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Topographical relationship between β-amyloid and tau protein epitopes in tangle-bearing cells in Alzheimer disease

(molecular pathology/amyloid fibrils/paired helical filaments)

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ABSTRACT **Double-labeling immunohistochemistry was used to investigate the topographical relationship between β-amyloid and tau protein epitopes present in cells bearing neurofibrillary tangles found in the hippocampal formation of patients with Alzheimer disease. An antiserum raised against the amino terminus of β-amyloid stained numerous tangle-bearing cells and other bodies ("extracellular tangles"), but double labeling showed that the β-amyloid staining is invariably peripheral to that of the tau-positive tangle proper. This and other results suggest that the extracellular amyloid plaques and the intracellular neurofibrillary tangles are biochemically distinct but may result from related pathological events that originate at the level of the nerve cell and lead to its degeneration.**

Abundant senile plaques and neurofibrillary tangles constitute the major neuropathological characteristics of Alzheimer disease (1, 2). The 43-amino acid β-amyloid is the main protein constituent of the extracellular senile plaques (3, 4). It is derived from a family of precursors with the properties of transmembrane proteins and the β-amyloid sequence is located toward the carboxyl-terminal end of the precursor, where part of it is present in the putative transmembrane region (5). The microtubule-associated protein tau forms a component of the core of the paired helical filament (6–8), the major constituent of the largely intracellular neurofibrillary tangles (9). At present, the pathogenetic relationships between plaques and tangles are not understood.

In the preceding paper (10), we have used double-labeling immunohistochemistry to demonstrate a close association between extracellular β-amyloid deposits and neurofibrillary tangles in the hippocampal formation in Alzheimer disease. Moreover, we have shown that antisera directed against different parts of β-amyloid can be obtained that stain almost exclusively either amyloid plaques without cores or amyloid plaques with cores, or, unexpectedly, large numbers of pyramidal cells, as well as the neuritic components of plaques and neuropil threads. Most previous studies that have used antibodies raised against different portions of β-amyloid have observed labeling only of diffuse amyloid deposits and senile plaques, but not of tangle-bearing objects (11–16). However, in addition to extracellular amyloid staining, staining of some tangle-bearing cells or bodies was reported (17–19), and this was used as an argument in favor of the view that β-amyloid also forms the paired helical filament (17, 19).

In the present paper, we have used double-labeling immunohistochemistry to investigate the topographical relationship between β-amyloid and tau protein epitopes in tangle-bearing cells in the hippocampal formation of Alzheimer disease patients. We show that β-amyloid is spatially distinct from, and invariably peripheral to, tau protein staining. This leads us to propose a conjectural relationship between plaques and tangles.

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MATERIALS AND METHODS

A polyclonal antiserum raised against a synthetic peptide derived from human β-amyloid and two polyclonal antisera raised against different parts of the sequence common to known human tau protein isoforms were used throughout this study. BR88 is a polyclonal rabbit antiserum that was raised against amino acids 1–12 of β-amyloid (10), whereas BR133 and BR135 are polyclonal rabbit antisera that were raised against amino acids 1–16 and 323–335 of the longest known human tau protein isoform (20).

Tissue blocks containing the hippocampal formation from four patients who had died with a histologically confirmed diagnosis of Alzheimer disease (65, 72, 76, and 79 years old) and from two control patients who had died without neurological or psychiatric disorders (74 and 78 years old) were dissected <6 hr after death and kept frozen at −70°C until use. They were immersion fixed in 4% paraformaldehyde for 48 hr and kept in 30% sucrose in phosphate-buffered saline at 4°C. Sections (40 μm) were cut on a freezing microtome and processed free-floating for double-labeling immunohistochemistry exactly as described in the previous paper (10). In some of the experiments described here, the sections were treated with 86% formic acid for 5 min (21). Specificity of the staining was confirmed by concurrent processing of tissue sections in which one or the other primary antibody was omitted or adsorbed for 6–12 hr at room temperature with 10 μg of synthetic peptide per ml. Omission of the second primary antibody allowed us to determine whether the second secondary antibody cross-reacted with any of the first step antibodies or whether any residual peroxidase activity had remained from the first step and reacted with the chromogen of the second staining. The staining pattern for each antibody used in the double labeling was also tested in parallel in serial sections by single labeling.

RESULTS

The effect of formic acid treatment on the staining with BR88 and BR133 or BR135 was investigated by pretreating alternate tissue sections with formic acid (Fig. 1). A substantial increase in the number of BR88-positive structures was observed in formic acid-treated sections (Fig. 1*b*) when compared with untreated sections (Fig. 1*a*). No such increase was observed in tissue sections stained with BR133 or BR135. Formic acid pretreatment was used in all subsequent double-labeling experiments.

Double labeling with BR88 first and BR133 second indicated the presence of a large number of small compact tangle-bearing cells labeled by BR133 alone and of a large number of nerve cells labeled by BR88 alone, with the latter ranging from small compact to large distended (Fig. 2*a*). On average, the BR88-positive cells were larger than the cells

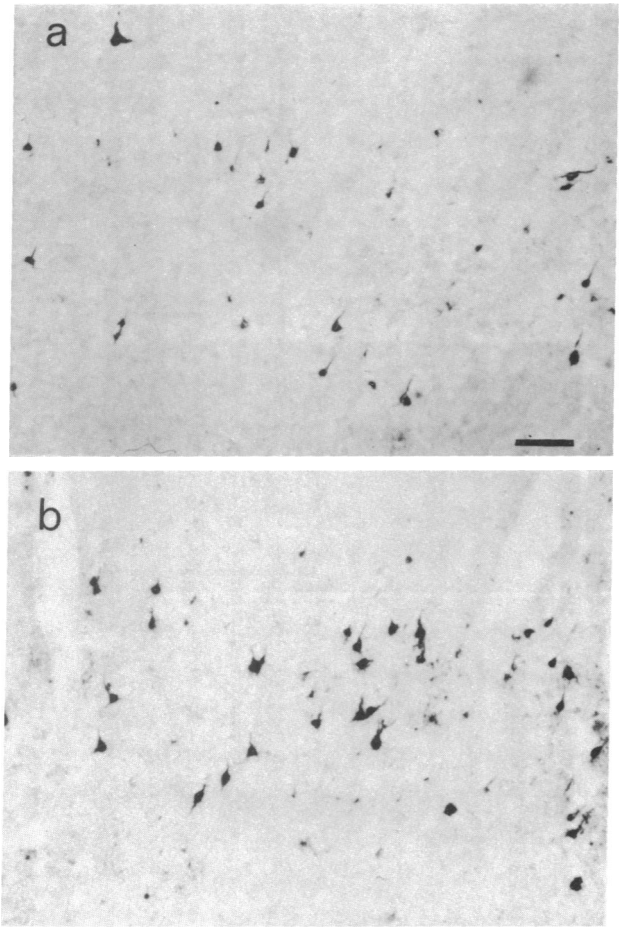

FIG. 1. The effect of formic acid pretreatment on the number of BR88-positive structures in subiculum/entorhinal cortex from Alzheimer disease patients. (*a*) No formic acid pretreatment. (*b*) Formic acid pretreatment. Tissue sections were treated with 86% formic acid for 5 min prior to immunostaining. (Bar = 270 μm.)

stained by BR133 alone (Fig. 2*a*). In general, the immunoreactive nerve cells were either blue or brown (Fig. 2*a*), giving the impression of two nonoverlapping populations. However, a number of tangle-bearing cells could also be labeled by BR133 (as the second primary antibody) when the BR88 staining was confined to a limited part of the cell (Fig. 2 *b*,1 and *c*,3). Importantly, the BR88 staining was always peripheral to the BR133 staining (Fig. 2 *b* and *c*).

Fully double-labeled nerve cells were only observed when the order of the primary antibodies was reversed—i.e., when BR133 or BR135 was used first and BR88 was used second (Fig. 2 *d* and *e*). This strongly suggests that the peripheral location of the BR88 epitope gives a reaction product that blocks access of the anti-tau antibodies to the interior of the cell when BR88 is applied first and when the BR88-positive material covers the whole outside of the cell (as in most cases shown in Fig. 2*a*).

These fully double-labeled pyramidal cells had the characteristic appearance of intracellular tangles (Fig. 2 *d* and *e*), and by focusing the microscope the BR88-positive material could be seen to be on the outside and the BR133-positive tangle could be seen to be on the inside of the cell. Fully double-labeled objects with the fibrous appearance of extracellular tangles were also found when the primary antibodies were applied in the order stated (Fig. 2 *f* and *g*). Close examination of a large number of double-labeled apparently intact tangle-bearing cells and also of the large-diameter extracellular tangles showed that in both types of object tau protein staining was present inside the more peripheral amyloid staining (Fig. 2 *d–g*). Control experiments showed the double labeling to be specific.

DISCUSSION

Single-labeling experiments showed that formic acid pretreatment produces a substantial increase in the number of BR88-positive immunoreactive structures; no such increase was observed with BR133 or BR135. Similar results have previously been obtained with β-amyloid antibodies that stain extracellular β-amyloid deposits (21). Although the precise mechanism by which formic acid effects the increased staining is not known, it is thought to represent a characteristic of extracellular amyloid staining. The present results indicate that formic acid pretreatment also enhances the cellular β-amyloid pathology.

Double labeling with BR88 and BR133 or BR135 demonstrated the presence of tangle-bearing objects that were either single labeled by BR133 or BR135 or double labeled by both antibodies. The single-labeled tangle-bearing objects were invariably small and appeared to be intact pyramidal cells, whereas double-labeled tangle-bearing objects were of all sizes, consistent with their predominant extracellular nature. This is supported by the finding that these large and distended double-labeled objects had the characteristic fibrous appearance of extracellular tangles penetrated by astrocytic processes (22, 23). However, some nerve cells were double labeled with BR88 and BR133, indicating that apparently intact, but tangle-bearing, cells can also be associated with BR88 staining. In addition to fully double-labeled cells, a number of BR133-positive cells were found that were only partially stained by BR88. These cells probably represent intermediates between cells positive only for BR133 and fully double-labeled cells.

Importantly, in all cases studied, the region of staining of β-amyloid sequences was outside that of tau staining. Moreover, the order of the antibodies was important, in that fully double-labeled objects were only observed when tau antibodies were used first. The inability of BR135 or BR133 to penetrate when BR88 was used first is presumably due to the 3,3-diaminobenzidine precipitate covering the outside of the cell and thus this observation also indicates that β-amyloid sequences are peripheral to tau. It therefore appears that β-amyloid sequences are in a close topographical relationship to tau protein in some tangle-bearing nerve cells; however, the present results do not support the views quoted earlier (17, 19) that tangles are made of β-amyloid and emphasize the need to distinguish between what is on and what is in a cell.

Fig. 3 summarizes the staining patterns of the various objects observed in this and the preceding paper and outlines a conjectural relationship between plaques and tangles. Antiserum BR88 stains an amyloid protein that is probably still dispersed over the cell surface in tangle-bearing BR133- and BR135-positive nerve cells and that may consist of partially proteolyzed β-amyloid precursor from which the β-amyloid has not yet been generated as a discrete protein (Fig. 3*b*; illustrated in Figure 3*b* of the preceding paper). The same cells do not stain with 4G8 or BR89, indicating that these antibodies only stain β-amyloid in a condensing or condensed state. This suggests that the cleavage of β-amyloid precursors that produces the amino terminus of the β-amyloid represents an early event in the amyloid pathology of Alzheimer disease.

Interestingly, it has been shown that the sequence consisting of the carboxyl-terminal 100 amino acids of β-amyloid precursors is amyloidogenic (24). The latter result could imply that the abnormal proteolysis of β-amyloid precursors leads to the formation of paired helical filaments, possibly through the abnormal phosphorylation of tau proteins (25). However, this possibility is not easy to reconcile with our

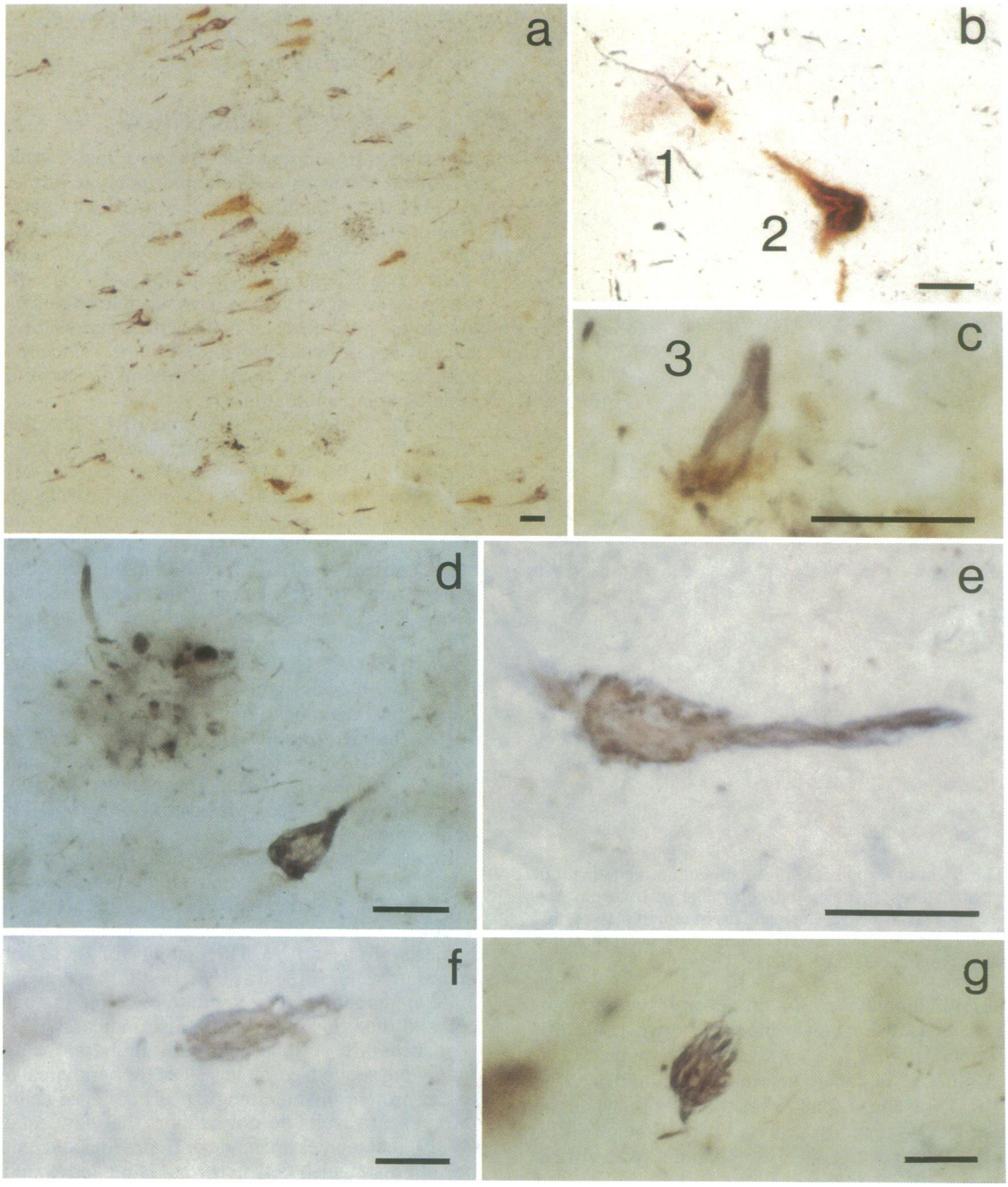

FIG. 2. Double labeling of subiculum/entorhinal cortex from Alzheimer disease patients with BR88 and anti-tau protein antibodies (BR133 and BR135). *(a) BR88 (brown) followed by BR133 (blue).* Numerous BR133-positive pyramidal cells are of normal size and shape and are therefore presumably intact tangle-bearing cells. On the other hand, while many of the BR88-positive nerve cells are of normal size and shape, a significant number are much enlarged, suggesting that the original cell may already be disrupted. In general, the whole cell stains either brown or blue, giving the impression of two nonoverlapping populations, but there are a number that are partially covered by both antibodies (see *b* and *c*). Nerve cells double labeled in their entirety are only observed when BR133 or BR135 is used first and BR88 is used second (see *d* and *e*). *(b and c) BR88 (brown) followed by BR133 (blue).* A number of tangle-bearing nerve cells are stained with both antibodies when the BR88-positive material does not cover the whole cell. 1, Part of the cell body is BR88 positive, whereas the region of the apical dendrite is only BR133-positive; 2, BR88-positive nerve cell of the enlarged type, as shown in *a*; 3, tangle-bearing BR133-positive cell partially covered with BR88-positive material. *(d and e) BR133 (d) or BR135 (e) (brown) followed by BR88 (blue).* Inversion of the order of antibodies with respect to *a–c* leads to double labeling of some apparently intact tangle-bearing pyramidal cells. The BR88-positive amyloid material is on the outside of the BR133- or BR135-positive tau material, as can be confirmed by focusing through the thickness of the 40-μm section. *(f and g) BR135 (brown) followed by BR88 (blue).* Extracellular tangles are stained by both antibodies when the order of antibodies used is the same as in *d* and *e*, and the BR88 staining is invariably peripheral to the BR135 staining. Inversion in the order of antibodies gives only BR88 staining, consistent with its peripheral location blocking access to the interior. (Bar = 35 μm.)

results, where a large number of small compact BR133-positive, but BR88-negative, tangle-bearing cells were found (Fig. 3*a*), whereas the converse was not the case. Indeed, the fact that many intact cells are tau positive yet BR88 negative (Fig. 3*a*) could be taken to mean that tangle formation precedes the cleavage in the β-amyloid precursor at the point in the sequence that is to become the amino terminus of β-amyloid. This may in fact be the case but we would hesitate to draw this conclusion from the limited number of histological markers presently available, which may not be sufficient

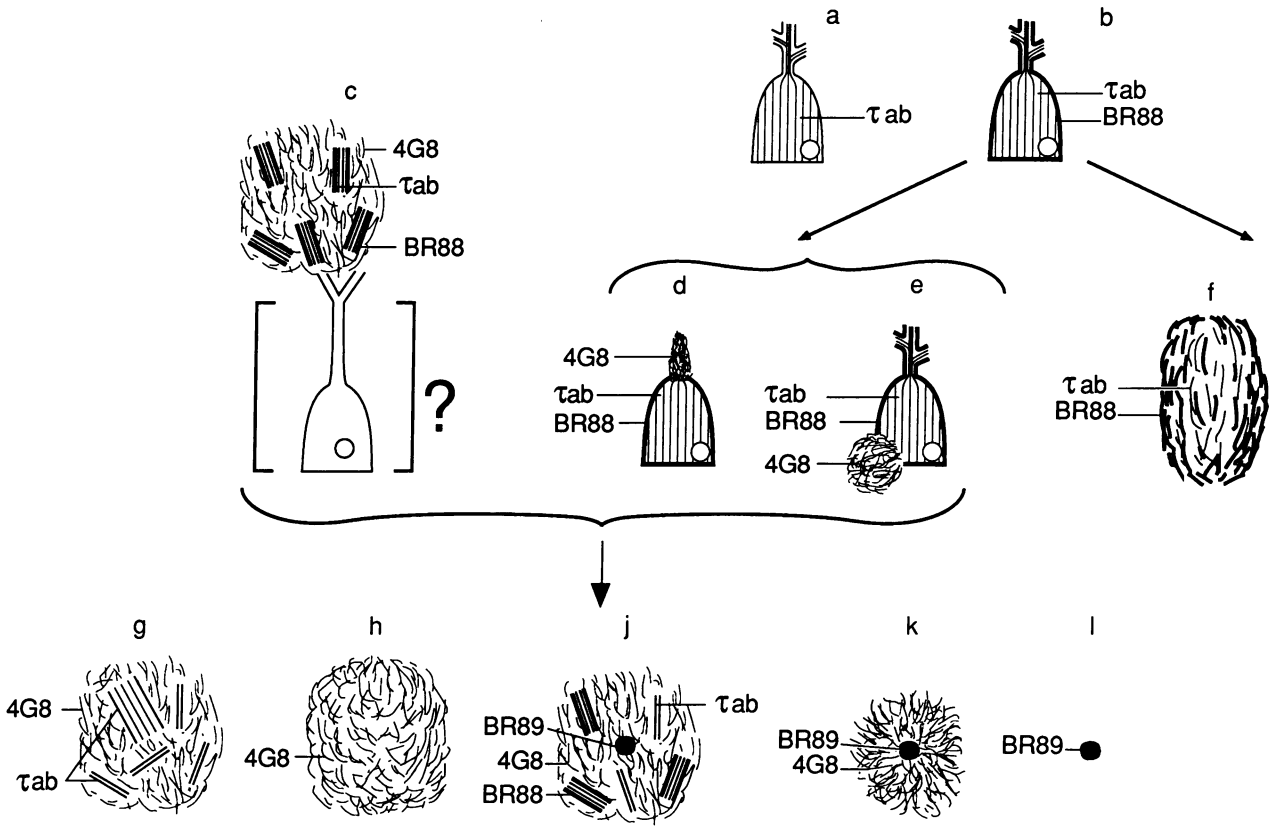

FIG. 3. Schematic representation of the double-immunolabeled cells and objects observed and outline of a conjectural relationship between plaques and tangles based on the findings reported here and in the preceding paper (see *Discussion* for a fuller explanation). The drawing is highly schematic and is not to scale. τab denotes antibodies against tau protein (BR133 or -135).

to mark out the very first stages of the development of amyloid pathology.

The present results suggest two possible fates for the BR133- and BR88-positive tangle-bearing cells, both resulting in the degeneration of the affected neurons. As shown in Fig. 3 *d* and *e* the first involves further cleavage of the dispersed, partially cleaved amyloid and its release and subsequent aggregation, resulting in BR88- and BR135-positive, intact looking tangle-bearing cells associated with deposits on the outside of released 4G8-immunoreactive material (cf. figure 2*a*, 3 and figure 2*b*, 1 of preceding paper); these deposits are BR88 and BR89 negative, consistent with the notion that they represent some intermediate between dispersed and fully condensed β-amyloid. The tangle-bearing cells then progressively degenerate, as evidenced by the frequent association of BR135-positive tangle fragments with 4G8-immunoreactive diffuse amyloid deposits; this leads to plaque formation (Fig. 3 *g* and *h*) and to the formation of plaque cores made of densely aggregated amyloid, which are identified by antiserum BR89 (Fig. 3 *j* and *k*). Examples of these objects are found in figure 2 of the preceding paper. Although the large size of the nerve cell makes it easy to observe these changes, plaque formation could also result from a similar process taking place in the neurites of the dendritic tree (Fig. 3*c*).

The second pathway involves the progressive degeneration of affected cells, but with the retention of BR88-positive material. The small compact BR88- and BR135-positive tangle-bearing cells enlarge to take the characteristic shape of large extracellular tangles, perhaps through the action of processes from the astrocytes that cover them (22, 23). Astrocytes also surround plaques (26), which contain microglia within them (27). It appears probable that glial cells are required for the complete processing of β-amyloid precursor and the possible turnover of extracellular tangles.

It is implied in our interpretation that the events leading to plaque and tangle formation are related. One might envisage a connection through a biochemical link between the cytoplasmic tails of β-amyloid precursors and components of the cytoskeleton, which, if disturbed, leads to abnormal changes in both systems. Tau protein probably constitutes only part of the core of the paired helical filament of Alzheimer disease and the nature of the remainder of the filament is as yet unknown. Whether tau directly contributes to the assembly of paired helical filaments or whether it becomes merely passively attached to the filament core inside remains to be discovered.

Against the above view is the reported absence of neocortical tangles and the presence of numerous amyloid plaques in some patients with Alzheimer disease (28, 29). However, the same patients had numerous plaques and tangles in the hippocampal formation, suggesting that more than one mechanism may lead to amyloid plaque formation in some patients with Alzheimer disease. Abundant plaques and tangles are also found either alone or in combination in various brain regions in diseases other than Alzheimer disease. Thus, β-amyloid deposits are observed in cerebral blood vessels and brain in the Dutch form of cerebral amyloidosis (30, 31) and in cerebral blood vessels in arteriovenous malformations (32), whereas tau protein-immunoreactive neurofibrillary tangles are seen in subcortical regions in progressive supranuclear palsy (33) and in Hallervorden-Spatz disease (34). β-Amyloid precursors and the microtubule-associated tau proteins are found normally in nerve cells throughout the central nervous system. The substrates for plaque and tangle formation are thus ubiquitously distributed and it may not be surprising if the two abnormalities are found either alone or

in combination in different brain regions in various diseases. What distinguishes the different diseases may be the mechanisms leading to plaque or tangle formation. It follows that in a disease such as the Dutch form of cerebral amyloidosis amyloid plaques can exist in the absence of neurofibrillary tangles, whereas in Alzheimer disease both abnormalities are found.

Taken together, the major result of the present study is that some nerve cells containing neurofibrillary tangles in their interior have exposed on their surface epitopes for the amino-terminal region of β-amyloid that are not found in normal cells. The presence of the latter probably results from the proteolytic cleavage of β-amyloid precursor while still anchored in and dispersed over the cell surface and thus presumably represents an early stage in the formation of β-amyloid. This suggests that senile plaques and neurofibrillary tangles, although biochemically distinct, may be closely related in origin.

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