Kinetics of Intraocular Tumor Necrosis Factor and Interleukin-6 in Endotoxin-Induced Uveitis in the Rat

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Purpose. To determine the kinetics of tumor necrosis factor (TNF) and interleukin-6 (IL-6) in serum and aqueous humor of rats with different susceptibilities to endotoxin-induced uveitis (EIU), after footpad injection of lipopolysaccharide (LPS).

Methods. Samples were collected from EIU-susceptible Lewis rats and EIU-resistant Brown Norway (BN) rats for up to 72 hours after LPS injection. Specific bioassays were used to measure TNF and IL-6 activity. Northern blot analysis was used to assess intraocular IL-6 mRNA expression.

Results. High levels of TNF and IL-6 were detected in serum of both rat strains early after LPS injection. A second rise in serum TNF was observed at 18 to 20 hours in Lewis rats only. In aqueous humor of Lewis rats, high levels of TNF and IL-6 were observed early after LPS injection (2 to 8 hours) and concomitant with maximal uveitis (18 to 24 hours). Low levels of TNF and IL-6 were found in aqueous humor of BN rats. Ocular IL-6 mRNA was detected at the same time as IL-6 activity was measured in aqueous humor.

Conclusions. The results of this study indicate that both TNF and IL-6 may play a role in the pathogenesis of EIU. The early release of TNF in aqueous humor during EIU suggests that this cytokine may serve as an initial mediator of intraocular inflammation. Furthermore, Northern blot analysis indicates that IL-6 is produced locally during EIU. Invest Ophthalmol Vis Sci. 1994;35:1100-1106.

Although the exact pathogenic mechanisms underlying uveitis are poorly understood, cytokines, which are considered important mediators of immunologic and inflammatory responses, appear to be involved in this disorder. Several cytokines, including tumor necrosis factor-α (TNF), interleukin-1 (IL-1), IL-2, IL-6, and interferon-γ (IFN-γ), have been detected in ocular material obtained from patients with uveitis. Further evidence indicating that cytokines mediate uveitis is derived from experimental models.

We previously analyzed the role of IL-6 in endotoxin-induced uveitis (EIU). EIU can be induced in certain susceptible rats by systemic injection of endotoxin, the lipopolysaccharide (LPS) component of gram-negative bacterial cell walls. This response is maximal at 24 hours after injection and is characterized by an infiltrate of polymorphonuclear cells in the anterior segment, vasodilatation of iris vessels, and protein extravasation into the aqueous humor of the eye. EIU may serve as a useful model for acute anterior uveitis in humans. EIU in young Lewis rats was accompanied by high levels of intraocular IL-6, compared to serum levels, and these intraocular IL-6 levels correlated with the severity of uveitis. Moreover, preliminary studies revealed that resistance to EIU, observed in aged Lewis rats and Brown Norway (BN) rats (regardless of age or weight), is associated with the absence of intraocular IL-6 at 20 to 24 hours after LPS injection. The release of other inflammatory cytokines in the eye during EIU has not been described.

A number of cytokines, including TNF, IL-1α, IL-1β, IL-6, IL-8, and IFN-γ, have been shown to induce uveitis when injected intraocularly in animals.
vitreal injection of IL-6 in Lewis rats induces acute uveitis that resembles the response to endotoxin, according to clinical and immunohistologic analysis. This indicates that IL-6 may participate in the pathogenesis of EIU as a proinflammatory mediator. TNF appears to elicit an almost identical response after intravitreal injection in rabbits, with extravasation of plasma proteins into the aqueous humor of the eye and cells infiltrating the anterior segment.

These data raise the following questions: (1) What are the kinetics of TNF and IL-6 release during EIU in serum, compared to aqueous humor? (2) Is resistance to EIU in BN rats associated with the absence of TNF and IL-6 in serum and/or the eye? (3) Is intraocular IL-6 the result of leakage from the circulation or of intraocular production? The results of this study indicate that both TNF and IL-6 play an important role in the pathogenesis of intraocular inflammation.

MATERIALS AND METHODS

Experimental Design

In this study, 45 young, male, inbred Lewis and 45 young, male, inbred BN rats (Harlan CPB, Zeist, The Netherlands), weighing approximately 150 g, were used. All rats were treated according to the ARVO Resolution on the Use of Animals in Research. LPS (Salmonella minnesota LPS, Lot 89F4007, Sigma Chemical Co., St. Louis, MO) was dissolved in sterile, pyrogen-free saline 0.9% at a concentration of 2 mg/ml. During systemic anesthesia by intramuscular injection of 1 mg fluanisone and 30 mg phenylbutyl citrate (0.1 ml Hypnorm, Janssen Pharmaceutica, Goirle, The Netherlands), 50 /l LPS was injected into each hind footpad. At each time point, that is, before LPS injection (time 0) and 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 48, and 72 hours after LPS injection, uveitis was graded clinically, aqueous humor and blood samples were collected, and eyes were enucleated. Each group consisted of three Lewis and three BN rats.

The eyes were examined clinically with a slit lamp and the uveitis was graded using the following scoring system: iris hyperemia (grades 0 to 2), flare (0 to 1), hypopyon (0 to 1), and miosis (0 to 1). The maximum possible score was 7. Blood was withdrawn during systemic anesthesia by intracardiac puncture, allowed to clot at room temperature, and centrifuged to obtain the serum. Aqueous humor was obtained as described previously. Briefly, during systemic and local anesthesia, with the latter induced by one drop of tetracaine hydrochloride 0.5% (Bournonville Pharma, Almere, The Netherlands), the anterior chamber of the eye was punctured with a 30-gauge needle connected to a syringe, and aqueous humor was collected. The aqueous fluid from both eyes of one rat was pooled. All serum and aqueous humor samples were stored at —20°C before TNF and IL-6 levels were determined. The total protein concentration was determined in aqueous humor samples with the Bradford method, using bovine serum albumin (Sigma Chemical Co.) as a standard. After euthanization by intracardiac injection of 60 mg pentobarbital sodium (0.4 ml Nembutal; Algin, Maassluis, The Netherlands), the eyes were excised and immediately stored at —70°C until RNA extraction.

Cytokine Assays

TNF was measured by a cytotoxicity assay, using a subclone of WEHI-64.13 cells, as previously described. Briefly, 2 × 10^4 WEHI-64.13 cells were cultured in 200 /l RPMI-1640 medium, supplemented with 25 mM HEPES, 10% BCS, 50 /M /-mercapto-ethanol, 100 IU/ml penicillin, 100 /g/ml streptomycin, and serial dilutions of serum, aqueous humor, or recombinant murine TNF standard (Genzyme, Cambridge, MA) in 96-well microtiter plates (Nunc, Roskilde, Denmark). After culturing for 20 hours, the cells were labeled by a 4-hour pulse with 0.5 pCi 3H-thymidine (Amersham, Amersham, United Kingdom), nuclei were harvested, and incorporated radioactivity was counted. The sensitivity of the assay was 0.2 pg/ml of TNF.

Levels of IL-6 were assayed by measuring the proliferation of IL-6-dependent B-9 cells, as previously described. In each experiment, the values were related to a standard; 1 U/ml is the concentration that leads to half-maximal 3H-thymidine incorporation in the assay.

RNA Preparation and Northern Blot Analysis

Total RNA was purified according to the rapid procedure described by Chomczynski and Sacchi. The eyes of the three Lewis rats or three BN rats of each time point were homogenized in guanidine thiocyanate, and total RNA was extracted by phenol-chloroform. Twenty micrograms of RNA per lane was electrophoresed in formaldehyde agarose gels and transferred to nylon membranes (Nytran, 0.45 /m; Schleicher & Schuell, Dassel, Germany) according to standard procedures. After fixation of RNA, the membranes were prehybridized at 65°C in 10% dextran sulfate, 1% sodium dodecyl sulfate, 1 M NaCl, 10 mM EDTA, and 250 /g/ml ssDNA. Hybridization was performed in the same solution with a 32P-labeled murine IL-6 cDNA probe. Membranes were washed with 2X SSC, 1X SSC, and 0.5X SSC (1X SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) at 65°C, and exposed to x-ray film (X-Omat; Kodak, Rochester, NY) with intensifying screens. Ethidium bromide-stained gels and blots were used to ensure that equal amounts of RNA had been loaded.
RESULTS

Uveitis Score and Intraocular Protein Extravasation

The first clinical signs of uveitis were observed 6 hours after footpad injection of LPS in Lewis rats (Fig. 1a). In each group of Lewis rats, LPS induced an almost identical bilateral uveitis. The response increased at 18 hours and reached its maximum at 20 to 24 hours, with iris hyperemia, miosis, flare, and cells in the anterior chamber, forming a hypopyon. At 72 hours, the response declined, but several signs of uveitis (hyperemia, flare, and cells in the anterior chamber) still were observed.

The protein concentration of aqueous humor from Lewis rats (0.27 mg/ml at 0 hour) first increased at 4 hours (1.2 mg/ml), rose at 20 hours, and peaked at 24 hours (4.2 mg/ml) (Fig. 1b).

Although no clinical signs of uveitis could be observed in BN rats at any moment after LPS injection (Fig. 1a), the protein concentration in aqueous humor increased during the period investigated. The concentration at 72 hours (0.61 mg/ml) was almost seven times the concentration at 0 hours (0.09 mg/ml) (Fig. 1b).

Kinetics of TNF and IL-6 in Serum and Aqueous Humor During EIU

TNF and IL-6 were not detectable in serum and aqueous humor of Lewis rats before LPS injection (Fig. 2a). LPS induced the release of TNF in serum, and maximal TNF levels were detected at 2 hours (1567 pg/ml). At 8 hours, the serum levels had returned to preinjection level. TNF reappeared in serum of Lewis rats between 18 to 20 hours, but the levels were lower than during the initial TNF release (382 pg/ml at 20 hours). At 72 hours, serum TNF had again reached the preinjection level. IL-6 was detected at 2 hours in serum of Lewis rats, and peaked at 4 hours.
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(18,250 U/ml), 2 hours after the first serum TNF peak (Fig. 2a). Serum IL-6 returned to the preinjection level at 12 hours, and did not increase in response to the second peak of serum TNF (at 20 hours) in Lewis rats.

LPS induced a biphasic release of TNF in aqueous humor of Lewis rats. The level of TNF in aqueous peaked at 4 hours (813 pg/ml) and 22 hours (2315 pg/ml) (Fig. 2b). High levels of IL-6 were observed in aqueous humor of Lewis rats early after LPS injection, at 4 hours (11,056 U/ml) and 8 hours (5080 U/ml), respectively (Fig. 2b). After returning to the preinjection level, between 10 and 16 hours, IL-6 reappeared at 18 hours and peaked 2 hours after the second aqueous humor TNF peak, at 24 hours (13,196 U/ml), the moment at which the uveitis score and aqueous humor protein concentration were maximal.

**TNF and IL-6 Release in BN Rats After LPS Injection**

LPS induced almost identical kinetics of TNF and IL-6 release in serum of BN rats (Fig. 3a), as compared to Lewis rats. Peak levels of TNF (2593 pg/ml) and IL-6 (29,166 U/ml) were observed at 2 and 4 hours, respectively. Serum TNF returned to preinjection level at 10 hours, serum IL-6 at 14 hours; however, a second rise in serum TNF at approximately 20 hours after LPS injection, as found in Lewis rats, was not observed.

In aqueous humor of BN rats, TNF and IL-6 were detected at several points in time after LPS injection (Fig. 3b), but the levels were low compared to the levels in aqueous humor of Lewis rats. The highest level of TNF in aqueous humor was found at 10 hours (80 pg/ml), and a significant level of IL-6 was observed in aqueous humor of BN rats at 6 hours only (3357 U/ml).

**Intraocular IL-6 mRNA Expression After Footpad Injection of LPS**

IL-6 mRNA was not detected in ocular extracts of Lewis or BN rats before LPS injection. In Lewis rats, LPS induced intraocular IL-6 mRNA expression at several points in time (Fig. 4). Expression of IL-6 mRNA was observed at the same points in time that IL-6 activity was found in the aqueous humor. At 2 to 4 hours, five IL-6 mRNA species were observed, with molecular weights of 2.4, 1.2, 1.0, 0.8, and 0.5 kilobases (kb), respectively. At 24 hours, the IL-6 probe hybridized only with the mRNA species of 2.4 and 1.2 kb.

In BN rats, intraocular IL-6 mRNA expression was observed at 2 to 8 hours (Fig. 5). Five mRNA species were observed, with molecular sizes of 2.4, 1.2, 1.0, 0.8, and 0.5 kb, respectively. IL-6 mRNA expression was not observed at other time points.

**DISCUSSION**

The current study demonstrates that EIU in the rat is accompanied by high intraocular levels of TNF and IL-6. In serum of EIU-susceptible Lewis rats, as well as EIU-resistant BN rats, TNF and IL-6 appeared early after LPS injection. The peak of TNF at 2 hours is consistent with the results obtained by others who studied EIU or other LPS-mediated models in the rat. The IL-6 peak at 4 hours is in accordance with the results of our previous work, and with results obtained in human volunteers after LPS injection. The observed time lag between the release of TNF and IL-6 also has been observed in LPS-treated baboons and human volunteers, and in patients with meningococcal infection, and suggests that TNF may be an intermediate between the LPS challenge and the IL-6 response in this stage of EIU.

An unexpected observation was the second peak of serum TNF at 18 to 20 hours in Lewis rats, which
was not followed by a subsequent release of IL-6 in serum. The trigger of this second TNF release is unknown. It is striking that this second release of TNF coincides with the exacerbation of uveitis. Influx of plasma TNF and IL-6 into the anterior chamber during the breakdown of the blood—aqueous barrier that accompanies EIU may not only have contributed to the levels of these cytokines in aqueous humor, but also to the ocular inflammatory response.

In aqueous humor of Lewis rats, a biphasic release of both TNF and IL-6 was observed after LPS administration, with peaks early after LPS injection, during the stage in which clinical signs of uveitis were absent or only mild, and concomitant with maximal uveitis. Northern blot analysis revealed intraocular IL-6 mRNA expression in Lewis rats both soon after LPS injection and at 24 hours, the same moments that IL-6 activity was detected in aqueous humor. Using reverse transcriptase—polymerase chain reaction-assisted mRNA analysis, we have confirmed these results (De Vos et al., manuscript in preparation). This observation indicates that IL-6 is produced locally, and may explain the higher levels in aqueous humor than serum observed in the current and previous studies.\(^5,7\)

\[\text{The meaning of TNF and IL-6 in aqueous humor concomitant with maximal uveitis, observed in the current study and in a repeat experiment (data not shown), is unclear. This holds as well for the second release of serum TNF. Further experiments in which TNF and IL-6 activity is blocked after initiation of EIU are required to determine the contribution of these cytokines to the inflammatory response.}\]

The source of these cytokines in the eye remains to be determined. It seems plausible that soon after LPS injection, ocular resident cells (ie, macrophages, endothelial, and epithelial cells) are responsible for the presence of these cytokines in aqueous humor, whereas infiltrating cells, such as polymorphonuclear cells and monocytes, may contribute to the level of these cytokines during the inflammatory stage. For example, retinal pigment epithelial cells have been shown to produce IL-6 in vitro in response to various stimuli, including IL-1\(\alpha\), IL-1\(\beta\), and TNF-\(\alpha\).\(^{26-28}\) In situ hybridization analysis is required to identify the cells in the eye that produce TNF and IL-6 during EIU.

Although no clinical signs of uveitis were observed in BN rats after LPS injection, these rats did manifest an ocular response, as evidenced by an intraocular IL-6 mRNA expression, elevated protein concentrations, and low levels of TNF and IL-6 in aqueous humor. The latter observation indicates that resistance to EIU in BN rats is not associated with the absence of these cytokines in the eye. The different susceptibility to EIU of Lewis and BN rats appears to be the result of local differences (ie, in the blood–aqueous barrier), because BN rats and Lewis rats showed similar TNF and IL-6 serum kinetics early after LPS injection, and, like Lewis rats, BN rats develop severe uveitis in response to intravitreally injected LPS (data not shown). Breakdown of the blood–aqueous barrier may be initiated by binding of circulating LPS or cytokines to vas-
cular endothelial cells of the uvea, leading to activation of endothelial cells and ultimately to increased vascular permeability and the triggering of other ocular resident cells. In BN rats, vascular endothelial cells or other cells in the uvea may not have been activated by circulating LPS or cytokines. Further studies are needed to compare the ability of vascular endothelial cells and other resident uveal cells of EIU-resistant and EIU-susceptible rats to respond to LPS. Resistance to EIU of BN rats also may be related to differences in the number of certain ocular resident cells, compared to Lewis rats. In this regard, it has been reported that BN rats contain fewer mast cells in the anterior uvea than Lewis rats, and that the number of these cells correlates with susceptibility to EIU. It has been hypothesized that inflammatory mediators released by these cells may be important in the development of EIU.

The pattern of intraocular IL-6 mRNA species observed in this study appears to be more complex than previously described. Northemann et al originally described cultured rat peritoneal exudate cells expressing two IL-6 mRNA species, with molecular weights of 2.4 and 1.3 to 1.2 kb, due to alternative polyadenylation. These two mRNA species also were detected in the spleen, liver, kidney, lung, and bowel 2 hours after systemic LPS injection in rats. In addition, in the latter study a third IL-6 mRNA band, between the 2.4 and 1.2 kb species, was observed in some experiments. The three additional IL-6 mRNA species we observed may originate from tissue- or cell-specific and/or trigger-specific expression. In this regard, it has been shown that different IL-6 mRNA species are expressed by human monocytes or fibroblasts, and that mRNA species of TNF-α and IFN-α of different molecular weights are expressed in response to different inducers. Further research is required to determine the origin of these mRNAs, and whether these species code for functional proteins.

TNF has been postulated to serve as an inducer of IL-6 in several models. Administration of TNF in vivo induced circulating IL-6. Furthermore, anti-TNF antibodies suppress the release of IL-1 and IL-6 during bacteremia. The observed pattern of systemic and intraocular TNF and IL-6 release suggests that the interactions between TNF and IL-6 during EIU are more complex. The early release of TNF in aqueous humor during EIU suggests that TNF may serve as an initial mediator of ocular inflammation. We are currently studying the uveitogenic effect of intravenously injected TNF in rats, as well as the effect of neutralizing anti-TNF antibodies during EIU. It is anticipated that such studies will help to elucidate the exact contribution of TNF to EIU, and the network in which TNF interacts with IL-6 and other cytokines during EIU.

Key Words
endotoxin-induced uveitis, rats, cytokines, tumor necrosis factor, interleukin 6

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