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p3 β-Amyloid Peptide Has a Unique and Potentially Pathogenic Immunohistochemical Profile in Alzheimer's Disease Brain

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The presence of β-amyloid in brain tissue is characteristic of Alzheimer's disease (AD). A naturally occurring derivative of the β-amyloid peptide, p3, possesses all of the structural determinants required for fibril assembly and neurotoxicity. p3-specific antibodies were used to examine the distribution of this peptide in brain. p3 reactivity was absent or sparse in aged non-AD brains but was prevalent in selected areas of AD brain in diffuse deposits and in a subset of dystrophic neurites. p3-reactive dystrophic neurites were found both independent in the neuropil and associated with plaques. Little or no reactivity was observed to amyloid cores in classical plaques or to amyloid in the cerebral vasculature. The exclusive appearance of p3 reactivity in AD brain plus the selective localization of p3 reactivity to abnormal structures in the temporal lobe limbic system suggests that p3 may be a contributing factor to AD pathology. (Am J Pathol 1996, 149:585–596)

The deposition of β-amyloid is a central and early event in the pathogenesis of AD. Genetic evidence, both human[1,2] and transgenic,[3–5] supports this contention, as do histological studies of AD brain tissue primarily from individuals with Down's syndrome.[6–9] The production of β-amyloid has been shown to result from normal cellular metabolism,[10–12] and recently we have demonstrated that this peptide is a non-obligatory minor degradative by-product of β-amyloid precursor protein (β-APP) catabolism.[13,14] The enzymology of β-amyloid formation remains unclear except that two proteolytic processing steps are required to liberate β-amyloid from the backbone of β-APP. The proteinase(s) that generates the amino terminus of the peptide is referred to as β-secretase and that producing the carboxy terminus is referred to as γ-secretase.

Another β-APP processing activity exists, α-secretase. This single processing event releases the bulk of the precursor from its membrane-bound state such that it can be secreted into extracellular compartments.[15–17] The α-secretase cleavage occurs near the transmembrane domain of β-APP within the β-amyloid domain. The primary α-secretase site of proteolysis has been mapped between residues 16 and 17 of the amyloid domain,[18] although minor cleavages have also been observed adjacent to this major site.[13] A by-product of α-secretase action is an 8.0-kd fragment consisting of the carboxy-terminal portion of the β-amyloid domain followed by the cytoplasmic domain and carboxy terminus of β-APP. Although the α- and β-secretase cleavage steps originate independently, producing proteolytic fragments of 8.0 kd and larger amyloidogenic carboxy-terminal fragments, respectively, all of these fragments are substrates for γ-secretase[14] (Figure 1). As a consequence, an amino-terminally truncated 3-kd peptide, p3, is generated together with the complete β-amyloid peptide.[10,12,14,19] Both 4-kd β-amyloid and p3 peptides are secreted from cultured cells and both are identical from residues ~17 to 39 to 43.[10,12]

The biological and physical properties of β-amyloid have been under intensive investigation. Because the peptide can be chemically synthesized,

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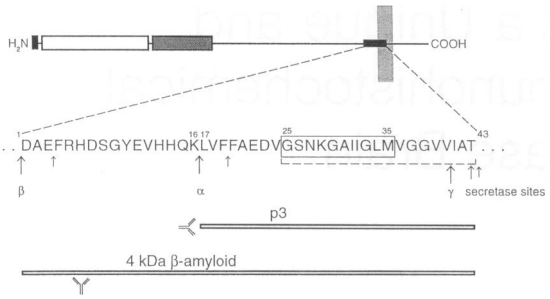

Figure 1. *Schematic representation of β-APP-derived β-amyloid and p3 peptides. The amino acid sequence of the β-amyloid domain is shown with α-, β-, and γ-secretase processing sites indicated by* **arrows**; *the* **large arrows** *represent major cleavage sites and the* **smaller arrows** *represent minor sites. The p3 peptide and β-amyloid peptides are denoted in conjunction with their sequences. A frequently used neurotoxic synthetic peptide is indicated by the boxed sequence (residues 25 to 35) and the amyloid fibril nucleation and assembly sequence (residues 25 is 43) is denoted by the* **dashed underline**. *The p3 and 4.1 antibody epitopes, for p3 and β-amyloid peptides, respectively, are each designated with antibody symbols. The epitope recognized by 4.1 MAb was described in Higgins et al.*[4]

many studies have focused on identifying the structural determinants required for self-aggregation and insolubility. Numerous investigations have demonstrated that a variety of different synthetic β-amyloid peptides can form fibrils *in vitro*.[20,21] Although both amino- and carboxy-terminal β-amyloid synthetic fragments have been shown capable of fibril formation,[22–28] other important features of amyloid were often absent such as insolubility and fibril stability. More detailed and rigorous analyses of subdomains within the β-amyloid sequence have subsequently identified the hydrophobic carboxy-terminal domain as the critical region for seeding amyloid aggregation,[29] for forming a β-sheet structure,[30,31] and for insolubility.[31] These physical features of amyloid can be recreated with synthetic peptide spanning amino acids 26 to 42[29,31] and with a minimal sequence encompassing residues 25 to 35.[21] It should be noted that these biophysical features are derived from *in vitro* analyses and the relationship of these parameters to those occurring *in vivo* has not been elucidated.

The β-amyloid peptide has also been shown to possess biological activity. Yankner et al[32] first identified the neurotoxic effects of synthetic β-amyloid peptides using cultured hippocampal rat neurons. A carboxy-terminal peptide fragment containing residues 25 to 35 was demonstrated to possess the toxic effects for neurons when applied at micromolar concentrations. Subsequent to this original study, the neurotoxic effects of β-amyloid have been extensively characterized *in vitro* and *in vivo*. It has been determined that the neurotoxic effect elicited *in vitro* was highly dependent on the aggregated fibrillar state of the amyloid peptide.[21,33–35] Other investigations have revealed that β-amyloid peptides (frequently β-amyloid 25 to 35) can induce neuronal apoptosis,[36,37] can increase the vulnerability of neurons to excitotoxic damage[38,39] and to injury by glucose deprivation,[40] and can participate in microglial activation resulting in secretion of neurotoxic substances.[41] These biological activities of the β-amyloid peptide have been interpreted to explain the neuronal dysfunction and loss observed in AD.

The p3 peptide possesses the sequences critical for peptide aggregation and toxicity to neurons; however, the potential pathogenicity of this naturally occurring β-amyloid derivative has not been addressed. To begin to understand whether or not p3 contributes to the disease process, we generated antibodies that selectively recognize p3 but not β-amyloid. These p3-specific antibodies were used to survey AD and age-matched control brain tissue using immunohistochemistry. We report that p3 immunoreactivity is associated with AD pathology and that the profile of stained structures is distinct from those reacting with β-amyloid antibodies.

Materials and Methods

Production and Characterization of p3 Antisera

Three rabbits were repeatedly immunized with a synthetic peptide containing residues 17 to 28 of the β-amyloid sequence. A cysteine residue was added to the carboxy terminus of the peptide to enable conjugation to keyhole limpet hemocyanin using *N*-maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzenesulfonic acid (Bachem California, Torrance, CA). Samples of sera were collected before peptide immunization and at 10-day intervals after each immunization. The sera were analyzed by enzyme-linked immunosorbent assay for reactivity with a set of synthetic peptides, including β-amyloid 1 to 28, 17 to 28, and 18 to 28. All peptides were prepared using an Applied Biosystems peptide synthesizer 430A (Foster City, CA), purified by high pressure liquid chromatography, and characterized using both amino acid composition and amino-terminal microsequence analyses. All peptides were solubilized in H_2O containing 20% isopropanol. Serial dilutions of each serum sample were made and reacted with 100 μl of a 1 μmol/L solution of each peptide. Bound antibody was detected with horseradish-peroxidase-conjugated anti-rabbit secondary antibody and the chromogenic substrate solution *O*-phenylenediamine and H_2O_2 in citric phosphate

buffer, pH 5. For immunohistological competition experiments, each peptide was incubated at a final concentration of 30 μmol/L with a 1:1000 dilution of p3 antibody 2 or 3 in phosphate-buffered saline containing 0.2% gelatin. After incubation for 60 minutes at 37°C and overnight at 4°C, the antibody-peptide solutions were used in the standard single-antibody immunohistological protocol using AD brain sections. Characterization of the p3 antibody against native β-amyloid peptides and β-APP carboxy-terminal fragments was made using immunoprecipitation. Human 293 cells, transformed to express β-APP695, were radiolabeled with [^{35}S]methionine/cysteine after which conditioned medium and lysates were prepared according to the methods described by Higaki et al.[14] Immunoprecipitation of β-amyloid peptides was made with a 1:50 dilution of p3 antiserum and a 1:50 dilution of an antiserum, BA2, raised to β-amyloid 1 to 40. Immunoprecipitation of β-APP carboxy-terminal fragments was made with a range of dilutions of p3 antisera from 1:10 to 1:1000, all with identical results, and with a standard 1:100 dilution of two β-APP carboxy-terminal antisera, BC1 and BC3. BA2 and BC1 antibodies have been described.[14] BC3 antiserum was generated to an identical peptide and in an equivalent manner as BC1. Polyacrylamide gel analysis of immunoprecipitates was performed as described.[14]

Origin and Preparation of Tissue Samples

Brains were obtained from clinically and neuropathologically diagnosed cases or from individuals free of neurological disease. Human tissue fixation (including methacarn treatment) and embedding was carried out as previously described.[42,43]

Immunohistochemistry

Immunohistochemistry with the p3 rabbit sera was performed with 1:1000 and 1:2000 dilutions of antibody. β-Amyloid staining was conducted with a 1:25 to a 1:100 dilution of the monoclonal antibody 4.1 MAb. Staining with antisera directed against the carboxy terminus of β-APP was conducted with a 1:1000 dilution of antisera and optimally with methacarn-fixed tissue. Formic acid treatment of tissue sections was made for all other immunostaining reactions. The single-primary-antibody immunocytochemistry procedure used for both rabbit and murine antibodies has been described.[4,43] Vectastain ABC Elite kits (Vector Laboratories, Burlingame, CA) designed for horseradish peroxidase detection of primary rabbit or mouse antibody reactivity were used as appropriate. Double-labeling reactions were performed as previously described[42] using both peroxidase antiperoxidase and avidin-biotin complex methods. The tau mouse monoclonal antibody (clone tau-1) was obtained from Boehringer Mannheim (Indianapolis, IN). Silver staining was performed according to standard methods. All stained sections were assessed by three investigators who were blinded to the diagnosis.

Method for Quantifying Plaques

Tissue blocks were chosen from the medial temporal lobe and from the neocortex. From each block, adjacent or closely spaced sections were immunostained with the 4.1 or the p3 reagents. On the temporal lobe sections, the amygdala was demarcated based on cytoarchitecture and contained portions of the lateral, lateral basal, medial basal, and cortical nuclei of the amygdala. Quantification was performed using a standard research microscope with a 9 × 9 ocular reticle grid at a magnification of ×100. Each grid covered a field on the section of 250 μm × 250 μm. Within the amygdala, all fields were examined and the number of stained plaques within each field counted. For the cortex, the same quantification system was used, except that sampling was performed at three different cortical locations on each slide. At each location, twelve 250-μm × 250-μm fields were examined, constituting a radial section through the six cortical layers. All plaques within each field were quantified. A total of 323 fields were examined on the p3-immunostained sections, and the corresponding 323 fields were examined on the MAb-4.1-immunostained sections by aligning the sections using anatomical landmarks such as vessels.

Results

Generation and Binding Specificity of p3 Antibodies

To begin to address the potential role of p3 in AD, we sought to generate specific antibodies that would be capable of differentiating this peptide from the 4-kd β-amyloid peptide. Rabbits were immunized with a synthetic peptide designed to mimic the free amino terminus of p3 produced by α-secretase cleavage. Sera from two rabbits displayed highly specific immunoreactivity for the synthetic peptide representing p3 (ie, residues 17 to 28) but little reactivity to a peptide representing β-amyloid, ie, residues 1 to 28

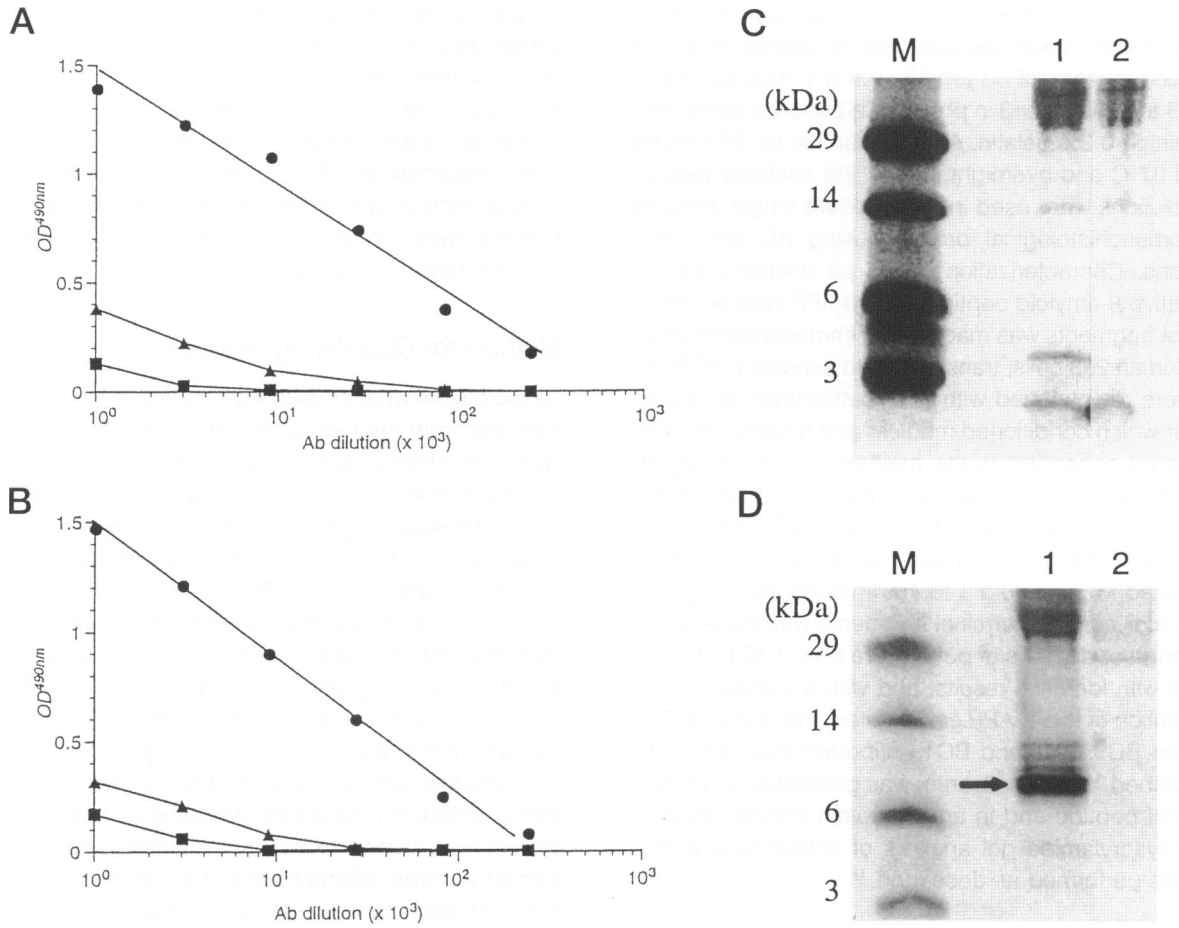

Figure 2. *Reactivity of p3 antisera to synthetic β-amyloid peptides.* A *and* B: *Binding of p3 antisera 2 and 3, respectively, to different β-amyloid synthetic peptides at increasing dilutions of antibody.* ●, *reactivity to a β-amyloid peptide spanning residues 17 to 28;* ■, *reactivity to a peptide spanning residues 18 to 28;* ▲, *reactivity to residues 1 to 28. Identical molar concentrations of each peptide were used for each assay point.* C: *p3 immunoprecipitation of [^{35}S]methionine-labeled 3-kd β-amyloid peptide from medium conditioned by cultured cells expressing both 4- and 3-kd peptides* (lane 2). *The control antibody, BA2* (lane 1), *recognizes the carboxy terminus and immunoprecipitates both peptides.* D: *Immunoprecipitation of [^{35}S]methionine-labeled cell lysates with a β-APP carboxy-terminal antibody, BC1* (lane 1) *and with a 1:50 dilution p3 antibody* (lane 2). *The* arrow *indicates the 8.0-kd carboxy-terminal fragment. Molecular weight protein markers are indicated.*

(or 1 to 40; data not shown; Figure 2, A and B). The specificity of the two sera for the amino terminus of p3 was further demonstrated by their lack of reactivity to a synthetic peptide that has a single residue deleted at the amino terminus of p3 (ie, residues 18 to 28; Figure 2, A and B). In addition, the preimmune sera from each rabbit had virtually no reactivity to any of the test peptides (data not shown). The specificity of the p3 antibodies were further demonstrated by their ability to only immunoprecipitate the 3-kd β-amyloid peptide from medium conditioned by cultured cells secreting both 4- and 3-kd peptides (Figure 2C). Based on these data, the antibodies appeared specific for the major form of p3 peptide. We therefore proceeded to examine brain tissue by immunohistochemistry using these antibodies. A schematic illustration of p3 reactivity, as well as the β-amyloid monoclonal antibody used for comparison in this study, 4.1 MAb, is presented in Figure 1. The 4.1 MAb has been previously reported to be highly specific for β-amyloid when used for immunohistology and reacts with a complete spectrum of β-amyloid-containing structures.[4,42,43]

p3 Reactivity Associated with AD Pathology and Distinct from β-Amyloid Reactivity

A brief survey of several AD brain tissue samples indicated that the two p3 antisera produced identical staining patterns. Consequently, we elected to conduct our larger analysis of brain p3 immunoreactivity with only one of the antibodies (antibody 3). The preimmune serum, employed at equivalent dilutions, was evaluated on AD tissue from 9 cases. Preimmune serum staining was essentially negative ex-

Table 1. *Quantification of Different p3 Immunoreactive Structures*

	Case 94R7, number of stained structures		Case 94R13, number of stained structures	
	Amygdala	Cortex	Amygdala	Cortex
p3				
Diffuse	20	43	50	34
Diffuse/granular	32	7	53	3
Neurites associated with plaques	20	2	16	0
Dense cores	4	0	1	0
Plaque halo	13	4	5	0
Total	89	50	125	37
MAb 4.1 Total	110	527	225	336

p3 and 4.1 MAb staining was performed on two different AD cases and for two anatomical regions, the amygdala and neocortex. Specific p3 immunoreactive structures were quantified as detailed in Materials and Methods.

cept for obvious corpora amylacea immunoreactivity. Nonspecific staining of corpora amylacea was not unexpected as such staining has been found to occur frequently using random sera (our unpublished observations). Tissue sections from the temporal lobe including the amygdala, hippocampus, and parahippocampal gyrus from 7 control aged brains (mean age, 76 years) obtained from individuals with no neurological disease, with incidental Lewy body disease, multiple sclerosis, and Parkinson's disease were surveyed for p3 immunoreactivity. Sections from the same anatomical area from 23 AD brains were also characterized (mean age, 77 years). Neocortical sections were also examined in 12 AD cases. Both control and AD cases had complete neuropathological evaluations. In addition, multiple sections from several different anatomical brain regions were evaluated for 6 of the AD cases.

From this survey, we found that p3-immunoreactive structures were present only in brains showing amyloid plaque formation as revealed by immunoreactivity with the β-amyloid antibody 4.1 MAb or by a modified Bielschowsky silver staining method. This included all AD cases and two cases diagnosed with Parkinson's disease that also had plaques. Figure 3 shows sections from the hippocampi of two AD cases analyzed in this fashion. Classical plaques of β-amyloid were revealed with the 4.1 MAb and silver staining (Figure 3, A and D). p3 immunoreactivity on adjacent sections showed a different staining pattern from that obtained for plaques. Two types of p3 reactivity were frequently observed (Figure 3). The p3 antibody was seen to stain structures resembling dystrophic neurites as displayed in Figure 3, B and C, or diffuse extracellular deposits as displayed in Figure 3, E and F. Immunoreactivity to these three common structures could be competed with prior incubation of the p3 antiserum with 17 to 28 synthetic peptide, but not with 1 to 28 peptide (data not shown). Quantification of the type of p3 structures stained was made for two AD cases (Table 1). In the neocortex, diffuse deposits constituted the majority of the p3-reactive structures in both cases (86 and 92%). In the amygdala, p3 diffuse deposits were in lower proportions (22 and 40%) whereas diffuse granular deposits and plaque-associated structures were the predominating stained entities. Diffuse granular deposits were defined as unorganized punctate material in contrast to diffuse deposits, which had a light dusting appearance. Both structures have a circular morphology.

In general, p3 and β-amyloid immunoreactivities were not coincident. This is particularly evident in Figure 3, D and E, where the clustered pattern of silver-stained plaques in the center of the panel is absent in the p3-stained section. Rather than colocalize with β-amyloid, the majority of p3 staining appears independent of plaques and can be seen to surround plaque-dense regions. Only one case was found in which both the p3 antibody and β-amyloid 4.1 MAb similarly stained mature plaque cores. This case was unusual in that it had a preponderance of dense core plaques in which the cores were extremely large. Nevertheless, only a very minor subset of these plaques displayed p3 reactivity as compared with many plaques that were 4.1 MAb positive but p3 negative. It should be noted, however, that faint dusting of the plaque corona was also seen in this case, similar to that in Figure 3C. From two cases more typical of AD analyzed in Table 1, only 5 plaques with dense cores were stained with p3 antiserum in the 323 fields quantitated. When examined with 4.1 MAb, these 323 fields contained 911 immunoreactive structures, many of which were mature cored plaques.

Double-staining reactions with p3 and with 4.1 antibodies further illustrated the non-overlapping distribution of the p3 and β-amyloid peptides (Figure 4, A and B). Typically, the 4.1 MAb reactivity revealed a typical array of β-amyloid deposits

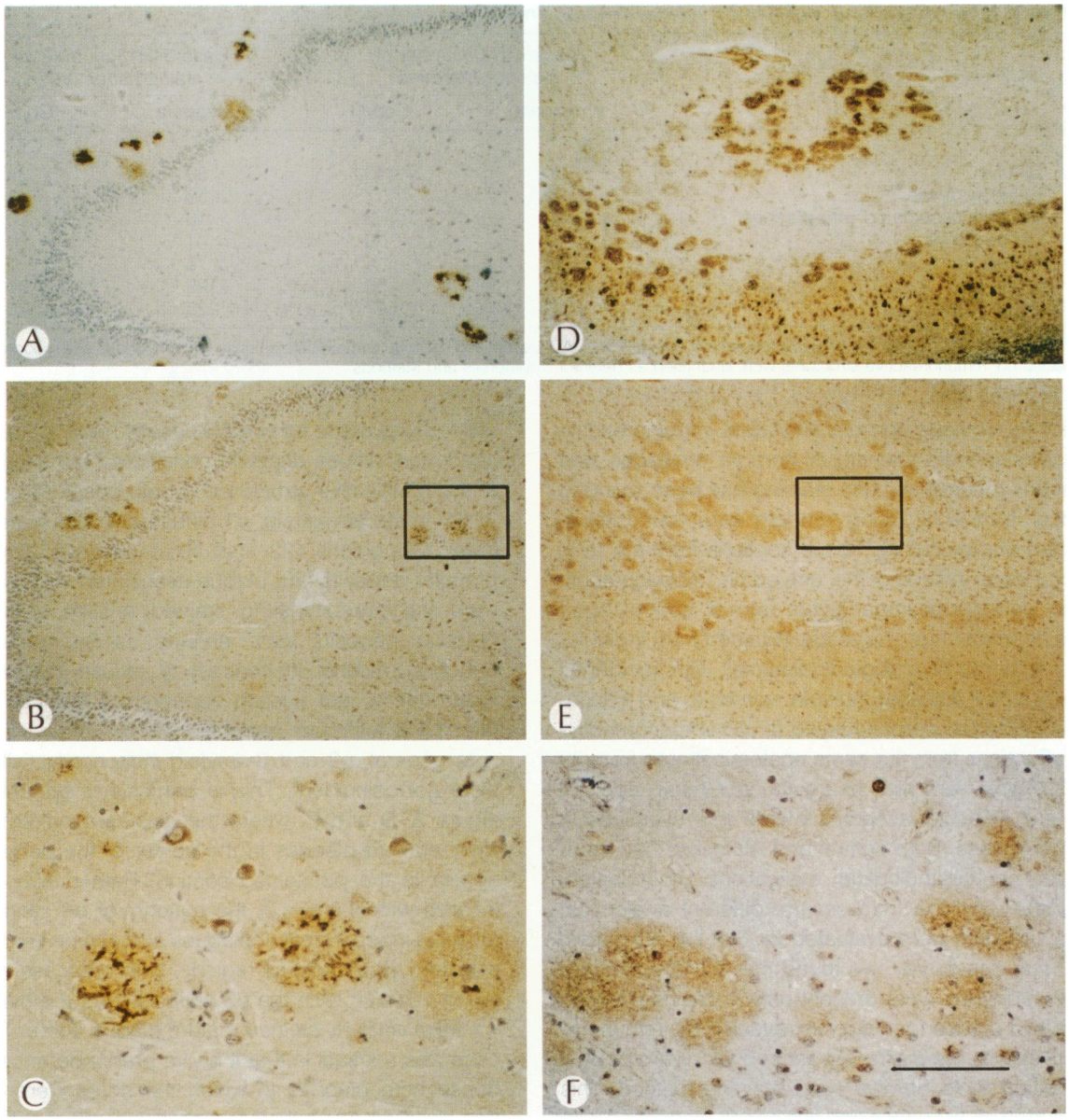

Figure 3. *p3 immunoreactivity in AD hippocampi.* **A:** *β-Amyloid staining using 4.1 MAb.* **B** *and* **C:** *p3 staining of an adjacent section. The boxed region of* **B** *is shown at greater magnification in* **C** *to reveal p3-positive neuritic structures associated with plaques.* **D:** *Modified Bielschowsky silver staining of plaques and tangles.* **E** *and* **F:** *p3 staining of an adjacent section.* **F** *is a greater magnification of the boxed area in* **E.** **A** *to* **C** *are from AD case 94A35, and* **D** *and* **E** *are from AD case 94A79. Scale bars, 150 μm* (**A, B, D,** *and* **E**) *and 30 μm* (**C** *and* **F**).

ranging from diffuse to mature plaques with cores and halos. The p3 reactivity illustrated in Figure 4 showed staining of dystrophic neurites and also vesicular structures scattered throughout the neuropil. These p3-reactive vesicular entities were seen in many of the AD brains examined. A second double-staining experiment was conducted employing p3 and tau protein antibodies to further explore the neuritic structures that were observed to be associated with the amyloid plaques. We found that p3 and tau appeared to be co-localized with some plaque-associated dystrophic neurites (not shown); with others, tau and p3 were independent (Figure 4C). Neurofibrillary tangles were also revealed with the tau antibody, which was distinct from p3 immunoreactivity (Figure 4D).

p3 and β-APP Carboxy-Terminal Domain Antisera React with Different Structures

The p3 peptide derives from two proteolytic processing steps, α-secretase cleavage, which occurs first, followed by γ-secretase cleavage. Hence, an 8.0-kd

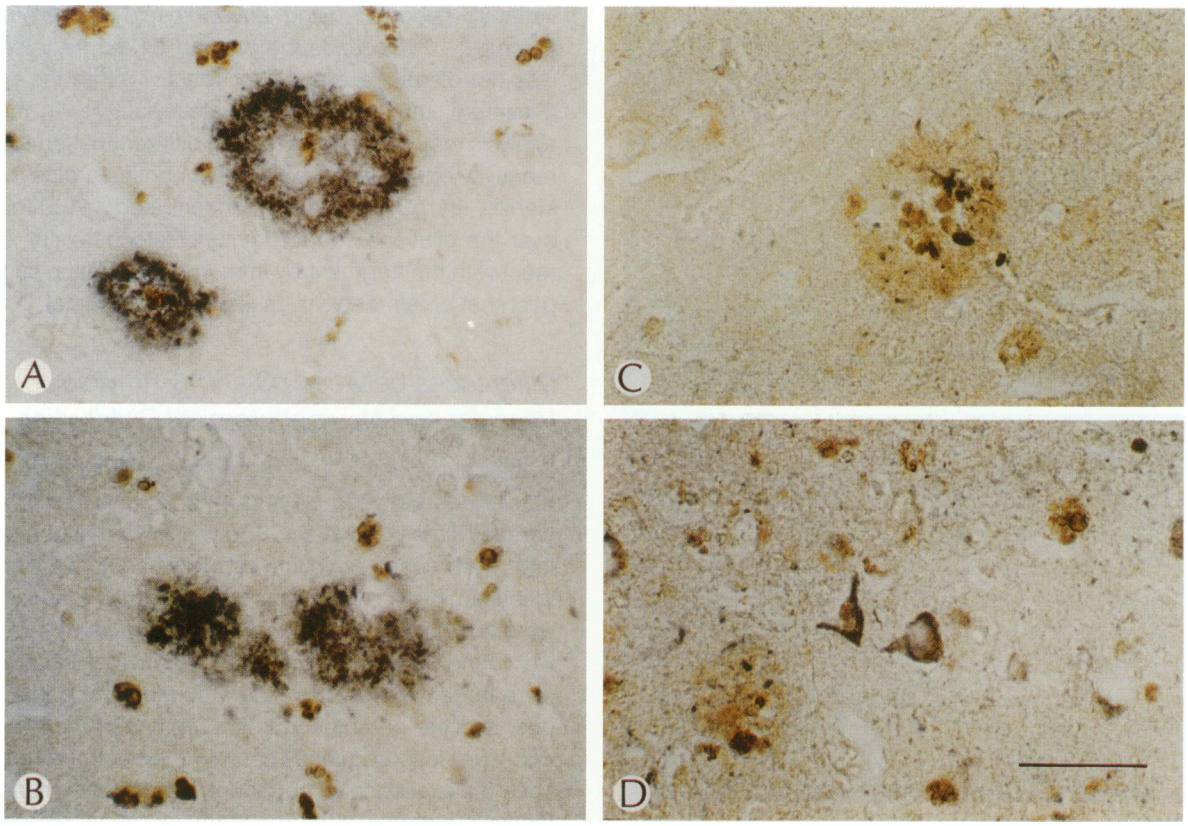

Figure 4. *Double-staining reactions for p3 and β-amyloid and p3 and tau.* **A** *and* **B:** *p3 immunoreactivity visualized with the brown chromogen identifies dystrophic neurites in the presence of 4.1 MAb immunoreactivity of β-amyloid plaque amyloid visualized with the purple chromogen.* **C** *and* **D:** *p3 immunoreactivity of dystrophic neurites by the brown chromogen in combination with tau immunoreactivity visualized with the purple chromogen. All sections were derived from AD case 88A88. Scale bar, 15 μm.*

carboxy-terminal fragment of β-APP, which results from only α-secretase processing and which is the immediate precursor of p3,[14] bears an identical amino terminus as p3. The p3 antisera used in this study were generated to the amino terminus of p3 and, therefore, could potentially recognize the 8.0-kd carboxy-terminal fragment as well as p3. We evaluated this possibility by two independent methods, immunoprecipitation and immunohistology. In Figure 2D, p3 and a β-APP carboxy-terminal antiserum were assayed for their ability to immunoprecipitate the 8.0-kd α-secretase fragment from mammalian cell lysates. Only the antiserum BC1, raised to the carboxy terminus of β-APP, was found to precipitate the 8.0-kd fragment. The p3 antisera, tested across a broad range of dilutions, were not able to react with the α-secretase fragment. For immunohistological analysis, BC1 and a second antiserum raised to the carboxy-terminal domain of β-APP were used to compare p3 reactivity in AD brain tissue. Ten additional AD cases were examined yielding similar results with each β-APP carboxy-terminal antiserum.

We found the pattern of p3 and carboxy-terminal staining to be different. This is evident in Figure 5, which shows three AD cases stained with both antibodies. The carboxy-terminal antibodies reacted with a variety of structures that were not stained by p3 antibodies such as cell bodies, neurofibrillary

Table 2. *Quantitative Analysis of p3 Immunoreactivity in the Amygdala* Versus *the Neocortex*

Region	Case 94R7			Case 9AR13		
	Number of structures stained		MAb 4.1/p3 ratio	Number of structures stained		MAb 4.1/p3 ratio
	p3	MAb 4.1		p3	MAb 4.1	
Amygdala	484	646	1.3	194	420	2.1
Cortex	517	4,715	9.4	322	3,004	9.3

p3 and 4.1 MAb staining was performed and the total number of immunoreactive structures was quantitated for two AD cases and for two brain regions, the amygdala and cortex.

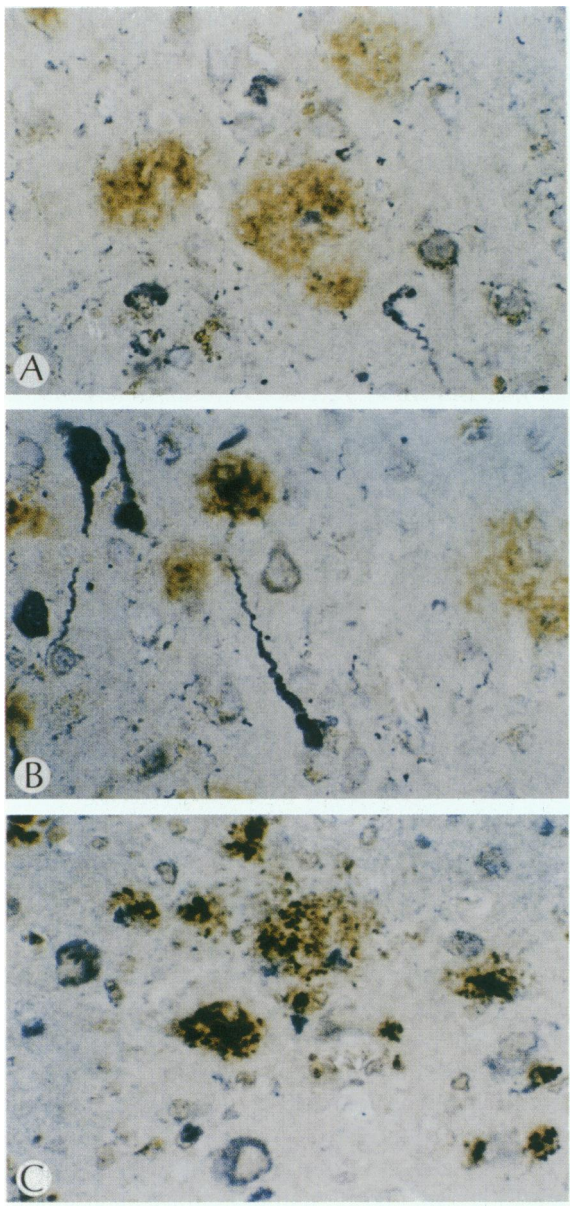

Figure 5. *Double-staining reactions for p3 and β-APP carboxy-terminal domain. Immunoreactivity to the carboxy-terminal domain of β-APP was visualized with the purple chromogen after staining with BC1 antiserum. Immunoreactivity to p3 was visualized using a brown chromogen identifying a different pattern of stained structures than that obtained with BC1. Each panel was a different AD case* (**A**, *C34C1;* **B**, *C24C3;* **C**, *C26B3*). *Sections in* **A** *and* **B** *were from neocortical tissue, and* **C** *was from the amygdala. Scale bar, 15 μm.*

tangles, and neuropil threads. Another difference was in the absence of carboxy-terminal staining of diffuse and granular deposits that were detected by p3 antibodies. Both antibodies stained dystrophic neurites. Typically, we found that p3 and BC1 staining were not coincident, although they may co-localize in a subset of dystrophic neurites (Figure 5, A and C). The structures recognized by the β-APP carboxy-terminal antisera agree with those previously and variously reported using antibodies to this β-APP domain.[44–48] Because the purified 8.0-kd fragment was not directly assessed for reactivity with the p3 antiserum, the possibility of cross-reactivity in this analysis may exist. However, the different patterns of reactivity observed for p3 and BC1 antisera plus the inability of p3 antiserum, concentrated >100-fold over the dilution used for immunohistochemistry, to react with the fragment by immunoprecipitation suggest that cross-reactivity is likely to be minimal.

Differences between p3 and β-Amyloid Vascular and Neuroanatomical Distribution

In the course of this analysis we noted other distinct differences between p3 and 4-kd β-amyloid immunoreactivities in AD brain. Specifically, we observed that, in AD cases exhibiting cerebrovascular amyloid identified by 4.1 MAb reactivity, vascular deposits were not stained by the p3 antibody (Figure 6). Also, we found that the p3-immunoreactive structures were largely observed in the amygdala, hippocampus, and the parahippocampal regions of the brain and were less frequently seen in the neocortex despite the presence of abundant β-amyloid-immunoreactive plaques (Figure 6). (Other brain regions were not surveyed for p3 reactivity.) Furthermore, as noted in Table 1, p3 immunohistochemistry highlighted deposits considered less developed in the evolution of neuritic plaques in the cortex than in the amygdala and hippocampus. A quantitative analysis of two AD cases in Table 2 revealed close ratios (1.3 and 2.1) of β-amyloid plaques to p3-stained structures in the amygdala, in contrast to the neocortex where nearly a 10-fold difference (9.4 and 9.3) was seen between the frequency of β-amyloid plaques and p3-reactive structures. The paucity of p3 structures in the cortex compared with the amygdala is also noted in Table 1. These observations further emphasize the different profiles of p3 and β-amyloid peptide distribution in AD brain.

Discussion

Two β-amyloid peptides are naturally produced by mammalian cell processing, the 4-kd β-amyloid peptide and its 3-kd truncated derivative p3. Although the amino termini of these two peptides originate through apparently different biological events, produced by either β- or α-secretase action, they are ultimately co-processed by γ-secretase and co-secreted from the cell.[14,19] The common γ-secretase proteolytic processing consequently produces two

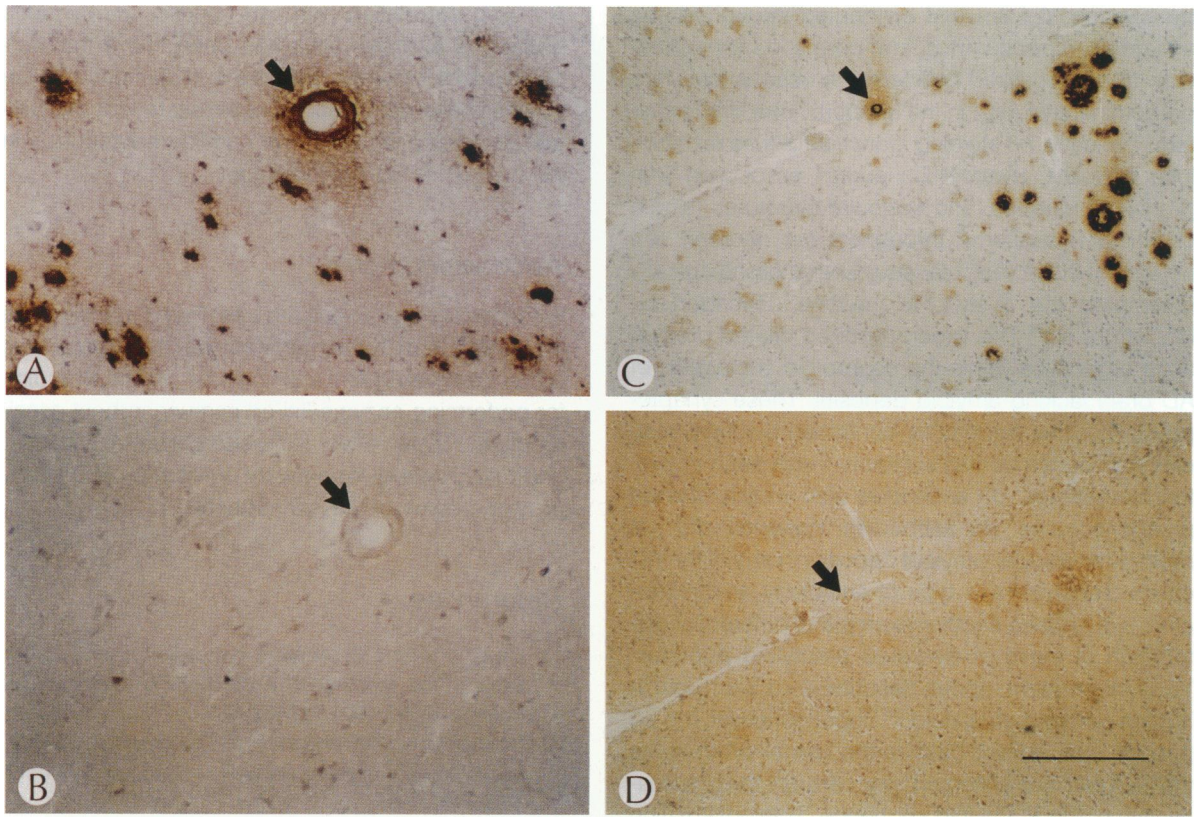

Figure 6. *Absence of p3 reactivity to cerebrovascular and neocortical amyloid.* **A** *and* **C:** *β-Amyloid staining using 4.1 MAb and AD cases C45D1 and 94A53, respectively.* **B** *and* **D:** *p3 staining of adjacent tissue sections to* **A** *and* **C,** *respectively.* **Arrows** *denote β-amyloid-laden vessels. Scale bar, 30 μm.*

peptides with identical carboxy domains and termini. The shared hydrophobic carboxy terminus of both peptides contains critical molecular determinants of the β-amyloid sequence that have been shown to drive amyloid fibril assembly and promote a β-sheet structural conformation *in vitro*.[29–31] This same domain has also been shown to possess neurotoxic properties[32,34] provided it is displayed in a fibrillar structure.[21,35] The issue of whether p3 participates in the pathogenesis of AD, however, has not been addressed. Presumably, this is because the 4-kd β-amyloid peptide is the most common form in AD brain,[49–52] although recently Gowing et al[53] reported the isolation of p3 (residues 17 to 42) from diffuse amyloid deposits of AD brain. The lack of antibodies that can differentiate p3 from 4-kd β-amyloid has also been a limitation in resolving the existence and possible role of p3.

We describe here the first immunohistochemical analysis of the p3 peptide in brain. To conduct this study, p3-specific antibodies were generated, characterized, and used to evaluate brain tissue from AD and aged controls. The specificity of p3 immunoreactivity was demonstrated in several ways: 1) the staining could be obtained only with the immune serum; 2) two different p3 antisera produced identical staining patterns; 3) the immunoreactivity could be competed only by synthetic p3 homologues; 4) the staining profiles of p3 and 4-kd β-amyloid antibodies were very different; 5) p3 reactivity was present only in brains with AD histopathology; and 6) p3 antisera selectively immunoprecipitated the 3-kd, but not the 4-kd, β-amyloid peptide. We also conclude that p3 reactivity could not be attributed to the 8.0-kd carboxy-terminal β-APP fragment derived from α-secretase action as the p3 antibodies do not immunoprecipitate this fragment nor do they produce the same staining pattern in AD brain as do antibodies to the carboxy terminus of β-APP, p3 staining notably lacking cellular and neurofibrillary reactivity. Hence, we conclude that the results from p3 immunohistochemistry are specific and cannot be attributed to artifact or to 4-kd β-amyloid immunoreactivity.

We observed that the profile of p3 immunoreactivity was different from that seen for 4-kd β-amyloid. The 4-kd β-amyloid pattern of staining in AD brain included diffuse deposits, preplaques, plaques, and

cerebrovascular amyloid distributed throughout the amygdala, hippocampus, and cortex. p3 immunoreactivity was localized to different structures that were predominantly found in the temporal lobe limbic system. Typically, p3 staining identified diffuse extracellular deposits, dystrophic neurites associated with β-amyloid plaques, and vesicular structures (single or clusters) scattered throughout the neuropil. We noted that these vesicular structures morphologically resembled the dystrophic neurites described by Masliah et al.[54] This group reported dilated presynaptic organelles within the neuropil in sublocales of AD brain lacking amyloid deposits. These synaptophysin-positive structures contained swollen vesicles and dense bodies similar to those seen in dystrophic neurites. p3 antibodies also weakly stained plaque coronas. p3 antibodies, however, did not react with vascular amyloid deposits in any of the AD cases displaying this type of pathology. In the literature, we noticed another example of differential distribution of these co-secreted β-amyloid peptides. Two independent reports describe 4-kd β-amyloid in human cerebrospinal fluid,[55,56] but curiously, the p3 peptide was absent despite the use of procedures that would have detected any carboxy-terminal derivative of the β-amyloid sequence. This observation further emphasizes the notion that p3 and 4-kd β-amyloid are influenced by different events after leaving the cell.

We can only speculate as to the reasons for the differences seen in the structural and anatomical distribution between p3 and 4-kd β-amyloid. A critical role may be played by the amino-terminal domain of the β-amyloid sequence, which is missing from p3. For instance, β-amyloid amino-terminal specific intermolecular binding, such as to heparan sulfate proteoglycan[57] or to apolipoprotein E,[58] might alter the intra- or extracellular localization of either of the β-amyloid peptides. Differential clearance of the peptides might also be relevant. However, we found that both p3 and 4-kd peptides secreted *in vitro* are degraded at a similar rate in culture.[59]

We initiated this investigation with the purpose of elucidating the potential role of p3 in AD pathogenesis. The results suggest that this abbreviated form of β-amyloid may be important. Several of the observations described in this study support the potential pathogenic candidacy of p3. The p3 peptide histopathology is prevalent in the temporal lobe limbic system. This system is intimately associated with the cardinal clinical features of AD and is affected early in the course of the disease. Also, p3 is a component of some dystrophic neurites, some of which are seen in the absence of β-amyloid plaques in the AD brain. A strong correlation between neuritic and synaptic alterations and dementia has been found that surpasses that between plaques and dementia.[60] Together, these observations necessitate that this natural β-amyloid derivative be considered seriously in the context of AD pathobiology.

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