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Demonstration of microglial cells in and around senile (neuritic) plaques in the Alzheimer brain

An immunohistochemical study using a novel monoclonal antibody*

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Summary. A monoclonal antibody, termed AD11/8, reactive to microglial cells, was produced by immunization of mice with partially purified amyloid fibrils of senile (neuritic) plaques. With immunoperoxidase staining on human tissues, AD11/8 also recognized macrophages in the red pulp of the spleen, Kupffer cells in the liver, and macrophages in the bone marrow. The results show that AD11/8 recognizes the antigens associated with mononuclear phagocytes lineage. In normal brains a few resting microglial cells were stained in gray matter, and less frequently in white matter. In senile dementia of the Alzheimer type numerous microglial cells were stained intensively and they often formed clusters in gray matter. By double immunostaining with AD11/8 and a polyclonal antibody against synthetic amyloid β-protein, clustered microglial cells were observed in and around senile plaques with amyloid deposits. Some amyloid plaque cores were surrounded by microglial cell processes. These results indicate that microglial cells may play an important role in senile plaque formation.

Key words: Monoclonal antibody – Microglial cells – Alzheimer's disease – Senile plaques – Immunohistochemistry

One major pathological feature of Alzheimer's disease (AD) and senile dementia of the Alzheimer type (SDAT) is the presence of numerous senile plaques in the brain tissue. The density of senile plaques correlates with the severity of dementia [4]. Deposits of amyloid fibrils are considered as the most important component of senile plaques, together with the degenerative neurites and reactive glial cells [9, 37, 38].

In previous immunohistochemical studies, some components of immunoglobulin [7, 19, 32], complement factors [7, 19] and α_1-antichymotrypsin [1] have been demonstrated in association with amyloid of senile plaques. Recently the purification of amyloid fibril protein from senile plaques was reported [2, 25]. The amino acid composition and N-terminal amino acid sequence of the purified protein (4,200 dalton) were identical to those of cerebrovascular amyloid β-protein [12]. These results suggest that amyloid fibrils are composed of β-protein, but other closely associated components may also take part in amyloid fibril formation. The production of monoclonal antibodies to isolated amyloid fibrils is one strategy which may help to identify unknown components or factors involved in the latter process. This report describes the immunohistochemical characterization of a new monoclonal antibody AD11/8, which was raised against partially purified amyloid fibrils of senile plaques. Unexpectedly, AD11/8 showed reactivity with microglial cells in normal and diseased human brains. Further, we have used this antibody to study the relationship between microglial cells and senile plaque formation in AD and SDAT brains.

Materials and methods

Purification of amyloid fibrils from senile plaques

Human brains from cases Alzheimer's disease were frozen at −70°C. Cortical gray matter (100 g) was homogenized in sucrose buffer A (1 M sucrose, 0.05 M Tris-HCl, pH 7.6) at a sample to buffer ratio of 1:4 (wt/vol). The homogenate was centrifuged at 15,000 rpm for 60 min at 4°C. The pellet was suspended in sucrose buffer B (2 M sucrose, 0.05 M Tris-HCl, pH 7.6) and then centrifuged as before. The pellet was resuspended in 80 ml of 0.05 M Tris-HCl (pH 7.6) with 9 mg of subtilisin, and incubated for 1 h at 37°C. 0.5 mg of

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phenylmethylsulfonyl fluoride (PMSF) was added to the subtilisin suspension, and stirred for 1 h at room temperature. The suspension was centrifuged as before. The pellet was then digested with 6 mg of collagenase in 25 ml of 0.05 M Tris-HCl (pH 7.6) for 1 h at 37° C. After centrifugation, the pellet was resuspended in 10 ml of 1% SDS, 0.05 M Tris-HCl (pH 7.6), and boilded for 5 min. The SDS suspension was again centrifuged and the pellet was suspended in 80% formic acid for 16 h at room temperature. This suspension was used as antigen after lyophilization. The purity of amyloid fibrils was assessed by polarization microscopy after Congo red staining.

Production of monoclonal antibody

The partially purified amyloid fibrils (3.5 mg) were sonicated in 1 ml of phosphate-buffered saline (PBS), and used as immunogen. Two BALB/c mice each received a subcutaneous injection of 0.15 ml of immunogen emulsified with an equal volume of Freund's complete adjuvant. Seventy-three days later, one of the mice was given an intraperitoneal booster injection of immunogen without adjuvant. Three days after the second injection, fusion was performed according to the method of Köhler and Milstein [23]. Supernatants of growing hybridomas were tested for reactivity by immunohistochemical staining with tissue sections of SDAT as described below. Subcloning was carried out twice by the limiting dilution method. The clones were grown as ascites tumors in BALB/c mice injected intraperitoneally with 0.5 ml of pristane (Aldrich) 5 days prior to injection of cloned cells. The immunoglobulin subclass of AD11/8, determined by Ouchterlony immunodiffusion analysis, was shown to be of the IgM type.

Tissue samples

For immunohistochemical studies, human brains were obtained from AD (one case), SDAT (six cases), acute Herpes encephalitis (one case), hemorrhagic infarction (three cases), and non-neurological cases (three cases). The ages of demented patients ranged from 71 to 89 years (average: 82 years). Normal tissues from another five cases, including liver, spleen, lung, bone marrow, and lymph node, were also examined. Tissues were fixed in 10% buffered formalin and embedded in paraffin by standard procedures.

Small blocks of frontal and occipital cortex were also collected from four SDAT patients. They were embedded in Tissue-Tek O.C.T. Compound (Miles Lab.), followed by snap freezing in liquid nitrogen. Cryostat sections were cut at 6 μm, and fixed with acetone for 7 min.

Immunohistochemical reagents

A monoclonal antibody EBM/11 (DAKO) for a pan macrophage marker was compared in this study with the reactivity of AD11/8 antibody. A monoclonal antibody Hy 12-29-1L, produced in this laboratory, was used to demonstrate the glial fibrillary acidic protein (GFAP) in astrocytes [15]. A rabbit antiserum (Rb758) raised against a synthetic peptide consisting of residues 1–24 of amyloid β-protein [39] was used to demonstrate amyloid deposits in senile plaques.

Immunoperoxidase staining

Immunoperoxidase staining was carried out using the avidin-biotin complex peroxidase method according to Hsu et al. with some modification [17]. Deparaffinized tissue sections were incubated with 0.3% H_2O_2-methanol for 30 min to inhibit endogenous peroxidase. After washing in Tris-buffered saline (TBS), sections were overlaid with 10% normal horse serum (NHS) to block non-specific binding sites. Some sections were pretreated with 90% formic acid for 10 min before incubation with 10% NHS. The sections were incubated at room temperature with AD 11/8 antibody (ascites fluid diluted at 1:500 with TBS) for 1 h. The sections were washed three times in TBS, then overlaid with biotinylated anti-mouse IgM for 1 h. They were washed again, and incubated overnight with avidin DH-biotinylated peroxidase complex. The peroxidase activity was visualized with 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris buffer (pH 7.6) for 5 min. The sections were counterstained with hematoxylin.

Double staining

To visualize the localization of the amyloid in senile plaques and microglial cells in the same section, the immunoperoxidase staining for microglial cells was carried out, followed by an indirect immunofluorescent technique using antiserum to β-protein. Using a fluorescence microscope in combination with a standard bright-field system, the green fluorescence for amyloid and the peroxidase-stained microglial cells could be observed in the same field.

Results

Preparations of senile plaque amyloid fibrils from the brains of Alzheimer's disease were used to produce monoclonal antibodies. The preparations consisted of more than 70% amyloid fibrils, with lipofuscin, microvessel fragments, and a few neurofibrillary tangles as minor components.

Unexpectedly, one supernatant out of those from 380 hybridomas specifically stained microglial cells in paraffin-embedded tissue sections. Other supernatants reacted with astrocytes, neuronal components, blood vessels, or lipofuscin. Cells stained with the monoclonal antibody AD11/8 were considered to be microglial cells, as these were morphologically indistinguishable from microglial cells demonstrated by silver impregnation [5, 6], enzyme histochemical techniques [18, 28], and lectin histochemical methods [24, 35]. AD11/8 showed positive reactivity with the microglial perikarya and their processes. These cells exhibited variations in their shapes and sizes, and their degree of immunoreactivity.

In non-neurological cases microglial cells were rarely observed, and positive cells exhibited a small round or elongated nucleus with scanty perinuclear cytoplasm, from which a few short processes or ramified fine processes were extended (Fig. 1a). These are presumably resting microglial cells. They were often observed in the vicinity of blood vessels. In white matter microglial cells with a few processes were occasionally found along the axis of fiber tracts. Microglial cells were more frequently observed in the brain of demented cases compared with normal ones. Some of these cells appeared to be reactive microglial cells with swollen cytoplasm, which were intensely

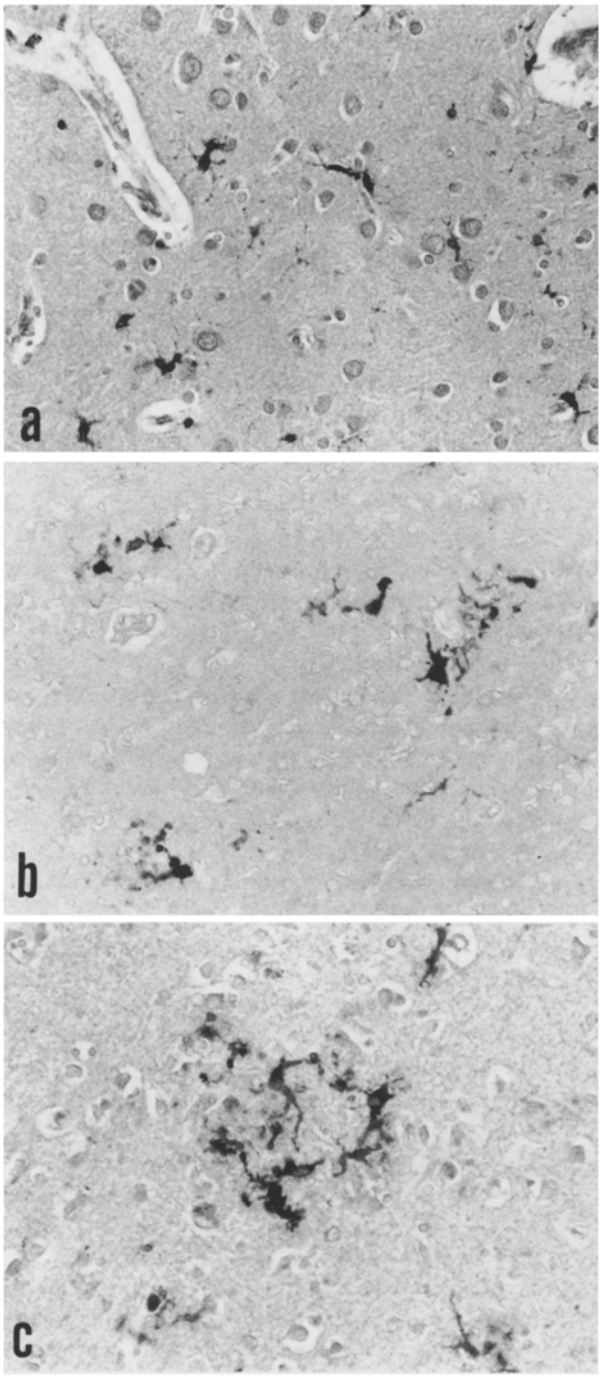

Fig. 1a–c. Formalin-fixed paraffin-embedded sections of human brains, immunostained with AD11/8. **a** Microglial cells in age-matched normal brain, counterstained with hematoxylin, ×66. **b** Microglial cells in senile dementia of the Alzheimer's type (SDAT), no counterstaining, ×50. **c** Microglial cells in SDAT, counterstained with hematoxylin. Several reactive microglial cells form cluster in gray matter, ×66

stained by AD11/8 (Fig. 1b). The numbers of microglial cells varied from area to area. Several reactive microglial cells formed clusters in gray matter (Fig. 1c). By double immunostaining with anti-synthetic amyloid β-protein (Rb 758), and AD11/8, microglial cells were found in and around senile plaques with amyloid deposits (Fig. 2a–c). Some of the microglial cells were closely attached to the senile plaque amyloid and occasionally their processes covered the amyloid core (Fig. 2d). In a few instances, small amyloid deposits adjacent to microglial cells were observed with the double-staining method using AD 11/8 and Rb758 (Fig. 2e). These pictures suggest that amyloid fibers may be produced by microglial cells.

GFAP-positive astrocytes were also observed around the amyloid plaques. However, they were found at the periphery of plaques, and GFAP-positive astrocytic processes were rarely seen in the center of plaques (Fig. 2f).

In the patients with hemorrhagic infarction and acute Herpes encephalitis, a large number of brain macrophages with foamy vacuoles were intensely stained by AD11/8. But, larger balloon-like cells, demonstrable by hematoxylin-eosin, were faintly or negatively stained by AD11/8. There was an increased number of reactive microglial cells adjacent to the necrotic areas (Fig. 3).

In cryostat sections, positively stained microglial cells were not found either in brains of non-neurological or demented cases. In contrast, microglial cells reactive with EBM/11 (DAKO), a monoclonal antibody to human macrophage, were shown only in cryostat sections. Interestingly, cells reactive to EBM/11 were much more numerous in cerebral white matter of normal and demented brains. Superimposed by the staining with anti-β-protein, EBM/11-positive cells also appeared around senile plaques as observed with AD11/8 (not shown).

In spleen, red pulp macrophages with long, fine processes were stained with AD11/8, but follicular macrophages were not (Fig. 4a). In liver, Kupffer cells lining the hepatic sinusoids were intensely stained with AD11/8 (Fig. 4b). The number of stained cells varied from case to case. Cells reactive to AD11/8 in bone marrow also seemed to be macrophages and they showed a large, rounded appearence. Among such organs as lung and lymph node, AD11/8 stained only mononuclear phagocytes and the immunoreactive proportion varied from specimen to specimen.

With formic acid pretreatment, the immunhistochemical reactivity was enhanced for the cells positive with AD11/8 and these cells showed more extended and branched processes.

Discussion

A monoclonal antibody (AD11/8) was reactive to microglial cells in formalin-fixed paraffin-embedded

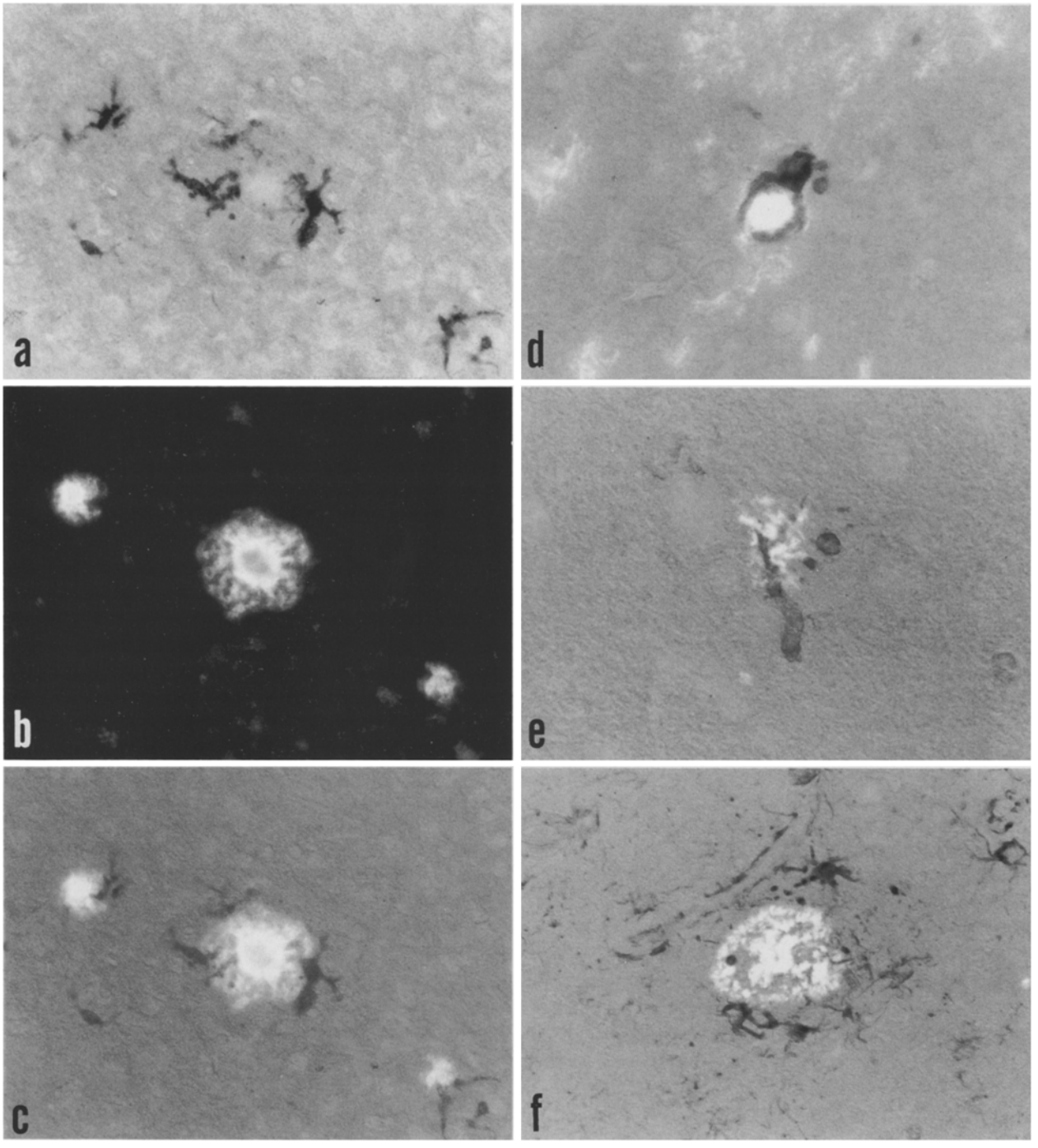

Fig. 2a–f. Double staining of peroxidase-labeled microglial cells (**a**, **c–e**) or astrocytes (**f**) and fluorescein isothiocyanate-labeled β-protein (**b–f**). **a** Microglial cells in SDAT brain, ×100. **b** Same area as photograph **a**. Senile plaque amyloid is stained by anti-β-protein. **c** Microglial cells are demonstrated around a senile plaque with amyloid deposits. Some processes of microglial cells are attached to the amyloid core. **d** Processes of microglial cells surround the amyloid core, suggesting phagocytosis, ×200. **e** Wispy amyloid material seems to be emanated from the processes of microglial cells, ×200. **f** Astrocytes labeled by anti-glial fibrillary acidic protein also surround the senile plaque, ×100

tissue sections. Since all of the macrophage-specific antibodies so far reported fail to react with paraffin-embedded tissues, AD11/8 will prove to be a useful reagent for routine immunohistochemical staining of microglial cells in brain and mononuclear phagocytes in other organs.

Del Rio-Hortega [6] first proposed that microglial cells were of mesodermal origin, derived from blood

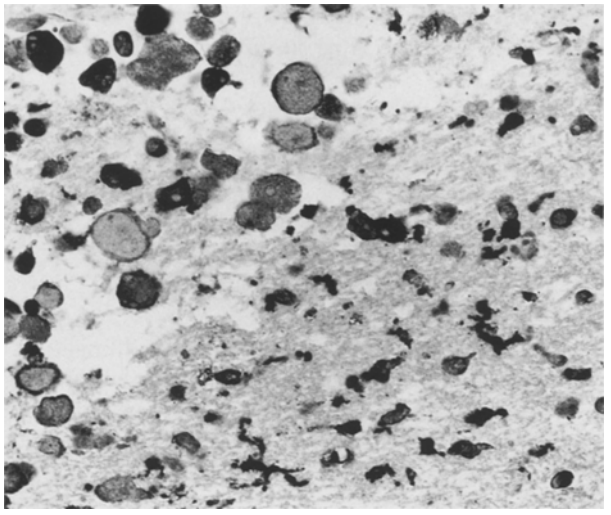

Fig. 3. Necrotic areas in cerebral infarction. AD11/8 intensely labels round macrophages and reactive microglial cells, but reacts weakly or not at all with larger cells, counterstained with hematoxylin, × 66

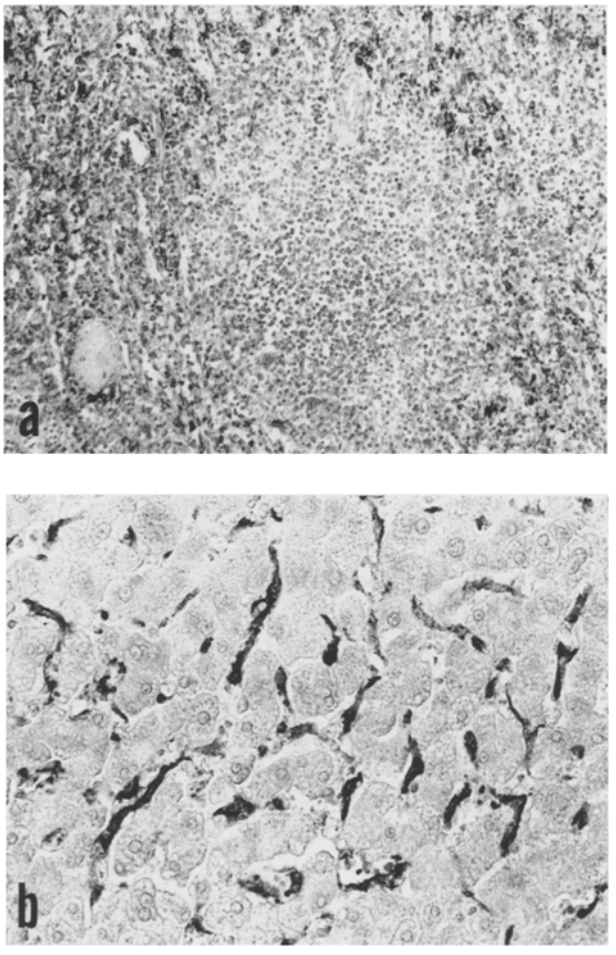

Fig. 4a, b. Immunostaining with AD11/8 for spleen and liver, counterstained with hematoxylin. **a** Macrophages in red pulp are stained, × 33. **b** Kupffer cells lining the hepatic sinusoids are also labeled, × 66

monocytes, and differentiated to resting microglia in brain parenchyma. The mesodermal origin of microglial cells has been widely accepted, but a neurodermal origin has also been proposed [10, 22, 34]. Since immunohistochemical studies using mononuclear phagocyte-specific markers have failed to stain microglial cells in adult brain, some authors have concluded that these cells are not derived from blood monocytes [29, 40].

Recently, however, rat monoclonal antibodies Mac-1 and F4/80, specific for mouse myelomonocytic differentiation antigen and for mature mouse macrophages, respectively, have proved to show reactivity to microglial cells in developing and adult mouse brain [26, 30]. Furthermore, the monoclonal antibody EBM/11, raised against lung macrophages, reacts with macrophages and microglial cells in human brain [8, 16]. AD11/8 also recognized cells belonging to the mononuclear phagocytes. These results provide evidence for the mesodermal origin of microglial cells.

The reason why an antibody reactive to microglial cells was produced by injection of partially purified amyloid as the immunogen is not clear at present. However, we assume that microglial cell components were present either in association with amyloid fibrils or as other non-associated contaminants. As we observed, microglial cells are often closely associated with senile plaques, i.e., these cells occasionally surround amyloid deposits. AD11/8 exhibited no staining in cryostat sections, but reacted only in paraffin sections, especially after pretreatment with formic acid. This suggests that AD11/8 recognized a denatured antigen of microglial cells. The staining patterns of microglial cells with AD11/8 were different from those with pan macrophage marker EBM/11. In white matter of normal and SDAT brains, EBM/11-positive cells were more numerous compared with AD11/8. In addition, EBM/11 was also reactive with perivascular cells and leptomeningial cells. Thus microglial cells unreactive to AD11/8 may exist, especially in white matter.

AD11/8 showed an especially intense immunoreactivity with reactive cells associated with pathological lesions. In SDAT brains, degenerating neurons, neurofibrillary changes and senile plaque formation may act as the stimuli to resting microglial cells, causing them to be transformed into reactive microglial cells. In our study it was confirmed that several reactive microglial cells were located in and around senile plaques and their processes frequently surrounded the central amyloid core. There is a possibility that the antigen recognized by AD11/8 may be involved in

amyloid formation. Recently with the use of monoclonal antibodies to HLA-DR (the class 2 glycoprotein), McGeer et al. [27] also found positively stained microglial cells in gray matter of SDAT brains, particularly concentrated in the area of senile plaques. Such microglial cells, bearing HLA-DR, may reflect the function as antigen-presenting cells, such as those observed in acute experimental allergic encephalomyeliits and multiple sclerosis [31, 41].

In previous immunohistochemical studies it was found that complement C1q, C4, and C3 were closely associated with amyloid fibrils of senile plaques [7, 20]. It is well known that binding of C3b to membrane antigens or immune complex activates macrophages. This mechanism may be available to microglial cells. Namely, these cells, activated by C3 on the amyloid fibrils, adhere to and attempt to phagocytose amyloid deposits.

In double-stained SDAT sections, some microglial cells seemed to be producing a small amount of amyloid fibrils from their soma or processes into the extracellular spaces. In systemic amyloidosis, the cells belonging to the mononuclear phagocytic system (MPS) are associated with the formation and deposition of amyloid fibrils. Fuks and Zucker-Franklin [11] have shown in an in vitro study that the precursor of amyloid A protein (SAA) is processed by Kupffer cells and impairment of these cells causes incomplete processing of SAA, which results in the production of AA peptide. They proposed that AA peptide polymerized into amyloid fibrils on the surface of the plasma membrane. In the central nervous system, microglial cells and pericytes belong to MPS. Therefore, it is interesting to speculate that these cells are able to process the brain amyloid precursor protein and that faulty processing results in formation of the amyloid fibrils. Recently, in several laboratories complementary DNA clones encoding brain amyloid β-protein have been characterized [14, 21, 33, 36] and the deduced full-length amino acids sequence of the precursor was predicted [21]. Using the in situ hybridization technique, it was shown that mRNA encoding amyloid β-protein was expressed in subpopulations of neurons in normal and AD brains [3, 13]. Therefore, it can be hypothesized that brain amyloid β-precursor protein produced by neurons may be completely degraded by microglial cells in normal brain, but aberrant processing or failure of full degradation of the precursor proteins leads to the accumulation of abnormal or amyloid proteins in AD. Finally, extracellar amyloid fibrils may be produced from the plasma membrane of microglial cells.

In the present study with light microscopic immunohistochemistry, it remains unclear whether or not the microglial cells produce the amyloid proteins or amyloid fibrils, so that additional electron microscopic studies, and in vitro studies are required.

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