

## MHC class II positive microglia and lymphocytic infiltration are present in the substantia nigra and striatum in mouse model of Parkinson's disease

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**Abstract.** We have studied MHC class II antigen expression and lymphocytic infiltration during dopaminergic neurone degeneration produced by intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Microglial activation was observed in the striatum and in the substantia nigra (SN) in this model. We noticed a marked increase of MHC class II antigen expression on microglia and T-cell recruitment in these regions after MPTP treatment. B-lymphocytes were not observed. T-cell infiltration predominantly consisted of CD8+ cells at every time point but CD4+ cells were present too. More than a half of the observed lymphocytes showed strong staining of CD44 antigen. Our findings suggest a possible immune system involvement in the pathological process following MPTP intoxication.

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## INTRODUCTION

Degeneration, as any other direct or indirect injury in the central nervous system, leads to the activation of microglia and astrocytes. Glial activation includes an increase in the number of glial cells, cytokine secretion, phagocytosis, and increase in the content of some intracellular proteins (Perry and Gordon 1988, 1991). Activated glia have been shown to express MHC class II antigens (Vass and Lassmann 1990). In autoimmune diseases of the CNS, such as multiple sclerosis and its animal model, experimental allergic encephalomyelitis (EAE), it has been suggested that microglia may contribute to the inflammation process as antigen presenting cells (Hayes et al. 1987, Butter et al. 1991). In Alzheimers and Parkinsons diseases increased number of microglia were observed expressing MHC class II antigens and complement receptors, and a small number of T-lymphocytes around impaired neurones (McGeer et al. 1988, 1993). In addition, MHC class I antigens were upregulated in the striatum in the brains of parkinsonic patients (Mogi et al. 1995). However, the purpose of immune reactivity in neurodegeneration is not clear. In order to examine possible immune system involvement in the toxic degeneration of neurones we have examined the expression of MHC class II molecules and the phenotype of lymphocytes in the substantia nigra and striatum at the mouse model of Parkinsons disease (PD) produced by intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Heikkilä et al. 1992).

## METHODS

We used the PD mouse model as described elsewhere (Ricaurte et al. 1984).

C57Bl/6 mice, weighing 30-35 g, 8-12 months old, received four intraperitoneal injections of MPTP-HCl (Sigma) dissolved in 0.9% NaCl (10 mg/kg), at one hour intervals, for a total dose of 40 mg/kg. Control mice were injected with 0.9% NaCl.

The animals, 5 for each time point, were sacrificed on days 1, 2, 3, 7, 14 and 21 after MPTP intoxication. Animals were deeply anaesthetized with chloral hydrate (400 mg/kg i.p.) and transcardially perfused with heparinized 0.9% NaCl solution followed by 2% paraformaldehyde-lysine-periodate fixative (PLP) (McLean and Nakane 1974). The brains were removed, post-fixed in PLP for 4-6 hours, than immersed in 20% sucrose solution overnight at 4°C and rapidly frozen. For MHC stain-

ing, mice were perfused only with heparinized 0.9% NaCl, brains removed and immediately frozen (free-frozen sections). Horizontal sections, 20 µm thick, were cut on a cryostat through striatum and SN, picked up on gelatinised slides, and dried before immunohistochemical staining.

Every tenth section was stained with hematoxyline-eosine. Intervening sections were saved for staining with antibodies for: (1) the receptor for C3bi complement factor (rat anti-CR3, 1:5000, Serotec) which recognizes microglia and macrophages; (2) CD3 (rat anti-CD3, 1:500, Serotec) a marker for T lymphocytes; (3) CD4, CD8 (rat anti-CD4, 1:100, rat anti-CD8, 1:100, Serotec) to differentiate T-cell phenotype; (4) CD40 (rat anti-CD40, 1:100, Serotec) which recognizes B lymphocytes and macrophages; (5) CD44 (rat anti-CD44, 1:100, Serotec); (6) MHC class II (rat anti-Ia, 1:500, Boehringer-Mannheim) which recognizes MHC class II antigens of our mice strain.

Frozen sections were dried at room temperature and rinsed in phosphate buffered saline (PBS) containing 0.1% Triton X 100. Free-frozen sections after drying were post-fixed in 4% paraformaldehyde for 5 min, 50% acetone/100% acetone/50% acetone - 2 min each and rinsed in PBS.

The binding of primary antibody was revealed by an avidin-biotin-peroxidase method with reagents supplied by Vector Laboratories (USA) and stained in diaminobenzidine (DAB). The sections were dehydrated, cleared in xylene and mounted.

Double staining was performed to show that MHC class II antigens were in fact on microglia (CR3 positive cells). After staining microglia with anti-CR3 antibody (with DAB as indicator) slices were incubated in 3% H<sub>2</sub>O<sub>2</sub> solution to block peroxidase activity of the first staining. Next, they were incubated in the second primary antibody (anti-Ia antibody) and followed the same steps with avidin-biotin-peroxidase reagents and benzidine dihydrochloride (BDHC) as the second indicator.

The intensity of CR3 and MHC class II staining was estimated. The number of CD3, CD4, CD8 [CD3(+), CD4(+), CD8(+)] lymphocytes in the SN were evaluated as follows. Five animals for each time group were taken and three different levels of the SN per animal were chosen: ventral tegmental area level, and 100 µm above and below. Sections were coded for counting. Cells were counted using drawing tubes at six fields of vision per section, summed for each animal and cell density per 1 mm<sup>2</sup> was calculated.

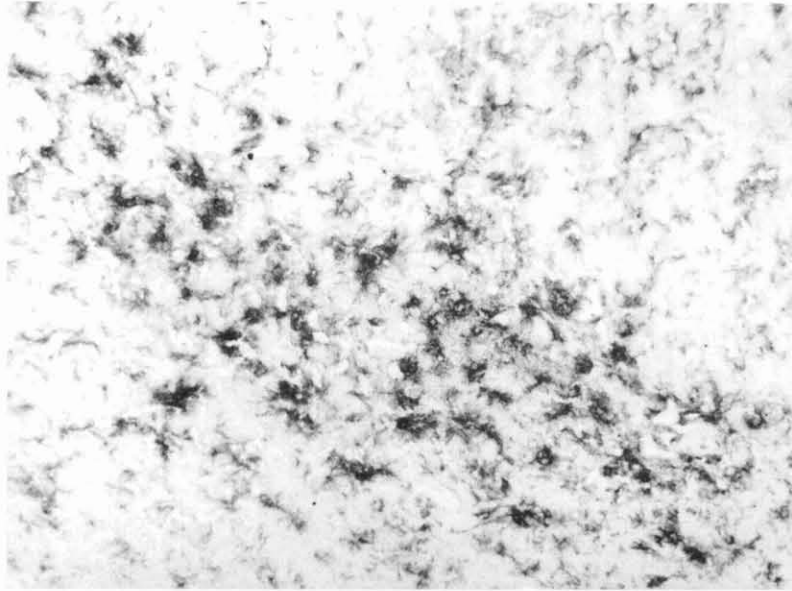


Fig. 1. Activated microglia in the pars compacta of the substantia nigra stained with anti-CR3 antibody - MPTP treated mouse 2 days after intoxication (magnification x 200).

## RESULTS

### CR3 staining

In brain tissue from control mice, the expression of CR3 was easily detectable throughout the brain on the resting microglia. From the first day following MPTP administration, we observed an increase in the staining intensity in the whole striatum, and from the second day

in the SN. The morphology of glial cells changed: the cell bodies became larger, processes were thicker, shorter, less ramified and the labelling with anti-CR3 antibody was stronger. The maximum of this reaction was seen in the striatum and SN from 1-3 days after MPTP treatment (Fig. 1). On the 7th day there were no more activated microglia in the striatum. In the SN on the 7th day, the reaction diminished but was still observable until the 14th day after MPTP administration. On the

TABLE I

CR3 and MHC staining on microglial cells in control mouse brain and after MPTP treatment

	Control	1d	2d	Following MPTP treatment			
				3d	7d	14d	21d
<b>CR3</b>							
SN	+	+	+++	++++	+	+	+
striatum	+	+++	++++	++++	+	+	+
<b>MHC class II</b>							
SN	--	--	--	(+)	+	+++	+
striatum	--	--	--	(+)	+	+	+

The intensity of the staining on glial cells in the tissue was estimated as follows: --, no staining detected; (+), faint or variable staining; +, staining easily detected; + +/+++/+ + + +, staining elevated to increasing extent. Perivascular cells and choroid plexus cells are not included.

21st day CR3 staining was similar to that in the control brains (Table I).

### MHC class II antigens expression

In control mouse brain, MHC class II was detectable only in the choroid plexus and on some perivascular cells. In the first days following MPTP administration, only the perivascular cells were more intensely labelled with anti-MHC class II antibody, and were easily detect-

able in SN and striatum. Some cells of glial morphology expressing MHC antigens were found in the SN on the 7th day (Fig. 2). On the 14th day, the number of these cells increased but was diminished on day 21. In the striatum, there were fewer MHC-positive glial cells than in the SN, but the pattern of staining followed that of the SN (Table I). The morphology of the majority of MHC-positive cells resembled microglia (Fig. 2D), and this was confirmed using double immunostaining (data not shown).

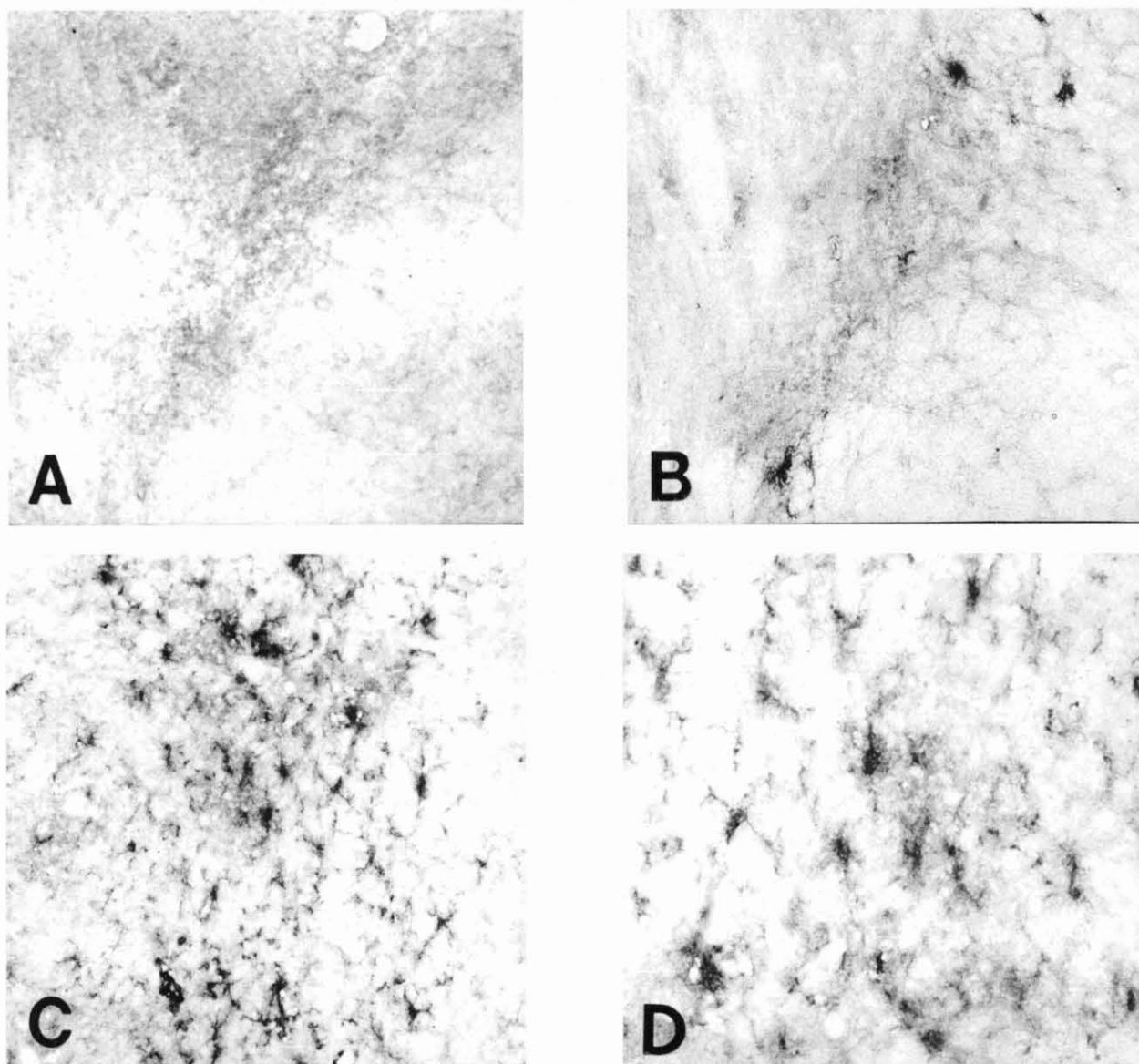


Fig. 2. MHC class II staining in the pars compacta of substantia nigra. Control mouse (A); MPTP, treated mouse at 7 days (B) and 14 days (C) after intoxication (magnification x 200). Cells of the morphology of microglia stained for MHC class II 14 days after MPTP administration (D) (magnification x 400).

### Lymphocytes' infiltration

In the normal mouse brain, we detected very few lymphocytes in the SN or striatum. T-lymphocytes (CD3+ cells) were seen in the SN, mainly pars compacta, from the 1st day following MPTP administration. The number of the CD3+ cells was most increased on the 2nd and the 7th day after MPTP treatment, then the reaction diminished. The preponderance of T-cells was of the CD8+ phenotype at every time point, and the pattern of their re-

action was the same as that of CD3+ cells. CD4 + lymphocyte infiltration was of a lower magnitude, with only one maximum on the 2nd day following treatment (Table II). In the whole striatum we also detected a significant infiltration of T-lymphocytes but it was not so pronounced as in the SN. In the SN, the cells infiltrated the whole pars compacta, but in the striatum they were located only next to blood vessels (Fig. 3). More than half of the infiltrating lymphocytes expressed very strong staining of CD 44 antigen. We were not able to find any

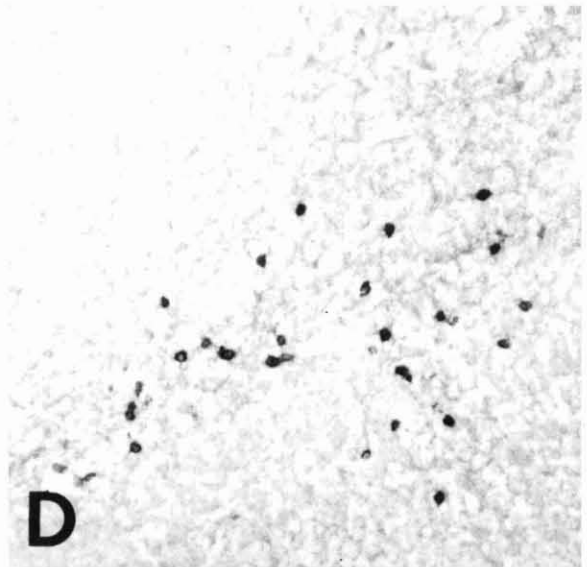
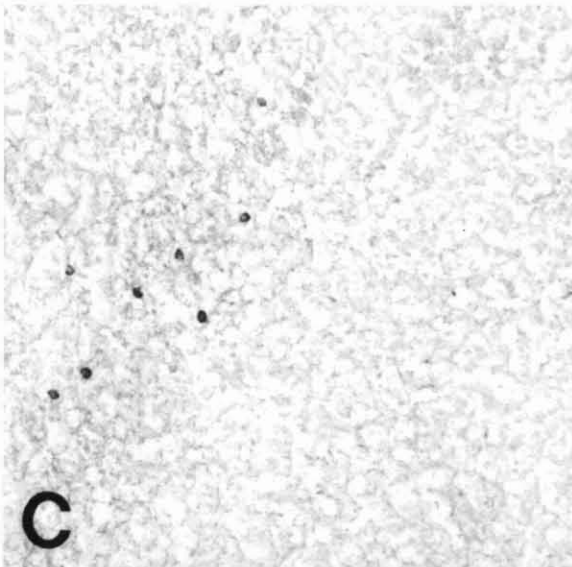
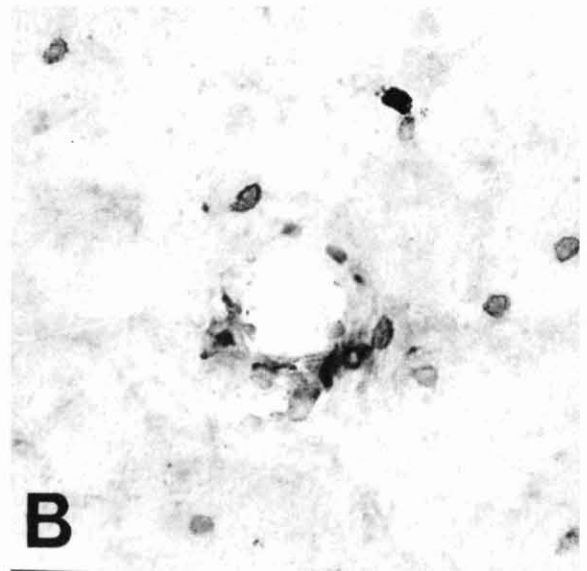
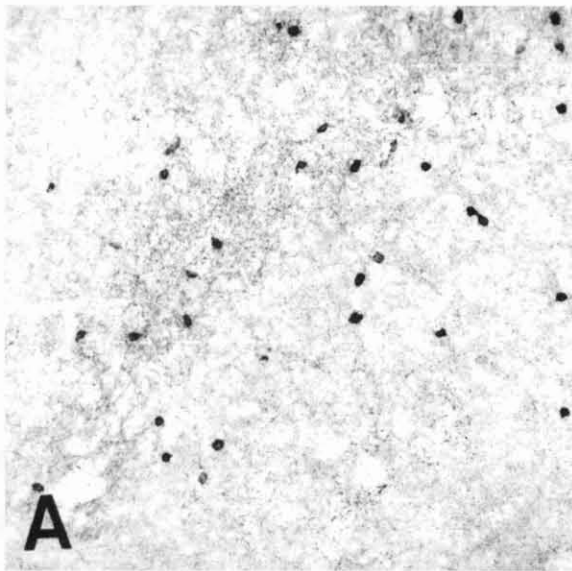


Fig. 3. Lymphocyte infiltration 7 days after MPTP administration. CD3, positive cells in the pars compacta of the substantia nigra (A) (magnification x 200). Lymphocytes (CD3+ cells) in the striatum located around the blood vessels (B) (magnification x 600). Clear preponderance of CD8 - positive lymphocytes (D) over CD4 - positive cells (C) (pars compacta of SN) (magnification x 200).

TABLE II

Lymphocyte infiltration in the substantia nigra of MPTP treated and control mice

	Control	1d	2d	Following MPTP treatment			
				3d	7d	14d	21d
CD 3	--	+	+	++++	++++	+	+
CD 4	--	--	+	+	+	+	--
CD 8	--	+	+	+	++++	+	+
CD 44	--	+	+	+	+++	+	+

Lymphocytes were counted in the SN using a drawing tube: from six fields of vision per one slide from three slides of the mouse brain as we described previously (Członkowska et al. 1996). The number of cells were estimated per 1 square mm as follows: --, < 5 cells; +, 5 - 30 cells; ++, 30 - 60 cells; +++, 60 - 100 cells; +++, >100 cells.

CD40+ cells that might exclude B-lymphocytic infiltration. Neutrophils were also not observed in hematoxyline-eosine staining.

## DISCUSSION

In the present study using anti-CR3 antibody as a microglia marker (Graeber et al. 1988), we confirmed the previous observation that MPTP-induced degeneration of nigrostriatal neurones leads to microglial activation (Francis et al. 1994, Członkowska et al. 1996). Additionally we found an upregulation of MHC class II antigens and the presence of lymphocyte infiltration in the SN and in the striatum.

In control mouse brains, MHC class II antigens were detectable only on perivascular cells and in the chorioid plexus. Streit et al. (1989b) described a similar distribution of MHC-positive cells in the normal rat brain, and the increased expression of these molecules after retrograde degeneration of the facial nerve in that nerves nucleus. MHC antigen expression was seen upregulated mainly in autoimmune processes but also was described in other experimental models such as ischemia, kainic acid lesions (Akiyama et al. 1988, Morioka et al. 1992b, Finsen et al. 1993) and central nervous system injury (Moffett et al. 1994). Cellular localisation of these antigens indicated that perivascular cells and microglia and also astrocytes (Vass and Lassmann 1990) were able to express MHC class II molecules. In our study MHC-positive glial cells were predominantly microglia beside some other cells of glial morphology not anti-CR3-labelled that were probably astrocytes. Activation of astrocytes in the mouse striatum was observed from the 2nd

day until the 6th week after MPTP treatment (Stromberg et al. 1986, O'Callaghan et al. 1990, Francis et al. 1994).

It was previously shown that MHC-positive microglia were able to present antigens to CD4+ cells and in this way could initiate an immune reaction (Lassmann et al. 1991, Shrikant and Benveniste 1996). However, in the absence of lymphocyte activation, the increased expression of MHC antigens may be considered as another activation marker of glial cells, or it might play some other function (Streit et al. 1988a).

Leukocyte recruitment was observed in areas of a leaky blood-brain barrier - in the cerebral cortex following a stab wound (Flaris et al. 1993), trauma (Giulian et al. 1989), or in an infarcted area (DuBois et al. 1985). During degeneration processes, such as Wallerian degeneration of the optic nerve (Lawson et al. 1994), or neural damage in the rat facial nucleus following facial nerve section, leukocytes were not noticed (Rothwell 1995). In these animal models, as in our own study, the blood-brain barrier was not damaged. McGeer et al. (1993) described a small number of T-lymphocytes around the lesions in Alzheimer disease. In our study we observed lymphocyte infiltration in the areas of degeneration induced by MPTP, and this reaction was associated with microglial activation. The largest infiltration was observed on the 2nd and 7th day. However the highest number of CD3+ and CD8+ cells was noticed on the 7th day. The number of CD4+ cells was always 2-4 times lower than CD8+ cells, and was most prominent at the beginning of the pathological process. In multiple sclerosis and early EAE lesions T-lymphocytes infiltrate were predominantly of the CD4+ subpopulation, whereas CD8+ cells were seen in preponderance in later stages

(Weller et al. 1996). CD4<sup>+</sup> lymphocytes were always observed at the beginning of the immune reaction, as they appeared to stimulate other lymphocytes and enhance inflammation by secreting an array of cytokines. While we couldn't compare a mechanism of tissue injury in an immune-mediated process to that in toxic degeneration, we observed some similarities in the time course of CD4<sup>+</sup> and CD8<sup>+</sup> cells infiltration.

Lymphocytes that we observed infiltrating the injured structures had to be activated to cross the intact blood-brain barrier. As documented in EAE, lymphocytes do not need antigen presentation or do not have to specifically recognise CNS-antigen to enter the brain (Cross et al. 1990). Since the lymphocytes' recruitment we observed followed microglial activation, it is possible that the lymphocytes' infiltration occurred in response to some agents secreted by microglia. Microglia are able to produce agents enhancing, for example, expression of adhesion molecules, activating chemokines and that way to stimulate lymphocytes to enter the brain (Dalakas 1995).

As in other tissue lymphocytes which infiltrate the brain usually belong to the memory cell population. In the early stages of EAE the infiltrate contain mainly non-specifically activated memory T-cells characterised by strong expression of LFA-1, ICAM-1 and CD44 molecules and low expression of CD45RB in mouse (Engelhardt et al. 1995, Weller et al. 1996). Cells which recognise any CNS antigens may be then activated but others undergo apoptosis. In fact, in our study, we found CD44 staining on more than half of the observed lymphocytes at all time points. However it is too less to state that the observed lymphocytes belonged to such an activated memory cell population.

It is too early to postulate that the observed lymphocytic infiltration played any role in the degenerating process after MPTP treatment. In order to undergo further activation lymphocytes needed antigen presenting cell expressing not only MHC antigens but also costimulatory molecules such as ICAM-1, VCAM-1 and B7 (Shrikant and Benveniste 1996). We found upregulation of MHC class II antigens at the same time as CD4<sup>+</sup> cells infiltrated the SN and striatum. The activation of CD8<sup>+</sup> cytotoxic lymphocytes require however, MHC class I antigen expression and we don't know if this occurred after MPTP treatment. It has been shown that dying neurones express MHC class I antigens (Neumann et al. 1995). In this regard, it was interesting that the peak of CD8<sup>+</sup> cell infiltration was on the day of the most pro-

nounced dopaminergic neurone damage (Bieganowska et al. 1993, Członkowska et al. 1996).

Although the presence of T-lymphocytes and MHC antigens on glial cells is not sufficient to determine that an immune-mediated inflammation takes part in the pathological process (Morioka et al. 1992a), our preliminary findings indicate that the immune reaction might contribute to the neurodegeneration following the MPTP intoxication.

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