Induction of mRNA for Tumor Necrosis Factor-α and Interleukin-1β in Mice Brain, Spleen and Liver in an Animal Model of *Shigella*-Related Seizures

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Key words: *Shigella*, seizures, cytokines, tumor necrosis factor-α, interleukin-1β, animal model

Abstract

Background: The pathogenesis of neurological symptoms, the most common extraintestinal complication of childhood shigellosis, is unclear. To elucidate the mechanisms involved, we developed an animal model and demonstrated that TNFα and IL-1β play a role.

Objectives: To determine whether TNFα and IL-1β genes are expressed in the brain following peripheral administration of *Shigella dysenteriae* 60R.

Methods: Expression of mRNA for TNFα and IL-1β was examined in the brain structures (hypothalamus and hippocampus) and peripheral organs by reverse transcriptase polymerase chain reaction, at different time points after intraperitoneal injection of *S. dysenteriae* sonicate.

Results: In our animal model of *Shigella*-related seizures, TNFα and IL-1β mRNA were induced in the brain, spleen and liver already 1 hour after injection of *S. dysenteriae* sonicate. The expression of TNFα and IL-1β mRNA in spleen, hippocampus and hypothalamus decreased after 6 h and increased again at 18 h post-injection.

Conclusions: Local production of TNFα and IL-1β in the brain may be involved in the enhanced seizure response of mice after administration of *S. dysenteriae.* It is possible that intracerebral production of TNFα and IL-1β plays a role in neurological disturbances of human shigellosis.

IMAJ 2000;2:86–90

Neurological disturbances are the most common extraintestinal manifestations of shigellosis in children [1,2]. Of these, convulsions are best documented and appear in 12 to 45% of hospitalized children [2,3]. Other symptoms include severe headaches, confusion and hallucinations, which are often referred to as encephalopathy [1,3,4]. Although usually self-limited, neurological symptoms can be fulminant, leading rapidly to unconsciousness and death [5].

The pathogenesis of the *Shigella*-associated neurological symptoms is unclear. Shiga toxin, the main toxic product of *Shigella dysenteriae*, has been implicated in neurotoxicity since its administration to animals caused paralysis and death [6]. However, most *Shigella* strains currently associated with seizures do not produce Shiga toxin [7,8]. The role of other cytotoxins is unclear, as is the importance of other bacterial virulence traits [8–10].

Elucidation of the mechanisms involved in the neurological manifestations of shigellosis has been hampered by the lack of an animal model that reproduces the neurological manifestations of human infections. We recently developed such a model in mice [11]. It is based on *Shigella*-induced increased sensitivity of mice to the proconvulsant pentylenetetrazole, which induces seizures by its antagonistic activity at the γ-aminobutyric acid (GABA) receptor/benzodiazepine/chloride ion channel complex. Modulation of the severity and rate of PTZ-induced seizures by *Shigella* components and host mediators was used as a measure of their involvement in the neurological processes.

Employing this model we recently demonstrated that TNFα and IL-1β play a major role in the sensitization of the mice to PTZ-induced seizure after administration of *S. dysenteriae* 60R [12]. Plasma TNFα and IL-1β increased in *Shigella*-treated mice while pretreatment with antibodies to murine TNFα or IL-1β prior to *Shigella* administration abolished the increased seizures response [12].

To further investigate the mechanisms by which TNFα and IL-1β enhanced the susceptibility of mice to PTZ-induced seizures after administration of *S. dysenteriae*, we

TNFα = tumor necrosis factor alpha
IL-1β = interleukin 1 beta

PTZ = pentylenetetrazole

undertook the present study to determine whether in our model these cytokines are also induced locally in the brain. To this end, we examined the expression of mRNA for TNFα and IL-1β by the reverse transcriptase polymerase chain reaction in the hypothalamus and hippocampus as well as in the non-neuronal organs, liver and spleen, at different time points after intraperitoneal injection of *S. dysenteriae* sonicate.

Methods

ICR outbred male mice, 25–28 days old and weighing 18–25 g, were maintained under standard conditions.

Preparation of bacterial sonicate

Strain 60R of *S. dysenteriae* serotype 1 was grown in syncase broth for 48 hours with shaking, lysed by sonication and filter-sterilized as described [11]. The bacterial sonicate was analyzed for protein content, cytotoxic activity and lethality in mice [11]. Intraperitoneal injection was performed in a dosage of 4 lethal dose 50.

Determination of lipopolysaccharide concentration in *S. dysenteriae* sonicate

LPS concentration was measured by the Limulus amoebocyte lysate assay method [13]. We thank Eliezer Solomon of Qualified Control Lab. Biotechnology General (Q.C. B.T.G) Scientific Park, Nes Ziona, Israel, for performing the assay.

Isolation of mice total tissue RNA

After decapitation, the brain, spleen and liver were rapidly removed. For localization within the brain tissue, the hypothalamus and hippocampus were dissected and immediately frozen in liquid nitrogen. For each experiment we used five hypothalami and five hippocampi taken from mice that were treated identically, allowing sufficient extraction of total tissue RNA. Tissues were kept at –70°C until RNA isolation. After tissue homogenization, total cellular RNA was extracted with a solution of guanidine isothiocyanate and phenol (Trizol Gibco BRL Life Technologies, Grand Island NY, USA) based on the methods described [14]. Total tissue RNA concentration was measured by spectrophotometric adsorbence (260/280 nm) and its quality was verified by demonstration of mice ribosomal RNA on agarose gel electrophoresis with ethidium bromide.

Analysis of tissue mRNA by RT-PCR

We used 2.5 μg of purified total RNA as a substrate for single-stranded cDNA synthesis, with M-MLV reverse transcriptase (200 U) oligo d (T) primers (0.6 μg) and dNTPmix (1 mM), all purchased from Promega (Madison, WI, USA). The reaction was performed at 42°C for 50 minutes, followed by 5 minutes at 94°C. An aliquot of 6 μl cDMA mix was used to amplify cDNA encoding TNFα and IL-1β and β-actin. The PCR primers used are shown in Table 1. The incubation solution contained: Taq polymerase buffer, $MgCl_2$ (2 mM), dNTP (200 μM), Taq polymerase (1.25 U) (all purchased from Promega, Madison, WI, USA), and the sense and anti-sense specific primers (0.2 μM of each one), in a final volume of 25 μl. The mixture was overlaid with mineral oil and the PCR was run for 30 cycles (unless otherwise specified). Each cycle consisted of denaturation for 1 min at 94°C, annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension for 7 min, and storage at 4°C, in DNA thermal cycler (Perkin Elmer Cetus, USA). A separate single reaction was performed for each gene. An aliquot of 7 μl of the PCR product was electrophoresed in a 2% agarose gel containing ethidium bromide, and photographed.

Table 1. Oligonucleotides primers for PCR used in the study.

Cytokine	Sense primer	Antisense primer	Size of the product
TNFα	5'-TCTCATCAGTTCTATGGCCCC	*	212
IL-1β	5'-TTGACGGACCCCAAAAGATG	**	204
β-Actin	5'-GACTACCTCATGAAGATCCTGACC	***	423

The size of the PCR product obtained by amplification of cDNA (mRNA) is given:

* GGG AGTAGACAAGGTACAAC-3'

** AGAAGGTGCTCATGTCCTCA-3'

*** TGATCTTCATGGTGCTTAGGAGCC-3'

Results

Detection of TNFα and IL-1β mRNA in different organs

Intraperitoneal injection of *S. dysenteriae* serotype 1 strain 60R sonicate markedly increased TNFα mRNA in the liver and spleen, and to a lesser extent in the whole brain, 1 h after the injection. Representative results are shown in Figure 1a. IL-1β mRNA was also markedly increased in liver and spleen of mice 1 h after *Shigella* administration. However, we could not clearly demonstrate an induction of mRNA for IL-1β in the whole brain [Figure 1b]. Saline-treated mice were used as controls. In these mice no expression of TNFα and IL-1β mRNA was detected (data not shown).

TNFα and IL-1β expression in discrete brain structures

For a more thorough study of TNFα and IL-1β production in discrete brain regions relevant to seizure activity, we examined the induction of mRNA for these cytokines in the hypothalamus and hippocampus. An expression of mRNA for TNFα and IL-1β was observed in both structures 1 h after i.p. injection of *S. dysenteriae* sonicate. As shown in Figures 2 and 3, the expression of TNFα and IL-1β was more extensive in the hippocampus than in the hypothalamus.

LPS = lipopolysaccharide

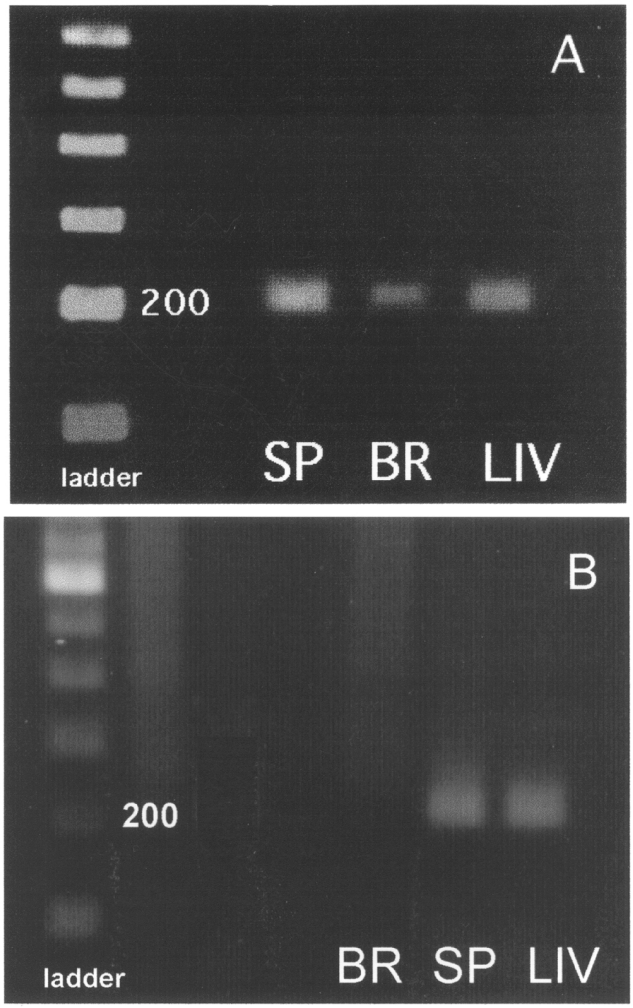

Figure 1. Photograph of representative results of RT-PCR products from spleen, brain and liver of mice amplified 1h after i.p. injection of *S. dysenteriae* sonicate, after electrophoresis on 2% agarose gel and staining with ethidium bromide.
[A]. TNFα. The first column shows the amplification in the liver, the second in the brain and the third in the spleen. A 100 base pair DNA ladder is shown on the left.
[B]. IL-1β. The first column corresponds to the amplification in the liver, the second to the spleen and the third to the brain. A 100 base pair DNA ladder is shown on the left.

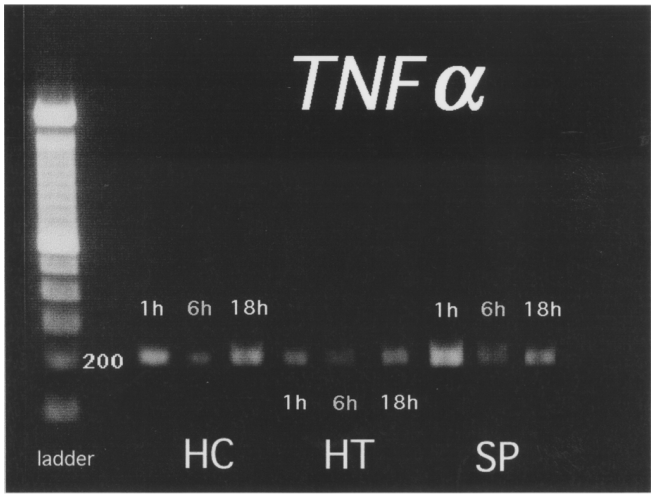

Figure 2. RT-PCR for TNFα of RNA from hippocampus, hypothalamus and spleen of mice amplified 1 h, 6 h, and 18 h after i.p. injection of *S. dysenteriae* sonicate. Photograph of products electrophoresed on 2% agarose gel and stained with ethidium bromide.

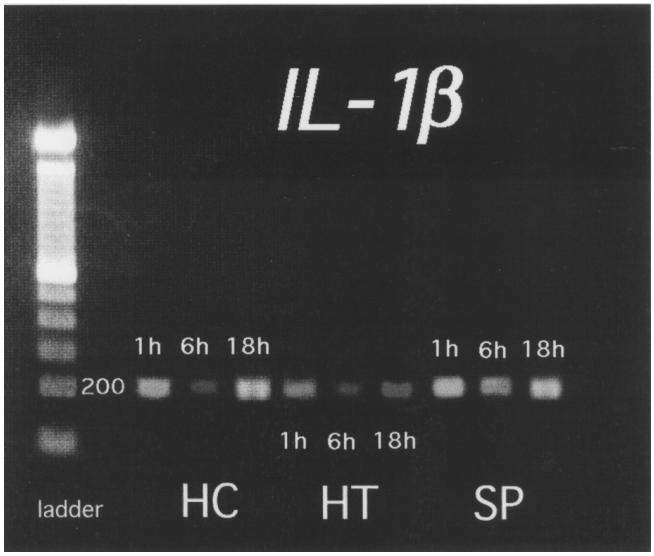

Figure 3. RT-PCR for IL-1β of RNA from hippocampus, hypothalamus and spleen of mice amplified 1 h, 6 h, and 18 h after i.p. injection of *S. dysenteriae* sonicate. Products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

Kinetics of TNFα and IL-1β induction in discrete brain structures and spleen

The expression of mRNA for TNFα and IL-1β was examined at 1, 6 and 18 h after a systemic injection of *S. dysenteriae* sonicate. To obtain the best comparison of mRNA expression at different time points, we compared the expression of TNFα in the hippocampus 1 and 18 h after injection as amplified by different number of cycles. As shown in Figure 4, the most pronounced difference was obtained with 30 PCR cycles and this was therefore used in all further experiments. In the hippocampus and hypothalamus the expression of both TNFα and IL-1β exhibited a similar pattern. After an early induction, already 1 h after injection there was a decrease in mRNA expression of TNFα and IL-1β at 6 h. This expression increased again at 18 h post-injection, exceeding the level at 1 h. As in the brain structures, a similar pattern of mRNA induction for TNFα and IL-1β was observed also in the spleen. There was a reduction in the expression of both cytokines at 6 h after *S dysenteriae* sonicate injection and a second increase in mRNA level at 18 h post-injection. The results are demonstrated in Figure 2 (for TNFα) and Figure 3 (for IL-1β).

Discussion

In a previous study we demonstrated that TNFα and IL-1β are involved in the processes that lead to the enhanced susceptibility of mice to PTZ after administration of *S.*

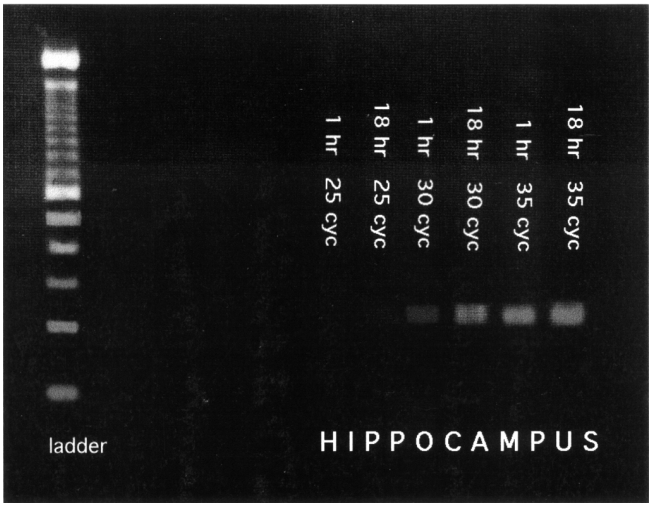

Figure 4. Comparison of RT-PCR products for TNFα in the hippocampus, 1 and 18 h after i.p. injection of S. dysenteriae sonicate amplified by different number of cycles. A 100 base pair DNA ladder is shown on the left. Photograph of products electrophoresed on 2% agarose gel containing ethidium bromide.

dysenteriae sonicate [12]. In the present study we showed that mRNA for both TNFα and IL-1β is also induced locally in the hippocampus and hypothalamus after intraperitoneal injection of *S. dysenteriae* sonicate. Although cytokines can stimulate the central nervous system from the periphery through the afferent nerves [15], it is most probable that the local production of TNFα and IL-1β contributes to the pathological processes inside the brain.

Pro-inflammatory cytokines have been implicated in the pathogenesis of shigellosis in peripheral organs. The production of TNFα and IL-1β was demonstrated in the human colon, and their increased secretion was correlated with the extent of the inflammatory reaction and the severity of tissue damage in the colon [16]. Induction of TNFα mRNA was also observed in mice after injection of Shiga toxin [18]. It has been postulated that in hemolytic uremic syndrome, a complication that often occurs after infection with *S. dysenteriae* or with certain *Escherichia coli* strains which produce-Shiga like toxins, the locally produced TNFα acts in concert with Shiga toxin to exert the renal damage.

It is unclear whether the induction of TNFα and IL-1β in our model is evoked in response to LPS, to Shiga toxin, or perhaps to both. An increase in TNFα and IL-1β expression in the brain after systemic injection of LPS has been reported in several studies [17,19,20]. However, in all of those studies, except one [17], the dose of LPS used was 1–6.5 mg/kg, which was much higher than in our study. The amount of LPS in the sonicate of *S. dysenteriae* injected, as assessed by the LAL method, was 0.5–5 μg/mouse (20–200 μg/kg). Moreover, Satta et al. [20] reported that a low dose of LPS (250 μg/kg) increased IL-1β mRNA only 8 h after intraperitoneal injection, in contrast to the rapid response that we observed in our study.

The changes in the expression of TNFα mRNA with time, which was found in our study, are in agreement with the results of Breder et al. [21] who showed an expression of TNFα in various regions of the brain 1.5 h after i.p. injection of LPS (50 μg/mouse), a decrease at 6 h, an increase at 9 h, and peak expression at 18 h. Interestingly, the kinetics of mRNA for IL-1β within the brain resembles that of TNFα. To the best of our knowledge, this is the first study to demonstrate a second elevation in IL-1β mRNA 18 h after injection.

In conclusion, induction of mRNA for TNFα. and IL-1β in the hippocampus and hypothalamus implies that *in situ* production of these cytokines is involved in the enhanced seizures response of mice after administration of *S. dysenteriae* sonicate. It is possible that intracerebral induction of TNFα and IL-1β is also involved in the emergence of the neurological disturbances in human shigellosis.

LAL = limulus amoebocyte lysate assay

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One can never consent to creep when one feels an impulse to soar.

Helen Keller

Capsule

DNA repair experts

High levels of ultraviolet and ionizing radiation can cause extensive DNA damage and chromosomal breakage that can prove lethal to an organism. White et al. determined the genome sequence of Deinococcus radiodurans, the most radiation-resistant bacterium known. The sequence analysis suggests that several factors contribute to radiation resistance: a large number of DNA repair genes, many of which are redundant; polyploidy, which can allow homologous recombination of double-strand DNA breaks; and the presence of a system that exports damaged nucleotides from the cell. The bacteria's natural resistance to radiation may prove useful in the bioremediation of contaminated radiation and toxic sites.

Science 1999;286:1571