Acute and Chronic 1-Methyl-4-Phenyl-1,2,3, 6-Tetrahydropyridine Administrations Elicit Similar Microglial Activation in the Substantia Nigra of Monkeys

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Abstract
Increasing evidence suggests a pivotal role for neuroinflammation in the pathogenesis of Parkinson disease, but whether activated microglia participate in disease progression remains unclear. To clarify this issue, we determined the numbers of activated microglial cells in the substantia nigra pars compacta and ventral tegmental area of monkeys subacutely and chronically exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Monkeys in the subacute MPTP treatment group were killed 1 week after the last MPTP injection; chronically treated monkeys were killed either 6 or 35 months after the last MPTP injection. Subacute MPTP administration induced loss of dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area and microglial activation in the same areas. Chronic MPTP treatment resulted in greater dopaminergic neuron depletion in both treatment groups. Both groups of chronic MPTP-treated monkeys showed increased numbers of activated microglial cells in the substantia nigra pars compacta that were similar to those of the subacute MPTP treatment group. These results indicate that microglial activation seems to be induced mainly by the toxic effects of MPTP and that it does not further progress once the toxin administration has been terminated. This suggests that the progressive degeneration of nigral cells in Parkinson disease may not necessarily be associated with progressively increased microglial activation.

Key Words: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Activated microglia, Dopaminergic cells, Monkeys, Parkinson disease.

INTRODUCTION
A major neuropathologic feature of Parkinson disease (PD) is the progressive degeneration of midbrain dopaminergic (DA) neurons (1). Although the etiology of this disorder remains unknown, inflammatory mechanisms have been proposed as initiators or pathogenetic factors that promote the neuronal damage that characterizes this entity. Some investigators also claim that inflammation might contribute to disease progression.

The involvement of activated microglia in the pathogenesis of PD was initially suggested by the observation of reactive microglial cells in the substantia nigra (SN) of brains of patients with PD (2). Subsequently, robust profusion of activated microglial cells was observed in the SN of humans exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (3) and in rodents treated with rotenone and 6-hydroxydopamine and in MPTP-treated monkeys (4–7). The presence of activated microglia, even several years after termination of the neurotoxin exposure, has been interpreted as indicating that they are involved in the perpetuation of neuronal degeneration. Moreover, the delayed and progressive loss of DA neurons induced by intracerebral delivery of lipopolysaccharide, which induces a microglial reaction but not neurotoxicity, supports the hypothesis that microglial activation might be sufficient to induce neuronal degeneration (7, 8).

Activated microglia produce various inflammatory cytokines such as interleukin 1-β and tumor necrosis factor, which are increased in the SN, striatum, and cerebrospinal fluid of patients with PD (9–11). Alternatively, activated microglia might also participate in the degeneration of DA cells by their generation of free radicals (12–14) and other proinflammatory molecules such as cyclooxygenase 2 (Cox-2) (14, 15). Indeed, upregulation of inducible nitric oxide synthase and Cox-2-containing amoeboid microglial cells has been observed in the SN of PD patients but not in control subjects (15, 16). Moreover, Cox-2 inhibitors provide protection against MPTP- and 6-hydroxydopamine–induced toxicity in rodents (17, 18), and Cox-2–knockout mice are resistant to the toxic effects of these neurotoxins (19, 20).

The production of inflammatory molecules along with the substances released from the dying DA cells might result in amplification and perpetuation of the inflammatory
processes that lead to the irreversible destruction of SN DA neurons (21, 22). For example, neuromelanin released from damaged pigmented neurons could stimulate reactive oxygen species and Cox-2 generation by activated microglia (23–26). It has recently been reported that in the presence of activated microglia, a low concentration of α-synuclein, the main component of Lewy bodies, provokes damage of DA neurons (27).

Although all these data clearly confirm that microglia plays a central role in the degeneration of DA neurons, it is still unclear whether microglial activation is an early critical process (8) or whether it merely constitutes a secondary response to a primary insult of unknown origin. To obtain new insight into the role of microglia in the degeneration of DA cells, we studied the patterns of microglial activation in monkeys after subacute and chronic MPTP exposure. In addition, chronic MPTP–treated animals that had been given similar amounts of MPTP but were killed at different times after the last MPTP dose; this permitted assessment of the possibility that the numbers of activated microglial cells might continue to increase despite cessation of MPTP administration.

MATERIALS AND METHODS

Animals

Young (3–4 years old) male Cynomolgus monkeys (Macaca fascicularis) (n = 16) were studied. The animals were housed in cages under controlled temperature conditions (21°C ± 1°C), humidity (55% ± 5%), air replacement (16 times per hour), and light (08.00–20.00). They were fed fresh fruit and commercial pellets and had free access to water. Experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), for the care of laboratory animals. Animals were divided into 4 groups. The control group (Group 1, n = 3) was untreated. The subacute group (Group 2), 6 months (Group 3), and 35 months (Group 4) after the last MPTP dose. A detailed description of the regimen of MPTP administration and the degree of parkinsonism each monkey exhibited are summarized in the Table.

Behavioral Assessment

The MPTP-induced parkinsonism was assessed according to a disability rating scale for nonhuman primates that independently scores from 0 (normal) to 3 (maximal disability) (28). The main parkinsonian features exhibited by MPTP-treated monkeys are bradykinesia, posture, tremor (intensity and duration), freezing and feeding, balance from 0 to 2, and spontaneous activity from 0 to 5; thus giving a total maximal disability score of 28. In the acute MPTP-treated monkeys, evaluations were performed immediately before death. Motor deficits in animals chronically exposed to MPTP were evaluated weekly until death.

Histological Analysis

Before death, the animals were deeply anesthetized with ketamine and midazolam and transcardially perfused with 4% paraformaldehyde in PBS. Brains were postfixed overnight in the same fixative at 4°C and then immersed in 30% sucrose in PBS. Coronal sections, 40-μm thick, were cut on a freezing microtome, collected in antifreezing solution (0.125 mol/L PBS, 15% dimethylsulfoxide, and 15% glycerin) and stored at −20°C until subsequent analysis.

Table. Characteristics of the Animals

<table>
<thead>
<tr>
<th>Animal Group and MPTP Treatment</th>
<th>Animals</th>
<th>Individual Doses, mg/kg</th>
<th>Duration of MPTP Treatment</th>
<th>No. MPTP Doses</th>
<th>Cumulative Dose, mg/kg</th>
<th>Degree of Parkinsonism</th>
<th>Survival Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n = 3; control)</td>
<td>Cyn-1</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>Cyn-2</td>
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<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Cyn-3</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Group 2 (n = 5; subacute)</td>
<td>Cyn-4</td>
<td>0.25</td>
<td>4 Weeks</td>
<td>4</td>
<td>1</td>
<td>Normal</td>
<td>1 Week</td>
</tr>
<tr>
<td></td>
<td>Cyn-5</td>
<td>0.25</td>
<td>4 Weeks</td>
<td>4</td>
<td>1</td>
<td>Normal</td>
<td>1 Week</td>
</tr>
<tr>
<td></td>
<td>Cyn-6</td>
<td>0.25</td>
<td>4 Weeks</td>
<td>4</td>
<td>1</td>
<td>Normal</td>
<td>1 Week</td>
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<tr>
<td></td>
<td>Cyn-7</td>
<td>0.25</td>
<td>4 Weeks</td>
<td>4</td>
<td>1</td>
<td>Normal</td>
<td>1 Week</td>
</tr>
<tr>
<td></td>
<td>Cyn-8</td>
<td>0.25</td>
<td>4 Weeks</td>
<td>4</td>
<td>1</td>
<td>Normal</td>
<td>1 Week</td>
</tr>
<tr>
<td>Group 3 (n = 5; chronic)</td>
<td>Cyn-9</td>
<td>0.25–0.55</td>
<td>5 Months</td>
<td>13</td>
<td>5.35</td>
<td>Severe</td>
<td>6 Months</td>
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<tr>
<td></td>
<td>Cyn-10</td>
<td>0.25–0.60</td>
<td>6 Months</td>
<td>15</td>
<td>6.55</td>
<td>Severe</td>
<td>6 Months</td>
</tr>
<tr>
<td></td>
<td>Cyn-11</td>
<td>0.25–0.60</td>
<td>15 Months</td>
<td>32</td>
<td>15.30</td>
<td>Severe</td>
<td>6 Months</td>
</tr>
<tr>
<td></td>
<td>Cyn-12</td>
<td>0.25–0.60</td>
<td>6 Months</td>
<td>14</td>
<td>5.95</td>
<td>Severe</td>
<td>6 Months</td>
</tr>
<tr>
<td></td>
<td>Cyn-13</td>
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<td>10 Months</td>
<td>21</td>
<td>8.30</td>
<td>Severe</td>
<td>6 Months</td>
</tr>
<tr>
<td>Group 4 (n = 3; chronic)</td>
<td>Cyn-14</td>
<td>0.25–0.50</td>
<td>6 Months</td>
<td>13</td>
<td>4.55</td>
<td>Severe</td>
<td>35 Months</td>
</tr>
<tr>
<td></td>
<td>Cyn-15</td>
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<td>6 Months</td>
<td>14</td>
<td>5.6</td>
<td>Severe</td>
<td>35 Months</td>
</tr>
<tr>
<td></td>
<td>Cyn-16</td>
<td>0.25–0.55</td>
<td>10 Months</td>
<td>19</td>
<td>8</td>
<td>Moderate</td>
<td>35 Months</td>
</tr>
</tbody>
</table>

Cyn: cynomolgus; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
One section containing the SN from each animal was taken every 7 consecutive sections and stained with thionine to visualize the boundaries of adjacent nuclei.

**Immunocytochemistry**

Free-floating immunocytochemistry and immunofluorescence techniques were used for histological analysis. Slices were rinsed 3 times for 10 minutes in 0.01 mol/L PBS in between steps.

**Single-Labeled Immunocytochemical Techniques**

Tissue sections were washed in double-distilled water and 0.01 mol/L PBS to remove the cryoprotectant solution and incubated in PBS with 0.02% H$_2$O$_2$ (Merck, Darmstadt, Germany) for peroxidase inhibition. They were then incubated in 0.01 mol/L buffered citrate pH 9.0 for 30 minutes at 80°C for antigen retrieval. The sections were then incubated overnight at 4°C in a solution of PBS containing 10% normal goat serum, 0.1% Triton X-100 (Sigma, St Louis, MO), and the antibody to either tyrosine hydroxylase (TH, mouse IgG; 1:5000; Chemicon International, Temecula, Calif) or to HLA-DR (mouse IgG, 1:200, DakoCytomation, Glostrup, Denmark). The sections were then incubated with the appropriate biotinylated secondary antibody and processed with an avidin-biotin complex kit (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif) for 35 minutes at room temperature. Staining for peroxidase was performed in buffer acetate-imidazole (0.125 mol/L acetate and 0.01 mol/L imidazole, pH 7.2) using 0.05% 3,3’-diaminobenzidine tetrahydrochloride (Sigma), 0.001% H$_2$O$_2$, and 0.1% nickel.
ammonium sulfate (BDH, Poole, UK). The TH- and HLA-DRα-immunostained sections were counterstained with Nissl and methyl green, respectively. Finally, slices were mounted under a glass coverslip using DPX (BDH) and examined under normal light.

Double-Labeled Immunocytochemical Techniques

Coronal tissue sections were rinsed and permeabilized following the protocol used for immunocytochemistry previously described. They were then incubated in 0.06% K2MnO4 to reduce the autofluorescence of the tissue. After antigen retrieval with 0.01 mol/L citrate buffer, pH 9.0, at 80°C for 30 minutes, the sections were incubated overnight at 4°C in a mixture of anti-TH (1:5000) and anti–HLA-DRα (1:200) antibodies. After washing, the sections were incubated for 2 hours in a combination of secondary antibodies coupled to fluorescent markers anti-mouse IgG Cy3 conjugate (1:500; Amersham Biosciences, Buckinghamshire, UK) and Alexa Fluor 488 donkey anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR) diluted in PBS. The sections were then coverslipped with Vectashield mounting medium (Vector Laboratories) to prevent rapid bleaching. Doubly labeled cells were detected in confocal images obtained using a laser scanning microscope 510, equipped with 3 lasers (LSM 510/Meta; Zeiss, Götingen, Germany).

Stereology

Stereological analysis was performed using an Olympus BX51 microscope with a monitored x-y-z stage linked to the CAST-GRID software package (Olympus, Glostrup, Denmark) and applying the optical fractionator method (29). The numbers of TH-immunostained cells were quantified in the SN pars compacta (SNpc) and in the ventral tegmental area (VTA) of control and MPTP-treated animals, as previously described (30). For HLA-DRα–positive cell counting, the SN and the VTA were delineated at low magnification (4X objective) on each immunostained section. Series of 6 sections that were regularly spaced at intervals of 840 μm and covering the entire SNpc from the rostral tip back to the caudal end were counted in each animal. From a random start position, a counting frame was superimposed on the image; DA neurons were sampled using a ×63 lens, with the nucleolus used as the sampling unit. The section thickness was previously determined in a pilot study showing that stained sections with an initial thickness of 40 μm shrink to a final thickness of approximately 15 μm after dehydration and coverslipping; this enabled the use of a 5-μm-high dissector, taking into consideration the need to safeguard distances above and below the dissector (31). A minimum of 100 cells was sampled according to the rules of the optical dissector method (32). The percentage of sampling fraction and the size and spacing between counting frames were set to achieve an error coefficient generally less than 0.1.

Statistical Analysis

Data were analyzed using a non-parametric Mann-Whitney U test for independent groups with significance defined as p < 0.05.
activated microglial cells were surrounded by neighboring TH-ir neurons (data not quantified) (Fig. 4).

The counts of activated microglia showed that in control monkeys, very few cells expressed the HLA-DRα in the SNpc (Fig. 5A) and the VTA (Fig. 5B). In the subacute and chronic MPTP treatment groups, there were significantly increased numbers of cells HLA-DRα-positive cells both in the SNpc (p < 0.01; Fig. 5A) and the VTA (Group 2, p < 0.01; Groups 3 and 4, p < 0.05; Fig. 5B). The subacutely treated animals displayed increases in numbers of activated microglial cells similar to those in both groups of chronic treatment animals (Fig. 5A), and counts were significantly increased in VTA (p < 0.05) (Fig. 5B). The numbers of activated microglial cells in the SNpc and VTA were similar in the 2 chronic MPTP groups (Figs. 5A, B). Thus, both acute and chronic MPTP administration significantly increased the numbers of activated microglial cells in the SNpc and VTA. This increase was of similar magnitude in the SNpc of all MPTP animal groups and seemed to be independent of the MPTP dose and the survival time after the last MPTP injection. Interestingly, both groups of chronic MPTP-treated monkeys showed similar numbers of surviving DA cells in the SNpc and VTA.

DISCUSSION

Previous studies have assessed the extent of the microglial response in monkeys treated with MPTP (6, 7, 33). In these studies, however, MPTP was exclusively administered in a chronic regimen, whereas we compared acute and chronic MPTP administration. Moreover, death at different time points after the last MPTP injection in the chronically treated animals allowed us to determine whether the inflammatory process was affected over time, although no additional MPTP doses were given.

We found that subacute and chronic MPTP treatment elicited not only DA neuronal death in the SNpc and VTA, but also an inflammatory reaction characterized by increased

FIGURE 3. The HLA-DRα-immunoreactive cells (black arrows) in the substantia nigra pars compacta of a control and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys. (A) Control, (B) subacute treatment, (C, D) chronic MPTP-treated monkeys killed 6 months (C) and 35 months (D) after the last MPTP dose. Immunoreactive cells display the typical morphology of microglia in the control monkey; the microglial cell is located close to a blood vessel (arrowhead). Methyl green counterstain. Scale bars = 50 μm; inset scale bars = 50 μm.
numbers of microglia that expressed an activated HLA-DR-positive phenotype (34, 35). Accordingly, in all groups of animals, HLA-DR-positive cells displayed the characteristic morphology of activated glial cells, that is, hypertrophic, shortened, and thickened cell processes (36). Confocal images in the MPTP-treated monkeys showed activated microglia in the vicinity of nigral DA neurons. This supports previous findings of their localizations proximal to degenerating DA cells and may implicate them in the disease pathogenesis (2).

Inflammation and microglial responses are thought to play important roles in the degenerative process of PD (37–39). Indeed, a sustained microglial reaction in the SN has been described in patients with PD (2) and in humans and monkeys intoxicated with MPTP (3, 6, 7, 33). For example, Barcia et al found an increased microglial reaction in monkeys chronically treated with low doses of MPTP and killed 1 year after the last injection. They pointed out that the degenerative process was still active despite the lack of any agent triggering cell death and suggested that the microglial reaction was associated with long-term degeneration of DA cells (7). On the other hand, Hurley et al (33) advocated a disconnection between DA cell degeneration and the pattern of microglial activation in MPTP-treated monkeys because sites with different degrees of cell loss showed equally increased numbers of activated microglial cells, which is consistent with our results. The same authors suggested that the pattern of microglial reactivity was also independent of the behavioral consequences of MPTP-induced damage. Again, our results seem to be in accordance with these findings because the increased number of activated microglial cells was similar in all our MPTP-treated monkeys regardless of the disability rating scale, the degree of DA cell death, or the survival time after the last MPTP dose.

The subacutely treated monkeys displayed a significant DA cell loss, which was increased further in the 2 chronically treated groups. Interestingly, the latter groups showed comparable numbers of surviving DA cells irrespective of the survival times, indicating the absence of an ongoing degenerative process despite the presence of large numbers of activated microglia. In keeping with this, we have previously reported the absence of an active degenerating process in the SN of these MPTP-treated monkeys (40). Therefore, it seems that the extent of DA cell death is closely related to the total amount of MPTP, that is, the higher the quantity of administered neurotoxin, the greater the DA cell loss in the SNpc and the VTA. McGeer et al (6) had proposed that microglial activation in the SN of chronically MPTP-treated monkeys might be an episodic process or might even burn out. Indeed, microglia can respond rapidly or organize for responses by other cells that can be triggered within minutes after stimulation (41, 42).

We found similar numbers of activated microglia in the SN of the monkeys after acute and chronic exposure to MPTP despite different survival times and MPTP doses. We propose that in our animals, microglial activation would first be induced by the toxic effect of MPTP, and that subsequently,
the degeneration of DA neurons induced by the toxin might act as a perpetuating factor. Once MPTP administration is interrupted and the neuronal death ceased, the number of activated microglial cells was not further increased. We have demonstrated apoptotic microglial cells in the SN in these monkeys (40) that might contribute to the consistency in numbers of activated microglia, irrespective of the MPTP dose and DA nigral cell loss.

Some studies have shown that microglia release prosta-glandins and other proinflammatory molecules such as Cox-2, and we recently reported that monkeys subacutely treated with MPTP display upregulation of Cox-2 in the DA neurons of the SNpc (30). Cyclooxygenase 2 expression decreased to baseline values once MPTP administration was terminated. These results also argue against the hypothesis that MPTP-induced DA cell death increases as a result of microglial activation that, in turn, would release inflammatory molecules such as Cox-2. Altogether, our results suggest that these inflammatory processes are independent of each other. We conclude that activated microglial cells might be the consequence of the toxic effect exerted by MPTP and the progressive degeneration of nigral cells, and it is unlikely that they activate inflammatory molecules such as Cox-2.

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REFERENCES


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