a neuropathologically verified AD patient according to Selkoe and Abraham (1986).[34]

†† Organon Teknika-Cappel, West Chester, PA.

‡‡ 1% normal goat serum in 0.1 mmol/l TRIS saline, pH 7.6.

It is of considerable interest that by subjecting IMR32 cells to the 'differentiation' condition, the degree of PHF-associated epitope expression in terms of their immunostaining intensity could be significantly increased (Table 2). This increase in immunocytochemical reactivity for PHF in IMR32 cells was further verified by the enhanced SABC technique, not only with the aforementioned anti-PHF immunoprobes but also with the rabbit anti-PHF serum (anti-PHF [DS]) donated by Dr. D. J. Selkoe (Figure 2 and Table 3). Results obtained with the enhanced immunostaining technique were qualitatively similar, but the immunocytochemical reactivity was far more intense (Tables 2 and 3). The immunoreactive intensity of IMR32K cells grown in the 'differentiation' medium rose from 1.00 ± 0.11 to 2.96 ± 0.02 with anti-PHF (ICN) antibody and from 0.18 ± 0.10 to 2.08 ± 0.17 with anti-PHF (SY) antibody, the monoclonal antibody donated by Dr. S.-H. Yen. Under all three culture conditions, the immunocytochemical reactivity of these cultured cells was significantly greater than that observed with the immunocytochemical controls (Table 3). Statistical analysis of the data with the absorbed antibody control indicated that the value did not differ significantly from that obtained with antibody diluent (Table 3). This enhanced technique therefore makes the distinction between the immunostaining intensity of these neuroblastoma cells exposed to the differentiation medium from those of the replicate cultures under the other culture conditions readily evident with a two- to fourfold margin in estimated immunostaining indices (Table 3). It is noteworthy, however, that histochemical techniques such as Bielschowsky's silver impregnation, thioflavin S, and Congo red staining failed to demonstrate any tinctorial characteristics indicative of NFT formation in these cultured cells.

Ultrastructurally, IMR32 cells accumulated discrete paracrystal-like arrays of fibrils in their neuritic extensions (Figure 3) and occasionally in their perikarya as well after their exposure to the differentiation medium. These materials appeared as noncompact, wavy arrays of homogeneous filaments (Figure 3B) spatially segregated from other cytoplasmic organelles (Figure 3B and C). Structural continuity was discernible between these filaments and normal cytoskeletal elements, such as microtubules (Figure 3D). The constituent filaments within these fibrillary arrays (Figure 3E) had a diameter of 11.5 ± 0.2 nm (range, 7 to 14 nm; N = 100). Some of these filaments appeared to run in pairs and wind around each other to give configurations reminiscent of PHF, although not as compact (Figure 3E). These paired fibrils measured 29.2 ± 0.8 nm (range, 25 to 35 nm; N = 17) at their maximum width, with an internode periodicity of 88.2 ± 2.4 nm (range, 75 to 100 nm; N = 17) (thus referred to hereafter as PHF-like filaments). In light of their unique ultrastructural features, viewing of these PHF-like fibrils by tilting the specimen stage was performed and appeared to exclude the possibility that the twisted pairs of fibrils observed could be produced artifactually by overlapping filaments at different levels.

In addition, immunoelectron microscopic studies with a colloidal gold-labeling procedure were also carried out to identify directly the subcellular structures that are responsible for the demonstrated expression of PHF-associated epitopes. The immunogold-labeling studies of these IMR32K cells with anti-PHF (ICN) antibody revealed that there was no discernible immunolabeling of the nucleolus (not shown), nuclear matrix, nuclear envelope, and cytoplasmic organelles such as mitochondria, endoplasmic reticulum, and cytoskeletal components, except

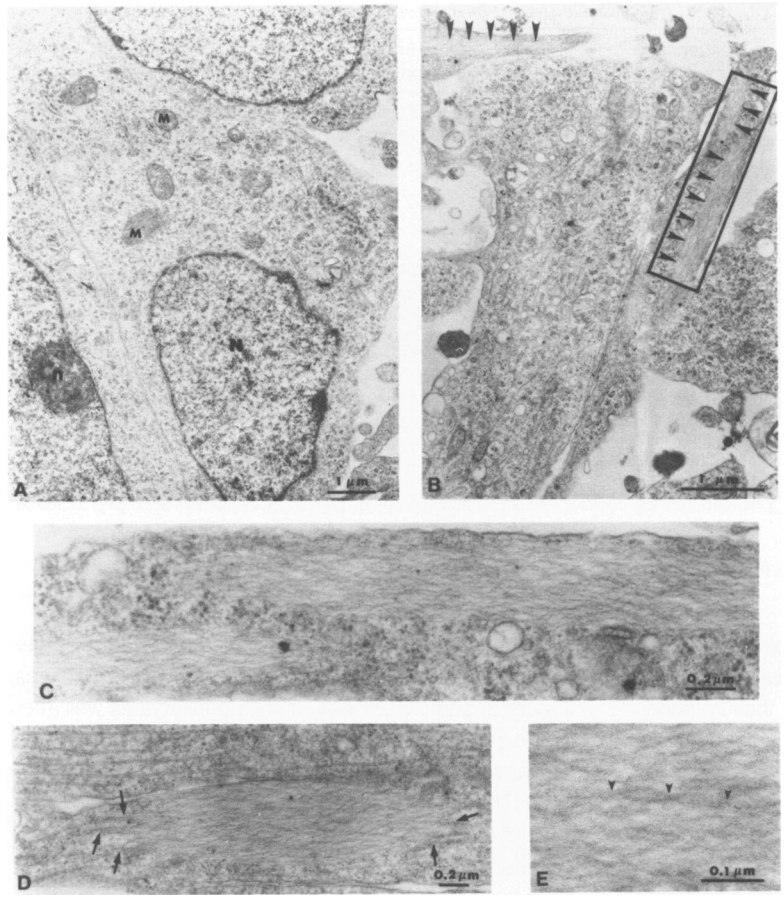

Figure 3. *Transmission electronmicrographs of cultured IMR32 cells after their exposure to the differentiation medium. The human neuroblastoma cells were cultured in the differentiation medium for 7 days and subsequently processed for* in situ *transmission electron microscopy.*[31] **A:** *Perikarya of IMR32 cells cultured in the differentiation medium (×13,800). The morphologic features of perikarya of these cells did not appear any different from those of the culture exposed to the maintenance medium (data not shown). M, mitochondrion; N, nucleus.* **B:** *Neuritic extensions of cells as depicted in* **A**, *with abundant cytoskeletal elements (×18,800). Arrowheads indicate the discrete collections of paracrystal-like arrays of wavy fibrillar bundles spatially segregated from other organelles in the cytoplasm.* **C:** *Higher magnification of the rectangle depicted in* **B** *(×71,000).* **D:** *The paracrystal-like array of filamentous materials in a neuritic process, which bears structural continuities with microtubules (indicated by arrows) (×40,500).* **E:** *Arrays of the filamentous bundles exhibit the structural configuration resembling that of PHF in the affected neurons of AD brains (×181,700). Small arrowheads point at the characteristic constrictions of a twisted pair of helices with an apparent internode periodicity of 88 nm.*

for a subset of fibrillar structures (Figure 4A). Interestingly, three types of fibrillar structures in these neuroblastoma cells were selectively immunolabeled by this PHF immunoprobe at the ultrastructural level (Figure 4B through D): fibrillar bundles that appeared as aggregates of 10-nm filaments (Figure 4B), scattered cytoskeletal fibrils that appeared thickened (Figure 4C), and filamentous tufts (Figure 4D). Within the elaborate cytoskeletal meshwork of these cells, aggregates of 10-nm filaments (occasionally accompanied with microtubules) that appeared to form fibrillar bundles were selectively decorated by electron-dense colloidal gold particles (Figure 4B). Numerous scattered cytoskeletal fibrils that appeared thickened were also immunolabeled by electron-dense immunogold particles (Figure 4C). It is noteworthy that a significant proportion of these fibrils appeared to be coated with variable amount of fuzzy amorphous materials of yet unknown nature (Figure 4C). The coating material makes definitive identification of some of these cytoskeletal fibrils difficult. There are also fibrillar materials that were selectively decorated by immunogold particles, usually 3 to 7 in number, lining up continuously on one side of a fibrillar structure (Figure 4C). The diameter of these fibrils measured invariably larger than that of normal cytoskeletal components, although they seemed to appear with much heterogeneity in terms of their dimension and ultrastructural characteristics. There were immunolabeled filamentous tufts (Figure 4D), which consist of a homogeneous array of 8- to 12-nm filaments. Like the discrete arrays of homogeneous PHF-like filaments noted above, these immunolabeled filamentous tufts were also spatially segregated from other cytoplasmic organelles. However, the texture of these tufts appeared far less compact than that of the array of PHF-like filaments; and paired helices were never observed in these tufts. It remains possible that the detergent treatment before fixation, which was necessary to make these cells permeable for subsequent immunogold-labeling, might have altered certain ultrastructural features, such as formation of paired helices and texture of the fibrillary array, without affecting other characteristics of the PHF-like filaments.

It is noteworthy that the topographic distribution of electron-dense colloidal gold immunolabels on selectively decorated fibrils were mostly clustered at locales where fibrillar structures converge or aggregate to form bundled cytoskeletal filaments (Figure 4B). Some immunolabeled domains appeared with a regular interval of approximately 100 nm (depicted by arrows in Figure 4C) on gold-decorated fibrils.

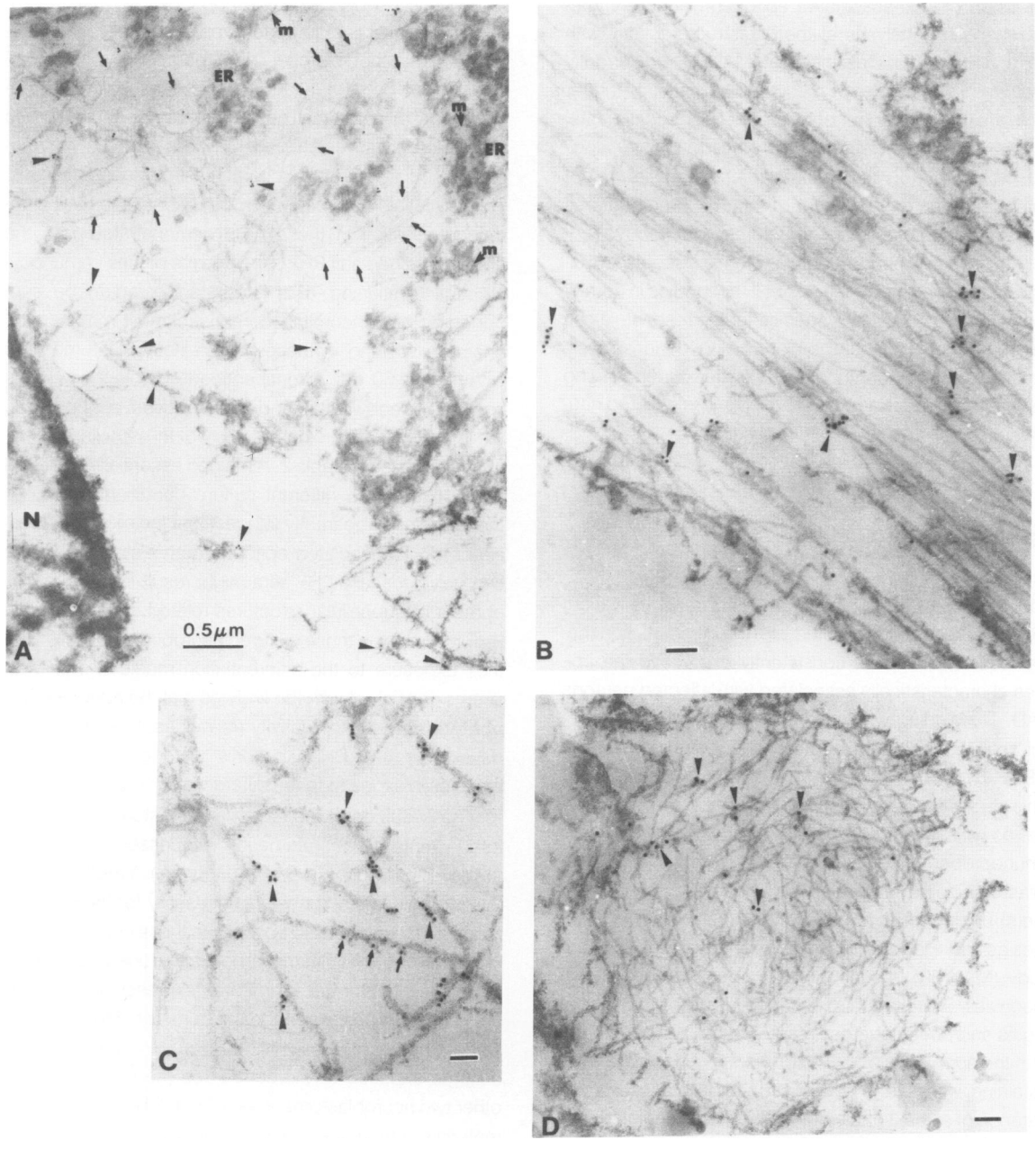

Figure 4. *Immunoelectron microscopy of intracellular fibrillar structures within Triton-permeabilized IMR32 cells using the anti-PHF (ICN) antibody. The human neuroblastoma cells were cultured in the differentiation medium for 7 days, permeabilized with Triton X-100, followed by immunogold labeling with the anti-PHF (ICN) antibody and subsequently processed for* in situ *transmission electron microscopy.*[31] **A:** *Perikaryon of a Triton-permeabilized IMR32 cell (×26,400). The nuclear matrix (N), nuclear envelope, mitochondria (m), endoplasmic reticulum (ER), and most cytoskeletal fibrils (arrows) were devoid of any immunogold labeling. In contrast, some fibrillar structures, which appeared in aggregates or bundles, were selectively decorated with electron-dense colloidal gold particles (arrowheads).* **B:** *Fibrils, which appeared aggregated to form bundled filaments, were distinctly decorated by immunogold particles (arrowheads) within the elaborate cytoskeletal meshwork of a Triton-permeabilized neuroblastoma cell (×51,300).* **C:** *Microtubules and other cytoskeletal fibrils, which appeared thickened or aggregated into bundles (arrowheads), were selectively decorated by this immunogold-labeling procedure (×55,400). Arrows denote an apparent periodicity of spacing between the immunogold-labeled domains on a microtubule.* **D:** *A subset of fibrils (arrowheads) within a spatially segregated tuft, which consisted of an homogeneous array of filaments, were selectively immunolabeled (×55,000). Scale bar, 0.5 μm.*

And it is interesting to note that only a fraction of the fibrils that constitute the homogeneous filamentous tufts (Figure 4D) were selectively immunolabeled. These filaments were decorated by the electron-dense immunogold particles at the structures where aggregation or crosslinking of fibrillar structures seemed evident (arrowheads in Figure 4D).

To demonstrate that the development of selective immunolabeling on these fibrillar structures results from the

expression of PHF-associated epitope(s), further immunogold-labeling analyses at the ultrastructural level with other anti-PHF antibodies, including MAb 39, are currently underway. To characterize these fibrillar materials further and examine whether or not normal cellular constituents, such as tau, high-molecular weight microtubular-associated protein MAP2, and neurofilament subunits, might contribute to the development of the PHF-like structures or of other abnormal fibrillar structures noted above, colocalization experiments at both photomicroscopic and ultrastructural levels using the anti-PHF antibodies in combination with immunoprobes specific for the respective cellular components in question are being done as well. These studies may contribute to a better understanding of how the PHF-like filaments develop in this cultured neuroblastoma cell model.

Discussion

Cultured human neuroblastoma IMR32 (and its variant IMR32K) cells, when incubated with the differentiation medium described above, consistently expressed the epitopes characteristically associated with affected neurons of AD. This finding was substantiated by a panel of distinct antibody probes with established immunoreactivity for PHF. Both anti-PHF (ICN) and anti-PHF (DS) sera used in this study have been found to recognize the PHF structures as well as tau proteins.[39] Therefore, by using these immunoprobes, it is likely that we monitored in these neuroblastoma cells the development of not only certain epitope(s) unique for PHF but the accumulation of tau epitopes as well. However, the monoclonal anti-PHF (SY) antibody (MAb 39) is such a highly specific probe that it recognizes only epitope(s) associated with neurofibrillary tangles and does not cross-react with any antigens present in the normal brain.[8,43] The presence of positive immunostaining with this antibody strongly indicates the appearance of at least one epitope uniquely associated with PHF in these neuroblastoma cells when exposed to the differentiation medium. And it is noteworthy that this is the only human neuroblastoma cell line that consistently exhibited PHF-associated immunoreactivity among the three lines tested. Whether or not the selective vulnerability of this particular neuroblastoma line to development of such cytoskeletal abnormalities is in any way relevant to that of specifically affected neurons is unknown at present. Nonetheless, these interesting features appear to make IMR32/IMR32K line a convenient experimental model system to be exploited in the future studies for exploring the mechanism that leads to the intracellular accumulation of fibrillar materials recognized by anti-PHF antibodies.

Mounting evidence has strongly indicated that tau proteins constitute an important part of the PHF structure,[11–15,44] either by direct incorporation into the PHF core[45–47] or by integration in the PHF-associated structures.[6,11–15,48] Like other neuroblastoma cells or neuroectodermal derivatives,[49–51] IMR32 cells express microtubule-associated proteins characteristic of neural tissues. This expression therefore may be responsible for the immunoreactivities in these cells demonstrated with the aforementioned anti-PHF sera in terms of their immunocytochemical staining. This notion appeared to be supported by the concomitant demonstration of immunocytochemical staining in these human neuroblastoma cells with the Alz-50 monoclonal antibody (Table 2), which is known to react with tau and its derivatives.[40,41] However, the immunocytochemical reactivity of these cells with Alz-50, as depicted in Table 2, remained essentially unaltered regardless of the different culture conditions used. All these findings apparently suggest that the elicited expression of PHF-associated epitopes demonstrable with the two polyclonal anti-PHF sera might result from the development of subcellular structures related in some way to PHF formation in these cultured neuroblastoma cells after their exposure to the differentiation medium. This view seems consistent with the findings that the augmentation of MAb 39 immunoreactivity as well as the concomitant development of PHF-like fibrils were demonstrable in these cells exposed to the differentiation medium.

The IMR32 cells, like other neuroblastoma cells in culture, extended elaborate neuritic processes on exposure to the differentiation medium (Figure 2). Whether or not this morphologic response is necessary for the development of PHF-associated epitopes in these cells is unclear. However, the cytoplasmic arborization does not appear to be sufficient for the augmented expression of PHF-associated epitopes, since the same morphologic features without increased immunostaining were noted in replicate cultures exposed to the serum-free medium and in the other two neuroblastoma lines, which did not develop any materials immunoreactive with anti-PHF probes.

It is well established that a specific structural configuration is essential for developing certain tinctorial characteristics with some histochemical stains (eg, beta-pleated sheet configuration is required for Congo red birefringence). It is tempting to speculate that the accumulated fibrillar structure in these cells may not yet have attained the structural requirements necessary for demonstrating the specific staining patterns observed with NFT of AD brains. It thus is plausible that the accumulation of abnormal fibrils in these cells was still at a relatively early phase of development that renders the material undetectable with current histochemical staining techniques. As a result, it takes a rather sensitive technique such as the im-

munocytochemical staining used in this investigation to bring out the cytoskeletal alterations in these cells.

The development of immunocytochemical staining demonstrated with this panel of anti-PHF antibodies seems to coincide with the appearance of PHF-like fibrillar materials observed ultrastructurally in these cultured human neuroblastoma cells. The characteristic dimension and configuration makes these filamentous materials reminiscent of PHF even though the texture of the fibrillar arrays is not as compact. Furthermore, viewing of these paired fibrils by tilting the specimen stage in an electron microscope excluded the possibility that the twisted pairs of fibrils observed could result artifactually from overlapping filaments at different focal levels.

Immunoelectron microscopic analyses with the anti-PHF (ICN) antibody (which recognizes tau proteins as well as PHF) by a immunogold-labeling procedure correlate the intracellular accumulation of abnormal cytoskeletal structures with the concomitantly elicited immunocytochemical reactivity in these cultured human neuroblastoma cells after their exposure to the differentiation medium. The selectivity in immunolabeling by this PHF immunoprobe at ultrastructural level is readily evident by its discriminating decoration of only a subset of fibrillar elements with colloidal particles amongst a wide variety of cellular organelles. It is interesting to note that there were three types of fibrillar structures that became selectively immunolabeled by this anti-PHF antibody. The predominant fibrillar structures that were selectively immunolabeled amongst the elaborate cytoskeletal meshwork of these cells were numerous aggregated or bundled fibrils consisting primarily of intermediate filaments. Occasionally, microtubules were also found as part of these immunolabeled aberrant cytoskeletal structures. In addition, some immunogold-decorated microtubules appeared scattered in the cytoplasmic domain and coated with variable amount of "fuzzy" amorphous materials. It is not surprising to find microtubules selectively decorated by the electron-dense colloid gold particles, since the immunochemical recognition of tau proteins by this antibody might occur and be responsible for this immunolabeling, as discussed above. Immunolabeling of tau protein by the anti-PHF (ICN) immunoprobe on some microtubules is suggested by the following observations: appearance of immunogold particles at locales where twiglike sidearms projected from microtubules, existence of spacing periodicity of immunogold particles on labeled microtubules (arrows in Figure 4C), and aggregation or bundle formation of cytoskeletal fibrils. This seems consistent with the recent finding of Hirokawa et al[52] that tau forms a short armlike projection from microtubule with periodic spacings and crosslinks adjacent microtubules. Whether or not tau proteins might have contributed to certain "fuzzy" coats on these immunolabeled fibrils as was described for the external fuzzy covering of PHF[44] remains unclear. Colocalization experiments with specific immunoprobes directed against tau may help to resolve this issue.

It is intriguing to note that there were immunolabeled fibrils whose width exceeds that of any normal cytoskeletal elements. These fibrillar materials appeared to consist of tightly adherent or aggregated filaments. In addition, the immunolabeling pattern of three to seven immunogold particles lining up on one side of the fibrillar structure is unusual. It is tempting to speculate that these abnormal fibrils probably result from the formation of tightly adherent filaments such as the PHF-like filaments noted above. Co-localization experiments using immunoprobes monospecific for distinct cytoskeletal constituents will probably help in delineating the identity of these abnormal fibrillar structures.

Interestingly, there were immunolabeled filamentous "tufts" that appear to share structural characteristics with the arrays of PHF-like filaments noted above, such as 8 to 12 nm in filament diameter, structural homogeneity of constituent filaments, and spatial segregation from other cytoplasmic organelles. The texture of these tufts, nevertheless, appeared far less compact than that of the array of PHF-like filaments, and the filaments in these tufts never formed paired helices as observed occasionally with the PHF-like filaments. It is plausible that permeabilization of cultured neuroblastoma cells with Triton X-100, which made the cytoskeletal components accessible in the immunogold-labeling procedure, may have resulted in this discrepancy in their structural appearances as observed. However, it is noteworthy that only a subset of fibrils within these tufts were immunolabeled and the filaments that were selectively labeled appeared aggregated or bundled. Therefore, it should be noted that, despite distinct ultrastructural characteristics that these immunolabeled fibrillar materials appeared to display, all (except the scattered microtubules that appeared thickened) seem to share a common attribute, which is aggregation of adjacent fibrillar structures.

Topographically, it is intriguing also that colloidal gold particles on these selectively immunolabeled fibrils appeared at locales where cytoskeletal structures converged or aggregated to form bundled fibrils (arrowheads in Figure 4A to C). Furthermore, most immunogold particles on the labeled fibrillar structures were clustered in distribution. It is also interesting to note that the number of immunogold particles at the labeled sites appears to be more than the number of filaments involved in aggregation or formation of bundles. Whether this peculiar topographic distribution of immunolabels results from the clustered epitopes on the filaments, which results in aggregation of these fibrillar materials, or from the distinct epitopes brought to their proximity passively as a result of fibrillar aggregation, can not be ascertained at this stage.

This cell culture system thus may provide a convenient model system for studying aspects related to PHF formation, such as expression of PHF-associated epitopes and sequence of events leading to the development of PHF-like ultrastructures, which are hard to examine in AD brain obtained at autopsy.

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