Acta Neuropathol (1993) 86: 337 – 344

Acta Neuropathologica

Immunohistochemical study of microglia in the Creutzfeldt-Jakob diseased brain

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Received: 25 January 1993/Revised, accepted 1 April 1993

Abstract. Immunohistochemical techniques have been used to investigate microglial reaction in Creutzfeldt-Jakob diseased (CJD) brains. Autopsy cases of six patients with CJD and age-matched controls were studied. Formalin-fixed, paraffin-embedded brain tissue samples were stained with antibodies against major histocompatibility complex (MHC) class II antigen (Ag), leukocyte common antigen (LCA), CDw75, CD68 and glial fibrillary acidic protein. Of the patients with CJD, two with a subacute spongiform encephalopathic type and short-survival periods after onset of the disease showed an increased number of reactive microglia labeled with anti-MHC class II Ag or LCA in the affected cerebral cortex. In advanced cases of the panencephalopathic type of CJD, in which both cerebral atrophy and astrocytosis were marked, the increase of reactive microglia was small. Some vacuoles developing in the neuropil of the CJD patients were surrounded by MHC class II Ag- or LCA-immunoreactive microglial cells. The number of ramified microglia in the affected lesions was decreased, although their number in the hippocampus was not affected. These results indicate that microglia can frequently be involved in the process of CJD and may be activated at the early stage of the disease.

Key words: Creutzfeldt-Jakob disease – Immunohistochemistry – Leukocyte common antigen – Major histocompatibility complex class II antigen – Microglia

Creutzfeldt-Jakob disease (CJD) is a human neurodegenerative disease with characteristic features, such as a fatal outcome mostly within 1 year of onset, that can be transmitted to laboratory animals [9]. Characterized by the triad of alterations consisting of spongiform change, neuronal loss, and astrocytic proliferation, the neuropathology of CJD has been described in detail in previous studies [16, 18, 22, 25, 27]. However, the role of microglial reaction at any stage of the disease has not received much attention [34], even though a few patients have shown the presence of microglia and macrophages in the cortical lesions [16, 21, 22, 26–28].

Under pathological conditions, microglia are probably the most responsive cellular element in the central nervous system (CNS) [10]. With recent advances in immunohistochemistry, microglia, originally demonstrated by silver impregnation methods [4], can now be labeled with certain markers in paraffin sections [5, 13, 19, 28, 30, 33]. One of these markers, the major histocompatibility complex (MHC) class II antigen (Ag), has been shown to be present on microglia, mainly on the "reactive" or "activated" ones, in a number of pathological conditions including infectious and neurodegenerative diseases [20, 29]. Therefore, microglial reaction should be demonstrable immunohistochemically in paraffin sections of autopsied brains with CJD.

In the present study, we investigated the morphological changes of microglia and their relationship to the disease process in the brains of patients with CJD by an immunohistochemical method employing various antibodies (Abs).

Materials and methods

Subjects

Autopsied brains from six patients with CJD (Table 1) were examined. No immunologically active therapies (i.e., cytokine infusions) had been performed in any of these patients. The brains were fixed for various intervals (case 1, 9 days; case 2, 3, undetermined; case 4, 9 months; case 5, 1 month; case 6, 7 months) in formalin. Control brain samples were obtained at autopsy from seven patients (aged 58–78 years; fixation time, 1 day-7 months) who died of carcinoma. Neuropathological diagnoses of the controls were six cases of normals and one case of brain abscess secondary to sepsis. After fixation, the tissues were routinely

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Table 1. Summary of the cases of Creutzfeldt-Jakob disease (CJD) studied

Patient profile	Case: 1	2	3	4	5	6
Age (Years)	64	80	57	55	68	65
Sex	M	F	F	F	F	M
Duration of illness (months)	2	5	12	12	20	26
CJD subtype	SSE	SSE	PE	PE	SSE	PE
Brain weight (g)	1420	950	930	800	1000	880
Cerebral atrophy	−	++	+++	+++	+	+++
Degeneration of white matter	−	−	+++	++	−	+++
Lesions of gray matter:						
spongiform change	++	+++	++	+	+++	+
neuronal loss	+	++	++	+++	++	++
astrocytosis	+	++	+++	+++	+	+++

F, Female; M, male; SSE, subacute spongiform encephalopathy; PE, panencephalopathic type
+++, Severe; ++, moderate; +, mild; −, none

dehydrated in a graded ethanol series and embedded in paraffin. Various regions of the CNS from one case of CJD (case 1) were processed for this study. From other cases, representative sections of the temporal lobes, including the hippocampus, were examined.

Immunohistochemical procedures

Sections from the blocks were cut at a thickness of 6 μm and mounted on glass slides precoated with 0.1 % poly-L-lysine (Sigma, St. Louis, Mo.). Before application of the primary Abs, protease treatment using 0.05 % solution of protease XXVII (Sigma, P-4789) was performed for LN-1 staining, and sometimes for the labeling of both LCA and CD68. Specific data for each of the primary Abs employed are shown in Table 2. The biotin-streptavidin immunoperoxidase method (Nichirei, Tokyo, Japan) and the peroxidase-antiperoxidase (PAP) method (Dakopatts, Glostrup, Denmark) that were used have been described previously [30]. The reaction products were visualized in solution containing 0.01 M imidazole, 0.02 % (w/v) 3,3′ diaminobenzidine, 0.005 % (v/v) H_2O_2, and 0.05 M TRIS-HCl (pH 7.6). After immunostaining, the sections were counterstained lightly with hematoxylin.

Nomenclature

The nomenclature regarding microglia and macrophages in the CNS remains inconsistent. In this study, we applied the terms "ramified microglia", "reactive microglia", and "macrophage". The term "ramified microglia" refers to the small glial cells with highly branched cell processes, while "reactive microglia" have fewer branches, thicker cell processes, and rounded cell bodies. The term "macrophage" is used to refer to rounded phagocytes filled with lipid droplets, which are usually recognizable in paraffin sections and with routine stains.

Results

Immunoreactivity

LN-1 Ab and anti-MHC class II Abs (LN-3, CR3.43) labeled microglia as previously described [29, 30], with the predominant affinity for "ramified" microglia and "reactive" microglia, respectively. LCA Ab usually showed weak staining for both types of microglia, regardless of the duration of fixation time. However, in contrast to anti-MHC class II Abs, LCA Ab labeled the microglia even after prolonged fixation (in cases 2, 4, and 6).

KP 1 [12], a marker for macrophages, was rarely reactive with microglia in normal and pathological human brain, and was inconclusive for the identification of microglia due to the intracytoplasmic, paranuclear localization of the Ag.

Microglia in CJD

The patient designated as case 1, who died earliest (2 months after onset of the disease) and whose brain was fixed for almost same period as a control patient with brain abscess, showed a marked increase of MHC class II Ag-positive microglia in the CNS. These microglia had differing cell morphology but, in most, the morphology was compatible with reactive microglia at the light microscopic level (Fig. 1a). The increase of MHC class II Ag-positive microglia was seen in all layers of the cortical gray matter where a large number of degenerating neurons were seen with routine staining. MHC class II Ag-positive microglial processes were often observed in the rim of small vacuoles in the

Table 2. Primary antibodies used in the immunohistochemical procedures

Antibody (clone)	Specificity	Source	Dilution
LN-1	Sialoantigen (CDw75)	NI[a]	1:2
LN-3	HLA-DR (29–33 kDa)	NI	1:2
CR3/43	HLA-DR	DA[b]	1:50
2B11 + PD7/26	LCA	DA	1:20
KP1	CD68 (110 kDa)	DA	1:100
Anti-GFAP	GFAP (40–55 kDa)	Nakazato[c]	1:1000

[a] Nichirei, Tokyo, Japan
[b] DAKOPATTS, Glostrup, Denmark
[c] See [24]

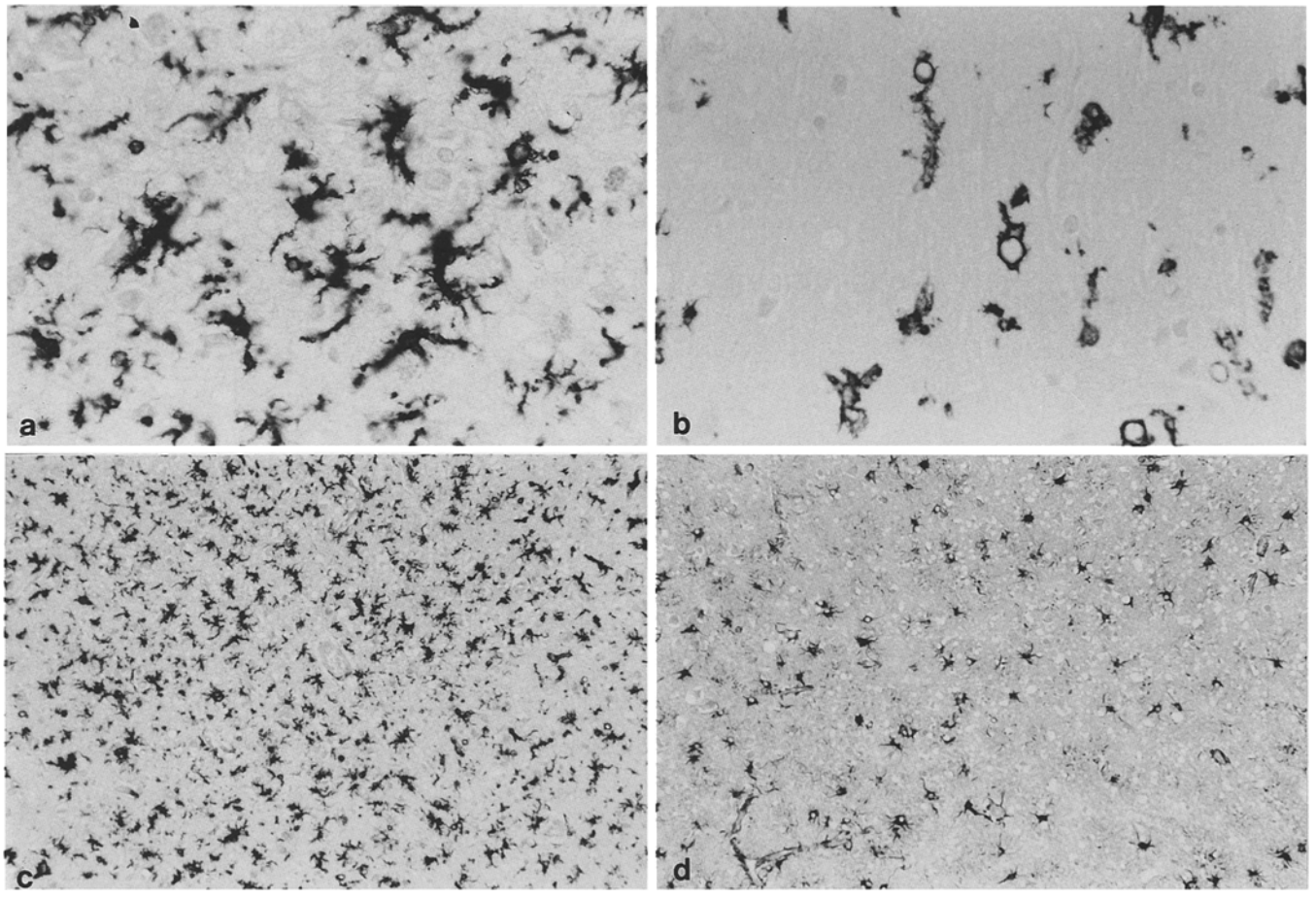

Fig. 1a–d. Paraffin sections of brain tissue from the Creutzfeldt-Jakob disease (CJD) patient with the shortest survival period (case 1). **a** Cerebral gray matter manifested many LN-3-positive microglia in the neuropil. **b** LN-3 immunoreactivity was often seen in the rim of vacuoles in the neuropil in the cerebellar molecular layer. **c, d** Sections of the corresponding area in the temporal gray matter were stained with LN-3 (**c**) and anti-GFAP (**d**). All immunostained sections were counterstained with hematoxylin. **a, b** ×330; **c, d** ×80

neuropil (Fig. 1b). Topographically, the increase of MHC class II Ag-positive microglia was widespread, but most were located in the frontal and temporal lobes and thalamus (Fig. 2). The degree of histological change (spongiform change and astrocytosis) in the cerebrum was more severe in the frontal and temporal lobes. However, MHC class II Ag-positive microglia (Fig. 1c) were more numerous than GFAP-positive astrocytes (Fig. 1d) in the affected cortex. Neither tissue destruction nor inflammatory cell infiltration was present in any parts of the brain.

In the other patient (case 2) with a relatively short survival period after onset (5 months), LCA staining showed a marked increase of reactive microglia in all layers of the temporal gray matter (Fig. 3a, b). In this case, hypertrophic astrocytes were also markedly increased in the cortex.

Even in a patient who survived longer (case 5), an increase of MHC class II Ag-bearing reactive microglia was observed in the cortex (Fig. 4a, b). The total number of reactive microglia in this case was less than that in cases 1 and 2. In case 5, however, a number of reactive microglia with shorter cell processes and less evident ramification were focally present in a markedly spongy cortex.

In the advanced cases of CJD (cases 3, 4 and 6), in which both cerebral cortical atrophy and astrocytosis were marked, the increase of reactive microglia was mild (Fig. 5a, b). Routine neuropathological examinations in all these cases revealed extensive white matter degeneration, and they were then classified as being the panencephalopathic (PE)-type of CJD [22]. The white matter contained numerous macrophages which were labeled with Abs against MHC class II Ag, LCA, and CD68 (Fig. 5c). The immunostaining disclosed gray matter infiltration of macrophages in all the PE-type brains. However, this was observed primarily in the deep layers and was less marked than the astrocytic response (Fig. 5d).

Histological change of the hippocampus was minimal or absent in all cases. Our immunohistochemical study showed that, while a number of LN-1-positive ramified microglia were present in the hippocampus, definite increase of reactive microglia and macrophages was

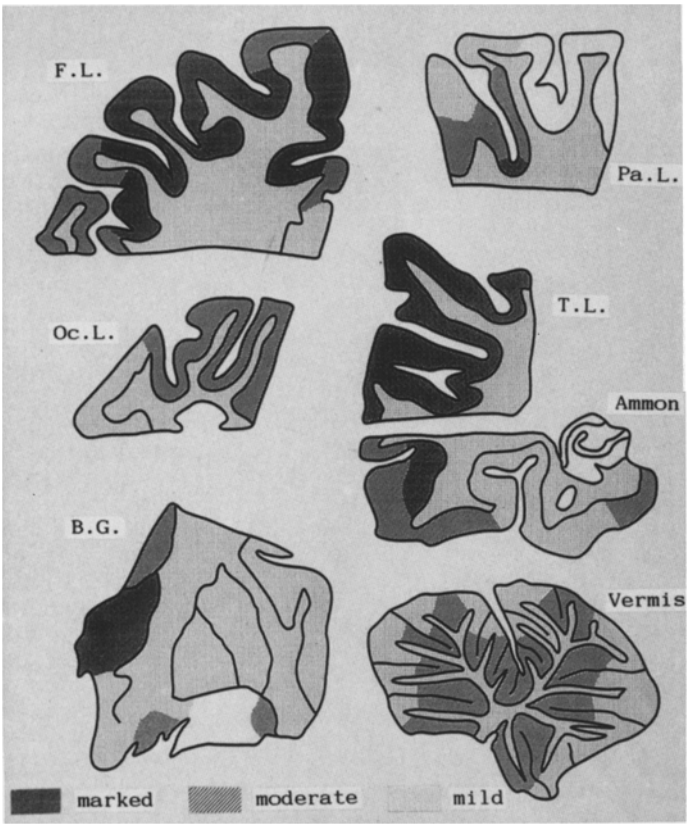

Fig. 2. Schematic distribution of LN-3-positive microglia in the brain of a CJD patient (case 1). *F. L.*, frontal lobe; *Pa. L.*, parietal lobe; *Oc. L.*, occipital lobe; *T. L.*, temporal lobe; *Ammon*, Ammon's horn; *B. G.*, basal ganglia; *Vermis*, cerebellar vermis

absent (Fig. 6a, b). In contrast, the number of ramified microglia was decreased in the affected cortex in all the cases.

The reactions of microglia, macrophages and astrocytes in each CJD case examined in this study are summarized in Table 3.

Control

Cerebral cortical tissue sections of age-matched controls showed numerous ramified microglia labeled with LN-1 Ab or anti-LCA Ab. The MHC class II-immunoreactive microglia were observed in various numbers in the white matter of normal brains, including a brain fixed for 7 months. In the gray matter, however, little or no class II immunoreactivity was detected in microglia. Even in subject with brain abscess, class II Ag staining showed scattered microglia in the gray matter.

Discussion

Judged from our previous studies [29, 30] and other recent studies [11, 20], microglia appear to be the major CNS cell type expressing the MHC class II Ag, and MHC class II Ag may be a marker for activation of microglia [19]. Previous data demonstrate the constitutive expression of LCA on microglia in the resting as well as the reactive state, although reactive microglia have been shown to stain more intensely than resting microglia [2]. There was no definitive expression of MHC class II Ag or LCA on GFAP-positive astrocytes in human brain sections with well-preserved cell morphology [2, 19, 29]. In this study, therefore, we could identify microglia by microscopical cell morphology, the presence of class II Ag or LCA, and the absence of GFAP in all cases examined. In particular, the application of Abs against class II Ag and LCA, which are available in formalin-fixed, paraffin-embedded tissue sections, enabled us to demonstrate the increase of microglial cells in brains of CJD patients.

The microglia which increased in brain sections of CJD patients were microscopically compatible with reactive microglia, which also have been referred to as "activated" or "progressive" microglia in previous stud-

Table 3. Summary of the reaction of microglia, macrophages and astrocytes[a]

	Case: 1	2	3	4	5	6
Reactive microglia	+++	+++	+	+	++	+
Macrophages	–	–	++	++	–	+
Hypertrophic astrocytes	+	++	+++	+++	+	+++

[a] From the lesions of the temporal gray matter
+++, Many; ++, some; +, few; –, none

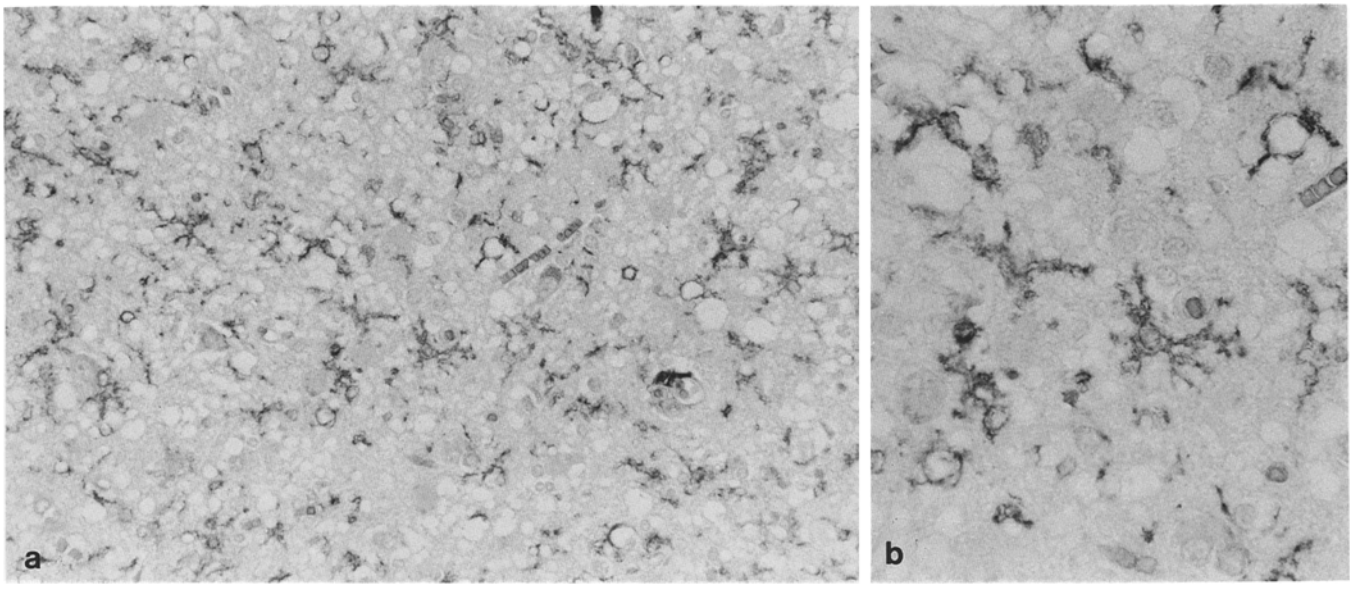

Fig. 3a, b. Temporal gray matter in a CJD patient (case 2) with a relatively short survival period after onset (5 months). Note that there are many LCA-positive reactive microglia (**a**). **b** Higher magnification of **a.** Immunoperoxidase with hematoxylin. **a** ×230; **b** ×460

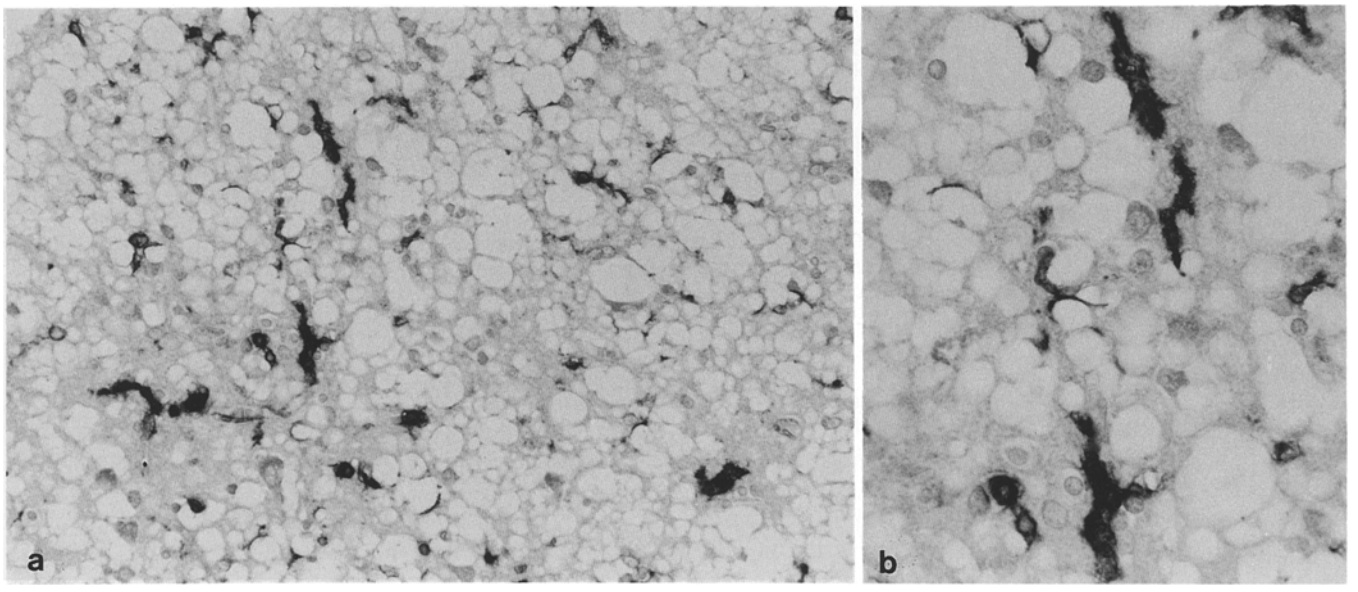

Fig. 4a, b. Temporal gray matter in a CJD patient (case 5) who survived relatively longer. Fewer and less ramified microglia labeled with LN-3 were seen in the cortex, which manifested severe spongy degeneration (**a**). **b** Higher magnification of **a.** Immunoperoxidase with hematoxylin. **a** ×230; **b** ×460

ies [32]. Rio-Hortega [4] described these cells as transition forms, intermediates in shape between the dendritic microgliocyte and the rounded phagocyte filled with lipid droplets or iron. From a viewpoint of Rio-Hortega, reactive microglia should have various morphological forms, and may be indistinguishable from ramified microglia at the microscopic level. In fact MHC class II Ag or LCA was found to exist in some of the ramified forms of microglia in this study. Concerning the reactive microglia, our MHC class II Ag staining indicated no significant difference of morphology between CJD cases and cases with other neurological diseases, as had been described previously [20, 29]. In this study, however, class II Ag-bearing microglial cells from a patient who had survived relatively longer showed less evident ramification. Thus, the morphological changes of reactive microglia may be related to the duration of the CNS disease, not to the pathogenesis. On the other hand, in our present and in previous studies, the number of class II Ag-positive reactive microglia throughout the gray matter was much higher in cases of early CJD than in normal and other pathological human brains. The reason for failure of anti-class II Ag in our studies to label numerous microglia throughout the gray matter in

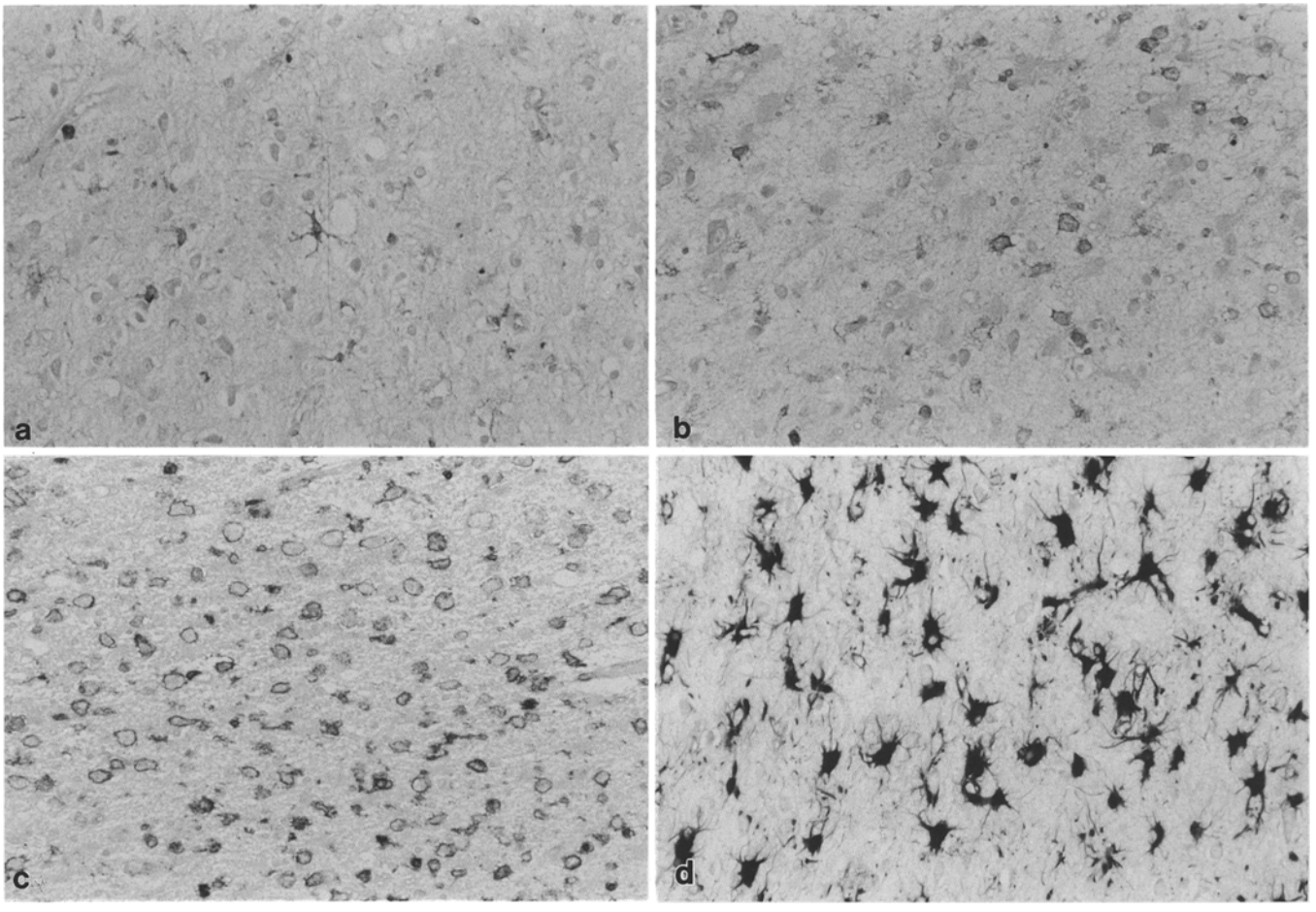

Fig. 5a–d. Immunohistochemical study of a CJD patient who died at an advanced stage and had extensive white matter degeneration (case 6). LCA staining revealed a small number of reactive microglia in the middle (**a**) and deep (**b**) layers of the temporal gray matter, but many macrophages were present in the white matter (**c**). Note the marked increase of GFAP-positive hypertrophic astrocytes in the gray matter (**d**). Immunoperoxidase with hematoxylin. **a–d** ×170

control patients who died of chronic debilitating illness or infectious disease, in contrast to the report of Mattiace et al. [19], remains unknown, but may be due to the difference of Abs used in the immunohistochemical studies. In terms of the morphology, the distribution and the degree of increase, microglial reaction in CJD patients, particularly in patients with short-survival periods, may be characteristic among human CNS diseases.

Based on the results of immunostaining and the lack of tissue destruction in the subacute spongiform encephalopathic (SSE) type of CJD patients, we consider that ramified microglia transform into the reactive form in the lesions in the gray matter in CJD. Our study suggests that as CJD progresses, the degree of microglial activation decreases. Our immunostaining showed some macrophages in the gray matter in brains of advanced cases of CJD. However, all the cases were of the PE type of CJD. Judged from previous studies [1, 14], a large number of the macrophages in the gray matter seems to be derived from blood monocytes, attracted from the bloodstream to extensive, destructive lesions of the white matter. The infiltration of macrophages into the gray matter has been rarely reported, even among previous neuropathological studies of CJD [16, 18, 22, 27]. Thus, we consider that most of transformed, reactive microglia are unlikely to develop into lipid-laden macrophages.

Our topographical study of microglial response showed that it was the gray matter which was mostly affected, and that the degree of the increase was similar to that of histological changes (spongiform change, astrocytic reaction). Moreover, microglial activation was minimal in the hippocampus, where the degree of pathological changes is known to be much milder, than in the cerebral cortex [31]. Thus, microglial activation is considered to take part in the primary lesions of CJD. Based on the observation of the tissue distribution of reactive microglia, and on the observation that microglial reaction was associated with a number of degenerating nerve cells, primary damage to neurons may be attributed to microglial activation.

In the context of glial response in CJD, both microglia and astrocytes were found to participate in the formation of primary brain lesions. However, our findings highlight the temporal difference between the changes of these cells in the disease process. Briefly, microglial reaction may occur earlier, whereas astrocytic

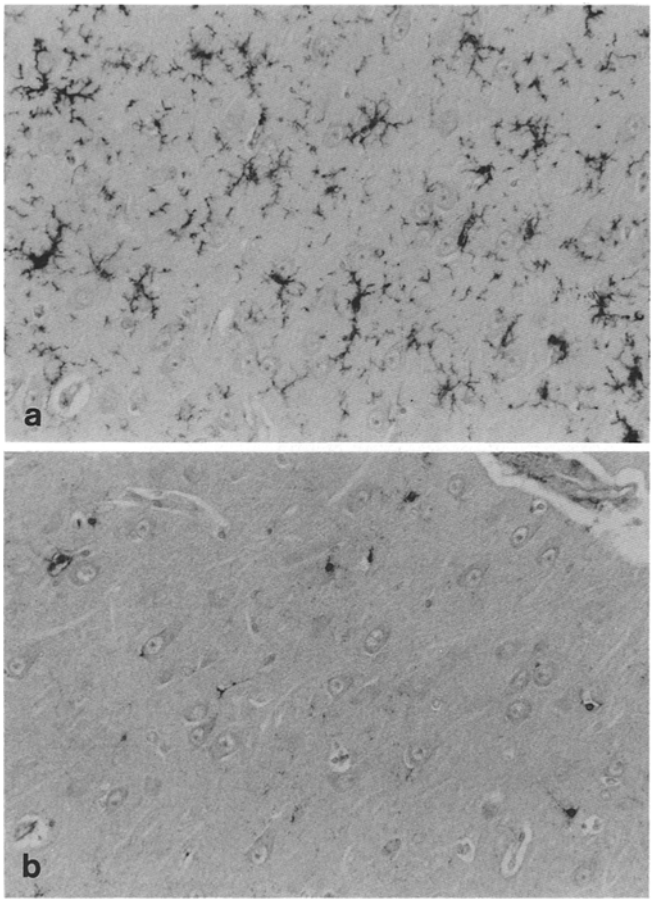

Fig. 6a, b. Immunohistochemical study of the hippocampus in a CJD patient (case 5). Numerous ramified microglia were stained with LN-1 (**a**), while no LN-3-positive cells were seen (**b**). Immunoperoxidase with hematoxylin. **a, b** ×150

reaction may follow but also persist at an advanced stage of CJD. Masters and Richardson [18] suggested that astrocytosis predominated in CJD patients who survived more than 5 months. Determination of the exact time of microglial activation awaits further studies.

This study demonstrated that the vacuoles developing in the neuropil of the CJD patients were surrounded by processes of MHC class II Ag- or LCA-immunoreactive microglial cells, and that the vacuoles were sometimes seen in the cytoplasm of swollen microglia. Electron microscopic studies on the cerebral cortex of chimpanzees afflicted with CJD revealed phagocytosis of degenerated cytoplasmic processes by proliferated glial cells, and most of the phagocytes were identified as microglial cells having a cytoplasm devoid of filaments but rich in ribosomes [15]. Therefore, our study supports the contention that microglia may be involved in the phagocytosis of the affected neuronal process in CJD. Recent studies have suggested that microglia may secrete some factors that are trophic for neurons, including interleukin-6 [8] and nerve growth factor [17]. The secretory products of microglia may also play important roles in the maintenance of the normal CNS [6]. Any increase in production of cytokines may convert a normal trophic action into a toxic property [3]. Thus, it is deduced that the abnormality in the secretory products of microglia may lead to further damage to the nerve cells in CJD.

It has been established that the causative agent in scrapie replicates in the lymphoreticular system, notably in the spleen [7]. However, it was reported that cell-mediated immunity plays no role in the pathogenesis of mouse CJD [23]. In this study, there was no leukocytic infiltration in the brain, such as described in the literature [16, 34], and no evidence of abnormality of the immune system in the CJD patients examined (data not shown). However, it remains to be determined whether MHC class II Ag expression on microglia has an effect on the pathogenesis of CJD or on an immune response in the CNS.

Acknowledgements. The authors are grateful to Drs. Syoji Hoshi, Toshio Fukuda, Masatoshi Hasegawa and Masaru Kojima for generously providing some of the materials used in this study. We also thank Masako Kohno and Kohji Isoda for their skillful technical assistance.

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