Recognition of tau epitopes by anti-neurofilament antibodies that bind to Alzheimer neurofibrillary tangles

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ABSTRACT

Eleven anti-neurofilament (anti-NF) monoclonal antibodies were studied for their reactivity with heat-stable, microtubule-associated proteins and Alzheimer neurofibrillary tangles (ANT). On immunoblots of NF proteins, the antibodies recognized epitopes that were variably sensitive to Escherichia coli alkaline phosphatase. Eight of the antibodies showed reactivity with ANT and decreased binding to electroblotted NF after phosphatase treatment. The same eight antibodies reacted with tau proteins from bovine and rat brain; binding to tau proteins was also substantially reduced by phosphatase. Of the eight antibodies that bound to animal tau proteins, five also bound to tau proteins from normal human brain. All of the antibodies that bound to animal tau proteins stained ANT in frozen tissue sections. Brief treatment of tissue sections with trypsin in most cases enhanced antibody binding to ANT. All antibodies that lacked reactivity with tau proteins failed to bind ANT. Phosphatase treatment of Alzheimer tissue sections did not change the immunoreactivity of ANT and neurites in senile plaques with ANT-reactive, anti-NF antibodies, except for two antibodies that showed decreased binding to ANT. In contrast, axonal staining was decreased or eliminated by phosphatase treatment, similar to the response of electroblotted NF and tau proteins. These results suggest that (i) staining of ANT by anti-NF antibodies may be due to cross-reactivity of anti-NF with epitopes in tau proteins, (ii) the epitopes in axons, NF, and tau are sensitive to the effect of phosphatase, whereas the majority of those in ANT are not, and (iii) some of the epitopes in ANT that are shared with NF and tau proteins are not readily accessible to antibody binding.

Neurofibrillar tangles in Alzheimer disease (ANT) are relatively insoluble structures (1) composed of bundles of paired helical filaments (2). Immunocytochemical studies have demonstrated that ANT contain epitopes recognized by antibodies to neurofilament (NF) (3-9) and heat-stable microtubule-associated proteins: MAP2 (10, 11) and tau (12-17). These results suggest that several cytoskeletal components may be involved in the formation of ANT. However, recent evidence showing that different cytoskeletal proteins may share common epitopes (7, 18) has cast some doubt on the hypothesis that ANT are composed of a complex mixture of cytoskeletal elements.

All anti-tau antibodies so far tested, but only some antibodies to NF or MAP2, react with ANT. Moreover, immunization of animals with partially purified ANT produces anti-ANT antibodies that react with tau (12, 13, 17, 19, 20), rather than NF, suggesting the possibility that tau may be an important antigenic component of ANT (12-17). In this study we present evidence that anti-NF antibodies that bind to ANT share epitopes with tau proteins.

By using phosphatase treatment (21) in combination with immunohistochemical and immunocytochemical studies, we show that in NF and tau proteins the epitopes shared with ANT are phosphate-dependent. In ANT, the binding of anti-NF antibodies in most cases is not affected by phosphatase. This raises the possibility that apparent crossreactivity of anti-NF monoclonal antibodies with tau and ANT may be due to binding to phosphorylated epitopes in possibly unrelated phosphoproteins.

MATERIALS AND METHODS

Monoclonal Antibodies. Six anti-NF mouse antibodies, designated NP8, NP12, NP14, NP15, NP16, and NP18, were produced by hybridomas that have been recombined twice. The immunogen was brain homogenate from the basal nucleus of Meynert from patients with Alzheimer disease (22). Other anti-NF antibodies used included 07-5 (5, 6, 21) and SMI-32 (8) from Ludwig Sternerberger and Sternberger-Meyer, Inc., respectively; RT97 originally from John Wood (23); and two antibodies (BM160, BM200) purchased from Boehringer Mannheim. For comparison, an antibody to ANT (AB635) that recognizes tau epitopes (17, 19) and an antibody to bovine brain tau (Tau-1), generously provided by Lester Binder (24), were also used. Monoclonal supernatants were used undiluted or diluted 1:5 and ascitic fluid antibodies were diluted 1:100 to 1:2000.

Material. Calf brains were obtained fresh from the slaughterhouse. Human material from normal and Alzheimer disease patients was obtained at autopsy performed 5–8 hr postmortem.

Microtubules and Other Preparations. Microtubules (one cycle) were prepared from calf and rat brains using a modification (25) of the method of Shelanski et al. (26). This fraction was boiled for 5 min to produce a heat-stable microtubule fraction (27). Application of the method on human tissue did not yield an appreciable amount of microtubules. The pellet obtained after one cycle of assembly contained mostly NF, whereas the supernatant contained tau proteins. This supernatant (containing no glycerol) was boiled (27), dialyzed against polymerization buffer, concentrated on an Amicon apparatus, and applied to a phosphocellulose column (28). Fractions eluted with 0.8 M NaCl were tested on immunoblots with anti-tau antibody (Tau-1). The fractions reacting strongly with the antibody were pooled, dialyzed, and concentrated.

NF-enriched samples from calf brain or from normal human brain were obtained during preparation of microtubules. In the case of calf brain, a pellet fraction from the first cold cycle was used, whereas in human, a pellet from the first warm cycle was used. Protein content was determined by the method of Lowry et al. (29).

Gel Electrophoresis, Immunoblotting, and Phosphatase Treatment. Electrophoresis was performed on NaDodSO4/polyacrylamide gels (10%) using the system of Laemmli (30).

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Abbreviations: ANT, Alzheimer neurofibrillary tangle(s); MAP, microtubule-associated protein; NF, neurofilament(s); NF-H and NF-M, high and middle molecular weight NF triplet proteins.

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In the case of NF, Coomassie blue-stained protein bands of high and middle molecular weight triplet proteins (NF-H and NF-M) were dissected from gels, placed on a new gel, and subjected to a second electrophoresis. Proteins separated on gels were electrophoretically transferred to nitrocellulose paper (31). Immunoblotting was carried out by a procedure reported earlier (19) using 5% nonfat milk to block nonspecific binding. The bound immunoglobulins were detected with avidin-biotin peroxidase (Vector Laboratories, Burlingame, CA) and diaminobenzidine as chromogen. Some nitrocellulose strips containing electroblotted proteins were treated with 10–50 units of Escherichia coli alkaline phosphatase (Sigma; type III) per ml for 2 hr as before (7).

**Immunofluorescence and Immunoperoxidase.** Frozen sections of human brain (right hippocampus at the level of the lateral geniculate nucleus) were fixed in acetone for 10 min and incubated with antibodies at room temperature for 2 hr or 16–18 hr at 4°C. Bound antibodies were detected with rhodamine-conjugated anti-mouse immunoglobulin (Cappel Laboratories, Malvern, PA) at 1:100 (immunofluorescence) or either mouse monoclonal peroxidase-antiperoxidase reagent (Sternberger-Meyer, Jarrettsville, MD) or avidin-biotin peroxidase (Vector Laboratories) with diaminobenzidine as chromogen. In immunofluorescent studies the immunostained sections were counterstained with 0.01% thioflavine S, a reagent that binds to ANT and emits green fluorescence under ultraviolet illumination. Some sections, prior to incubation with antibodies, were treated with 0.5% trypsin (GIBCO) for 10 min or with phosphatase as for immunoblots.

**RESULTS**

The results are summarized in Table 1.

**Table 1. Reactivities of anti-NF antibodies with tau and ANT**

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**Immunoblots.** All 11 anti-NF antibodies in these studies reacted with either one or both high molecular weight NF proteins (NF-H and NF-M) of human origin (Fig. 1 and Fig. 2, lane e). The antibodies also recognized NF proteins from bovine and rat brain (data shown partially in Fig. 3). One of the anti-NF antibodies (NP18) has been characterized in a previous study and found to bind to NF-H and NF-M of human and animal origin (7). Treatment of electroblotted proteins with phosphatase prevented or significantly reduced binding of all antibodies to NF-H and NF-M, except for BM160 and SM132. The binding of BM160 to NF-M was not
significantly altered and the binding of SM132 to NF-H and NF-M was largely improved by phosphatase (Fig. 1B). NF proteins, regardless of phosphatase treatment, were in no case recognized by Tau-1 (16, 24) or Ab635 (17, 19), even at the loading of 76 μg of NF protein per lane (not shown).

The heat-stable microtubule fraction from bovine and rat brain contained MAP2, tau proteins, and a small amount of heat-stable NF proteins (mostly NF-M) and their fragments (Fig. 2, lanes b and c). On immunoblots, six of the anti-NF antibodies (NP8, NP14, NP15, NP18, 07-5, RT97) reacted strongly with proteins comigrating with bovine and rat tau proteins (Fig. 3A and B). NP12 and NP16 also stained tau, but less intensely. Other anti-NF antibodies (SM132, BM200, BM160) showed no reactivity with tau proteins. The proteins comigrating with tau were stained with two anti-tau antibodies (Tau-1 and Ab635), offering suggestive evidence that these proteins were indeed tau, rather than degradation products of NF.

The human tau preparation contained protein bands in the Mr 50,000–62,000 region and several polypeptides with Mr <50,000 (Fig. 2, lane d). All of the bands were recognized by Tau-1 and Ab635 (Fig. 3C). Therefore, we concluded that the bands below Mr 50,000 were most likely degradation products of Mr 50,000–62,000 tau proteins. Three anti-NF antibodies (NP14, 07-5, RT97) reacted with most of the tau bands, including the lower molecular weight bands; NP12 and NP15 reacted only with some of them (Fig. 3C). Human tau proteins were stained weakly, or not at all, by NP8, NP16, and NP18. Human tau proteins were not stained by any of the antibodies that also failed to recognize animal tau proteins (SM132, BM200, BM160).

Phosphatase treatment of electroblotted tau proteins from rat, bovine, and human brain resulted in decreased staining by all of the eight anti-NF antibodies that formerly bound to tau proteins (data not shown). This treatment did not affect immunoblots of the three anti-NF antibodies that did not react with tau.

**Immunofluorescence.** All anti-NF antibodies stained axons in white matter and gray matter neurites. Double-labeling studies with immunofluorescence and thioflavine S demonstrated that five anti-NF antibodies (NP8, NP14, NP18, 07-5, RT97) stained ANT in frozen sections (Fig. 4A and B). Among them the staining was most consistent and intense with two antibodies (07-5 and RT97). A small number of ANT were weakly stained by NP12, NP15, and NP16 (Fig. 4B). The immunofluorescent pattern of NP12, NP15, and NP16 was markedly changed by a brief preincubation of tissue sections with trypsin. After trypsinization, these antibodies showed bright staining of ANT (Fig. 4C). This treatment also enhanced the axonal staining and increased the number of ANT stained by NP8 and NP18. No appreciable staining of ANT could be detected with three other anti-NF antibodies (BM200, BM160, SM132). Trypsinization had no effect on these anti-NF antibodies.

**Immunoperoxidase.** The higher sensitivity of the immunoperoxidase method, in comparison to immunofluorescence, allowed detection of some ANT as stained by NP12, NP15, and NP16. Staining of ANT by anti-NF monoclonal antibodies was enhanced by pretreatment of frozen sections with trypsin, and results were similar to those with immunofluorescence. Two of the three antibodies that lacked reactivity with tau proteins or with ANT (BM200 and BM160) showed weak staining of neurites in some senile plaques that was not noted with immunofluorescence.

Phosphatase treatment eliminated axonal staining by six (NP8, NP14, NP16, NP18, 07-5, RT97) of the eight anti-NF antibodies that bound to ANT (Fig. 5B and D). Phosphatase had no effect on the staining of ANT (Fig. 5A and C), except for two antibodies, which either showed decreased staining (NP16) or no ANT staining (07-5). Phosphatase treatment did not affect axonal staining by NP12, NP15, SM132, and BM160 (data not shown).

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**Fig. 2.** Coomassie blue-stained 10% gel of different preparations from human and animal tissues. Lane a, microtubule fraction from bovine brain after one cycle of assembly; lane b, heat-stable fraction of microtubules from bovine brain, 20 μg of protein; lane c, heat-stable fraction of microtubules from rat brain, 9 μg of protein; lane d, heat-stable tau preparation from normal human brain, 60 μg of protein; lane e, NF preparation from the same normal human brain as in lane d; lane f, molecular weight standards (Sigma) shown as Mr x 10^3.

**Fig. 3.** Immunoblots of heat-stable fractions from different species with anti-NF antibodies. (A) Bovine brain. (B) Rat brain. (C) Normal human brain. The Coomassie blue stain of these fractions is shown in Fig. 2, lanes b, c, and d, respectively. The locations of NF-H and NF-M and the region of tau polypeptides or their fragments are indicated.
and axons stain proteins that do not bind to any of the human tau proteins on immunoblots. The other five antibodies recognize either some (NP12, NP15) or all tau proteins (NP14, 07-5, RT97). It is possible that certain (phosphorylated) epitopes in human tau are labile to postmortem effects since tau proteins obtained from bovine brain with an 8-hr postmortem interval failed to react with several of the anti-NF antibodies (unpublished observation). Alternatively, some epitopes may be only expressed in animal tau. This question may be resolved by obtaining tau proteins from human brains with shorter postmortem intervals.

The amount of protein necessary for detection of NF proteins by immunoblotting is barely visible on Coomassie blue-stained gels. By comparison of immunoblots it becomes apparent that more tau protein than NF has to be loaded on the gel to be detected by several anti-NF antibodies. In case of tau from animal sources, there is a 2-fold difference (on total protein basis). This is by no means overloading. In case of tau preparation from human, the difference is higher than that due to a presence of degradation products (presumably tau). Preliminary ELISA reveals an ~10-fold difference in detection of human NF and tau proteins by NP14, 07-5, and RT97. This may be due to a better preservation of epitopes in NF as compared to tau. In addition, the epitopes may be present in higher concentrations in NF than in tau.

Results of immunohistochemical and immunocytochemical studies with phosphatase treatment show that the anti-NF antibodies that bind to ANT react with phosphate-dependent epitopes in NF and tau proteins. Both proteins may exhibit several such epitopes since they are known to contain from

DISCUSSION

This study demonstrates crossreactivity of several anti-NF antibodies (NP8, NP12, NP14, NP15, NP16, NP18, 07-5, RT97) with tau proteins from bovine and rat brain. Anti-NF antibodies that recognize epitopes shared with animal tau proteins stain ANT in tissue sections. In contrast, anti-NF antibodies that do not crossreact with tau proteins (SM132, BM200, BM160) do not stain ANT. This difference is not due to variations in antibody titer since on immunoblots the staining of NF by these antibodies is similar. The results suggest that staining of ANT by the anti-NF antibodies may be due to crossreaction with tau epitopes.

This relation, however, is less certain when crossreactivity with human tau protein is considered. Tau preparations purified from adult human brain contain several polypeptides within the expected Mr of tau (50,000–62,000) (25, 32, 33) and a number of low molecular weight proteins, presumably degraded tau (13–15, 20). Three (NP8, NP16, NP18) of the eight anti-NF antibodies that react with ANT and animal tau do not bind to any of the human tau proteins on immunoblots. The other five antibodies recognize either some (NP12, NP15) or all tau proteins (NP14, 07-5, RT97). It is possible that certain (phosphorylated) epitopes in human tau are labile to postmortem effects since tau proteins obtained from bovine brain with an 8-hr postmortem interval failed to react with several of the anti-NF antibodies (unpublished observation). Alternatively, some epitopes may be only expressed in animal tau. This question may be resolved by obtaining tau proteins from human brains with shorter postmortem intervals.

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Results of immunohistochemical and immunocytochemical studies with phosphatase treatment show that the anti-NF antibodies that bind to ANT react with phosphate-dependent epitopes in NF and tau proteins. Both proteins may exhibit several such epitopes since they are known to contain from
2 (tau) to 23 (NF-H and NF-M) phosphate groups or more per polypeptide (25, 34–36).

In contrast to antibodies to phosphate-sensitive epitopes, anti-NF antibodies that recognize nonphosphorylated epitopes do not react with ANT or crossreact with tau protein. Although additional studies with more antibodies to nonphosphorylated epitopes in NF are needed, it nevertheless raises the possibility that crossreaction between NF, tau, and ANT may be due to sharing of phosphorylated epitopes.

One of the anti-NF antibodies in this study that reacts with a phosphate-dependent epitope shared by ANT and tau proteins (RT97) was shown previously to stain nuclei of cultured neurons and to react with histones (37), highly phosphorylated proteins. We have also noted that some other anti-NF antibodies (NP14, NP16) stain nuclei of neurons and glia in frozen sections after trypsin treatment (38), and the same phenomenon is seen with 07-5 (data not shown). Not all phosphorylated epitopes in NF proteins, however, are shared by tau and/or ANT. One of the antibodies in this study (BM200) to a phosphate-dependent epitope does not crossreact with tau protein or bind to ANT.

Abnormal phosphorylation of NF or accumulation of phosphorylated NF in neuronal cell bodies has been considered to be involved in the formation of ANT (5, 8). This was based on the staining of ANT with anti-NF antibodies that recognize phosphate-dependent epitopes that are normally restricted to axons. In considering the broad crossreactivity between different cytoskeletal proteins presented here and in previous studies (7, 18), this view apparently requires modification. It should be noted that in normal neurons tau epitopes are distributed in axons, whereas in ANT-containing neurons tau epitopes are found in cell bodies and in fine neuronal processes. This may suggest a failure of tau or tau-reactive epitopes to enter the axon.

The results of our studies support the idea that ANT contain phosphoproteins. Whether the phosphoproteins are actually tau and/or NF remains to be proven. It is interesting that most of the ANT epitopes recognized by anti-NF antibodies (NP8, NP14, NP18, RT97), unlike their counterparts in NF or tau proteins, are not sensitive to phosphate. Only two epitopes in ANT, recognized by 07-5 and NP16, are phosphate sensitive. A similar observation has been made with 07-5 (5). Thus, some phosphorylated epitopes in ANT behave similarly to their counterparts in normal cytoskeletal proteins, but others do not. The inability of phosphate to remove certain phosphate groups from ANT suggests that these phosphate-dependent epitopes in ANT may be packed (or distributed) differently than in the normal protein, limiting their access to phosphate, or they are parts of some unrelated phosphoproteins.

Immunocytochemical studies demonstrate that some ANT epitopes are exposed and accessible to antibody binding without any prior treatments, whereas others are not. Since brief trypsin treatment reveals these latter epitopes, it suggests that they may be protected or covered by proteinaceous material. Ultrastructural studies of ANT often show granular electron-dense material in addition to paired helical filaments. Whether this granular material might hinder the binding of certain antibodies or prevent action of phosphate requires further investigation. Alternatively, trypsin may cleave a critical hinge or loop region in a protein component of ANT and thus expose the epitope. Further studies of the structure of ANT are needed to determine the arrangement of phosphate-dependent tau and/or NF epitopes in ANT.

Note. Nukina et al. (39) have also reported on Alzheimer disease neurofibrillary tangles.