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Detection of HLA-DR on Microglia in the Human Brain Is a Function of Both Clinical and Technical Factors

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Detection of HLA-DR, a class II major histocompatibility antigen, on glial cells is dependent not only on duration and type of tissue fixation and processing, but also on clinical factors. Glial cells labeled by anti-HLA-DR were consistent with microglia by light microscopic and ultrastructural criteria, and were colabeled with other microglial markers, including LN-1, Leu-M5, and leukocyte common antigen (LCA). In young and elderly subjects who died suddenly, anti-HLA-DR labeled microglia in the white matter, but far fewer cells in the gray matter. In subjects who died of chronic debilitating illness, such as Alzheimer's disease and carcinomatosis, anti-HLA-DR labeled numerous microglia throughout both the gray and white matter. In Alzheimer's disease, microglia were aggregated in compact senile plaques, but loosely associated with diffuse amyloid deposits. These results suggest that HLA-DR may be constitutively expressed in white matter, but induced in gray matter microglia in chronic disease states or in association with amyloid deposits. (Am J Pathol 1990, 136:1101-1114)

Several studies have demonstrated that in certain conditions microglia may express the class II major histocompatibility (MHC) antigen, HLA-DR. A cell surface glycoprotein involved in cell recognition, HLA-DR is required for certain immune-mediated events.[1,2] Originally thought to be restricted to cells of the immune system such as lymphocytes, macrophages,[3] and activated T cells,[4] recent studies have demonstrated a wider distribution of this antigen. In particular, it has been localized on endothelial,[5-9] endocrine,[9,10] epithelial,[5,8,11] and central nervous system (CNS) cells.[5,6,12,13] Within the CNS, conflicting results have been reported concerning HLA-DR immunoreactivity on white and gray matter microglia of both normal brains and those with neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[6,7,12-23]

The controversy as to whether HLA-DR immunoreactivity exists in the normal human brain appears to be partially related to technical factors. Immunocytochemical localization of cell surface antigens such as HLA-DR has been difficult using routinely processed postmortem human brain tissue, because prolonged exposure to formalin masks or destroys the epitope.[19,24,25] Thus, most early studies were based on acetone-fixed cryostat sections,[21,23,26] but often the morphology was suboptimal. This may have contributed to possible misidentification of HLA-DR-immunoreactive cells in the normal CNS.[5,6,11]

More recently, several laboratories have demonstrated that HLA-DR can be detected in paraformaldehyde-fixed, microtome sections when the exposure to fixatives is limited.[17,19,20,22] These studies, however, have not considered the possibility that clinical variables, such as the terminal state of the subject, may be a confounding factor in the extent of HLA-DR immunoreactivity. Recent studies in multiple sclerosis have suggested that detection of cytokines in brain tissue is dependent on intercurrent infections.[27] Because cytokines from the systemic circulation may be involved in inducing or upregulating HLA-DR,[28,29] clinical factors need to be considered in interpreting immunocytochemical results in human autopsy tissue.

The present report describes a systematic examination of fixatives and fixation duration parameters that optimally preserve HLA-DR in postmortem human brain tissue. With these improved techniques, it is apparent that anti-HLA-DR, and some antibodies to monocytes/macrophages, can label an extensive array of microglia in both the gray and white matter, and that clinical factors may

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be significant in determining the level of expression of HLA-DR.

Materials and Methods

Tissue

Tissue was obtained at autopsy with postmortem intervals of less than 7 hours (mean, 6.3 hours) from Alzheimer cases (n = 15; two men, 13 women; mean, 6.4 hours; mean age, 76.6 years) and non-neurologic controls (n = 14; three men, 11 women; mean, 6.3 hours; mean age, 67.8 years). Clinical features are summarized in Table 1. Presence of senile plaques and neurofibrillary tangles in at least five cortical sections was determined with thioflavin S fluorescent microscopy. Diagnostic criteria for Alzheimer's disease included those proposed by Khachaturian et al.[30]

Vibratome Sections

Tissue blocks from the midfrontal cortex (Brodman's area 9) were fixed by immersion in 4% paraformaldehyde, periodate lysine-paraformaldehyde (PLP),[31] Bouin's solution, or formalin for 1, 5, and 10 hours. Tissue fixed for extended times (24 hours, 48 hours, and 72 hours) was processed in some cases. After the indicated duration, tissue was transferred from the fixative into 30% sucrose TRIS-buffered saline (TBS). Vibratome sections (40 to 60 μ) were cut in cold TBS.

Frozen and AMeX Sections

At the time of autopsy, tissue blocks from midfrontal cortex adjacent to tissue submitted for Vibratome sections were fixed by immersion overnight in ice-cold (4°C) acetone and subsequently embedded in paraffin according to the AMeX procedure.[25] In addition, tissue blocks from the hippocampus were frozen in OTC (Lab-Tek Products, Naperville, IL).

Antibodies

Antibodies used in this study, their specificity, source, and dilution are listed in Table 2. To verify the results with HLA-DR, two other commercially available antibodies to HLA-DR (DAKO and ICN) were used. The ICN antibody labeled both perivascular microglia and dendritic microglia. The DAKO antibody gave essentially the same results as the Becton-Dickinson antibody; however, the Becton-Dickinson antibody could be used in a more dilute form and it was used for these studies.

Immunocytochemistry

Vibratome

Vibratome sections were processed using a modification of the Mason and Sammons procedure.[32] Sections were incubated for 30 minutes in 0.25% Triton X-100 and 3% hydrogen peroxide (H_2O_2) in 0.01 molar (M) TBS, followed by a blocking step for 1 hour in 5% powdered milk TBS to reduce nonspecific staining. All primary and secondary antibodies were diluted in 5% milk TBS. Between the primary, secondary, and diaminobenzidine (DAB) steps, sections were washed (5 × 5′) in TBS. The reaction product was visualized by DAB (0.3 mg/ml) dissolved in 100 mmol/l TRIS base (pH 7.4) with 0.03% H_2O_2. Sections were incubated in the primary antibodies overnight at 4°C and in IgG subclass-specific secondary antibodies (Fisher Sci. Co, Fair Lawn, NJ) conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. Sections were mounted on glass slides, dehydrated through a series of graded alcohols, and coverslipped with Permount (Fischer).

Double Immunostaining

With the exception that sections were incubated with a mixture of two primary antibodies overnight at 4°C, the procedure was identical to the single immunoperoxidase procedure. After DAB, sections were washed briefly in TBS (5′), then incubated in the second subclass specific antibody (1 hour), washed (5 × 5′) in TBS, and visualized with 4-chloronaphthol (2 mg/ml) with 0.05% H_2O_2 and, because of the solvent-sensitive nature of 4-chloronapthol,[33] directly coverslipped with Gelvatol (Air Products and Chemicals, Inc; Allentown, PA).

Some sections from each case were initially processed for immunoperoxidase staining (as above) and then counterstained with thioflavin S (0.001%) for 7 minutes, sequentially dehyrated through a series of graded alcohols, and coverslipped with Fluoromount (Biomedical Specialties, Santa Monica, CA).

Frozen and AMeX Sections

After fixation of frozen sections in acetone for 10 minutes, sections were incubated with 0.3% H_2O_2 for 20 minutes. Nonspecific antibody binding was blocked with 5% nor-

Table 1. *Clinical and Pathologic Features of Cases*

Age (yrs)	Sex	Postmortem delay (hrs)	Duration of dementia (yrs)	Amyloid deposits	Ratio of HLA-DR to Leu-M5 positive microglia		Activated microglia	Clinical features
					WM	GM		
Cardiovascular								
1/2	F	10	—	0	2+/3+†	0/3+†	0	Metabolic myopathy; SIDS
29	F	5.5	—	0	2+/3+†	0/3+†	0	Familial cardiomyopathy acute PE
61	M	6	—	1+ (focal)	3+/3+	1+ (focal)/3+†	1+	ASCVD; COPD; sudden death
62*	F	12	—	1+ (focal)	3+/3+	1+ (focal)/3+†	1+	ASCVD; DM; s/p CVA; Died in sleep
73*	M	5	—	1+ (focal)	3+/3+	2+/3+†	1+	Parkinson's; HTN; acute MI
85*	F	6	—	1+ (focal)	3+/3+	2+/3+†	1+	DM; CHF; Acute cholecystitis
91*	F	1.5	—	3+	3+/3+	2+/2+	1+	Acute CVA
96*	F	8.5	—	2+	3+/3+	3+/3+	1+	HTN; Sudden death (?PE)
Chronic Debilitating Illness—Pneumonia or Sepsis								
53	F	1.5	—	0	3+/3+	3+/3+	1+	Disseminated ovarian CA; Depressive psychosis; pneumonia
69	F	6	—	0	4+/4+	3+/3+	1+	Disseminated pancreatic CA; hepatic abscess; hepatic encephalopathy
70	F	12	—	0	3+/3+	3+/3+	0	Metastatic colon CA; manic-depressive psychosis; pneumonia
80*	M	2	—	1+ (focal)	3+/3+	3+/3+	1+	ASCVD; disseminated prostatic CA
88*	F	6.5	—	2+	3+/3+	3+/3+	2+	Severe rheumatoid arthritis; CHF
91	F	5	—	0	3+/3+	3+/3+	1+	Pre-leukemia; anemia; ASCVD
Alzheimer's Disease—Cardiovascular								
68	F	6	6	3+	3+/4+†	3+/4+†	3+	ASCVD; died in sleep at home
77	F	6.5	20	3+	4+/4+	4+/4+	3+	Seizures; UTI; sudden death in nursing home
79	F	6	4	3+	3+/3+	3+/3+	2+	Sudden death in nursing home
82	F	4	12	3+	4+/4+	4+/4+	3+	ASCVD; sudden death
86	M	6	5	3+	NA/3+	NA/3+	2+	ASCVD; sudden death in nursing home
Alzheimer's Disease—Pneumonia or Sepsis								
62	F	5	13	3+	3+/3+	3+/3+	3+	Decubiti; pneumonia
72*	M	6	13	3+	4+/4+	3+/4+†	3+	Cachexia; pneumonia; septicemia
73	M	14	NA	3+	3+/3+	3+/3+	2+	ASCVD; COPD; pericarditis; pneumonia
74	F	6	13	3+	3+/3+	3+/3+	3+	Septic shock; pneumonia
76	F	6.5	8	3+	3+/3+	3+/3+	2+	Decubiti; pneumonia
78	F	2	10	3+	4+/4+	4+/4+	3+	CHF; Small bowel obstruction; septicemia
81*	F	5.5	3	3+	4+/4+	3+/3+	2+	Pneumonia; febrile illness
88	F	4	6	3+	3+/NA	3+/NA	2+	ASCVD; s/p CVA; cholelithiasis pancreatic abscess; septicemia
Alzheimer's Disease—Carcinoma and Pneumonia or Sepsis								
75	F	5	NA	3+	3+/3+	3+/3+	3+	Anal CA; septicemia
79	F	15	5	3+	4+/4+	3+/4+†	3+	Lung CA; pneumonia
85*	F	4.5	5	3+	4+/NA	4+/NA	2+	Disseminated breast CA; ASCVD; pneumonia

* Prospectively studied individual.

† Cases with more microglia detected by Leu M-5 than with HLA-DR.

NA, not available; WM, white matter; GM, gray matter; F, female; M, male; SIDS, sudden infant death syndrome; PE, pulmonary embolus; ASCVD, atherosclerotic cardiovascular disease; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; s/p, status-post; CVA, cerebrovascular accident; HTN, hypertension; MI, myocardial infarction; CHF, congestive heart failure; UTI, urinary tract infection; CA, cancer.

Density of microglia, activated microglia, and amyloid deposits was estimated by two of the authors (LAM and DWD) using a subjective rating scale: 0, none; 1+, a few; 2+, some; 3+, many; 4+, very many. Activated microglia had morphologic features as shown in Figure 1.

Table 2. *Antibodies*

Antigen		Source	Dilution	Isotype
HLA-DR	—	BD	1:50	IgG2A
Leu-M5	—	BD	1:25	IGgG2B
HAM56	—	Enzo	1:1000	IgM
EBM11	—	DAKO	1:25	IgG1
LCA	CD45	DAKO	1:50	IgG1
LN-1	—	ICN	neat	IgM
NP-7	—	PD	1:10	IgG1
GFAP	—	DAKO	1:100	IgG1

PD, Peter Davies. BD, Becton Dickinson, Mountain View, CA. DAKO, Dako Corp., Santa Barbara, CA. Enzo, Enzo Diagnostics, New York, NY. ICN, ICN Biomedicals, Inc., Costa Mesa, CA.

mal goat serum. Primary antibodies were incubated for 2 to 4 hours at room temperature or overnight at 4°C. Mouse monoclonal antibodies were detected by the ABC method using a commercial kit (Vector Lab, Burlingame, CA).[26] AMeX sections were deparaffinized in xylene for 20 minutes, followed by acetone for 10 minutes, and rehydrated in phosphate buffer before immunostaining as above. Before coverslipping, some sections were counterstained with thioflavin S.

Controls

Negative controls included the omission of the primary antibody. Positive controls included incubations with unrelated antibodies, including mouse monoclonal antibodies to neurofilaments,[34] Alzheimer neurofibrillary tangles,[35] and rabbit antisera to ubiquitin[36] or a beta amyloid synthetic peptide (BetaSP).[37] In addition, sections of lymph node were stained with anti-HLA-DR. Other markers, such as Leu-M5, leukocyte common antigen (LCA), LN-1, and EBM11, which used other subclass-specific immunoglobulins, not only controlled for nonspecific staining by the secondary antibody, but also were independent markers for microglia (Table 2). To control for nonspecific binding of mouse immunoglobulins by the secondary antibody, some sections were incubated in human serum.[38]

Semiquantitative Evaluation

Sections of frontal cortex from each case that displayed optimal staining with anti-HLA-DR (usually tissue-fixed for 5 hours in PLP) were evaluated blind to clinical information and scored for immunolabeled microglia (0 = none, 1+ = few, 2+ = some, 3+ = many, 4+ = very many). Adjacent sections were similarly evaluated with Leu-M5 immunostaining. Presence of activated microglia was noted and scored on a three-point scale, because they were not as numerous. Cells considered to be 'activated' were similar to those shown in Figure 1. The presence and extent of amyloid deposits was determined on sections stained with beta amyloid antibodies.[37]

Immunoelectron Microscopy

Free-floating sections immunostained by anti-HLA-DR were trimmed to 1-mm squares that contained either white or gray matter. Sections were postfixed in osmic acid (Dalton's solution) for 2 hours, dehydrated in progressive grades of alcohol and propylene oxide, and then embedded flat in epoxy resin. One-micron-thick sections were scanned for areas of interest, then ultrathin sections were cut and examined with a transmission electron microscope without additional staining.

Results

Analysis of Optimal Staining Techniques

Similar to other studies, the preservation of HLA-DR immunoreactivity using a commercially available antibody to HLA-DR was a function of fixation.[21] Paraformaldehyde, PLP, in addition to fixatives commonly used in routine pathology such as formalin and Bouin's solution, preserved HLA-DR immunoreactivity in immersion-fixed human brain tissue when the duration of fixation was curtailed. Tissue that had been fixed for more than 5 hours generally showed diminished staining. In many cases, little or no HLA-DR immunoreactivity could be detected after 10 hours of 4% paraformaldehyde fixation. Just as critical was the choice of fixative. Although 4% paraformaldehyde resulted in adequate staining across the shorter fixation durations, fixation for 5 hours in PLP and 1 hour in Bouin's solution produced the best morphologic detail. Fixation in Bouin's solution was far superior to that of PLP in terms of tissue integrity. When the duration of fixation was short, HLA-DR immunoreactivity was preserved even in formalin. At 1, 5, and 10 hours of formalin fixation, HLA-DR staining was comparable to the other fixatives. At 24 and 48 hours, the staining was still intense and distinct, but by 74 hours, there was a total absence of staining. This was in marked contrast to another putative microglia marker, LN-1,[39,40] which stained microglia after even prolonged (3 weeks) fixation, but stained predominantly astrocytes in short fixation conditions.[41]

Distribution, Morphology, and Identification of HLA-DR–immunoreactive Glia

In sections with optimal labeling, HLA-DR–immunoreactive glia formed an extensive reticular array in both the

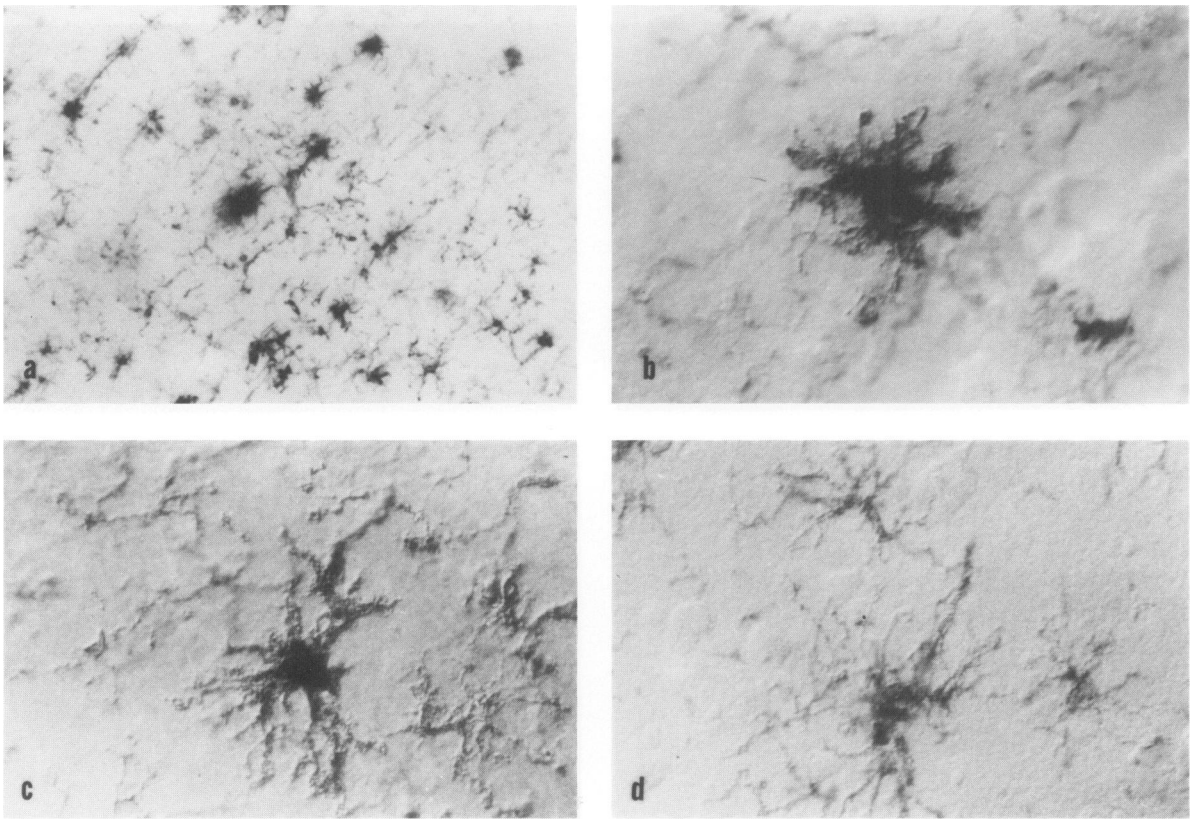

Figure 1. *Microglia with morphologic features of activation are present in the cortex of Alzheimer's disease. At low magnification* (a), *many HLA-DR immunoreactive cells are seen. Some clusters of dense HLA-DR immunoreactivity are also present. At higher magnification* (b), *the clusters of cells have highly ramified cell processes. Similar activated microglia are detected with other microglial markers, such as Leu-M5* (c) *and LCA* (d) (a, ×170, b–f, ×340).

gray and white matter. In controls, the HLA-DR–immunoreactive glia formed a reticular array that was most prevalent in the white, with fewer labeled cells in gray matter (Figure 2). In the youngest control, HLA-DR immunoreactivity was less intense in white matter microglia, despite numerous microglia seen with other microglial markers such as LN-1, Leu-M5, and LCA. The HLA-DR–immunoreactive glia often were elongated and bipolar, with processes that were divided into 'secondary' and 'tertiary' branches. Processes were thicker proximal to the cell body and thinner at the more distal branches.[42] The processes were free ending and specifically did not form vascular end feet. These cells had the morphologic characteristics of 'resting' or 'ramified' microglia.[42] Another type of HLA-DR–immunoreactive cell encountered in gray matter, especially in Alzheimer's disease and elderly controls, had fewer branches, rounded cell bodies, and thicker cell processes (Figure 1). These cells were morphologically consistent with 'activated' microglia,[42] and were often associated with amyloid deposits, demonstrated with an antibody to beta amyloid (Figure 3c, d) or with thioflavin S (Figure 4).

Several independent markers for macrophages and astrocytes confirmed that HLA-DR–immunoreactive glia (Figure 1a, b) were microglia and not astrocytes. Leu-M5, a monoclonal antibody to C3b receptor that has been previously reported to stain microglia[19,43] and macrophages,[44] immunostained microglia best in tissue fixed for 10 hours in 4% paraformaldehyde. There was a total absence of immunostaining with Leu-M5 by 24 hours, irrespective of the fixative. Furthermore, very little or no Leu-M5 immunoreactivity could be detected in tissue fixed with Bouin's solution, regardless of duration. Microglia stained with Leu-M5 were similar to HLA-DR–labeled cells in terms of distribution and morphology (Figure 1c). Previously reported to stain 'activated' microglia[22] and macrophages[45,46] LCA (CD45) labeled both 'resting' and 'activated' microglia, in addition to perivascular leukocytes, across a wider range of fixations and durations than anti–HLA-DR (Figure 1d). Other antibodies against less well-defined monocyte and or macrophage antigens, such as HAM56[39,47] and EBM11,[14,16,43] labeled cells similar to HLA-DR–immunoreactive cells (not shown). In addition to staining a small number of microglia, particularly those in amyloid plaques, HAM56 also reacted extensively with endothelia and in this respect resulted in a staining pattern similar to *Ricinus communis agglutinin* (RCA).[24,26,48] The morphology of cells stained with EBM11 was comparable

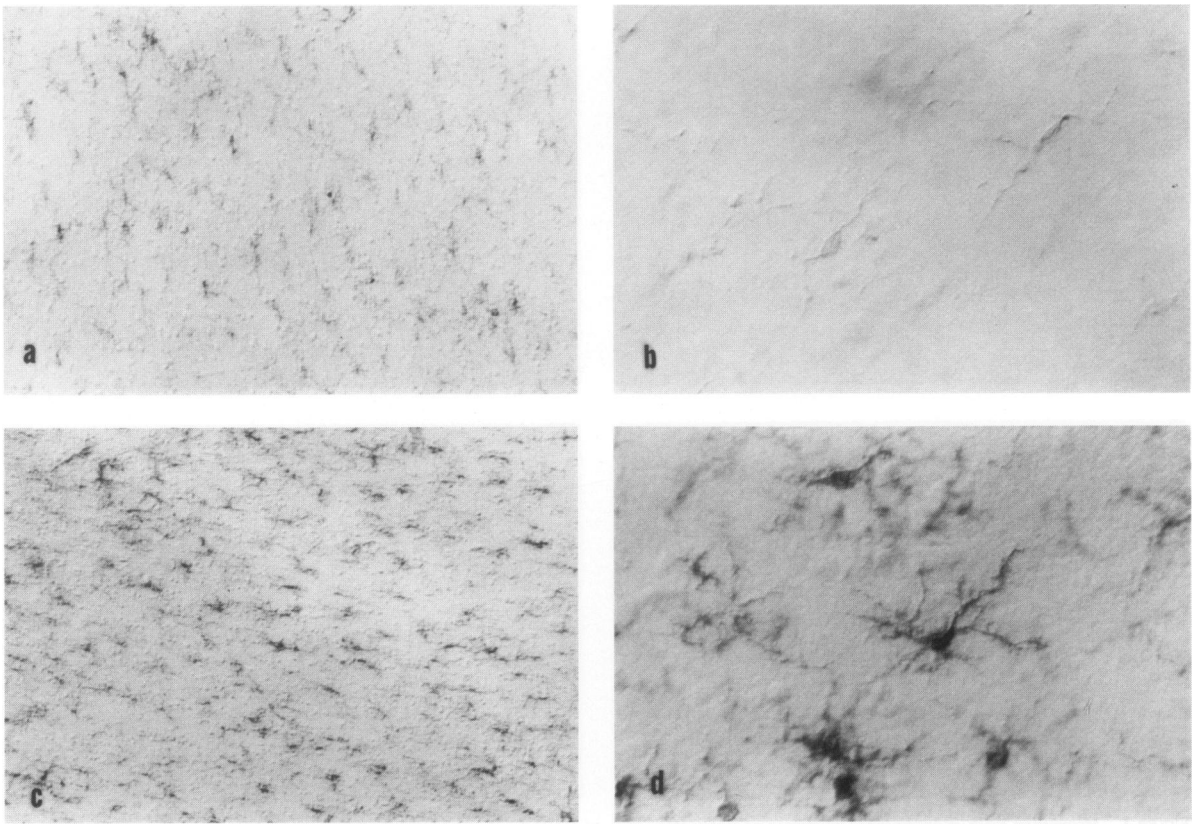

Figure 2. *HLA-DR immunoreactive microglia in white matter* (a and c) *and gray matter* (b and d) *of young* (a and b) *and elderly* (c and d) *control subjects. Note that extensive microglia labeling is present in the white matter of both young and old subjects, but that far fewer microglia are present in the gray matter in the young than in the old subject. The elderly subject died suddenly, but had many amyloid deposits in the cortex* (a and c, ×*180;* b and d, ×*360*).

to Leu-M5, except that the antigen appeared to be cytoplasmic and granular,[43] and far fewer cells were stained. In contrast to the granular cytoplasmic staining with EBM11, Leu-M5 and anti-HLA-DR detected cell surface antigens and thus provided better morphologic detail. An important distinction between anti-HLA-DR and other microglial markers is that these other markers (LN-1, LCA, and Leu-M5) stained an extensive array of microglia in both the gray and white matter, irrespective of the clinical state (see below).

Markers for monocytes (Leu-M1, Leu-M3, MAC-1), natural killer cells (Leu-7, MAC-1), B cells (Leu-14{CD22}), or activated T cells (Leu-M1; IL-2{CD25}) were not reactive with microglia at either the dilutions recommended by the manufacturer or at higher concentrations, or on frozen sections fixed briefly in acetone (not shown).

To confirm that the HLA-DR–immunoreactive cells were not astrocytes, sections were stained with antibodies to glial fibrillary acidic protein (GFAP). Double immunolabeling demonstrated two distinct cell populations (Figure 3a). Astrocytes had a different morphology and tissue distribution than microglia. They were stellate-shaped cells with long straight and relatively unbranched processes that often ended on blood vessels forming perivascular end feet. They were dense throughout the white matter and also numerous in the glial limitans of subpial and perivascular areas. In Alzheimer's disease, astrocytes were located at the periphery of senile plaques, while HLA-DR–immunoreactive microglia were located in the center (Figure 3b).

Immunoelectron Microscopy

With immunoelectron microscopy, HLA-DR–immunoreactive glia were small cells with elongated or irregularly shaped nuclei and condensed chromatin (Figure 5). The cytoplasm contained large heterogenous dense bodies and lipofuscin, but few, if any, recognizable filaments. The cell processes were long and irregular, with focal nodular swellings filled with heterogenous dense bodies (Figure 5). The HLA-DR–immunoreactive cells also included perivascular microglia, but not cells recognizable as astrocytes, oligodendrocytes, or neurons. Also included in the HLA-DR–immunoreactive cells were perineuronal satellite cells whose cytoplasmic membranes were in apposition

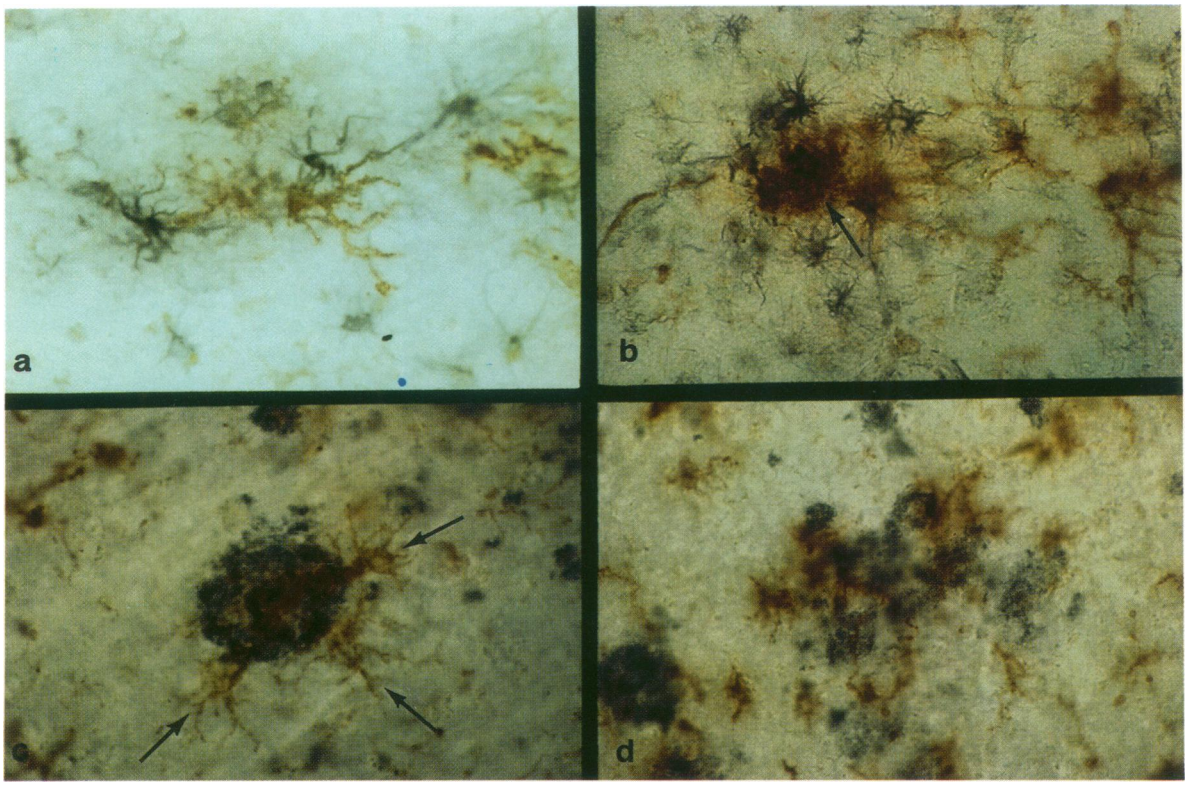

Figure 3. *Double-labeling immunocytochemistry with anti-HLA-DR plus anti-GFAP* (a and b) *and anti-HLA-DR plus an antibody to a beta amyloid synthetic peptide (BetaSP)* (c and d). *The chromogen for anti-HLA-DR is diaminobenzidine (brown) and for anti-GFAP and BetaSP, 4-chloronaphthol (blue-gray).* a: *Note two separate cell types with respect to morphology. HLA-DR-immunoreactive microglia have branching cell processes.* b: *In Alzheimer's disease, HLA-DR-immunoreactive microglia form dense clusters (arrow) surrounded by astrocytes.* c: *A cluster of activated HLA-DR-immunoreactive microglial cells (arrows) colocalizes with a compact amyloid deposit.* d: *Diffuse and punctate amyloid deposits are associated with contiguous HLA-DR-immunoreactive microglial processes* (a–d, ×380).

to neuronal cytoplasmic membranes (Figure 6). Membranes of both cell types were not well preserved, but peroxidase reaction product was linearly disposed between the two cells types. The HLA-DR–immunoreactive cells in the white matter were not directly associated with myelin sheaths.

In Alzheimer's disease, HLA-DR–immunoreactive cells had similar features (Figure 7). Some of the labeled cells had peroxidase reaction product on cell processes directly abutting extracellular amyloid fibrils (Figure 7b). In some cases, the peroxidase reaction product decorated extracellular fibrils (Figure 7c), but diffusion of immuno-

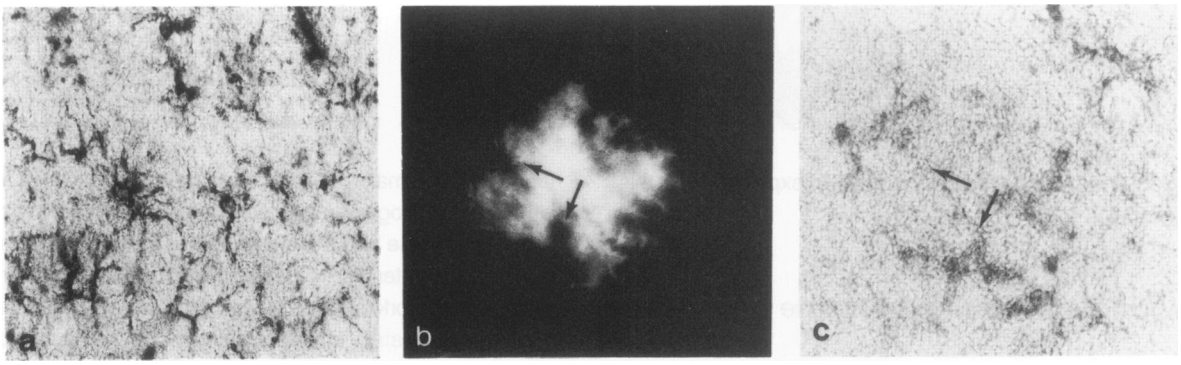

Figure 4. *Double labeling of AMeX section from Alzheimer's disease with HLA-DR and thioflavin S for amyloid.* a: *HLA-DR-immunoreactive microglia are similar in morphology to those detected in Vibratome sections.* b: *A dense amyloid deposit is apparent with thioflavin S with fluorescent microscopy and fluorescein filters. Arrows denote HLA-DR-immunoreactive microglia.* c: *The same section and area as* (b) *under bright field shows a cluster of HLA-DR-immunoreactive microglia (arrows) clustered in the vicinity of the dense amyloid deposit* (a–c, ×490).

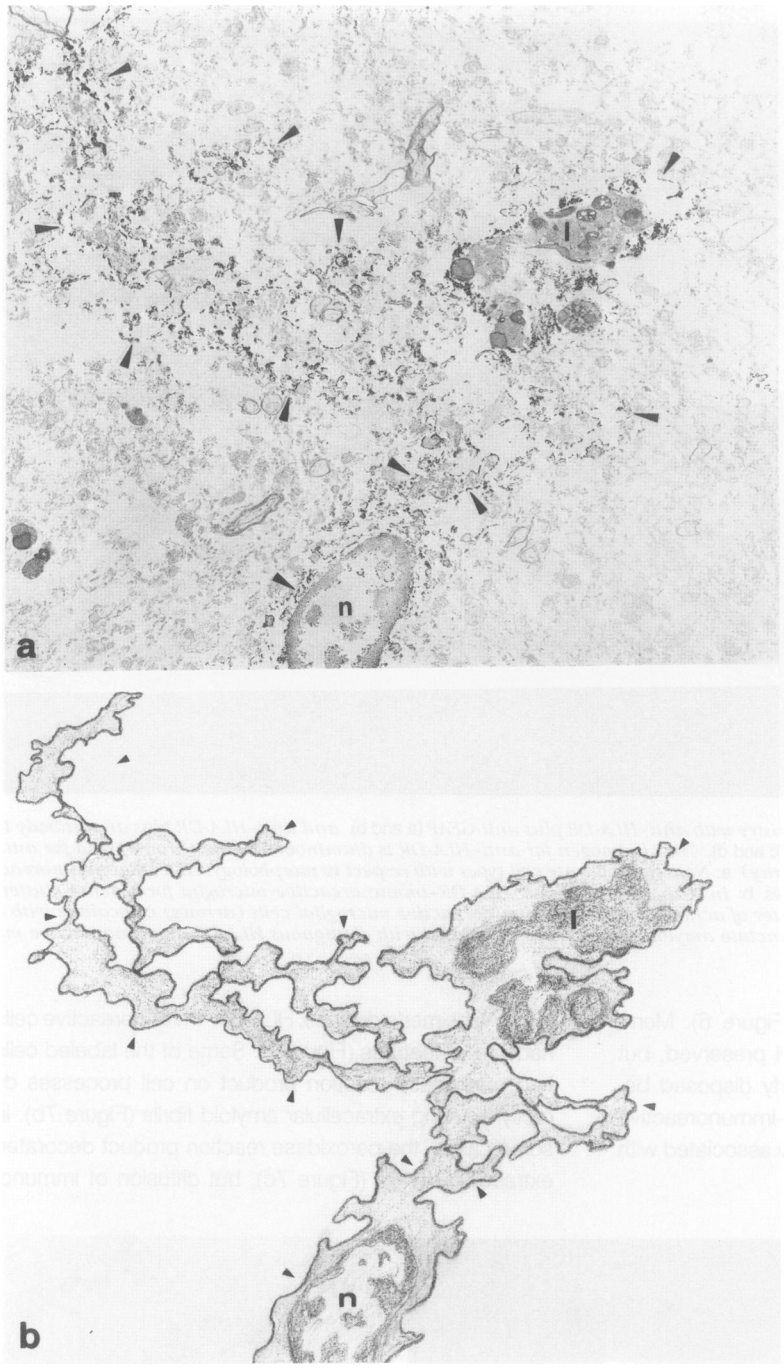

Figure 5. *Immunoelectron microscopy. HLA-DR–immunoreactive cells in the gray matter have a highly branched dendritic cell morphology. Immunoperoxidase reaction product is present on membranes that are partially disrupted due to postmortem artifact. An overlay tracing of the immunoreactive cell is shown in* **b**. *Note that focal cytoplasmic expansions of some of the processes contain dense bodies and lipofuscin. Arrow heads delineate cell borders. (n = nucleus; lipofuscin = l) (×8000).*

globulins could not be excluded as an explanation for this finding.

Microglia in Alzheimer's Disease

Microglia detected by anti–HLA-DR or the other macrophage antibodies did not show significant differences in Alzheimer's disease in comparison to controls with respect to optimal duration and type of fixation. Similarly, there were no major differences in the distribution, density, or morphology of microglia in the white matter in Alzheimer's disease and the elderly nondemented controls. In the white matter, HLA-DR–positive microglia formed an extensive network that was similar to that in controls. Cells were approximately equidistant from one another and were free ending. Variability in HLA-DR immunoreactivity in the gray matter, to some extent, could be accounted for by the clinical state (Table 1). Elderly subjects with isolated amyloid deposits who died suddenly of cardio-

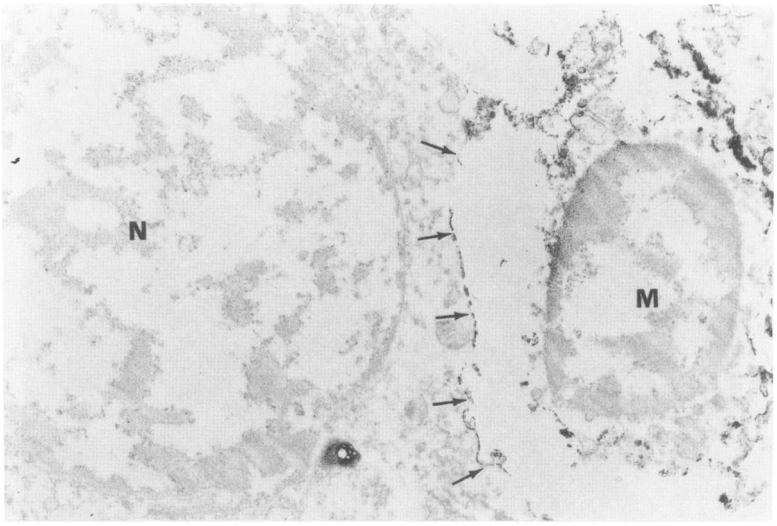

Figure 6. *Immunoelectron microscopy of HLA-DR–immunoreactive satellite cell. The satellite cell (M) has denser nuclear chromatin than the neuron (N). Note that linearly arranged peroxidase reaction product representing the two opposing cellular membranes.*

vascular disease or pulmonary emboli had sparse HLA-DR immunoreactivity in the gray matter. The HLA-DR–immunoreactive microglia were restricted to areas in the neocortex with amyloid deposits in these subjects (Figure 8). In contrast, with other microglial markers, such as Leu-M5 and LN-1, numerous microglia were labeled throughout the gray matter. In isolated amyloid deposits, these other markers also labeled cells resembling activated microglia (Figure 8). Conversely, those individuals dying of infectious diseases complicating chronic debilitating diseases, such as disseminated cancer, had extensive HLA-DR–immunoreactive microglia in the neocortex, even in the absence of amyloid deposits (Figure 9).

In Alzheimer's disease, HLA-DR–immunoreactive glia formed 'clusters' in areas of dense amyloid deposition. The surrounding areas were relatively free of microglia. Colocalization of microglia and amyloid was demonstrated in sections double stained with BetaSP (Figure 3c, d) or thioflavin S (Figure 4). Some of the HLA-DR–immunoreactive material appeared to be deposited with the amyloid and was not clearly associated with cell processes, comparable to immunoelectron microscopy results (Figure 7). Activated microglia were preferentially labeled by HAM56 and EBM11 (not shown). In addition to differences in microglial distribution in Alzheimer's disease, the cells in the gray matter also displayed morphologic changes. There were fewer microglia with delicate and branched cell processes. Over compact amyloid deposits, in particular, microglia appeared to be aggregated, and the cell bodies were swollen with thick and less extensive processes (Figures 2 and 3c). In contrast, microglial cell bodies that more often retained their delicate, branched appearance were not specifically aggregated over diffuse plaques either in Alzheimer's disease or in the nondemented elderly controls (Figure 3d).

Discussion

Immunoreactivity of HLA-DR is highly sensitive to both the duration and choice of fixative.[19,21] Fixation for brief periods (up to 5 hours) in PLP or Bouin's solution gave the most consistent results with immunostaining. Vibratome sections provided greater morphologic detail and more consistent staining of microglia with anti–HLA-DR than did paraffin[49] or frozen[19] sections. Although not comparable to vibratome sections in the morphologic detail of HLA-DR–immunoreactive cells, AMeX-processed paraffin sections appeared to be superior to frozen sections. In addition, when optimal methods are employed, microglia were reactive with a battery of monocyte/macrophage markers that were previously undetected in paraffin sections. This inability to detect microglia on paraffin sections with monocyte/macrophage markers has contributed to the notion "that microglia are a separate population of neuroglia."[15]

Microglia immunoreactive with HLA-DR, LCA, or Leu-M5 could be distinguished from astrocytes because of their distinctive morphology at both the light- and electron-microscopic level,[50] the absence of colocalization with GFAP, and their differential distribution in senile plaques. Specifically, microglia were centrally located in compact amyloid deposits,[17,19] while astrocytes were located in the periphery.[19,20,26]

Astrocytes have been reported to express HLA-DR and to have antigen-presenting capabilities in a number of other diseases,[51,52] including multiple sclerosis. The HLA-DR–immunoreactive astrocytes in either controls or Alzheimer's disease were not observed in the present study nor in a recent study of multiple sclerosis.[54] Similar to previous reports,[17,21,23] occasional HLA-DR–immunoreactive endothelial cells and frequent HLA-DR–immunoreactive

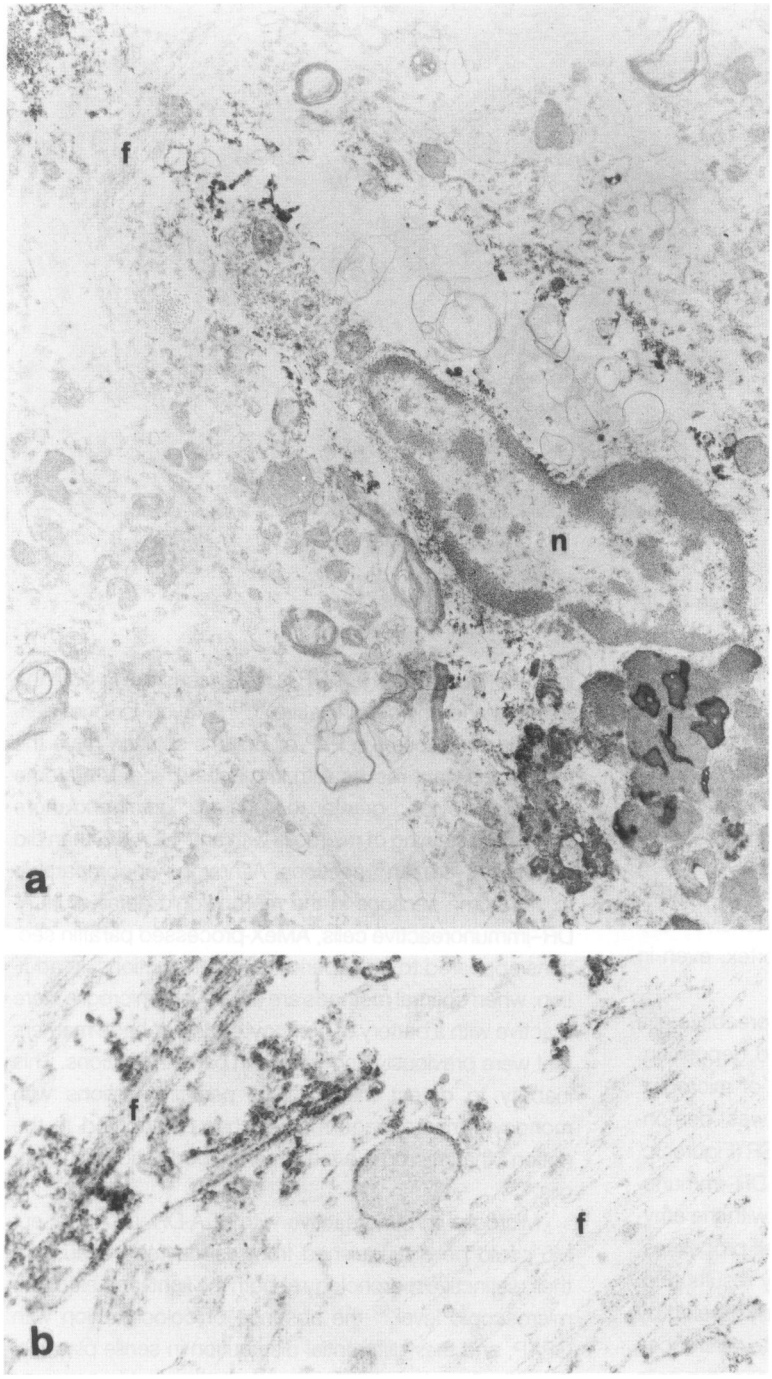

Figure 7. *An HLA-DR–immunoreactive cell in Alzheimer's disease* **(a)** *shows similar nuclear and cytoplasmic features as in Figure 5 except for close cellular apposition of cell borders with amyloid filaments (×10,000). In some foci of amyloid deposition (n = nuclear; f = filaments)* **(b)**, *peroxidase reaction product decorates extracellular amyloid filaments. Such decoration may be a result of antibody or chromogen diffusion, as amyloid filaments were only labeled in association with adjacent cellular labeling (n = nuclear; f = filaments) (×26,000).*

perivascular microglia were observed at both the light- and electron-microscopic level.

Numerous HLA-DR–immunoreactive microglia were observed in all cases, with more cells in the white matter than the gray matter, especially in young controls. Reports of the extent of HLA-DR–immunoreactive microglia in the white matter have been particularly inconsistent, ranging from a few HLA-DR–positive microglia in age-matched controls[17,19,49] to many in both Alzheimer's disease[17,19–23] and neurologic normals.[21,23] Rogers et al (1988)[21] have reported a linear increase with age of HLA-DR reactivity on white matter microglia. This has lead to the speculation that there is an upregulation of HLA-DR reactivity on white matter as a function of age. In the present study, however, an extensive array of HLA-DR–immunoreactive microglia was present in the white matter in all cases, with only a slight increase with age. This suggests that the HLA-DR reactivity in white matter matter may be constitutive.

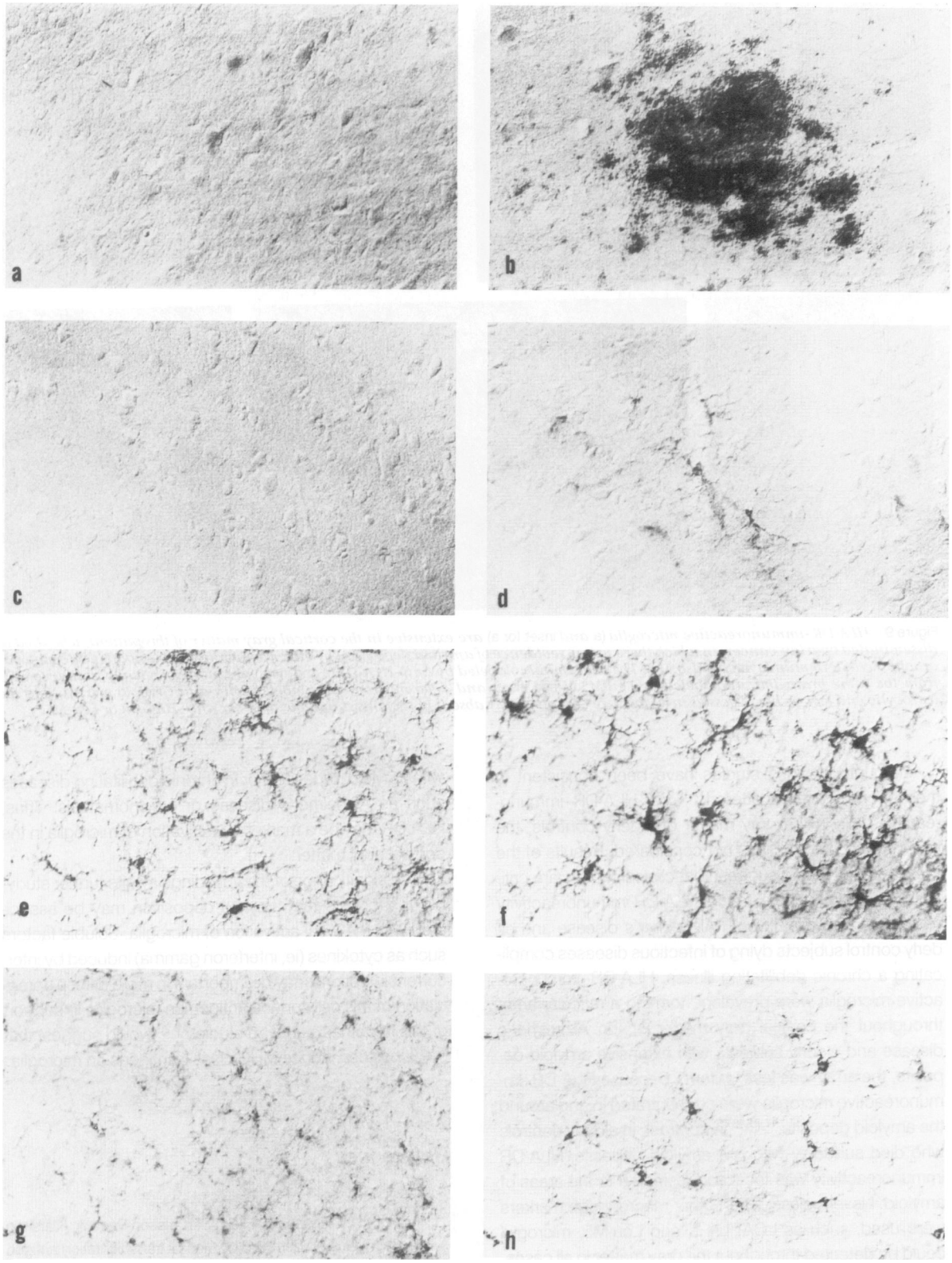

Figure 8. *Immunocytochemial study of a previously healthy, nondemented prospectively studied elderly subject who died suddenly of cardiovascular illness.* **a and b:** *The cortex had focal amyloid deposits that were reactive with BetaSP* **(b)** *and more extensive areas that were devoid of amyloid* **(a). c and d:** *Microglia with HLA-DR immunoreactivity colocalized with amyloid deposits* **(d),** *whereas areas without amyloid were devoid of HLA-DR–immunoreactive microglia.* **e–h:** *Many microglia were detected in cortical areas with amyloid deposits* **(f and h),** *but also in cortical areas without amyloid deposits* **(e and g)** *with two separate markers for microglia (LN-1,* **e and f;** *Leu-M5,* **g and h**). *The microglia in the areas with amyloid deposits had morphologic features that were suggestive of activation, including more prominent, shorter, and less extensively branched cell processes* **(a–h,** $\times 170$).

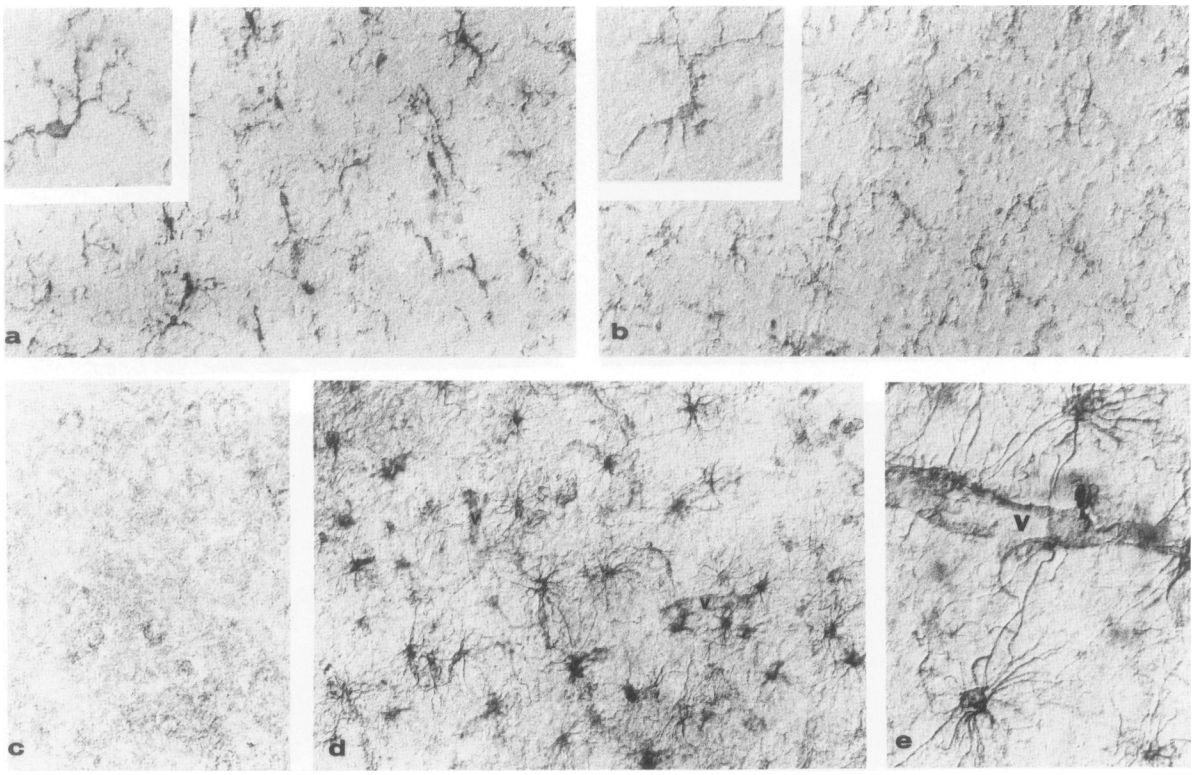

Figure 9. *HLA-DR–immunoreactive microglia* (a *and* inset for a) *are extensive in the cortical gray matter of this patient, who died of disseminated ovarian cancer, in which there was no evidence of amyloid deposition by either conventional histopathologic methods or with BetaSP immunocytochemistry* (c). *The cells immunolabeled by anti–HLA-DR* (a and inset) *are morphologically similar to cells from the same brain immunolabeled by Leu-M5* (b and inset) *and different from cells labeled with anti-GFAP* (d and e). *Note the astrocytic end feet on blood vessels in* (e) *that are conspicuously absent in sections stained with either anti–HLA-DR or Leu-M5.*

Although previous studies have been consistent in their reports of a few scattered[17,19,21–23] HLA-DR–immunoreactive cells in the gray matter of elderly controls, the role of clinical factors was not considered. Results of the present study would suggest that clinical factors are critical in determining the extent of HLA-DR immunoreactivity in cortical microglia. In both Alzheimer's disease and elderly control subjects dying of infectious diseases complicating a chronic debilitating illness, HLA-DR–immunoreactive microglia were prevalent, forming a reticular array throughout the cortical gray matter.[17–23] In Alzheimer's disease and elderly subjects with extensive amyloid deposits, the array was less uniform, because HLA-DR–immunoreactive microglia were concentrated in and around the amyloid deposits.[17,21,49] In contrast, in elderly controls who died suddenly with few amyloid deposits, HLA-DR immunoreactivity was focal and restricted to the areas of amyloid. Nevertheless, when other macrophage markers were used, such as LCA, LN-1, and Leu-M5, microglia could be detected throughout the gray matter in all cases, irrespective of age, disease, and terminal condition.

In summary, HLA-DR appears to be constitutively present on microglia in the white matter and induced or upregulated on microglia in the gray matter in association with amyloid deposition or in chronic debilitating diseases such as Alzheimer's disease or carcinomatosis. Thus, HLA-DR may be a marker for activation of microglia in the cortical gray matter.

The significance of these findings awaits further study, but it suggests that amyloid deposition may be associated with the local activation of microglia. Soluble factors such as cytokines (ie, interferon gamma) induced by intercurrent infections may be important in more diffuse upregulation of this cell surface antigen on microglia. In support of this hypothesis, *in vitro* studies[28,29] would suggest that cytokines can induce MHC class II antigens on microglia.

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