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Alzheimer's Disease

A Double-Labeling Immunohistochemical Study of Senile Plaques

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The senile plaque is one of the histopathologic changes that characterizes Alzheimer's disease and the aging brain. The histopathology of senile plaques was studied using double-labeling immunohistochemistry and lectin histochemistry with thioflavin S fluorescent microscopy in 9 cases of Alzheimer's disease, 2 nondemented elderly individuals, and 3 individuals with non-Alzheimer primary degenerative dementias. Every plaque that was visualized with thioflavin also had amyloid, but not all thioflavin-positive plaques contained neurites that could be recognized with specific monoclonal antibodies to paired helical filament, tau, or neurofilament epitopes. Some neurofilament-positive neurites were not visualized with thioflavin, but almost all tau-positive neurites were colabeled with thioflavin. Microglia were associated with most plaques. Most plaques were also surrounded by fibrous astrocytes. These results suggest that amyloid may be the common feature that defines senile plaques, but that other elements may be more specific for Alzheimer's disease, because extensive neuritic degeneration was seen only in Alzheimer brains and not in either nondemented elderly individuals with senile plaques or in non-Alzheimer dementia cases. (Am J Pathol 1988, 132:86–101)

THE SENILE PLAQUE is perhaps the most characteristic histopathologic feature in Alzheimer's disease and senile dementia of the Alzheimer type (AD/SDAT). Because some elderly demented patients may have neocortical senile plaques but no neurofibrillary tangles, and have all other biochemical and morphometric abnormalities of patients with both plaques and tangles,[1] one could argue that senile plaques are the *sine qua non* of AD/SDAT. Senile plaques are, however, also detected in the brains of aged individuals who do not suffer from dementia,[2] and in the brains of some aged mammals.[3,4]

Senile plaques are heterogeneous structures[5,6] with a distribution in the brain that is nonuniform. In the neocortex they are most numerous in higher order association cortices, where they are concentrated in the middle and upper cortical laminae, suggesting that they may derive from degeneration of cortico-cortical afferents.[7,8] They are considerably less frequent in primary motor and sensory cortices. Regions of the brain that, almost without exception, have many senile plaques include the hippocampus and amygdala.[9] They are also present in the diencephalon[10] and the cerebellum[11,12] more frequently than was once suspected.

Various staining methods can be used to detect senile plaques that reflects their compositional heterogeneity. They can be demonstrated with stains for neurofibrous elements such as the Bodian and Bielschowsky stains. These stains reveal the degenerating neuritic processes commonly found in the periphery

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of plaques. Plaques can also be seen with stains for sulfated acid mucopolysaccharides, such as Alcian blue.[13] They are perhaps best visualized with stains for amyloid, including Congo red[14] and thioflavin S.[15] The latter staining method is sensitive, but less specific for amyloid than Congo red.[15] Thioflavin S stains elastic fibers, myelin, and filaments in ependymal and choroid plexus epithelium, elements that are clearly neither amyloid nor amyloidlike.[15] Thioflavin also labels many neurites in senile plaques, presumably due to binding to amyloidlike fibrillar elements such as paired helical filaments (PHF)[4,5,16] that are the major component of neurofibrillary tangles.[17]

This study used double-labeling immunohistochemical methods and antibodies or lectins to stain neurites, amyloid, and glia in an investigation of the pathogenesis of senile plaques. Our results suggest that amyloid may be the feature that is common to all plaques, sometimes to the exclusion of significant neuritic involvement.

Materials and Methods

Nine cases of Alzheimer's disease, 3 cases of non-Alzheimer primary degenerative dementia, and 2 cases of nondemented old people were studied. Table 1 lists pertinent clinical features.

At the time of autopsy 0.5-cm-thick sections of hippocampus, including the parahippocampal gyrus, at the level of the lateral geniculate nucleus were rapidly frozen on a freezing bar in a cryostat kept at a constant temperature of −20 C. Consecutive 7–10-μ-thick sections were cut, air-dried, and kept at −20 C until staining was performed.

Table 1—Clinical Features

Age	Sex	Post-mortem interval (hours)	Pathological findings
Alzheimer's disease			
80*	M	6	Alzheimer's disease with cerebellar and basal ganglia plaques; amyloid angiopathy; lacunar infarct in caudate; incidental Lewy bodies
80	F	12	Alzheimer's disease with basal ganglia plaques
60	M	6	Alzheimer's disease with basal ganglia and cerebellar plaques; amyloid angiopathy; anoxic changes
78	M	10	Alzheimer's disease; subdural membrane
76*	F	10	Alzheimer's disease with basal ganglia plaques
84*	M	3	Alzheimer's disease
86	F	4	Alzheimer's disease
78	F	13	Alzheimer's disease
76*	F	10	Alzheimer's disease
Non-Alzheimer primary degenerative dementia			
53	F	8	ALS-dementia
74*	M	3	Non-Pick lobar atrophy (frontal)
54	M	8	Non-Pick lobar atrophy (frontal)
Nondemented elderly			
73	M	8	Hypertensive vascular disease; lacunar infarcts in basal ganglia and thalamus
89*	F	3	Senile changes of the Alzheimer type (plaques only type)

* Indicates patient prospectively studied with neuropsychological and neurological assessments.

Immunofluorescence

The sections were fixed in acetone for 10 minutes and washed in 10 mM phosphate buffered saline, pH 7.4 (PBS), before incubation in 5% normal serum to block nonspecific staining. Diluted antibodies or monoclonal supernatants were incubated for 2 hours at room temperature. Antibody binding was detected with rhodamine-conjugated F_{ab} immunoglobulin directed to the appropriate species (goat anti-rabbit, 1:100, Axell; rabbit anti-mouse, 1:100, Cappell). The sections were then counterstained with thioflavin S (0.01% in 10% phosphate-buffered formaldehyde) for 10 minutes, decolorized with 70% ethanol, and mounted in glycerol. Sections were incubated without primary antibodies as negative controls.

Lectin histochemistry was used to identify microglial cells. The lectin, *Ricinus communis* agglutinin I (RCA-I), has been shown in previous studies to be a specific marker for microglial cells in the brain of normal humans.[18] Rhodamine-labeled RCA-I (Vector Labs) at 5 μg/ml was incubated on sections for 4 hours at room temperature. Control slides were incubated without the lectin or were incubated with the lectin after absorbing the lectin with beta-D-galactose. Some sections were treated with neuraminidase (Sigma, 5 U/ml, in pH 5.0 acetate buffer, at 37 C). The slides were counterstained with thioflavin S as above.

Lectin Peroxidase/Amyloid Double Staining

Four Alzheimer cases, 1 case of non-Alzheimer primary degenerative dementia, and 1 nondemented old person were studied with lectin peroxidase methods. The tissue was fixed and embedded using the AMeX method.[19] At the time of autopsy, 0.5-cm-thick sec-

tions of frontal cortex, visual cortex, hippocampus, and basal forebrain were placed in acetone and fixed overnight at −20 C, then 15 minutes at 4 C, and finally at room temperature for 15 minutes. The tissue was clarified in absolute methyl benzoate for 30 minutes and xylene for 30 minutes, then embedded in paraffin. Seven-micron-thick sections were deparaffinized in xylene and acetone before incubation in buffer. Nonspecific peroxidase reaction was blocked with 0.3% H_2O_2 for 30 minutes. Tissue sections were incubated with biotinylated RCA-I (Vector Labs) at 5 μg/ml for 4 hours at 37 C or for 18 hours at 4 C. Lectin binding was detected with the avidin–biotin peroxidase complex from a Vector ABC kit. The chromagen for this step was diaminobenzidine. The sections were counterstained with antiserum to a synthetic peptide of cerebral amyloid using the peroxidase-anti-peroxidase method. The second chromagen was 4-chloronapthol rather than diaminobenzidine.

Antibodies

Several antisera raised in rabbits to synthetic peptides derived from the amino acid sequence of the amyloid beta protein[20] were used. These were generous gifts of Dr. J. Kates (University of California at San Francisco) (antisera 3980 and 3981)[21] and the Cetus Corporation, San Jose, CA, (antisera 1a and 2a). All 4 antisera gave essentially similar staining patterns. In this report antibody 3981 was used at a dilution of 1:100.

A rabbit antiserum to glial fibrillary acidic protein (GFAP) was the generous gift of Dr. J. E. Goldman (Columbia University) and was used at a dilution of 1:250.

Mouse monoclonal antibodies used in this study have been characterized previously.[22,23] Antibody 39 (Ab39) was used to label paired helical filaments. This antibody was generated in mice immunized with Alzheimer neurofibrillary tangle (ANT) preparations. It binds to high molecular weight proteins that are excluded from sodium dodecyl sulfate (SDS)-polyacrylamide gels in Alzheimer's disease, but does not recognize any normal proteins of either human or animal brains.[22,23] Immunoelectron microscopic studies have localized the epitope to PHF.[24] Antibody 175 (Ab175) was similarly generated. It, however, binds to several midmolecular weight proteins in both Alzheimer and normal brain homogenates that comigrate with tau proteins.[22] It also binds to bovine tau proteins.[23]

NP18 is a monoclonal antibody that has been characterized extensively.[25] It binds to a phosphorylated neurofilament epitope that is present in the carboxy terminal, head domain of the 200 and 160 kd neurofilament proteins. It does not bind to the 68 kd neurofilament protein. It, like many (if not all) neurofilament antibodies that bind to Alzheimer neurofibrillary tangles, cross-reacts with tau proteins from both human and animal brains.[26] NP18 binds to both axons and neurofibrillary tangles in frozen sections and routinely fixed paraffin sections.[27]

NP15 is a monoclonal antibody generated at the same fusion as NP18.[28] It binds to a phosphorylated epitope in the high molecular weight (210 and 160 kd, but not 68 kd) neurofilament proteins. In contrast to NP18, the phosphorylated epitope is relatively insensitive to the effects of alkaline phosphatase.[26,27] It stains axons in white matter even after treating the sections with alkaline phosphatase, but only reacts with neurofibrillary tangles if sections have been pretreated with trypsin.[26] It also shows some cross-reactivity with tau proteins from animals, but not to human tau proteins.[26] Since trypsin is not used in these current studies, NP15 recognizes only neurofilament epitopes.

Quantitation

Semiquantitative evaluation of immunostaining was attempted. The slides were examined with fluorescein filters to identify thioflavin-stained senile plaques of a particular morphologic subtype (see below); the same plaque was then examined on the rhodamine filter to assess the intensity of antibody or lectin binding. The amount of fluorescence of each plaque was scored on a 0–3+ basis depending on the number of processes or intensity of amyloid staining within the boundaries of the plaque, as defined by thioflavin. Over 1500 individual plaques were scored. The scores were tabulated per plaque subtype; the average score was rounded off for each antibody and plaque subtype (see Table 2). A score of less than 0.5 was rounded to zero.

Nomenclature

The terminology for senile plaque subtypes is that of Terry and Wisniewski.[6] Plaques with well-defined amyloid cores, a zone of absent staining, and a "neuritic" halo are called "classical" plaques. Those apparently composed only of an amyloid core are called "burnt-out." They have also been called "compact senile plaques."[15] Plaques without a well-defined amyloid core are "primitive." In the course of this and other studies of senile plaques,[29,30] it has become apparent that plaques without well-defined amyloid

Table 2—Summary of Immunohistochemistry and Lectin Histochemistry

	Senile plaque subtype			
	Very primitive	Primitive	Classical	Burnt-out
3981 (antiserum to amyloid beta-protein)	+++	+++	+++	+++
RCA-I (lectin that stains microglia)	++	++	++	+
GFAP (antiserum that stains fibrous astrocytes)	+	+++	+++	+
Ab39 (monoclonal antibody to epitope in PHF)	+	+++	++	−
Ab175 (monoclonal antibody to epitope in tau and PHF)	++	+++	+++	−
NP18 (monoclonal antibody to epitope in neurofilament, tau and PHF)	+	++	++	+
NP15 (monoclonal antibody to epitope in neurofilament)	+	++	+	+

	Staining of neurofibrillary tangles and cell processes			
	Neurofibrillary tangles	Axons	Gray matter neurites	
			Thioflavin (+)	Thioflavin (−)
Ab39	+++	−	++	−
Ab175	++	−	+++	+
NP18	+	++	+	++
NP15	−	++	−	++

RCA-I, *Ricinus communis* agglutinin I; GFAP, glial fibrillary acidic protein; PHF, paired helical filament; Thioflavin (+), processes were stained by both thioflavin S and the antibody; Thioflavin (−), processes were stained with the antibody, but not thioflavin.
See text for semi-quantitative scoring system for senile plaque subtype. Scoring of neurofibrillary tangles and processes was subjective.

cores can be subdivided into those with "neurites" and those without "neurites." The latter plaques have been referred to as "very primitive plaques"[31] or as "pale senile plaques."[15] They often have a reticulated or granular staining pattern on thioflavin fluorescence, while the "primitive plaque" has coarse neurites in addition to the granular staining. These 2 types of plaques can be clearly distinguished with Bielschowsky's staining method (Figure 1) and less easily with thioflavin S fluorescent microscopy. This distinction may be of importance since senile plaques with neurites (Figure 1a) are more common in demented subjects, while very primitive plaques (Figure 1b) may be found in great numbers in nondemented elderly individuals.[29]

Results

Results of antibody and lectin staining of senile plaques with respect to senile plaque subtype are summarized in Table 2.

3981 (Amyloid)

All senile plaques that could be identified with thioflavin, regardless of their morphologic subtype and including very primitive plaques, were immunostained with antibodies to the synthetic peptide of cerebral amyloid (Figure 2). The staining pattern was essentially the same as thioflavin fluorescence, except that neuritic processes and neurofibrillary tangles were not labeled. Very primitive plaques seemed to lack discrete neurites on thioflavin staining; both thioflavin and antibody staining was reticular or finely granular. Burnt-out plaques and amyloid in blood vessels (not shown) were also immunostained. In the non-Alzheimer degenerative dementias and in the normal elderly individuals, only a few senile plaques were stained. We did not see staining of neurofibrillary tangles with the antibody to synthetic peptide.

RCA-I (Microglia)

Both fluorescent histochemistry of frozen sections and fluorescent and peroxidase histochemistry on paraffin (AMeX) sections gave essentially similar results; however, the cellular characteristics had better definition on paraffin sections. Virtually every senile plaque that was stained with thioflavin or the antibody to synthetic peptide (3981) had a microglial cell or cell processes in the plaque (Figures 3 and 4). This was true even for the very primitive plaques (Figure 4) and burnt-out plaques that had no apparent neuritic element on thioflavin S. In plaques with compact amyloid cores, the lectin sometimes showed weak binding to the amyloid. RCA-I staining was eliminated by preabsorbing the lectin with beta-D-galactose. Neuraminidase treatment of sections also diminished lectin binding.

The cells labeled with RCA-I had delicate branching processes (Figure 4). These cells were more prevalent in gray than white matter, and they had the classical morphology of microglial cells with silver carbonate impregnations.[38,39] They were often closely related

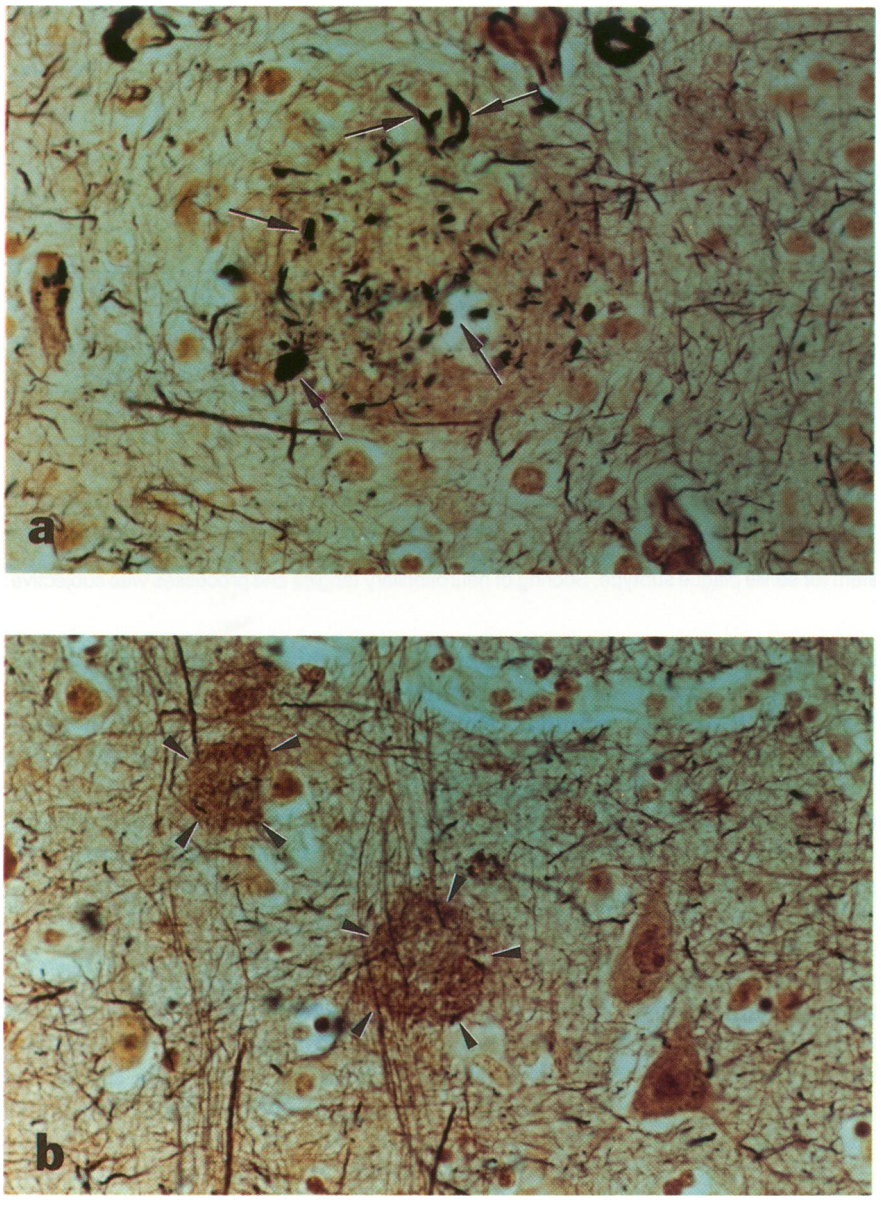

Figure 1—Senile plaque without amyloid cores as seen with Bielschowsky's silver impregnation method. The primitive plaque in a from a demented individual has coarse, black neurites (arrows) on a background of light brown, granular staining; while the "very primitive plaque" in b from a nondemented elderly individual has light brown, granular staining (arrowheads), but no apparent neurites, except for thin processes that seem to be passing through the plaque. (×480)

to blood vessels and sometimes resembled pericytes. Double-labeling studies with fluorescein-conjugated antiserum to detect GFAP and rhodamine-conjugated avidin to detect biotinylated RCA-I showed conclusively that RCA-I was not staining astrocytes (not shown).

RCA-I also stained blood vessel endothelial cells in both the Alzheimer and control cases. It was possible to distinguish between microglial and endothelial cells in almost all cases by morphologic features. Control cases also had microglial staining, but less than in Alzheimer's disease. The staining of blood vessels by RCA-I revealed that most, if not all, senile plaques were closely related to and sometimes centered on blood vessels (Figure 4). Further studies are in progress to evaluate the quantitative relationship of blood vessels to senile plaques.

GFAP (Fibrous Astrocytes)

The Alzheimer brains had increased astrocytic gliosis in the cortex compared to nondemented individuals. In addition to subpial and perivascular astrocytes, many senile plaques were labeled. Most of the senile plaques that were labeled with GFAP had thioflavin-positive neuritic elements or amyloid cores, either a

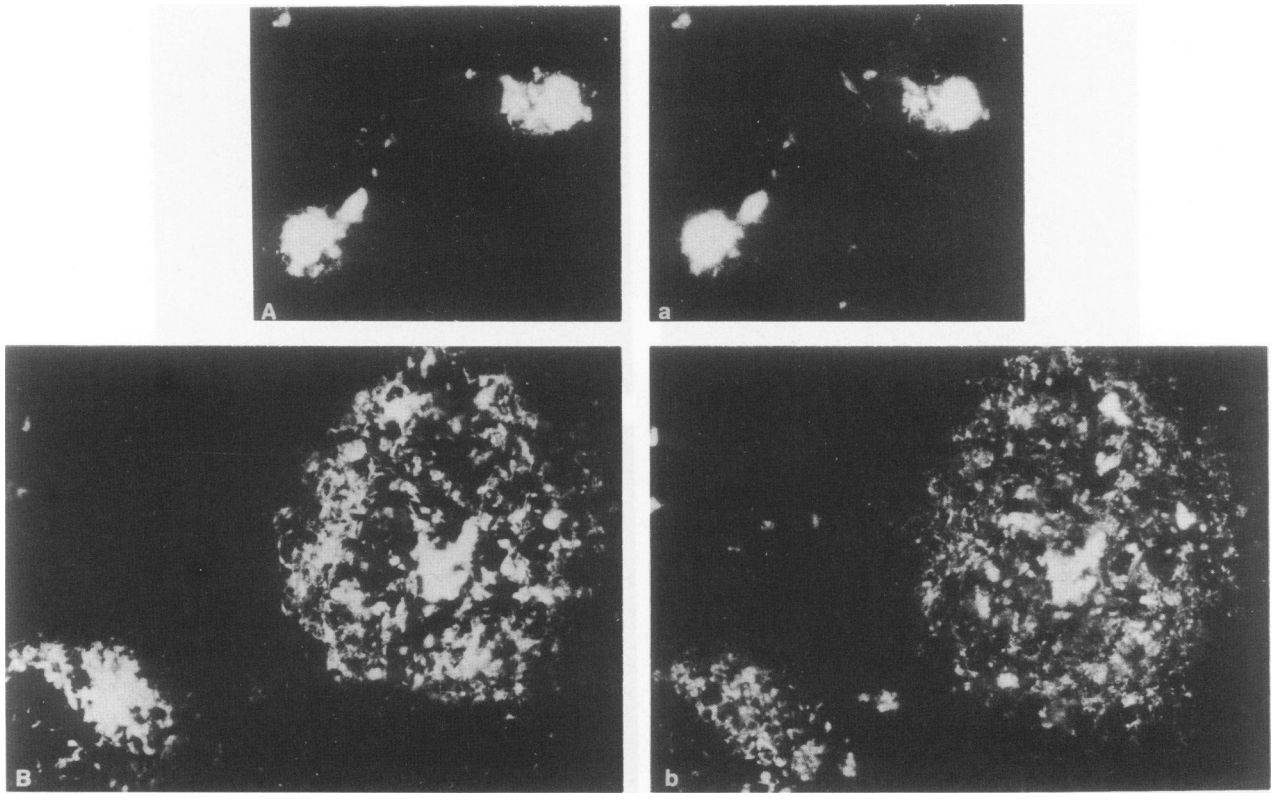

Figure 2—A and B are stained with antiserum to amyloid synthetic peptide (3981) and visualized with rhodamine filters; a and b are the same slides counterstained with thioflavin S and visualized with fluorescein filters. (×448) Note concordance of staining in A and a, and in B and b. In A, compact amyloid deposits define two burnt-out plaques. In the classical plaques in B amyloid is dispersed in a circumscribed area with a compact central core.

single central core or multicentric cores. In most cases cell bodies of fibrous astrocytes were located at the periphery of the plaque (Figure 5) from which they sent penetrating, finger-like processes into the center. Most of the very primitive plaques had little GFAP staining.

Normal brains had GFAP staining confined to the subpial region and the white matter. The non-Alzheimer dementia cases had prominent astrocytic gliosis in both the gray and white matter.

Ab39 (Unique PHF Epitopes)

Ab39 immunostained virtually every neurofibrillary tangle in the Alzheimer brain and the few hippocampal and parahippocampal neurofibrillary tangles in the nondemented individuals. Neurites stained with Ab39 were present in only a fraction of senile plaques (Figure 6). Primitive and burnt-out plaques had few and sometimes no detectable Ab39-positive neurites. Primitive and classical plaques with thioflavin-positive neurites usually had many stained neurites. Senile plaque neurites stained by Ab39 were usually thick and coarsely fibrous. Almost without exception the neurites that were stained with Ab39 also were, at least weakly but more often intensely, stained with thioflavin S. In nondemented individuals, Ab39 stained a few neurites only in a small proportion of some plaques. Nothing was immunostained in the non-Alzheimer dementia cases.

Ab39 labeled many neurites in the neuropil of the Alzheimer brain that were not clearly associated with senile plaques. These neurites were also colabeled with thioflavin. Neurites not associated with plaques were rarely seen in old normals. They were absent from the non-Alzheimer dementia cases.

Ab175 (Epitope in PHF and Tau)

The staining of Alzheimer brains with Ab175 was striking, in that innumerable small, delicate processes in the neuropil of the hippocampus and parahippocampal cortex were labeled (Figure 7). The majority of these fine neurites were also stained with thioflavin, but some were clearly not fluorescent with thioflavin. Most of the neuropil neurites were not specifically associated with senile plaques as identified with thioflavin. On the other hand, primitive and classical plaques often had dense accumulations of Ab175-pos-

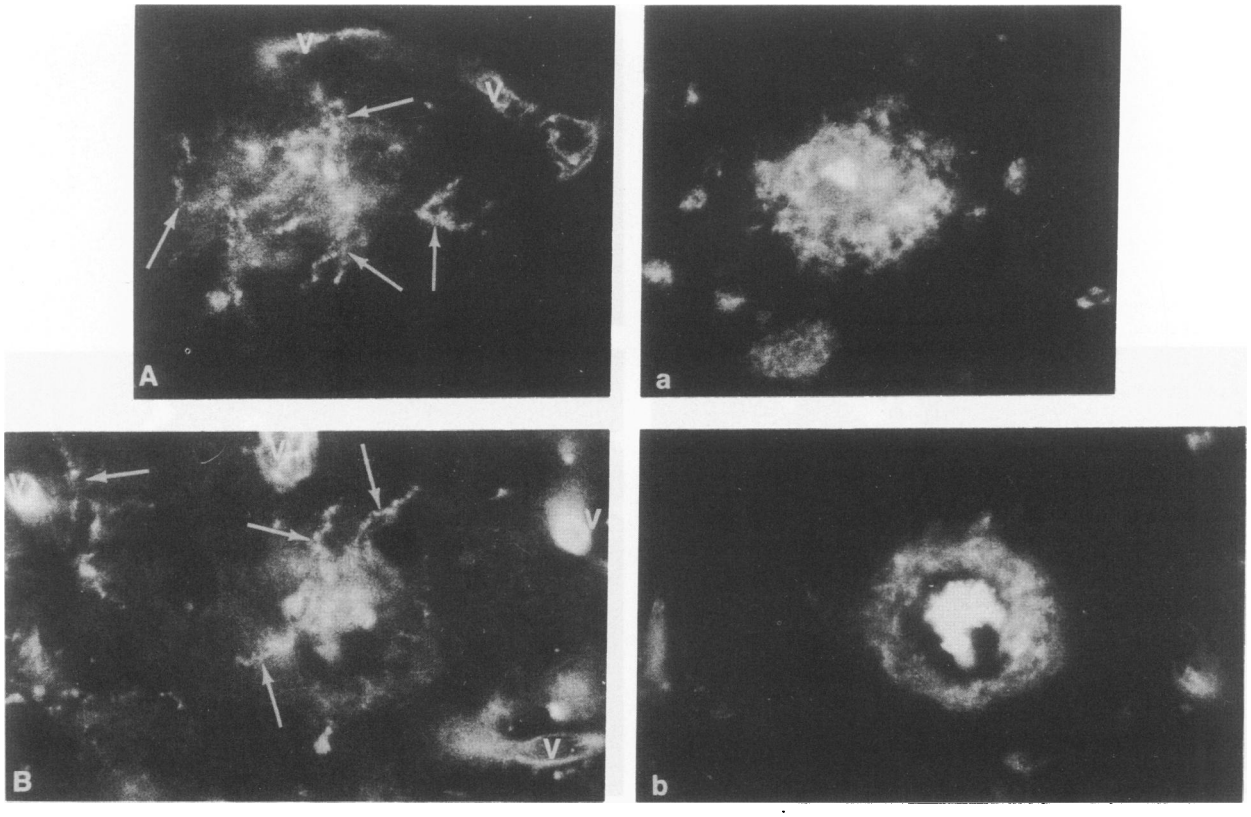

Figure 3—A and B are stained with rhodamine-conjugated RCA-I (a lectin that binds to microglial and endothelial cells) and visualized with rhodamine filters; a and b are the same sections as A and B counterstained with thioflavin S and visualized with fluorescein filters. (×448) RCA labels cells with delicate, branching processes (arrows) (A and B) that are in the vicinity of amyloid deposits that are either dispersed (a) or compact (b). Blood vessels are indicated (v).

itive neurites. Burnt-out plaques usually lacked Ab175-positive neurites. Very primitive plaques usually had no more Ab175-positive neurites than the surrounding neuropil.

The Ab175-positive neuropil neurites were most numerous in areas with many senile plaques and neurofibrillary tangles and almost completely absent from the white matter and areas with fewer tangles and plaques, such as the endplate of the hippocampus. They were most dense in the subiculum and CA-1 of the hippocampus.

Ab175 stained only some (about 50–75%) neurofibrillary tangles. It did not stain anything in non-Alzheimer degenerative dementia. In elderly nondemented individuals Ab175-positive neurites were found only in association with plaques. They were, however, far less abundant than in Alzheimer's disease.

NP18 (Phosphatase-Sensitive NF Epitope)

NP18 labeled axons in the white matter and neurites in the gray matter of both Alzheimer and control brains. Axons were unstained with thioflavin, and most of the neurites in the gray matter were also thioflavin-negative (Figure 8). NP18 stained some neurofibrillary tangles (about 50%) and some neurites in senile plaques that were thioflavin-positive and coarsely fibrous similar to those stained with Ab39 (Figure 8). NP18-positive, thioflavin-negative processes in senile plaques were thin and delicate and often seemed to be axons passing through the plaque (Figure 8). Most burnt-out plaques and very primitive plaques had no NP18 neurites, except for thin processes passing near the lesion. Some NP18-positive processes were present in senile plaques in normal individuals. These were largely negative with thioflavin.

NP15 (Phosphatase-Resistant NF Epitope)

NP 15 labeled axons in the white matter and neurites in the gray matter of both Alzheimer and control brains that were similar to NP18 in that they were nonreactive with thioflavin. In several of the cases the axons in the white matter showed swellings that were consistent with axonal spheroids. NP15 did not stain neurofibrillary tangles. This result was similar to previous studies with this antibody, in which neurofibril-

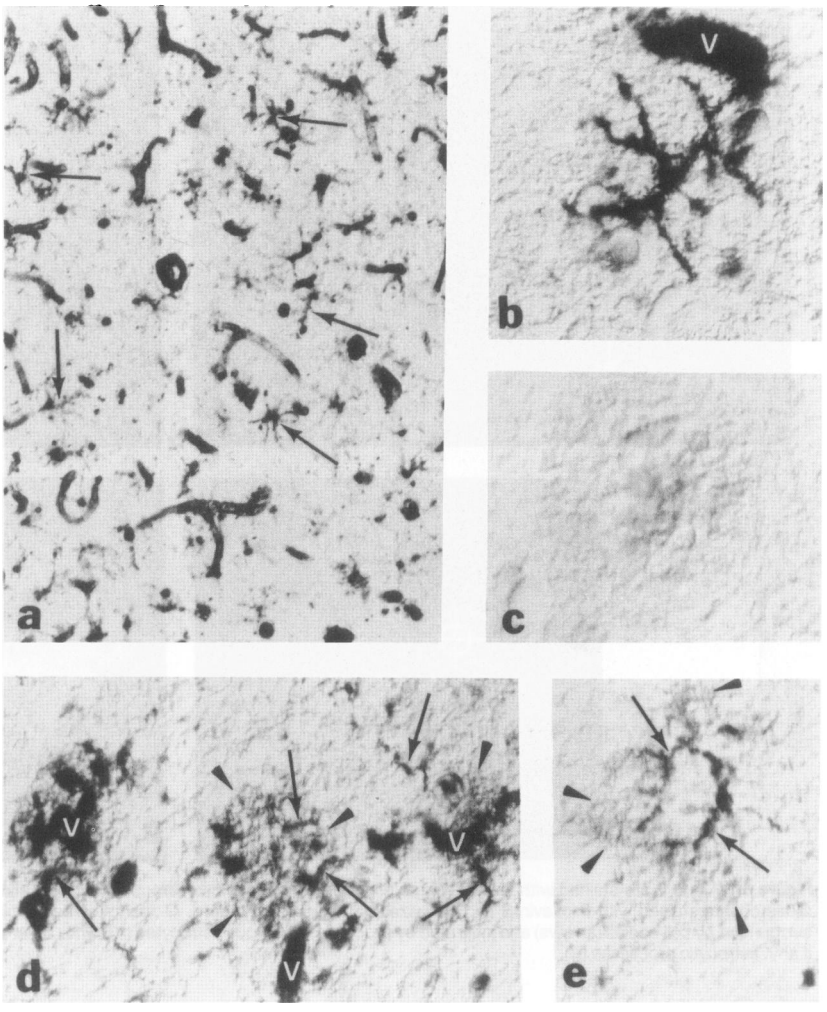

Figure 4—Panels a and b are lectin peroxidase stained sections that have been processed with the AMeX method and stained with avidin-labeled RCA-I, demonstrating cells with branching processes that are often closely associated with blood vessels. (a, ×112; b, ×448) In c the section was incubated without the lectin. Similar staining was obtained if the lectin was preabsorbed with 0.2 M beta-D-galactose. (×448) In d and e the sections are double-labeled using 4-chloro-naphthol as the chromagen to visualize amyloid (antiserum 3981; PAP method) and diaminobenzidine to visualize microglia (RCA-I ABC peroxidase method) (×448). The amyloid antibody stains finely granular, light gray material (arrowheads) in very primitive plaques that are closely related to microglial cell processes (arrows) and sometimes to blood vessels (v).

lary tangle staining was only observed if the sections were preincubated with trypsin.[26]

Most senile plaques were not labeled by NP15, but some senile plaques, particularly primitive plaques and classical plaques, contained bulbous NP15-positive, thioflavin-negative neurites (Figure 9). An occasional burnt-out plaque also contained NP15-positive neurites. Some of the neurites showed segmental swelling in the region of the plaque with a normal caliber in the adjacent parenchyma. More commonly, NP15-positive neurites seemed to be displaced by the senile plaque, so that the neuritic density in the plaques was less than in the surrounding neuropil, with some neurites deviating from their apparent course in the vicinity of the plaque (Figure 9).

Discussion

Amyloid and Microglia in Senile Plaques

This double-labeling immunohistochemical study of senile plaques in Alzheimer's disease demonstrated that despite the morphologic heterogeneity of senile plaques on thioflavin and Bielschowsky stains, they all contained amyloid and microglial cells or their processes. Not all senile plaques contained neurites recognized by antibodies to unique paired helical filament epitopes, antibodies to epitopes common to tau and paired helical filaments, and antibodies to neurofilaments. Although most senile plaques had fibrous astrocytic components, there were exceptions.

Recent clinicopathologic studies in this laboratory of a group of prospectively followed elderly subjects, some of whom were demented and some of whom were not, found that some elderly subjects have great numbers of senile plaques without dementia.[29,30] Further study of this group of patients, some of whom are included in the present report, has revealed that senile plaques in both demented and nondemented subjects contain amyloid, but only the demented subjects have a significant number of plaques with neurites that can be identified with Bielschowsky's stain or immunocytochemistry (manuscript in preparation).

In light of these findings, the present results take on

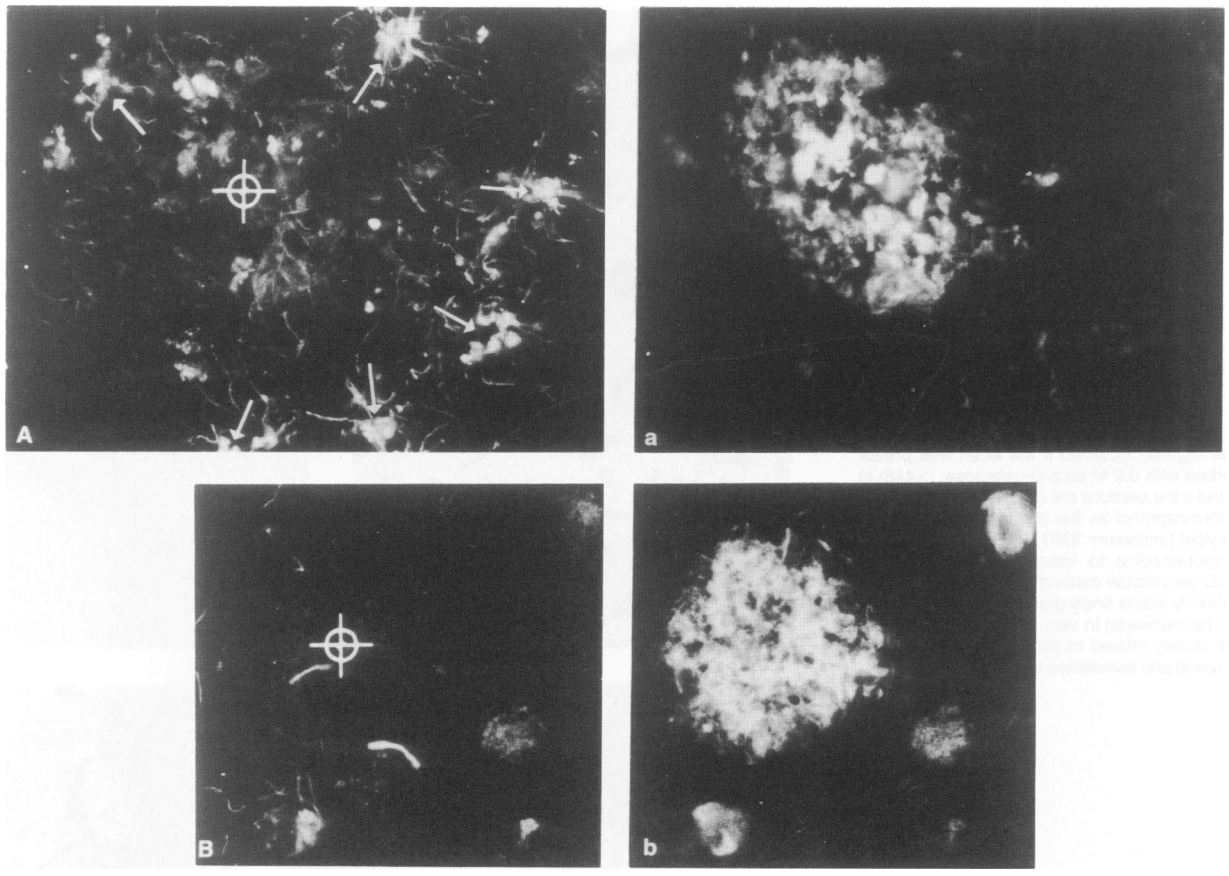

Figure 5—A and B are stained with anti-GFAP, an antibody that recognizes fibrous astrocytes, and visualized with rhodamine filters; a and b are the same slides counterstained with thioflavin S and visualized with fluorescein filters. ⊕ marks center of plaque. (×448) Note that some primitive plaques in a are surrounded by cell bodies (arrows) and infiltrated by processes of fibrous astrocytes (A), while other plaques, such as the very primitive plaque in b, have few, if any, astrocytic processes (B).

added significance, for they show that the feature common to senile plaques, amyloid, may be a manifestation of aging rather than Alzheimer's disease. Our results are consistent with a large retrospective study of human brains demonstrating that cerebral amyloidosis is a manifestation of aging[32] and an analysis of the proportion of senile plaques that contain amyloid compared with those with argyrophilic neurites,[33,34] which also suggested that amyloid deposition was a function of aging, while neuritic change was more disease-specific.

One of the consistent manifestations of aging in both humans and animals is degeneration and loss of dendrites.[35-37] The role of microglial cells in this process is largely unexplored at present, however, one of the major functions of microglia may be synaptic remodeling.[38,39] It is commonly believed that microglia are the cells responsible for generating cerebral amyloid,[5,6,40] analogous to their role in amyloid deposition in experimental Creutzfeldt–Jakob disease and scrapie.[40,41] Although the origin of microglial cells is still a matter of dispute,[42,43] recent studies have shown that microglial cells can be labeled with specific lectins.[18,38,39,44] We have found RCA-I to be a label that reliably stains cells with morphologic features entirely consistent with microglia and not consistent with other known types of glial or nonglial cells.[44] Furthermore, double-labeling shows that the RCA-positive cells are not astrocytes.

One of the cell types that has been shown to contain messenger RNA (and presumably the actual protein) for the precursor protein of cerebral amyloid is cerebral neurons.[45] The precursor protein has a number of properties consistent with it being a membrane protein.[46]

Combining these previous observations with our own findings in demented and nondemented individuals studied with lectin and antibody immunohistochemical studies leads to the hypothesis that cerebral amyloid may be a byproduct of microglial processing of synaptic elements that degenerate as a manifestation of aging of the brain, and that the precursor pro-

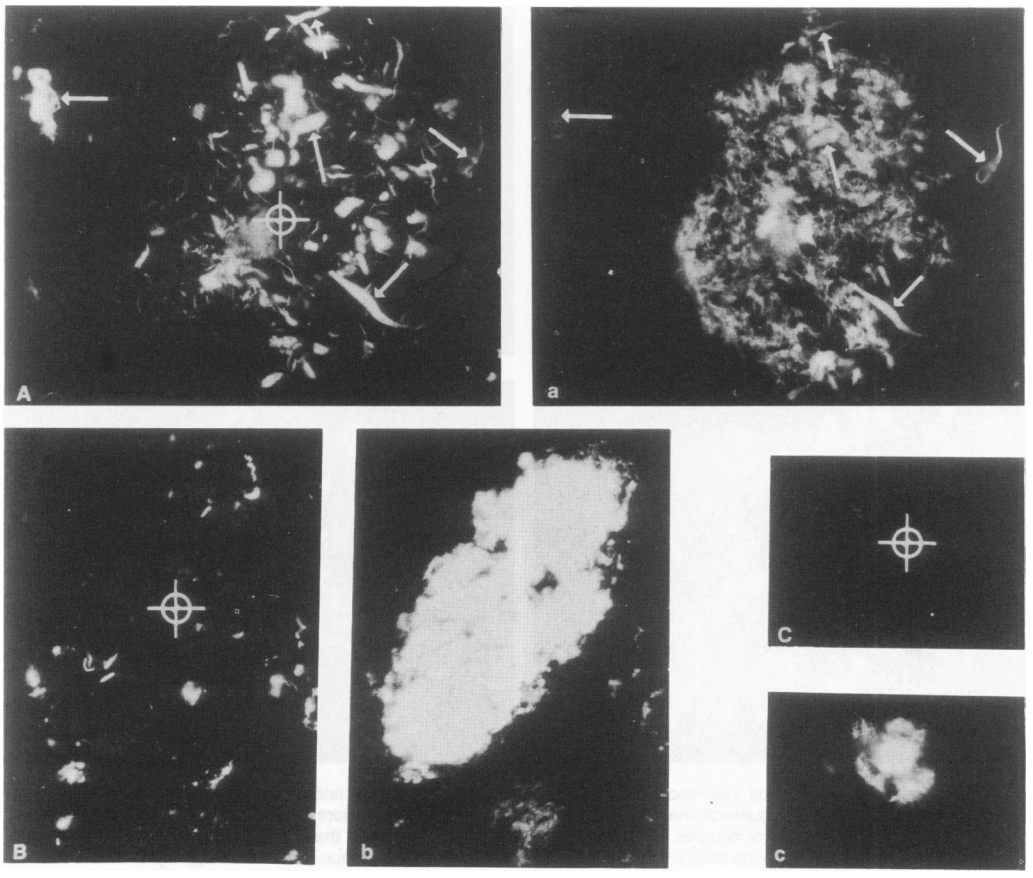

Figure 6—A, B, and C are stained with an antibody that recognizes a unique epitope in paired helical filaments (Ab39) and visualized with rhodamine filters; a, b, and c are the same slides counterstained with thioflavin S and visualized with fluorescein filters. Φ marks center of plaque. (×448) In the primitive plaque in A and a note correspondence of thioflavin-positive neurites with neurites stained with Ab39 (arrows). In the very primitive plaque in b and the burnt-out plaque in c amyloid deposits are present with few (B) or no (C) Ab39-stained neurites.

tein for amyloid may be a component of synapses. Immunohistochemical studies will be necessary to demonstrate the presence of the precursor protein in synapses. More importantly, our results suggest that in Alzheimer's disease additional factors are operative in the generation of abnormal neurites, both in the neuropil and the plaque.

Our double-labeling lectin–immunohistochemistry results demonstrate that finely dispersed cerebral amyloid may be directly contiguous with microglial cell processes. At this time it is not possible to state whether this represents a reaction of microglial cells to amyloid or production of amyloid by these cells. Our results are similar to those of Probst et al, who have shown that very primitive plaques usually contain RCA-positive cells.[47] These authors, however, argue that very primitive plaques, despite weak fluorescence with thioflavin S, do not contain amyloid.[47] (They did not perform immunocytochemical studies with antibodies to amyloid or to synthetic peptides of the amyloid beta-protein.) Our results suggest that "pale" ("very primitive") plaques, as first described by Schwartz, represent "amyloid deposits" in the "reticular ground substance" of the neuropil[15] consistent with Dirvy's original hypothesis of the pathogenesis of senile plaques[48]; we have shown with double-labeling methods that these plaques contain both amyloid and microglial cells.

Neuritic Change in Alzheimer's Disease

Studies with our monoclonal antibodies, as well as those with other antibodies, have clearly shown that one of the major pathologic changes in the Alzheimer gray matter is extensive neuritic degeneration not related specifically to senile plaques.[49–51] This is seen particularly well with antibodies to tau protein or epitopes shared with tau proteins and PHF, such as our Ab175, which binds to a nonphosphorylated epitope in both paired helical filaments and tau proteins from both human and animal brains.[22,23] The present study shows that in some instances amyloid of senile

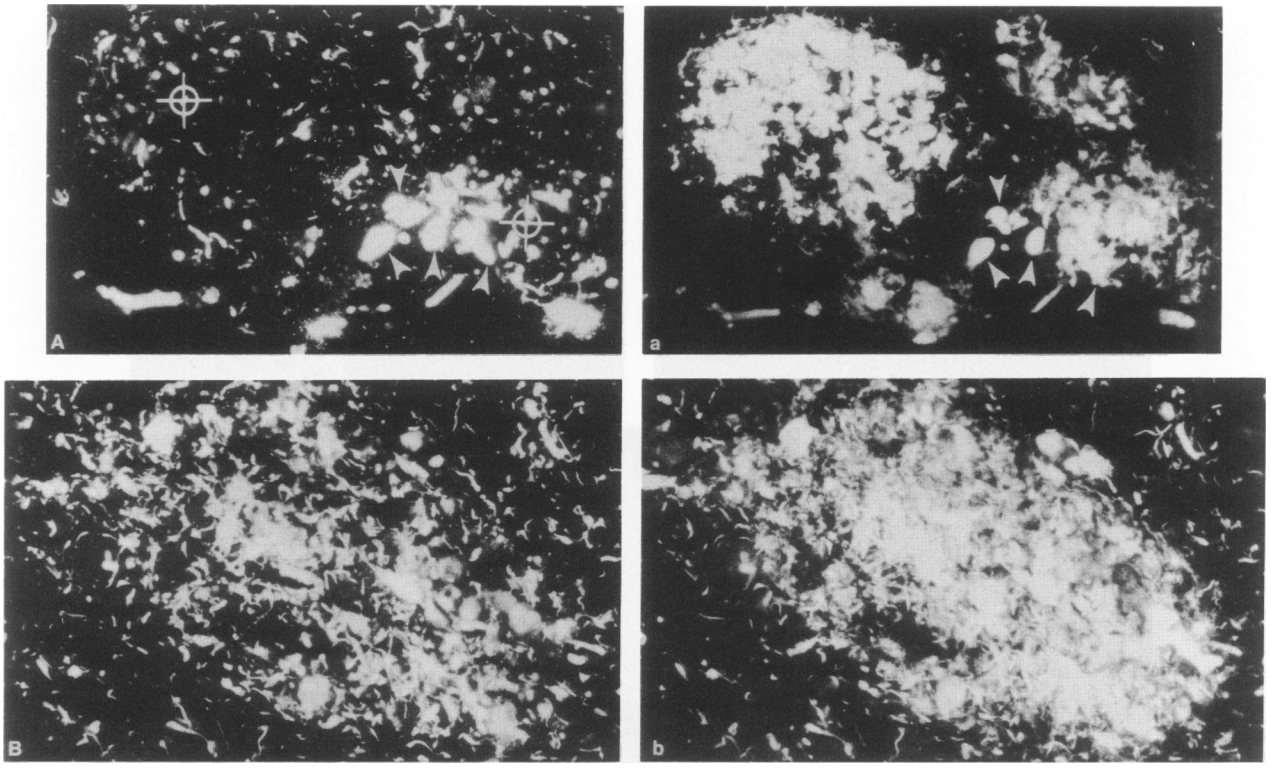

Figure 7—A and B are stained with an antibody that recognizes an epitope shared between tau and paired helical filaments (Ab175) and visualized with rhodamine filters; a and b are the same slides counterstained with thioflavin S and visualized with fluorescein filters. ⊕ marks center of plaque. (×448) In the 3 primitive plaques in A notice that the density of neurites in the plaque is not significantly greater than in the surrounding neuropil and that Ab 175 stains neurites (arrowheads in A) that are also stained with thioflavin (a). In the primitive plaque in B the plaque has a dense aggregate of fine neurites, most of which are also detected with thioflavin (b). Additional staining with thioflavin is due to amyloid in the plaque.

plaques seems to be independent of extensive neuritic degeneration, since the number of neurites in some plaques (very primitive and burnt-out types) is equal to (or sometimes even less than) the number of neurites in the surrounding neuropil.

Studies with Ab175 cannot resolve whether these neurites contain PHF, since it also reacts with normal tau protein. That the majority of the processes, both within the plaque and the surrounding neuropil, that are labeled with Ab175 are also stained with thioflavin suggests that they probably have PHF. They may, thus, be analogous to the so-called "neuropil threads" that have been shown to contain PHF.[52] Ab39 is an antibody that recognizes a unique epitope in PHF (and abnormal straight filaments in progressive supranuclear palsy)[22-24] and it labels only a fraction of the neuropil neurites. Furthermore, immunocytochemical studies in mouse scrapie have demonstrated that abnormal plaque neurites may contain tau epitopes without PHF.[53] The extensive neuritic change found in the hippocampus of Alzheimer's disease may represent an alteration in tau proteins in the neurites.

An unanswered question is whether the neuritic change observed in Alzheimer's disease is a qualitative or quantitative difference from that seen in normal aged individuals. In this regard it is important to note that similar neurites are seen in the hippocampus of nondemented individuals, but to a far lesser degree, and usually associated with senile plaques. Another question that this raises is whether amyloid itself may play a role in neuritic degeneration. Since amyloid can be detected in some cases apparently independent of neuritic changes, one may postulate that the increased synaptic remodeling, possibly precipitated by major head trauma, toxic factors, or an unconventional infectious agent, may lead to elaboration of excessive amounts of the amyloid protein, which induces neuritic degeneration. In normal aging the rapidity of amyloid deposition may be slower and thus less neuritic response ensues. Alternatively, there may be individual variability in the susceptibility to the possible neurotoxic effects of amyloid. Future studies are needed to address the question of whether amyloid has neurotoxic properties.

Our findings are at variance with the hypothesis that amyloid in senile plaques is derived from the same protein that composes PHF,[54] since it was not uncommon to find amyloid deposits independent of PHF-type neuritic change.

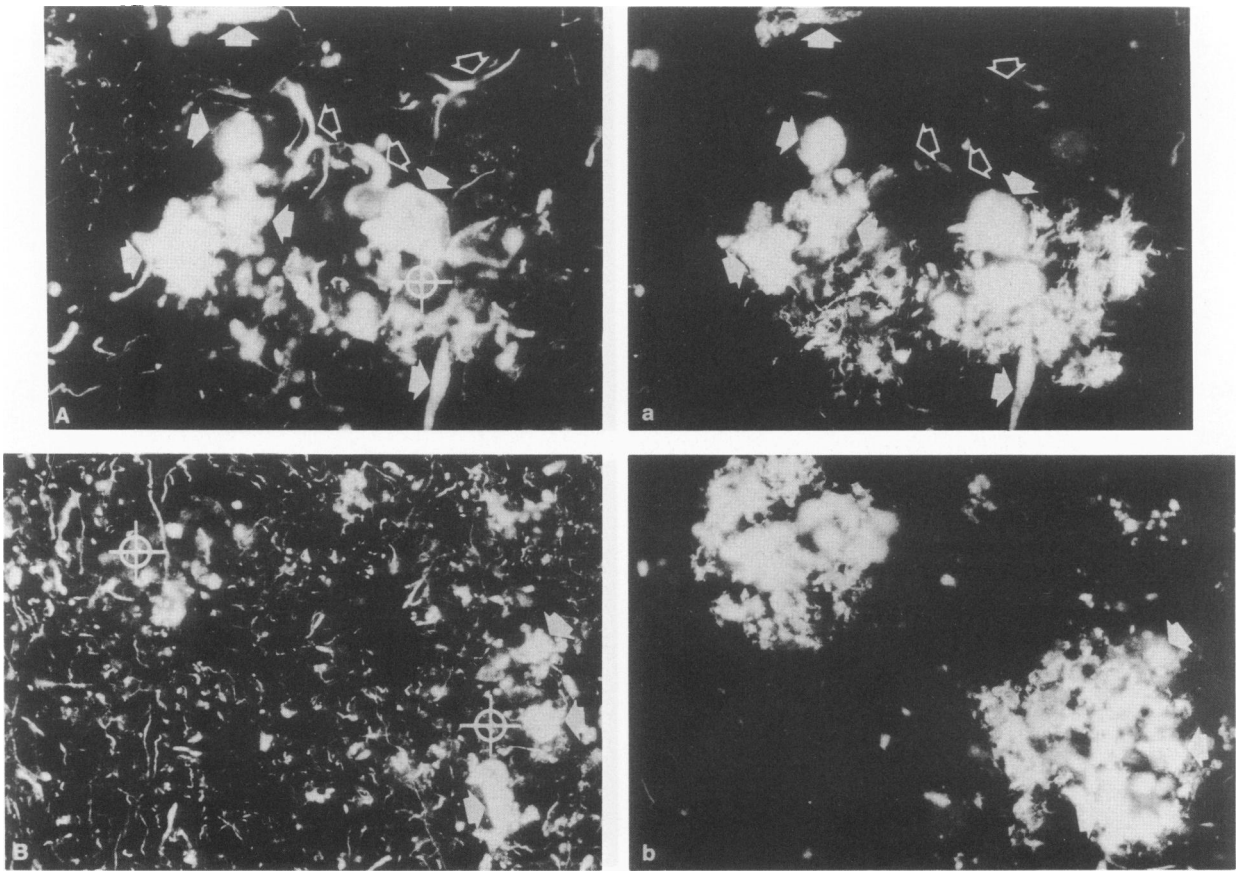

Figure 8—A and B are stained with an antibody that recognizes a phosphorylated epitope shared between neurofilaments, tau, and paired helical filaments (NP18) and visualized with rhodamine filters; a and b are the same slides counterstained with thioflavin S and visualized with fluorescein filters. ⊕ marks center of plaque. (×448) In the classical plaque in A notice that some large, coarse neurites are stained by both the antibody and thioflavin (solid arrows), while others are only stained with NP18 (open arrows). Most of the thioflavin-positive neurites are coarse and incorporated into the plaque, while the thioflavin-negative neurites are thinner and more peripheral. In the primitive plaques in B most of the NP18 neurites are thin, not specifically associated with the plaque, and also negative on thioflavin (b). Only those larger more coarse neurites in the plaque are thioflavin-positive (arrows).

Senile Plaques with Amyloid Without Apparent Neurites

The present study suggests that both very primitive plaques and burnt-out plaques may actually represent amyloid deposits in the neuropil without appreciable neuritic elements. We have not excluded the possibility that other markers may reveal a neuritic component in these plaques that has heretofore gone unnoticed. Both very primitive and burnt-out plaques may be different morphologic manifestations of the same process. One might speculate that amyloid is initially deposited in the brain in a diffuse form that becomes compacted into a core by the action of local factors. This may account for the regional variability in plaque types. For example, burnt-out plaques are more common in primary cortices and the cerebellum and in lower cortical layers, while plaques with diffuse amyloid deposits are more common in upper cortical layers.

Furthermore, our results suggest that most of the amyloid in senile plaques is not in the form of a compact core; it is more commonly diffusely distributed. These studies must be interpreted with caution, because it is possible that the staining we observed may be due to the presence of intracellular, nonfilamentous proteins antigenically related to the beta protein, rather than extracellular amyloid fibrils. Immunoelectron microscopic studies will be necessary to resolve this issue. Nevertheless, the staining patterns we have shown are similar to those with a monoclonal antibody to synthetic peptide of the beta protein.[55]

Both our study and the study with monoclonal antibodies to beta protein[55] employed frozen sections. We have noted that when standard paraffin sections are used, far less amyloid is detectable. The amyloid core and cerebrovascular amyloid are, however, well-stained. Sections processed with the AMeX method[19] give staining similar to frozen sections, that is, most

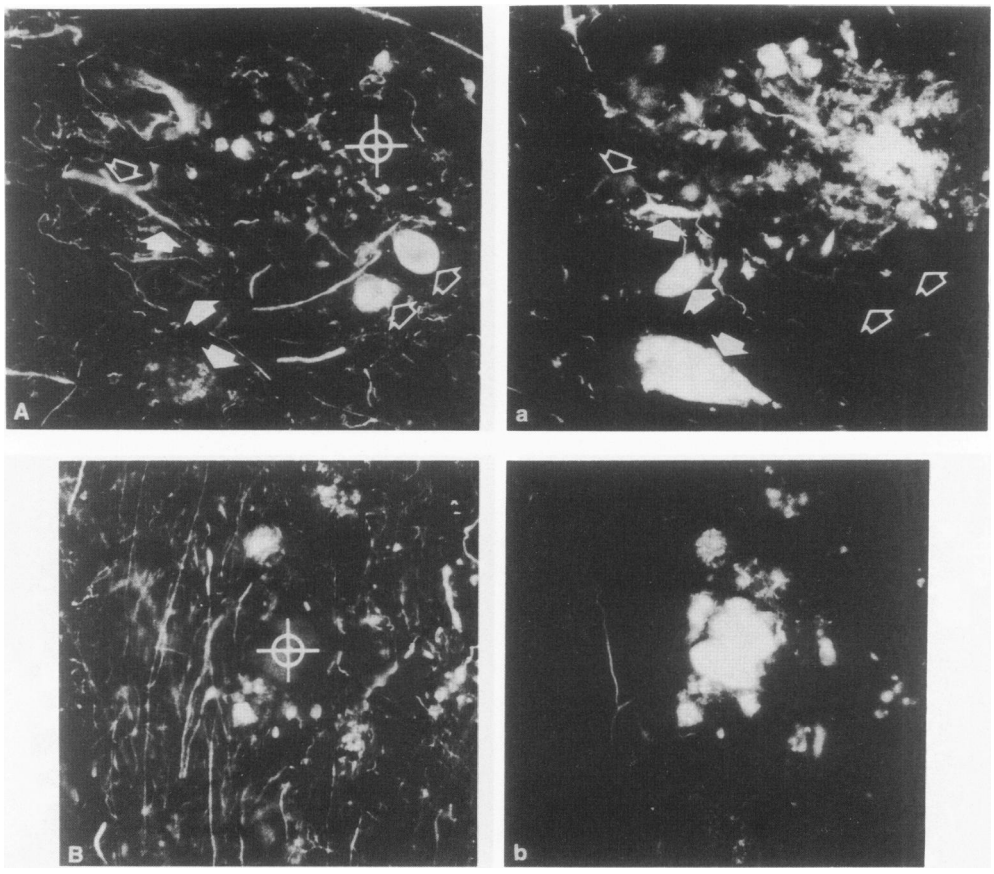

Figure 9—A and B are stained with an antibody that recognizes a phosphatase-insensitive epitope of neurofilaments (NP15) and visualized with rhodamine filters; a and b are the same slides counterstained with thioflavin S and visualized with fluorescein filters. ⊕ marks center of plaque. (×448) In A and a note the nearly complete discordance of NP15 and thioflavin staining. Thioflavin-positive neurites and neurofibrillary tangles (a) (solid arrows) are not stained with NP15; conversely, both bulbous and fine processes stained by NP15 (A) (open arrows) are not stained with thioflavin (a). In B, thioflavin-negative, NP15-positive processes seem to be displaced from the region where amyloid is deposited in the neuropil (b).

plaque amyloid is diffuse and granular. Adjacent sections stained with Bielschowsky stain and amyloid synthetic peptide antibodies have shown that the brown, finely granular staining observed with Bielschowsky stain corresponds to amyloid (not shown). Thus, the "plaquelike" lesions ("pale senile plaques," "very primitive plaques") that have been described in the brain of a nondemented individual with the Dutch form of familial cerebral amyloidosis almost certainly represent amyloid deposits.[56] The lack of dementia in Dutch hereditary amyloidosis cases is entirely consistent with our findings, since neuritic degeneration and neurofibrillary tangles were not described.[56] This reiterates that amyloid beta protein can accumulate in the brain in the absence of dementia.

Diversity of Antigenic Determinants of Senile Plaque Neurites

Although many neurites in senile plaques may contain PHF, other types of neurites are also present. Some of these neurites are not stained by thioflavin. Some of the thioflavin-negative neurites can be immunolabeled with antibodies to neurofilament. Our study employed 2 different probes for neurofilament, 1 cross-reacted with PHF and the other did not. The former antibody stained both thioflavin-positive and-negative processes; the latter antibody only stained thioflavin-negative processes. Antibodies that react with unique epitopes in PHF and those that bind to phosphorylated neurofilament epitopes in PHF seemed to bind to thick, coarsely fibrous, thioflavin-positive neurites, presumably those with PHF.

Thioflavin-negative neurites were either thin, long processes that appeared to be in axons that were either passing through or nearby the plaque or bulbous and spherical, resembling axonal swellings seen in a number of conditions that interfere with axoplasmic transport of neurofilaments or that occur at the margin of focal injuries to white matter. One may speculate that these neurofilament-containing neurites represent "by-stander" axonal elements, while tau-reactive and

PHF-containing neurites are intrinsic to the disease process. It is interesting to note that in aged animals many of the neuritic processes in senile plaques are similar to these.[58] Namely, they are stained by neurofilament, but not PHF-specific antibodies. Our results suggest that a similar phenomenon may occur in humans. It is worth noting, however, that these bulbous neurites were always a minor component of the plaque and were not detected in the majority of plaques.

Astrocytes in Senile Plaques

Fibrous astrocytes were a common feature of most plaques, especially those with well-formed neuritic elements and/or compact amyloid cores. Their presence in the gray matter of Alzheimer's disease has been described previously and is decidedly abnormal.[58] Plaques composed primarily of amyloid deposits (very primitive plaques and burnt-out plaques) had far fewer glial processes. These results suggest that the astrocytic component of the plaque may be secondary to other components of the plaque and not a necessary precondition for plaque formation. Since astrocytes respond to brain injuries in a timely fashion, they also suggest that plaques composed of amyloid deposits only may evolve before those with neuritic elements. It seems unlikely that astrocytes play a major role in plaque formation given their absence in some plaques.

Is Amyloid Significant in the Pathogenesis of Alzheimer's Disease?

Recent studies demonstrating that the gene for cerebral amyloid is present on the same chromosome (chromosome 21)[59] as the gene for familial Alzheimer's disease[60] have raised a great deal of interest in the study of cerebral amyloid and its relationship to Alzheimer's disease. If amyloid is closely related to the fundamental pathology of Alzheimer's disease, then amyloid should be present in all cases and in all senile plaques. Our results support the primary role of amyloid in senile plaques, however, it seems that neuritic degeneration, both localized to the plaque and also more widely distributed in the Alzheimer neuropil, may be the morphologic feature more closely associated with dementia.

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