Interleukin-1α, Interleukin-1β, and Tumor Necrosis Factor Gene Expression in Endotoxin-Induced Uveitis

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Purpose. To localize and determine the levels of interleukin-1α (IL-1α), interleukin-1β (IL-1β), and tumor necrosis factor (TNF) gene expression in the process of endotoxin-induced uveitis (EIU) in rats.

Method. EIU was induced by lipopolysaccharide (LPS) injection in male Lewis rats weighing 150 to 200 g. The levels of IL-1α, IL-1β, and TNF gene expression in the iris-ciliary body (ICB) were quantified by a semiquantitative polymerase chain reaction (PCR) method; in situ hybridization histochemistry was carried out to localize the gene transcripts.

Results. Little expression of IL-1α, IL-1β, and TNF genes was observed in normal ICB. IL-1α gene expression began to increase (about 10-fold greater than that of the control) as early as 1 hour after the LPS treatment, reached a peak (about 100-fold) at 3 to 6 hours. A second peak (60-fold) was observed at 24 hours, and the expression returned to near basal levels (3-fold) at 48 hours. Expression of IL-1β and TNF genes showed a pattern similar to that of IL-1α. Three hours after LPS treatment, IL-1β and TNF genes were found by in situ hybridization histochemistry to be expressed by "histiocyte-like" cells in the stroma of the ICB. None of these genes were detected in the control rats.

Conclusions. Expression of IL-1α, IL-1β, and TNF genes was dramatically up-regulated in the process of EIU. These genes were found to be expressed in "histiocyte-like" cells in the ICB, and may have an important role in EIU. Invest Ophthalmol Vis Sci. 1994;35:1107-1113.

Endotoxin-induced uveitis (EIU) is one of several animal models of ocular inflammation. In this model, severe inflammation occurs in the anterior segment of the eye after peripheral administration of lipopolysaccharide (LPS), whereas changes in other organs are minimal. Because LPS induces a variety of inflammatory signals and events, many factors could contribute to the pathogenesis of EIU. Among the chemical mediators that promote and perpetuate EIU, cytokines are thought to play an important role. They are usually produced at the site of inflammation and have the ability to mediate inflammatory responses including prostaglandin release, leukocyte adhesion, and production of other cytokines. Importance of interleukin-6 in EIU was recently reported, but it is still not well known how EIU is induced or how cytokines affect this process.

Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are candidates for a role in the promotion of EIU, although anti-TNF antibody or cytokine antagonists do not suppress EIU. Local production of these cytokines in the process of EIU has not been well documented. There are only recent preliminary reports on the up-regulation of IL-1 and TNF genes in EIU.

Herein, we report that a semiquantitative polymerase chain reaction (PCR) analysis shows that LPS up-regulates interleukin-1α (IL-1α), interleukin-1β (IL-1β) and tumor necrosis factor (TNF) gene expression in the iris-ciliary body (ICB) prior to the inflammatory reaction of EIU. We also show that the cells expressing these genes in the ICB are likely to be macrophage or "histiocyte-like" cells, as revealed by in situ hybridization technique.
MATERIALS AND METHODS

Rat and EIU Induction

Male Lewis rats (6 to 8 weeks old, 150 to 200 g) were used in these experiments, all of which conformed to the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. Lipopolysaccharide (LPS, Salmonella Minnesota, Sigma, St. Louis, MO) was dissolved in endotoxin-free saline at a concentration of 2 mg/ml. One hundred μl of this solution was injected into each hind footpad of the rats, for a total of 200 μg of LPS per rat. Control rats were injected with the same volume of endotoxin-free saline. The anterior segment of the eyes was observed with a slit-lamp biomicroscope to document the development and progression of uveitis at 3, 6, 9, 12, 24, 48, and 72 hours after LPS injection. Severity of uveitis was estimated and scored by clinical features, including iris hyperemia, pupil miosis, cellular infiltration and protein extravasation into the anterior chamber. Signs of uveitis were first seen at 6 hours in most cases, reached a peak at 24 hours, and had cleared by 72 hours, similar to observations described in previous reports.1'2 No inflammatory signs were observed in the control rats treated with endotoxin-free saline.

Quantification of the Protein in the Aqueous Humor

Aqueous humor was collected with a 27-gauge needle under an operating microscope at 3, 6, 9, 24 and 48 hours after the LPS treatment. The total protein content in the aqueous humor was quantified with BCA protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Collection of the ICB

Eyes were enucleated from the rats at 0, 0.5, 1, 3, 6, 9, 12, 24, and 48 hours after the injection of LPS. The eyes were cut into two pieces along the equator, and the posterior segment was discarded. The ICB was collected with fine forceps and the RNA contained therein was extracted immediately. The ICB was similarly collected from saline treated rats at 6 hours after treatment.

Extraction of RNA and cDNA Synthesis

Total RNA was isolated from the ICB by the acid guanidinium thiocyanate-phenol chloroform extraction (AGPC) method.11 Each RNA sample was extracted from the pooled ICBs of both eyes of each animal. The extracted RNA was quantified after which the template cDNA was synthesized with a First Strand cDNA synthesis kit (Pharmacia-LKB, Uppsala, Sweden).

Polymerase Chain Reaction

PCR was carried out by the method of Saiki et al12 with slight modifications. The following conditions were used: denaturation, 95°C for 30 seconds; annealing, 55°C for 30 seconds; extension, 72°C for 60 seconds. The reaction was initiated by adding two units of Taq DNA polymerase, after which 20 to 35 cycles were carried out, depending upon the target gene. DNA thermal cycler and Taq DNA polymerase were purchased from Perkin-Elmer Cetus (Norwalk, CT). The primers used in this experiment are GCCCTAGCATTCTGAAACTGC (sense) and TGAACTCGTGTTGACGATCC (antisense) for IL-1α, GACC-TGTCTTTGAGCCTGAC (sense) and TTCTAGCTGGAAGCCTGAGT (antisense) for IL-1β, AGAACTCCAGCCGCTGTCGTG (sense) and CCT- TGCTCTTGAGGAGGAGACC (antisense) for TNF, and TGGCCACAGTCAAAGCTGAG (sense) and CCTCTGAGTGCCAGTGATGG (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).13-16 The nucleotide sequence of cDNA for rat IL-1β was kindly provided by Ohtsuka Pharmaceutical (personal communication, Tokushima, Japan).

Sequencing of the PCR Product

PCR products were separated by 2% agarose gel electrophoresis and bands of expected sizes (289 bp for IL-1α, 330 bp for IL-1β, 367 bp for TNF, and 388 bp for GAPDH) were extracted. The extracted DNA was subcloned into the pBluescript II vector (Stratagene, La Jolla, CA). Before subcloning, the vector was treated by Eco RV and the T-vectors were made by Taq polymerase and deoxymethylene triphosphate.17 Nucleotide sequencing of the subcloned DNA was carried out according to the dideoxynucleotide chain termination method18 using a Sequenase ver. 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, OH). Double strand template DNAs were denatured by alkaline treatment and the sequencing reaction was initiated by adding T3 and/or T7 primer.

Semi-Quantification by PCR of the Levels of Cytokine Gene Expression

To quantify changes in the levels of cytokine gene expression in EIU, PCR was carried out in a semiquantitative manner as previously described.19 In this method, 2 μCi of radiolabeled dCTP was added to the PCR reaction mixture. The PCR products were electrophoresed, and the bands were excised and the radioactivity incorporated into the DNA was measured by Cerenkov scintillation counting. A standard curve was drawn from the radioactivities by serial dilution of the template cDNA. The relative quantity of the expressed gene in the cDNA was calculated by this standard curve. The cDNA concentration was first normal-
ized by PCR with the primers for GAPDH, an enzyme that is constitutively expressed. Similar experiments were done using specific primers for IL-1α, IL-1β, and TNF to determine the relative expression during the course of the EIU.

**In Situ Hybridization**

For in situ hybridization, the eyes were enucleated 3 hours after LPS or normal saline injection. The eyes were prefixed by perfusion with phosphate-buffered 4% paraformaldehyde, and postfixed overnight in the same fixative. Eyes were then dehydrated in a graded alcohol series and embedded in paraffin. The horizontal sections of 5 μm thickness were pretreated, hybridized and washed, according to the procedure of Noji et al.20

The rat IL-1β cDNA clone was a generous gift from Ohtsuka Pharmaceutical.14 For the synthesis of antisense and sense RNA probes, the rat IL-1β cDNA (full length, about 1.4 kbp) was subcloned into the pBluescript II vector. The radiolabeled RNA probes were transcribed with T7 or T3 RNA polymerase with α-35S UTP (Amersham Japan, Tokyo, Japan). The transcription was carried out using the Riboprobe Gemini II Core System and T3 RNA polymerase (Promega, Madison, WI). Partial alkaline hydrolysis was done at 60°C to yield about 100 to 150 bp. Similarly, RNA probes for rat TNF were made using a 0.7 kbp cDNA fragment. This cDNA (nucleotide No. 812-2433 of genomic DNA) was synthesized by PCR, subcloned to pBluescript II, and confirmed by sequencing. For autoradiography, the slides were immersed in Kodak (Rochester, NY) NTB3 emulsion (diluted 1:1 with water), air-dried, and exposed for 4 weeks at 4°C. The slides were developed and counterstained with Nissl staining.

**RESULTS**

**Aqueous Humor Protein Concentration**

The aqueous protein concentration in normal rats was 2.2 ± 0.3 (mean ± SEM, n = 3) mg/ml. There was no increase in the protein content 3 hours after LPS treatment; at 6 hours after treatment however, protein concentration in the aqueous humor was increased to 5.0 ± 0.5 (n = 3) mg/ml. The protein reached peak

[FIGURE 1. Protein content in the aqueous humor of LPS-treated rats. Protein concentration was measured by using a BCA protein assay reagent. The protein concentration 3 hours after LPS treatment was the same as that of the control. A maximal protein reaction was observed at 24 hours. Data are represented by mean ± SEM (n = 3).]

[FIGURE 2. Electrophoresis of PCR products for IL-1α, IL-1β, TNF, and GAPDH. An ethidium bromide stained agarose gel (2%) is shown. M, marker (fx 174 Hae III digest); lane 1, IL-1α; lane 2, IL-1β; lane 3, TNF; lane 4, GAPDH.]
levels at 24 hours after LPS injection (12.8 ± 0.8 mg/ml, n = 3) (Fig. 1).

**Gene Expression of IL-1α, IL-1β, and TNF in the ICB**

To define the changes in IL-1α, IL-1β, and TNF gene expression in the ICB that occur in EIU, a semiquantitative PCR procedure was carried out. PCR products of the expected size were obtained by PCR using specific primers for IL-1α, IL-1β, TNF and GAPDH cDNA in 3 hours LPS-pretreated rat ICB, as shown in Figure 2. Nucleotide sequencing and restriction pattern analysis revealed that the PCR products were indeed derived from the target cDNA sequences (graphic data not shown). Using control rat ICB, the same size PCR products were obtained but the expression was very little.

Semiquantitative PCR analysis was next carried out to determine changes in the levels of IL-1α, IL-1β, and TNF gene expression. In this experiment, control rats were each injected with 100 μl of endotoxin-free saline into their footpads 6 hours before the ICB was collected. The mean value of the results from two rats was used as the control level, and the levels of gene expression are shown as a ratio to this control. As shown in Figure 3, IL-1α gene expression was 1.2-fold that of the control at 0 hours after LPS injection, was 9.9-fold increased as early as 1 hour, and reached a peak at 3 hours (98-fold) to 6 hours (85-fold), and then decreased once. It then increased again at 24 hours (58-fold), then returned to basal levels at 48 hours (3.1-fold). IL-1β and TNF gene expression patterns were similar to that of IL-1α. They had two peaks, at 3 to 6 hours and at 24 hours, with the second peak being about half the magnitude of the first ones.

**In Situ Hybridization**

Localization of the mRNAs of IL-1β and TNF in the EIU rat was done by in situ hybridization. As shown in Figure 4, the signals of IL-1β was seen in the stroma of the iris and ciliary body in the LPS treated rat, the cells that expressed IL-1β were located in the stromal tissue of the iris and ciliary body, and had a rather large nucleus. A small number of cells that expressed IL-1β mRNA were found also around the limbal blood vessels. Under higher magnification and in the hematoxylin-eosin stained section, the cells that expressed IL-1β mRNA appeared to have vacuoles in the cytoplasm. In addition to ICB, IL-1β mRNA was detected in the choroid (photographic data not shown), but no signals were obtained in the cornea, sensory retina, retinal pigment epithelium, and sclera. In the control (saline treated) eye, IL-1β was not expressed in any segment (Fig. 5). Similar experiments using the sense RNA
probe were carried out, but no signal was detected (photographic data not shown).

Localization of TNF mRNA was examined with same methods, and signals were similarly detected around the same type of cells as was IL-1β (photographic data not shown).

**FIGURE 4.** In situ hybridization for IL-1β in LPS-treated rat eye. (A) Dark-field (original magnification, X25). (B) Bright field (original magnification, X50). (C) Bright field of the ciliary body. Open triangle, nonpigmented epithelial cells; closed triangle, pigmented epithelial cells (original magnification, X200). Positive signal was detected in the stromal tissue of ICB. Under higher magnification, the cells showed large nucleus.

**FIGURE 5.** In situ hybridization for IL-1β in control rat eye. (A) Dark field (original magnification, X25). (B) Bright field (original magnification, X50). No positive signal was observed.

**DISCUSSION**

EIU is an experimental model of ocular inflammation. In this model, there occurs severe, albeit transient, inflammation in the anterior segment of the eye. In addition to EIU, LPS injection induces drastic changes in many organs. Bone-marrow derived cells markedly respond to LPS, and release many inflammatory mediators into the blood. However, this cannot explain why there is such severe ocular inflammation in EIU. Moreover, injection of a small amount of LPS into the eye is known to cause similar inflammation in the eye. Therefore, the ocular inflammation in EIU could be both systemically and locally induced. Cytokines are one of the candidates of such signals in EIU. Among the cytokines, IL-1 and TNF are inflammatory substances that have many functions. These cytokines may play a role in the development of EIU. In fact, it was previously reported that intraocular administration of IL-1 or TNF cause severe inflammation.

In this study, we determined if there is up-regulation of IL-1 and TNF gene expression in EIU. A possible role for cytokines in EIU is indicated by the results of studies in which there was dramatic increase in IL-6
associated with EIU. Preliminary reports on the up-regulation of IL-1 and TNF genes in EIU are available. However, the participation of IL-1 and TNF in the process was not confirmed because Rosenbaum et al were unable to suppress the inflammatory response of EIU with either IL-1 receptor antagonist or a soluble TNF antagonist.

We have clearly demonstrated that IL-1α, IL-1β, and TNF gene expression occurs as early as 1 hour after peripheral administration of LPS. At the peak, which occurs at 3 to 6 hours, gene expression is upregulated 30- to 100-fold greater than that of the control (Fig. 3). The IL-1 and TNF produced in the ICB may play a role in the induction of EIU. It is interesting that when IL-1 and TNF gene expression reached this second peak at 24 hours, the ocular inflammation is subsiding. Because cellular infiltration in EIU is known to begin at about 8 hours after LPS treatment, the increased gene expression seen at 1 hour after LPS treatment cannot be explained by recruited cells.

The next question we wished to address was in regard to the types of cells in the ICB that produce such cytokines. Recent studies showed that many types of cells produce IL-1α, IL-1β, and TNF. In the present study, in situ hybridization histochemistry was carried out and showed that the cells that expressing IL-1β mRNA are located in the stroma of the ICB and choroid. These cells were found only in LPS-treated rats (Figs. 4 and 5). These findings agree with the dramatic upregulation of gene expression shown by our semiquantitative PCR analysis. These cells in the ICB have large nuclei and vacuoles in their cytoplasm (Fig. 4C). Fibroblasts, melanocytes, clump cells, mast cells, macrophages, and lymphocytes are known to be present in the stroma of human ICB. In addition, a large number of bone-marrow derived cells, most of which are macrophages. Judging from the location and morphology, the cells that we observed in the rat ICB are likely to be macrophages or “histiocyte-like” cells. But double staining of the section by using macrophage markers is still needed to draw an unequivocal conclusion. Because no cellular recruitment takes place 3 hours after the LPS-treatment, the cells in the ICB which express IL-1β and TNF genes are thought to be already present in normal ICB. Our finding that aqueous humor protein content had not increased at 3 hours, at which time the in situ hybridization study was performed, is consistent with this hypothesis. The level of serum TNF increases rapidly after LPS injection (peak = 1.5 hours), so “histiocyte-like” cells within the ICB may be secondarily stimulated by other cytokines.

From our results, we conclude that IL-1α, IL-1β and TNF genes are expressed locally in the ICB by “histiocyte-like” cells. IL-1 and TNF have many functions that can directly and indirectly promote inflammation, so it seems logical to hypothesize that locally produced IL-1, TNF, or both could induce EIU.

**Key Words**
endotoxin-induced uveitis, interleukin-1, tumor necrosis factor, iris-ciliary body, macrophage

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**References**