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Long-lasting perivascular accumulation of major histocompatibility complex class II-positive lipophages in the spinal cord of stroke patients: possible relevance for the immune privilege of the brain

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Abstract Six cases of middle cerebral artery occlusion are presented in which the cellular changes accompanying descending degeneration of the lateral corticospinal tract were studied at different time points (5 days–10 years) following the insult. Microglia and perivascular cells were found to ingest large amounts of myelin degradation products, while expressing high levels of major histocompatibility complex (MHC) class II molecules. Activation of perivascular macrophages, as indicated by increased class II expression, lasted for many years and appeared to follow down-regulation of both phagocytic activity and class II expression on parenchymal microglia. TUNEL labeling was absent from both microglia and perivascular cells at all time points investigated. Indirect evidence is presented that microglia may transfer myelin degradation products to the perivascular space. Perivascular cells which express MHC class II molecules constitutively do not appear to leave the perivascular compartment in large numbers and could release myelin degradation products into the cerebrospinal fluid. The possible immunological consequences of these findings are discussed with respect to their possible relevance for antigen presentation and autoimmune central nervous system disease.

Key words Apoptosis · Demyelination · Microglia · Perivascular cells · Wallerian degeneration

We would like to dedicate this paper to Prof. Georg W. Kreutzberg on the occasion of his 65th birthday.

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Introduction

The immunological changes associated with Wallerian degeneration in the spinal cord are poorly understood. In Wallerian degeneration, breakdown of the distal axon is followed by degradation and removal of myelin with large amounts of lipid and associated proteins being cleared from the central nervous system (CNS). Both glial cells and macrophages have been implicated in this process [2, 6, 7, 23]. Specifically, it has been suggested that there is a redistribution of lipid droplets between glial cells and macrophages [6]. The macrophage population involved in this process is most likely heterogeneous with the majority of cells derived from intrinsic microglia [8, 31]. In a series of studies on the rhizotomized cat spinal cord in which the Marchi method was used, Franson and Ronnevi [7] have shown that microglia incorporate collapsed myelin which is transformed into myelin bodies and subsequently into lipid droplets. They demonstrated that months following the insult large numbers of macrophages may still be found in the vicinity of blood vessels possibly draining the tissue area where myelin degradation is taking place [6, 8]. These findings fit well with the view of Penfield [28], who suggested that following phagocytosis of cellular debris, microglia cells migrate to the vessels, deliver their cytoplasmic contents through the vessel walls or into the perivascular space and return to a renewed phagocytic activity. Perivascular macrophages, which are distinct from microglia including juxtavascular cells [10, 22], have been shown to be involved in the removal of broken down myelin [6, 8]. These macrophages of the blood-brain interface are located exterior to the basal lamina of the blood vessel proper and outside of the glial lamina limi-

tans, i.e., in the perivascular space. The cells were termed 'perivascular cells' [21] and have recently found much attention as they are most likely involved in antigen presentation at the blood-brain interface [12, 16]. However, their role in the immunological changes associated with Wallerian degeneration in the spinal cord is poorly understood. We have studied the time course of expression of immunomolecules known to be associated with activated microglia/brain macrophages and perivascular cells in the human spinal cord undergoing pyramidal tract degeneration. Time points studied ranged from 5 days to 10 years following middle cerebral artery occlusion. A dramatic and long-lasting increase was observed in the number of major histocompatibility complex (MHC) class II-positive perivascular cells containing large amounts of myelin degradation products, raising the important question of their immunological relevance during Wallerian degeneration.

Materials and methods

Subjects

Six cases with a history of middle cerebral artery infarction (5 days, 6 weeks, 16 weeks, 3 years, 4 years and 10 years following middle cerebral artery occlusion) were obtained at the Institute of Neuropathology of the University of Munich. Detailed summaries of the case reports are given in Table 1.

Neuropathological examination

Brain and spinal cord tissue was fixed in 3.7% formaldehyde solution for at least 1 week at room temperature. Microscopical examination was carried out using 3-μm-thick paraffin sections taken from the relevant cortical areas, brain stem and spinal cord. Hematoxylin and eosin (H&E) and cresyl violet-Luxol fast blue were used as routine stainings. In addition, frozen sections of spinal cord (cases 1–5) and medulla oblongata (case 6) were stained with Sudan III.

For immunocytochemistry, paraffin sections were obtained from cervical, thoracic and lumbar spinal cord (cases 1–5) as well as medulla oblongata (case 6). Endogenous peroxidase activity was quenched in 7.5% H_2O_2 for 5 min. Following incubation in 0.01 M phosphate-buffered saline (PBS) for 15 min, sections were incubated in 1% rabbit normal serum for 30 min. Incubation with the following monoclonal antibodies was carried out at 4° C overnight (dilution 1:100 in PBS): CR3/43 (anti-MHC class II; Dako, M 775) [14], Ki-M1P (Seikagaku, no. 93401) [27], LC (Dako, M 701), UCHL1 (Dako, M 742), and L26 (Dako, M 0755).

Antibody binding was detected using biotinylated anti-mouse rabbit immunoglobulin (Dako, E 354, 1:400) and peroxidase-conjugated streptavidin (Dako, P 397; 1:1000). Visualization was carried out with 1 mg/ml 3,3'diaminobenzidine (Dako, S 3000) and 0.05% H_2O_2 in 0.05 M TRIS-HCl, pH 7.6, containing 0.15 M NaCl.

Table 1 Case reports (*a.m.* ante mortem)

	Age (years)	Sex	Event of stroke a.m.	Clinical findings	Main autopsy findings	Cause of death
Case 1	62	f	5 days	Cardiac insufficiency, bypass surgery 5 days a.m., intraoperative left middle cerebral artery occlusion, minimal therapy	Severe general arteriosclerosis, acute myocardial infraction, biventricular myocardial hypertrophy; thrombosis of right femoral and right jugular vein, paracentral pulmonary embolism, pulmonary edema	Cardiac failure caused by pulmonary embolism and myocardial intrafraction
Case 2	44	m	6 weeks	Acute left hemiplegia, right-sided leg-pronounced hemiparesis; cranial CT scanning: infarction of right middle cerebral artery and right basal ganglia hemorrhage; pneumothorax, staphylococcal sepsis	Recurrent paracentral and acute centralpulmonary embolism, cor pulmonale, thrombosis of right and left femoral veins	Recurrent paracentral and acute central pulmonary embolism
Case 3	85	f	16 weeks	Right femoral neck fracture 5 months a.m.; myocaridal infarction and left-sided cerebrovascular insult 4 months a.m.; total hip replacement, postoperative progressive cardiocirculatory failure	Severe general arteriosclerosis, arteriosclerotic heart disease; severe osteoporosis; pulmonary emphysema	Cardiac failure
Case 4	86	m	3 years	For 3 years left-sided hemiparesis and sensory disturbance after stroke, 1 day a.m. embolectomy in left superficial femoral artery, postoperative bradycardia and asytolia	Severe general arteriosclerosis, arteriosclerotic heart disease, old myocardial infarction; acute purulente bronchopneumonia; complete micronodular liver cirrhosis	Cardiocirculatory failure with acute bronchopneumonia
Case 5	66	m	4 years	Myocardial infarction 14 years a.m. right-sided stroke and left-sided hemiparesis 4 years a.m.; stenosing gastric cancer, cachexia; acute myocaridal infarction during gastrectomy	Severe general arteriosclerosis, arteriosclerotic heart disease; gastric adenocarcinoma; pancreatic fibrosis	Cardiocirculatory failure
Case 6	70	m	10 years	Apoplexia followed by right-sided hemiplegia and aphasia 10 years a.m.; myocardial infarction, cardiac pulmonary oedema 18 days a.m.; sepsis	Severe general arteriosclerosis, myocardial infarction; acute bronchopneumonia; cholangitis	Cardiocirculatory failure, sepsis

Table 2 Summary of neuropathological findings

	Event of stroke a.m.	Age (years)	Brain weight (g)	Large arteries of the base of the brain	Coronal sections	Midbrain, brain stem and spinal cord
Case 1	5 days	62	1380	Slight arteriosclerotic changes, fresh blood clot in the left middle cerebral artery with luminal occlusion	Anemic necrosis stage I affecting the complete area of the left middle cerebral artery, midlineshift from left to right side, narrowing of the left lateral ventricle	Tentorial herniation with multiple midbrain hemorrhages and hemorrhages in the tegmentum pontis; spinal cord without visible pathological changes
Case 2	6 weeks	44	1580	No pathological findings	Anaemic necrosis stage I-II affecting the area of the right middle cerebral artery, blood clot involving parts of the right basal ganglia	Signs of raised intracranial pressure; beginning axonal degeneration and demyelination in the right pyramidal tract
Case 3	16 weeks	85	1270	Severe arteriosclerosis	Necrosis stage II-III (4 mm) in the left capsula interna	Beginning axonal degeneration and myelin sheath damage in the left pyramidal tract; small necrosis stage II affecting the left nucleus proprius V, nucleus ambiguus and nucleus funiculi lateralis
Case 4	3 years	86	1200	Severe arteriosclerosis	Cystic necrosis stage III (4 cm × 4 cm) involving parts of the right capsula interna and parts of the putamen; small necrosis stage II-III in the right occipital lobe	Marked axonal degeneration and demyelination in the right pyramidal tract
Case 5	4 years	66	1400	Vertebral and basilar arteries with several ateriosclerosis, moderate arteriosclerosis of the internal carotid and middle cerebral arteries	Cystic necrosis stage III with complete destruction of the right capsula interna, putamen and globus pallidus	Severe axonal degeneration and myelin loss in the right pyramidal tract; cerebellomedullary herniation
Case 6	10 years	70	1050	Moderate general arteriosclerosis, severe arteriosclerosis of the left internal carotid artery	Cystic necrosis stage III with complete destruction of the left caudate, putamen, and capsula interna, parts of the pallidum and lateral parts of the thalamus	Nearly complete axonal degeneration and myelin loss in the left pyramidal tract

Quantitative image analysis

Quantitative evaluation of demyelination, MHC class II-positive cells and lipophages was performed using the image analysis software package Optimas 5.1 (Optimas Corporation, Seattle, Wash.). The right and left lateral corticospinal tracts were identified based on their anatomic location. For all stained sections to be analyzed, thresholds for the detection of specifically labeled cells were set individually using visual control. Perivascular and non-perivascular areas were selected and the respective tissue areas (0.062 mm^2; × 40 objective) determined based on the average value of three independent measurements. The resulting percent areas were calculated and compared between the normal and the affected side. Independent measurements were performed for perivascular and non-perivascular class II-immunoreactive cellular profiles as well as Sudan III-stained and Luxol fast blue-positive cells. Areas of profiles were defined with minimum boundaries of 10 pixels (cresyl violet-Luxol fast blue) and 20 pixels (CR3/43, Sudan III).

Electron microscopy

Formalin-fixed tissue blocks taken from the spinal cord of case 5 (Table 1) were post-fixed for 3 weeks in a 3.7% formaldehyde/0.75% glutaraldehyde-buffered (0.1 M, pH 7.4) fixation solution. Vibratome sections (Oxford vibratome, Technical Products International, St. Louis, Mo.) were made and post-fixed for 24 h. After several washes in cacodylate buffer (0.1 M, pH 7.4), small (1 mm^3) tissue blocks were cut and fixed in 1% Dalton's osmium overnight. Subsequently, the blocks were processed for Araldite embedding. Thin sections were contrasted using uranyl acetate and lead citrate.

Terminal transferase-mediated nick end-labeling (TUNEL)

Nick end-labeling of double-stranded DNA breaks was performed as described [20]. In brief, following proteinase K treatment, dewaxed and rehydrated tissue sections were incubated at 37° C for 1 h in 1.6 μM biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) and 0.08 U/μl terminal transferase (Boehringer Mannheim) in TdT buffer (3.0 mM TRIS, pH 7.2, 14 mM sodium cacodylate, 0.1 mM $CoCl_2$). TUNEL labeling was visualized using peroxidase-conjugated streptavidin and 3,3′-diaminobenzidine. Nuclei were counterstained with Nuclear Fast Red. Sections of tonsil and small intestine were used as positive controls in each series of labelings.

Results

Following occlusion of the middle cerebral artery, the pathological changes in the corresponding pyramidal tract ranged from visually nearly unaffected (5 days) to com-

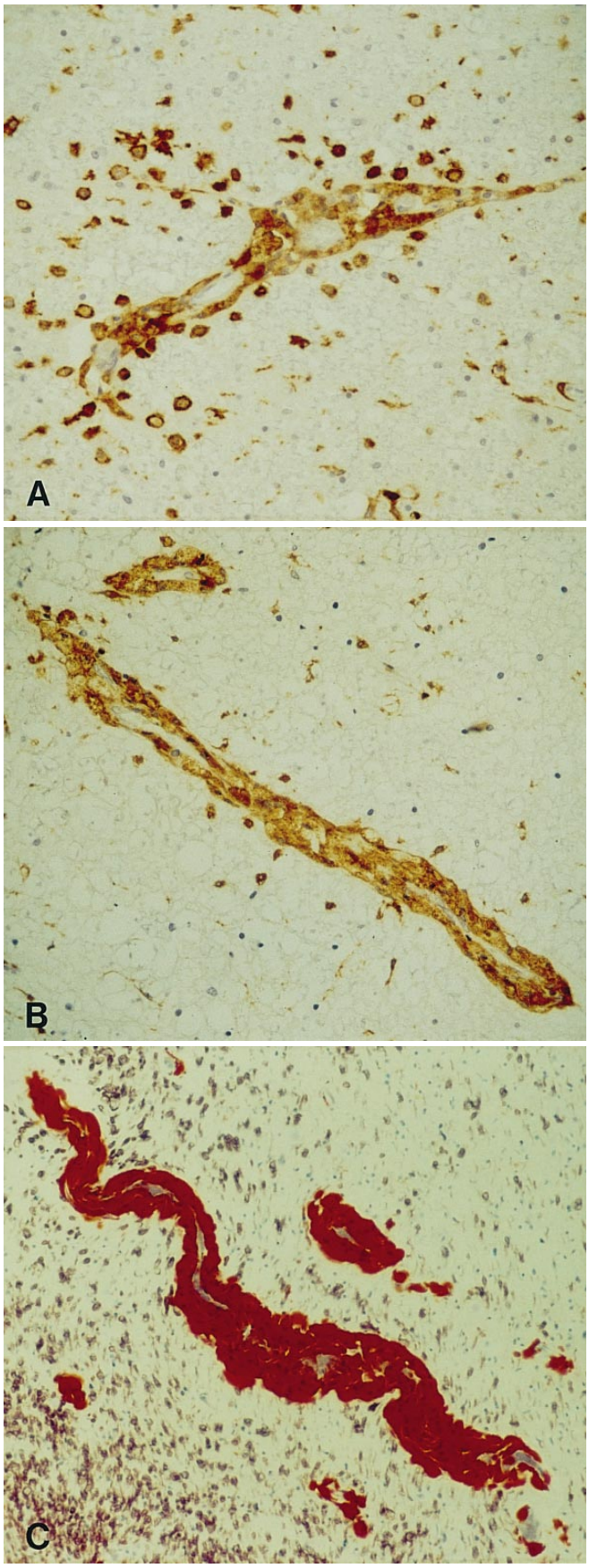

Fig. 1 **A**, **B** MHC class II immunoreactivity in the lateral corticospinal tract following infarction of the middle cerebral artery is prominent on perivascular macrophages and also present on some parenchymal cells (**A** case 4, **B** case 5). **C** Perivascular Sudan III staining of a blood vessel from the case shown in **B** (*MHC* major histocompatibility complex). **A** × 130; **B**, **C** × 70

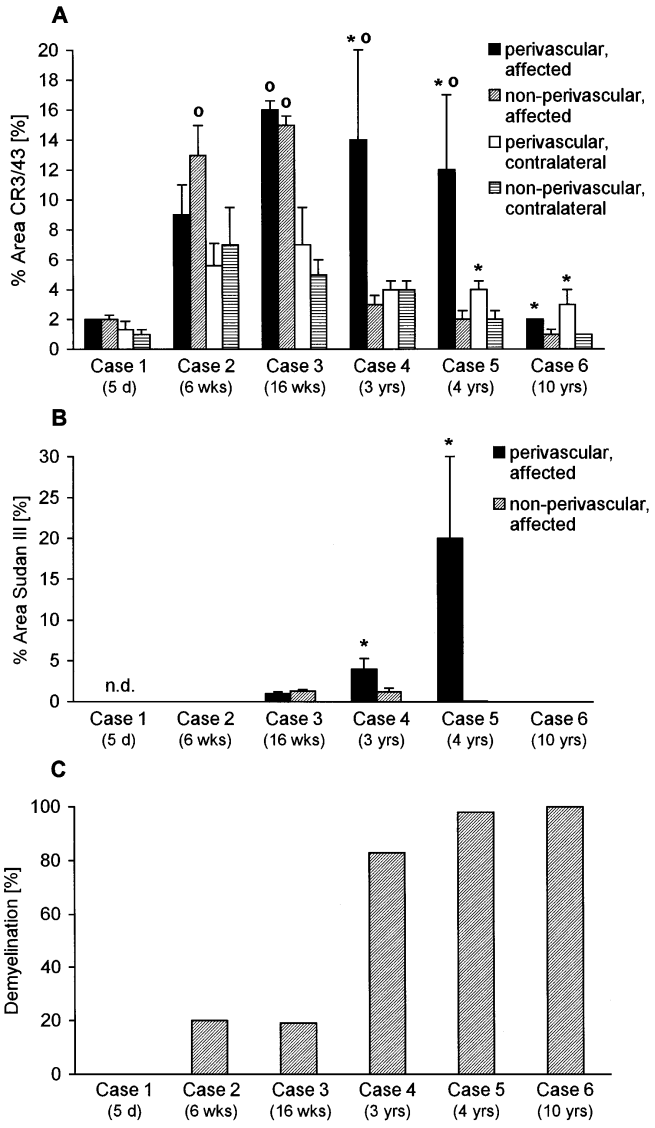

Fig. 2 Quantification of MHC class II- (**A**) and Sudan III- (**B**) positive profiles, and of demyelinated tissue areas (**C**) in the lateral corticospinal tract between 5 days and 10 years following occlusion of the middle cerebral artery. Percent areas of stained profiles were determined in three representative visual fields (0.062 mm^2) on the affected and unaffected side, respectively. Values are given as mean ± SD. *Asterisks* Significantly different from non-perivascular labeling, $P < 0.05$; ○ significantly different from contralateral side, $P < 0.05$ (Mann and Whitney U-test). *Numbers in parentheses* indicate the survival time after stroke involving the middle cerebral artery (*n.d.* not determined)

plete axonal degeneration and loss of myelin (after 10 years; Table 2). For quantitative evaluation of the different staining results, morphometric analysis was performed on the lateral corticospinal tract. While the percent area of

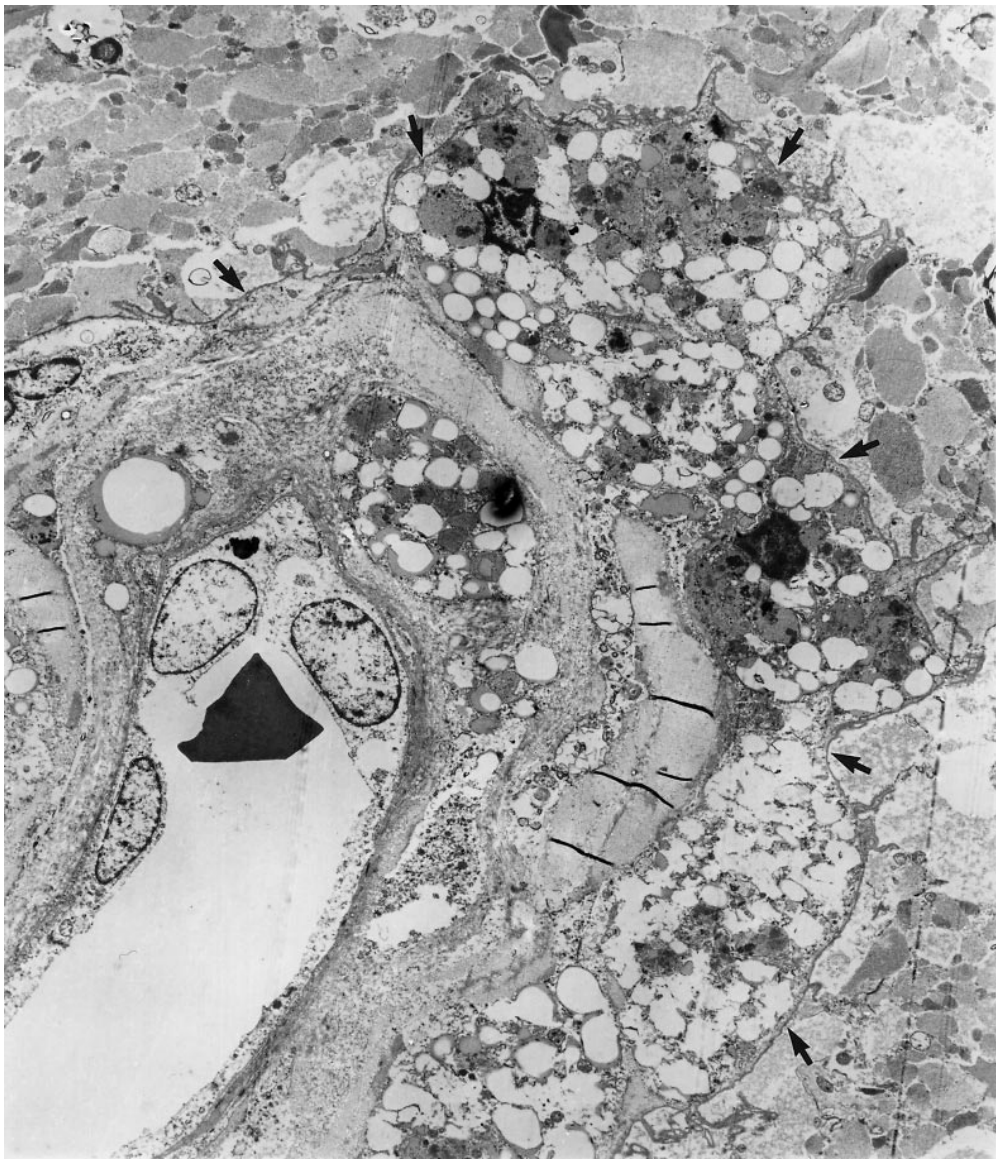

Fig. 3 Electron micrograph showing perivascular macrophages filled with lipid vacuoles (case 5, Tables 1, 2). The parenchymal basement membrane is marked by *arrows*. × 3600

demyelinated spinal fiber tracts did not exceed 20% up to 4 months after the insult (cases 1–3), demyelination exceeded 80% at 3 years (case 4) and approached 100% after 4 years (cases 5 and 6). Intense perivascular clustering of CR3/43-positive cells was observed in demyelinated tissue areas in cases 4 and 5 (Figs. 1, 2). This finding was statistically significant compared to non-perivascular and contralateral perivascular tissue areas ($P < 0.05$, Mann and Whitney U-test) (Fig. 2). As shown by electron microscopy, clustered cells met the morphological criteria of perivascular cells (Fig. 3) [10, 12]. The cells contained large numbers of lipid droplets, suggesting extended uptake and accumulation of myelin breakdown products in these cells over many years (Figs. 1, 2). A few macrophages with lipid droplets were also found in the adjacent meninges. At earlier stages of corticospinal tract degeneration, strong CR3/43 immunoreactivity of both brain macrophages and ramified microglia was also observed in parenchymal, i.e., nonperivascular tissue areas. The highest levels of perivascular and parenchymal class II expression was seen at 16 weeks after infarction (case 3, Fig. 2), but even 10 years after the ischemic injury, spinal cord labeling for MHC class II antigen had not returned to normal levels (case 6, Fig. 2). At the same time, up-regulation of perivascular class II expression was still detectable in the affected as well as in the unaffected lateral corticospinal tract. In cases 2–5, an increase in microglial activation was also seen on the contralateral side and in morphologically unaffected regions of the spinal cord. However, this labeling was much weaker than in the degenerating lateral pyramidal tract. Labeling of macrophages and microglia with Ki-M1P resulted in a slightly different overall labeling pattern since this antibody stains intracellular, lysosome-like structures (data not shown). In all cases only very few cells could be labeled for the LC, UCHL1, and L26 epitopes. Thus, there was no significant infiltration of lymphocytes. Apart from very few scattered cells, no TUNEL-positive structures were observed.

Discussion

Our study demonstrates that there is a strong but slow and transient increase in the number of perivascular macrophages in tissue areas of the spinal cord affected by Wallerian degeneration of the lateral corticospinal tract following infarction of the middle cerebral artery. The cells contain large amounts of myelin degradation products and simultaneously express high levels of MHC class II antigen. We interpret the observed perivascular clustering of lipid-laden, MHC class II-positive cells as an indication for transport of myelin degradation products from the CNS parenchyma towards blood vessels in the degenerated tissue (Figs. 1, 2) [7, 8]. This view is in line with the observation that the staining for lipids became stronger in perivascular macrophages with increasing duration of the degenerative process and that it was most pronounced when the largest number of MHC class II-positive macrophages had accumulated in the perivascular space (Fig. 3). The perivascular macrophages described in this study most likely derive from a population of resident cells of the perivascular space termed perivascular cells [10, 12, 13, 18]. Perivascular cells are physiologically renewed from the bone marrow in the adult [16]. Although there is little information on their life cycle, perivascular cells have been suggested to develop into microglia under certain conditions [37]. However, turnover of perivascular CNS macrophages is slow and trafficking of their precursors through the blood-brain interface seems limited [34]. Furthermore, it remains unknown whether perivascular cells actually reenter the vasculature upon completion of their tasks which are generally considered phagocytosis and clearance of cellular debris from the CNS [21, 25].

There are different possibilities to explain the increased number of perivascular lipophages around blood vessels during late stages of Wallerian degeneration. It is conceivable that ‘resident’ perivascular cells can undergo mitosis before or after incorporating myelin degradation products. However, experimental studies on the facial nucleus of adult rodents suggest that these cells are largely post-mitotic even in their activated state [11]. Alternatively, the cells may be principally able to enter the circulation or migrate to the meninges, but lose this capacity after the incorporation of myelin degradation products. The latter possibility is attractive from a teleological point of view since perivascular ‘capture’ of CNS autoantigen during the acute phase of demyelination could represent a protective mechanism against autoimmunization. A third explanation would be that parenchymal microglia take temporary residence in the perivascular space after changing their phenotype into that of perivascular cells. However, there is no evidence to support this hypothesis. Answers to these questions are potentially of great immunological relevance as cells of the CNS perivascular space can gain direct access to the peripheral immune system [26]. Ten years after the insult, the number of lipid-containing cells and the perivascular expression of MHC class II molecules had reached almost normal levels and most myelin debris which is transformed into lipid droplets [7] had been cleared from the parenchyma. The number of microglia was also comparable to control levels. Based on our negative results using the TUNEL technique, we do not believe that apoptosis contributed significantly to the observed decrease in the number of lipid-laden perivascular cells. Similarly, Jones et al. [17] could show that apoptosis does not represent a major mechanism in the population control of microglial cells in vivo. In view of these findings, and since microglial cells are functionally deficient with respect to myelin removal [30], we believe that at least some of the perivascular lipophages could have directly entered the bloodstream or left the CNS via the meninges.

Phagocytosis is one of the strongest stimuli for microglial cells to express MHC class II molecules which are of crucial importance for antigen-dependent T cell responses [3, 4]. It has been previously demonstrated that sites of direct and indirect brain injury acquire a predisposition for the development of autoimmune inflammatory lesions [19, 24]. Even in the absence of breakdown of the blood-brain barrier [15, 29], autoimmune encephalomyelitic lesions can be targeted to CNS tissue undergoing Wallerian degeneration [19]. However, Wallerian degeneration is not normally followed by autoimmune attack. Thus, the CNS must be somehow protected against unwanted presentation of myelin-associated antigens to T lymphocytes [36]. Recent studies have shown that in addition to MHC class II molecules, co-stimulatory factors such as B7 are required to confer complete antigen-presenting capacity to MHC class II expressing cells, which are then able to stimulate a proliferative T cell response [5, 35]. Since the expression of co-stimulatory molecules has been described in multiple sclerosis lesions but not in neurodegenerative conditions [1, 5], we speculate that co-stimulatory factors may be absent from lipophages during Wallerian degeneration and that antigen presentation, if it occurs, may lead to T cell anergy rather than proliferation [9, 32]. Clearly, secondary rather than primary T cell responses should be considered in this context [33]. In the light of this hypothesis, the very slow breakdown of CNS myelin and the associated expression of class II molecules may confer a biological advantage and help to maintain the immune privilege of the CNS. Similarly, the well-established low threshold of microglia and perivascular cells for the up-regulation of MHC class II molecules could serve a protective function.

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