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STAT mRNA kinetics in the central nervous system during autoimmune encephalomyelitis in lewis rats

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Abstract : To elucidate the molecular mechanisms of autoimmune inflammation in the central nervous system, we examined the expression and localization of STAT1, STAT3, STAT4 and STAT6 molecules during experimental autoimmune encephalomyelitis (EAE) by competitive PCR. In the present study, we quantitated IL-4 and IL-12 p40 mRNA by competitive PCR in the CNS during EAE. IL-4 mRNA was found at early and peak stages. On the other hand, the IL-12 p40 mRNA level reached maximal levels at the peak stage and still found at the recovery stage of the disease. We examined the kinetics of STAT mRNA in the CNS during EAE and demonstrated that STAT1 and STAT4 mRNA reached a maximal level at the peak stage of EAE, whereas STAT3 mRNA level increased gradually to the recovery stage. STAT6 mRNA increased rapidly at the early stage followed by gradual decrease till the recovery stage. Taken together, these findings suggest that STAT4 which was probably activated by IL-12 plays a pro-inflammatory role and that STAT3 which was activated throughout the disease course seems to serve as a transducer of anti-inflammatory signals.

Key words : STAT mRNA, experimental autoimmune encephalomyelitis, cytokine

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T-cell mediated, inflammatory demyelinatng disease of central nervous system (CNS) that serves as model for multiple sclerosis (MS). Activation of cells and subsequent secretion of cytokines play an important role in autoimmune and inflammatory diseases. Predominant expression of either Th1 or Th2 type cytokines has been associated with active inflammation. The Th1-type cytokines, interleukin-2 (IL-2), interferon-gamma (IFN-γ), interlukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) were highly upregulated in CNS lesions at the active stage of EAE [8, 12, 23]. Th2-type cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10) were upregulated at later stages and thought to counter-regulate Th1 cells and initiate the remission of EAE [2, 10, 19, 26]. However, it remains unclear which molecules activates these cytokines in the CNS of EAE.

To elucidate the immune interactions in the CNS during autoimmune inflammation, it is essential to know the upstream signal pathway of the cytokines released at a particular stage of autoimmune disease. Recently, the molecular mechanisms of the cytokine signal transduction pathway were elucidated [1, 5, 9]. Many cytokines that regulate immune responses activate specific members of STAT (signal transducers and activators of transcription) family of transcription factors [3, 6]. After the binding of cytokines with cytokine receptors, JAKs attached to the receptor are phosphorylated, which is followed by the binding and subsequent tyrosine phosphorylation of STAT molecules. The activated STATs form dimers, move to the nucleus and bind the target gene promoter region to induce gene transcription. It has also become clear

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that the type of STAT activated after the cytokine binding differs depending on the type of cytokine. IFN-γ preferentially activates STAT1, and IL-6 and IL-10 preferentially activate STAT3 [17]. Although the signal transduction pathway has been well characterized, the kinetics of STATs in organ-specific autoimmune inflammation is not well understood.

In the present study, we examined the expression and kinetics of STAT mRNA in the CNS during EAE. We chose STAT1, STAT3, STAT4 and STAT6 for the analysis because these STATs are thought to be highly involved in the cytokine signal transduction in autoimmune inflammation [11, 20, 24]. STAT2 and STAT5, on the other hand, seem to be less involved in the key processes in the inflammation [14]. It was revealed that STAT1 and STAT4, which was activated by IFN-γ and IL-12, respectively, play a pro-inflammatory role at the peak stage of EAE. However, STAT3 which was activated by IL-10, seems to serve as a transducer of anti-inflammatory signals during the recovery stage of the disease.

Materials and Methods

Rats and EAE induction

Lewis rats were purchased from SLC Japan (Shizuoka) and used at 8-12 weeks of age. Active EAE was induced in Lewis rats as described previously [18]. Each rat was injected in the hind footpads on both sides with an emulsion containing 100 μg of guinea pig myelin basic protein (MBP) in CFA (*M. tuberculosis* H37Ra, 5 mg/m*l*). The clinical stage of EAE was divided into four categories (grade 1, floppy tail; grade 2, mild paraparesis; grade 3, severe paraparesis; grade 4, tetraparesis or moribund condition) [15]. For mRNA analyses, rats were killed under ether anesthesia at different time points and several segments of the lumbar spinal cord were snap-frozen in OCT compound. Twenty sections, 20 μm thick, cut in a cryostat were used for RT-PCR analysis. In this study, the early, peak and recovery stages of EAE generally refer to day 10-11 (Grade 1), day 13-14 (Grade 3 or 4) and day 18-20 (Grade 0), respectively.

cDNA synthesis and PCR amplication

Total RNA was extracted from 20 frozen sections using RNAzol B (Biotecx Laboratories, Houston, TX). cDNA was then synthesized by reverse transcription using SuperScript Preamplification System (Life Technologies, Gaithersberg, MD) and amplified in a thermal cycler (Perkine Elmer, Norwalk, CT) using primer pairs for cytokines and STATs (Table 1). The cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min for 40 cycles in a DNA thermal cycler (Perkin Elmer, Norwalk, CT).

Competitive PCR

The levels of cytokine and STAT mRNA were

Table 1. Primer sequences used in this study

Cytokine & STATs		Sequence	Size of PCR product
IL-4	sense	5'-accttgctgtcaccctgttctg-3'	352
	antisense	5'-gttgtgagcgtggactcattcacg-3'	
IL-12(p40)	sense	5'-gaagaagatgacatcacctgg-3'	384
	antisense	5'-ctttggttcagtgtgaccttc-3'	
Stat1	sense	5'-cttgacaacaggagaagggag-3'	341
	antisense	5'-aagaggacgaaggtgcgatcg-3'	
Stat3	sense	5'-agagtcaaggagacatgcagg-3'	485
	antisense	5'-cagtcttgatgactaagggcc-3'	
Stat4	sense	5'-ctcagtggaatcaagtccaa-3'	472
	antisense	5'-ttctcctctcttcccttaagc-3'	
Stat6	sense	5'-ttcctgttcctggcccagaag-3'	463
	antisense	5'-agcgaatggacaggtctttg-3'	

quantified by competitive PCR as described previously [23]. Competitor DNA, a DNA fragment containing the same primer sequences as the target, was prepared using a PCR MIMIC Construction Kit (Clontech, Palo Alto, CA). In competitive PCR, the competitor and the target DNA were amplified using the same primer pair in the same reaction and the PCR products were distinguished by size. After amplification, the PCR products were electrophoresed on ethidium bromide-containing 1.5% agarose gels and were quantitated densitometrically using an FMBIO II image analyzer (Hitachi Software Engineering Co., Yokohama, Japan). When the densities of the target and competitor bands were equivalent, the relative amount of target mRNA was estimated from the amount of the competitor DNA added to the reaction.

Results

Quantitative analysis of IL-4 and IL-12 mRNA in the CNS during EAE

We examined the clinical course of EAE in rats immunized with GPBP/CFA. As shown in Fig. 1, all the rats immunized with GPBP/CFA developed clinical EAE. The mean maximal disease severity was 3.4.

To examine the signal transduction pathway of cytokines, it is essential to know the kinetics of key pro- and anti-inflammatory cytokines. In the present study, we further quantitated IL-4 and IL-12 mRNA by competitive PCR. As shown in Fig. 2A, IL-4 mRNA was found at early and peak stages. On the other hand, the IL-12 p40 mRNA level reached maximal levels at the peak stage and still found at the recovery stage of the disease (Fig. 2B).

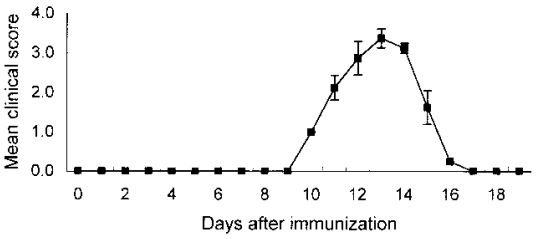

Fig. 1. Clinical course of the acute EAE. Acute EAE was induced by immunization with an emulsion containing 100 μg GPBP and CFA in the hind footpads. Rat developed the clinical signs on day 10, showed the maximal signs on 13 and 14 and recovered by day 17.

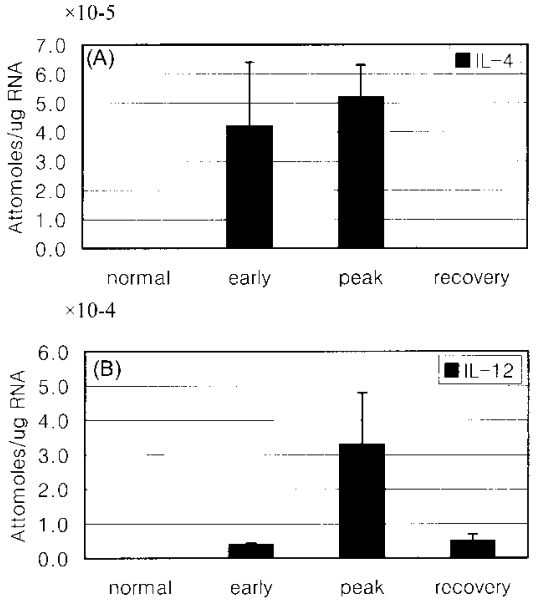

Fig. 2. Competitive PCR analysis of IL-4 (A) and IL-12 p40 (B) mRNA.

Kinetics of STAT1, STAT 3, STAT 4 and STAT 6 mRNA in the CNS

We examined the kinetics of STAT1, STAT3, STAT4 mRNA upon various stages of EAE. The expression of STAT1 (Fig. 3A) and STAT4 (data not shown) mRNA was significantly upregulated at the stage of peak and recovery of EAE. In contrast, STAT3 mRNA level increased gradually to the recovery stage (Fig. 3B).

As we saw a significant difference in the expression of STAT1, STAT3 and STAT4 mRNA in a stage dependent manner, we next performed quantitative analysis of STAT1, STAT3, STAT4 and STAT6 mRNA by the same method employed in the cytokine study in more detail. Before overall examination, we estimated the reliability of the method for quantitation of STAT mRNAs. Target DNA (STAT4) corresponding to 1 μg of total RNA was serially diluted 10 fold and each sample was amplified with a certain amount of competitor DNA. As shown in Fig. 4A, bands representing target DNA were recognizable in lanes 1-6 at the early stage of EAE. The densities of target and competitor DNA plotted on a log-log scale showed good linearity (Fig. 4B). Point 0 of the Log{At/As} indicates that target DNA is equal to competitor DNA. In this case, the amount of STAT4 cDNA was 0.00021 attomole (Fig. 4B).

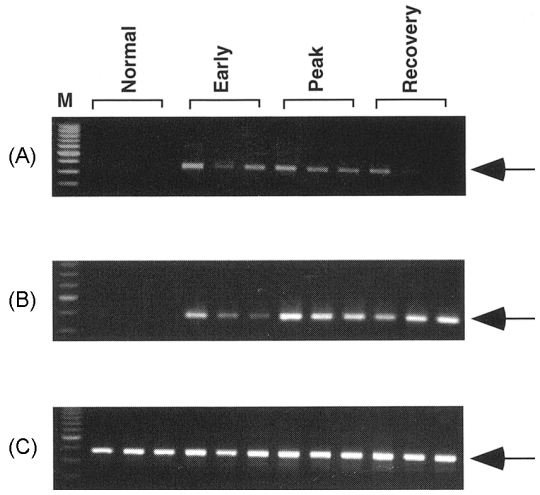

Fig. 3. Agarose gel profile of STAT1 (A), STAT3 (B) and β-actin (C) mRNA by RT- PCR upon various stages of EAE.

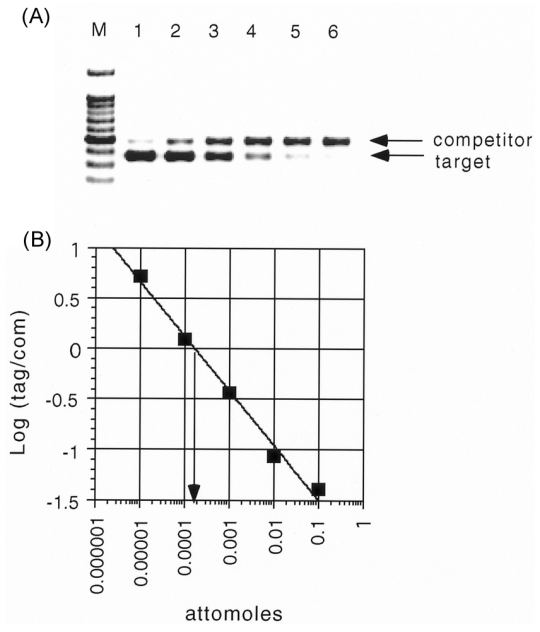

Fig. 4. Agarose gel profile of competitive PCR and determination of amount of STAT4 mRNA. Target DNA corresponding to 0.1 ug of total RNA was mixed with serially diluted competitor DNA(10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) attomoles corresponding to lanes 1, 2, 3, 4, 5, and 6 and amplified by the same primer pair(A). The density of each band was measured using a fluorescence image analyzer and ratios of target/competitor were plotted in log-log scale. In this case, the amount of target DNA was estimated to be 0.00021 attomole.

mRNA of STAT1, which is activated by pro-inflammatory cytokines such as IFN-γ, was upregulated gradually and reached a maximal level at the peak stage of EAE (Fig. 5A). STAT3, transducer of the signal by cytokines such as IL-6 and IL-10 peaked at the recovery stage (Fig. 5B). The kinetics of STAT4, which is activated by IL-12, was essentially the same as that of STAT1 (Fig. 5C). The level of STAT6 mRNA, transducer of IL-4, rapidly increased at the early stage of the disease and then declined gradually thereafter (Fig. 5D).

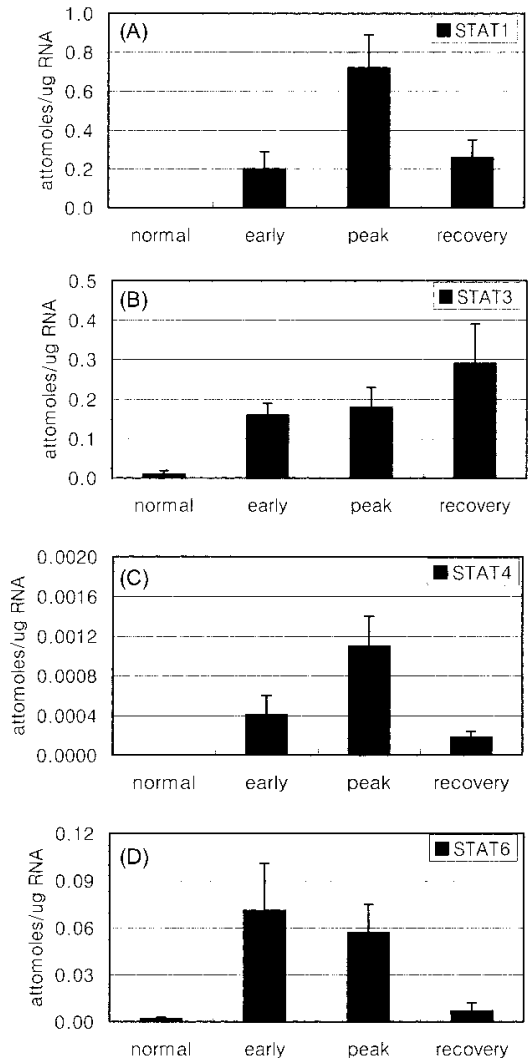

Fig. 5. Quantification of mRNA for STAT1 (A), 3 (B), 4 (C), and 6 (D). The results are expressed as the mean values +S.E.M. (attomoles) of three samples at each time point.

Discussion

In a previous study, we determined the amount of TNF-α, IFN-γ, IL-10 and TGF-β1 mRNA by competitive PCR during the course of acute EAE [23]. Pro-inflammatory cytokine mRNA, IFN-γ and TNF-α, was detectable mainly at the early stages of EAE. Although one of anti-inflammatory cytokines, IL-10 mRNA, was also found at the same stage, TGF-β1 reached a maximum at later stages [23]. In the present study, we examined the kinetics of STAT mRNA in the CNS during EAE development and demonstrated that STAT1 and STAT4 mRNA reached a maximal level at the peak stage of EAE, whereas STAT3 mRNA increased gradually to the recovery stage. STAT6 mRNA increased rapidly at the early stage followed by gradual decrease till the recovery stage.

Based on our previous [23] and present findings along with those in the literature, the interactions between the cytokine and STAT expression can be summarized as shown in Table 2. Since STAT1, STAT4 and STAT6 are activated mainly by stimulation with IFN-γ, IL-12 and IL-4, respectively [13, 22, 24], these cytokines are candidates for stimulators of each STAT. Although STAT3 is activated by many cytokines [17], we assumed that IL-10 [4, 25] is the cytokine most likely to activate STAT3. However, this does not rule out the possibility that pro-inflammatory cytokines such as IL-12 activate STAT3 [9] to upregulate suppressive abilities of some cell type. As reported by many groups, STAT4 is activated by IL-12 [1, 9, 11, 24]. In acute EAE, both IL-12 and STAT4 mRNA showed a maximal level at the peak stage. Since activated microglia induce T-cell proliferation in antigen-specific manner [16], the above findings suggest that the IL-12-STAT4 pathway accelerates autoimmune inflammation. There are very few reports demonstrating that IL-4 protein is actively produced in the normal and diseased CNS [7, 21] although IL-4 mRNA was detectable in this study (Fig. 1). STAT6 which is activated mainly by IL-4 [22] may be less involved in the inflammatory processes. Taken together, it is possible to speculate that this pathways are involved in the regulation of autoimmune inflammation in the CNS.

The present study on the expression and localization of STATs provides information about the downstream of cytokine-mediated activation. These findings suggest that cytokines that activate STAT3 and STAT4, possibly IL-10 and IL-12, are important in the development and convalescence of autoimmune inflammation in the CNS.

Acknowledgements

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Table 2. Relationship between cytokine and STAT expression

Cytokine		STAT	
Candidate cytokine	EAE stage	Corresponding STAT	EAE stage
IFN-γ[a)]	early[b)]	STAT1	peak[b)]
IL-10[a)]	early	STAT3	gradual increase
Il-12[c)]	peak	STAT4	peak
IL-4[c)]	early	STAT6	early-peak

a) Data from Tanuma *et al.* [1].
b) EAE stage showing a maximal level.
c) Low throughout the course of EAE.

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