Biphasic Ocular Inflammatory Response to Endotoxin-Induced Uveitis in the Mouse

De Fen Shen, PhD; Margaret A. Chang, BA; Dawn M. Matteson, MS; Ronald Buggage, MD; Alexander T. Kozhich, PhD; Chi-Chao Chan, MD

Objective: To examine the kinetics and mechanisms of endotoxin-induced uveitis in the mouse.

Methods: C3H/HeN mice were injected subcutaneously with 0.3 mg of Salmonella typhimurium lipopolysaccharide (LPS) in 0.1 mL of phosphate-buffered saline solution or phosphate-buffered saline solution alone in 3 separate experiments; mice were killed after 1, 3, 5, and 7 days. In 2 other separate experiments, mice were killed 1, 3, 6, and 24 hours after LPS injection. All eyes were collected for histological examination, immunohistochemical analyses, aqueous protein level determination, and reverse transcriptase–polymerase chain reaction for ocular interleukin (IL)1α, IL-6, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor mRNA (mRNA). Enzyme-linked immunosorbent assay was used to measure tumor necrosis factor α and IL-6 levels in aqueous and serum samples.

Results: Results were consistent for all experiments. Numbers of ocular inflammatory cells and levels of aqueous protein peaked 1 and 5 days after LPS injection. Control mice did not develop inflammation. Serum and aqueous IL-6 and ocular IL-6 mRNA levels peaked at 1 day and subsided at 3 days. However, ocular IL-1α, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor mRNA appeared, peaked, and subsided at 3, 5, and 7 days, respectively. Predominant infiltrating cells were neutrophils at 1 day and macrophages at 5 days. Although no ocular inflammatory cells were detected before 24 hours after LPS injection, tumor necrosis factor α mRNA was noticed at 1 hour, peaked at 3 hours, and disappeared at 6 hours and granulocyte-macrophage colony-stimulating factor mRNA was spotted only at 3 hours after LPS injection.

Conclusions: The ocular inflammatory response to C3H/HeN mouse endotoxin-induced uveitis is biphasic for 7 days. The first wave appears at day 1 and subsides by day 3. A second, higher peak appears at day 5. The 2 inflammatory waves are related to the kinetics of the different cytokines released in the eye. This is in contrast to the rat monophasic endotoxin-induced uveitis model, which has only one peak of intense inflammation associated with cytokine release.

Clinical Relevance: A biphasic inflammatory response associated with cytokine release lasting several days is observed in C3H/HeN mice with endotoxin-induced uveitis. Because human anterior uveitis has a tendency to be recurrent in nature, this might be a better experimental model.
MATERIALS AND METHODS

ANIMALS

In the first study, 3 separate experiments were conducted involving 120 female C3H/HeN mice (National Cancer Institute, Frederick, Md). For the second study, an additional 60 female C3H/HeN mice were used in 2 experiments to evaluate the early stage of EIU. All mice were 6 to 8 weeks old, weighed 18 to 20 g, and were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

INDUCTION OF EIU

In study 1, 0.3 mg of Salmonella typhimurium LPS endotoxin (Difco Labs, Detroit, Mich) in 0.1 mL of phosphate-buffered saline solution was injected into the hind footpads of 97 mice to induce EIU.8 The remaining 23 control mice were injected with 0.1 mL of phosphate-buffered saline solution in the same manner. Mice with EIU were killed 1, 3, 5, and 7 days after LPS injection. Control mice were killed 1 and 5 days after phosphate-buffered saline solution injection.

In study 2, mice were injected in the same manner as in the first study, but those with EIU were killed 1, 3, 6, and 24 hours after LPS injection and controls were killed 3 hours after phosphate-buffered saline solution injection.

HISTOPATHOLOGIC EXAMINATION

Eyes for histopathologic examination were immersed in 4% glutaraldehyde for 20 minutes and then fixed in 10% buffered formalin for at least 24 hours before being embedded in methacrylate. Vertical sections, 4 to 6 µm, were cut through the pupillary–optic nerve axis and then stained with hematoxylin-eosin. The infiltrating inflammatory cells in the anterior and posterior chambers were counted and identified histologically by 2 masked observers using a double-headed microscope. There were 8 to 10 mice in each group for each experiment. All collected data were recorded and analyzed using commercially available software (StatView 5.0; SAS Institute, Cary, NC). This program performs parametric analysis of variance with Bonferroni correction. Statistical significance was set at P<.05.

IMMUNOPATHOLOGIC ANALYSIS

Eyes for immunopathologic analysis were embedded in OCT (optimal cutting temperature) compound (Miles Inc, Elkhart, Ind) and then frozen at −70°C. They were cut into 8-µm sections and then stained for CD4, CD8, CD20 (B cell), and CD68 (macrophage) cell markers using the avidin-biotin complex method as previously described.21 Primary rat-antimouse antibodies were obtained from the following sources: CD4, Southern Biotechnology Associates Inc (Birmingham, Ala); CD8, Biodesign International (Kennebunk, Me); and CD68 and CD20, Serotec (Raleigh, NC). Proper isotype immunoglobulins were used as controls. The secondary antibodies used were a goat-antirat IgM (Accurate Chemical and Scientific Co, Westbury, NY) for CD4 and CD20 and a goat-antirat IgG (American Qualex, La Mirada, Calif) for CD8 and CD68. Slides were developed in 3,3’-diaminobenzidine (Mallinckrodt Inc, Paris, Ky) after incubation with the avidin-biotin-peroxidase complex (Vector Labs, Burlingame, Calif). A series of alcohol washes preceded counterstaining with 1% methyl green, after which

overall severity of ocular inflammation is lower in mice than in rats.

The exact mechanisms of EIU have yet to be elucidated, but cytokines seem to play an important role.7-13 Cytokines are protein signals released by immune cells, the endothelium, and other resident cells that serve as important mediators of inflammation. They are secreted in response to an antigenic stimulus and attract more leukocytes to the area. Cytokines such as tumor necrosis factor α (TNF-α),7,14,15 interleukin (IL) 6,7,16-18 and IL-1α,19,20 are likely to be involved in EIU. However, the kinetics of their release and their roles in determining the nature of the inflammatory response are still unclear. Determining the kinetics of inflammatory cell infiltration and cytokine release throughout EIU would provide valuable insight into the mechanisms of ocular inflammation in EIU and, ultimately, anterior uveitis in humans.

To investigate the mechanisms and kinetics of EIU in the mouse, we used a variety of techniques to analyze the cellular and cytokinetic properties of the ocular inflammation. We found that ocular inflammation in the C3H/HeN mouse with EIU occurred in a biphasic manner for 7 days. Neutrophils were the predominant infiltrating cells during the first wave of inflammation, but macrophages dominated the second wave. Levels of IL-6 peaked during the first wave of inflammation, but levels of IL-1α, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were highest during the second wave. Higher levels of aqueous protein were found during the first wave. The results indicate that the 2 inflammatory phases of EIU are mediated by different cytokines and inflammatory cells and that different therapies for the 2 phases might be needed to combat the inflammatory response.

RESULTS

All 3 experiments in the first study showed similar, repeatable results. Ocular inflammation occurred in mice with EIU, but no inflammation was present in control animals. The results of the 2 experiments in the second study were also consistent with each other.

HISTOPATHOLOGIC EXAMINATION

In the first study, the numbers of inflammatory cells in the eyes of mice with EIU were high 1 day after LPS injection, then decreased at 3 days. However, the num-
slides were assessed and graded (0-3+) according to the number of positively stained cells.

PROTEIN ANALYSIS

Aqueous humor was aspirated from the remaining eyes and pooled. Levels of protein in the aqueous were measured using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, Ill).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assay was performed on the remaining aqueous humor to assess the levels of IL-6, TNF-α, and GM-CSF. Serum samples were also collected and pooled from each time point to assay for IL-1α and β, TNF-α, and IL-6. Kits for IL-6, TNF-α, GM-CSF, IL-1α, and IL-1β protein were obtained from Biosource International (Camarillo, Calif). The sensitivities of the kits were as follows: IL-1α and IL-1β, 15.6 pg/mL; TNF-α, 38 pg/mL; IL-6, 20 pg/mL; and GM-CSF, 15 pg/mL.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

After aspiration, eyes from each time point were pooled and underwent RNA purification as described by Chomczynski and Sacchi.22 They were homogenized in Solution D (guanidinium thiocyanate, 4 mol/L; sodium citrate, 25 mmol/L; 0.5% sarcosyl; and β-mercaptoethanol, 0.1 mol/L), then RNA was extracted by phenol-chloroform. After digestion with deoxyribonuclease, RNA was quantified by measurement of deviation of optical density at a wavelength of 260 nm. Ten micrograms of RNA was used to synthesize complementary DNA using the Superscript II RNase H-Reverse Transcriptase System (Life Technologies, Grand Island, NY) with Random Primers (Promega, Madison, Wis). Polymerase chain reaction amplification of the complementary DNA was then performed as previously described.23 Briefly, 0.5 µg of complementary DNA was added to 20 mmol of each deoxynucleoside triphosphate, 1.3 to 3.0 mmol of magnesium chloride, 3 pmol of 32P-labeled sense primer, 3 pmol of antisense primer, 1 µL of GeneAmp 10× PCR buffer (Perkin-Elmer Corp, Hayward, Calif), 0.5 U of AmpliTaq Gold polymerase (Perkin-Elmer Corp), and enough water to adjust the total reaction volume to 10 µL. Each reaction sample was incubated in a Hybaid PCR Express Thermal Cycler (Hybaid Lt, Middlesex, England). They were preheated for 9 minutes at 94°C and then underwent 40 cycles of 45 seconds at 94°C; 45 seconds at 54°C, 55°C, or 57°C; and 1 minute at 72°C. Each sample was then kept at 72°C for 7 minutes. The magnesium chloride concentration and annealing temperature for each sample were as follows: 3.0 mmol/L and 54°C for IL-6, 1.5 mmol/L and 53°C for IL-1α, and 1.5 mmol/L and 57°C for TNF-α and GM-CSF. Sense (S) and antisense (AS) primers were synthesized by GeneProbe Technologies (Gaithersburg, Md) and were composed of the following sequences: IL-1α-S: 5′-TTACAGTGAAAAACGAAGA-3′, IL-1α-AS: 5′-TGTTTGTCGACATCCTGA-3′, IL-6-S: 5′-TTCCCTCTCGGAAGAGACT-3′, IL-6-AS: 5′-TGTATCCTCTAGAAAGCCT-3′, TNF-α-S: 5′-TCTCTGCTACGTGAAAATTCCGGG-3′, TNF-α-AS: 5′-ACTGGCCAGATTTAGCCTGACG-3′, GM-CSF-S: 5′-TTCCCTGAGTATTGTTGCT-3′, GM-CSF-AS: 5′-TGGATTCAAGCGTGGCGT-3′. The expected sizes of the products are as follows: IL-1α, 416 base pairs (bp); IL-6, 432 bp; TNF-α, 513 bp, and GM-CSF, 430 bp.

At 7 days, the numbers of macrophages decreased, and no CD4, CD8, or B cells were found (Table 2).

AQUEOUS PROTEIN ASSAYS AND ENZYME-LINKED IMMUNOSORBENT ASSAY

Relative to controls, mice with EIU had aqueous protein levels that were highest at 1 day, disappeared at 3 days, reappeared with a smaller peak at 5 days, and subsided at 7 days (Table 3). Aqueous IL-6 levels peaked at 1 day and subsided at 3 days, with no reappearance at 5 and 7 days (Table 3). Aqueous TNF-α and GM-CSF levels were undetectable at all time points.

SERUM ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum IL-6 level peaked at 3 hours (13 050 pg/mL), stayed high at 1 day (2962 pg/mL), then dropped at 3 days (343 pg/mL) in mice with EIU. The level of IL-1α was highly elevated during the early hours (309, 1274, and 767 pg/mL at 1, 3, and 6 hours, respectively), and high TNF-α levels (2012 pg/mL) were found 1 hour after LPS injection.
Ocular IL-6 messenger RNA (mRNA) levels peaked at 1 day in mice with EIU and dropped by the third day after LPS injection (Figure 2). Tumor necrosis factor α mRNA was absent at 1 day but appeared strongly at 3 days in mice with EIU. It slowly decreased in the fifth and seventh days of inflammation (Figure 2). On the other hand, both IL-1α and GM-CSF appeared at 3 days, peaked at 5 days, and decreased at 7 days (Figure 2).

In the second study, TNF-α mRNA in the eyes appeared at 1 hour, intensified at 3 hours, and vanished at 6 hours after LPS injection. Granulocyte-macrophage colony-stimulating factor mRNA was detected only at 3 hours after LPS injection. No IL-1α or IL-6 was detectable 1, 3, or 6 hours after LPS injection (Figure 3).

**Table 1. Number of Ocular Inflammatory Cells in Mice With EIU**

| Days After LPS Injection | 1       | 3       | 5       | 7       |
|-------------------------|---------|---------|---------|---------|
| Experiment 1†           | 22 ± 6  | 14 ± 5  | 102 ± 27| 39 ± 12 |
| Experiment 2†           | 32 ± 4  | 12 ± 1  | 127 ± 22| 53 ± 7  |
| Experiment 3†           | 35 ± 3  | 11 ± 3  | 63 ± 4  | 11 ± 2  |
| Controls                | 3 ± 1   | NT      | 1 ± 0   | NT      |

* Data are given as mean ± SE. EIU indicates endotoxin-induced uveitis; LPS, lipopolysaccharide; and NT, not tested.
† Eight to 10 mice per group for each experiment.

We showed that the numbers of inflammatory cells and the levels of protein in the eyes of C3H/HeN mice with EIU peak 1 and 5 days after LPS injection, with a trough at 3 days, suggesting that the ocular inflammatory response to EIU in the C3H/HeN mouse, the most susceptible strain for EIU, is biphasic for 7 days.

The first, smaller wave of inflammation peaks 1 day and subsides 3 days after LPS injection and is characterized by the influx of mostly neutrophils and fewer macrophages into the posterior vitreous and iris and a sharp peak in ocular IL-6 mRNA and serum IL-6 levels and elevation of the aqueous protein level. This resembles the acute form of human anterior uveitis. The second, larger wave of inflammation peaks 5 days after LPS injection and declines by the seventh day. Macrophages are the predominant infiltrating cells at this time and are present in ocular anterior segments, accompanied by neutrophils and a few cytotoxic CD8 T cells. This is similar to the cellular profile of human subacute anterior uveitis. High levels of ocular TNF-α, IL-1α, and GM-CSF message are found in this wave, but IL-6 is absent. The level of aqueous protein during the second wave is slightly lower than that in the first wave.

Although TNF-α and GM-CSF mRNA are readily detectable in the eye 1 to 3 hours after LPS injection, they decrease at 6 hours, before ocular inflammation (EIU) even develops. Serum TNF-α and IL-1α levels are only measurable during the early hours after LPS injection. The early presence of these 2 cytokines represents the immediate response to endotoxin and might not be re-
High levels of aqueous IL-6 have been observed 1 hour after receiving a subcutaneous injection of LPS. IL-6 is a multifunctional cytokine produced by many cells, including monocytes, macrophages, and endothelial cells. It is now believed to be a chemoattractant in neutrophil-mediated lung and heart reperfusion injuries.

The different cellular profiles of the 2 waves of inflammation seem to be related to the kinetics of different cytokines released in the eye. Inflammatory mediators such as LPS are known to trigger the release of proinflammatory cytokines such as IL-6, which is involved in the inflammatory response. The early persistent elevation in the mRNA expression of multiple cytokines, including TNF-α, IL-1α, IL-6, IL-10, interferon γ, monocyte chemoattractant protein-1, macrophage inflammatory protein-1, and IL-1RA, has been reported in the anterior uvea of rats with EIU.

The early persistent elevation in the mRNA expression of multiple cytokines, including TNF-α, IL-1α, IL-6, IL-10, interferon γ, monocyte chemoattractant protein-1, macrophage inflammatory protein-1, and IL-1RA, has been reported in the anterior uvea of rats with EIU. With the exception of IL-10, the ocular mRNAs of these cytokines are detected almost concurrently with the onset of EIU. Because cytokines are known to modulate each other’s release, it is surprising that the pattern of cytokine release in rat EIU is not more “cascadelf.” For example, IL-1α and TNF-α are traditionally referred to as “master” cytokines in light of their ability to govern the expression of several other cytokines, including IL-6. However, IL-6 protein is found even 2 hours before TNF-α in the rat eye with EIU. These observations suggest that the roles of cytokines in EIU might be different from those expected initially.

In the C3H/HeN mouse eye, IL-6 mRNA is expressed at 24 hours, 2 days before the reappearance of TNF-α and the presence of IL-1α message. The second appearance of TNF-α mRNA occurs 3 days after LPS injection, accompanied by low levels of IL-6 and GM-CSF mRNA. The trough in cellular inflammation at 3 days probably corresponds to the lag time during which inflammatory cells are in the process of responding.

### Table 2. Subtypes of Ocular Inflammatory Cells in Mice With EIU

| Days After LPS Injection | 1 | 3 | 5 | 7 |
|-------------------------|---|---|---|---|
| Neutrophils             | +++| ± | ++| ± |
| Macrophages             | ++| ± | ++| + |
| T lymphocytes           | - | - | + | - |
| B lymphocytes           | ± | - | - | - |

Grading was determined arbitrarily as follows: - , no staining; ±, trace staining; +, mild staining; ++, moderate staining; and ++++, abundant staining.

### Table 3. Aqueous Protein and IL-6 Levels in Mice With EIU

| Days After LPS Injection | 1 | 3 | 5 | 7 |
|-------------------------|---|---|---|---|
| Aqueous protein, mg/mL† | 2.07| 0.03| 1.01| 0.64|
| IL-6, pg/mL‡           | 434| 100| <48| <48|

Grading was determined arbitrarily as follows: - , no staining; ±, trace staining; +, mild staining; ++, moderate staining; and ++++, abundant staining.

**Table 2.** Subtypes of Ocular Inflammatory Cells in Mice With EIU

**Table 3.** Aqueous Protein and IL-6 Levels in Mice With EIU

Figure 2. Reverse transcriptase–polymerase chain reaction amplification showing the kinetics of indicated cytokine messages in eyes of mice with endotoxin-induced uveitis (EIU). Lane 1 indicates 1-day control; lane 2, 5-day control; lane 3, 1-day EIU; lane 4, 3-day EIU; lane 5, 5-day EIU; lane 6, 7-day EIU; and lane 7, negative control. Ocular messenger RNA products were determined using 32P-labeled complementary DNA probes specific for each mouse cytokine and were run in a polyacrylamide gel. These results are representative of all 3 experiments. IL indicates interleukin; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte-macrophage colony-stimulating factor; and HRPT, hypoxanthine guanine phosphoribosyltransferase.
ing to the new cytokine signals. The level of ocular TNF-\(\alpha\) mRNA plateaus at 5 days; in contrast, IL-1\(\alpha\) and GM-CSF mRNA levels rise 5 days after LPS injection. The second, stronger wave of cellular infiltration is composed predominantly of macrophages in the anterior segments and is correlated with the peak in IL-1\(\alpha\) and GM-CSF levels and the sustained high level of TNF-\(\alpha\) mRNA. This synchronous cytokine release bears close resemblance to the pattern of cytokine release in the rat with EIU.8,12

Macrophages are known to secrete IL-1\(\alpha\), TNF-\(\alpha\), and GM-CSF and their messages and are attracted by these cytokines.34-36 Thus, it is possible that the source of these cytokines at 3 days is the initial population of macrophages that appeared in response to endothelial IL-6 during the first wave. In turn, these cytokines might recruit more macrophages and other inflammatory cells to the anterior chamber, iris, and ciliary body of the eye so that a larger peak of cellular infiltration occurs at day 5. Along with the decrease in the number of infiltrating cells at day 7, a decline in the mRNA levels of IL-1\(\alpha\), TNF-\(\alpha\), and GM-CSF is evident. Our findings in this study are in agreement with those of de Vos and colleagues,8 who argue against an early cascadelike induction of intraocular cytokine production during EIU. Instead, they hypothesize that these mediators might affect each other’s production at later stages of the inflammatory response.

In summary, EIU in the C3H/HeN mouse produces a biphasic inflammatory response associated with cytokine release lasting several days. This is in contrast to the rat EIU model, which shows only one main peak of inflammation 24 hours after LPS injection. Because human anterior uveitis has a tendency to be recurrent in nature, the mouse might be a better model than the rat. Further investigations focusing on other mouse strains, analyses of other inflammatory mediators, and various specific targeting agents for cytokines are currently being undertaken in our laboratory and might shed new light on the mechanisms and management of human uveitis.

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Reprints: Chi-Chao Chan, MD, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Building 10, Room 10N103, 10 Center Dr, Bethesda, MD 20892-1857 (e-mail: ccc@helix.nih.gov).

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