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Accumulation of Intercellular Adhesion Molecule-1 in Senile Plaques in Brain Tissue of Patients with Alzheimer's Disease

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The still unsolved pathogenesis of Alzheimer's disease (AD) has been the subject of extensive speculation. Some years ago, a local acute phase reaction involving production of interleukin-1 (IL-1) and IL-6 was proposed as the triggering event in AD. Since it has been reported that these cytokines induce expression of intercellular adhesion molecule-1 (ICAM-1), we analyzed AD brain tissue cryosections for the presence of ICAM-1 by immunostaining and for ICAM-2 expression as a control. In senile plaques a marked diffuse or granular staining for the ICAM-1 domains 1, 4, and 5 was observed, whereas ICAM-2 expression was observed in microglial cells. Immunoprecipitation analysis demonstrated the presence of a 85 kd ICAM-1 molecule in AD frontal cortex. Our findings indicate that ICAM-1 accumulates in senile plaques as a complete 5-domain molecule at a relatively early stage of senile plaque formation. Our results are in support of a cytokine-mediated pathogenesis of senile plaque formation. (Am J Pathol 1994, 144:104–116)

Typical neuropathological features of Alzheimer's disease (AD) comprise the presence of numerous senile plaques, neurofibrillary tangles, and cerebrovascular amyloidosis (CA) in various regions of the cortex, hippocampus, and amygdala of the brain.[1]

Several components of senile plaques (including the classic, diffuse, and burned-out types of plaques) have been identified in the past few years. The 4.2-kd amyloid β protein (Aβ) is the major constituent of the diverse types of senile plaques and of CA.[2–4] It has been assumed that the degenerative lesions in AD arise by the deposition of Aβ, which may be formed by proteolytic processing of the amyloid precursor protein (βPP) in the endosomal-lysosomal system.[5–11] Apart from Aβ, a number of Aβ-associated proteins[12] have been identified by immunostaining of Alzheimer brain specimens. Thus, the presence of α-1-antichymotrypsin (ACT),[2,13] complement factors,[14] serum amyloid P-component[15] and heparan sulfate proteoglycans[16,17] in all types of senile plaques and in CA could be demonstrated. Also α_2-macroglobulin (α_2-M), C-reactive protein, and IL-6 have been found in different subsets of senile plaques in Alzheimer brains.[18]

The possible role of each of these components in the pathogenesis of AD has been the subject of extensive speculation. In particular, the presence of the acute phase proteins ACT, α_2-M, and C-reactive protein and of IL-6 in the senile plaque has drawn attention to the possibility of an IL-1- or IL-6-mediated pathogenesis of AD, with several components within the senile plaque originating from a cerebral acute phase response.[19,20] Both IL-1 and IL-6 are known as inducers of the acute phase reaction and there is indirect evidence that the synthesis of both βPP and ACT might be induced by IL-6.[21,22]

In cultured human endothelial cells, IL-1 upregulated the expression of both βPP mRNA[23] and ICAM-1 protein.[24] In tissue, both IL-1 and IL-6 induced the expression of, among other molecules, ICAM-1 on the vascular endothelium.[24,25] Unlike ICAM-1, the recently discovered adhesion molecule ICAM-2 is constitutively expressed on vascular endothelium, lymphocytes, and monocytes and is not inducible by several cytokines.[26,27] On the basis of these previous findings, we decided to analyze a series of brain tissue samples from patients with AD, senile dementia of the Alzheimer type (SDAT), non-Alzheimer dementia (NAD) and from control cases

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for expression of ICAM-1 on brain cortical capillaries. For this purpose, we used a set of anti-ICAM-1 monoclonal antibodies (MAbs) raised in our own laboratory, in addition to a series of already established anti-ICAM-1 MAbs. Antibodies directed against the closely related ICAM-2 molecule were used as controls.

Both anti-ICAM-1 and anti-ICAM-2 antibodies showed the expected vascular staining, but with anti-ICAM-1, a marked reaction with the senile plaques in Alzheimer brain tissue was observed as well. Besides, a variable ICAM-2 reactivity was observed with microglial cells. An accumulation of ICAM-2 positive microglial cells in senile plaques was observed.

Materials and Methods

Tissue Samples

Brain tissue from patients with clinically diagnosed and neuropathologically confirmed AD (n = 7), SDAT (n = 13), NAD (n = 6), and from normal controls (n = 8) were obtained from autopsy material with postmortem delays between 20 minutes and 9 hours. Neuropathological diagnosis of AD or SDAT was based on established criteria.[28] Differentiation between AD and SDAT was based on the age of onset of dementia. The age of 65 was chosen as critical value to differentiate between AD and SDAT. Tissue samples from parietal, frontal, and temporal cortex and hippocampus were frozen in liquid nitrogen immediately after removal (Table 1).

Table 1. *Brain Tissue Investigated**

Case no.	Sex	Age (yr)	PMD (h)	Diagnosis	Region
1	F	16	4.5	Control	F
2	M	73	9.0	Control	F
3	F	66	6.0	Control	F
4	M	49	3.5	Control	F
5	F	96	3.5	Control	F
6	F	85	2.5	Control†	F
7	M	81	3.0	Control	F
8	F	87	5.5	PD	F
9	F	73	0.7	NAD	T
10	M	87	2.5	PD, NAD	F
11	M	79	0.8	NAD	P
12	F	64	3.0	NPH, NAD	F
13	F	68	3.0	NAD	F
14	F	85	2.0	NAD	F
15	M	65	1.1	AD	P
16	F	69	1.3	AD	F
17	F	67	0.3	AD	F
18	F	72	0.7	AD	F
19	F	63	2.0	AD	F
20	M	59	3.0	AD	F, HC
21	M	64	3.0	AD	F, T
22	F	93	1.3	SDAT	P
23	F	89	1.6	SDAT	P
24	F	87	0.7	SDAT	T
25	F	77	0.8	SDAT	P
26	F	77	0.6	SDAT	F
27	F	78	0.5	SDAT	F
28	M	79	1.0	SDAT	F
29	F	90	1.3	SDAT	F
30	F	88	3.0	SDAT	F
31	F	94	4.0	SDAT	F, T
32	F	83	5.0	SDAT	F, HC
33	M	84	5.0	SDAT	F, HC
34	F	81	3.5	SDAT	F, P

* PMD, post-mortem delay; NAD, non-Alzheimer dementia; AD, Alzheimer's disease; SDAT, senile dementia of Alzheimer type; PD, Parkinson's disease; NPH, normal pressure hydrocephalus; F, frontal cortex; T, temporal cortex; P, parietal cortex; HC, hippocampus.
† Non-demented person with numerous plaques in cortex.

Antibodies

MAb CL203 was a kind gift of Dr. S. Ferrone (Valhalla, NY)[29]; MAb RR1/1 and CA7 were a kind gift of Dr. R. Rothlein (Boehringer Ingelheim, Ridgefield, CO); MAb P3.58 clones 11, 14, and 19 were a kind gift of Dr. J. Johnson (München, Germany); MAb WT32 was a kind gift of Dr. W. Tax (Nijmegen, The Netherlands); MAb CBR-IC2/2 was a kind gift of Dr. T. Springer (Boston, MA); MAb 6D5 was a kind gift of Dr. C. Gahmberg (Helsinki, Finland); MAbs H3/5–47, A10/33, EN7/44, RM3/1, and 27E10 and the pAb MRP8 and MRP14 were kind gifts of Dr. C. Sorg (Münster, Germany). MAb 4B9 was a kind gift of Dr. T. Carlos (Seattle, WA)[30]; MAb ENA-1 was a kind gift of Dr. J. Leeuwenberg (Maastricht, The Netherlands)[31]; MAb NKI-L12 was a kind gift of Dr. C. Figdor (Dutch Cancer Institute, Amsterdam, The Netherlands); Anti-Aβ antibodies were purchased from Boehringer Mannheim, Almere, The Netherlands, and affinity-purified anti-Aβ polyclonal antibodies were a kind gift of Dr. W. E. Van Nostrand, Irvine, CA (Table 2).

Hybridoma Production

Seven-week-old female BALB/c mice were injected intraperitoneally with 5×10^6 tumor necrosis factor-α (TNF-α)-stimulated (1 nmol/L for 72 hours) human umbilical vein endothelial cells (HUVEC) in 0.25 ml PBS on the left side of the body, and the same amount of cells in 0.25 ml CFA on the right side of the body. The HUVEC were washed twice with PBS before they were released from the culture flask by treatment with 0.02% EDTA in PBS and harvested with a rubber policeman. After 2 months the mice received, on four subsequent days, a boost of 3×10^6 TNF-α-stimulated HUVEC in 0.3 ml PBS (intravenously). On the fifth day splenocytes were obtained by removing and mincing the spleen of an immunized animal. Hybridomas were generated by

Table 2. *Antibodies Used in this Study and Their Specificities*

Antibody	MAb/pAb	Specificity
PN-E4	MAb	Domain 1 of ICAM-1
PN-E8	MAb	Domain 1 of ICAM-1
PN-E12	MAb	Domain 1 or 2 of ICAM-1
RR1/1	MAb	Domain 1 of ICAM-1
CL203	MAb	Domain 4 of ICAM-1
CA7	MAb	Domain 5 of ICAM-1
P3.58 Cl11	MAb	Domain 5 of ICAM-1
P3.58 Cl14	MAb	Domain 5 of ICAM-1
P3.58 Cl19	MAb	Domain 5 of ICAM-1
CBR-IC2/2	MAb	ICAM-2
6D5	MAb	Domain 1 of ICAM-2
NKI-L12	MAb	α-chain of LFA-1 (CD11a)
6F2	MAb	GFAP
4B9	MAb	VCAM-1
ENA-1	MAb	E-selectin
PN-E2	MAb	Endoglin
PAL-E	MAb	Uncharacterized endothelial antigen
H3/5–47	MAb	Uncharacterized endothelial antigen
A10/33	MAb	Uncharacterized endothelial antigen
EN7/44	MAb	Uncharacterized endothelial antigen
RM3/1	MAb	Uncharacterized monocyte/macrophage antigen
27E10	MAb	Uncharacterized monocyte antigen
OKT9	MAb	Transferrin receptor (CD38)
W6/32	MAb	MHC class I
WT32	MAb	CD3
MRP8	pAb	Uncharacterized macrophage antigen
MRP14	pAb	Uncharacterized macrophage antigen
vWF-FVIII	pAb	von Willebrand factor

fusing splenocytes with the SP2/0 fusion partner. An ELISA on unstimulated versus TNF-α-treated HUVEC was used to screen hybridoma culture supernatants. Hybridomas showing a differential reaction pattern were analyzed more closely.

ELISA

HUVEC were seeded in gelatin-coated 96-well microtiter plates (Costar, Cambridge, MA) at a density of 2×10^4 cells/well, and allowed to grow to confluence in the appropriate medium for 3 days. In some experiments cells were incubated with TNF-α (1 nmol/L, a kind gift of Boehringer Ingelheim) during culture in the microtiter plate. Cells were fixed with 0.025% glutaraldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature, and the wells were incubated with 1% gelatin in PBS for 30 minutes at 37 C to prevent nonspecific binding of antibody. After washing with PBS the wells were successively incubated for 30 minutes at 37 C with MAb and with peroxidase-labeled goat anti-mouse immunoglobulin heavy and light chain (Nordic, Tilburg, The Netherlands), both diluted 1:500 in PBS/1% gelatin. Enzyme substrate was then added (8 mg/ml 5-aminosalicylic acid (Sigma, St. Louis, MO) in 50 mmol/L phosphate buffer with 0.025% H_2O_2). Color development was measured after 30 minutes by reading the OD at 450 nm in a Titertek ELISA plate reader (Flow Laboratories, Irvine, Scotland).

Transfection of COS Cells and Flow Cytometry Analysis

ICAM-1 deletion mutants were generated by ligation of polymerase chain reaction fragments coding for different parts of the ICAM-1 molecule. Guided by the assignment of domain boundaries by Staunton et al,[32,33] polymerase chain reaction primers were made to generate different ICAM-1 fragments, which were ligated by a newly introduced *Eco*RV restriction site. These fragments were constructed by 10 cycles of polymerase chain reaction amplification by using human ICAM-1 cDNA[34] as template. The human ICAM-1 cDNA in the CDM8 expression vector[35] was kindly provided by Dr. B. Seed (Harvard Medical School, Boston, MA). In this way, cDNA was generated coding for ICAM-1 deletion mutants lacking the NH_2-terminal domains, domain 1 or 2. The ligated fragments were digested with appropriate restriction enzymes, gel purified, and ligated into the CDM8 expression vector. Appropriate clones were identified by restriction mapping. COS-7 cells were transfected with different ICAM-1 cDNAs in the expression vector CDM8. Anti-ICAM-1

MAbs were analyzed using flow cytometry as described by Bloemen et al[36] (in case of COS cells transfected with complete ICAM-1 cDNA) or by intracellular staining (in case of COS cells transfected with ICAM-1 deletion mutants).

Immunohistochemical Staining

Sections from frozen tissue (4 μ) on aminopropyltrietoxysilane coated glass slides were fixed with acetone for 10 minutes at room temperature. The sections were incubated with appropriate dilutions of the primary antibodies for 1 hour at room temperature or overnight at 4 C in the dark. All dilutions were made in PBS containing 1% bovine serum albumin, which also served as a negative control. The preparations were washed twice for 8 minutes with PBS and incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (dilution 1:80, DakoPatts, Glostrup, Denmark) for 30 minutes at room temperature in the dark. The peroxidase reaction was performed by incubating the sections for 5 to 8 minutes with 0.6 mg/ml diaminobenzidine (DAB, Sigma) in PBS with 0.015% H_2O_2. Nickel ammonium sulfate was added to this solution where indicated, resulting in a purple reaction product. Where indicated, the slides were incubated for 5 minutes in 0.5% $CuSO_4$ in saline and again washed with tap water, counterstained with hematoxylin for 1 to 3 minutes, and thoroughly washed with tap water for 10 minutes. After dehydration in 96% alcohol and xylol, the preparations were enclosed in Permount. Adjacent sections were used when the staining patterns obtained with the MAb had to be compared with the anti-Aβ staining or with each other.

Double immunostaining was performed as described previously[37] with some modifications. After the DAB/Ni reaction of the first cycle the sections were treated for 30 minutes with 0.5% H_2O_2 in PBS to destroy residual peroxidase activity. Subsequently the sections were incubated with a mixture of unlabeled rabbit anti-mouse antibodies (DakoPatts) and FITC-labeled sheep anti-mouse antibodies (Cappel, Boxtel, The Netherlands) to block residual free binding sites on the mouse antibodies from the first cycle. The blocking was checked by immunofluorescence microscopy and by omitting secondary antibodies from the first cycle. By doing so, it could be verified that the blocking was successful. The second staining cycle was performed as described above without the addition of nickel ammonium sulfate in the DAB mixture, resulting in a brown reaction product. Reversing the order of incubation of the primary antibodies did not affect the results.

Anti-Aβ Immunostaining

Sections from frozen tissue (4 μ) on aminopropyltrietoxysilane-coated glass slides were fixed in acetone for 4 minutes, followed by another 6 minutes of fixation in acetone supplied with 0.15% H_2O_2 to block endogenous peroxidase. Sections were then treated with 50% formic acid for 15 to 30 minutes, washed in PBS, and incubated with 20% normal swine serum for 10 minutes. Primary rabbit anti-Aβ antibody solution at 20 μg/ml (Boehringer Mannheim) or 1:300 (gift from W. E. Van Nostrand) was incubated overnight at 4 C in the dark. The preparations were washed twice for 8 minutes with PBS and incubated with swine anti-rabbit antibodies (dilution 1:20, DakoPatts) for 30 minutes, washed again in PBS, and incubated with peroxidase anti-peroxidase complex (dilution 1:800, DakoPatts) for 30 minutes. The peroxidase reaction was performed as described above.

Thioflavin S Staining

Sections stained for ICAM-1 were subsequently stained with thioflavin S as described.[38] In short, sections were rinsed in 50% ethanol and stained for 5 minutes with 0.01% thioflavin S (Sigma) in 50% ethanol. Subsequently, the sections were washed twice in 50% ethanol and twice in water.

Immunoprecipitation and SDS-PAGE

To determine the molecular weight of the MAb, HUVEC were metabolically labeled for 4 hours with 150 μCi [^{35}S]methionine (Amersham, Houten, The Netherlands) in 5 ml methionine-free Eagle's minimal essential medium, supplemented with 15% dialyzed fetal calf serum, gentamicin, and glutamine, after an initial incubation for 4 hours in the same medium without [^{35}S]methionine. After three washes with PBS, cells were removed from the tissue culture flask non-enzymatically (see above) and treated with lysis buffer (0.1% sodium dodecyl sulfate, SDS; 1% Nonidet P-40, NP-40; 0.5% Na deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, PMSF; 1 mmol/L benzamidine, 500 IE/ml aprotinin in PBS). After centrifugation to remove nuclear debris, radiolabeled HUVEC lysates were immunoprecipitated essentially as described by Tamura et al.[39] Briefly, Costar 96-well EIA plates were coated with 100 μl of a 100 μg/ml solution of affinity-purified goat anti-mouse antibody (Cappel, Boxtel, The Netherlands) for 18 hours at 4 C. After washing with PBS/0.05% Tween-20, specific MAb was added to the wells

and incubated for 4 hours at 4 C, followed by another washing step. Wells were then incubated with 200 µl/well PBS/1% bovine serum albumin/1% normal mouse serum for 2 hours at room temperature, and, after washing, radio-labeled cell lysate was added at 2×10^6 cpm/well. After incubation of the plate for 18 hours at 4 C, wells were washed extensively with lysis buffer. Bound antigen was extracted from the wells by incubation with sample buffer for 15 minutes at 60 C and analyzed by SDS-PAGE. Gels were dried and exposed for autoradiography.

For ICAM-1 immunoprecipitation from brain tissue, small pieces of frontal cortex (SDAT: $n = 3$; control: $n = 3$) were thawed, homogenized in lysis buffer without SDS, NP-40, and Na deoxycholate, and left for 30 minutes at 0 C. After centrifugation at 12,000 × *g* for 10 minutes, the pellet was treated with complete lysis buffer for 30 minutes at 0 C. After centrifugation the supernatants were radioiodinated as follows. To four Iodobeads (Pierce, Rockford, IL) in 200 µl PBS, 1.0 mCi $Na^{125}I$ was added and allowed to react for 5 minutes. 200 µl of lysate were added and incubated for 15 minutes. Unbound ^{125}I was separated from the labeled proteins on a Sephadex G-25 column. Radiolabeled proteins were eluted with lysis buffer, incubated on a pretreated ELISA plate, and subsequently analyzed as described above. TNF-α-stimulated HUVEC expressing high levels of ICAM-1 were used as controls and were harvested as described above. The endothelial cell lysate was labeled with 0.5 mCi ^{125}I and processed as described.

Results

Generation and Characterization of ICAM-1-Specific MAbs

MAbs were raised against TNF-α-treated HUVEC. Three MAbs were selected that showed a stronger reaction with TNF-α-stimulated than with unstimulated HUVEC in the ELISA. These MAbs, designated as PN-E4, PN-E8, and PN-E12, immunoprecipitated a 96-kd antigen from TNF-α-stimulated HUVEC. A molecule with identical M_r was recognized by the anti-ICAM-1 MAb CL203 (Figure 1).

To confirm the nature of the antigen recognized by the MAb, binding studies were performed with COS cells transiently transfected with cDNA encoding for ICAM-1. The four MAbs reacted positively with these transfected COS cells (Table 3). Transfection of COS cells with ICAM-1 cDNA deletion mutants lacking a specific domain revealed that the MAbs PN-E4 and

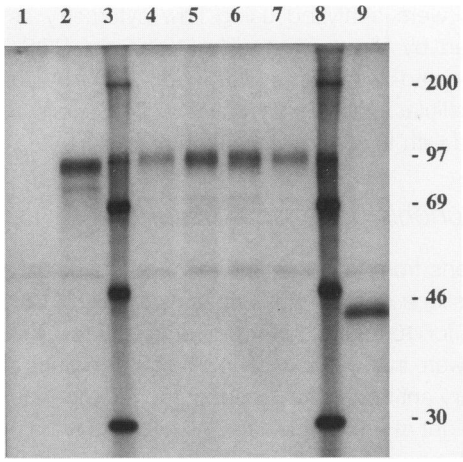

Figure 1. *Analysis of indirect immunoprecipitates by SDS-PAGE under reducing conditions. Autoradiography of a dried 10% gel containing ^{35}S-labeled endothelial cell lysates precipitated by:* lane 1: *anti-CD3 MAb WT32 (negative control);* lane 2: *MAb PN-E2, directed against endoglin on endothelial cells[60];* lanes 3 *and* 8: *molecular weight markers;* lane 4: *MAb PN-E4;* lane 5: *MAb PN-E8;* lane 6: *MAb PN-E12;* lane 7: *MAb CL203;* lane 9: *MAb W6/32. Molecular sizes of the standards are indicated at the right in kilodaltons.*

Table 3. *Binding of Anti-ICAM-1 mAb to COS Cells Transfected with ICAM-1 cDNA*

Antibody	% Positive	Mean Fluorescence Intensity
Control	10.9	54
PN-E4	39.6	513
PN-E8	42.8	465
PN-E12	40.5	681
CL203	43.5	663

PN-E8 are reactive with domain 1 of the ICAM-1 molecule, and MAb PN-E12 is directed against an epitope between domains 1 and 2 or is reactive with parts of both domains (data not shown). Immunohistological analysis on a number of tissues with inflammatory lesions showed identical staining patterns with all four MAbs (not shown). From these results we concluded that the MAbs PN-E4, PN-E8, and PN-E12 are directed against ICAM-1.

ICAM-1 Immunohistochemistry

Serial frozen sections of brain tissue of 13 SDAT patients, 7 AD patients, 6 NAD patients, and 8 controls (Table 1) were immunostained with 9 different anti-ICAM-1 MAbs (CL203, CA7, PN-E4, PN-E8, PN-E12, the P3.58 clones 11, 14 and 19, and RR1/1), with two different anti-ICAM-2 MAbs (CBR-IC2/2 and 6D5) and several MAbs directed against other adhesion molecules or control antigens (Table 2). In 12 of 13 SDAT and in all 7 AD tissue specimens, with all anti-ICAM-1 MAb foci with a diffuse or diffuse granular staining were observed in cortical

gray matter from the frontal, parietal, and temporal regions and in the hippocampus. These foci co-localized exactly with Aβ-positive senile plaques (Figure 2, A-D). Incidentally, fibrillar ICAM-1 staining projecting from the diffusely stained plaques was observed (Figure 2). In the one case of SDAT that was negative for ICAM-1 staining, no senile plaques were present in the available frozen tissue specimen. In NAD and control tissue, no staining with anti-ICAM-1 MAb was observed, except for a few cases where a sporadic positive spot was present. This finding is explained by the appearance of incidental senile plaques in brains of elderly controls without dementia.[28] No plaque-associated staining was observed when brain tissue sections were tested with a large number of unrelated monoclonal or polyclonal antibodies (Table 2), including antibodies against ICAM-2, which is a molecule functionally closely related to ICAM-1, and with 35% homology with the two amino-terminal domains of ICAM-1.

Anti-ICAM-1 MAb stained the classic, diffuse, and burned-out type of senile plaques as is shown by comparison with anti-Aβ stain in serial sections (Figure 2, E and F). From the micrographs in Figure 2 it is also evident that the amyloid deposits in a se-

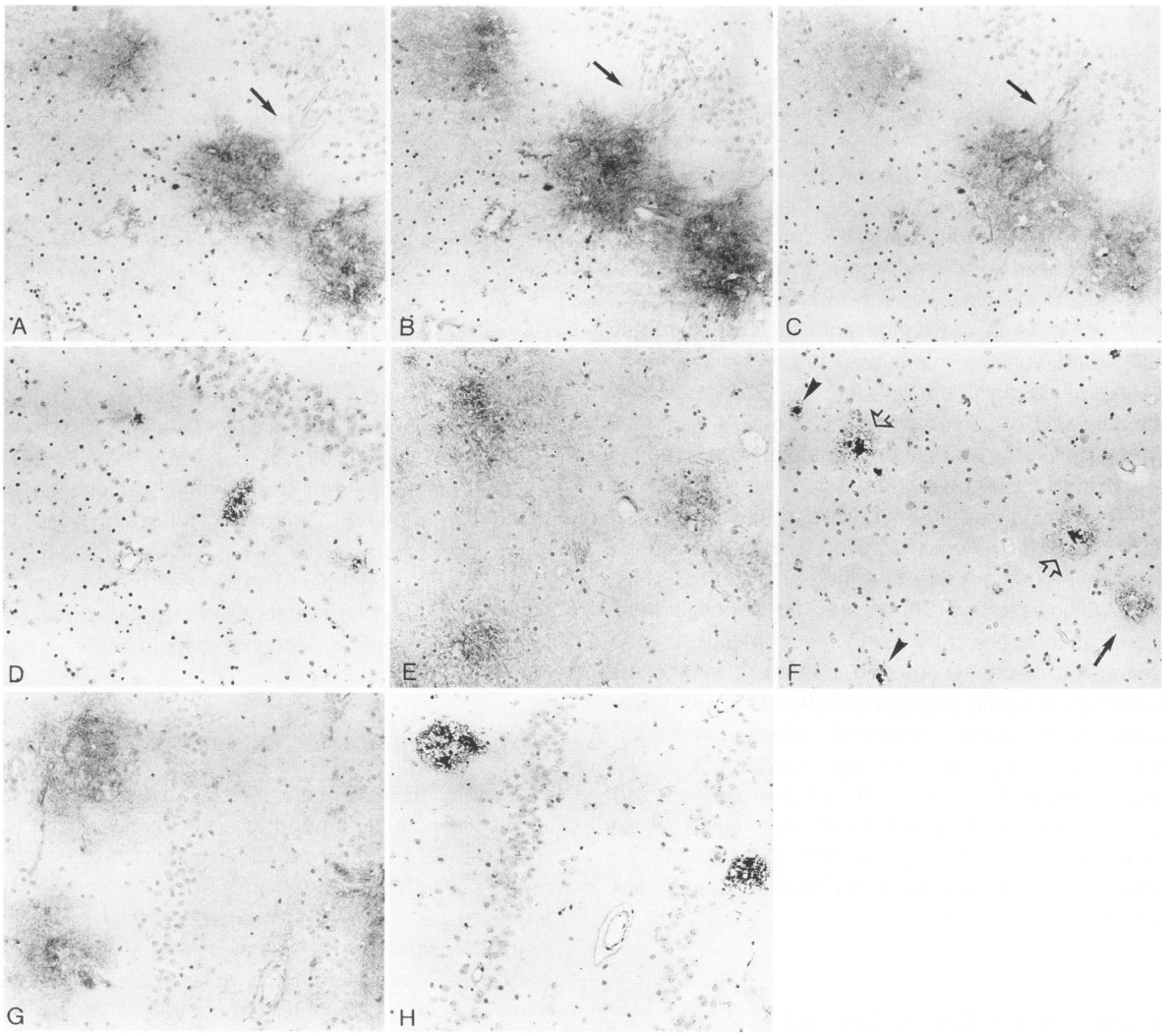

Figure 2. *Comparison of indirect ICAM-1 and Aβ immunostaining in serial cryosections of AD brain tissue.* **A-D:** *Anti-ICAM-1 MAb stain senile plaques in AD brain tissue. Serial sections of hippocampus of AD brain stained with MAb CL203* (**A**), *MAb PN-E12* (**B**), *MAb PN-E8* (**C**), *or anti-Aβ* (*Boehringer Mannheim*) (**D**). *Arrows indicate a plaque from which fibrillar ICAM-1 staining is extending from the plaque into the surrounding tissue.* **E-F:** *Classic plaques* (*open arrows*), *a diffuse plaque* (*closed arrow*), *and burned-out plaques* (*arrowheads*) *are all labeled with anti-ICAM-1 MAb. Note that anti-ICAM-1 MAbs stained a larger tissue area compared with anti-Aβ antibodies. Sections of frontal cortex of AD brain stained with MAb CL203* (**E**) *or with anti-Aβ* (*Boehringer Mannheim*) (**F**). **G-H:** *An ICAM-1 positive spot is not labeled with anti-Aβ antibodies* (*Boehringer*). *Serial sections of hippocampus of AD brain stained with MAb CL203* (**G**) *or with anti-Aβ* (**H**). *All sections were stained with DAB as chromogen and counterstained with hematoxylin. Magnifications:* **A-F,** *×150;* **G-H,** *×140.*

nile plaque are surrounded by a large tissue area affected by ICAM-1 accumulation. In addition, Figure 2, G and H, show an example of a plaque-like spot which was positively labeled by anti-ICAM-1 MAb, but not by the commercially available anti-Aβ antibody. It may be possible that this antibody was not sensitive enough to detect low levels of Aβ in early plaques. Therefore, we checked the results by thioflavin S staining, which is a sensitive method of detecting amyloid, and by staining with a highly sensitive affinity-purified non-commercial anti-Aβ antibody (see Materials and Methods). Using these techniques, all ICAM-1 positive structures appeared to co-localize with Aβ accumulation, indicating that all plaques, including those containing low levels of Aβ, are positive for ICAM-1 staining as well (data not shown).

We were able to demonstrate the presence of ICAM-1 in senile plaques by MAbs directed specifically against domain 1 (PN-E4, PN-E8, and RR1/1), domain 4 (CL203), and domain 5 (CA7 and P3.58) of the ICAM-1 molecule, implying that the complete ICAM-1 molecule containing all 5 domains is present.

In all cases examined, the anti-ICAM-1 antibodies stained the endothelium and, incidentally, perivascular cells in capillaries and larger blood vessels, with varying intensities, when stained in a series of sections under identical conditions. No staining was observed when the anti-ICAM-1 MAbs were applied to routinely formalin-fixed and paraffin-embedded tissue. Also, no neurofibrillary tangles or CA were visualized by antibodies against ICAM-1.

As controls, we also studied several other tissue specimens (kidney, intestine, cerebrum) affected by non-Aβ amyloidosis. These amyloid deposits were never accompanied by ICAM-1 accumulation, indicating that this phenomenon is specific for the Aβ formation in senile plaques (results not shown). Besides, other neuropathologically affected brain tissues lacking Aβ deposits (eg, neuropathologically diagnosed patients with Parkinson's disease and normal pressure hydrocephalus) also failed to reveal plaque-like staining with anti-ICAM-1 MAb as observed in Alzheimer brain tissue (results not shown).

Immunoprecipitation Analysis

To confirm the presence of ICAM-1 molecules in Alzheimer frontal cortex tissue, extracts of tissue samples were prepared, radioiodinated, and immunoprecipitated with anti-ICAM-1 MAb. Figure 3 shows that MAb PN-E12 detected a specific 85-kd band which is within the range of M_r described for ICAM-1. The other anti-ICAM-1 MAb did not detect a specific band in this assay. This was probably due to the lower affinity of these MAbs for ICAM-1 as can be seen from the immunoprecipitation of ICAM-1 from HUVEC. From control brain tissue lysates, no detectable ICAM-1 could be precipitated (not shown), probably indicating that the amount of vascular ICAM-1 extractable from control brain tissue was below the detection threshold.

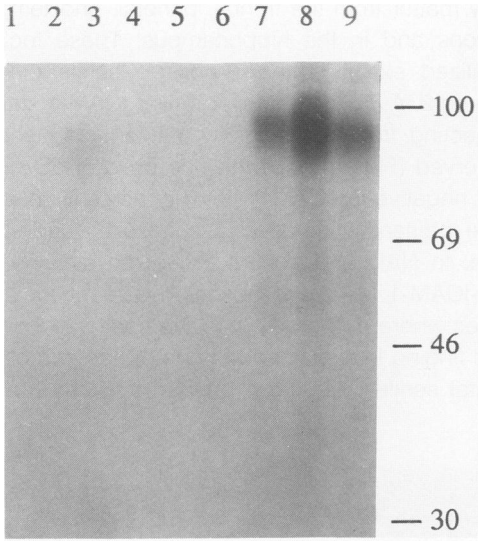

Figure 3. *Analysis of radioiodinated lysates of SDAT frontal cortex* (lanes 1–6) *or endothelial cells* (lanes 7–9) *by SDS-PAGE under nonreducing conditions. Autoradiography of a 10% gel after 4 weeks of exposure. A 85-kd form of the ICAM-1 molecule is immunoprecipitated by MAb PN-E12 from SDAT frontal cortex.* Lane 1: *MAb W6/32;* lanes 2 *and* 7: *MAb CL203;* lanes 3 *and* 8: *MAb PN-E12;* lanes 4 *and* 9: *MAb PN-E8;* lane 5: *MAb PN-E4;* lane 6: *MAb CA7. Molecular sizes of the standards are indicated at the right in kilodaltons.*

ICAM-2 Immunohistochemistry

The MAb CBR-IC2/2 and 6D5 showed staining of small, mononuclear, and predominantly dendritic cells dispersed in the neural tissue and in a perivascular position in control and Alzheimer gray and white matter (Figure 4A). Morphologically similar cells were stained by the anti-LFA-1α MAb NKI-L12 (compare Figure 4A and 4B), while distinct cell types were stained by anti-GFAP and anti-ICAM-2 MAb (Figure 4, A and C). On basis of these findings, the ICAM-2 positive cells were considered as microglial cells. However, we could not completely exclude that a small subset of astrocytes also contributed to the observed ICAM-2 expression (Figure 4C). A remark-

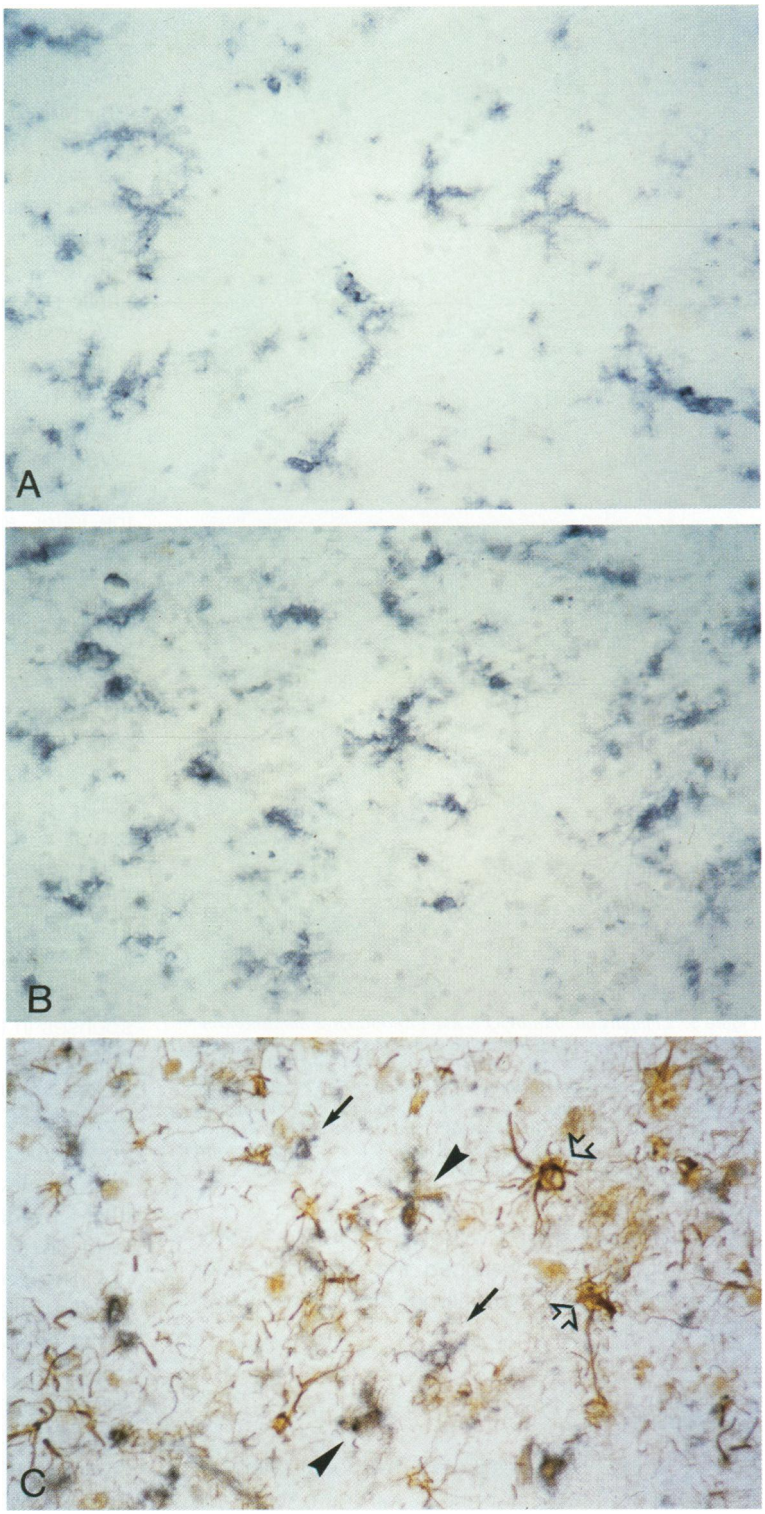

Figure 4. *Staining of AD brain tissue with microglial and astrocyte markers. Cryosections were stained with MAb NKI-L12 (anti-LFA-1α), MAb CBR-IC2/2 (anti-ICAM-2), and MAb 6F2 (anti-GFAP). Microglial cells were stained for ICAM-2* (**A**) *and LFA-1α* (**B**). **C:** *Double-staining of AD brain tissue with anti-ICAM-2 (purple) and anti-GFAP (brown). Note that most of the ICAM-2 positive cells (closed arrows) are not stained by the astrocyte marker GFAP (open arrows). A small number of cells showed staining for both markers (arrowheads). All sections were stained with DAB as chromogen with or without the addition of nickel ammonium sulfate (purple and brown reaction product, respectively). Magnifications ×375.*

able accumulation of microglial cells was observed in senile plaques as is shown in Figure 5A. Serial sections stained for ICAM-2 and ICAM-1, respectively, clearly showed the differences between the cell-associated ICAM-2 staining and the diffuse ICAM-1 staining in senile plaques (Figure 5, A and

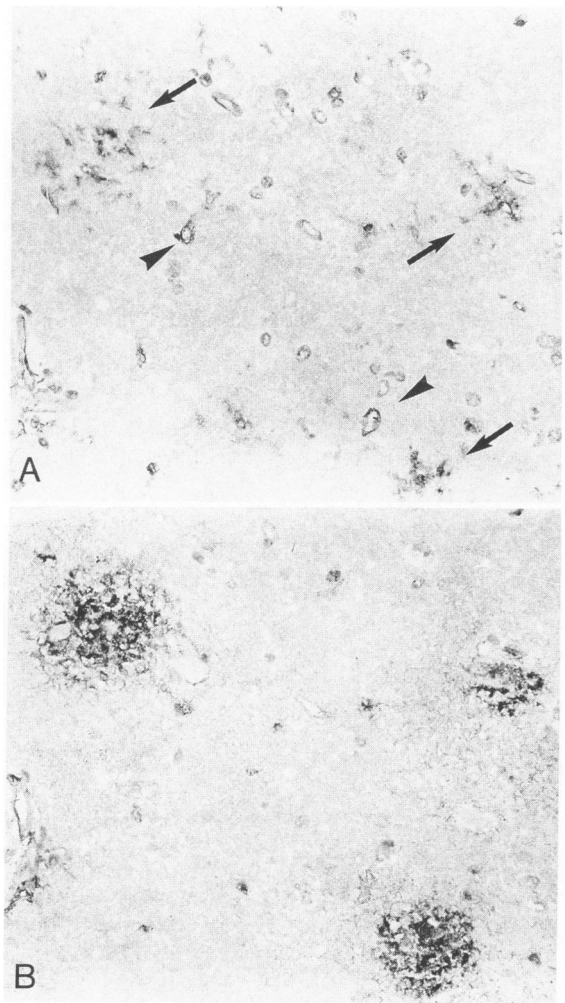

Figure 5. *Comparison of indirect ICAM-1 and ICAM-2 immunostaining in cryosections of AD brain tissue. Serial sections of AD brain tissue, stained with anti-ICAM-2 MAb CBR-IC2/2* (**A**) *and anti-ICAM-1 MAb PN-E8* (**B**). *Note the accumulation of microglial cells stained for ICAM-2 in senile plaques* (*arrows*). *Also, capillary endothelial cells were stained for ICAM-2* (*arrowheads*). *MAb PN-E8 strongly stained senile plaques. All sections were stained with DAB as chromogen, and were counterstained with hematoxylin. Magnification ×250.*

B). Similar as observed with the anti-ICAM-1 MAb, the anti-ICAM-2 MAb stained the endothelium of capillaries (Figure 5A) and larger blood vessels (not shown).

Discussion

In this study we investigated the localization of ICAM-1 and ICAM-2 in brain tissue from Alzheimer and control patients by immunohistochemical staining of cryosections. By immunoprecipitation, the identity of the ICAM-1 molecule from SDAT brain tissue was confirmed.

ICAM-1 was detected in all subsets of senile plaques, including classic, diffuse, and burned-out plaques, and thus co-localized with fibrillar as well as non-fibrillar Aβ. Anti-ICAM-1 MAb even stained a small population of plaques containing low levels of Aβ. Moreover, ICAM-1 was found in tissue surrounding Aβ deposition in senile plaques, suggesting that larger areas of brain tissue are involved in senile plaque formation than would be expected on the basis of Aβ distribution. These findings indicate that ICAM-1 aggregation may be an early event in senile plaque formation.

As expected, ICAM-2 was expressed on the cerebrovascular endothelium, but, in contrast to ICAM-1, was not observed diffusely in senile plaques. Furthermore, ICAM-2 reactivity was found in microglial cells in gray and white matter in control brain tissue as well as in Alzheimer brain tissue. In the latter, an accumulation of microglial cells was observed in and around senile plaques.

ICAM-1 and ICAM-2 are both cell-surface glycoproteins belonging to the immunoglobulin superfamily of adhesion molecules and they are expressed on various cell types. ICAM-1, consisting of five immunoglobulin-like domains, is expressed on endothelial cells, leukocytes, tissue macrophages, and dendritic cells.[25] ICAM-2, a two-domain molecule, is expressed on endothelial cells, lymphocytes, and monocytes.[27] Both molecules play an important role in the tethering of neutrophils, lymphocytes, and monocytes to the vessel wall endothelium, which is a key event allowing both normal recirculation of lymphocytes and extravasation of these cells into inflamed tissue. Local immune responses in tissue resulted in an increase of ICAM-1 on a wide variety of cells.[25,40] In cell cultures, ICAM-1 could be upregulated on a variety of cells by inflammatory factors like lipopolysaccharide, interferon-γ, TNF, and IL-6.[25,41–44]

Only a few studies report about the distribution of ICAM-1 in the brain. ICAM-1 is expressed at a low level on cerebral endothelial cells,[45,46] but could not be demonstrated on pericytes or smooth muscle cells.[45] In inflammatory and necrotic lesions in the central nervous system, ICAM-1 may be upregulated on brain mononuclear, endothelial and microglial cells.[45] In brains from multiple sclerosis patients, multiple sclerosis plaques showed an increased number of ICAM-1 positive microvessels.[45] In a few reports, the presence of ICAM-1 in senile plaques was mentioned.[37,47,48] Our results do not support the observation of Akiyama et al[37] that the majority of diffuse senile plaques are negative for ICAM-1. In our study, all types of senile plaques (diffuse, classic, and burned-out) were ICAM-1 positive. A small number of plaques showed fibrillar

staining for ICAM-1, which extended from the stained plaque. These fibrils may be the processes of a subpopulation of reactive astrocytes as described previously.[37]

Compared to ICAM-1, much less is known about ICAM-2 and hardly any study has been published about the distribution of ICAM-2 in the central nervous system. It has been described that, apart from vascular staining, reactivity with ICAM-2 MAb is not observed in brain tissue.[27,48] In the present study, however, in both control and AD brain tissue, ICAM-2 reactivity was also found in microglial cells localized in a perivascular position as well as dispersed in the neural tissue.[49] In senile plaques an accumulation of ICAM-2-positive microglial cells was seen, probably due to an activation of microglial cells by a yet unknown mechanism or a recruitment of these cells to the site of senile plaque formation. The presence of microglial cells in senile plaques was demonstrated previously by staining with MAbs against the α and β chains of LFA-1, Mac-1, and p150,95[37,47] and their possible involvement in senile plaque formation has been discussed.[48,50] A minor contribution of astrocytes to the observed ICAM-2 reactivity could not be completely excluded, however.

Initially, ICAM-1 was described as a membrane-bound cell adhesion molecule, but it has also been detected in a soluble form.[51,52] The ICAM-1 in senile plaques neither seems to be cell-associated nor it is likely to be in a soluble form. We immunoprecipitated a 85-kd form of the ICAM-1 molecule from brain tissue of Alzheimer patients but not from control brains. The molecular weight of ICAM-1 varies between 55 kd and 114 kd, depending on the degree of glycosylation.[53] The precipitated ICAM-1 molecule probably contains all five domains and is, in view of its M_r, rather highly glycosylated. The presence of the entire 5-domain molecule does not exclude that the ICAM-1 in senile plaques has the primary structure of soluble ICAM-1, but we assume that the molecule has been aggregated into an insoluble form. The exact nature of the ICAM-1 aggregates and their interaction with other plaque components remain unknown, however. It is possible that the ICAM-1 in senile plaques interacts with its counterstructures LFA-1, Mac-1, and p150,95 expressed by microglial cells, but a more cell-associated distribution pattern of ICAM-1 would then be expected.

The question arises whether the ICAM-1 deposited in senile plaques is of vascular or of neural origin. In all cases examined, anti-ICAM-1 MAb stained the endothelium (and incidentally perivascular cells) in the microvasculature of the brain tissue. ICAM-1 is expressed at the luminal side of endothelial cells. When the blood-brain barrier is intact, it seems less likely that the ICAM-1 in senile plaques is derived from an endothelial origin. ICAM-1 might be of neural origin as well and this idea is supported by the finding that cultured neural cells[41] and astrocytes[54] can be induced to express ICAM-1 by mediators like TNF, IL-1, and IFN-γ. So far we do not have direct evidence for an increased neural ICAM-1 production and subsequent deposition in senile plaques in Alzheimer's disease.

Some years ago, a cerebral acute phase reaction was proposed as a pathogenic mechanism of Alzheimer's disease.[13,19] Upon stimulation by bacteria, viruses or immune complexes, cytokines like IL-1, IL-6, and TNF are produced to induce this complex reaction. These cytokines also induce expression of ICAM-1. We found ICAM-1 in all types of plaques, either containing fibrillar or nonfibrillar Aβ, as detected by sensitive Aβ immunohistochemistry and thioflavin S stain.[38,55] However, the ICAM-1 depositions covered a larger area than Aβ does, and anti-ICAM-1 MAb also stained plaque-like structures containing low levels of Aβ. This seemed to indicate that ICAM-1 deposition is a relatively early event in senile plaque formation. The presence of acute phase-related proteins (like α_2-M, C-reactive protein, IL-6[18] and ACT[2,13]) together with the presumed early appearance of ICAM-1 in senile plaques as described here, favors the suggestion that a cerebral acute phase response caused by an increased IL-1 or IL-6 production in the brain is the underlying mechanism for the early accumulation of ICAM-1 and aggregation of Aβ and other plaque components, although a neuroinflammatory effect of Aβ itself cannot be excluded.[56] Support for this hypothesis comes from the observations that astrocytes[57] and microglial cells[58] are potential sources of IL-1 or IL-6. In addition, it was reported very recently that treatment of Alzheimer patients with anti-inflammatory drugs might slow the advance of dementia.[20] This study gave clinical support to the idea that Alzheimer's is a chronic inflammatory disease.

From our results it appeared that ICAM-1, together with ACT[2,13,59] and complement proteins,[14,59] may be regarded as proteins specifically associated with Aβ deposits. These proteins are, in contrast to heparan sulfate proteoglycans and P-component, not associated with various other types of amyloid formation. ICAM-1 staining therefore seems to be an early and specific indicator of senile plaque formation.

In our hands, the ICAM-1 staining proved to be a useful instrument for detecting senile plaques that was comparable to staining with Aβ antibodies on frozen sections. However, more study is needed to establish the use of anti-ICAM-1 MAbs as diagnostic markers in Alzheimer's disease.

Taken together, a series of events starting with the acute phase reaction may lead to the accumulation of various plaque components and may also lead to an abnormal processing of βPP, resulting in the deposition of Aβ in senile plaques and in the cerebrovasculature. Synthesis of IL-1 or IL-6 and the subsequent induction of acute phase proteins and ICAM-1 requires previous stimulation of the IL-1 or IL-6 producing cells. Until now, it is not known what factor may initiate these reactions in brains of patients who ultimately develop Alzheimer's disease.

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