Acta Neuropathol (2003) 106 : 518–526
DOI 10.1007/s00401-003-0766-2

REGULAR PAPER

**Kazuhiro Imamura · Nozomi Hishikawa
Makoto Sawada · Toshiharu Nagatsu · Mari Yoshida
Yoshio Hashizume**

Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains

Received: 2 January 2003 / Revised: 5 May 2003 / Accepted: 4 August 2003 / Published online: 25 September 2003
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Abstract There are numerous observations confirming that microglia expressing major histocompatibility complex (MHC) class II molecules are associated with the central nervous system (CNS) in aging and pathological conditions. In this study, we investigated the distribution of MHC class II-positive microglia in Parkinson's disease (PD) brains. The number of MHC class II-positive microglia in the substantia nigra (SN) and putamen increased as the neuronal degeneration of the SN proceeded. These cells were also ICAM-1 (CD54) and LFA-1 (CD11a) positive. The number of activated microglia not only in the SN and putamen but also in the hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex in PD was significantly higher than that in the normal control. Most activated microglia persisted regardless of the presence or absence of Lewy bodies. They were frequently associated not only with α-synuclein-positive Lewy neurites, but also with TH-16-positive dopaminergic and WH-3-positive serotonergic neurites, as well as MAP-2- and SMI-32-positive neurites. These activated microglia were also positive for TNF-α and interleukin-6, which are known to have a neuroprotective function. We conclude that MHC class II-positive microglia are a sensitive index of neuropathological change and are actively associated with damaged neurons and neurites.

Keywords Parkinson's disease · Microglia · MHC class II antigen · ICAM-1 · Cytokines

K. Imamura (✉)
Department of Neurology, Okazaki City Hospital,
3-1 Goshoai, Kouryuuji-cho, Okazaki, 444-8553 Aichi, Japan
Tel.: +81-564-218111, Fax: +81-564-255531,
e-mail: katy@syd.odn.ne.jp

N. Hishikawa
Department of Neurology, Nagoya University School of Medicine,
Nagoya, Aichi, Japan

M. Sawada · T. Nagatsu
Institute for Comprehensive Medical Science,
Fujita Health University,
Japan Science and Technology Corporation, Toyoake, Aichi, Japan

M. Yoshida · Y. Hashizume
Department of Neuropathology,
Institute for Medical Science of Aging, Aichi Medical University,
Aichi, Japan

Introduction

Microglia qualify as immunocompetent cells in the central nervous system (CNS) by virtue of their ability to express major histocompatibility complex (MHC) class II antigens. Microglia with a resting or ramified morphology seldom express those antigens, although in elderly human tissue MHC class II-positive microglia are very occasionally found in gray matter [45]. Since up-regulation of MHC class II antigen is an early consequence of activation, the threshold of detection is reached prior to the onset of visible morphological change. MHC class II expression on microglia is also up-regulated in pathological situations where microglia are activated [27, 28, 29, 40, 45]. Parkinson's disease (PD) is one such condition. The salient pathological features of PD are selective neuronal loss presumably by apoptosis [16, 17, 24, 43] and the presence of Lewy bodies (LBs) in the affected regions. The presence of activated microglia and the absence of reactive astrocytosis in the substantia nigra (SN) of patients with PD suggest microglial involvement in the pathological process of dopaminergic neurons [35, 36, 40]. In PD brains, degenerating neurites have also been detected in the brain stem, especially in the dorsal vagal nuclei [14], hippocampus [13] and amygdala [6, 21]. Gai et al. [14] speculated that a demonstration of extensive ubiquitin-positive degenerating neurites might provide a clue to disease activity at the time of death. In this study, we showed MHC class II-positive activated microglia to be widely distributed in the affected regions, frequently in association with α-synuclein-positive Lewy neurites (LNs) and monoaninergic neurites in PD patients' brains.

Table 1 Clinical presentation and neuropathoiogical findings of PD and control cases (*PD* Parkinson's disease, *NFT* neurofibrillary tangle, *SP* senile plaque, *LB* Lewy body, *SN* substantia nigra)

		Age	Sex	Duration (years)	NFT	SP	Neuron loss SN	LB counts			
								Hippocampus	Transentorhinal	Cingulate	Temporal
PD	1	81	M	14	I	–	+	20	13	11	0
	2	75	F	13	I	±	+++	30	22	26	2
	3	84	M	11	I	±	+++	28	17	37	4
	4	86	M	11	I	–	+++	20	12	16	3
	5	71	M	8	I	±	+++	11	10	7	1
	6	67	M	2.5	I	–	+++	22	15	19	1
	7	79	M	13	II	±	++	26	7	28	1
	8	69	M	1.1	I	–	+	10	5	7	0
	9	71	M	12	I	–	+++	21	3	5	0
	10	74	M	9	I	–	++	10	3	7	0
	11	81	M	8	I	–	++	21	2	3	1
	12	72	M	4	I	–	+	14	14	13	0
NC	13	73	F	–	I	–	–	0	0	0	0
	14	74	M	–	–	–	–	0	0	0	0
	15	75	M	–	–	–	–	0	0	0	0
	16	81	M	–	–	–	–	0	0	0	0

Materials and methods

Subjects

Autopsied brains from 12 clinically and neuropathologically confirmed cases of PD (ages at death 67–86 years, mean 75.8 years) and four age-matched individuals (ages at death 73–81 years, mean 75.8 years) were used in this study. All PD patients had presented clinically with resting tremor, rigidity and akinesia. Neuropathologically, the brain specimens showed neuronal loss in the SN, locus ceruleus and dorsal vagal nuclei. LBs appeared in the SN, locus ceruleus, dorsal vagal nuclei and neocortex. There were few or no neurofibrillary tangles (NFT) or senile plaques (SP). The clinical data of PD and control cases were summarized in Table 1. The distribution and frequency of LBs were evaluated according to the consensus criteria for pathological diagnosis of DLB [39].

Conventional histopathology

All brains were removed within 12 h of death and immersed in 20% neutral-buffered formalin. Fixation periods of all brains were within 3 weeks. Each brain part was sliced into 5-mm-thick sections along various planes: cerebrum in the frontal plane, brain stem and spinal cord in the horizontal plane, and cerebellum in the sagittal plane. The tissues were embedded in paraffin and sectioned at 10-µm thickness. For routine histological examinations, each section was stained with hematoxylin and eosin (H-E) and the Klüver-Barrera (K-B) method. Selected sections were also stained according to the Bodian and Holzer methods. In all cases, additional sections taken from the cerebrum, brain stem, cerebellum and spinal cord were stained according to the Gallyas-Braak (G-B) method.

Immunohistochemical staining

The 10-µm-thick sections were deparaffinized and rehydrated according to the standard procedures for immunohistochemistry. They were then subjected to microwave treatment for 30 min in 0.01 M citrate buffer at pH 6.0, removed from the buffer to cool down to room temperature, and treated for 20 min with 0.3% H_2O_2 solution in 0.01 M phosphate-buffered saline at pH 7.4. After blocking, they were then incubated for 74 h at 4°C with primary antibodies, treated with biotinylated second antibodies (DAKO, Carpinteria, CA) for 1 h at room temperature, and incubated with avidin-labeled horseradish peroxidase (DAKO) for 1 h at room temperature. Peroxidase labeling was visualized by a brief incubation in 0.01% 3.3 diaminobenzidine and 0.1% H_2O_2 in 0.05 M TRIS-HCl buffer at pH 7.6. Nuclei were counterstained with hematoxylin. Double immunostaining was also performed. The first cycle was carried out as mentioned above, and stained sections were again given a microwave treatment for 30 min in 0.01 M of citrate buffer at pH 6.0. The second immunohistochemical cycle was carried out similarly to the first except that it was incubated with avidin-labeled alkaline phosphatase (DAKO), and immunolabeling was visualized by incubation in first red. For double immunofluorescence labeling, the cerebral hemispheres were fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. Three small blocks were dissected from the putamen, cingulate cortex and hippocampus, and were cut into 10-µm-thick sections on a vibratome. The first cycle was carried out using the tyramide signal amplification (TSA) method (NEN Life Science Products, Boston, MA) and was visualized with Alexa Fluor 488 (emission peak 519 nm; Molecular Probes, USA). Then the second cycle was carried out using the avidin-biotin combination method and was immunofluorolabeled with Alexa Fluor 568 (emission peak 603 nm). The primary antibodies used in this study were monoclonal antibody to human pan macrophage (Ki-M1p) (clone VI-20) at a dilution of 1:100 (Seikagaku Corporation, Tokyo, Japan), monoclonal antibody to human HLA-DP, DQ, DR (clone CR3/43) at a dilution of 1:100 (DAKO, Glostrup, Denmark), monoclonal antibody to human LFA-1, α-chain (CD11a) (clone MHM24) at a dilution of 1:50 (DAKO), monoclonal antibody to human ICAM-1 (CD54) (clone W-CAM-1) at a dilution of 1:100 (Chemicon, Temecula, CA), monoclonal antibody to human tyrosine hydroxylase (clone TH-16) at a dilution of 1:400 (Sigma, St. Louis, MO), monoclonal antibody to human tryptophan hydroxylase (clone WH-3) at a dilution of 1:400 (Sigma), monoclonal antibody to human MAP-2 (clone HM-2) at a dilution of 1:100 (Sigma), monoclonal antibody to human non-phosphorylated neurofilament (clone SMI-32) at a dilution of 1:100 (Sternberger Monoclonals, Lutherville, MD), polyclonal goat antibody to human α-synuclein (C-20) at a dilution of 1:100 (Santa Cruz Biotech, CA), polyclonal goat antibody to human TNF-α at a dilution of 1:100 (DAKO Japan, Kyoto, Japan), and polyclonal goat antibody to human interleukin (IL)-6 at a dilution of 1:100 (DAKO Japan).

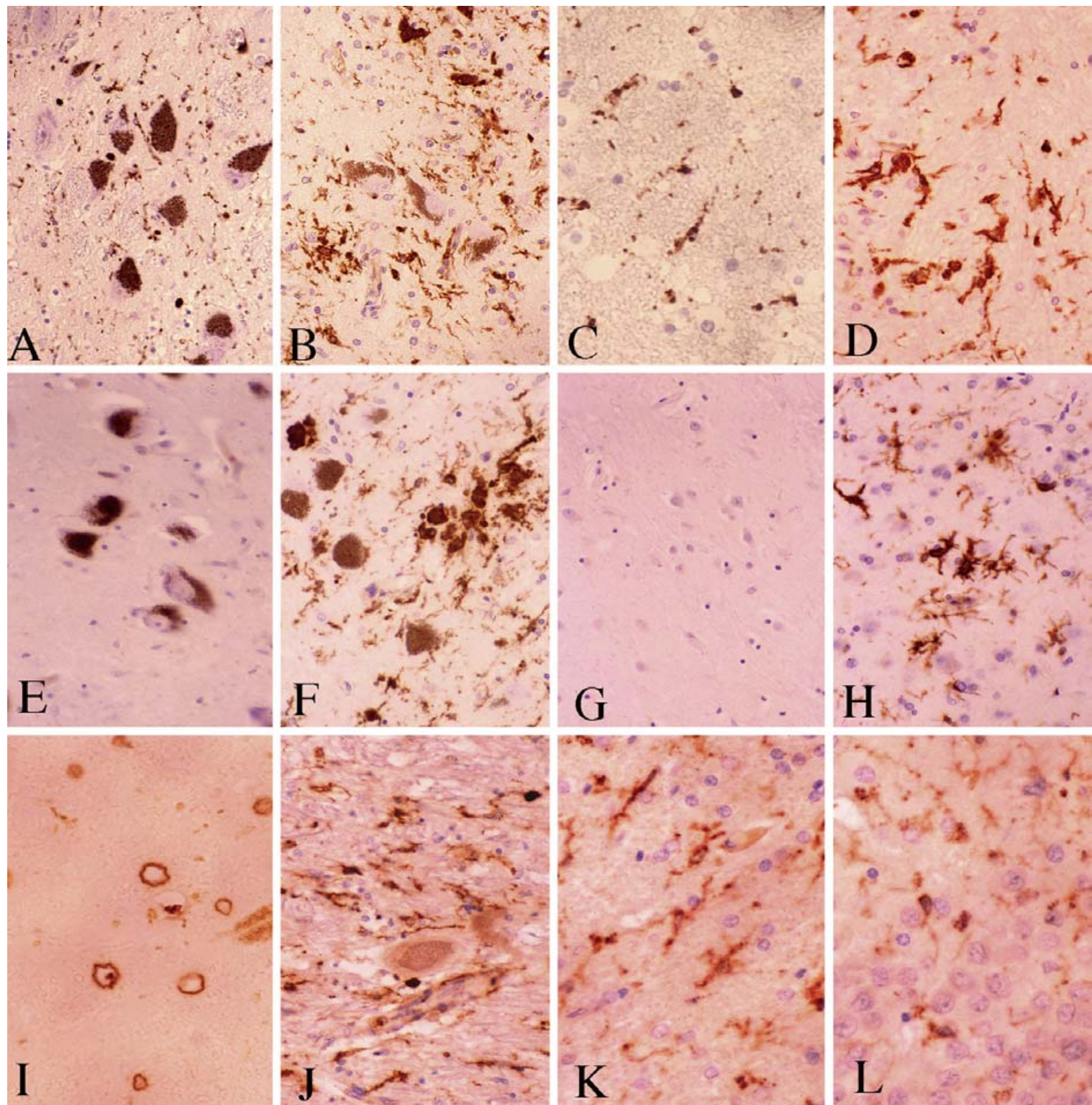

Fig. 1 Distribution of resting and activated microglia in normal and PD brains investigated by immunohistochemistry. In normal brains, Ki-M1p-positive resting microglia are seen in the SN (**A**) and putamen (**C**). In PD brains, increased number of Ki-M1p-positive microglia are seen in the SN (**B**) and putamen (**D**). A few MHC class II (CR3/43)-positive microglia are seen in the SN (**E**) and putamen (**G**) in normal brains. In PD brains, many CR3/43-positive microglia are seen in those same regions (**F**, **H**). In the SN of normal brains, only endothelial cells are positive for ICAM-1 (**I**). Many ICAM-1-positive microglia are seen in the SN (**J**), putamen (**K**) and hippocampus (**L**) in PD brains (*PD* Parkinson's disease, *SN* substantia nigra, *ICAM* intercellular adhesion molecule). **A–I** ×157, **J–L** ×235

Western blot analysis

TRIS-buffered saline insoluble fraction of human peripheral blood mononuclear cells (PBMC) with or without stimulating with lipopolysaccharide (LPS) and brain tissues (putamen) from PD patients were solubilized in 0.5 M TRIS-HCl pH 6.8 containing 10% SDS and electrophoresed on 10% SDS-PAGE gel, and then transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with monoclonal antibody to human HLA-DP, DQ, DR (clone CR3/43) (DAKO), polyclonal goat antibody to human TNF-α (DAKO Japan) and polyclonal goat antibody to human IL-6 (DAKO Japan), then treated with biotinylated second antibodies (DAKO) for 1 h at room temperature, and incubated with avidin-labeled horseradish peroxidase (DAKO) for 1 h at room temperature and then ECL Western blotting detection reagent (Amersham, UK) to visualize protein bands on X-ray films.

Quantification

The degree of neuronal cell loss in the SN was determined by counting the average number of pigmented neurons on one side of the five different midbrain sections of K-B stain, and was graded from PD I–III: PD I >150, PD II 150–75, PD III <75. Average numbers of HLA-DP, DQ, DR (CR3/43)-positive cell counts in the SN, putamen, hippocampus, transentorhinal cortex, cingulated cortex and temporal cortex were calculated as the sum of reactive mi-

croglia in five ×200 fields of the five different sections. Stat View (Abacus, Cary, NC) was used for statistical analysis. Differences were analyzed by the Wilcoxon test. Statistical significance was confirmed using backward elimination at a probability value of 0.05.

Results

We investigated the distribution of resting and activated microglia in normal and PD brains, excluding PD brains with Alzheimer's disease pathology so as to investigate only the pure form of PD pathology (Table 1). In normal brains, many Ki-M1p-positive resting microglia were seen in the SN and putamen (Fig. 1A, C). In PD brains, higher numbers of Ki-M1p-positive ramified microglia were seen in the SN and putamen (Fig. 1B, D). A few CR3/43-positive microglia were seen in the SN and putamen in normal brains (Fig. 1E, G). In PD brains, many CR3/43-positive ramified microglia were seen in those regions (Fig. 1F, H). In normal brains, endothelial cells were positive for ICAM-1 (Fig. 1 I), whereas many ICAM-1-positive microglia were seen in the SN, putamen, and hippocampus in PD brains (Fig. 1 J–L).

Quantitative analysis of the mean number of CR3/43-positive microglia in the SN and putamen of PD and normal control (NC) subjects indicated that patients with PD were shown to have a significantly higher number of CR3/43-positive microglia compared with NC. The cell count of CR3/43-positive microglia in PD increased as the neurodegeneration of pigmented cells in the SN advanced, but there was no statistical difference in CR3/43-positve microglia counts among PDI, PD II and PD III (Fig. 2).

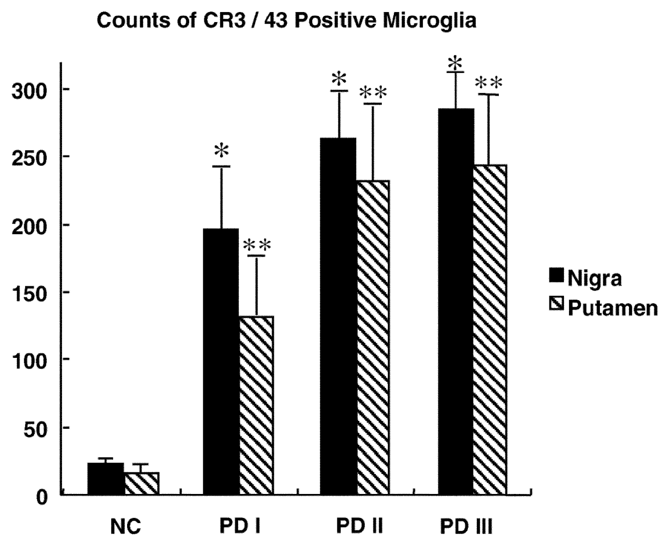

Fig. 2 Bar graphs showing a comparison of the average numbers ± SEM of MHC class II (CR3/43)-positive microglia in the SN (*black bars*) and putamen (*striped bars*) in PD and normal control (*NC*) (number/HPF). Patients with PD have a significantly higher number of CR3/43-positive microglia compared with NC (*P<0.001, **P<0.005). The cell count of CR3/43-positive microglia in PD increases as the neurodegeneration of pigmented cells in the SN progresses, but there is no statistical difference in CR3/43-positve microglia counts among PDI, PD II and PD III (*HPF* high-power field)

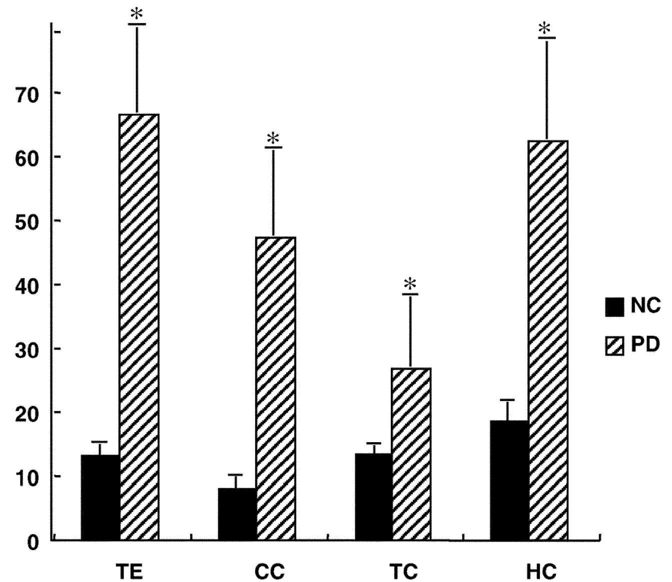

Fig. 3 Bar graphs showing a comparison of the average numbers ± SEM of MHC class II (CR3/43)-positive microglia in the limbic system and neocortex in PD (*striped bars*) and NC (*black bars*) (number/HPF). Patients with PD have a significantly higher number of CR3/43-positive microglia in the hippocampus (*HC*), transentorhinal cortex (*TE*), cingulate cortex (*CC*) and temporal cortex (*TC*) compared with NC (*P<0.01), but there is no statistical difference in CR3/43-positive microglia counts among HC, TE, CC and TC in PD brains

Moreover, quantitative analysis of the mean number of CR3/43-positive microglia in the limbic system and neocortex in PD and NC indicated that the former had a significantly higher number of CR3/43-positive microglia in the hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex, but there was no statistical difference in CR3/43-positve microglia counts among these places (Fig. 3).

Double immunostainings with CR3/43 and TH-16 are shown in Fig. 4A–C. In normal brains, there were many TH-16-positive dopaminergic neurites and a few CR3/43-positive microglia (Fig. 4A). In PD brains, however, many CR3/43-positive microglia were associated with TH-16-positive neurons or neurites (Fig. 4B, C). Double immunostainings with CR3/43 and WH-3 were shown in Fig. 4D–F. In normal brains, there were many WH-3-positive serotonergic neurites and a few CR3/43-positive microglia (Fig. 4D), whereas many CR3/43-positive microglia were associated with WH-3-positive neurites in PD brains (Fig. 4E, F). Double immunostaining with CR3/43 and α-synuclein showed that CR3/43-positive microglia were associated with about 20% of α-synuclein-positive LBs. However, most activated microglia flourished regardless of LBs (Fig. 4G). On the other hand, activated microglia were frequently associated with α-synuclein-positive LNs (Fig. 4H). There was no correlation between the number of LBs and the number of CR3/43-positive microglia in PD brain cortices (data not shown). Double immunostainings

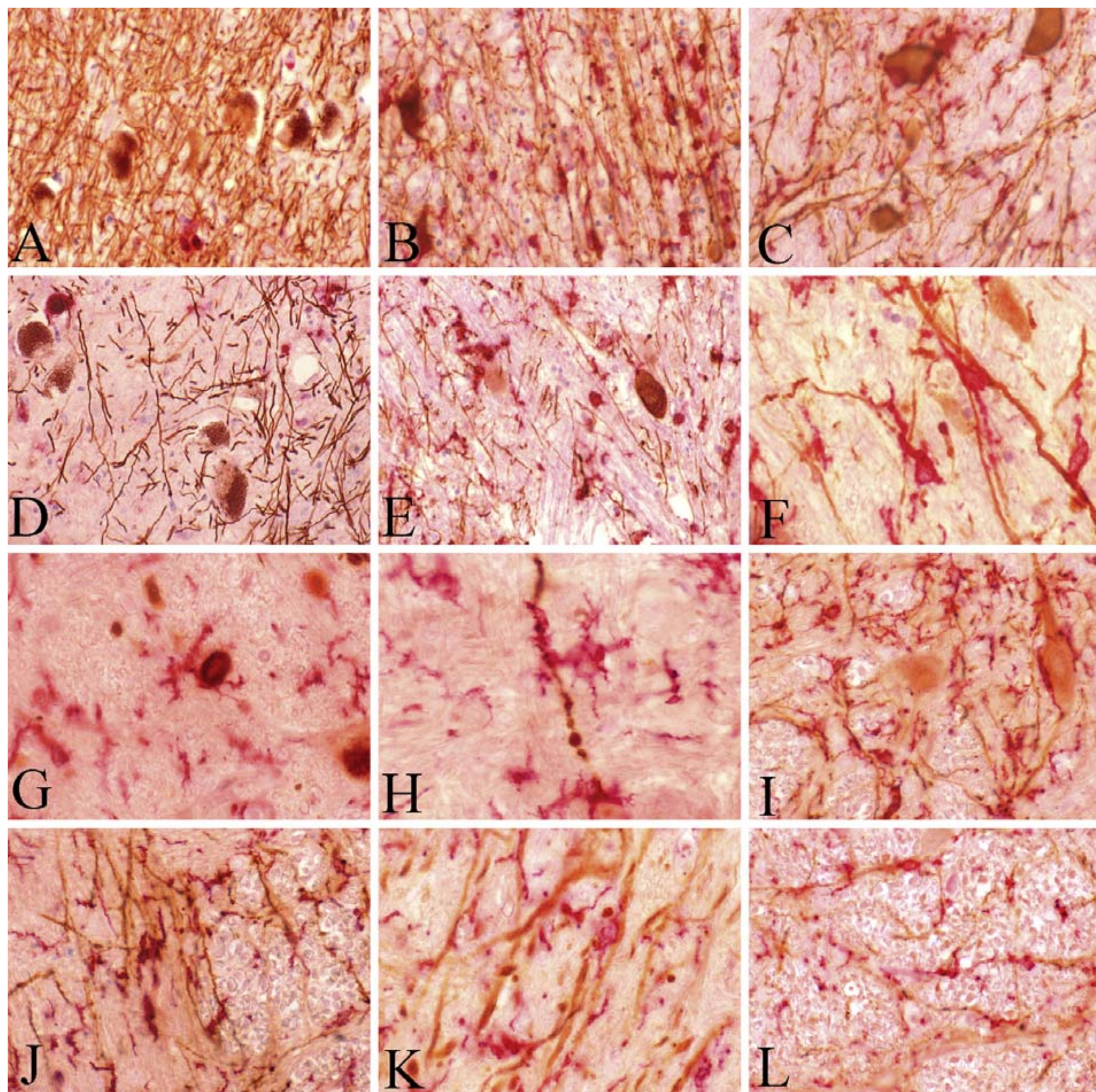

Fig. 4 Double immunostaining with MHC class II (CR3/43) and TH-16 are shown in **A**–**C**. In the SN of normal brains, there are many TH-16-positive dopaminergic neurites (*brown*) and a few CR3/43-positive microglia (*purple*) (**A**). In the SN of PD brains, many CR3/43-positive microglia (*purple*) are associated with TH-16-positive neurons or neurites (*brown*) (**B**, **C**). Double immunostaining with CR3/43 and WH-3 are shown in **D**–**F**. In the SN of normal brains, there are many WH-3-positive serotonergic neurites (*brown*) and a few CR3/43-positive microglia (*purple*) (D). Many CR3/43-positive microglia (*purple*) are associated with WH-3-positive neurites (*brown*) in the SN of PD brains (**E**, **F**). Double immunostaining with CR3/43 and α-synuclein in the cingulated cortex of PD brains shows that CR3/43-positive microglia (purple) are associated with about 20% of α-synuclein-positive LBs (*brown*) (**G**). Activated microglia (*purple*) were frequently associated with α-synuclein-positive LNs (*brown*) (**H**). Double immunostaining with CR3/43 and MAP-2 in the SN (**I**) and in the pons (**J**) shows that many CR3/43-positive microglia (purple) are associated with MAP-2-positive neurites (*brown*) in PD brains. Double immunostaining with CR3/43 and SMI-32 in the cingulate cortex (**K**) and in the pons (**L**) shows that many CR3/43-positive microglia (*purple*) are associated with SMI-32-positive neurites (*brown*) in PD brains. **A**–**E**, **I**, **J**, **L** ×157; **F**–**H**, **K** ×235

with CR3/43 and MAP-2 (Fig. 4I, J) or SMI-32 (Fig. 4K, L) showed many CR3/43-positive microglia were associated with MAP-2- or SMI-32-positive neurites in PD brains.

Double immunofluorostainings with ICAM-1 and LFA-1 showed that almost all activated microglia were positive for both antibodies in the putamen of PD brains (Fig. 5A–C). Double immunofluorostainings with CR3/43 and TNF-α or IL-6 showed that CR3/43-positive microglia were also positive for TNF-α (Fig. 5D–F) or IL-6 (Fig. 5G–I) in the putamen of PD brains.

Western blots of PD patients PBMC and brain tissue homogenates showed CR3/43 protein as 34- and 28-kDa bands, IL-6 protein as a 21-kDa band and TNF-α protein as a 17-kDa band (Fig. 6).

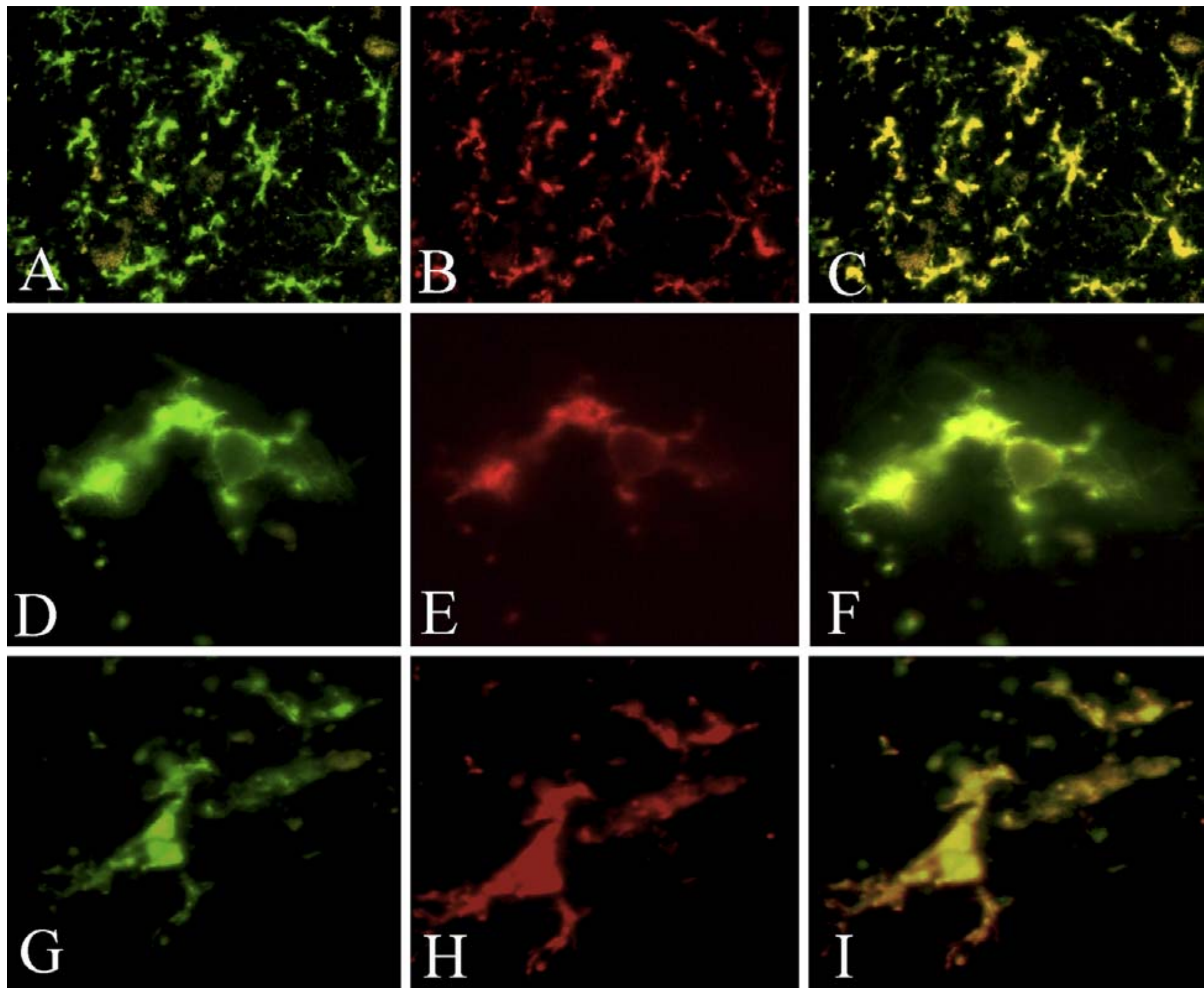

Fig. 5 Double immunofluorostaining with ICAM-1 (*green*) (**A**) and LFA-1 (*red*) (**B**) in the putamen of PD brains shows that almost all activated microglia are positive for both antibodies (**C**). Double immunofluorostaining with TNF-α (*green*) (**D**) and CR3/43 (*red*) (**E**) in the putamen of PD brains shows that CR3/43-positive microglia are also positive for TNF-α (**F**). Double immunofluorostaining with IL-6 (*green*) (**G**) and CR3/43 (*red*) (**H**) in the putamen of PD brains reveals that CR3/43-positive microglia are also positive for IL-6 (**I**). **A–C** ×157; **D–I** ×314

Discussion

Microglia comprise the largest population of phagocytes associated with the CNS. It is now widely accepted that these cells are of mononuclear, phagocyte lineage [19, 20, 28]. Cells of such a lineage enter the developing nervous system during embryogenesis, and are involved in the removal of cells undergoing apoptosis as a normal component of brain development. During brain maturation, the macrophages within the parenchyma adopt a highly differentiated morphology and phenotype [45]. Although microglia do not constitutively express MHC class II antigen in human brains, they are readily up-regulated by aging as well as by many forms of CNS pathology, including neurodegenerative diseases. MHC class II expression is necessary for antigen presentation to $CD4^+$ T cells; recent in vivo studies, however, demonstrated that microglia and perivascular macrophages are unable to initiate a primary immune response in the CNS microenvironment [44]. The factors that regulate MHC class II expression on microglia are poorly understood. Although microglia are the most sensitive cells to interferon-γ-induced up-regulation of MHC class II expression, there is no endogenous expression of interferon-γ within the CNS, and there are few or no T cells recruited, thus making it unlikely that MHC class II expression on microglia is driven by T cell-secreted interferon-γ. One possibility is that this is a consequence of activation via K^+ channels [25]. On the other hand, recent studies suggest that Cl^- channels rather than K^+ channels may be of relevance [7]. IgG deposits in neurons are one of the first changes to be observed in the CNS of mutant SOD1 mice. The source and role of IgG are still unclear, but it is possible that IgG accumulation in neurons is one of the factors triggering the activation of microglia [2].

Adhesion molecules are important for the transendothelial migration of inflammatory cells and their adhesion to damaged neurons after CNS injury [9]. Among adhesion molecules, much interest has been focused on ICAM-1

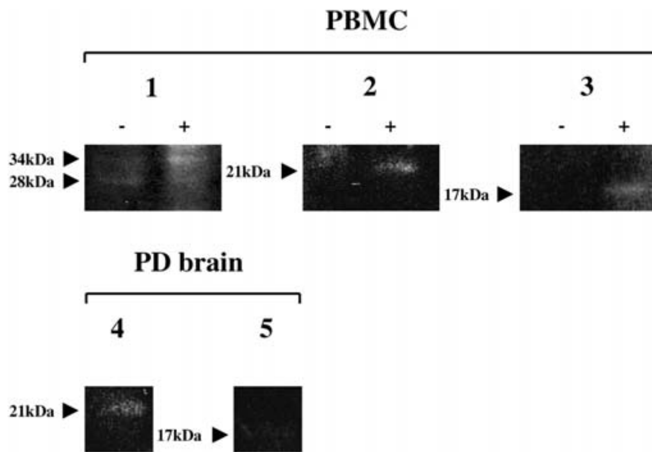

Fig. 6 Western blots was performed for CR3/43 (*1*), IL-6 (*2* and *4*) and TNF-α (*3* and *5*) antibodies with homogenates of PBMC and brain homogenates of PD patients. PBMC(-) indicates no stimulation and PBMC(+) indicates stimulation with LPS. The CR3/43 antibody shows the 34- and 28-kDa bands in PBMC. The IL-6 antibody shows the 21-kDa band and TNF-α antibody shows the 17-kDa band in both samples (*PBMC* peripheral blood mononuclear cells)

(CD54). Activation of ICAM-1 in relationship to neuronal injury has been demonstrated after facial nerve axotomy [55], spinal cord injury [21], Alzheimer's disease [1] and amyotrophic lateral sclerosis [37]. In an experimental model of neurodegeneration, an increase in ICAM-1 expression was one of the first pathological changes to be observed, and strong ICAM-1 immunoreactivity was found on microglia and later on astrocytes [2]. In this study, we showed an increased expression of ICAM-1 on activated microglia; another striking observation was the co-expression of LFA-1 and ICAM-1 on the majority of activated microglia in PD brains. The expression of both receptors and counter-receptors on the same cells suggests that activation events could be bi-directional and triggered via microglia/monocyte contact. These results show that ICAM-1 expression might be important to the adhesive functions of microglia as well as for the recruitment of additional cell types such as monocytes to the site of neuronal injury.

In dementia with LBs (DLB), the presence of cortical LBs may be the most specific pathological marker of disease. Mackenzie [31] demonstrated a positive correlation between the number of MHC class II-positive microglia and LBs in different brain regions, showing that MHC class II-positive microglia frequently extended their processes to degenerated neurons with synuclein-positive LBs. Microglia in contact with neural somata displayed an activated appearance and began proliferating, suggesting that direct contact between damaged neurons and microglia is one of the causes of microglia activation [52]. In fact, microglia in contact with damaged neurons do not appear to elicit neurotoxic effects but may actually enhance the recovery of neurons. However, microglia are sometimes associated with neurons in normal brains while exhibiting a resting appearance with small somata, and survive to become stimulating factors for the proliferation of microglia. This suggests that healthy neurons in the normal brain can not activate microglia, even though they are associated with them. In this study, we showed that MHC class II-positive microglia were diffusely distributed in the nigrostriatal system, limbic system and cerebral cortex in PD brains, but that they were contacted with only about 20% of cortical LBs. Moreover, there was no correlation between the number of LBs and the number of activated microglia. Thus, we speculate that there are other possible candidates for the activation of microglia. α-Synuclein-positive neurites comprise one such candidate, as well as the many activated microglia associated with damaged neurites. In addition, there were many activated microglia in association with TH-12- or WH-3-positive monoaminergic neurites and MAP-2- or SMI-32-positive neurites. Our observation showed that association with damaged or residual neurites is the leading candidate for the activation of microglia.

Many studies indicate the vulnerability of neurons to the potential toxicity of microglia that produce neurotoxic substances including superoxide anion, nitric oxide (NO), glutamate and pro-inflammatory cytokines [10, 11, 19, 26, 27, 30, 38]. The presence of microglia expressing TNF-α, IL-1β and other cytokines in the SN of PD brains has been reported previously [19] and Nagatsu et al. [43] have reported the presence of an increased concentration of TNF-α, IL-1β and IL-6 in the striatum of PD by ELISA. In this study, we showed by immunohistochemistry that activated microglia in the putamen expressed TNF-α and IL-6. Although these data may suggest an involvement of the pro-inflammatory cytokines secreted by microglia in the degeneration of dopaminergic neurons in PD, it is also recognized that TNF-α and IL-6 have neurotrophic mechanisms [3, 4, 12, 50]. To date, glial cells are acknowledged to possess neurotrophic properties that are essential for the survival of dopaminergic neurons [42]. Among them, glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), which can be released by activated microglia, seem to be the most potent factors in supporting SN dopaminergic neurons [4]. Although reactive microglia in the vicinity of neuronal injury are generally considered to be involved in the removal of debris from degenerating neurons [41, 49], the exact contribution of microglial activation to synaptic recovery and remodeling is not yet clear. It has been hypothesized that activated microglia exacerbate neuronal injury through the synthesis and secretion of agents that potentiate synaptic overactivity and aggravate the primary insult [15, 23, 36]. Another intriguing possibility is that microglial activation is a brain-protective mechanism designed to limit neurodegeneration and improve synaptic recovery following damage. In support of this, data from recent studies have indicated that microglial activation can attenuate excitotoxic or ischemic injury in rodents [5], prevent apoptosis in vitro [53], and increase neurite outgrowth and functional recovery following injury [29, 46, 47]. Thus, it can be hypothesized that microglia are recruited to areas of neuronal injury to phagocyte degenerating neurons, where they release trophic factors to support the remaining healthy neurons, thus facilitating synaptic regrowth following injury.

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