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The brain immune response in human prion diseases. Microglial activation and microglial disease. I. Sporadic Creutzfeldt-Jakob disease

Grażyna M. Szpak[1], Eliza Lewandowska[1], Waldemar Lechowicz[1], Teresa Wierzba-Bobrowicz[1], Jerzy Kulczycki[2], Ewa Bertrand[1], Elżbieta Pasennik[1], Jerzy Dymecki[1]

[1]Department of Neuropathology, [2]Department of Neurology, Institute of Psychiatry and Neurology, Warsaw, Poland

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Abstract

A study of microglial activation and its contribution to the CNS immune response was performed on the brain autopsy material of 40 patients with definite sporadic Creutzfeldt-Jakob disease (sCJD). Spatial patterns of microglial activation and prion protein disease-associated (PrPd) deposition were compared in cerebellar and cerebral cortices using immunohistochemical (IHC) activation markers. Morphological phenotype forms of microglial cells in activation stages were assessed immunohistochemically (IHC). The immune inflammatory response dominated by microglia was found to be a characteristic feature in CJD. Differences in the intensity and patterns of microglial activation corresponded to variable patterns of PrP deposition, whereas the morphological phenotype forms of microglia were specific for activation stages. The presence of activated microglial cells in the various activation stages regardless of illness duration indicates continuous microglial activity and microglial contribution to the spread of infection for the whole symptomatic period of the disease. Remarkable vacuolar degeneration changes of numerous microglial cells in different activation stages including homing stage may suggest dysfunction of microglial immune surveillance in human sCJD that can significantly contribute to transmissible spongiform encephalopathy (TSE) pathogenesis.

Key words: *sporadic Creutzfeldt-Jakob disease, human transmissible spongiform encephalopathy, prion disease, activation microglia, degeneration microglia, PrP deposition, immunohistochemistry.*

Introduction

It has been contended for a long time that prion infection in transmissible spongiform encephalopathies (TSE) or prion diseases does not elicit a brain immune response. Although plaque-associated accumulation and proliferation of microglial cells in various TSE forms have been recognized [7,22,23,38] however, no humoral (antibody against prions) or cellular (classic brain inflammatory infiltrates) immune responses have been detected [1,21,33]. The first reports of brain inflammatory microglial response to scrapie agent (PrP^{sc}) appeared in the last decade when new immunohistochemical (IHC) microglial activation markers and pretreatment methods led to a growing number of investigations of the immune microglial functions on the scrapie CJD model

Communicating author:

Dr Grażyna M. Szpak, Zakład Neuropatologii, Instytut Psychiatrii i Neurologii, 01-913 Warszawa, Sobieskiego 9; tel.: +48 22 458 26 33, e-mail: szpak@ipin.edu.pl

[4,11,14,52]. However, the notion that the central nervous system (CNS) has an unusual immune system of its own, comprising a network of microglial cells, has become accepted only recently [1,40].

In the central nervous system (CNS) two forms of immune response are recognized: innate – responsible for immediate reactions to foreign invaders; and adaptative – responsible for the results in long-term immunological memory. Activated microglia are cellular initiators of the innate and then adaptative brain immune response, whereas astrocytes appear to counterbalance microglial activation, including regulation microglial function and deactivation [19]. It is widely accepted that neuronal immune surveillance maintains downregulation of immune cells in CNS tissues [32]. Although several recent reports suggest that highly versatile immunocompetent microglia are population surveillanced the neuronal microenviroment, is capable of initiating both the inflammatory and anti-inflammatory responses [40].

The pathogenic mechanisms of TSE have not been fully elucidated; however, the quantity of data on microglial activation involvement in human TSE pathogenesis is limited [2,34,48]. To determine the involvement of microglial immune response in CJD pathogenesis we performed an IHC study on brain microglial activation patterns and stages in the most often observed form of sCJD with presence of PrP deposition but without amyloid plaques [16]. This study could lead to better understanding of the microglial contribution to spreading PrP infection, the immune surveillance of neuronal protection and damage and dysfunction of microglial immune surveillance during progressive TSE cascade of tissue changes.

Materials and methods

The study was performed on brain autopsy material of 40 definite sCJD patients (aged 27-72 years; illness duration 2-24 months) and from 10 age-matched control patients with no neurodegenerative brain changes. For light microscopy, samples obtained from brains fixed in 4% paraformaldehyde buffered to pH 7.4 were decontaminated in 96% formic acid for 30 min. after fixation and embedded in paraffin. Histological (H-E, Congo red, PAS, luxol fast blue/cresyl violet-Klüver-Barrera) and IHC reactions in 8 μm sections were performed. Hydrated autoclaving pretreatment at 121°C for 60 min. to enhance the immunoreactivity for PrP was applied [24]. Definite sCJD diagnosis was confirmed immunohistochemically and coexistence of CJD and other neurodegenerative diseases was excluded in the preliminary study. Furthermore, we performed a preliminary review of various IHC markers' brain inflammatory response (perivascular lymphocyte infiltrations were observed only occasionally) and all cases associated with amyloid PrP- and A β-immunoreactive plaques were excluded from the present part of investigations using: monoclonal PrP/3F4 (DAKO, 1:50), LCA/CD45, A β, and GFAP; and polyclonal tau and ubiquitin antibodies [43].

In the present study the IHC identity of resting and activated microglia was confirmed using a panel of mAb: MHC class II (HLA-DP, DQ, DR), CD68, PCNA and Ki67. The diaminobenzidine chromogen (DAB) in the avidin-biotin-complex (ABC) method or DAB/Fast red chromogens in the streptavidin/biotin amplification method in single- or double-immunostaining were applied. Distribution, pattern and interaction between activated HLA-positive microglia and PrP-positive deposition assessed IHC as "regional pattern changes" using a modified scoring scale ranging from (1+) for a few activated cells, (2+) for dispersed cells, to (3+) for severe diffuse microgliosis (x200). Phenotype forms and stages of activated microglia were analyzed IHC and compared with the multistep grade immune response of microglial activation in human and animal brains from in *vivo* and *in vitro* studies [34,37,39].

Results

In all control species, immunoreaction with PrP antibodies was negative and the perivascular and a few (1+) parenchymal CD68 and HLA-positive microglial cells were found occasionally. In all CJD cases, considerable variability of brain atrophy, from mild to advanced stage, usually higher grade in cerebral than in cerebellar samples, was shown (Fig. 1A-B). In some cases, cortical ribbon of diffuse but not uniform intensity PrP immunoreactivity in cerebral and cerebellar samples was observed (Fig. 1C- F).

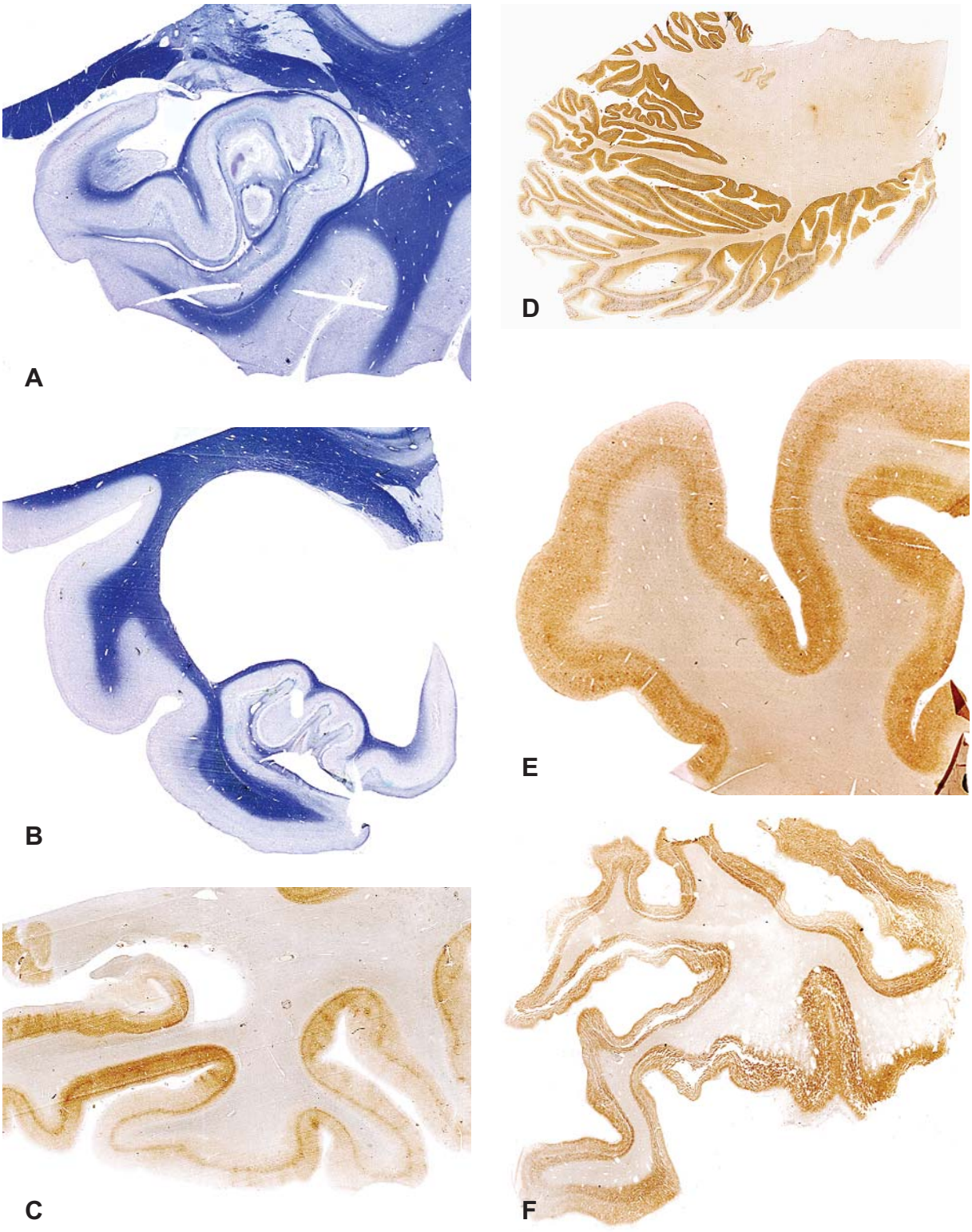


Fig. 1. Sporadic CJD. Macroscopic cerebral and cerebellar samples. **A-B.** Mild (A) and advanced (B) stage of brain atrophy in temporal lobe. Fast luxol-blue/cresyl violet (Klüver-Barrera). **C-F.** Cortical ribbon of the diffuse PrP immunoreaction. Temporal (C), cerebellar (D), frontal (E) and occipital (F) samples; PrP/3F4

Relationship between PrPd immunoreactive deposition and microglial activation patterns

A few Purkinje cells in the cerebellar cortex and neocortical neurons only occasionally showed fine-granular positive cytoplasmic immunoreaction, whereas PrP-positive extracellular cortical deposition were found in all 40 cases in the cerebellar cortex and at least in one region of the cerebral cortex (results of white matter study not shown).

In the cerebellar cortex, deposition of diffuse pattern coexisted in two cortical layers (synaptic in molecular and coarse in granular layer) in 26 cases. The synaptic pattern of the diffuse deposition resembled synaptophysin immunoreactivity, whereas coarse deposits both diffuse and mixed with plaque-like aggregations showed stronger PrP reaction (Fig. 2A-C).

In almost all these cases, diffused (3+) or dispersed (2+) pattern of microglia activation was observed. The spatial relation of the highest density diffuse inflammatory reaction (3+) and the strongest PrP diffuse synaptic immunoreactivity, regardless of the presence of granular non-amyloid plaque-like depositions, was found (Fig. 2C-D). In 12 cases, PrP deposition of mixed types, focal, patchy and plaque-like non-congophylic aggregation coexisted with predominant diffused depositions of synaptic or coarse patterns and corresponded to microglial activation of dispersed pattern (2+) (Fig. 2E-F). Parenchymal and perivascular microglial activation (1+) was found only in two cases with synaptic deposition and usually corresponded to regional dot-like coarse deposits of the lowest intensity in the Purkinje cell layer (Fig. 2 G).

In the cerebral cortex, in 14 cases with diffuse synaptic deposits, the highest accumulation of activated microglia, more frequently diffuse (3+) than dispersed (2+), was shown. The highest variability of density and distribution of microglial activated cells in the temporal lobe was noted. In the frontal cortex, diffuse PrP deposition corresponded to diffuse microglial activation (3+) and only in four of these cases did diffuse astrogliosis (3+) predominate over microglial activation (2+). In the occipital cortex, diffuse microglial activation (3+) was shown in 13/14 cases, whereas in the visual cortex, laminar accentuation of microglial activation diffuse pattern predominated in the same cases (Fig. 2 H-I). Activation (1+) of parenchymal and perivascular microglia was found in only one case. Very strong focal and coarse perivacuolar PrP deposits coexisting with numerous and large clusters of morula-like spongy change predominated in cortical layers III-VI in 18 cases and in 8 other cases, associated with diffuse deposition of synaptic pattern or granular plaque-like aggregations. In some cases with mixed perivacuolar and synaptic PrP immunoreactivity, focal accumulation of the activated microglial cells at site vacuolar clusters coexisted with areas of diffuse (3+) microglial activation corresponding to regions of diffuse PrP deposition synaptic type. Numerous activated microglial cells and usually single astrocytes were found along tissue fibres between vacuoles (Fig. 2J-L).

Graded microglia inflammator response

In control and occasionally in CJD cases, perivascular and a few parenchymal ramified microglial cells with thin processes were found (Fig. 3A). In all CJD cases, morphological transformation of microglia from the ramified resting state cells with fine and thin processes to activated forms of various phenotype were found in both cerebellar and cerebral cortices. Strong HLA immunoreactivity of microglia with hypertrophy and retraction of microglial processes corresponded to the easiest activation features of “alert and transformation stage” (Fig. 3B). A relatively small number of microglial cells immunoreactive with IHC proliferation markers were found. Double immunolabelling for PCNA and HLA revealed PCNA immunoreactivity of nuclei in some cells dispersed at site, diffuse accumulation of activated ramified or rod microglial cells and particularly in microglial doublets or small clusters that indicated the “proliferation stage” (Fig. 3C-D). Sometimes PCNA-immunoreactive cell doublets developed “bushy” hyperramified morphology, with multiple hypertrophic and partly retracted processes. The presence of numerous poorly ramified microglial “rod cells” with elongated nuclei corresponded to “migratory or locomotory stage” (Fig. 3 E). The large number of activated microglial cells with various sized protrusions of their processes surrounding

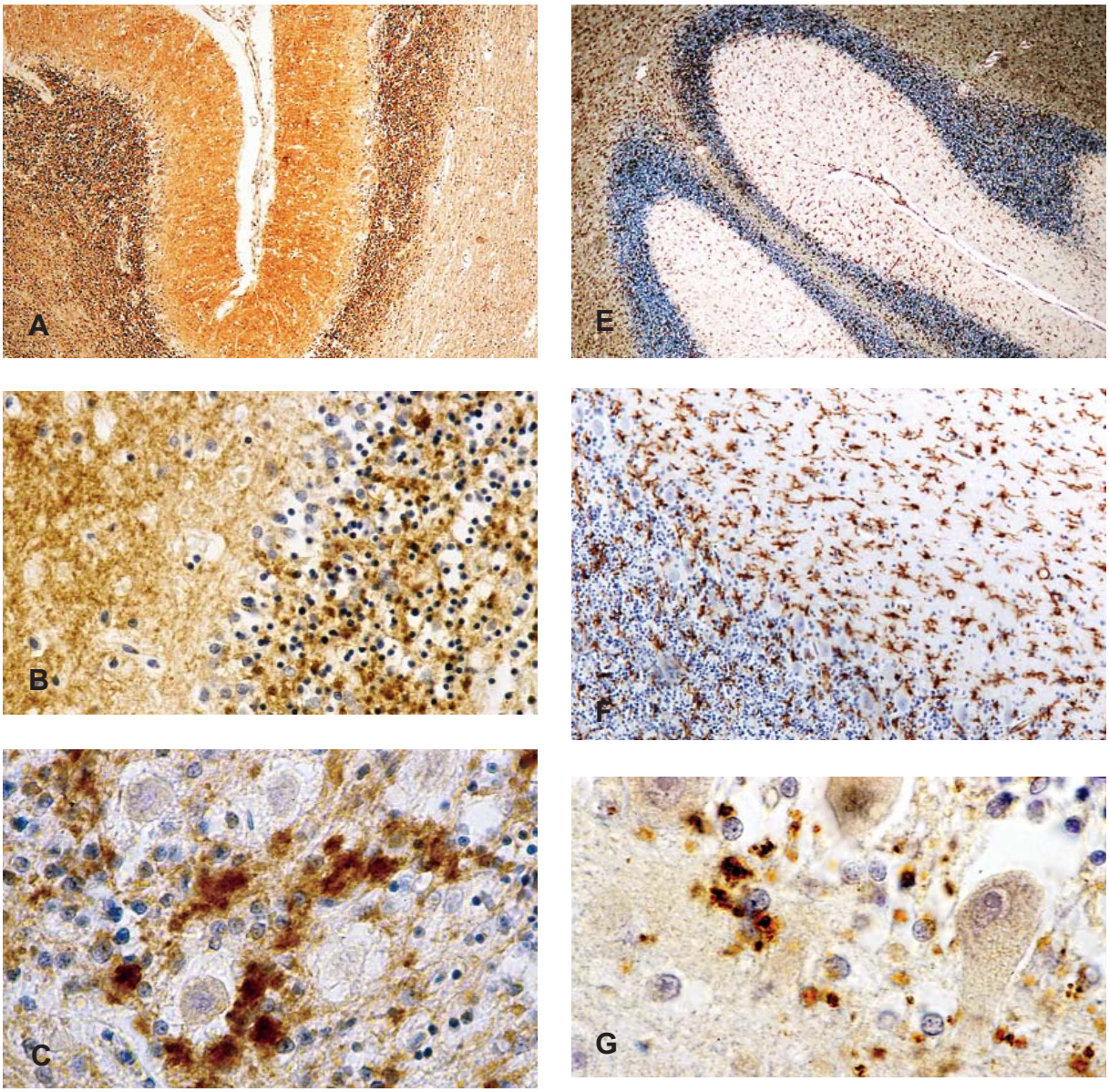
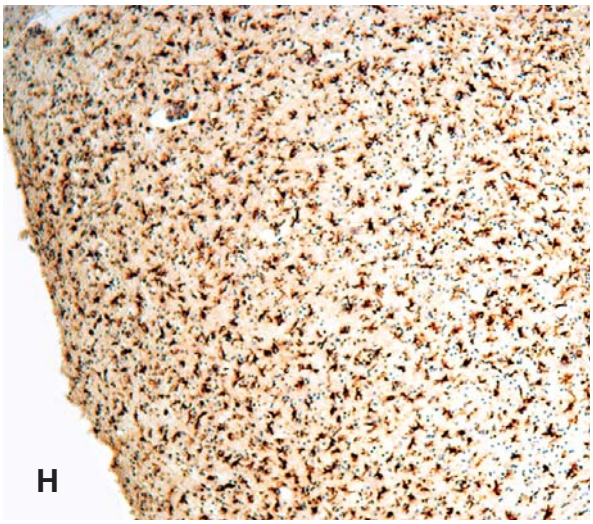
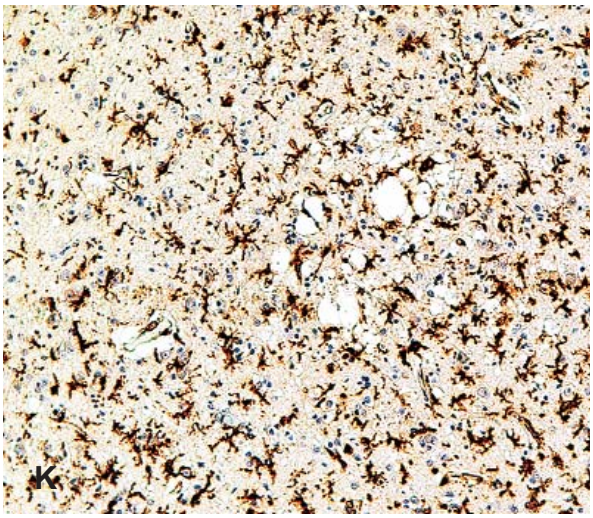
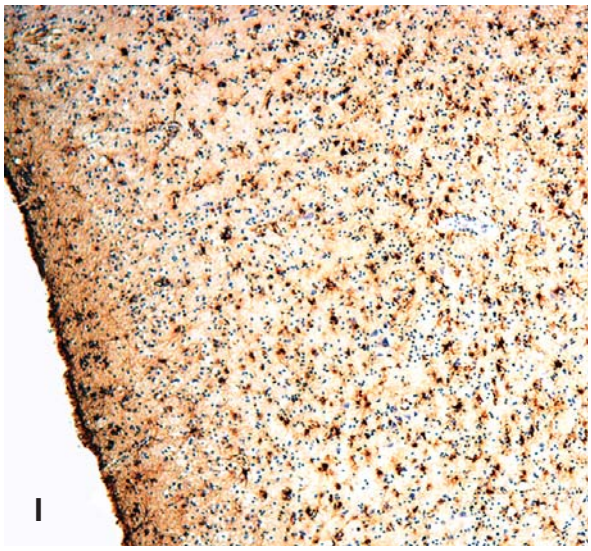
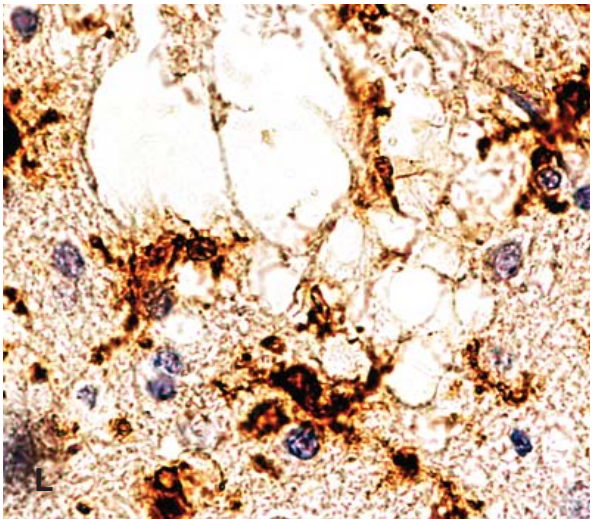


Fig. 2. Relationship of prion protein (PrP/3F4) and microglia activation (HLA) patterns. **A-G.** The cerebellar cortex. A. Synaptic pattern of diffuse deposition in molecular layer. B. Synaptic (molecular layer) and coarse (granular layer) pattern of diffuse deposition. C. Mixed patterns of coarse – diffuse and focal perineuronal/patchy and plaque-like deposits. D. Diffuse pattern of microglial activation (3+) in all cortical layers. E-F. Dispersed pattern of microglial activation (2+) in molecular and granular layer (F-in higher magnification). G. Dot-like pattern of coarse deposits associated with Purkinje cells









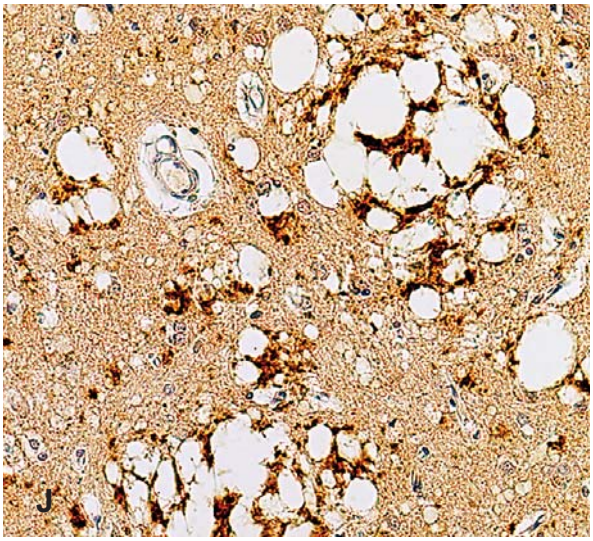


Fig. 2. continued. Relationship of prion protein (PrP/3F4) and microglia activation (HLA) patterns. **H-L.** The cerebral cortex. H. Diffuse pattern of microglial activation (3+) in occipital cortex. I. Microglial activation (3+); laminar accentuation of microglial cells. Visual cortex. J. Perivacuolar pattern of PrP coarse deposition in the frontal cortex. K. Mixed patterns of microglial activation: perivacuolar and dispersed (2+) in the frontal cortex. L. Perivacuolar pattern of microglial activation in higher magnification. Bars represent: A – 25 μm, B – 12 μm, C – 9 μm, D, E – 25 μm, F – 12 μm, G – 9 μm, H, I – 25 μm, J – 8 μm, K – 15 μm, L – 8 μm

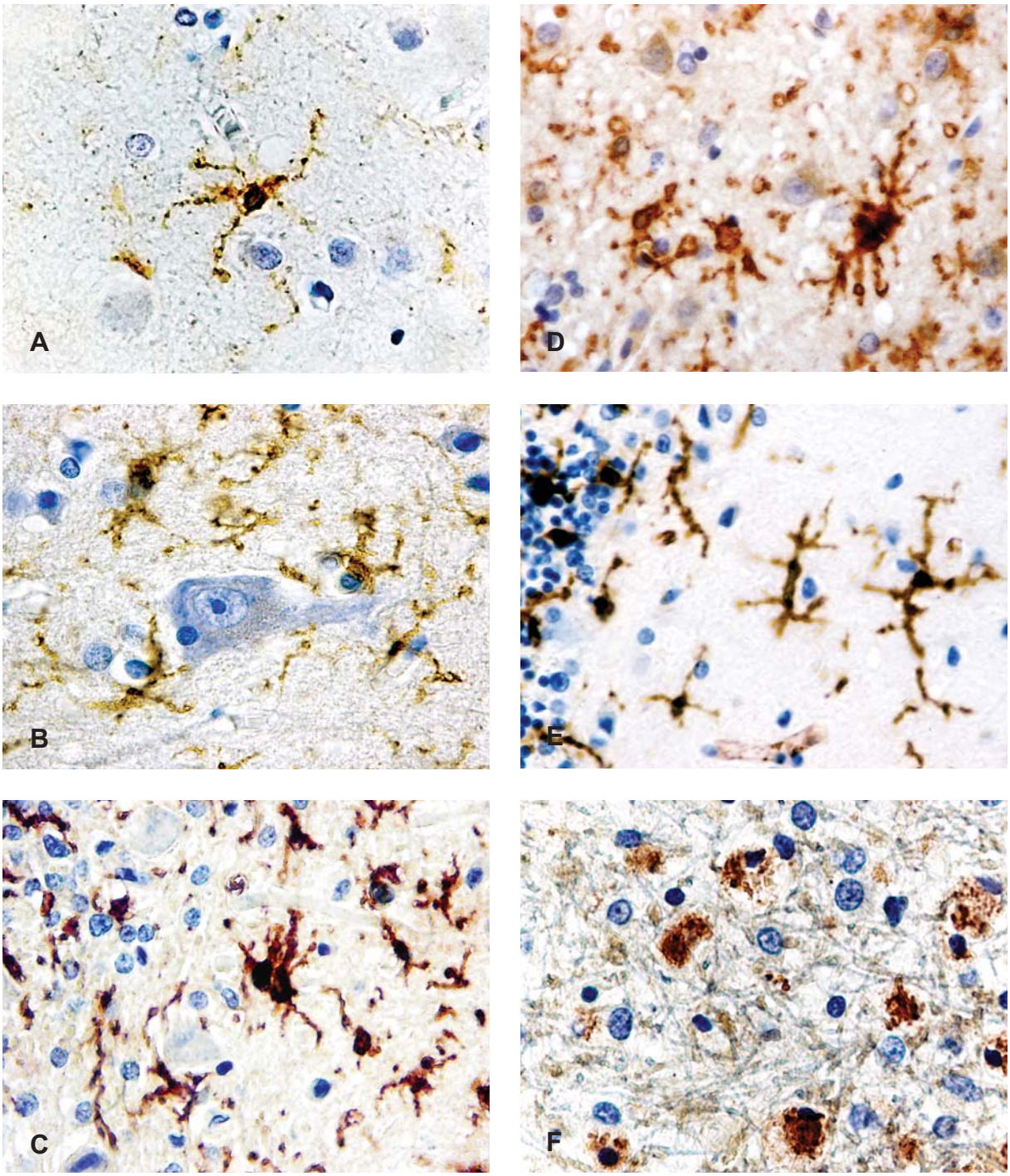


Fig. 3. Various phenotypic forms of microglia at different stages of activation and degeneration, IHC study. **A.** control case, B-L. CJD cases. A. CD68-immunoreactive ramified microglial cell in the resting state with scanty cytoplasm and thin processes; **B.** Alert stage; Increased HLA expression and accumulation of microglial cells transforming from resting to activation state; **C.-D.** Proliferation stage; double HLA/PCNA immunoreaction on microglial doublets and microrosets with PCNA-positive nuclei; **E.** Locomotory stage; elongated rod cells with scanty ramification, HLA/CD31; F. Phagocyte stage; PrP expression on macrophage-like, HLA-positive cells in double immunoreaction PrP/HLA

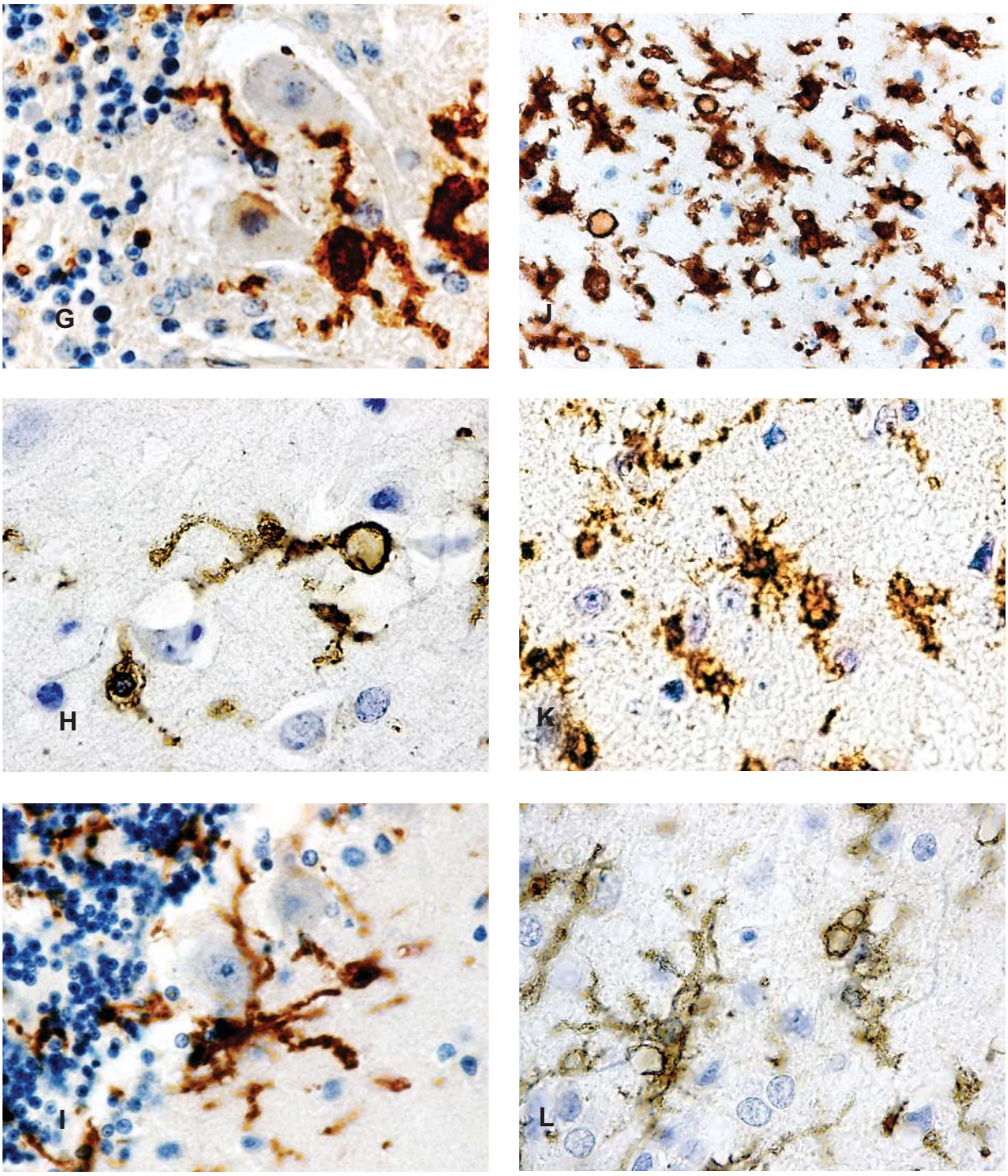


Fig. 3. continued. Various phenotypic forms of microglia at different stages of activation and degeneration, IHC study. **G-I.** Homing stage; microglial cells and their processes in close contact with healthy and damaged, cerebellar and cerebral neuronal cells, HLA; **J-L.** Degenerative changes of microglia - "microglial disease"; microglial cells at various activation stages display marked intraplasmatic vacuolation with decreased expression HLA (L)

normal, degenerating or dying neurons or other glial cells corresponded to the "homing and motility stage" (Fig. 3 G-I). Macrophage-like, large and round cells of ameboid phenotype, phagocyting the tissue remnants, indicated the "phagocytic stage". Reduction of the number of microglial cells and positive PrP/HLA double-immunoreaction of microglia were found only in this stage (Fig. 3 F). Except for this activation stage, observed usually in cases of long-lasting disease, other presented phenotype forms of microglia were observed in all activation stages regardless of illness duration.

Microglial processes surrounding the outer rim of small dispersed cortical vacuoles were frequently observed in both the cerebellar and cerebral spongy change (Fig. 3 J). Additionally, a large number of microglia of both ramified and rod cells, not only ameboid ones, displayed marked intraplasmatic vacuolation. Intraplasmatic vacuoles of different size and variable deformations along short or elongated processes of numerous activated microglial cells were shown. These striking degenerative changes were found regardless of illness duration in the same microglial cells which revealed different activation forms and stages (Fig. 3 J-K). In some cases, progression of degenerative changes leading to lack of processes and lower intensity of immunoreactive markers of microglial activity was noted (Fig. 3 L).

Discussion

A special form of brain inflammatory response dominated by microglia was found to be a characteristic feature of immune CNS response in sCJD. Prominent accumulation of strong MHC class II expressing activated microglia at site cerebellar and cerebral cortical PrP deposition and a spatial relationship between patterns of microglial activation and PrP deposition have been shown. Different phenotype microglial forms corresponded to various activation stages. The graded immune response in sCJD was dominated by a large number of microglia in homing activation stage and remarkable vacuolar degenerative changes of activated microglial cells, independent of age of patients and illness duration.

Our results support the hypothesis of the special form of brain immune response to prion protein infection of microglial cells characterized by association of activation, degeneration and potential dysfunction of immune surveillanced microglia population [4,8,17]. This inflammatory brain response, dominated by microglial cells presenting strong expression of MHC class II and leukocytes antigens (LCA) with or without a slight contribution of the classic T-cell response, represents a special form of brain innate immune response [40]. This indicates that CJD may rank with diseases characterized by chronic neuroinflammation with no overt T-cell involvement. It may be a manifestation of immune natural tolerance to the cellular isoform of prion protein (PrPc) due to identical sequence composition of the protein amino acids of isoforms cellular PrPc and disease-related PrPd [3,21].

The presented inflammation-like glial immune response included in our study two populations of brain cells, microglia and astroglia, having the ability of immune activation. Activated microglia are initiators of the innate and then adaptative brain immune response, whereas astrocytes regulating brains homeostasis appear to counterbalance microglial activation, including microglial function and deactivation [19,49]. Reactive astrocytosis represents one of the morphological hallmarks of TSE [15], whereas only recently has microglial activation and accumulation been found among the criteria for TSE [16].

We observed a special or "atypical" form of brain response, also dominated by microglia but of different intensity and pattern, in our previous investigations of CNS disorders [27,42,44-46,51]. Differences in microglial activation cascade of brain innate immune response and in potential contribution to neurodegeneration pathogenesis may be due to different trigger mechanisms of activation. Microglial activation may be primary or secondary relative to the temporal and spatial relations between damaged neuronal and microglial cells.

Our previous study of microglial response to non-transmissible brain amyloidoses supported the hypothesis of secondary microglial activation due to reveal of microglia from suppressive immune surveillance of damaged neuronal cells [32,46]. However, our present study of brain response to sCJD – transmissible amyloidosis – supports the hypothesis of a key role of early microglial PrP infection and impairment activation, although the pathogenic mechanisms of TSE have not been fully elucidated. It is generally accepted that conformed

and transmissible isoform of prion protein-associated disease (PrPd) [28] is an infectious agent forming brain deposition and leading to loss of neuronal cells, although it is not determined to date whether neuronal death is due to neuronal replication of neurotoxic PrPd in parallel loss of PrPc function [14,33,35] and/or PrP-activation of infected microglia preceding apoptotic death of infected neurons [4,8,11,18,47]. In the majority of cases of sCJD study, there was an inflammatory response with a strikingly large number of activated cells (quantitative data not shown). Although the mitotic activity of microglia was not so great, IHC proliferation markers in nuclei of doublets and small microglial clusters were noted. In TSE animal models, early activation and proliferation of microglia already in the incubation period of disease were present and some recent data indicate the stimulation of mitotic activity and recruitment of microglial cells by prion protein [13,30,52]. However, the results of our study are not comparable because our CJD material was collected from autopsy brains in terminal state of human disease different from CJD models' duration of illness, incubation period and blood-barrier state. The major pool of microglia may be in in terminal period of disease in postmitotic stage [12] and even in apoptotic state [our ultrastructural data not shown], whereas the striking accumulation of activated microglia in our study could be a result of earlier mitotic microglial activation and later recruitment of macrophages in the final stage of disease. It has recently been documented that microglial accumulation and activation may be detected *in vivo by* molecular imaging in CJD patients [31].

Variable phenotype forms of activated microglial cells were thoroughly observed IHC in this study. Our results show that all forms of microglial transformation observed in sCJD from resting to activated cells correspond to phenotype forms of multistep microglial activation recognized in the graded response to CNS injury from *in vivo* or *in vitro* monitoring studies in confocal microscopy [37,39]. It is generally accepted that the microglial phenotype cells presented in various activation stages in our material are capable of proliferation, phagocytosis and locomotory functions. Activated microglia may then have a significant contribution to spread of CNS infection [5,13,48].

Our finding that a relatively high proportion of activated microglia are in the "homing stage" regardless of illness duration supports the hypothesis of microglial immune surveillance significant for a better understanding of disease pathogenesis [4,6]. In this stage, close intercellular interactions between microglial and neuronal cells and between glial cells were previously detected IHC and ultrastructurally in some cases in the CJD study of brain biopsy [29] and brain autopsy material [26,43]. In our present study, the "motile" processes of microglial cells were in close contact with damaged and normal neuronal cells in various phases of the homing stage. This agrees with the results from *in vivo* and *in vitro* studies of CNS-damage no-TSE models [6,8,9,36,39]. According to the concept of the above-quoted authors, microglia in the homing stage are selector cells of immune surveillance. They are able to distinguish between degenerating and surviving neurons, then selectively respond to signals from lethally injured neurons and eliminate them. In the homing stage microglia can no longer promote inflammatory cascade, but they facilitate the elimination of damaged cells and protect healthy neurons. Depending on the primary or secondary mechanisms of activation, surveillance functions of immunocompetent microglial cells can be beneficial and protective or destructive for CNS tissue.

Motile ability of microglia is a characteristic feature not only of cells in the homing stage. Contrary to the classic terminology, "resting" microglia cells observed in *in vivo* study are in a continuous state of motion corresponding to the motility/homing and migratory stage of activated microglia facilitating a remarkable degree of alertness to microenvironment alterations during surveillanced immune functions of microglia in ramified resting state in the healthy CNS [6,37].

Apart from predomination of the homing microglial activation stage, the widespread coexistence of microglial activation and vacuolar degeneration and sometimes apoptosis features were noted. Association of degenerative and activate changes in some microglial cells with various activation stages suggests a direct mechanism of microglial activation and the potential dysfunction of microglial surveillance by PrP infection when microglia are a target of TSE agent [4,20]. Vacuolar degeneration of activated microglia with decreased HLA expression and apoptosis could appear as special and specific to TSE

changes [17,38], different and regardless from age-related microglial dystrophic changes [25,41] and from degenerative microglial changes found in the other, probably immune-mediated CNS disease [50,51]. In addition, primarily infected microglial cells can also be secondarily infected the results in their phagocytic-scavenger functions [4,5,18].

In the light of our study and data from literature, the degenerative changes of activated microglia support the hypothesis of a key role of dysfunction of microglial immune surveillance in TSE pathogenesis [4,17,40]. It is possible that,degenerative changes characteristic for TSE could be evidence markers of "disease of microglia" resulting in impaired surveillance function of elimination and protection of neurons in the homing activation stage. It seems that microglia as "sensor cells" keeping on the alert and immune surveillance of the neuronal microenvironment may be primary targets, carriers and secondary reservoirs of TSE pathogenic prion protein agents and potential targets for therapeutic interventions in at least some forms of CJD.

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