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Increased senile plaques without microglia in Alzheimer's disease*

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Summary. To clarify the association of microglia with senile plaques, the brains from 13 patients with Alzheimer's disease (AD) and 23 nondemented aged controls were investigated immunohistochemically by a double-labeling method using anti-β-protein antiserum and anti-ferritin antibody, which is a recently reported microglia marker. In addition, a quantitative analysis was performed. The senile plaques which appeared initially in the nondemented aged controls consisted of a diffuse type without any amyloid cores and these were found in the group aged 50–59 years. The great majority of them were found to contain no ferritin-positive microglia. The number and proportion (percentage) of microglia-containing diffuse plaques increased with age. Classical and compact plaques began to appear in the brains of the group aged 70 years and over, and practically all of them contained microglia. These results suggest that microglia are not associated with initial plaque formation, but correlate with amyloid core formation. In AD, the most prominent feature was that the diffuse plaques, which contained either no or only a few ferritin-positive microglia, increased markedly.

Key words: Senile plaque – Microglia – β Protein – Immunohistochemistry – Alzheimer's disease

Senile plaques are one of the most conspicuous pathological findings of Alzheimer's disease (AD), and are also detected, to a lesser degree, in the brains of aged individuals who do not suffer from dementia [32]. Senile plaques are composed mainly of amyloid fibrils, degenerative neurites and reactive cells such as astrocytes, microglia and macrophages [13, 31, 34]. The amyloid fibrils both in senile plaques and vascular deposits consist of polymers of a 4 to 5 kDa protein subunit [3, 17], termed the β protein (also referred to as the A4 protein). The β protein is the cleavage product of a precursor resembling a cell-surface receptor [11]. Little is known, however, about the process of amyloid formation in AD.

Of all reactive cells which make up the senile plaques, microglia have been identified as playing an important role in the formation of senile plaques [4, 5, 22, 26, 35, 36], because microglial cells are frequently found in and around the senile plaques. Wisniewski et al. [36] suggested that the microglia of the plaques are amyloid-forming cells. On the other hand, Glenner et al. [4] proposed that proteolytic cleavage by microglia of β protein produces the amyloid cores of senile plaques. Our previous ultrastructural study showed the close relationship between macrophages (microglia) and the production of amyloid fibrils in the brains of Creutzfeldt-Jakob disease-infected mice [6], which is another type of amyloid, prion protein [27].

The conventional classification of senile plaques, using silver impregnation and electron microscopic observation together with the assumption of the developmental association among them made by Wisniewski and Terry [34], is as follows: the primitive plaques, composed of degenerative neurites with no amyloid cores, were considered to evolve into classical (mature) plaques, composed of an amyloid core surrounded by degenerative neurites. The classical plaques were then thought to degenerate into compact (burned-out) plaques, composed of only an amyloid core. The use of β protein immunostaining coupled with formic acid enhancement [14], however, revealed not only these senile plaques and the vessels with amyloid angiopathy but also perivascular deposits, subependymal deposits and various sizes of plaque-shaped diffuse deposits, which were not detected by Congo red staining or silver impregnation [24]. These diffuse deposits, described as very primitive plaques [33], amorphous non-congophilic plaques [29] or diffuse plaques [37], are thought to be the initial stages of plaque formation [8, 29, 33, 37]. However, both the correct se-

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quence and process of plaque evolution are still under discussion.

We investigated senile plaques found in the brains of patients with AD and those in nondemented aged controls by utilizing a double-immunolabeling method using anti-β protein antiserum and affinity-purified light (L)-ferritin-specific antibody. The latter was recently shown to be a useful marker for microglia in formalin-fixed, routine paraffin sections [10]. In this report, we describe (1) the microglia association patterns with each type of β protein immunoreactive deposit, (2) the relationships of the numbers and proportion (percentage) of senile plaques associated with microglia in regard to the nondemented aged controls, and (3) in regard to AD.

Materials and methods

Materials

Thirteen patients (age ranging from 33 to 75 years; mean $\pm$ SD, 59.8 $\pm$ 11.7), who fulfilled the established criteria for AD [12], were examined. All of them had a presenile (under 65 years) onset of dementia at 51.3 $\pm$ 11.1 years (mean $\pm$ SD). For the nondemented controls, patients with clinically proven dementia were excluded. From 69 nondemented patients, 23 cases (ages ranging from 50 to 93 years: mean $\pm$ SD 72.7 $\pm$ 11.8) were selected because of the presence of senile plaques (nondemented aged controls). These 23 cases were previously shown to be in the typical range of density and distribution of plaques [24]. Cerebral tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-μm-thick sections. We examined the following sites: frontal, parietal, temporal, occipital and insular cortices and parahippocampal gyrus.

Double immunohistochemistry

Anti-β protein antiserum and affinity-purified L-ferritin antibody used in this study, were generated and characterized as described [10, 24], respectively. The unlabeled antibody/biotin-streptavidin method (Stravigen kit, Biogenix Laboratories, Dublin, Calif.) was used to immunostain the sections. After deparaffinization, the endogenous peroxidase was blocked. To enhance the β protein immunoreactivity, a formic acid pretreatment [14] was done for 5 min, followed by washing with tap water and then with Tris buffer (50 mM Tris-HCl, pH 7.6). The preparations were incubated overnight at 4° C with the primary antibody solution, which consisted of affinity-purified L-ferritin antibody (0.3 μg/ml). The following steps were carried out using the Stravigen kit. The standard mixture, consisting of 0.01% diaminobenzidine and 0.003% H_2O_2 in 50 mM Tris buffer, gave a brown reaction product. After washing with glycine buffer (100 mM glycine-HCl, pH 2.2) for 2 h, followed by washing with tap water and finally Tris buffer, the preparations were incubated overnight at 4° C with the secondary antibody solution, which consisted of diluted anti-β protein antiserum (1:2000). The following steps were also carried out using a Stravigen kit. Sections were incubated in a solution of 0.05% diaminobenzidine and 0.02% cobalt chloride for 5 min and then for an additional 3 min after addition of 0.0003% H_2O_2 [7]. A bluish-black or dark-bluish reaction product was formed. Counterstaining was performed briefly with hematoxylin.

Quantitative analysis

The senile plaques were classified as shown in the results and counted separately under 200-fold magnification. Counting was done in a randomly selected area of 20 to 60 mm^2 so as to cover the whole cortical layers of the respective sites. The number density of plaques for each site was expressed as the number of plaques per area of 10 mm^2. The mean plaque density of each case was obtained by averaging the densities of the plaques at all five sites examined. The microglia positivity of the plaques in each site was calculated as the microglia-positive plaque counts divided by the total plaque counts (in %) within an investigated area. The mean microglia positivity of the plaques in each case was obtained by averaging the microglia positivity in all five sites examined. Not only the plaques in which the whole structure of the microglial cell bodies and processes were involved, but also those in which microglial cell bodies and/or processes were partially involved and those to which the microglia were intimately attached were all defined as being microglia-positive plaques.

Statistical analysis

With regard to the plaque density, one-way analysis of variance was performed to test the equality of the group means and then the Bonferroni's *t*-test was performed for multiple comparisons between the group means.

Results

Microglia association patterns with various amyloid deposits

Immunostaining using anti-β protein antiserum, coupled with formic acid pretreatment [14], revealed a positive immunoreaction in the various types of deposits as described [24]. Affinity-purified L-ferritin antibody stained not only small, bipolar or multipolar cells with a scanty cytoplasm and fine delicate processes, presumably resting microglia, but also intensely stained the microglia which appeared to be reactive microglia with swollen cytoplasm and fewer delicate branching processes [1, 10, 25]. The co-localization of amyloid deposits and ferritin-positive microglia was more clearly and readily visualized in the formalin-fixed, paraffin-embedded sections by double immunostaining using anti-β protein antiserum and anti-L-ferritin antibody (Fig. 1).

1. Diffuse plaque: these consisted of an amyloid fibril deposit without any core formation (smaller plaques corresponded to type 1 and larger ones to type 2 deposits in our previous study [24]). There were various forms of plaques consisting of those containing no microglia, only a small number of microglia, a larger number of microglia or clustered microglia (Figs. 1 B, C, D). When senile plaques were involved with microglia either in increasing numbers or in clusters, there was a tendency for the more reactive microglia and less resting microglia to appear.
2. Classical plaque: most plaques had various numbers of microglia in the ring or corona around the amyloid cores or closely attached to the amyloid cores. Clusters of microglia were occasionally observed around the amyloid cores or in the corona (Fig. 1 E). Amyloid cores were rarely involved with the microglia. Most of the microglia seen in these plaques were reactive microglia with swollen cytoplasm and few branching processes.
3. Compact plaque: the plaques consisted exclusively of amyloid cores and various numbers of microglia, most

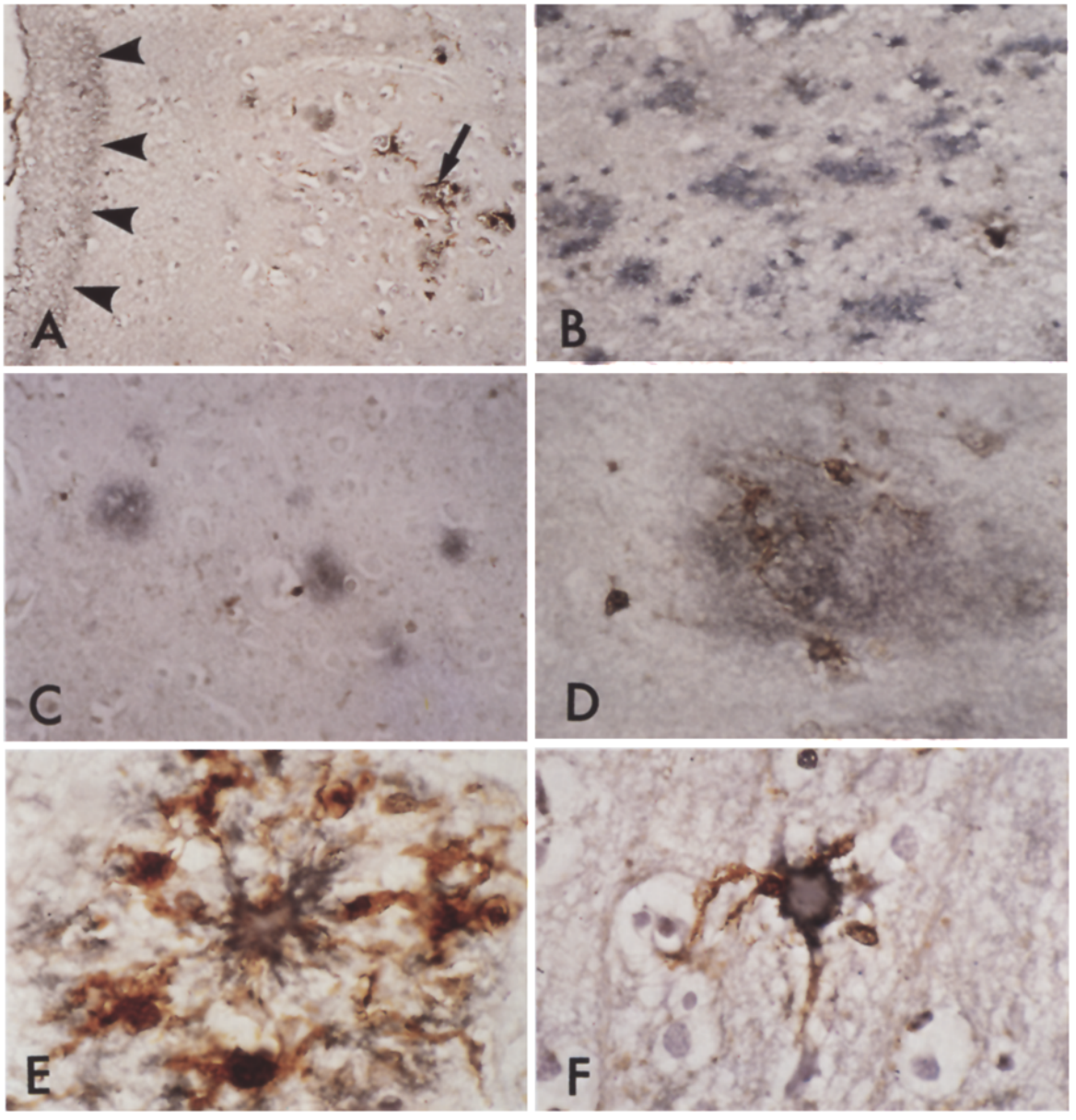

Fig. 1. **A** Double immunostaining for anti-β protein with diaminobenzidine (DAB) and $CoCl_2$ and affinity-purified L-ferritin antibody with DAB. **A** and **B** show the parahippocampal gyrus from a patient with Alzheimer's disease (AD). **A** β Protein amyloid deposits (*bluish-black*) involved with a small number of ferritin-positive microglia (*brown; arrow*), but no microglia in superficial deposits (*arrowheads*). **B** A large number of diffuse plaques without microglia. **C–F** Parahippocampal gyrus from nondemented aged controls. **C** A small number of diffuse plaques without microglia, a 56-year-old person. **D** A few microglia with a scanty cytoplasm and fine blanched processes, presumably resting ones, involved in a diffuse plaque, a 64-year-old person. **E** Clusters of a large number of reactive microglia with swollen cytoplasm and less delicate branched processes in a typical plaque, an 80-year-old person. **F** A few reactive microglia attached to a compact plaque, an 80-year-old nondemented person. **A** ×113; **B, C** ×226; **D–F** ×565

of which were reactive microglia, closely attached to the amyloid core (Fig. 1F).

4. Perivascular deposits: fibrillary amyloid deposits surrounded the vessels [24]. These deposits rarely contained microglia, which were few in number and appeared to be resting microglia with a scanty cytoplasm. A cluster of microglia was seldom seen in the region of these deposits.

5. Superficial deposits: amyloid deposits in the subpial and subependymal areas [24] were very rarely found to contain a small number of microglia which appeared to be resting.

6. Vascular deposits: amyloid deposits were seen in the vascular walls corresponding to amyloid angiopathy [24]. Microglia, most of which, appeared to be resting, were occasionally observed on and around the vascular walls, but not in clusters.

Perivascular, superficial and vascular deposits had much less apparent association with the microglia than did diffuse, classical and compact plaques. In addition, some smaller and larger diffuse plaques showed similar results as to microglial association. Therefore, quantitative analyses were performed for large diffuse plaques

Table 1. Density of microglia-containing senile plaques in patients with Alzheimer's disease (AD) compared with nondemented aged controls[a]

	Age (*n*)	Diffuse plaque			Classical plaque			Compact plaque		
		Grade 1	Grade 2	Grade 3	Grade 1	Grade 2	Grade 3	Grade 1	Grade 2	Grade 3
Control	50–59 (4)	0.8 ± 0.2*	0.1 ± 0.2**	0	0	0	0	0	0	0
	60–69 (4)	3.9 ± 1.1*	0.9 ± 0.7**	0	0	0	0	0	0	0
	70–79 (7)	6.3 ± 3.7*	2.7 ± 2.6**	1.6 ± 2.1	0.1 ± 0.1	2.6 ± 2.6	0.9 ± 1.0	0	1.0 ± 0.0	0.2 ± 0.4
	80–93 (8)	13.9 ± 5.8*,d,c	7.1 ± 4.9***,c	4.4 ± 3.8	1.3 ± 0.9	4.7 ± 3.9	0.9 ± 0.6	1.0 ± 0.7	2.2 ± 0.3	0.3 ± 0.4
AD	33–75 (13)	53.7 ± 20.1	52.1 ± 39.9	13.5 ± 20.1	1.5 ± 2.1	5.7 ± 5.0	1.4 ± 1.5	1.0 ± 2.0	2.1 ± 1.7	0.5 ± 1.1

[a] Values are mean (± SD) plaque counts/10 mm^2; see text for grades 1, 2 and 3
[b] Control, nondemented aged controls; *n* = number of cases
[c] $P < 0.05$ compared with 50s age group of control
[d] $P < 0.05$ compared with 60s age group of control
* $P < 0.001$; ** $P < 0.01$; *** $P < 0.05$ comparing AD and control in each age group

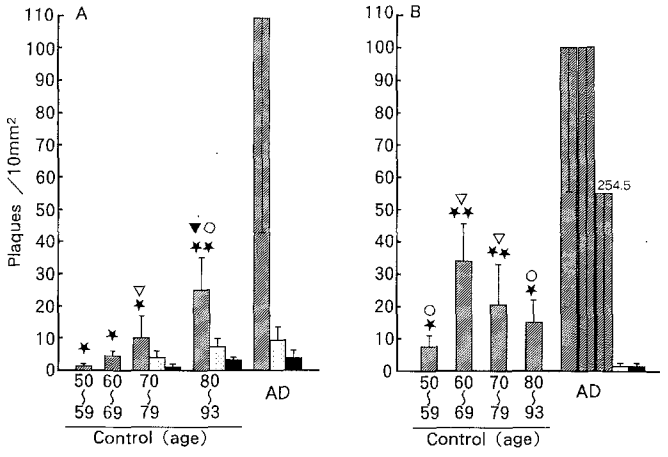

Fig. 2. Number density of microglia-positive (**A**) and microglia-negative (**B**) plaques for each type of senile plaque. Results are expressed as mean ± SD. ▨: Diffuse plaque; ▩: classical plaque; ■: compact plaque; *: $P < 0.01$; ** $P < 0.05$ comparing AD with nondemented aged controls in each age group; ▽ $P < 0.05$ and ▼ $P < 0.01$ compared with 50s age group of nondemented controls; ○ $P < 0.05$ compared with 60s age group of nondemented controls

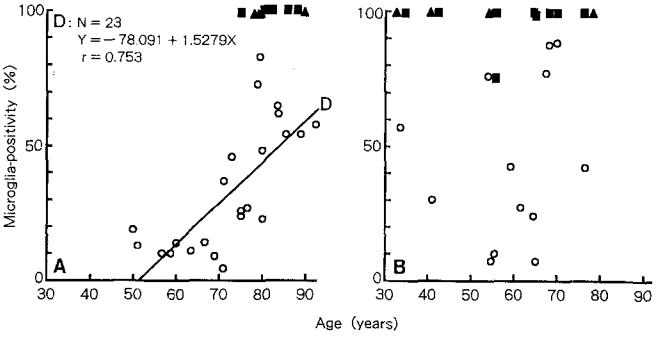

Fig. 3. Relationship between age and microglia positivity of diffuse plaque (○), classical plaque (■) and compact plaque (▲) in nondemented aged controls (**A**) and patients with AD (**B**). *Solid line*: regression line for diffuse plaques. N = *n*, number of subjects

(larger than 20 μm in diameter), classical plaques and compact plaques.

Nondemented aged controls

It was diffuse plaques without cores that initially appeared in the brains of patients who died in their 50s. The density of microglia-positive diffuse plaques tended to increase with age (Fig. 2A), while the density of microglia-negative diffuse plaques tended to decrease after reaching their highest levels in the 60s age group (Fig. 2B). The great majority of diffuse plaques in the 50s age group were found to contain no microglia (Fig. 1C). The microglia positivities of diffuse plaques in the 50s and 60s age groups were very low, but increased with age and a significant positive correlation was also observed (Fig. 3A) between the microglia positivity of diffuse plaques and age, with the correlation coefficient (*r*) being 0.753 ($n = 23$, $P < 0.001$).

Microglia-positive plaques were further divided into three grades according to the severity of microglial reaction involved. Grade 1 plaque: a plaque containing very few (one or two) cell bodies or microglia processes; grade 2 plaque: a plaque containing few (three to five) cell bodies or processes of microglia but not in a cluster (Fig. 1D, F); grade 3 plaque: a plaque containing many (more than six) cell bodies or processes of microglia or clusters of many microglial cells (Fig. 1E). Grade 3 diffuse plaques were not found in the 50s and 60s age groups but appeared initially in the 70s age group, and grades 1 and 2 diffuse plaques (Fig. 1D) showed a gradual increase in density with age.

As for classical and compact plaques, they appeared in the brains of the 70s age group and over, and no correlation was observed between the density of microglia-positive plaques and age. Classical and compact plaques were shown to have virtually a 100% of microglia positivity and most of them were grade 2 and 3 plaques (Fig. 1E, F).

Alzheimer's disease

Every type of diffuse, classical and compact plaque was observed in all age groups over 30s of patient with AD

(Fig. 3B). No correlation, however, was observed between age and density of microglia-positive or -negative diffuse, classical and compact plaques. Moreover, no correlation was observed between age and microglia positivity of their plaques (Fig. 3B).

The densities of microglia-positive plaques of each type were compared among the AD and nondemented aged controls in the different age groups (Figs. 2A and Table 1). For microglia-positive diffuse plaques, AD patients showed a significantly high density, compared with nondemented aged controls in each age group (Fig. 2A). Of all microglia-positive diffuse plaques, the densities of grade 1 and 2 diffuse plaques were significantly higher than those of the nondemented aged controls (Table 1). The density of microglia-negative diffuse plaques (Fig. 1B) was significantly high in AD, compared with those in the nondemented aged controls in each age group (Fig. 2B). In AD, in particular, the density of microglia-negative diffuse plaques was much higher than that of microglia-positive diffuse plaques.

As for classical and compact plaques, microglia-positive plaques showed no significant differences in the density between AD and nondemented aged controls in each age group (Fig. 2A). Classical and compact plaques had nearly a 100% microglia positivity (Fig. 3B). Most of microglia-positive plaques were grade 2 and 3 plaques, but did not show any significant differences in the densities between AD and nondemented aged controls (Table 1). However, classical and compact plaques without microglia were very scarce in AD, as in the nondemented aged controls.

Discussion

β Protein immunostaining coupled with formic acid pretreatment [14] is considered to have the highest sensitivity and specificity for recognizing several types of senile plaques among all of the methods reported to date [8, 14, 23, 24, 37]. Demonstration of microglia has been performed by immunological and cytochemical staining [5, 9, 10, 15, 16, 18–21, 26, 30]. Methods for labeling microglia with antibodies to macrophages or class II major histocompatibility antigens (HLA-DR) have been limited by the decreased reactivity of the epitopes in tissues which have been fixed in formalin for long periods and routinely embedded in paraffin [9, 18–20, 28, 29]. *Ricinus communis* agglutinin-1 (RCA-1) has been used most often for routine paraffin sections [5, 15, 16, 21, 26, 30]. However, the use of RCA-1 is not feasible for accurately recognizing microglia associated with senile plaques because RCA-1 stains the corona or the core in senile plaques [30]. On the other hand, anti-L-ferritin antibody, which we recently reported to be another marker for microglia applicable to formalin-fixed, routine paraffin sections, does not label senile plaques or endothelial cells [10]. Therefore, the double immunolabeling method used in this study is considered to be more suitable for the clear demonstration of the co-localization of amyloid deposits and microglia.

In nondemented aged controls, senile plaques were not detected in the brains of persons younger than 50 years of age but initially appeared in the brains of patients who died in their 50s [23, 33, 37], and all these plaques were identified as diffuse plaques. The majority of diffuse plaques seen in the 50s or 60s age groups were found to contain no microglia. The diffuse plaques appearing in these age groups of nondemented aged controls are considered to be still in the initial stages of plaque formation. Therefore, it is likely that diffuse plaques in the initial stage are not involved with microglia reaction.

Diffuse plaques increased in microglia positivity with aging. Among all microglia-positive diffuse plaques, those containing a larger number of microglia, i.e., diffuse plaques of higher grade also increased in density with aging, and those containing clusters of many microglia were observed only in the brains of persons in the 70s age group and over. Classical and compact plaques were detected only in the brains of persons in the 70s age group and over, and nearly all of them were found to contain microglia. Many diffuse plaques observed in the older brains can be considered ones that have persisted longer after they were formed. Classical and compact plaques are considered to be in more advanced stages of plaque evolution than diffuse plaques [34]. Therefore, the older the plaques, i.e., the longer the period after the plaques were formed, the more likely they are to be associated with microglia, and with even larger numbers of microglia.

The morphology of microglia is considered to be heterogeneous and determined by the microglial functional status [1, 25]. The majority of microglia involved in diffuse plaques observed in the brains of the 50s and 60s age groups were presumably resting microglia with a scanty cytoplasm and fine processes. In constrast, clustered microglia involved in diffuse plaques observed in the 70s age group and over were reactive microglia with swollen cytoplasms and few fine processes. Most of the microglia in typical and compact plaques were also reactive microglia. Therefore, the longer senile plaques persist, the larger the number of reactive microglia which tend to become associated with them. All the findings mentioned above suggest that there might be a close relationship between microglial reaction and aging or maturation of senile plaques, in respect to both its severity and function.

McGeer et al. [18] reported that the hippocampus of patients with senile dementia of Alzheimer type showed a significantly higher density of HLA-DR-positive reactive microglia than in nondemented individuals, and that most of these cells were present in the areas where many senile plaques were found. Our double-immunohistochemical study also revealed an increase of microglia-containing plaques in AD, however, the most striking feature of AD was the increase of plaques without microglia. Diffuse plaques without microglia were also plaques that initially appeared in the aging process of nondemented aged controls. Thus, senile plaques in the initial stage of plaque formation were abnormally increased in AD. These findings suggest that plaque generation in AD might develop at a higher speed than microglial reactions are able to follow.

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