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Immunodetection of the amyloid P component in Alzheimer's disease

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Summary. Amyloid P component (AP), a plasma constituent normally not found in brain parenchyma, has been immunohistochemically determined in brains from patients with Alzheimer's disease (AD). Tissue came from 11 clinically diagnosed and neuropathologically verified AD patients and from 6 normal aged controls. Positive labeling for AP was observed in amyloidotic blood vessels, senile plaques (SP) and neurofibrillary tangles (NFT). The immunoreactivity was specific for these AD-associated lesions and clearly revealed their morphological appearance. Affected blood vessels appeared to be mainly of the arteriolar type and were labeled abluminally in short segments. SP constituents such as amyloid fibrils, amyloid core and degenerative axonal and dendritic processes were positive for AP antiserum; the morphology and distribution of immunoreactive SP corresponded to previous descriptions. Labeling of NFT revealed the morphology of paired helical and straight filaments. In all cerebral areas studied, tangle-bearing neurons were immunoreactive to AP antiserum, suggesting that AP is involved in early cellular development of NFT. Given the large molecular weight of AP (about 220,000), these results point to a potential impairment of the blood-brain barrier in AD. Since AP is always present in systemic amyloidosis, its detection in cerebral amyloidosis associated with AD may suggest mechanisms common to the two disorders.

Key words: Alzheimer's disease – Amyloid P component – Amyloidosis – Neurofibrils

We have previously investigated the cerebral microvasculature in Alzheimer's disease (AD) and observed morphological alterations in the cerebral vessels that could impair the integrity of the blood-brain barrier [26, 27]. This posited dysfunction may lead to the abnormal presence of plasma constituents in brain parenchyma; the present study uses immunohistochemical techniques to detect and localize one such constituent in AD brains. Serum amyloid P component is a normal plasma glycoprotein present in human serum at a relatively stable concentration [19]. It is manufactured by hepatocytes and belongs to a family of plasma proteins known as pentraxins. It gives rise to amyloid P component (AP) and the two proteins share a similar structure [1, 21]. AP is a small but constant constituent in all forms of systemic amyloid deposits, which contain 90% amyloid fibrils and 10% AP [20]. In AD, cerebral amyloid deposits occur in senile plaques (SP), neurofibrillary tangles (NFT) and blood vessels. Although previous immunocytochemical studies [23, 31] have reported the presence of AP in vessel walls, its possible localization in plaques and NFT has remained controversial. We used peroxidase-antiperoxidase (PAP) immunohistochemistry to detect and localize AP in AD and control brains. Labeling of SP and NFT using antisera raised against human serum AP was observed. These results were not confounded by immunolabeling with antisera raised against the other human pentraxin C-reactive protein (CRP), or serum amyloid A component (SAA), a constituent of some forms of systemic amyloidosis. Preliminary results of this work have been reported previously [28].

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Materials and methods

Tissues

Tissue came from 11 brains of patients diagnosed with AD (78 ± 10 years) and from 6 brains of patients who died from non-neurologic causes (66 ± 7 years). All clinical diagnosis of AD were confirmed by independent postmortem neuropathological examination. The autolysis time ranged from 6 to 15.5 h

Table 1. Patients in the study

	Age	Sex	Autolysis time (h)	Diagnosis
Patient 1	66	M	14	AD
Patient 2	93	F	12	AD
Patient 3	80	M	14	AD
Patient 4	80	F	7.5	AD
Patient 5	74	F	12	AD
Patient 6	75	M	15.5	AD
Patient 7	81	F	7.5	AD
Patient 8	93	F	6.5	AD
Patient 9	74	M	8.5	AD
Patient 10	68	M	7	AD
Patient 11	71	M	6	AD
Control 1	75	M	14	Squamous cell carcinoma
Control 2	58	M	14.5	Histiocytic lymphoma
Control 3	61	M	14.5	Liver cirrhosis
Control 4	71	M	11	Adenocarcinoma
Control 5	70	M	12	Heart failure
Control 6	62	M	6	Lung cancer

AD, Alzheimer disease

(Table 1). The specimens were obtained from the National Neurological Research Bank, VA Wadsworth Medical Center, Los Angeles, California and from the Department of Neuropathology, Veterans Administration West Los Angeles Medical Center, Los Angeles, California.

Dissected areas included olfactory bulb and tract, frontal, parietal, and occipital cortices; hippocampus and the adjoining temporal cortex; amygdala, the nucleus basalis of Meynert and locus coeruleus. Fixation was performed by two different methods. In one, blocks from unfixed tissue were dissected, immersed in 4% paraformaldehyde (pH 7.4) for 2 h at 25° C, and then for 24–48 h at 4° C. They were rinsed in 0.12 M Millonig's phosphate buffer with 30% sucrose for cryoprotection and cut on a cryostat (40 μm section thickness). Sections were stored in 0.1 M Tris buffer (pH 7.4) prior to immunohistochemical processing. In the other fixation method, tissue blocks were obtained from brains which had been routinely processed for neuropathological examination. Briefly, brains were removed from skulls and perfused with 10% neutral buffered formalin via internal carotid and vertebral arteries for 5 to 10 min, then immersed in the same fixative for an average of 2 weeks. They were rinsed in running tap water for 24 to 48 h prior to sampling. Tissue blocks were dissected and stored in 10% neutral buffered formalin. They were rinsed in 0.1 M Tris (pH 7.4) prior to vibratome sectioning (40–50 μm section thickness).

Antisera

The antisera to AP were purchased from two different commercial sources. The antiserum from Dako Corporation, Santa Barbara, California (Product A302) was raised against AP isolated from human acute phase plasma. This polyclonal antiserum is the immunoglobulin fraction of rabbit serum which has been liquid-phase absorbed by the manufacturer with fractions of normal and acute phase plasma until only the P component precipitate was seen in crossed immunoelectrophoresis with 12.5 μl antiserum cm^2 in the gel and 2 μl human plasma in the well. The antiserum from Calbiochem Corporation, San Diego, California (Product 565191) was manufactured by immunization in rabbits with isolated, pure human AP. The manufacturer tested the specificity of the antiserum by (1) double immunodiffusion and immunoelectrophoresis against human serum, which yielded a single immunoprecipitation line against a protein antigen of alpha mobility, coresponding to known precipitation patterns of serum AP against AP antiserum [2, 3]; (2) specific immunohistochemical labeling of human tissues containing serum AP such as amyloid deposits; (3) preabsorption of this antiserum with isolated, pure human serum AP abolishing all immunocytochemical labeling.

The antiserum to human CRP was purchased from Dako Corporation, Santa Barbara, California (Product A073). The antigen was isolated from human serum with high levels of acute phase proteins. The specificity of the polyclonal antiserum was determined by the manufacturer using the same method as for AP antiserum. In this study, the antiserum to human CRP was tested at dilutions between 1:200 to 1:3000.

Antiserum to SAA was purchased from Calbiochem Corporation, San Diego, California (Product 566703). It was produced by immunization of rabbits with human SAA isolated from acute phase serum. The manufacturer tested the specificity of the antiserum by indirect immunofluorescence labeling of human AA-type amyloid deposits. The antiserum to SAA was utilized in our study, at dilutions ranging from 1:50 to 1:800.

Specificity of AP antisera

We have preabsorbed the antisera to human AP using either excess purified human serum AP, excess purified human SAA (Calbiochem/Behring Diagnostics, La Jolla, California – Products 565190 and 566700, respectively) or human immunoglobulin G (IgG) (Sigma Chemical Company, St. Louis, Missouri – Product 14506). The antigens were allowed to react with the antiserum at different dilutions and the resulting mixtures were used in immunohistochemical experiments. These tests for the specificity of human AP antisera used the rationale that the bindings of AP antiserum to purified excess antigen would make it unavailable for the labeling of tissue antigen. Moreover, they verified the specificity of the antiserum to human AP and not to other plasma components such as SAA or IgG.

Immunohistochemistry

The present study was based on a modified Sternberger's PAP method [29]. Endogenous peroxidase was eliminated by treatment in 0.3% methanolic hydrogen peroxide for 30 min. The sections were preincubated for 1 h at 25° C in 3% normal goat serum, and then transferred to the primary antiserum solution. The AP antisera had been tested at dilutions ranging from 1:200 to 1:6000. The sections were incubated in primary antiserum at 25° C for 2 h, and at 4° C overnight. The next day, they were transferred to secondary antiserum (goat anti-rabbit IgG antiserum-Sternberger-Meyer) diluted 1:400 for 1 h at 25° C and then placed in PAP solution (rabbit-Sternberger-Meyer) diluted 1:500 for 1 h at 25° C. Labeling was produced with 0.05% diaminobenzidine and 0.015% hydrogen peroxide in 0.075 M phosphate-buffered saline (pH 7.3). Between each step, the sections were rinsed in either 0.1 M Tris buffer (pH 7.4), or 0.075 M phosphate-buffered saline (pH 7.3). Sections were mounted on alcohol-gelatinized slides, dehydrated through increasing alcohol concentrations, cleared and coverslipped. Some sections were counterstained with toluidine blue or cresyl violet. Every immunohistochemical experiment, whether for AD brains or control brains, included control sections in which the primary

Table 2. Cortical and subcortical areas containing elements immunoreactive to human amyloid P component antisera

Areas	SP	NFT	Amyloidotic blood vessels
Olfactory bulb and tract	+	+	0
Frontal cortex	+	+	+
Parietal cortex	+	+	+
Occipital cortex	+	+	+
Hippocampal formation	+	+	+
Amygdala	+	+	+
Nucleus basalis	0	+	0
Locus coeruleus	0	+	+

SP, Senile plaques; NFT, neurofibrillary tangles; +, positive immunoreactivity; 0, not observed

Table 3. Immunoreactivity of Alzheimer's disease type-associated lesions to selected antisera

Antisera	SP	NFT	Amyloidotic blood vessels
AP antiserum	+	+	+
SAA antiserum	0	0	0
CRP antiserum	0	0	0
AP antiserum preabsorbed to human AP	0	0	0
AP antiserum preabsorbed to human SAA	+	+	+
AP antiserum preabsorbed to human IgG	+	+	+

AP, Amyloid P component; SAA, serum amyloid A component; CRP, pentraxin C-reactive protein; SP, senile plaques; NFT, neurofibrillary tangles; +, positive immunoreactivity; 0, negative immunoreactivity

antiserum was omitted and replaced with normal goat serum. Sections immediately adjacent to those used for immunohistochemistry were stained with either thioflavin-S or by Bielschowsky's reduced silver method for structural correlation purposes.

Results

The antiserum raised against human AP strongly labelled SP, NFT and amyloidotic blood vessels at dilutions up to 1:6000 in all AD brains (Table 2), with dilutions between 1:3000 and 1:4000 being used most often. A small number of immunoreactive NFT were also observed in one control case. These AD-associated lesions were positively stained with thioflavin-S and/or Bielschowsky's reduced silver technique on adjacent sections. Antisera raised against human CRP or human SAA failed to label the AD-associated lesions at any dilution tested (Table 3). In the preabsorption studies, only excess purified human AP blocked the antiserum to human AP (Table 3).

Where present, SP were immunoreactive for human AP in all selected cerebral regions from AD patients (Table 2). Three types of SP were labeled. One type consisted exclusively of clusters of dystrophic neurites forming a loose network and containing no other immunoreactive material (Fig. 1a). A second type of SP contained immunoreactive neurites with portions of NFT and amyloid fibrils. The amyloid fibrils varied in their labeling intensity from faint to dark. The plaque core, whenever present, was made up of an intensely immunoreactive surrounding rim and a less intensely reacting central region (Fig. 1b). The third type of SP displayed only an immunoreactive core with no extracellular immunoreactive material or surrounding neurites (Fig. 1c).

All three types of SP were numerous in neocortex, hippocampus, and amygdala: In the neocortex, immunoreactive SP were predominantly distributed in layer II, the lower portion of layer III, and layer V (Fig. 2a). The upper portion of layer III was devoid of immunoreactive SP; instead, immunoreactive neurites were often observed, forming a loose network which extended up to layer II. Immunoreactive SP were predominantly small (20 μm average diameter) and closely spaced in layer II, whereas they were larger (60–80 μm average diameter) and more widely spaced in layer III (Fig. 2c). They were occasionally seen in close relationship with blood vessels, investing short segments of arterioles with immunoreactive fibrils (Fig. 6a, b). In the hippocampus (Fig. 2b), immunoreactive SP were present in the subiculum where they were predominantly distributed in the stratum lacunosum moleculare with a lesser number in the stratum pyramidale. Occasional, small immunoreactive SP were also observed in the pyramidal layer of CA1 area, but not in CA2 or CA3. Large (60–80 μm average diameter) conspicuous immunoreactive SP were distributed in CA4 (Fig. 2d), also forming clusters in the stratum moleculare of the area dentata. SP in the amygdala were mostly distributed in the corticomedial nuclei, with no apparent organization.

NFT in all studied regions were labeled by the antisera to human AP (Table 2). The swirls formed by the intracellular paired helical filaments (PHF) and straight filaments were clearly visible by light microscopy (Fig. 3). The nucleus and portions of the neuronal cytoplasm which did not contain tangle formations were not immunoreactive. Thus, all immunoreactivity displayed by the NFT to AP antisera appeared to be associated with the intracellular (PHF) and/or straight filaments. In AP-labeled sections counterstained with toluidine blue or cresyl violet, tangle-

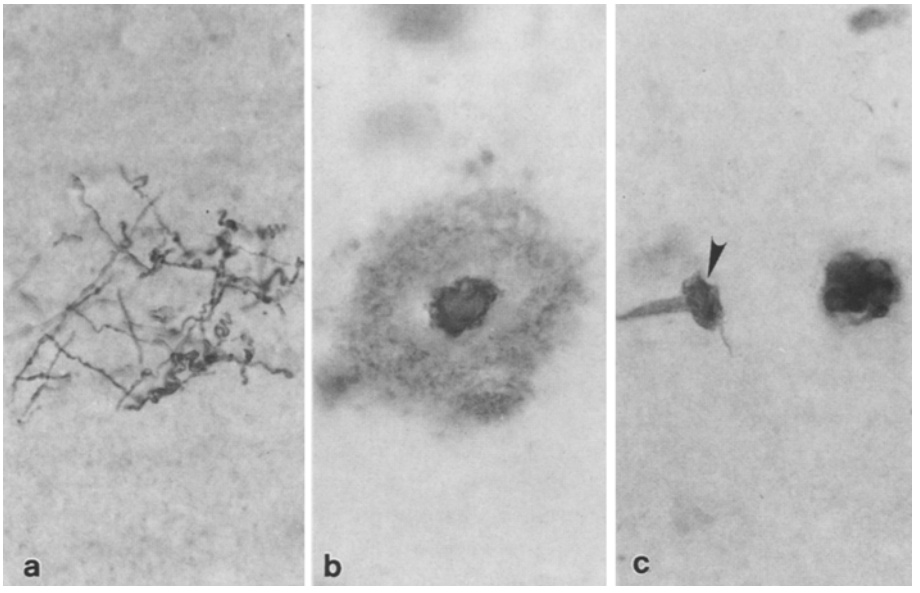

Fig. 1 a–c. Senile plaques (SP) labeled by amyloid P component (AP) antisera. **a** Neocortical SP containing only dystrophic neurites, ×360. **b** SP with amyloid fibrils and core, ×630. **c** SP displaying core only. Note adjacent AP-labeled neurofibrillary tangle (NFT; *arrowhead*), ×630

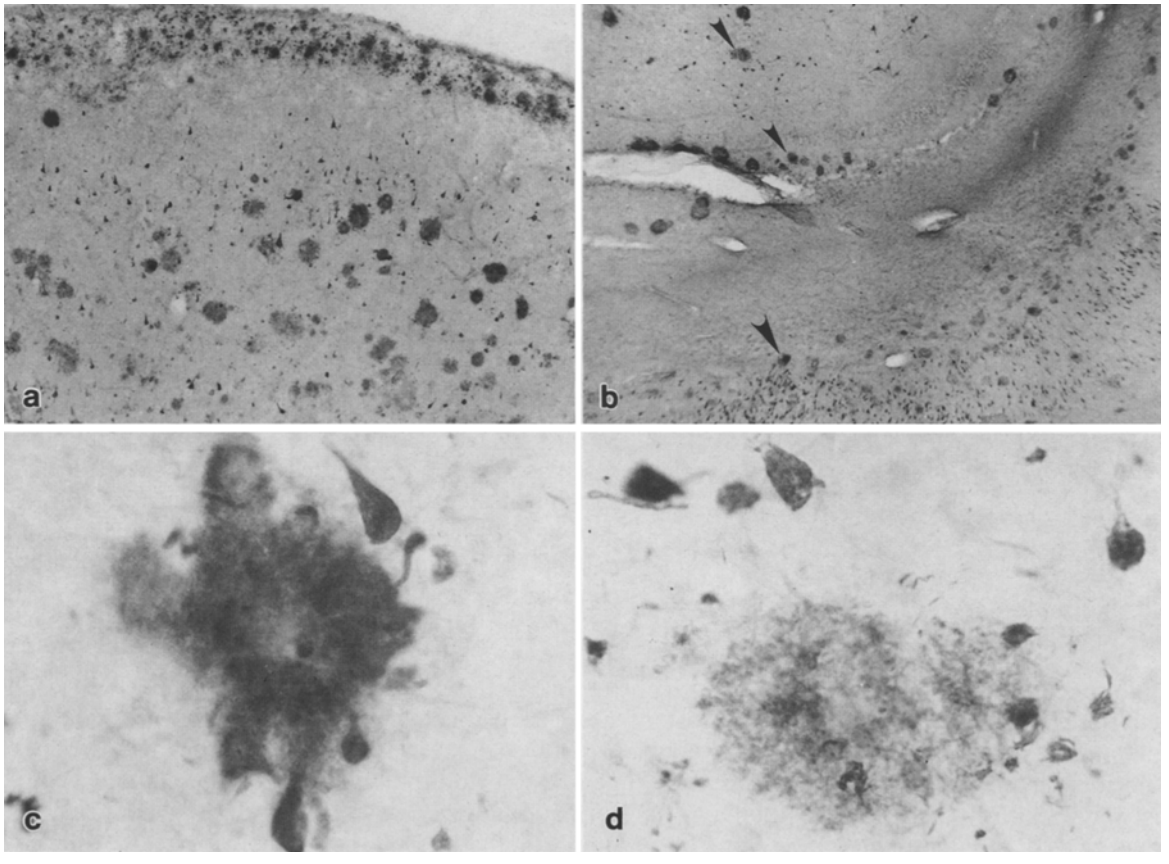

Fig. 2. a AP labeled neocortex. Note distribution of immunoreactive SP in cortical layers, ×40. **b** AP-labeled hippocampal formation. Note SP in subiculum, area dentata and CA4 (*arrowheads*), ×22. **c** SP in cortical layer III with labeled NFT, ×660. **d** AP-labeled SP in CA4 of hippocampus, ×288

bearing neurons in various states of degeneration were clearly demonstrated. They ranged from nerve cells containing slivers of immunoreactive NFT isolated in a small portion of the cytoplasm (Fig. 4a), through neurons whose cytoplasmic contents were displaced by significant NFT (Fig. 4b), to cells in which the somatic contents had disappeared, having been completely replaced by NFT extending into the proximal portion of dendrites (Fig. 4c).

In the olfactory bulb and tract, AP-immunoreactive NFT were observed in the anterior olfactory nucleus. Corpora amylacea were abundant in the olfactory tract but were not immunoreactive to human AP antisera. Amygdala and hippocampal formation showed the greatest amount of AP-immunoreactive NFT of all brain regions studied. NFT in amygdala were virtually limited to the corticomedial nuclei, while in the hippocampal formation, the majority of NFT were seen in subiculum and hippocampal fields CA1 and CA4. The AP-immunoreactive NFT presented the flame-shaped appearance, which can also be demonstrated by stains such as thioflavin-S or Bielschowsky's reduced silver method. The subicular area was usually heavily involved and almost all neurons contained NFT in various stages of development (Fig. 5a). Most of these tangle-bearing neurons were located in the pyramidal layer, as was the case in CA1. CA2 and CA3 contained occasional NFT, whereas CA4 contained many swirl-shaped, intensely labeled tangle-bearing neurons (Fig. 5b). In layer II of the entorhinal cortex, the islands of stellate cells contained NFT in all AD cases and one control case. They formed large clusters intensely labeled by AP antisera (Fig. 5c). In the neocortex, the majority of NFT were found in the pyramidal cells of layers III and V (Fig. 5d). They occurred sometimes in clusters in and around SP. NFT in the nucleus basalis were labeled by antisera to human AP and were shaped as swirls with the immunoreactivity extending into proximal portions of dendrites. In the locus coeruleus, NFT were abundant and appeared to be derived from neuromelanin-containing neurons.

Amyloidotic vessels, which stained for thioflavin-S on adjacent sections, were also labeled by AP antisera. These vessels were usually of arteriolar size with cortical penetrating arterioles most frequently affected. They were labeled in short segments which, at times,

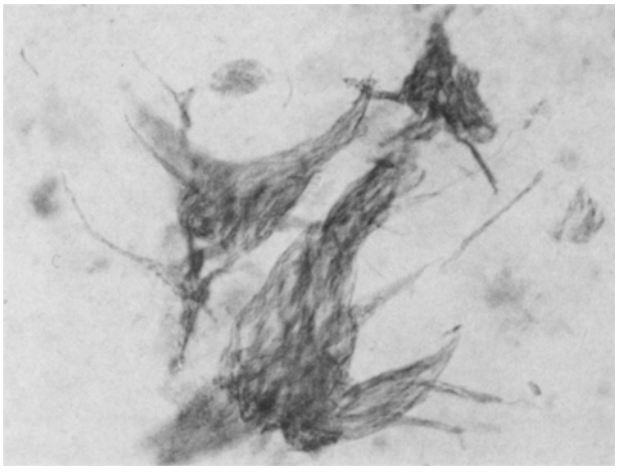

Fig. 3. AP-labeled NFT in layer II entorhinal cortex, ×640

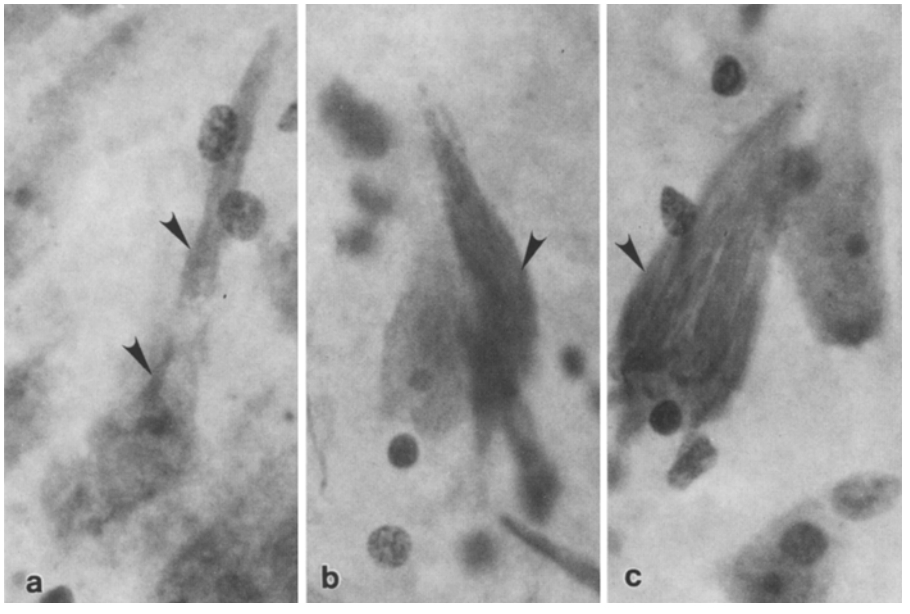

Fig. 4a–c. AP-labeled NFT in various stages of development. **a** Hippocampal neuron displaying slivers of NFT (*arrowheads*). **b** Increasing NFT (*arrowhead*) displace nucleus and normal cytoplasm. **c** Hippocampal neuron containing only NFT (*arrowhead*). Contrast with normal adjacent neuron. **a–c** Nissl counterstain, ×888

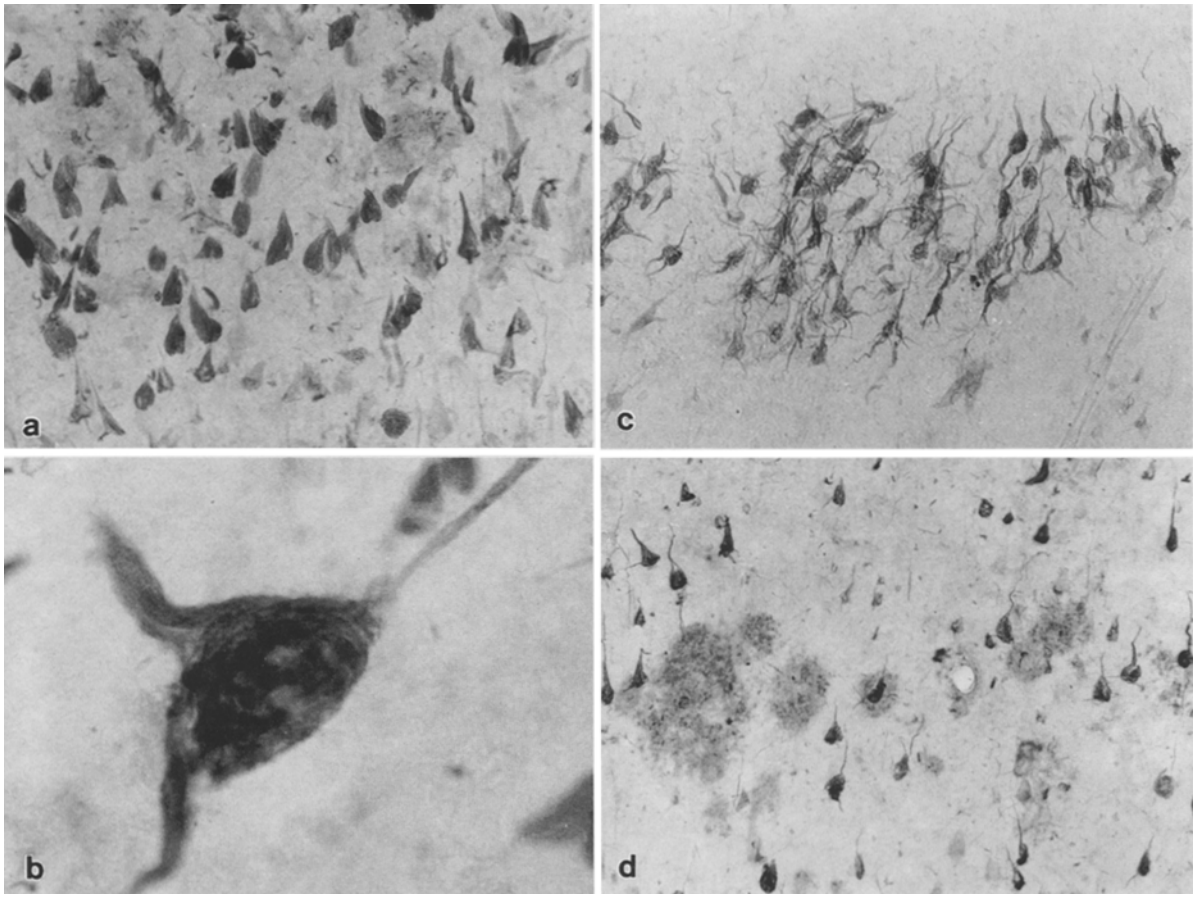

Fig. 5a–d. AP-labeled NFT in various cerebral areas. **a** Subicular tangle-bearing neurons, ×210. **b** Hippocampal CA4 NFT, ×1400. **c** NFT clusters in layer II entorhinal cortex, ×110. **d** NFT in layer III neocortex, ×120

were found to be in close relationship with SP (Fig. 6a, b). Light microscopic observations suggested that the affected segments were invariably labeled abluminally (Fig. 6c, d). They presented a contorted, "lumpy-bumpy" appearance similar to vessels described in a previous study using the scanning electron microscope [26]. It was not possible to identify the immunoreactive elements within the walls of these vessels by light microscopy.

Discussion

The results obtained support the specificity of the antiserum to human AP. Even though CRP shares as much as 66% homology in its primary structure with serum AP [17, 32], the antiserum raised against CRP did not label any of the AD lesions. Thus, the antisera raised against human AP did not cross-react with CRP. We have attempted to label the same lesions with SAA antiserum following a report that levels of this plasma component were elevated in Alzeimer's patients [6]. SAA has been implicated as the precursor of amyloid AA fibrils in some forms of systemic amyloidosis [7] but the antiserum did not label AD-associated lesions at any dilution tested, demonstrating that AP antisera were not cross-reacting with SAA. Furthermore, the antisera to human AP were blocked only by preabsorption with purified AP and not with other purified antigens (Table 3). Although the antisera to human AP tested in this study were obtained from two different commercial sources and were purified under different circumstances, both labeled the same structures: amyloidotic blood vessels, SP and NFT.

The morphology and distribution of the AP-labeled AD-associated lesions corresponded to the results obtained by previous studies [5, 8, 13, 14, 16, 18, 22, 24] utilizing other techniques for demonstrating SP, NFT and amyloidotic blood vessels. Neocortical SP had regional and laminar cortical distributions corresponding to those described previously [5, 18]. It has been proposed that the age of the plaque may be reflected by the size of its core [30]. Operating on this

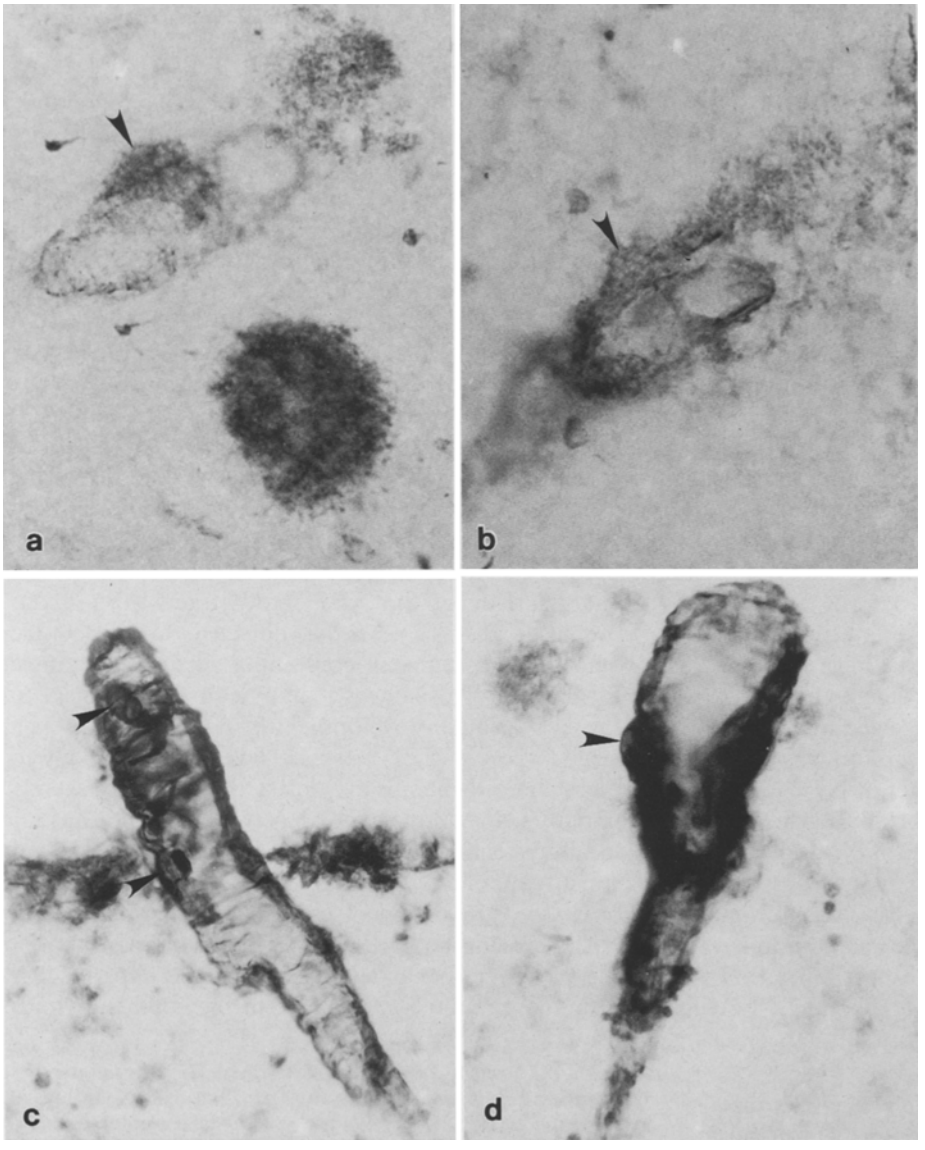

Fig. 6. a, b Note close relationship between SP (*arrowheads*) and AP-labeled blood vessels, ×375. **c, d** Note abluminal immunoreactive elements (*arrowheads*) in cortical penetrating arteriole (c) and intraparenchymal arteriole (d). **c** ×400, **d** ×640

thesis, a "young" plaque is made up entirely of swollen and degenerating axonal and dentritic processes, while a more "mature" plaque contains a well-defined extracellular amyloid core, surrounded by a halo of neurites. The isolated amyloid core is believed to characterize the "aged" plaque. Regardless of the sequence of progression, AP antisera labeled all three types of SP. The morphology and distribution of NFT also coincided with those described in previous studies [8, 13, 14, 16, 18, 22, 24]. NFT in the olfactory bulb were demonstrated in locations similar to those previously described [13, 16]. The vulnerability of hippocampal formation and entorhinal neurons to NFT formation is a consistent feature of AD and has been pointed out elsewhere [8]. NFT in neocortical pyramidal cells were distributed in layers III and V as reported by Pearson et al. [18]. Neurofibrillary swirls in the nucleus basalis of Meynert corresponded in morphology to those described by Saper et al. (24). Numerous NFT were also observed in the locus coeruleus and corresponded to the large amount of cell loss reported in this area [14]. Thus, the localization of AD-associated lesions by AP immunohistochemistry has many similarities to that obtained with other techniques and reinforces the presence of this plasma protein in AD brains.

As reported above, tissue from fresh brains and from those routinely processed for neuropathological examination displayed equally good results. In this regard, cryostat or vibratome sections yield better immunohistochemical results than paraffin-embedded sections [4]. Sections thus labeled can be counter-

stained with a Nissl technique for simultaneous observations of AD lesions and normal neural structures. Postmortem diagnoses of AD that rely on commonly accepted neuropathological criteria [10] may be performed by utilizing immunohistochemistry of AP antisera. This affords a reliable and sensitive method for detecting cerebral lesions associated with AD. Since our preliminary report [28], other researchers have obtained successful labeling results with the Dako Corporation AP antiserum [4, 9, 25].

The source of the human AP in AD-associated lesions is intriguing. It has been suggested that the detection of serum proteins in the brain parenchyma of AD patients may be due to postmortem leakage [15]. However, the AP labeling was always observed with AD-associated lesions and not in the normal parenchyma of control brains. Furthermore, it did not appear indiscriminately in all neurons but only in those containing tangle formations. These results lend credence to the interpretation that the antisera are labeling an antigen that has made its way in vivo into the AD lesions. Our negative labeling in all control brains (except one) and in normal neurons of AD brains suggest that AP can be detected only in lesions associated with AD. The one exception was a control case that exhibited a small number of NFT labeled in layer II stellate cells of the entorhinal cortex. Other brain regions were free of SP and NFT and thus this patient did not meet the criteria for a postmortem diagnosis of Alzheimer's disease. NFT are not unique to AD and they can be observed in small numbers in normally aged patients [30]. The possibility that AP may be expressed intracerebrally by tangle-bearing neurons is also being explored in our laboratory, using in situ hybridization methods.

At this time, it is reasonable to believe that AP originates systemically and enters the central nervous system from the vascular compartment. Serum AP is a normal plasma constituent and circulates freely in the body. It is manufactured abundantly by hepatocytes, which maintain a constant serum level. In systemic amyloidosis, it readily binds to amyloid fibrils of the AA and AL types [1]. Previous studies [23, 31] have reported the presence of AP in amyloidotic cerebral vessels and possibly in SP. AP in systemic deposits is identical in structure to serum AP and has been shown to be derived from deposition of the serum component [1]. In AD, distorted amyloidotic vessels may be focal points where an abnormal amyloid precursor has been deposited or a structural alteration has occurred. Circulating serum AP would be attracted to the developing vascular amyloid and deposited in the form of AP. Amyloid deposits are rich in calcium, and β-pleated sheets are present in all amyloid precursor proteins and fibrils. It has been demonstrated that AP has an affinity for peptide sequences containing calcium-binding carbonyl groups in appropriate spatial orientation such as β-pleated sheets [19]. This is also supported by the positive labeling of amyloidotic vessels, suggesting that potential blood-brain barrier impairment may allow an increasing amount of AP to penetrate the brain parenchyma and bind to amyloid deposits of SP. In fact, the extracellular amyloid fibrils and plaque cores were also labeled by the AP antisera used in this study. AP may be involved early in the plaque formation process since all three types of SP were labeled by AP antisera. The immunoreactivity displayed by the neurites and the NFT have not been observed in previous studies [23, 31]. Kidd et al. [11] have demonstrated that the amino acid compositions of the NFT and the amyloid plaque cores were the same. Using X-ray diffraction, Kirschner et al. [12] reported that NFT have the same cross-β conformation as the amyloid plaque core; it may be this characteristic of the NFT that attracts AP. In our preparations, AP can be detected in dystrophic neurites and neurons containing incomplete tangle formations. This suggests that AP may be crucial to the early cellular development of NFT. Since AP is a large molecule (mol. mass 220,000), it is not likely to cross the cell membrane unless the latter is severely impaired. In summary, AP has been detected thus far in all systemic amyloidoses as a small but constant constituent of amyloid deposits and our findings demonstrate its involvement in intracerebral amyloid deposits present in AD. This may suggest impairment of the blood-brain barrier in AD and perhaps mechanisms common to systemic and intracerebral amyloidoses.

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