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Ferritin is a component of the neuritic (senile) plaque in Alzheimer dementia *

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Summary. A strong immunoreactivity for ferritin was observed in the neuritic (senile) plaques in Alzheimer's disease hippocampus. The ferritin accumulation was almost exclusively associated with the microglia, which appeared to have proliferated greatly. These cells were also positive for HLA-DR, a putative marker for reactive microglia. In contrast, in the diffuse plaques, which were without neuritic pathology, the ferritin-stained microglia appeared to be normal. Microglia were seen frequently in contact with neurons undergoing neurofibrillary changes but only the tangles in the extracellular space were ferritin positive. No ferritin was detected, by Western blots, in paired helical filaments isolated from Alzheimer's disease brain, suggesting that ferritin was most likely weakly associated with and was not a constituent of these fibrils. No correlation between increased ferritin/microglia activity and blood-brain barrier leakage was detected. Ferritin, an iron-storage protein, might have a role in the formation of amyloid through the action of free radicals generated during the release of iron from the ferritin molecule. Alternatively, the ferritin/microglia system might be secondarily involved in the removal and processing of the amyloid.

Key words: Microglia – Amyloid – Ferritin – Paired helical filaments – Alzheimer's disease

Alzheimer's disease/senile dementia of the Alzheimer type (AD) is a progressive dementing disease of adult and late life. Its most characteristic neuropathological lesions are intraneuronal neurofibrillary tangles of abnormal fibrils, the paired helical filaments (PHF), and neuritic (senile) plaques in the neuropil. The most prominent component of the plaques is the amyloid, which is surrounded by or intermixed with degenerating or dystrophic neurites that frequently contain PHF. A neuritic plaque also contains one or more astroglia and microglia [20, 28]. The plaque amyloid consists of a mass of fine 4 to 8 nm fibrils, which are birefringent in polarized light when stained with Congo red [17]. These fibrils are composed of a 40–42 amino acid peptide, the A4 or β-peptide [9, 15]. Immunostaining with antibodies to the β-peptide has revealed, in addition to the amyloid accumulations in the center of the neuritic plaques, large numbers of diffuse amyloid-positive deposits in apparently unaltered regions of the neuropil [20, 24, 30]. At the electron microscopic level these plaques consist mostly of amorphous extracellular deposits and some fibrillar material reminiscent of amyloid fibrils [27]. The diffuse plaques are noncongophilic. They might represent either early stages in the process of amyloid formation or a variant that does not form the β-pleated sheets.

In addition to the histopathological changes, other abnormalities of the AD brain have been detected, primarily by biochemical or biophysical analysis. Of special significance to the AD neuropathology may be the trace metal aluminum, which has been found by X-ray microanalysis and wave-length dispersive spectrometry in the tangles and the plaque core amyloid [18, 19]. Interestingly, in a recent study, significantly higher amounts of ferritin-bound aluminum concomitant with slightly elevated levels of ferritin have been detected in two AD brains [6]. The increase of ferritin may be significant from different points of view. Ferritin, an iron-binding protein, also effectively binds other metallic ions such as zinc, beryllium, and aluminum and is, therefore, thought to play a role in the defense of the brain against toxic metals [13]. Furthermore, a role of ferritin via the release of Fe(II) in the peroxidative inactivation of enzymes, membranolysis, and nonenzymatic dephosphorylation has been postulated [13].

The immunocytochemical studies reported here show that ferritin is markedly increased in those microglia which are closely associated with the amyloid of the neuritic (senile) plaques and, to a lesser degree, with the neurofibrillary tangles in the extracellular space.

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Materials and methods

Antisera

Antisera to ferritin isolated from human brain and liver [6] were raised in New Zealand White rabbits. Two milligrams of ferritin in 1 ml of 5 mM Na_2HPO_4/Na_3PO_4, pH 7.0 was emulsified with 1 ml complete Freund's Adjuvant and injected subcutaneously at six separate sites on the rabbit's back. A single booster injection of 2 mg was given 6 weeks after the initial ferritin injection. After 1 month blood was collected at 2-week intervals over a period of 3 months. The IgG fraction was precipitated from the sera at 50% ammonium sulfate saturation (4° C) and was resuspended in phosphate-buffered saline to the original serum volume. For immunocytochemistry and immunoblots the anti-brain ferritin "serum" was used at 1:4000 and the anti-liver ferritin "serum" at 1:1000 to 1:2500.

The generation and characterization of rabbit antiserum to bovine microtubule-associated protein tau (92e, 1:8000) has been previously described [10]. Rabbit antiserum to glial fibrillary acidic protein (GFAP; 1:1000) was purchased from Accurate Chem., N.J. and to human IgG (1:3000) from DAKO Corp., Calif. Monoclonal antibodies anti-HLA-DR (monomorphic) and anti-common leucocyte antigen (CD45), were both from Becton Dickinson, Calif.; MRP 14 [2] was a gift from Dr. C. Sorg, University of Münster, Federal Republic of Germany.

Immunoblots

Immunoblots were carried out as previously described [11]. In short, protein samples were electrophoresed on SDS-polyacrylamide gels [8 × 6 × 0.75 cm, 5% – 15% acrylamide gradient, transferred to Immobilon membranes, (Millipore, Mass.)], and probed with the antisera. Bound antibodies were visualized using alkaline phosphatase-conjugated anti-rabbit immunoglobulin (BioRad, Calif.) and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as substrate.

Immunocytochemistry

For this study the following tissue was used: Hippocampus from nine neuropathologically confirmed AD cases (56 – 87 years), seven controls (27 – 83 years); both neurological and non-neurological cases, and a biopsy specimen from normal peritumoral tissue adjacent to a glioblastoma. All tissues had been fixed in neutral formalin for 3 to 14 days and embedded in paraplast. Tissue sections (6 µm thick) were deparaffinized in Histoclear (National Diagnostics, N.J.), rehydrated and incubated overnight at 4° C with the diluted antisera as previously described [10]. The bound antibodies were detected using either the Vectastain ABC kit (Vector, Calif.) or biotinylated species-specific anti-rabbit immunoglobulins (Amersham, Ill., 1:200) and avidin/horseradish peroxidase complex (Sigma, Mo.; 14 mg/ml) followed in each case by development with 0.05% (w/v) 3,3′-diaminobenzidine and 0.01% (v/v) H_2O_2. In double-staining experiments the sections were re-incubated with the second primary antibody and developed by the peroxidase-antiperoxidase technique [23] using as substrate 0.01% (w/v) 3,3′-diaminobenzidine, 0.6% (w/v) nickelammonium sulfate, 0.05% (w/v) imidazole and 0.0003% H_2O_2 (v/v) [25]. This substrate produced a dark purple color which could be differentiated from the brown color obtained after staining with the first primary antibody. Slides developed with this technique were washed in 10 mM phosphate buffer, pH 7.4, air dried and mounted in Permount (Fisher, N.J.). In some cases sections were counterstained with periodic acid-Schiff's reagent after the immune reaction to visualize the amyloid.

Results

On immunoblots, anti-brain ferritin and anti-liver ferritin sera labeled both the 19-kDa and 22-kDa subunit bands of isolated brain and liver ferritin (Fig. 1). No reactivity of the antiserum to liver ferritin was observed with a fraction rich in normal cytoskeletal proteins or to preparations of SDS-washed PHF that had been isolated by the long method of Iqbal et al. [12] (Fig. 1, panels B&C, respectively). In contrast, brain ferritin serum contained antibodies to ferritin and a variety of other brain proteins including the GFAP (Fig. 1, panel A). On tissue sections, the anti-brain ferritin serum labeled all astroglia strongly and indiscriminately. For this reason, in the immunocytochemical studies that follow, only anti-liver ferritin serum was used.

In biopsy material from human cortex and subcortical white matter, a prominent staining of oligodendrocytes including their fine processes was noted, similar to that described recently by Gerber and Connor [8]. In addition, somewhat weaker staining of microglial cells and of some astrocytes was found. In autopsy material of normal hippocampus, ferritin reactivity was almost exclusively associated with microglial cells (identified by their positive reaction with anti-CD45 and MRP 14), which were randomly distributed in the gray matter (Fig. 2a). In addition, occasional reactive astroglia were observed. In all these cases, some diffuse reactivity of oligodendroglia, especially in the white matter, was observed. The reaction, however, was weaker and less consistent compared with the biopsy material. The intensity of ferritin immunostaining varied considerably from tissue to tissue, suggesting that autolysis, fixation or other factors might influence the immunological preservation of the ferritin molecules. In tissues with inflammatory infiltrates such as brain tumors and multiple sclerosis, strong staining of macrophages was detected.

On sections of AD hippocampus, clusters of prominent, thick, strongly ferritin-positive processes were distributed abundantly over the area containing the neurofibrous degenerations and amyloid accumulations within the neuritic plaques (Fig. 2b). Frequently, these processes originated from small cells, most of which could be identified as microglia by their shape and characteristic elongated nucleus. In addition, cells with identical shape and distribution were stained with anti-CD45, anti-HLA-DR, or MRP 14 (data not shown). In all cases, staining of the structures in and around the plaques started to appear almost immediately after the sections were placed into the substrate and long before staining of other non-plaque-associated microglia occurred. Frequently, the arborized processes were seen to originate from expanded cell bodies with nuclei apparently rounder and slightly larger than those of the typical microglia. A comparison with the GFAP-positive cells showed, however, that the ferritin-positive cell population was clearly distinct from the typical astrocytes, which were mostly at the periphery of the plaques (Fig. 2c). In sections that had been counterstained with periodic acid-Schiff's reagent to visualize the amyloid, the ferritin-positive processes were always observed in close association with both the vascular amyloid and the amyloid in the center of the neuritic plaques. In plaques containing relatively sparse wisps of amyloid, the amyloid was interspersed with ferritin-positive cells and processes (Fig. 3a). The typical star-shaped amyloid in the center of the neuritic plaques

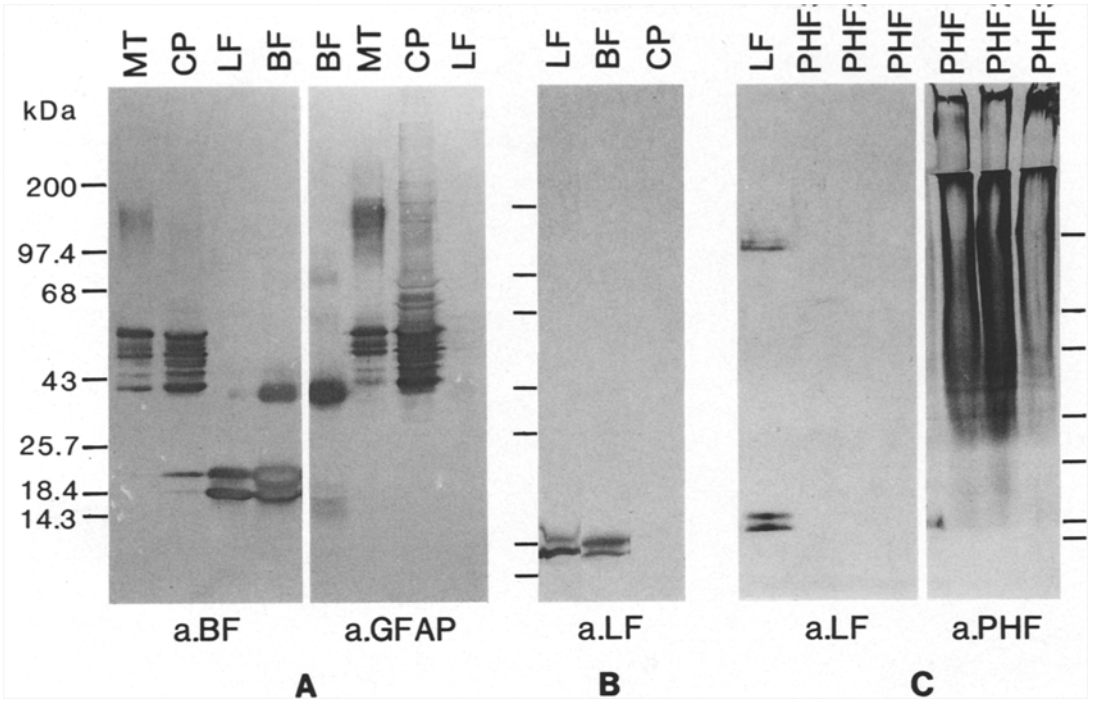


Fig. 1 A, B. Immunoblots of polypeptides separated by SDS-PAGE. **A** Immunostaining with anti-brain ferritin (*a.BF*) and anti-glial fibrillary acidic protein (*a.GFAP*) sera; **B** anti-liver ferritin (*a.LF*) sera, and **C** aLF and monoclonal antibodies 5-25/3-39 [26] mixture to PHF (*a.PHF*). The amounts of antigens electrophoresed per lane were: human brain microbubules (*MT*), 10 μg; cytoskeletal preparation (*CP*), 10 μg; liver ferritin (*LF*), 2 μg; brain ferritin (*BF*), 10 μg and 3 different preparations of PHF (*PHF1, PHF2, PHF3*), 6 μg each. CP was prepared by extracting with 8 M urea the 100000 *g* pellet of an Alzheimer's disease brain homogenate. LF sometimes is seen both in monomeric (19.5 kDa and 22 kDa) and oligomeric (around 200 kDa) forms by SDS-PAGE (**C**, lane *LF*)

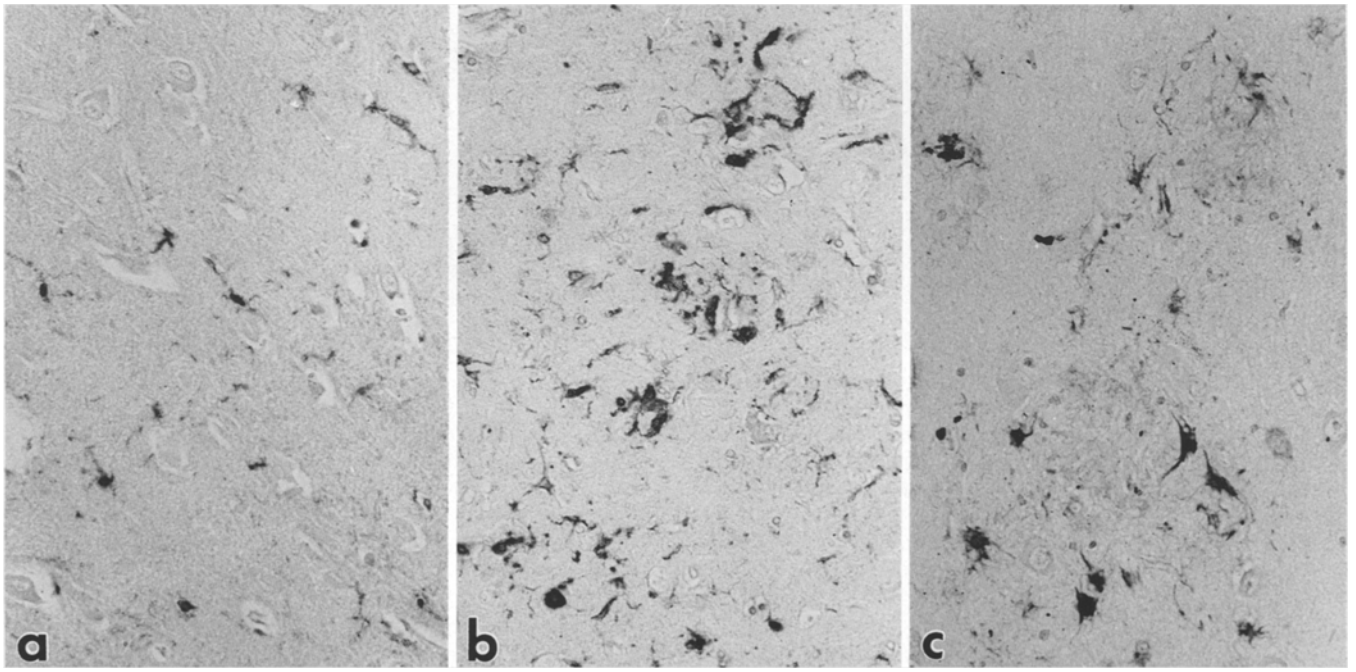
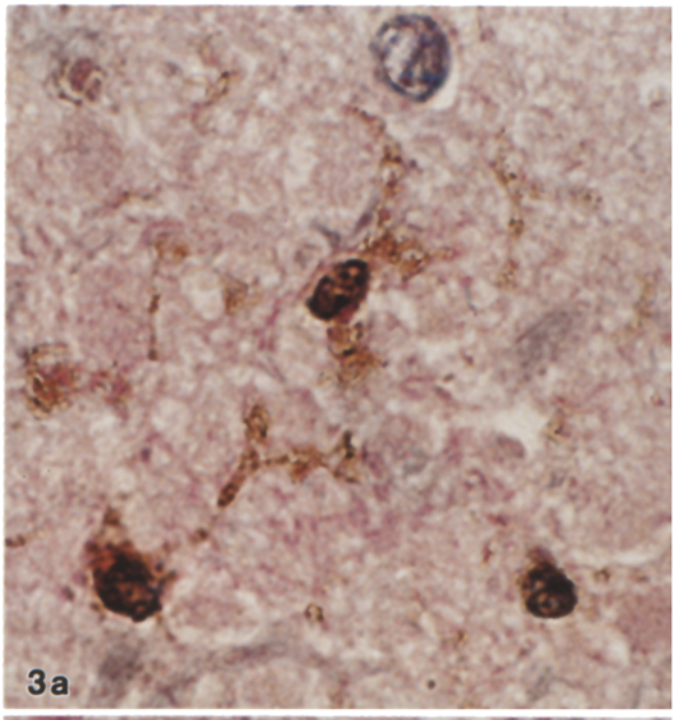
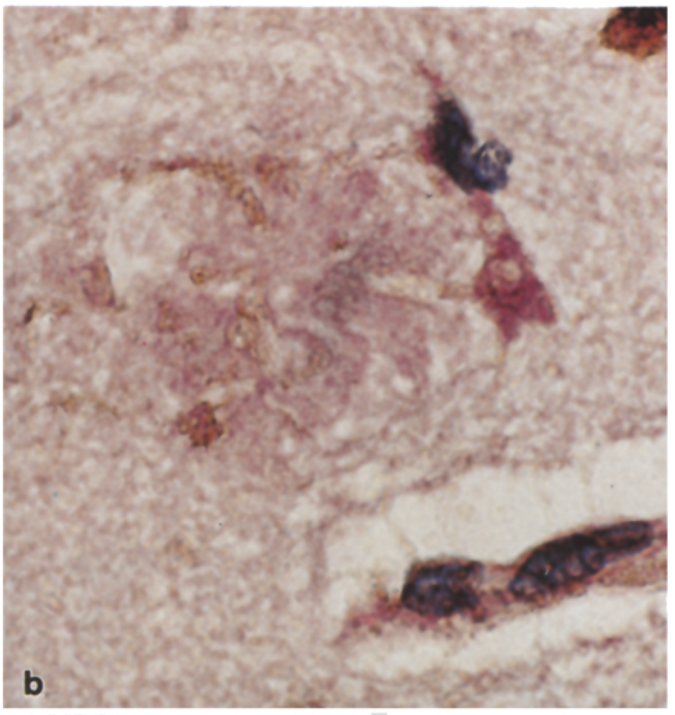
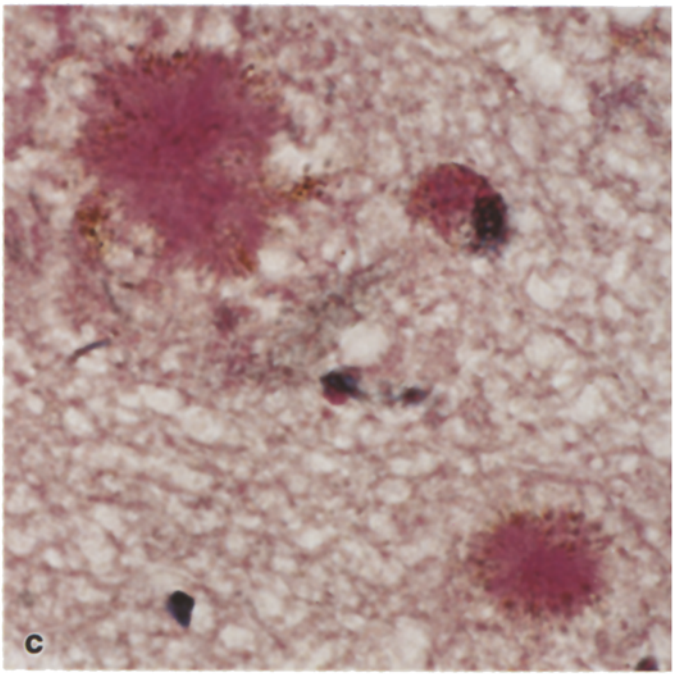


Fig. 2. Immunostaining for ferritin (**a, b**) and GFAP (**c**) in paraffin sections of parahippocampus: **a** ferritin-positive microglia in a histologically normal control, age 38 years; **b** ferritin-positive proliferated cells, mostly microglia, in an 85-year-old AD patient. Note the clusters of ferritin-positive structures within the plaques, whereas on immunostaining with antiserum to GFAP (**c**) the typical astroglia are observed mostly at the periphery of the plaques. **a–c** × 230







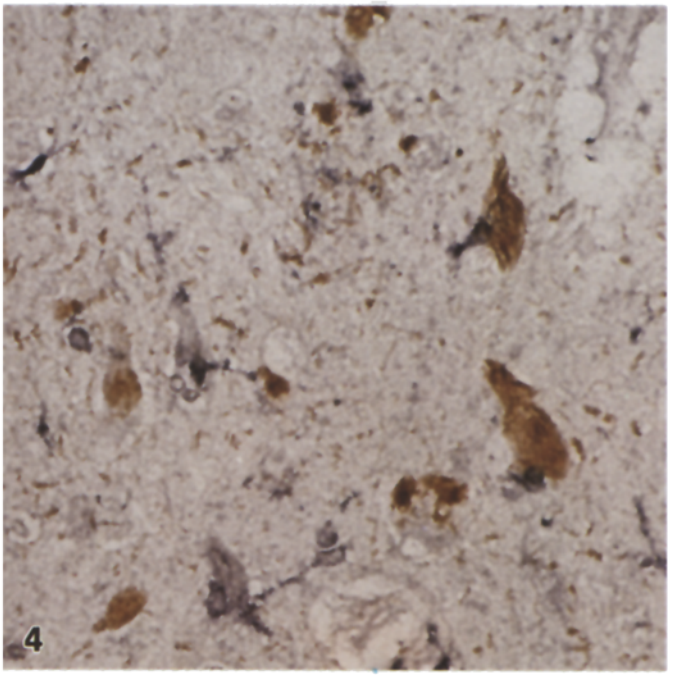


Fig. 3a–c. Ferritin immunoreactivity in plaque amyloid in an AD hippocampus. The immunostained structures are brown, the amyloid is stained red with periodic acid-Schiff's reagent and the nuclei are counterstained blue with hematoxylin. **a** Wisps of amyloid of immature plaque interspersed with ferritin-positive cells and their processes. **b** Ferritin-positive processes in typical amyloid star. At this stage usually no cell bodies are seen within the amyloid. **c** Tightly packed amyloid of so-called burnt-out plaques with ferritin-positive structures at the periphery. **a–c** ×1150

Fig. 4. Double-staining of AD hippocampus with antiserum to tau (*brown*) and to ferritin (*blue*). At *lower right* one seemingly intact brown tau-positive tangle is in close contact with a blue ferritin-positive structure. The brown tangle above is penetrated with several ferritin-positive processes. The two tangles at the *left* are fully ferritin positive with the one at the *lower left* connected to a microglial cell. ×460

contained fewer ferritin-positive processes and usually no ferritin-positive cells (Fig. 3b), whereas in the tightly packed amyloid of the so-called "burnt-out plaques", the ferritin activity was found only at the periphery, toward the ends of the amyloid fibrils (Fig. 3c). In contrast to the neuritic plaques, no accumulation of microglia was observed in the non-congophilic diffuse amyloid plaques in the apparently normal neuropil.

The relationship of the ferritin-positive cell population with the neurofibrous pathology was studied in

sections in which the PHF-containing structures, i.e., tangles, plaque neurites, and neuropil threads, had been visualized by immunostaining with antiserum 92e to tau [3, 10]. In these preparations, blue ferritin-positive processes, most of which originated from a microglia-like cell, were often seen connected with the seemingly intact brown tau-positive tangles. In addition, in the same fields, blue ferritin-positive, tangle-shaped structures reminiscent of tombstones, the extracellular end stage of the tangles, were frequently observed (Fig. 4). However, ferritin associated with the tangles observed by immunocytochemistry is probably only loosely associated because no ferritin reactivity was detected on Western blots even when a large amount of PHF was used (Fig. 1). Double staining with anti-GFAP serum revealed that most of the ferritin-positive tombstones in tissue sections were also positive for GFAP (figure not shown).

As in the hippocampus of AD cases, in the hippocampus of aged non-AD cases, ferritin-reactive structures were also seen in association with the amyloid in the neuritic (senile) plaques. However, in the cases studied so far, the numbers and the intensity of the immunostaining of the microglia and the proliferation of their processes appeared to be less than in AD cases.

To study the relationship of the ferritin-positive structures with the areas of blood-brain barrier damage, parallel sections were stained with anti-ferritin serum and, as an indicator for the permeability of the vessels, with an antiserum to human IgG. By these standards, in both AD and aged control cases alike, diffuse IgG deposits of varying dimensions around some vessels were observed, in agreement with earlier studies [1, 4]. However, no correlation between this staining and the distribution and intensity of ferritin-positive structures was observed (figure not shown).

Discussion

The present study shows that ferritin immunoreactivity, which is present in the normal brain in oligodendroglia [8] and microglia [14], is selectively and dramatically increased in the AD brain in what appears to be mostly activated microglia. Cells of similar shape to the anti-ferritin-positive cells observed here expressed HLA-DR and the leukocyte antigen CD45 and were stained by the macrophage marker MRP 14. Similar cells in neuritic (senile) plaques of AD brains have been described recently to express various monocyte/macrophage antigens [16, 21, 22]. Although the origin and nature of microglial cells is still a matter of debate, the expression of surface antigens involved in effector cell function, e.g., Fc receptors, complement receptors, HLA-DR and adhesion molecules [7, 16, 21, 22] and monocyte/macrophage-specific antigens [5], indicates that these cells are of mesodermic origin and are specialized forms of macrophages. As shown in the present study, these cells, like their blood-borne counterparts, also contain ferritin. The increase in ferritin reactivity in AD is primarily restricted to the areas of the greatest neurofibrillary pathology and is closely interconnected with both the tangles and the neuritic (senile) plaques.

Biochemical studies have shown that ferritin isolated from AD brain contains on molar basis five to six times more bound aluminum than that from control brains [6]. In light of our data showing the close interrelationship of the microglia with both tangles and plaques, it is conceivable that the ferritin-bound aluminum of the microglia might have contributed to the increased amounts of aluminum reported previously in tangles and plaques by X-ray microanalysis [18, 19]. In addition, more soluble ferritin was obtained from AD than from age-matched non-AD brains [6]. This finding is in agreement with the prominent ferritin staining of the proliferated microglia in AD brain sections observed in this study.

The prominence of ferritin in the stimulated microglia might be of functional consequence. Ferritin was first recognized as the major protein of iron storage and transport. Recent evidence suggests that this protein may have diverse biological functions [13]. Fe(III) stored in ferritin is released after reduction to Fe(II). The oxygenated free radicals generated during this reduction self-propagate by interacting with transition metal ions such as copper and iron. These free radicals are known to react with, and thereby modify, several cellular components. Proteins modified by such reactions would become inactive and more prone to proteolysis during phagocytosis by the microglia. The connection of the ferritin-positive microglial processes with the tombstones and the labeling of the tombstones themselves with ferritin most probably indicate the involvement of the microglia, in addition to the astroglial cells, in the removal of the tangle from the neuropil after death of the neuron.

The largest increase in ferritin immunoreactivity, however, seemed to be associated not with the tangles but with the amyloid of the neuritic plaques. This impression is based upon both the strong proliferation of the microglial processes and the increased intensity of the ferritin immunostaining in the plaques. The activation of the microglia in the vicinity of the plaques might indicate their involvement in the removal of blood components entering the neuropil because of an increased permeability of the capillaries within the plaques. However, previous immunocytochemical studies had demonstrated the presence of various blood proteins both within and outside the areas of the plaques in AD patients and aged controls [1, 4]. The proliferation of the ferritin-positive microglia might also reflect their active role in the formation of the amyloid, as suggested by Wisniewski et al. [29], who showed at the electron microscopic level amyloid fibrils within the distended cisternae of the rough endoplasmic reticulum of the microglia. In this case, the microglia might serve as processors of either internally synthesized or phagocytized amyloid precursor protein to β-peptide, which would polymerize into amyloid fibrils and then be deposited by these cells in the neuropil as plaque amyloid. However, at this point, it is difficult to link this scenario with the diffuse plaques, where most of the amyloid-reactive protein seems to be extracellular and the number and shape of the ferritin-positive microglia

appears to be normal. Is the initial event that induces the proteolysis of the precursor into amyloidogenic fragments outside the microglia? Are these cells only secondarily involved in the removal and processing of the amyloid? Or is there a second, defective population of microglia that because of their specific metabolic make up – possibly with the involvement of ferritin – would be instrumental in the production of the plaque amyloid? At this stage of our research, both possibilities are about equally likely, and significant further information is needed to consolidate any hypothesis.

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