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# Dynamics of expression of the mRNA for cytokines and inducible nitric synthase in a murine model of the Parkinson's disease

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**Abstract.** The inflammatory reaction and oxidative stress has been linked with PD. Proinflammatory cytokines promote neurodegeneration or neuroprotection in different animal models. In addition, these cytokines have been reported to increase iNOS expression. With the RT-PCR method we evaluated mRNA levels for IL1β, IL6, TNF, IFNγ, IL-10 and iNOS in the striatum of C57BL/6 mice after MPTP intoxication. The IL1β mRNA expression rapidly increased and peaked at 6 h. The first increase of mRNA for TNFα and IFNγ was noticed at 6-24 h and the second at the 7$^{th}$ day after MPTP intoxication. Two peaks of IL10 mRNA were seen, immediately (6 h) and at the 3$^{rd}$ day post MPTP injection. The peak of mRNA level for IL6 was observed at the 7$^{th}$ day. Expression of mRNA for iNOS peaked at 24h, started decreasing on the 3$^{rd}$ day, but was still present till the 14$^{th}$ day. Those findings suggest that cytokine network and iNOS may be involved in the development of immune changes accompanying degeneration of the nigrostriatal system.

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**Key words:** Parkinson's disease, neuroinflammation, cytokines, oxidative stress

## INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder in which dopamine (DA) neurons of the substantia nigra (SN) selectively degenerate, thus leading to a loss of their nerve terminals and DA deficiency in the striatum (Marsden et al. 1998) Although the pathomechanism by which these neurons degenerate is still unknown, there is increasing evidence from experimental and clinical studies for a possible involvement of immunological mechanisms in the etiopathogenesis of PD (Kuhn et al. 1997).

The neurodegenerative process observed in the nigrostriatal system in animal models of PD in which degeneration of nigral dopaminergic neurons was induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to the development of local inflammatory reaction results in glial activation accompanied by expression of inflammatory mediators and infiltration of circulating immune cells into the central nervous system (CNS) (Członkowska et al. 1996, Kurkowska-Jastrzębska et al. 1999, O'Callaghan et al. 1990).

Neuroinflammation is regulated by many signal molecules including cytokines. Cytokines are multifunctional proteins that in the CNS play a particular role in the normal development of the brain as well as in neuroimmunopathological processes following injury and neurodegeneration (Szelenyi 2001). Many investigators found markedly increased levels of pro-inflammatory cytokines such as IFNγ IL1β and TNFα that are expressed by glial cells in the nigrostratial regions of patients with PD (Hunot et al. 1999, Nagatsu 2002). This may have several implications for the pathophysiology of this disease. It seems very likely that the chronic production of these pro-inflammatory cytokines in high amounts may have a deleterious effect upon nigrostriatal dopaminergic regions. This concept is supported by the fact that anti-inflammatory agents, such as dexamethasone or indomethacin, have been shown to protect dopaminergic neurons against damage in the experimental models of PD (Kurkowska-Jastrzębska et al. 2002).

Recently, many research efforts have been focused on the explaining the molecular actions by which pro-inflammatory cytokines may induce neurotoxicity. One of the better characterized cytotoxic mechanisms induced by pro-inflammatory cytokines, such as IFNγ, IL1β and TNFα is induction and activation of the inducible nitric oxide synthase (iNOS, Chatterjee et al.1999; Akama et al.2000). Inducible nitric oxide synthase is a isoform of NOS which has been implicated in cellular toxicity in many cell systems including brain. Nitric oxide (NO) when generated in high quantities following induction of iNOS, combines with the superoxide anion to form highly reactive, death-inducing compounds such as peroxynitrite (Beal 1998; Dawson et al. 1998). It is well accepted that increased oxidative stress may play a prominent role in the neurodegenerative process.

Furthermore, TNFa through interaction with its receptor can activate an apoptotic transduction pathway in dopaminergic neurons (Hsu et al. 1996, Mogi et al.2000). The pro-inflammatory cytokines may also exert deleterious effects on the PD brain by stimulation of the reactive glial cells to expression of other inflammatory mediators such as complement and cyclooxygenase (COX) (Yamoamoto et al. 1995). On the other hand, there is multiple converging evidence to support neuroprotective effects of these molecules. Cytokines, such as IL6 or IL1β stimulate reactive astrocytes to synthesis of certain neurotrophic factors, thereby promoting axonal sprouting in the degenerated brain tissue (Ho et al. 1997). TNFα is a potent stimulator of survival factors such as calbindin, manganase-superoxide dismutase, and Bcl-2 proteins (Keller et al 1998; Mattson et al.1995). The TNFα-ceramide pathways that suppress the generation of free radicals may protect neurons from the damage (Hunot et al. 1997). This finding suggests that the pro-inflammatory cytokines also take part in the successful neuroregenerative process. The detailed study of the network of pro- and anti-inflammatory cytokines may help in better understanding of the role of these cytokines in the neurodegerative processes.

Our present study was aimed at studying the temporal changes in the mRNA expression of IL-10, IL6, IFNγ, IL1β, TNFα and iNOS in the striatum of C57BL/6 male mice, from 6 hours to 14 days after MPTP intoxication.

## METHODS

### Animals

Male adult C57BL/6 mice, 11-12 months old and 35-40 g of weight were used in this study. The animals were housed in plastic cages under a 12 h light/12 h dark cycle and had free access to food and water. Ambient temperature was maintained at 25 ± 2°C.

**Treatment**

MPTP-HCL (Sigma) was dissolved in sterile 0,9% saline, and was administrated in four intraperitoneal injections at 1-h intervals using a dose of 10 mg/kg body weigh. Control mice received sterile saline only. Animals were sacrificed by spinal cord dislocation and decapitated at: 6h, 1, 3, 7, 14 days after MPTP intoxication. Six to eight mice were killed at each time point. The striatial samples were prepared immediately after decapitation and stored at -80°C until use.

**RT-PCR (Reverse trancriptase - polymerase chain reaction)**

Total RNA was isolated from brain tissue using TRI reagent (Sigma), in accordance with the manufacturer's instructions. The RNA product was resuspended in 20 µl diethyl pyrocarbonate (DEPC)-treated water. The quality of RNA samples was confirmed by the electrophoresis of RNA through the 1,5 % agarose gel containing ethidium bromide and visualization by UV illumination. The RNA was stored at -70 ° C until used. Total RNA was reverse transcribed at 42°C for 1 hour with Moloney murine leukemia virus (MMLV) reverse transcriptase according to the instruction of the manufacturer of the reagent (Sigma). Following the RT reaction the cDNA products were stored at -20°C until use. The cDNA was amplified using adequate primers (Table I). Negative control reactions without template or MMLV reverse transriptase were included in parallel in the PCR amplification with primer set. As a control and to eliminate sample-to-sample differences in RNA extraction and conversion to cDNA, we amplified the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The thermal cycling parameters were as follows:

IL-6, IFNγ, IL1β, TNFα -95°C, 2 min; 94°C, 50 s; 60°C, 50 s, 35 cycles; and 72°C, 5 min;

IL-10: 94°C, 1 min; 60°C, 30 s; 72°C, 30 s, 30 cycles; and 72°C, 7 min.

iNOS: 94°C, 3 min; 94°C 1 min; 57°C 2 min; 72°C 3 min, 30 cycles and 72°C.

GAPDH: 94° C, 5 min; 94°C, 30s; 57,5°C, 45s; 72°C, 1 min; 30 cycles; and 72°C, 10 min. PCR products were separated on 1,5% agarose gels stained with ethidium

Table I

Specific primer pair sequences used in semiquantitative RT-PCR

| Amplicon | Oligonucleotide sequences (5'-3') | Size (bp) |
|---|---|---|
| IFNγ | AGC GGC TGA CTG AAC TCA GAT TGT AG<br>GGTC ACA GTT TTC AGC TGT ATA GGG | 243 |
| TNFα | GGC AGG TCT ACT TTG GAG TCA TTG C<br>ACA TTC GAG GCT CCA GTG AAT TCG G | 307 |
| IL-6 | CTG GTG ACA ACC ACG GCC TTC CCT A<br>ATG CTT AGG CAT AAC GCA CTA GGT T | 600 |
| IL-1β | TCA TGG GAT GAT GAT GAT AAC CTG CT<br>CCC ATA CTT TAG GAA GAC ACG GAT | 502 |
| IL-10 | ACC TGG TAG AAG TGA TGC CCC AGG CA<br>CTA TGC AGT TGA TGA AGA TGT CAA A | 237 |
| iNOS | ATG ACC AGT ATA AGG CAA GC<br>GCT CTG GAT GAG CCT ATA TTG | 367 |
| GAPDH | TGA AGG TCG GAG TCA ACG GAT TTG GT<br>CAT GTG GGC CAT GAG GTC CAC CAC | 493 |

bromide and recorded under UV light with camera linked to an image analyzer (One-descan, Scanalitics. Inc.). The result was evaluated as a relative unit determined by normalization of the optical density (OD) of cytokine band to that of the GAPDH band. Two or three cytokine PCR assays per sample were performed.

**Data analysis**

All results are expressed as means ± SE. Statistical comparisons were made using analysis of variance (ANOVA) and Mann-Whitney U-test. The value of $P<0.05$ was considered to indicate statistically significant differences between control and treatment groups.

## RESULTS

To evaluate the expression of cytokine mRNA for IFNγ, TNFα, IL1β, IL6 IL10 and iNOS in the stratium of male mice following intoxication with MPTP we used a semiquantitative RT-PCR method. All the values were normalized to GAPDH levels in corresponding samples. The GAPDH gene is expressed at a relatively constant level in cells and is commonly used in semiquantitative RT-PCR to assess the relative efficiency of each individual PCR. These results confirm the semiquantitative quality of the method and allow comparison of the kinetics of the cytokine mRNA production over time. The absence of PCR product from PCR when primers for GAPDH and water or total RNA were used instead of cDNA ensured that there was no contaminating exogenous DNA. Since DNA polymerase amplifies both cDNA and genomic DNA with equal efficiencies, the absence of PCR product from PCR when non-reverse-transcribed total RNA and primers for GAPDH were used confirmed that amplification was based solely on cDNA. The autoradiograms in Fig. 1 illustrated the expression of cytokine transcripts in the striatium of normal and MPTP-intoxicated mice.

RT-PCR analysis revealed the difference in kinetics of IFNγ, TNFα, IL1β, IL6, IL10 and iNOS mRNA expression following MPTP injection. In the control striatum very low baseline levels of mRNA for all investigated cytokines were detected. Rapid increases in the amounts of mRNA for pro- inflammatory cytokines: IFNγ, TNFα and IL1β we observed between 6h and 24h after MPTP injection. The peak of the IL1β gene expression was observed at the 6-h time point. The expression of mRNA for that cytokine slightly declined 24h after intoxication. After 3 days the level of mRNA for IL1β recovered to the baseline and the expression of IL1β mRNA continued at the same level in the 7$^{th}$ day after intoxication. At the 14-days time point we observed a moderate increase in IL1β gene expression (Fig. 2 C).

The kinetic of mRNA for IFNγ and TNFα showed a phasic pattern. Expression of the mRNA for these cytokines rapidly increased beginning at 6h and peaking at 24h after the MPTP injection. At the 3-days time point the level of mRNA for IFNγ (Fig.1 B) and TNFα (Fig. 2 A) recovered to the basaline (IFNγ) or was slightly enhanced (TNFα). After 7 days the amounts of mRNA for these proinflammatory cytokines increased again but it did not return to the levels observed at the 24-h time point. The expression of mRNA for IFNγ was minimal (as in the control) on the 14$^{th}$ day after intoxication. At this time point the expression of TNFα decreased, as compared to the 7$^{th}$ day, but it did not recover yet to the control level.

We noticed a moderate increase in the level of mRNA for IL6 between 6h and 3 days after MPTP intoxication. Increase of that mRNA peaked at the 7-days time point and after 14 days we still observed the elevated expression of mRNA for IL6 (Fig. 2 E).

Our study shows also a phasic pattern of the IL10 mRNA expression. However, that pattern differed from the patterns for IFNγ or TNFα. The first increase of the IL10 mRNA showed immediately - 6h after intoxication. At the 1-day time point we observed decrease in the amount of IL10 mRNA. The highest peak of IL-10 mRNA expression was noticed at the 3rd day after intoxication. The level of IL10 mRNA was also significantly elevated between the 7$^{th}$ day and 14$^{th}$ day post MPTP injection (Fig. 2 F).

In the control group baseline levels of mRNA for iNOS were very low. Their expression rapidly increased and was higher than in controls as early as at 6h after MPTP intoxication. Expression of the iNOS mRNA peaked at 24h, then it significantly decreased at the day 3 but it was visible till the 14$^{th}$ day (Fig. 2 D).

## DISCUSSION

In the present study, we have demonstrated using the RT-PCR method that administration of the MPTP neurotoxin induced expression of the IL1β, IL6, IL10, IFNγ and TNFα cytokines as well as the iNOS gene in

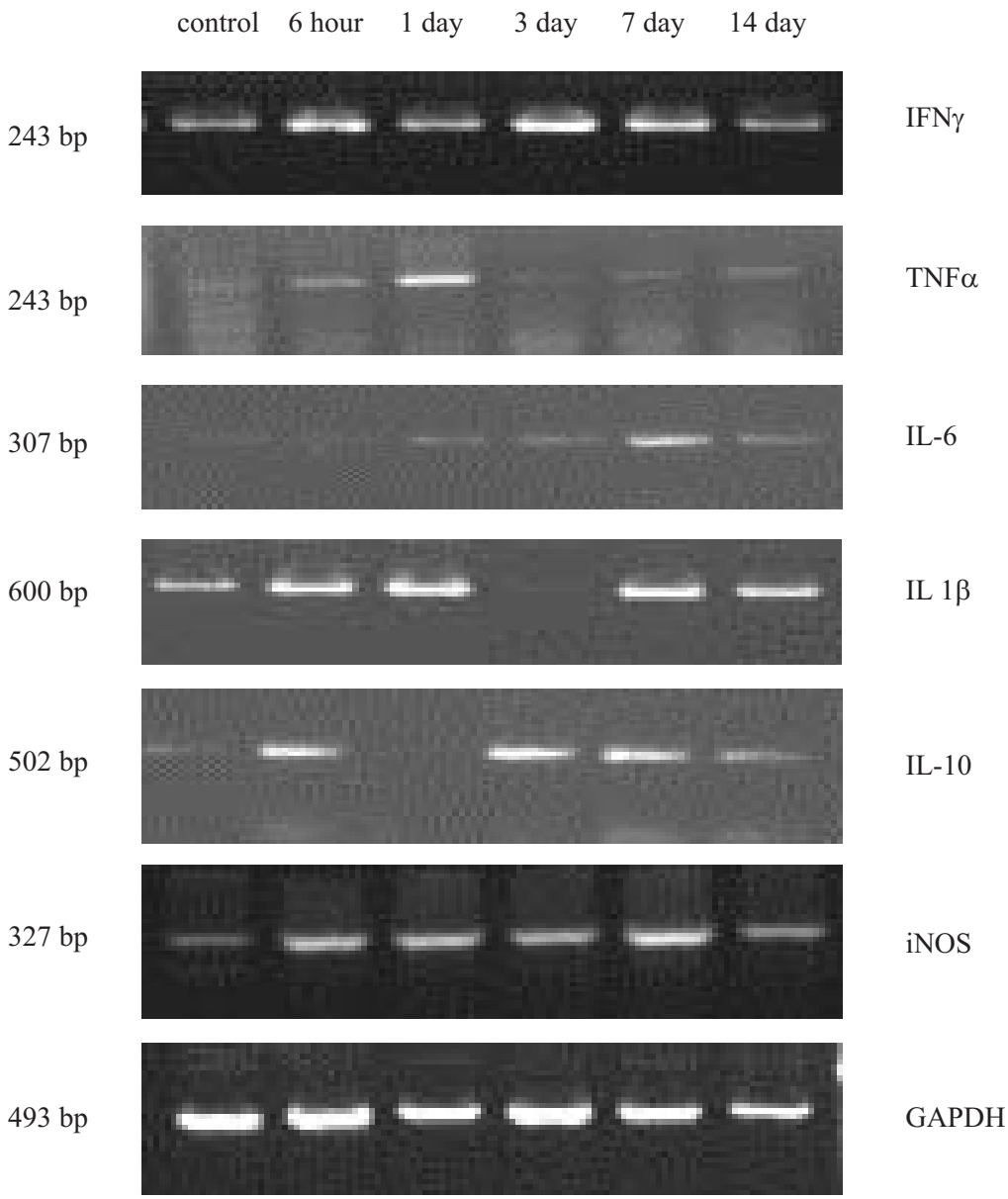


Fig. 1. Representative RT-PCR demonstrating the expression of TNFα, IFNγ, IL1β, iNOS, IL6, IL10 mRNAs in the striatum of C57BL/6 male mice at various times after the MPTP intoxication.

the mouse striatum. This method did not allow for showing the origin of particular cytokines and iNOS, but nevertheless measuring the mRNA levels allowed us to follow the dynamics of activation of some genes for inflammatory molecules genes.

There is growing evidence that the overproduction of proinflammatory cytokines by CNS cells contributes to pathophysiological changes seen in various neurological diseases and brain injury and that the major cellular source for these cytokines released is activated microglia (Banati et al., 1993). The proinflammatory cytokines released from activated microglia are considered to play an important role in initiation and progress of the neurodegenerative processes (McGeer et al. 1993, Kreutzburg 1996). In the previous studies it has been shown that beside dopaminergic neuronal involvement after MPTP intoxication, microglia and astrocytic reaction is also observed in the damaged regions

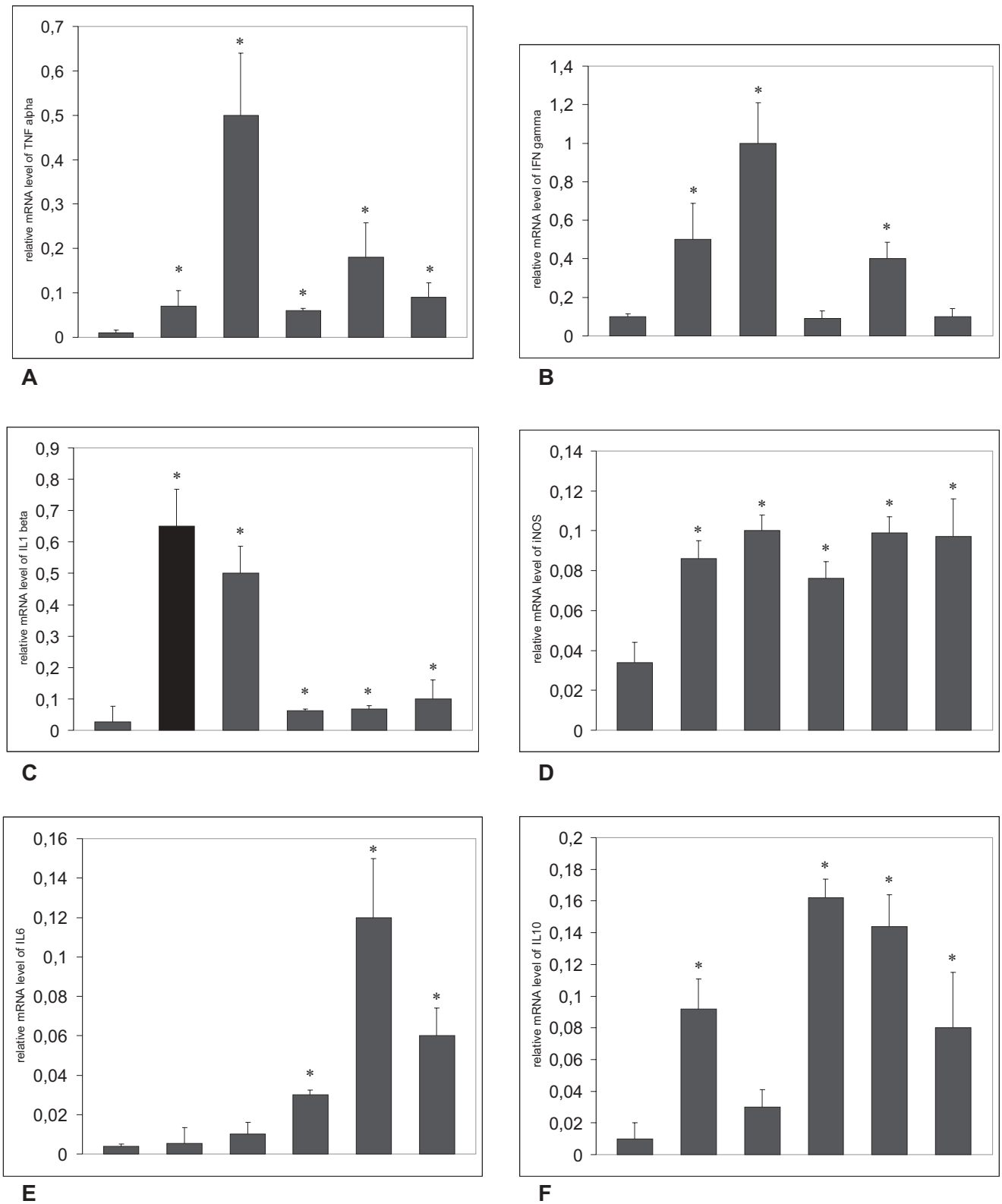


Fig. 2. Dynamic expression of the TNFα (A), IFNγ (B), IL1β (C), iNOS (D), IL6 (E), IL10 (F) mRNA in the striatum of C57BL/6 male mice at various times after the MPTP intoxication. Values are means ± SE (n = 6-8). $^{*}P<0.05$ compared with control mice.

(Kohutnicka et al. 1998). Our findings show that the MPTP intoxication causes rapid increase in mRNA expression for TNFα, IL-1β and IFNγ beginning 6h and peaking 24h after intoxication. Because the time course for expression of these cytokines parallels the time course for microglial activation in the striatum after MPTP injection (Kohutnicka et al. 1998, Kurkowska et al. 1999), it would appear that the activated microglia is a likely source of these cytokines in the MPTP model. They are likely to be the first cytokine proteins which are upregulated in the striatum after MPTP intoxication. This suggests that MPTP can potentially initiate a cascade of events leading to the induction of various cytokines that may interact synergistically to induce cell loss.

IL1β probably plays a crucial role in setting this cytokine network in motion under normal conditions, as it does in other inflammatory conditions and disease states (Lawrence et al. 1998). The present study demonstrated that induction of mRNA for this cytokine is starts rapidly and peaks at 6h after MPTP intoxication. These results may indicate the influence of the initial action of IL1β to further stimulate the subsequent pro-inflammatory cytokines' expression (Sairanen et al. 1997). IL1-β has been implied as a mediator of several forms of neurodegeneration, including ischemic, traumatic and excitotoxic brain injury (Rothwell et al. 1997). Several studies showed a rapid increase in the expression of IL1β in the microglial cells in response to the traumatic or ischemic injury (Goss et al. 1995, Holmin et al. 1997). It seems to be very probable that IL1β is not neurotoxic *per se* (Lawrence et al. 1998). However IL1β may act on glial cells that in turn produce neurotoxic and/or proinflammtory factors, thereby contributing to the neuronal damage. IL1β contribution might be related to the global inflammatory process that takes place after damage of the dopaminergic neurons, including induction of the microglial cells' activation and its intensity. However, neuronal death is not fully prevented by the antagonist of the receptor IL1β (IL-1ra), suggesting that this cytokine is only one of the components that together contribute to the striatal lesion (Lawrence et al. 1998).

TNFα is a potent neurotoxin and also a powerful inflammatory mediator (Akassoglou et al. 1997), that could initiate and maintain an inflammatory process in PD. Our results demonstrate a marked increase in the expression in mRNA of TNFα in the striatum already 6h after MPTP intoxication, with a peak at 24 h. Such a deleterious effect of TNFα may be mediated by a direct action involving receptors for the cytokine. TNFα elicits its biological effects through the activation of two distinct receptors, the p55 (TNFRI) and the p75 (TNFRII) (Louis et al. 1993, Deb et al. 1996). Sriram et al. (2002) using mice lacking both receptors for TNFα showed that they are completely protected against the MPTP-induced neurotoxicity. Theses data indicate that TNFα could be an obligatory component of the damage of dopaminergic neurons. (Sriram et al. 2002).

TNFα acting together with other proinflammatory cytokines, such as IL1, may also play an indirectly deleterious role by inducing expression of iNOS in the activated glial cells (Akama et al. 2000). Our studies reveal that expression of the iNOS mRNA rapidly increased and was higher than in controls as early as 6h after MPTP intoxication and peaked at 24h after the intoxication. NO generated by iNOS is thought to exert its toxic effect mainly by an interaction with the superoxide radicals, leading to formation of the peroxinitrite, a highly oxidizing molecule (Beckman et al. 1990). The presence of nitrotrosine in the SN of patients with PD, and especially in Lewy bodies, supports this hypothesis (Good et al. 1998).

In our study we observed significant MPTP-induced rapid increases in IFNγ mRNA expression with the peak at 24 h after intoxication. IFNγ is a classic T cell cytokine. However, data from the last decade intriguingly suggest that, under certain conditions, IFN may be also produced in the brain (Abbas et al. 2002). Expression of mRNA for IFNγ has been detected in human sensory neurons (Eneroth et al. 1992). The presence of IFNγ positive cells in the brain parenchyma of PD patients was also documented (Hunot et al. 1999). The function of IFNγ mRNA is probably related to the activation of microglial cells. It is also known that this cytokine may induce the synthesis of iNOS in activated microgilal cells (Reis et al. 2002). However the role of this cytokine in MPTP induced degeneration requires further investigation.

Expression of IL10 mRNA in the striatum after MPTP intoxication is an interesting subject, because it has many inhibitory effects on the proinflammatory response. It attenuates production of proinflammatory cytokines, such as TNF (Lee et al. 2002). In the present study, expression of the IL10 mRNA was noticed already 6h after the MPTP intoxication, but after 24 h that expression markedly decreased. After 3 days the expression of IL10 mRNA increased again and reaching

its peak, and it was still increased at the days 7 and 14 after intoxication. The early expression of this cytokine is probably due to attenuation of the inflammative reaction, which starts immediately after injury. Our results suggest that IL10 may have an important function to suppress activated microglia, by the attenuation of mRNA for TNF, IL1 or IFNγ expression, which is observed at the 3rd day post MPTP injection.

Present results are in good agreement with the general opinion that human microglia express not only proinflammatory, but also anti-inflammatory cytokines at the same time (Lee et al. 2002). Our results may indicate that activated microglia is a major cellular component causing inflammatory response in the striatum after the MPTP injection by producing proinflammatory cytokines, but microglia also may have an opposite function by producing IL10 that inhibits the function of microglia in the inflammatory response via autocrine negative feedback loop.

It is interesting that at the 7th day post MPTP injection we observed again an up-regulation of the mRNA transcripts for TNFα and IL1β. The genesis of this late response remained unclear, but it might indicate an involvement of these cytokines in the neuroregenerative processes (Chen et al. 1996, Taskien et al. 2000). The activated astroglial cells could be a source of the second wave of TNFα and IL1β mRNA expression. There are conflicting data regarding the effects and role of the TNFα in neurodegeneration. In vivo and in vitro studies provided conflicting data on the contribution of TNFα to neuronal death (Barone et al. 1997, Nawashiro et al. 1997). The reasons for these discrepancies are not clear, but may be related to the type of injury. The mechanism by which TNFα might produce its effects are not known, although the most likely is through activation of the transcription factor nuclear factor-kB (NFκB), which is thought to be predominantly involved in the neuroprotective action of TNFα (Venters et al. 2001). It seems to be very likely that TNFα may initially exert harmful effects but could improve recovery during the chronic post-injury period.

Recent studies have demonstrated that intrastratial administration of IL1β can enhance compensatory sprouting from residual DA neurons in the ventral segmental area of the midbrain and can induce behavioral improvement in hemiparkinsonian rats (Hansen et al. 1995). There is evidence showing that IL1β does not act directly to induce the survival and plasticity of the DA neurons and that it is possible that the IL1β stimulates the release of dopaminergic neurotophic factors from astrocytes.

In our studies we noticed a moderate increase of the level of mRNA for IL6. This mRNA increase peaked at the 7-days time point. After 14 days we still observed the elevated expression of mRNA for IL6. It seems to be very likely that the delayed increase of IL6 mRNA expression post MPTP intoxication may contribute to the neuroprotective action of IL6. Although IL6 overexpression is generally detrimental and can add to the pathology associated with several CNS disorders, there is evidence that IL6 may also have an anti-inflammatory immunosuppressive action. IL6 also inhibits IFNγ, IL1β or TNFα synthesis (Crowl et al. 1991). IL6 was suggested to play a key role in regulating neuronal survival (Campbell et al. 1998). Bolin at al. (2002) described the increased sensitivity of dopaminergic neurons to a neurotoxicant in the absence of IL6. This result indicates the neuroprotective activity of IL6 in the injured nigrostriatal system.

In conclusion, many cytokines are expressed after thte MPTP intoxication. Present findings suggest that the cytokine network should be studied in detail in the future. By blocking out different cytokines we can probably find out which of them are needed for successful regeneration and which of are harmful.

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