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Paired helical filaments from Alzheimer disease patients contain cytoskeletal components

(neurofibrillary tangles/senile dementia/immunocytochemistry)

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ABSTRACT **Neurofibrillary tangles from Alzheimer disease patients share antigenic determinants with neurofilaments and microtubule-associated proteins, as shown by light microscopy immunocytology. The present study addresses the issue of whether these determinants are located on the paired helical filaments or on other components of the neurofibrillary tangle. Sections from postmortem brains from Alzheimer disease patients were stained by using Bodian's silver method or immunostained by using poly- and monoclonal antibodies to neurofilaments and polyclonal antibodies to microtubules. Bodian's silver stain has an intense affinity for neurofibrillary tangles and has been shown to bind to specific domains of neurofilament subunits. The antibodies to neurofilaments used here immunostain most or all of the neurofibrillary tangles present in the sections whereas the antiserum to microtubule protein immunoreacted with about half of the neurofibrillary tangles. All of the antibodies as well as Bodian's silver stain reacted with the paired helical filaments. The epitopes that we have shown to be present in the paired helical filament, in contrast to the corresponding epitopes present in normal neuronal cytoskeleton, are insoluble in ionic detergent. It is concluded that these epitopes are integral components of the paired helical filaments and that, at least in part, paired helical filaments are derived from altered elements of the normal neuronal cytoskeleton.**

The two histologic lesions characteristic of brains from patients with Alzheimer disease are neuritic or senile plaques and neurofibrillary tangles (NFT) (1, 2). NFT are predominantly composed of paired helical filaments (PHF) (3) but straight 15-nm filaments, 10-nm intermediate filaments, dense granules of various sizes, and other structures are also present (4–6). Immunocytochemistry at the light-microscope level has shown that NFT have antigenic determinants in common with normal cytoskeletal components such as neurofilaments (NF) (7–12) and microtubule proteins (MTP) (13–16), as well as with noncytoskeletal components (17) and determinants not yet identified in any normal cell (18). Bodian's silver stain, which specifically binds to the NF subunits (11, 19), also reacts with NFT. The relevance of these findings to the composition of PHF has been questioned because of the heterogeneous nature of NFT (14, 20, 21) and because it has been reported that isolated NFT no longer immunoreact with antibodies to NF (18). In this ultrastructural study, we demonstrate that antigenic determinants present in NF and microtubule-associated proteins as well as Bodian's silver binding sites are integral PHF components. Our data indicate that PHF are derived from NF and possibly from other normal neuronal cytoskeletal elements.

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MATERIALS AND METHODS

Tissue Source. Tissue from five Alzheimer disease patients, aged 73, 76, 87, 88, and 90 years, all with a definite history of dementia and the presence at autopsy of a large number of NFT in the hippocampus and senile plaques in the neocortex was used. Tissue was fixed with 10% formalin in phosphate buffer, pH 7.0. Prior to use, it was rinsed in phosphate-buffered saline and 60-μm free floating sections were cut with a Vibratome (Oxford).

Antibodies. The following antibodies were used: (*i*) rabbit antiserum recognizing all three NF subunits (8), (*ii*) mouse antiserum recognizing all three NF subunits (12), (*iii*) monoclonal antibody 1.1 reacting with the 200-kDa NF subunit (11), and (*iv*) rabbit antiserum to chicken brain MTP (Miles). Preparations used for adsorption experiments were as follows: purified NF subunits (12), chicken brain MTP prepared by two cycles of assembly and disassembly (22), heat-stable microtubule-associated protein (23), and tubulin purified by phosphocellulose chromatography (23). For immunoblotting, polypeptides were separated on 5–15% polyacrylamide slabs, transferred to nitrocellulose (24), and immunostained using an indirect peroxidase procedure.

Immunostaining/Congo Red. Sections (5 μm) of paraffin-embedded Alzheimer disease hippocampus were immunostained using the peroxidase-antiperoxidase procedure (25) with 3′,3′-diaminobenzidine as peroxidase cosubstrate. The sections were treated for 30 sec with 1% OsO_4, rinsed in water, and counterstained with Congo red (26).

Immunostaining for Ultrastructure. Vibratome sections were processed according to the peroxidase-antiperoxidase procedure (25). Incubation with 3′,3′-diaminobenzidine was at pH 7.6 for <5 min. Sections were treated with 2% OsO_4 for 1 hr, rinsed, postfixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hr, dehydrated, and flat embedded in Spurr's medium. Thin sections were viewed at 60 kV in a JEOL 100C electron microscope.

Bodian's Silver Method. Vibratome sections were stained as described (19). Silver-stained sections were fixed and embedded as described for the immunoperoxidase procedure.

Detergent-Extracted NFT. Minced hippocampus, rich in NFT, was suspended in 20 vol of 1% $NaDodSO_4$ in 50 mM Tris·HCl, pH 7.6, for 18 hr at room temperature. The insoluble residue was smeared on glass slides and immunostained using the peroxidase-antiperoxidase procedure. Smears were embedded in Spurr's medium with a prehardened epoxy "dummy" placed over the area of interest. The preparations were detached from the glass slides by immersion in liquid nitrogen.

Abbreviations: NFT, neurofibrillary tangles; PHF, paired helical filaments; NF, neurofilaments; MTP, microtubule proteins.

Enriched NFT Fraction. Hippocampus was minced and further disrupted in a loose-fitting ground-glass homogenizer in 0.5% Triton X-100/2 mM EGTA/10 mM $MgCl_2$/50 mM Tris·HCl, pH 7.6, containing DNase I (Sigma) at 0.05 mg/ml. Following 30 min at room temperature, $NaDodSO_4$ was added to 1%. After 18 hr at 4°C, the homogenate was filtered through an 80-μm nylon mesh and centrifuged at 10,000 × *g* for 30 min. The pellet was suspended in 1% $NaDodSO_4$/50 mM Tris·HCl, pH 7.6, and layered on a gradient of 1.0, 1.2, 1.4, and 2.0 M sucrose in the same buffer and centrifuged at 150,000 × *g* for 3 hr. The 1.4–2.0 M interface contained most of the NFT.

Immunogold-Negative Staining. Colloidal gold (20 nm) was prepared and complexed to affinity-purified goat anti-mouse or anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) (27). Enriched NFT fractions were applied to carbon-coated nickel grids. Grids were floated on the primary antibody for 1 hr at room temperature, washed and floated on the appropriate IgG–gold complex for 1 hr at room temperature. Antibodies were diluted and washes were done with Millipore-filtered 1% bovine serum albumin/150 mM NaCl/20 mM NaN_3/20 mM Tris·HCl, pH 8.2. Grids were rinsed with distilled water and then stained for 5 min with 2% aqueous uranyl acetate, dried, and viewed at 80 kV.

RESULTS

All the antibodies used immunostained NFT at the light-microscope level. In paraffin sections prepared for the light microscope, all Congo red-positive NFT (28) were recognized by both antisera to NF as well as by the monoclonal antibody (Fig. 1 *a* and *b*). This contrasted with the antiserum to MTP, which reacted with about one-half of the NFT (Fig. 1 *c* and *d*).

Observation of the Vibratome sections at the light-microscope level showed a strong reaction in neurons containing NFT with all of the antisera used (Fig. 2*a*). At the ultrastructural level, the immunostained NFT (Fig. 2 *b–e*) were unmistakable when compared with NFT not immunostained or immunostained using as primary antibody normal goat serum, preimmune mouse and rabbit sera, monoclonal antibodies to NF not reacting with NFT (11), and, in the case of antibodies to NF, antisera adsorbed with purified NF subunits (12) (Fig. 2*f*). All the antibodies shown reacted with the PHF and the granular material of the NFT (Fig. 2); whether other NFT components, such as the 15-nm filaments, were also immunostained could not be established. The helical structure of PHF with a typical periodicity at 60–80 nm was evident in NFT immunostained with each of the antibodies (Fig. 2 *d* and *e*). A 60 to 80-nm periodicity of staining intensity along the PHF occasionally was noted for all the antibodies; whether this is related to a periodic distribution of antigenic determinants or to the morphology of the PHF (3) remains to be determined. Immunostaining of PHF was very intense with the antiserum to MTP (Fig. 2*e*). The antiserum reacted in immunoblots with tubulin and some of the high molecular weight microtubule-associated proteins but not with NF (Fig. 3). In normal human and animal tissue it stained cilia, axons, and apical dendrites. Staining of NFT was abolished by adsorption with chicken MTP or heat-stable microtubule-associated proteins but persisted after adsorption with phosphocellulose-purified tubulin (Fig. 3). These results indicate that the antigen common to NFT and MTP recognized by this antiserum is not tubulin but rather a heat-stable microtubule-associated protein.

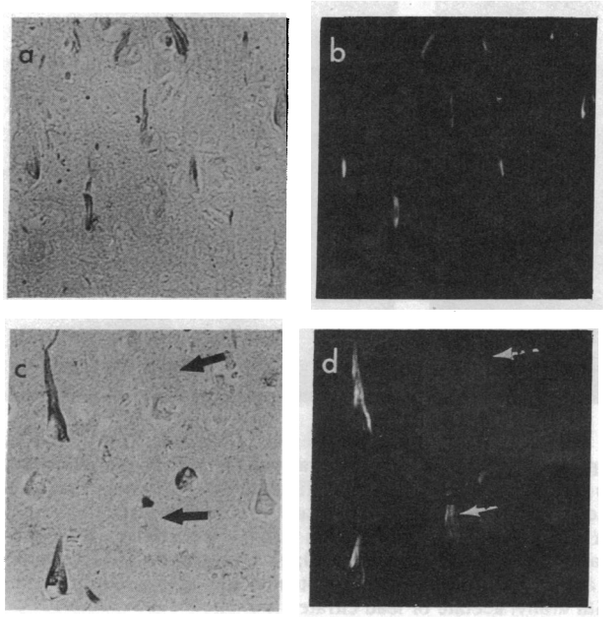

FIG. 1. Immunoperoxidase- and Congo red-stained section of hippocampus from a case of Alzheimer disease. In bright-field illumination (*a*), the monoclonal antibody to the 200-kDa NF subunit can be seen to immunostain essentially all Congo red-positive NFT (*b*) viewed in the same field with crossed polarizers. When antiserum to MTP is used, not all Congo red-positive NFT (*d*) are immunostained (*c*). Arrows indicate Congo red-positive NFT that were not immunostained. (×175.)

At the electron-microscope level, the Bodian's silver reaction resulted in a heavy decoration of PHF, of the granular components of the NFT (Fig. 4*a*), and of normal NF.

The sites responsible for binding the antibodies and Bodian's silver in NFT are not loosely attached to the PHF. After extraction with $NaDodSO_4$ for 18 hr, NFT reacted with each of the four antibodies and with Bodian's silver as seen at the light-microscope level. At the ultrastructural level, intense immunoreaction extensively covered the NFT filamentous components, some of which, however, still displayed a detectable periodicity (Fig. 4*b*). These filamentous components were definitely identified as PHF in negatively stained preparations at the electron microscope level. The preservation of the antigenic determinants recognized by the antibodies despite treatment with an ionic detergent was confirmed by using immunogold decoration of PHF isolated by $NaDodSO_4$ extraction. Such PHF showed helical periodicity as well as reactivity with all the antibodies (Fig. 5). The arrangement of the gold particles along the PHF appeared to be periodical; however, morphometric analyses are needed to confirm this observation.

DISCUSSION

The present results show that determinants present in NF and in heat-stable microtubule-associated proteins are integral components of PHF. The epitope in the 200-kDa NF subunit recognized by the monoclonal antibody used in this study as well as the silver-binding sites present in all NF subunits are located in the carboxyl-terminal tailpieces (29), a specific domain that distinguishes NF from other intermediate filaments (30). This highly charged domain, believed to mediate interaction of NF with other components of the neuronal cytoplasm, is therefore present in the PHF.

Previously, two arguments were used to question that the reaction of NFT with antibodies to NF occurred at the level of the PHF. The first, based on the finding that antibodies to NF no longer reacted with isolated NFT, was that the reaction occurred with NF undergoing transformation to PHF, whereas the latter were not recognized by antibodies to NF (18). The second was that the reactivity with NF was fortuitous, due to the occasional presence of normal NF

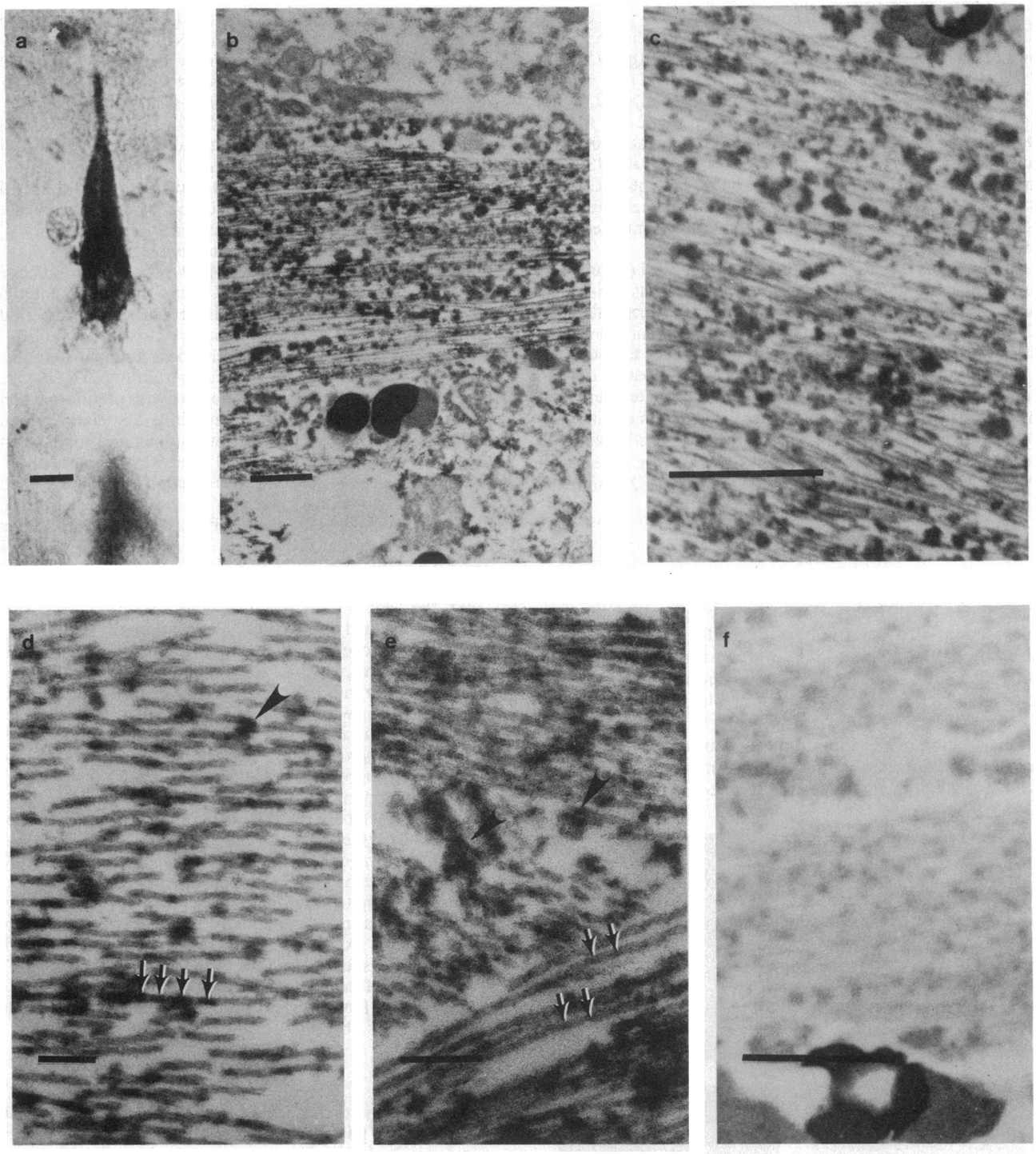

FIG. 2. Immunostaining of hippocampal sections from an Alzheimer disease patient with antibodies to NF and to MTP. (*a*) Intense immunostaining of a neuron containing a NFT seen at the light-microscope level in a 60-μm-thick Vibratome section. Mouse antiserum to NF was used at 1:500. (*b–f*) Electron micrographs showing that all the antibodies immunostain the filamentous as well as the granular components of the NFT. *b*, Monoclonal antibody to NF at 1:100; *c*, mouse antiserum to NF at 1:500; *d* and *e*, at higher magnification, most of the filamentous components of the NFT show the helical conformation of the paired helical filaments (arrows, periodical sites of intense immunoreaction occasionally seen; arrowhead, granular component; *d*, rabbit antiserum to NF at 1:600; *e*, antiserum to MTP at 1:100); *f*, adsorption of antisera abolished immunostaining (mouse antiserum to NF at 1:500 adsorbed with NF protein at 100 μg/ml). (Bars: a, 10 μm; *b*, *c*, and *f*, 1 μm; *d* and *e*, 0.2 μm.) None of the sections were stained with uranyl acetate or lead citrate.

within the NFT or to the polyspecificity of the antisera (14, 20, 21). The present findings rule out both these arguments.

The granular material containing antigenic determinants recognized by all our antibodies as well as the silver-binding sites remains to be identified. This material may be a postmortem degradation product since it has been described in ultrastructural studies of autopsy but not of biopsy tissue, where, instead, numerous polyribosomes have been found in association with PHF (5, 6). An interesting possibility is that the granular material represents degradation products of polyribosomes. Should this be the case, the reaction of this material with our antibodies may indicate a continuous

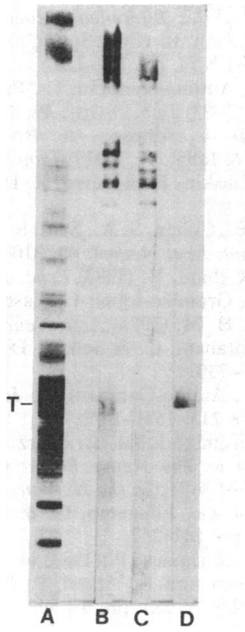

FIG. 3. Chicken MTP was electrophoresed on a 5–15% polyacrylamide gradient slab gel. Lanes: A, Coomassie blue stained; B–D, immunoblots with antiserum to chicken brain MTP, before (B) and after adsorption with tubulin purified by phosphocellulose chromatography (C) or with heat-stable microtubule-associated protein (D). Antiserum used in lanes B and C stained NFT in tissue sections while that used in lane D did not.

synthesis of PHF components by ribosomes located within the NFT.

The origin of the PHF remains a matter of hypothesis. Our current results as well as the results from another study (unpublished data) establish that PHF comprise sequences from NF and from microtubule-associated protein, as well as some sequences unique to PHF. Thus, PHF are a heterogeneous structure derived, at least in part, from components of the normal neuronal cytoskeleton. The presence of NF and microtubule-associated protein determinants in PHF suggests that the whole parent NF subunit and microtubule-associated protein are present. Our data, however, show that these components are present in an altered, insoluble form. It is difficult to envision how such diverse components may become insoluble and contribute to the formation of the PHF. Currently, the neuronal cytoskeleton is thought of as a complex three-dimensional meshwork where various types of sidearm connect NF with each other and with microtubules provided mainly by the tailpiece of the 200-kDa NF subunit and by microtubule-associated protein (31–35). The present finding that various NF determinants are an integral and consistent part of PHF as well as the observed continuity between PHF and 10-nm filaments (36, 37), probably NF, suggest that NF form the framework of the PHF. One may postulate that sidearms collapse and, as a result, the normal spacing between NF is lost, allowing two NF to wind around each other and generate the structure typical of PHF. This heterogeneous filamentous structure may then become insoluble because of progressive cross-link formation. The transformation to an insoluble form may change some of the antigenic determinants and give rise to new ones; as a result, PHF are recognized only by some antibodies to NF and microtubule-associated protein as well as by antibodies that do not recognize any normal neuronal component. This model would account for the variety of antigenic determinants, chemical characteristics, and structural complexity of the PHF.

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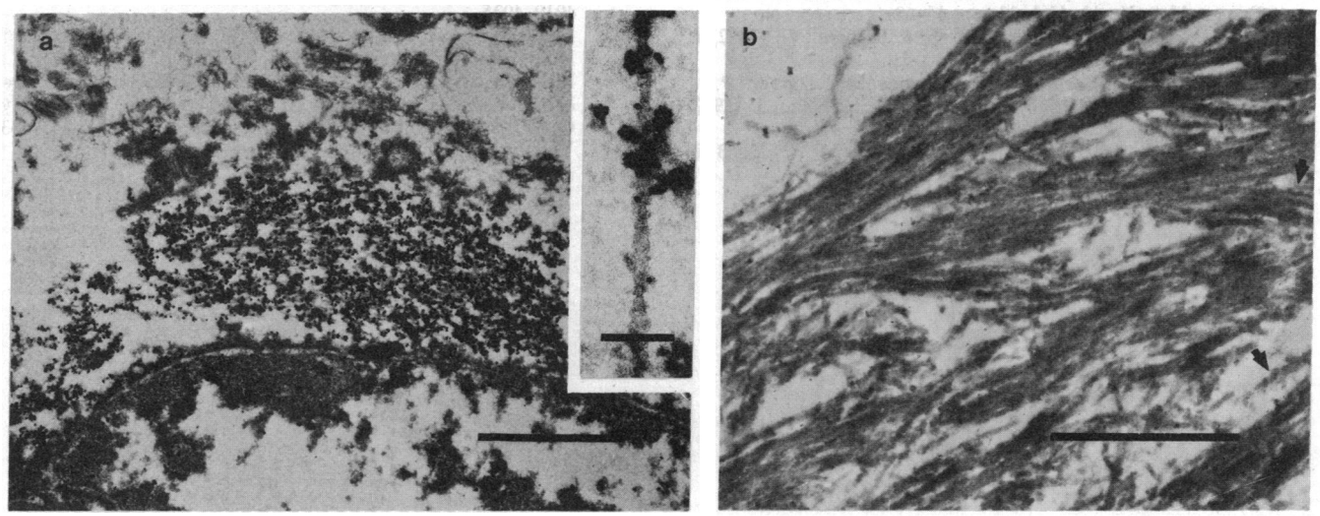

FIG. 4. (*a*) Electron micrograph of a NFT occupying part of the perinuclear cytoplasm and showing intense reaction with Bodian's silver stain. (Bar = 1 μm.) (*Inset*) A higher magnification showing the helical conformation of the PHF. (Thin section; uranyl acetate/lead citrate stain; bar = 0.1 μm.) (*b*) Immunoreaction enshrouding the filamentous components of NFT after extraction with 1% $NaDodSO_4$ for 18 hr. Arrows indicate an area displaying periodicity. Monoclonal antibody to NF was used at 1:100. (Section not stained with uranyl acetate or lead citrate; bar = 1 μm.)

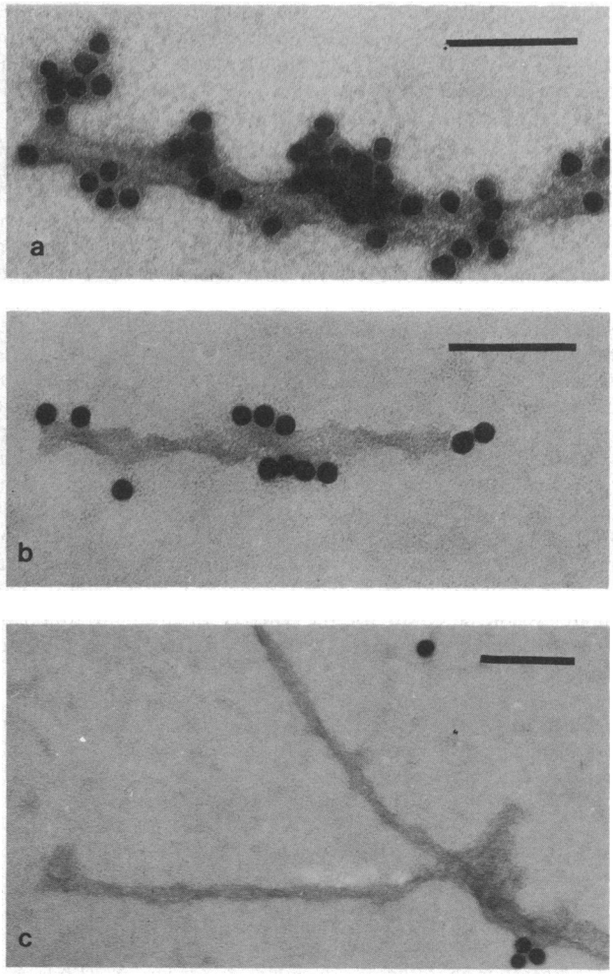

FIG. 5. Indirect immunogold decoration of $NaDodSO_4$-extracted PHF. Various primary antibodies were used. (*a*) Rabbit antiserum to NF. (*b*) Rabbit antiserum to MTP. (*c*) Preimmune rabbit serum. (Bar = 0.1 μm.)

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