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Rapid Communication

β Protein Immunoreactivity Is Found in the Majority of Neurofibrillary Tangles of Alzheimer's Disease

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The presence of dystrophic neurites in most extracellular neurofibrillary tangles (E-NFT) suggests a factor promoting neurite growth in E-NFT. Although the β-protein detected in E-NFT may fill that role, reports that only 2–10% of E-NFT contain β-protein whereas 80–100% contain dystrophic neurites suggested that β-protein does not play an important role. In this study, the authors used two antisera and one monoclonal antibody to β-protein to establish the effects of tissue preparation and formic acid enhancement on the detection of β-protein in E-NFT. We found that β-protein epitopes in E-NFT are sensitive to formaldehyde fixation and are best enhanced by 50% formic acid, whereas β-protein in senile plaques is best enhanced at higher formic acid concentrations. After treatment with 50% formic acid, β-protein was found in all E-NFT. Interestingly, after treatment with 10% formic acid, half of intraneuronal-NFT (I-NFT) also contained β-protein immunoreactivity. The finding that β-protein immunoreactivity in senile plaques, E-NFT and I-NFT is increased at different formic acid concentrations suggests that β-protein in each location is in a different conformation. In contrast, no β-protein immunoreactivity could be found in E-NFT of the brain stem, an area in which dystrophic neurites do not infiltrate E-NFT. These findings indicate a correlation between neuritic infiltration and presence of β-protein in E-NFT and suggests the two are linked in Alzheimer's disease for E-NFT as well as senile plaques. (Am J Pathol 1992, 140:283–290)

The relationship of the major pathologic lesions of Alzheimer's disease (AD), neurofibrillary tangles (NFT) and senile plaques (SP) is unknown. In early chemical analyses of NFT fractions, the 42 amino-acid β-protein was detected and claimed to be the subunit of NFT filaments.[1] Since β-protein is the subunit of the amyloid fibers at the core of the SP,[2] these findings suggested that both NFT and SP are derived by aberrant processing of the same parent protein, the amyloid precursor protein (APP). Yet, the presence of β-protein seems more likely due to contamination of the NFT fraction with amyloid since the subunit of NFT filaments has been found to be the microtubule associated protein τ.[3] Nevertheless, β-protein immunoreactivity has been noted in a few percent of extracellular-NFT (E-NFT), NFT that remain after the death of the neuron.[4–10] Ultrastructurally, these E-NFT are composed of 20 nm filaments with a diffuse amorphous layer where β-protein epitopes were localized.[6] However, the relative infrequency of β-protein in E-NFT suggested that β-protein deposition in E-NFT is a secondary phenomenon.[7] An alternative interpretation is that β-protein exists in a different form in E-NFT than in SP and that other detection techniques have to be used. Support for the latter contention is that we found β-protein in a greater number of NFT in acetone-fixed cryosections than in the formaldehyde-fixed paraffin-embedded tissue samples from the same case (Siedlak and Perry, unpublished observation). The present study addresses the effect of tissue preparation on the prevalence of β-protein immunoreactivity in both E-NFT and intraneuronal-NFT (I-NFT). Our findings indicate that β-protein immunoreactivity can be found in essentially all E-NFT and most I-NFT.

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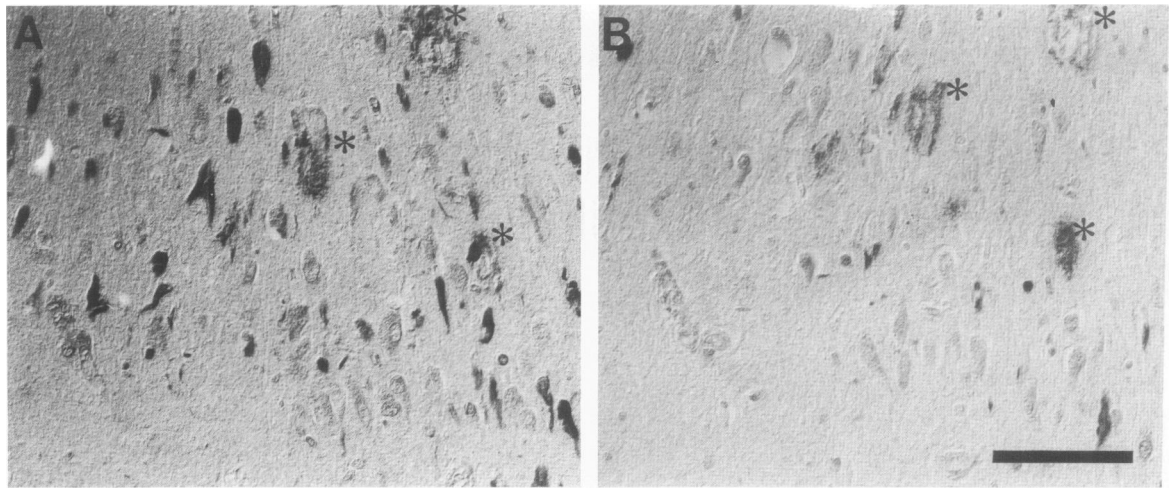

Figure 1. *Although many NFT are recognized by antibodies to β-protein (anti-β 1–42), in methacarn-fixed tissue* **(A)**, *postfixation with 3.7% formaldehyde* **(B)**, *greatly reduced NFT staining. In comparison, many SP were still recognized after formaldehyde fixation. All sections treated with 50% formic acid before immunostaining. Micrographs of fields defined by 3 SP(* to right) in adjacent sections. Scale bar = 100μm.*

Materials and Methods

Tissue

The hippocampus and adjacent temporal cortex or brain stem of seven AD cases were studied (ages 70, 72, 73, 82, 84, 85, 91 years). Clinical and pathologic diagnoses were based on established criteria.[11] Qualitatively identical results were obtained among all the cases, at least three cases, different for each parameter, were evaluated quantitatively for each of the parameters measured. Tissue taken at autopsy was fixed in methacarn (methanol 6: chloroform 3: acetic acid 1) for 16 hours before embedding in paraffin. Sections were cut at a thickness of 6μm. In some cases, hydrated tissue sections were postfixed with 3.7% formaldehyde in phosphate buffer for 30 minutes to assess the effects of formaldehyde fixation.

Immunoreagents

Three antibodies to synthetic peptides homologous to the β-protein were used: 1) rabbit antiserum to full length β-protein (anti-β 1–42) (1:100) (gift of Colin Masters);[12] 2) rabbit antiserum to the first 28 amino acids of β-protein (anti-β 1–28) (1:150) (gift of Blas Frangione);[13] 3) mouse monoclonal antibody to β-protein (4G8) (1:2000) whose epitope was mapped to β-protein sequences 17–24 (gift of K. S. Kim and Henryk Wisniewski).[14] Sections were pretreated with various concentrations of formic acid for 5 minutes before immunostaining. Absorption consisted of incubating the antibody with 1 mg/ml of a synthetic peptide homologous to β-protein sequences 1–42 (Peninsula Laboratories, Belmont, CA) at 4°C for 16 hours, followed by centrifugation and use of the supernatant for immunostaining. To assist in the interpretation of the staining pattern, on adjacent sections, we used probes specific for 1) E-NFT; basic fibroblast growth factor (bFGF)-binding which we have shown is a probe for heparan-sulfate proteoglycans in E-NFT;[15,16] 2) I-NFT; Alz-50, a monoclonal antibody to an epitope characteristic of a modified form of τ[3] which is present only in I-NFT[6,15] (gift of Peter Davies). APP immunoreactive neurites were localized with the monoclonal antibody 22C11 (1:250)[17] (Boehringer-Mannheim, Indianapolis, IN). The peroxidase-antiperoxidase method[18] with 33′ diaminobenzidine was used to visualize immunoreaction. Endogenous peroxidase activity was quenched by treating sections with 3% H_2O_2 in methanol. In double-stain experiments, involving antibodies to β-protein and APP, the additional antigen was localized by alkaline phosphatase-anti-alkaline phosphatase with fast blue as the substrate.[19]

Quantitation of Stained Structures

The number of E-NFT, I-NFT, and SP recognized by the various probes in adjacent serial sections, was deter-

Table 1. *Effect of Formaldehyde Fixation on β-protein Epitopes*

	Anti-β1-42		Anti-β1-28	
	NFT	SP	NFT	SP
Untreated	60	61	60	35
Formalin	7	52	3	20

Formaldehyde (3.7%) postfixation of methacarn-fixed hippocampal–entorhinal cortex tissue sections greatly reduced the number of NFT recognized by the antisera to β-protein while the number of SP recognized was less substantially reduced. Expressed as β-protein immunoreactive NFT or SP per mm^2, with regions evaluated for NFT and SP defined by different landmarks of serial sections taken from one patient. Pretreated with 50% formic acid.

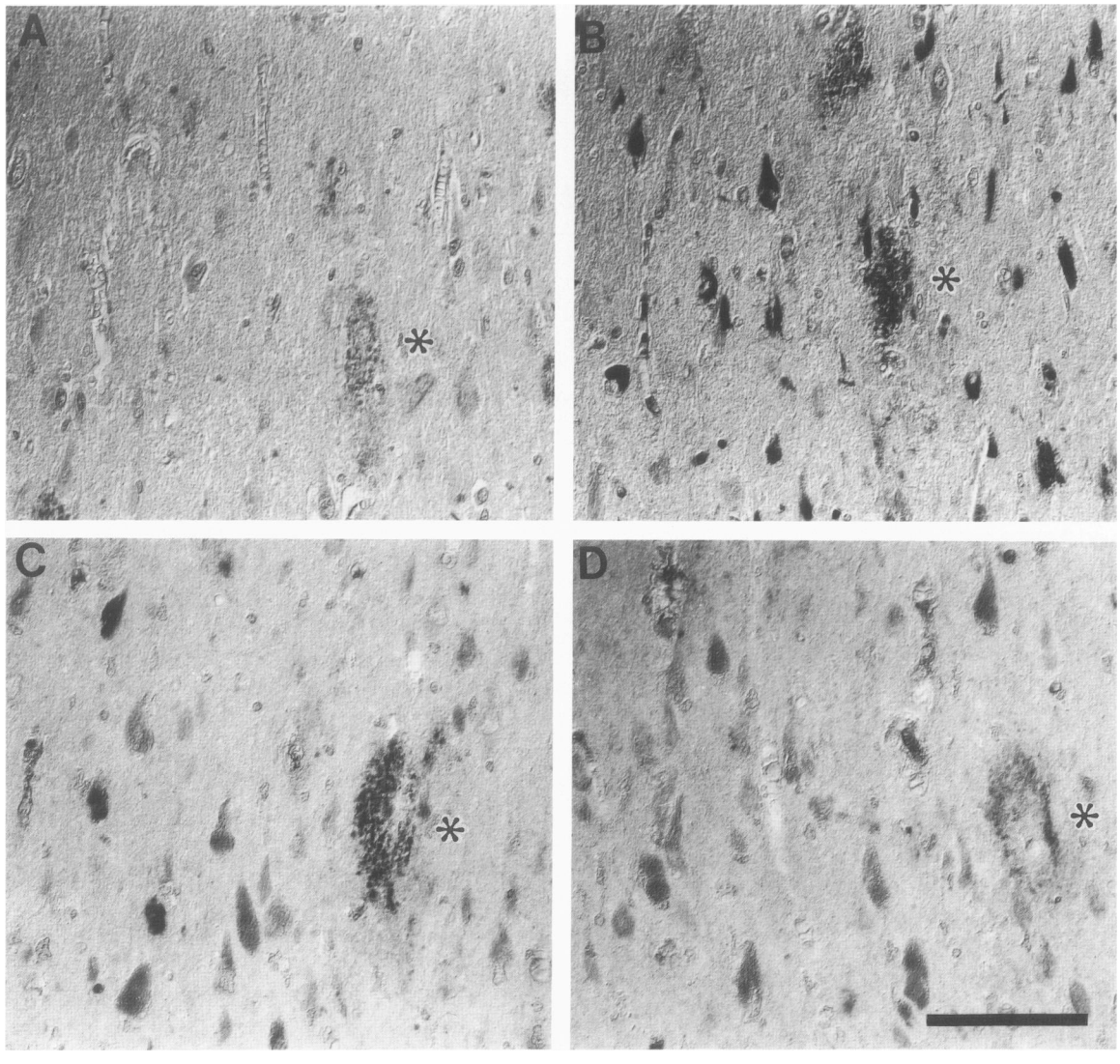

Figure 2. *The intensity of immunoreaction as well as type of NFT recognized by antibodies to β-protein (anti-β 1–42) is strongly dependent on the formic acid pretreatment. Although with no pretreatment* **(A)**, *no NFT were recognized, at 10%* **(B)**, *many I-NFT were recognized, note nuclei and compact NFT filaments. At 50% formic acid, both I-NFT and E-NFT were recognized whereas at 99% formic acid predominantly E-NFT were recognized, although less intensely than after 50% formic acid pretreatment. Micrographs of fields defined by an SP(* to right) in adjacent serial sections shown in order. Scale bar = 100μm.*

mined in five fields defined by landmarks of 0.199 mm^2 each (×250) of an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). Vessels and other landmarks were used to locate the same five fields in the subiculum within each group of adjacent sections. The fields evaluated for NFT and SP were nonoverlapping and were chosen in regions of maximum density for either lesion. In preliminary experiments, we stained a series of seven sections

Table 2. *Effect of Various Formic Acid Concentrations to Reveal β-protein Epitopes*

Formic acid	Anti-β1-42			Anti-β1-28			4G8		
	SP	E-NFT	I-NFT	SP	E-NFT	I-NFT	SP	E-NFT	I-NFT
99%	56	94	6	64	66	14	57	13	0
50%	76	97	15	45	67	41	49	11	3
10%	67	6	84	22	5	68	29	1	0
0%	46	0	9	26	0	13	15	0	0

Number of extracellular-NFT, intracellular-NFT, and SP recognized by three antibodies to β-protein in each adjacent serial section taken from hippocampal-entorhinal cortex tissue of a single patient fixed in methacarn and treated with various concentrations of formic acid for 5 minutes. The regions evaluated for NFT and SP were defined by different landmarks. Number per mm^2. Although the number of E-NFT recognized by the antibodies is similar after treatment with 50% or 99% formic acid, the intensity of the stain (Figure 2) is much greater after 50% formic acid treatment.

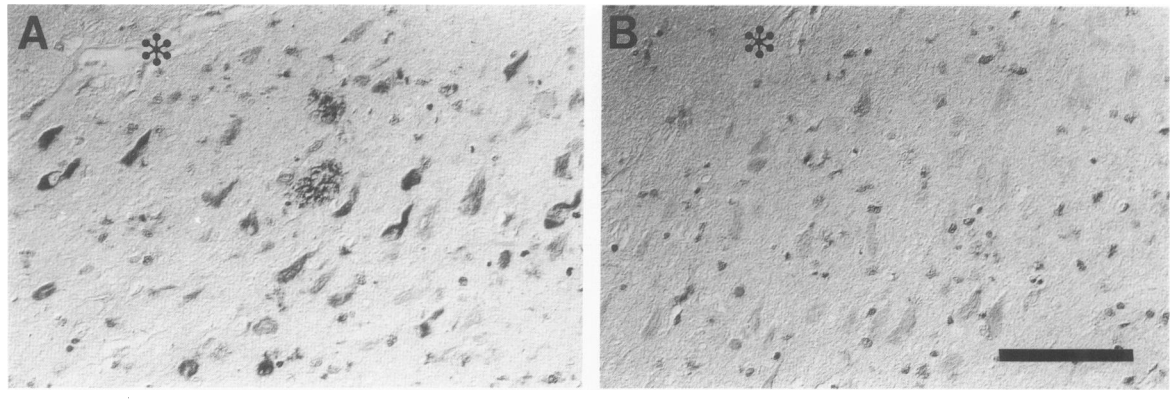

Figure 3. *The specificity of the antisera to β-protein for β-protein in NFT was determined by absorption. Although anti-β 1–42 recognized many NFT* **(A)**, *its absorption with 1 mg/ml of a peptide homologous to β-protein (1–42) blocked immunostaining of NFT as well as SP. Micrographs of fields defined by a large vessel(*) in adjacent sections. Scale bar = 100μm.*

with anti-β1–42 after 10% formic acid pretreatment, and found that the number of NFT obtained varied from section to section at most 11% from the mean. Differential interference contrast (Nomarski) was used to identify the cytoplasm and nucleus of I-NFT and disperse filaments of E-NFT.

Results

A large number of NFT were found to contain β-protein in paraffin-embedded sections of methacarn-fixed tissue. Formaldehyde fixation greatly reduced β-protein antigenicity in NFT while only reducing it slightly in SP (Figure 1, Table 1).

The number of NFT recognized by three antibodies to β-protein in methacarn-fixed tissue after treating with various concentrations of formic acid is shown in Figure 2 and Table 2. Although the number of E-NFT is similar after treatment with 99% or 50% formic acid, the NFT were more intensely stained after the 50% formic acid treatment (Figure 2), suggesting that concentrated formic acid may obliterate some β-protein epitopes in NFT. At 10–50% formic acid, many I-NFT were also recognized by the antibodies to β-protein. Similar results were obtained with the 2 antisera, whereas the monoclonal antibody recognized a lesser number of E-NFT and only few I-NFT. While most of the staining was of fibrillar structures in the E-NFT, the prominent granular stain of some may correspond to the localization of β-protein in amorphous material surrounding NFT filaments.[6]

Table 3. *β-protein Epitopes are Detectable in All E-NFT and Many I-NFT*

	Case 1		Case 2		Case 3	
	E-NFT	I-NFT	E-NFT	I-NFT	E-NFT	I-NFT
bFGF-binding	93	3	72	0	96	5
Anti-β1-42, 50% formic acid	79	12	76	29	92	11
Alz-50	2	58	3	98	2	73
Anti-β1-42, 10% formic acid	4	24	4	54	2	52

Comparison of number of E-NFT and I-NFT containing β-protein (anti-β1-42) immunoreactivity in each section of a series of adjacent serial sections taken from the hippocampus-entorhinal cortex of three patients with established markers for E-NFT (bFGF-binding) and I-NFT (Alz-50). Expressed as the number of E-NFT or I-NFT per mm^2 in the same area of adjacent sections. The data shows that the number of E-NFT binding bFGF and immunoreactive for β-protein is the same for this brain region. $P = 0.42$, Wilcoxon signed rank test.

The specificity of the two antisera to NFT was demonstrated by adsorption with synthetic β-protein (1–42) which greatly reduced anti-β 1–28 and blocked anti-β 1–42 staining of NFT and SP (Figure 3).

We compared the number of NFT recognized by antibodies to β-protein and a probe to E-NFT (bFGF-binding) or I-NFT (Alz-50) in adjacent serial sections (Table 3). After pretreatment with 50% formic acid, the number of E-NFT recognized by β-protein antisera ranged from 85–105% of the number of E-NFT that bound bFGF in the same area of an adjacent section.[15] We also counted the I-NFT that were β-protein immunoreactive after 10% formic acid. The greatest number of I-NFT were found β-protein immunoreactive after 10% formic acid with 41–71% of the number recognized by Alz-50 in an adjacent section (Table 3, Figure 4). Double stains with β-protein, Alz-50, and APP were also performed. We found that Alz-50 and β-protein immunoreactivity often colocalized to the same I-NFT although the overlap of the two colors made objective quantitative determination of the number of NFT recognized by each probe difficult (Figure 5A). In these double-stained preparations, APP-immunoreactive neurites in β-protein positive E-NFT was easily appreciated (Figure 5B).

In contrast to these findings made in the hippocampal region, in the brain stem region of the locus coeruleus and dorsal nucleus of raphae we found no β-protein immunoreactivity in E-NFT after pretreatment with formic

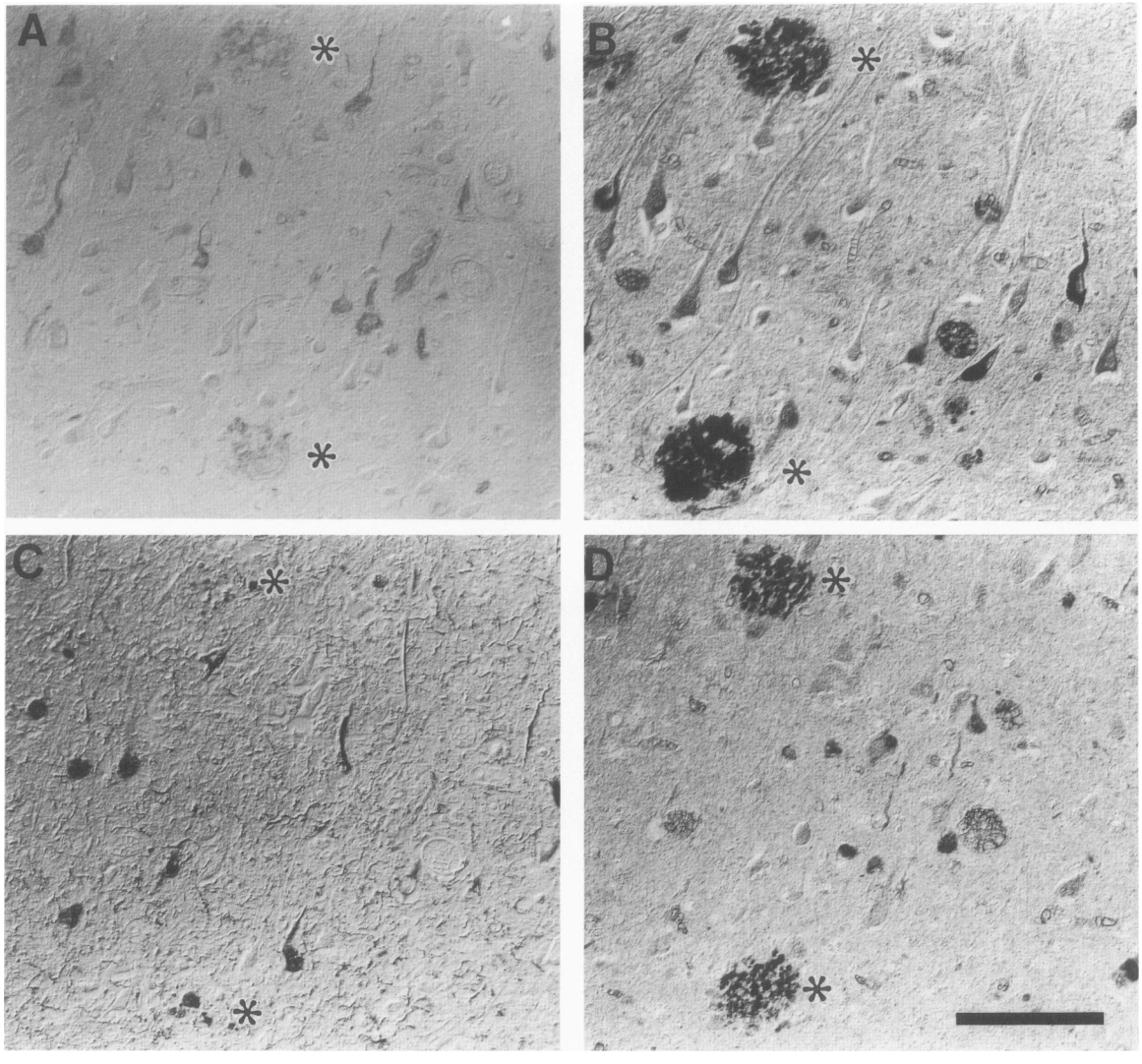

Figure 4. *Assessment of the proportion of E-NFT and I-NFT recognized after different formic acid pretreatments made by comparison to standards of bFGF-binding for E-NFT and Alz-50 immunoreactivity for I-NFT. Adjacent serial sections, shown in order, of a region defined by 2 SP(* to right) stained by each probe:* **(A)** *bFGF-binding;* **(B)** *anti-β 1–42 after 50% formic pretreatment;* **(C)** *Alz-50;* **(D)** *anti-β 1–42 after 10% formic acid pretreatment. Scale bar = 100μm.*

acid (10–99%) (Figure 6) whereas β-protein immunoreactivity was found in most I-NFT.

Discussion

This study demonstrates that β-protein immunoreactivity can be found in all E-NFT and some I-NFT. The probable reason β-protein was only reported in a small number of E-NFT in previous studies[4–10] is use of formaldehyde fixation and high concentrations of formic acid before immunostaining, both of which we found decreased β-protein immunoreactivity.

The association of β-protein with NFT may be indirectly mediated by heparan-sulfate proteoglycans (HSPGs) which are attached to NFT filaments.[15,16,20] APP, the precursor of β-protein, has been shown to bind to HSPG.[21] This APP may be processed *in situ* leaving insoluble β-protein on NFT filaments. Microglial and astrocytic infiltration of E-NFT could be critical for proteolytic processing of APP to β-protein.[22] After the neuron's death other features of the E-NFT may also be critical for β-protein deposition.

The differing formic acid pretreatment requirements for optimal detection of β-protein in E-NFT and I-NFT further suggests the β-protein epitopes and therefore the conformation or sequence differ in the two types of NFT. The β-protein staining of I-NFT may be due to binding of APP to NFT filaments consistent with previous reports[23,24] and this is possible since the 2 antisera used in our study recognize APP on immunoblots.[25] APP in I-NFT could be a source of β-protein in E-NFT but it alone may

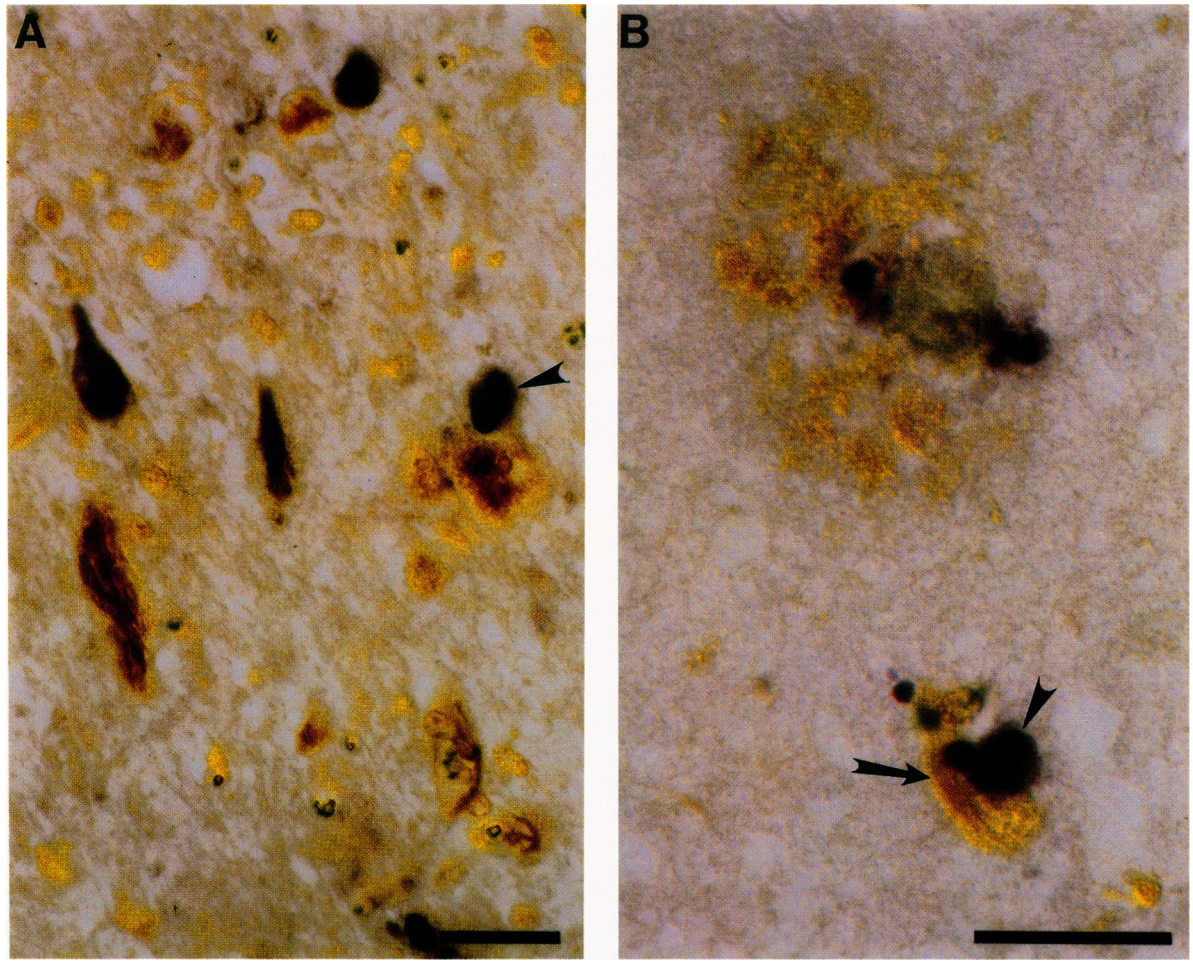

Figure 5. A: *β-protein (Anti-β1–42) immunoreactivity (brown), whereas seen alone in E-NFT, can also be seen in many I-NFT recognized by Alz-50 (blue), a marker of I-NFT. All but one (arrowhead) of the Alz-50 positive NFT shown also display β-protein immunoreactivity (brown-blue). It was usually difficult to objectively determine the presence of both markers in the same NFT, note the difficulty in seeing brown in the presence of blue.* B: *β-protein (Anti-β 1–42) immunoreactive E-NFT (brown), arrow, often contain APP-immunoreactive dystrophic neurites (blue), arrowhead, suggesting dystrophic neurites act as one source for β-protein in E-NFT. A similar pattern of APP-immunoreactive neurites in a β-protein deposit can be seen in an SP in the upper portion of the figure. Scale bars = 25μm.*

not be sufficient, since in neurons of the brain stem, β-protein immunoreactivity was detected in I-NFT but not in E-NFT.

In two recent studies,[26,27] dystrophic neurites were found in E-NFT, in one, 27–63% of E-NFT of the subiculum-entorhinal cortex were shown to contain APP-immunoreactive neuritic processes.[26] The present findings strengthen the connection between neuritic infiltration and presence of β-protein since the cortical E-NFT studied contain β-protein which has neurotrophic effects.[28] This, of course, does not rule out the contribution of other factors found in E-NFT and which promote neurite growth, e.g., bFGF,[15,16,29,30] in leading to neuritic infiltration. Although the initial deposition of β-protein on E-NFT may be from APP associated with I-NFT, dystrophic neurites containing APP may lead to subsequent β-protein deposition. Consistent with this interpretation, we could not find β-protein immunoreactivity in E-NFT of the locus coeruleus or dorsal nucleus of raphae, areas in which dystrophic neurites do not infiltrate E-NFT[26] even though I-NFT in these areas contain β-protein immunoreactivity. This may be because the β-protein immunoreactivity of I-NFT is soon removed after neuronal death

Table 4. *β-protein Epitopes are Not Detectable in E-NFT of the Brainstem*

	Case 1		Case 2	
	E-NFT	I-NFT	E-NFT	I-NFT
bFGF-binding	67	0	49	0
Anti-β1-42 50% formic acid	0	42	0	20
Alz-50	0	36	0	27
Anti-β1-42 10% formic acid	0	32	0	17

Comparison of the number of E-NFT and I-NFT containing β-protein (anti-β1-42) immunoreactivity in the entire dorsal nucleus of raphe in each section of a series of adjacent sections taken from two patients with established markers for E-NFT (bFGF-binding) and I-NFT (Alz-50). Expressed as the number of E-NFT and I-NFT in the same area of adjacent serial sections.

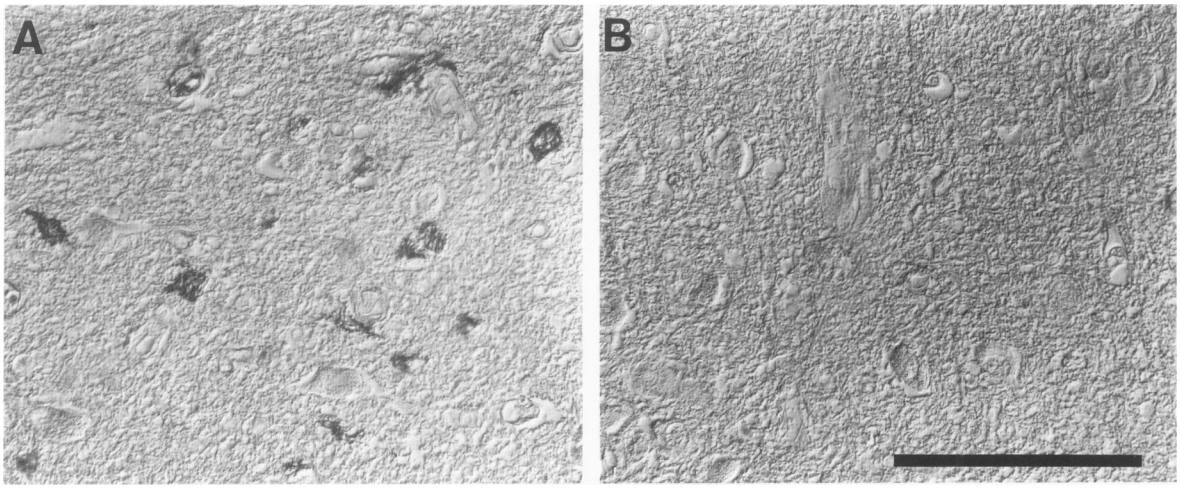

Figure 6. *Although many E-NFT could be seen in the dorsal nucleus of raphae, detected by b-FGF binding* **(A)**, *no β-protein (anti-β1–42) immunoreactivity was found in E-NFT in an adjacent section* **(B)**. *Scale bar = 100μm.*

and not replaced by β-protein derived from infiltrating neurites.

The mechanisms involved in remodeling E-NFT may be similar to those of the SP. First, E-NFT and SP contain dystrophic neurites, often with APP. Second, they are infiltrated by both astrocytes and microglia.[22] Third, they both contain many of the same associated proteins, e.g., α-1-antichymotrypsin, α-1-antitrypsin[3] and heparin sulfate proteoglycans. And finally, in this study we show the primary constituent of the SP core, β-protein, is found in each. Although SP and NFT are distinct structures, these parallels in the involved proteins and cellular elements suggest that the cellular and biochemical response to each may be similar.

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