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Dopamine, serotonin and noradrenaline changes in the striatum of C57BL mice following myelin oligodendrocyte glycoprotein (MOG) 35-55 and complete Freund adjuvant (CFA) administration

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Abstract. Many data suggest involvement of inflammation in neurodegeneration. However, the exact mechanisms of this cooperation are poorly understood. We have previously shown that induction of inflammatory reaction, both before and after injury of the striatum, affects regeneration of dopaminergic neurons. In the present research we studied the role of inflammatory reaction in non-injured striatum. We used myelin oligodendrocyte glycoprotein (MOG) 35-55 in complete Freund's adjuvant (CFA) to elicit experimental autoimmune encephalomyelitis (EAE) mice model. As determined by HPLC, striatal dopamine (DA) and serotonin levels in mice treated with either MOG 35-55 in CFA or CFA alone were significantly higher compared to vehicle-treated controls on 13th day after induction. The ratio of homovanilic acid/dopamine (HVA/DA) and 3, 4 dihydroxyphenylacetic acid/dopamine (DOPAC/DA) were significantly lower in the MOG and CFA groups on 13th day, indicating decreased DA metabolism. Noradrenaline (NA) concentration did not differ between groups. Moreover, the striatal mRNA IL-1β and TNF-α levels were elevated during induction phase of EAE in both groups, as determined by RT-PCR. Our data indicate regulatory connection between dopaminergic and immune systems.

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Key words: experimental autoimmune encephalomyelitis, MPTP, dopamine, serotonin

INTRODUCTION

Degeneration of nigrostriatal dopaminergic neurons is the cause of the main symptoms of Parkinson's disease (PD) (Samii et al. 2004, Schulz and Falkenburger 2004, Teismann and Schulz 2004). Whereas the primary reason of PD is not known, many data suggest the involvement of inflammation in the cell loss seen in neurodegenerative diseases (Araujo and Lapchak 1994, Bieganowska et al. 1993, Członkowska and Kurkowska-Jastrzębska 2002). In PD patients alteration of cytokine release from peripheral blood mononuclear cells (PBMC) and elevation of IL-1β and TNF a in the nigrostriatal dopaminergic system, as well as activation of glia were observed (Fiszer et al. 1991, Nagatsu et al. 2000). In animal model of the disease, evoked by 1-methyl-4 phenyl-1, 2, 3, 6-tetrahydropiridine (MPTP) intoxication, extensive inflammatory reaction, involving T lymphocytes influx, microglia and astroglia activation were raported in the striatum (Członkowska et al. 2000, 2001, 2002, Kurkowska-Jastrzębska et al. 1999a,b). We have previously shown that induction of inflammatory reaction in the central nervous system (CNS) by MOG 35-55 administration after nigro-striatal injury, decreased regeneration of dopaminergic neurons (Bałkowiec-Iskra et al. 2003). On the other hand, induction of inflammatory reaction before the nigro-striatal injury protected neurons from the injury (Bałkowiec-Iskra et al. 2004, Kurkowska-Jastrzębska et al. 2005, 2007). In the present study we used, described previously (Bałkowiec-Iskra et al. 2003, 2007), MOG 35-55-induced experimental autoimmune encephalomyelitis (EAE) model to study changes of neurotransmitters and selected cytokines concentration in the not-injured striatum during autoimmune/inflammatory reaction. Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS) that serves as a model for multiple sclerosis (MS) (Bradl and Linington 1996). For a vast majority of mouse strains, EAE is a chronic and monophasic disease with sparse inflammatory lesions in the CNS (Polman et al. 1986). C57BL mice are genetically susceptible to EAE, which can be induced in these animals by a single injection of the myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant (CFA) (Pal et al. 1999).

Neurotransmitters are considered to play a role in the CNS and immune system cooperation. Dopamine, noradrenaline and serotonin have been proved to act as immunomodulators (Jafarian-Tehrani and Sternberg 1999). Noradrenergic, dopaminergic and serotoninergic fiber endings are found among thymocytes in the thymic parenchyma and in the peri-arteriolar lymphoid sheaths of the spleen (White et al. 1983). Moreover receptors for all the three neurotransmitters have been found on lymphocytes, which mediate a multitude of immunomodulatory effects (Qiu et al. 2005).

Although multiple cytokines are elevated in response to CFA and during EAE we choose to assess the concentrations of IL-1β, IL-6, TNF-α and IFN-γ, because these cytokines are not only involved in the pathogenesis of PD but also play pivotal role in the cascade of events involved in the proinflammatory process (Viviani et al. 2004).

METHODS

Animals

We used C57Bl male mice, 8–12 weeks old, 20–25 g of weight.

Mice were divided into 3 groups, 6–9 animals each: (1) control group receiving injection of 0.9% NaCl in both flanks and nape area, in volume identical to used in MOG injection; (2) CFA group receiving subcutaneous injection in both flanks and nape area of CFA in deionized water solution, and introperitoneal pertusis toxin (PT) injection as MOG group; (3) MOG group receiving full induction of EAE.

Mice were bred in a local animal facility and were kept in standard conditions with full access to food and water during experiments. Supplementary food and water were provided on the cage floor for disabled animals.

All experiments and procedures were performed in accordance with the guidelines approved by the Ethical Committee of the Medical University of Warsaw.

Active induction of experimental autoimmune encephalomyelitis (EAE)

EAE was induced by s.c. flank injections of 150 mg of MOG 35–55 peptide (Neosystem, France) in com-

plete Freund adjuvant (CFA) (Difco, USA), enriched with 4 mg/ml *Mycobacterium Tuberculosis*, Difco Laboratories, USA). On day 0 and 2nd after immunization with MOG mice received intraperitoneal injections of 300 μg of PT (LIST Biological Labolatories, USA) which was prepared in distilled water in 1 μg/μl concentration.

Preparation of complete Freund adjuvant (CFA)

CFA solution (Difco Laboratories, USA) was prepared in distilled water in 1:1 proportion. Than solution was enriched with *Mycobacterium tuberculosis* (Difco Laboratories, USA) to the final concentration of 5 mg/ml.

Clinical assessment of animals

A clinical score was assigned daily for 13 days. The clinical score was graded on scale of 0 to 5 with graduations 0.5 for intermediate scores: (0) no clinical signs; (1) flaccid tail; (2) hind limb weakness and abnormal gait; (3) complete hind limb paralysis; (4) complete hind limb paralysis with forelimb weakness or paralysis; (5) moribund or deceased because of EAE. Intermediate scores were assigned if neurologic signs were of lower severity than typically observed.

In the present study animals were sacrificed by spinal cord dislocation either on the day of first signs of EAE (9th day after challange) or eventually when their clinical score was over 2 (13th day after challenge). To the further analysis only animals which clinical score was over 1 were taken (n=6).

HPLC analysis of noradrenaline, dopamine and serotonin content in striatum

HPLC evaluation was performed on the 9th and 13th day following CFA and/or MOG 35-55 administration (single analysis).

Left striata were rapidly dissected from brain tissue and then were weighed, homogenized in 1 ml ice cold 0.1 N HClO4 and centrifuged at 13 000 × g for 15 min. The supernatant was removed and filtered (0.2 μm pore size; Whatman, USA) and examined for its contents of DA, 5-HT3 and NA.

Standard samples of dopamine (RBI), its metabolite DOPAC (3,4 dihydroxyphenylacetic acid; RBI), HVA (homovanilic acid; Sigma, Germany), 5HT (5-hydroxytryptamine; Sigma, Germany), 5-HIAA (5-hydroxyindolacetic acid; Sigma, Germany), noradrenaline (RBI), and their tissue levels were measured using high-performance liquid chromatography (HPLC) with electrochemical detection and glassy carbon electrode. The electrochemical potential was set at 0.8V with respect to an Ag/Ag Cl reference electrode. The chromatograph system consists of an autosampler automatic injector (Knauer Basic Marathon), pump (Mini-Star K-500; Knauer, Germany), an electrochemical detector (L-3500A; Merck, Germany). The mobile phase comprised 32 mM sodium phosphate (Sigma), 39 mM citric acid (Sigma, Germay), 1 mM octan sulfonic acid (Aldrich, Germany), 54 μM ethylenediaminetetraacetic acid (EDTA, Sigma, Germany) in deionized, 18.3 mΩ polished water containing 0.15% acetonitrile (Merck, Germany) and 6.5% methanol (Merck, Germany).

Separation of monoamines was carried out with a C-18 column (250 mm × 4 mm reverse phase, Nucleosil, 5 μm particle size; Macherey-Nagel, Germany) and mobile phase flow rate maintained at 0.8 ml/min. Samples were quantified by comparison with standard solutions of known concentration using HPLC software and area under the peaks was quantified.

Data were collected and analyzed by Eurochrom 2000 for Windows (Knauer).

Cytokine evaluation

RT-PCR (*REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION*)

Total RNA was isolated from right striatum 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 use. 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

IFN-γ	5‘- AGC GGC TGA CTG AAC TCA GAT TGT AG - 3’	
	5’- GGTC ACA GTT TTC AGC TGT ATA GGG - 3’	- size (bp) 243
TNF-α	5’ - GGC AGG TCT ACT TTG GAG TCA TTG C - 3’	
	5’ - ACA TTC GAG GCT CCA GTG AAT TCG G - 3’	- size (bp) 307
IL-6	5’- CTG GTG ACA ACC ACG GCC TTC CCT A - 3’	
	5’- ATG CTT AGG CAT AAC GCA CTA GGT T - 3’	- size (bp) 600
IL-1β	5’- TCA TGG GAT GAT GAT GAT AAC CTG CT- 3’	
	5’- CCC ATA CTT TAG GAA GAC ACG GAT - 3’	- size (bp) 502
GAPDH	5’- TGA AGG TCG GAG TCA ACG GAT TTG GT - 3’	
	5’ - CAT GTG GGC CAT GAG GTC CAC CAC - 3’	- size (bp) 493

Plate 1. cDNA primers used in cytokine evaluation

–20°C until use. The cDNA was amplified using adequate primers (see Plate 1).

Negative control reaction without template or MMLV reverse transriptase was included in PCR amplification with primer set in parallel. As a control to eliminate variations for sample-to-sample differences in RNA extraction and convertion to cDNA, we amplified the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The thermal cycling parameters were as follows: IL-6, IFN-γ, IL1β, TNF-α – 95°C, 2 min; 94°C, 50 s; 60°C, 50 s, 35 cycles; and 72°C, 5 min; GAPDH – 94°C, 5 min; 94°C, 30 s; 57.5°C, 45 s; 72°C, 1 min; 30 cycles; and 72°C, 10 min. PCR products were separated on 1.5% agarose gels stained with ethidium 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. Three cytokine PCR assays per sample were performed.

Statistical analysis

Statistical significance of differences was assessed by one-way or two-way analysis of variances (Statistica). Differences between groups were assessed by Mann-Whitney *U* test. Results were considered significant at level of $P<0.05$.

List of abbreviations

CFA	complete Freund’s adjuvant
CNS	central nervous system
CSF	cerebro spinal fluid
DA	dopamine
DOPAC	3, 4 dihydroxyphenylacetic acid
EAE	experimental autoimmune encephalomyelitis
HPLC	high-performance liquid chromatography
HVA	homovanilic acid

IL-1β	interleukin 1β
MOG	myelin oligodendrocyte glicoprotein
MPTP	1-methyl-4 phenyl-1, 2, 3, 6-tetrahydropiridine
MS	multiple sclerosis
PD	Parkinson's disease
TNF-α	tumor necrosis factor
5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine

RESULTS

EAE course

Mice which received only CFA injection did not develop any signs of EAE, but only peripheral inflammatory reaction.

Clinical signs of EAE were observed in all the animals which received MOG 35-55.

In mice from the MOG group, EAE had a monophasic form. Specifically, on day 9th, the mean clinical score did not exceed 0.5 points. The mean clinical score reached its maximum (2.9 points) on day 13 (Fig. 1).

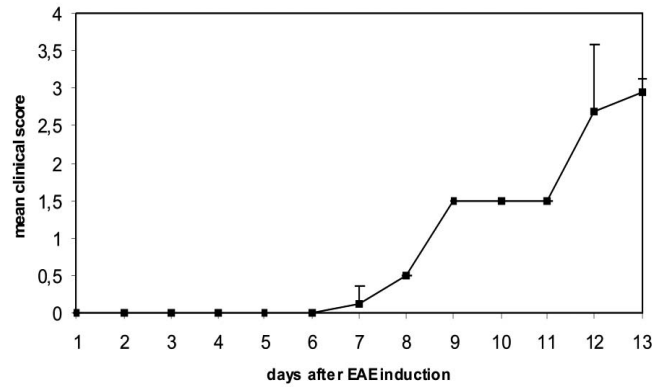

Fig. 1. EAE course. Mean scores of all the 8 animals with EAE induced followed for 13 days. First signs of EAE were observed on 9th day after EAE induction. From 9th to 13th day mean clinical score of mice gradually increased and eventually achieved above 3 points. Incidence from 9 to 10 day was 100%.

DA, NA and 5-HT content changes in striatum during EAE course

Concentrations of dopamine, noradrenaline and serotonin were determined in the preclinical phase (9 days after challenge) and in the acute phase of the disease (13 days after challenge) (Fig. 1).

Dopamine concentration in MOG group increased by 46% on 9th day (P<0. 01) and by 54% on 13th day (P<0.01), comparing to the control level. In CFA group, dopamine concentration increased but not achieved significantly higher level then in the control group on the 9th day and was higher by 86% on 13th day (P<0.0001). On the 13th day the increase in dopamine content in striatum was comparable in CFA and MOG groups (Table I).

NA concentration was not significantly different from the control level in both CFA and MOG groups. However, there was observed a slight tendency to NA content decrease in both groups which was deeper on 13th day than on 9th. Unfortunately differences in control group results impeded analysis of the results (Table I).

Serotonin concentration in both in MOG and CFA groups increased on the 13th day after challenge and was higher by 63% (P<0.002) in MOG group and by 68% (P<0.001) in CFA group comparing to the control level (Table I).

Metabolism of DA and 5HT during EAE course

The levels of DA's final metabolites – DOPAC and HVA did not follow dopamine increase. HVA content did not changed in either group but DOPAC content decreased on the day 13th after challenge in MOG and CFA groups.

The ratios of HVA/DA and DOPAC/DA were significantly lower in the MOG and CFA groups on 13th day (P<0.05), as compared to the control group, indicating a decrease in DA metabolism (Table II).

Metabolism of serotonin, as indicated by 5-HIAA/5-HT values, was not changed (Table II).

Cytokines mRNA expression in striatum during EAE

The mRNA level of four cytokines (IL-6, IFN-γ, IL-1β and TNF-α) was assessed in all animals groups in two time points (9 and 13 days after immunisation).

IL-6 and IFN-γ mRNA level was not changed during observation in both MOG and CFA groups in two time points (Fig. 2C,D).

IL-1β mRNA level was significantly increased in both CFA and MOG groups on 9th day (P<0.01). On 13th day after induction, no differences were found (Fig. 2A).

TNF-α mRNA level was significantly increased in MOG group (P<0.01) and in CFA group (P<0.05) on the 9th day, but not on the 13th day, as compared to

Table I

Noradrenaline, dopamine, serotonin and their metabolite concentrations (pg/mg tissue; mean ± SD)

Experimental group	NA	DA	DOPAC	HVA	5-HT	5HIAA
Control	344 ± 204	6404 ± 2478	4572 ± 1241	2554 ± 581	463 ± 128	305 ± 92
CFA 9	265 ± 43	8158 ± 1118	6810 ± 605*	3178 ± 412	500 ± 130	309 ± 57
MOG 9	272 ± 123	9319 ± 737*	5691 ± 1017	3100 ± 796	537 ± 46	334 ± 59
CFA 13	217 ± 41	11881 ± 726****	2027 ± 256*	2895 ± 318	776 ± 119***	456 ± 110*
MOG 13	206 ± 53	9829 ± 1224*	1452 ± 59*	2213 ± 361	753 ± 84**	436 ± 92*

Mean value of 6 animals/group/time point. *P<0.01 *versus* control; **P<0.002 *versus* control; ***P<0.001 *versus* control; ****P<0.0001 *versus* control.

Table II

Catecholamine and serotonin turnover

Group	HVA/DA	DOPAC/DA	5-HIAA/5-HT
Control	37.5 ± 6.14	62 ± 14.3	62.3 ± 11.6
CFA 9	39.36 ± 5.87	85.3 ± 18.3	67.2 ± 29
MOG 9	33.29 ± 8.37	61.8 ± 14.7	62.3 ± 9.58
CFA 13	24.56 ± 4.4[a,b]	17.2 ± 2.9[a,b,c]	59.13 ± 14
MOG 13	22.5 ± 2.27[a,b]	14.96 ± 2.36[a,b,c]	58.9 ± 17

Mean value of 6 animals/group/time point. [a]P<0.05 *versus* control; [b]P<0.05 *versus* CFA 9; [c]P<0.05 *versus* MOG 9.

control. In MOG group TNF-α mRNA level was significantly higher than in CFA group (P<0.01), indicating differences between groups developing inflammatory and autoimmune disease (Fig. 2B).

DISCUSSION

The main purpose of the study was to determine the effect of inflammatory reaction on the non-injured striatum. Our data showed that during preclinical phase of EAE concentration of DA and 5-HT increased. Additionally, during that phase of EAE the levels of pro-inflammatory cytokines: IL-1β and TNF-α mRNA were increased. CFA treatment caused comparable to MOG administration changes of catecholamine and cytokine mRNA levels. The results suggest that inflammatory/autoimmune reaction might affect non-injured striatum functioning.

The function of inflammation in the pathology of neurodegenerative disorders, including Parkinson's disease has been long studied (Kurkowska-Jastrzębska et al. 1999b, McGeer and McGeer 2004). However, it is still undetermined, whether inflammation causes, contributes to, is a consequence of or possibly even protects dopaminergic neurons from degeneration. Evidence for inflammation presence during degeneration of dopaminergic neurons in the substantia nigra (SN) include numerous reports of focal gliosis as well as increase in classical markers of inflammatory attack (Członkowska et al. 1996, Kohutnicka et al. 1998, Kurkowska-Jastrzębska et al. 1999b).

At the molecular level, a wide range of inflammatory mediators have been implicated in the PD pathophysiology. Upregulation of complement proteins C1-C9, increased levels of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) as well as the transcription factor NF-kB have been reported to be increased in SN in Parkinson's disease (Rozemüller et al. 2000). Similarly, increased level of IL-6 mRNA and many other cytokines has been reported in striatum of mice

treated with MPTP, an animal model of PD (Ciesielska et al. 2003).

At the cellular level, activated microglia has been widely implicated as cellular effectors of brain inflammation in neurodegenerative disorders. Degenerating DA pericarions are initially covered by astrocyte processes and surrounded by ramified microglia (Kurkowska-Jastrzębska et al. 1999b, Lewandowska et al. 1999). This data demonstrate that during PD or after the injury inflammation selectively targets DA system, not resolving, however, what role in the pathogenesis it plays.

We previously showed that during substantia nigra injury caused by MPTP additional vaccination with MOG 35-55 in CFA resulted in worse recovery and less dopamine content renewal (Bałkowiec-Iskra et al. 2003). In the present study we had no proof that inflammation itself may injure striatal dopaminergic system. It can be postulated, that changes in striatal DA and 5-HT together with cytokines mRNA level observed in the present study showed the specific reaction of the striatal system to the inflammatory reaction.

Pretreatment of animals with IL-1b has been reported to significantly increase DA concentration in the hypothalamus as well as to increase the noradrenergic catabolites in the medial hypothalamus (Dunn 1988, Kamikawa et al. 1998, MohanKumar et al. 1998).

IL-1β increases *in vivo* hippocampal serotoninergic functioning and hypothalamic NA, 5-HT and DA release (Shintani et al. 1995). IL-1β has been proved to increase levels of 5-HT in the anterior hypothalamus and in the hippocampus when administered systemically or into the brain. It has been shown that local administration of IL-1 β increases activity of 5-HT in the nucleus accumbens and hippocampus (Song et al.1999). IL-1β was also shown to increase the accumulation of the serotonin metabolite – 5-HIAA, in the hypothalamus (Dunn 1992) as well as in the prefrontal cortex and the hippocampus (Zalcmann et al. 1994). In our study an increase of IL-1β mRNA in the striatum correlated both with an increase of DA and 5-HT levels and a reduced DA turnover (decreased DOPAC/DA

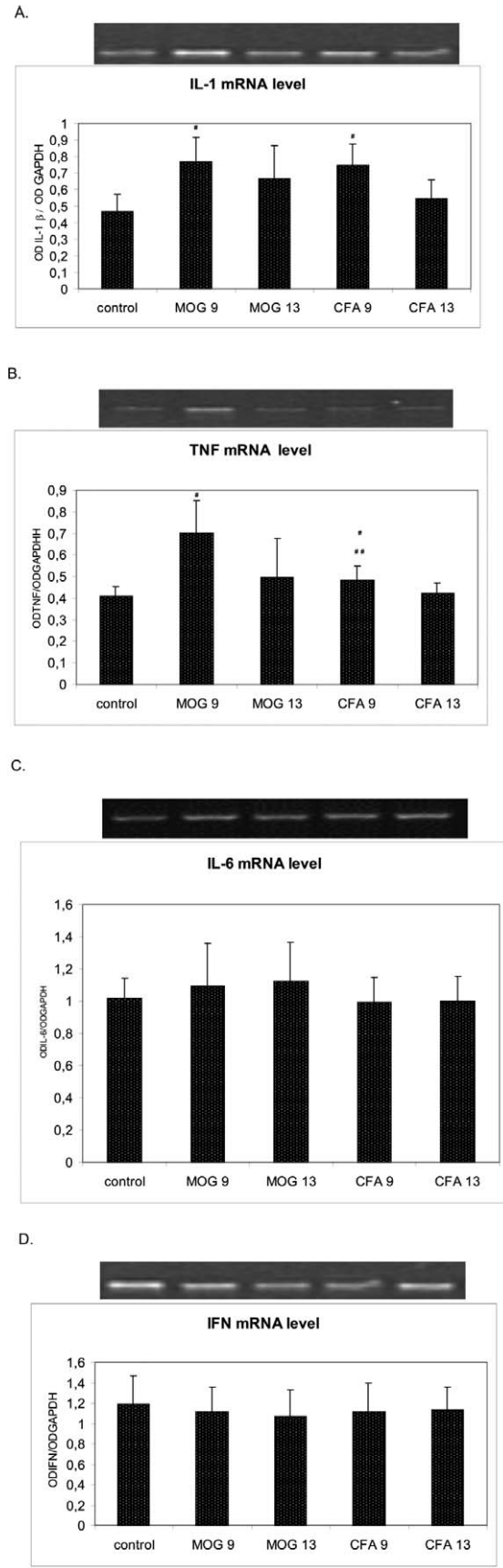

Fig 2. Cytokines mRNA levels in striatum in control and in both experimental groups. Mean value of 6 animals/group/time point. (A) IL-1β mRNA level, $^{*}P<0.1$ comparing to control; (B) TNF-α mRNA level, $^{*}P<0.1$ for MOG group, $^{*}P<0.5$ for CFA, comparing to control (group), $^{**}P<0.1$ comparing to MOG 9 group; (C) IL-6 mRNA level; (D) IFN-γ mRNA level.

ratio). Thus, it may be postulated, that IL-1β serves as a mediator of immune system to evoke neurotransmitter changes within the CNS.

Increased TNF-α production is observed in the CNS after damage due to traumatic injury, ischemia, infections as well as during diseases associated with degeneration of the CNS parenchyma (Wang and Shuaib 2002). *In vitro*, production of TNF-α by astrocytes may be enhanced in response to LPS treatment (Righi et al. 1989). In MS patients elevated levels of TNF-α in CSF have been shown to correlate with severity of the disease (Hofman et al. 1989). TNF-α has also been identified in macrophages, astrocytes, microglia and endothelial cells of acute and chronic active MS brain lesions (Selmaj and Raine 1988). In EAE model TNF-α concentration in CSF and serum also correlates with the peak of symptoms (Munoz-Fernandez and Fresno 1998).

In our study, TNF-α mRNA level was elevated in striatum 9 days after challenge, in the beginning of the clinical symptoms of EAE in MOG group, and also in CFA group indicating that both peripheral stimuli and inflammation in the CNS, induced observed changes. Higher TNF-α elevation in MOG mice than in CFA group might be associated with autoimmune reaction in the brain, however, it should not diminish on the 13th day. According to other studies mentioned above, TNF-α level in CSF and serum correlated well with the peak of symptoms, and in our study striatal TNF-α increased only at the beginning of the acute phase. It is however impossible to resolve at this point if TNF-α had any role in regulation of DA and 5HT production during EAE.

CONCLUSIONS

The present study showed changes of DA and 5-HT concentration in the non-injured striatum during preclinical and acute phases of EAE. We showed, that both DA and 5-HT concentration systematically grew during progression of the disease similarly as IL-1β and TNF-α mRNA levels. Both cytokines and neurotransmitters changes in the striatum were in answer to inflammation and were similar, irrespectively if the inflammation was only in periphery or in the brain. These indicate regulatory connection between nervous and immune systems. It can be postulated, that neurotransmitters and cytokines changes represent the regulation of nigrostriatal system by immune system.

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