Analysis of Interleukin-6 in Endotoxin-Induced Uveitis

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The mechanisms underlying the induction of intraocular inflammation in the rat model of endotoxin-induced uveitis (EIU) and the subsequent development of tolerance after repeated endotoxin injections are poorly understood. Interleukin-6 (IL-6) was measured in the aqueous humor and serum of Lewis rats after single and repeated injections of endotoxin into the footpad. After a single injection, a rise in serum and aqueous-humor levels of IL-6 was seen after 2 and 16 hr, respectively. The highest aqueous-humor level of IL-6 was seen 20 hr postinjection and was tenfold that seen in the serum sample taken at the same time, suggesting intraocular synthesis of this cytokine. Four hours later the most active uveitis and the highest total aqueous-humor protein level were observed. Repeated injection of endotoxin still resulted in a moderate but significant systemic release of IL-6 but no detectable IL-6 in the aqueous humor and the absence of uveitis. Intravitreal injection of endotoxin-free human recombinant IL-6 (10^{-10} U) in rats resulted in uveitis, resembling the ocular response to endotoxin. There appeared to be a prozone effect regarding the total aqueous-humor protein concentration. The largest amount of aqueous-humor protein was seen in the eyes injected with 10^2 U of IL-6, but increasing concentrations of intravitreal IL-6 showed a corresponding decrease in protein levels. In the fellow saline-injected eyes, a clear consensual response was observed with regard to the extravasation of protein, although the uveitic grade in these eyes was low or zero. Repeated intravitreal injection of IL-6 resulted in ocular unresponsiveness in nine of 11 rats. Intravenous injection of 10^5 U of IL-6 did not evoke uveitis in rats and did not result in detectable levels of IL-6 or increased levels of protein in the aqueous humor. It appears that IL-6 is a potential inflammatory mediator in EIU in the rat and that intraocular release of IL-6 rather than leakage from the serum is associated with the development of uveitis. The endotoxin tolerance observed in this model may be closely related to the absence of IL-6 in the aqueous humor which occurred after repeated endotoxin injection.

Endotoxin, the lipopolysaccharide (LPS) component of gram-negative bacterial outer membranes, is capable of inducing a dose-dependent inflammatory reaction in many species. Specific ocular effects of LPS have been reported after systemic or intraocular administration. Footpad injection of LPS in rats results in an acute bilateral intraocular inflammatory response mainly in the anterior uvea known as endotoxin-induced uveitis (EIU). It has been suggested that this phenomenon could serve as a model for some types of human uveitis such as those associated with seronegative arthritis, where infection with gram-negative bacteria may play a role in pathogenesis. Repeated footpad injections of LPS results in a state of refractoriness or LPS tolerance, and the rats do not develop uveitis. This phenomenon also occurs in the rabbit EIU model after repeated intravenous injections of LPS. Tolerance to the systemic effects of LPS has been well described in humans and experimental animals.

Although the ocular effects of LPS are well documented, the exact mechanisms of uveitic initiation and tolerance development are still unclear. Arachidonic acid metabolites such as prostaglandin E2, thromboxane B2, and leukotriene B4 have been implicated as important mediators in EIU. Lipoxygenase and cyclooxygenase antagonists, however, inhibit the inflammatory response only partially, suggesting that other mediators may be involved in the cellular response observed in EIU. Activation products of the complement system are unlikely candidates, however, since depletion of complement in vivo by cobra venom factor did not inhibit EIU in rats and rabbits.

A group of potent inflammatory mediators that has gained much interest in recent years is the group of interleukins (IL) or cytokines, especially IL-1, tumor...
necrosis factor (TNF), and IL-6. Both IL-1 and TNF have been shown to induce an acute ocular inflammatory response when injected into rabbit eyes. It is plausible that these and other cytokines are also released during EIU, since LPS is a well-recognized inducer of TNF, IL-1, and IL-6, both in vitro and in vivo. The role of IL-6 in EIU because, unlike IL-1 and TNF, it can be detected in many different biologic fluids without difficulty using a murine hybridoma bioassay. Furthermore, the availability of human recombinant IL-6, which is active in the rat in vivo, enabled us to test the uveitogenic properties of this cytokine after intravenous or intravitreal administration.

Materials and Methods

Rats and Reagents

Male inbred Lewis rats weighing approximately 150 g (6-8 weeks old) were used in this study. All animals were treated according to the ARVO Resolution on the Use of Animals in Research. The LPS (Salmonella minnesota, Lot 83F-4000) was obtained from Sigma (St. Louis, MO).

Human recombinant IL-6 (HrIL-6), purified to homogeneity as described previously, with a specific activity of 10^9 U/mg, was used for intravitreal and intravenous injections. As judged by the limulus assay with S. minnesota LPS as a standard, the diluted preparation of 0.1 mg/ml of HrIL-6 contained less than 30 ng endotoxin/ml.

Experimental Procedures

Measurement of IL-6 after LPS injection: The LPS was dissolved in sterile physiologic LPS-free (confirmed by the limulus assay) saline at a concentration of 2 mg/ml, and 100 µl was injected into one footpad, the total dose of LPS per rat being 200 µg. Control animals received an equal volume of saline.

Aqueous humor was collected essentially as described by Battacherjee et al. Rats were anesthetized with intramuscular 1 mg fluanison and 0.0315 mg fentanyl citrate (0.1 ml Hypnorm; Janssen, Goirle, The Netherlands). Then, under the operating microscope, the anterior chamber was punctured with a 27-gauge needle, and the aqueous humor was collected in calibrated microcapillaries. Great care was taken to ensure that no damage was caused to iris or lens. An intracardiac blood sample was taken at the same time the aqueous humor was collected.

Each serum and aqueous humor sample was pooled from four rats (eight eyes). Altogether ten groups of four rats were used. Samples were taken before LPS injection and 2, 4, 8, 16, 20, 24, 40, 48, and 139 hr afterwards with a separate group of rats used for each sample. Samples were also taken from two control groups of four rats at 4 and 24 hr after injection with saline.

Measurement of IL-6 after repeated LPS injection: The serum and aqueous humor samples were taken and pooled from a group of two rats (four eyes) before LPS injection and 1 day later. Next, the animals were reinjected with LPS on day 7 and samples collected on day 8. Samples were also taken on day 68 before a third injection of LPS and 24 hr later (day 69). In addition samples were taken from a control group of two rats using the same protocol except that sterile saline was used instead of LPS. The total protein concentration was determined in all aqueous samples by the method described by Bradford. Measurements of IL-6 were made on every serum and aqueous humor sample.

Intravitreal IL-6 injection: Intravitreal injections were done under the operating microscope similar to Forrester et al. An airtight system consisting of a 50-µl syringe attached to a repeating dispenser (both Hamilton, Reno, NV), fine (PE-10) polythene tubing, and a 30-gauge needle (without hub) were used to deliver precise 1-µl volumes. The rats were anesthetized as described.

Just before the injection, HrIL-6 was diluted in saline to final concentrations of 10, 10^2, 10^3, 10^4, or 10^5 U/µl. An intravitreal injection of 1 µl of HrIL-6 was given into the left eyes of five groups of three rats each with each group receiving a different concentration. All rats received 1 µl of saline in their fellow eyes. After 24 hr uveitis was graded clinically, and the aqueous humor was collected. Samples of aqueous humor from the left eyes and right eyes, respectively, were pooled for each group, and the protein concentration was determined.

The rats were allowed to recover and on day 14, the left eyes were reinjected with 10^3 U of HrIL-6, while the right eyes received saline. Uveitis was graded the next day.

Intravenous IL-6 injection: Three rats were injected with 10^3 U of HrIL-6 in the tail vein. The next day, the eyes were examined for signs of uveitis, and the aqueous humor was obtained to determine the levels of protein and IL-6.

Grading of uveitis: The eyes were examined biomicroscopically, and the uveitis graded using the scoring system in Table 1.

IL-6 bioassay: Levels of IL-6 were measured by a 3H-thymidine (3H-dThd) uptake assay using the murine hybridoma cell line B9, essentially as described by Aarden et al. Briefly, samples to be tested were titrated in twofold dilutions starting at 1:20 dilution, and 100-µl aliquots were placed in 96-well flat-bot-
Table 1. Scoring system for clinical evaluation of uveitis

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Grade of uveitis (score)</th>
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<tbody>
<tr>
<td>Iris hyperemia</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>3</td>
</tr>
<tr>
<td>Pupil</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Miosis</td>
<td>1</td>
</tr>
<tr>
<td>Exudate in anterior chamber</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Small</td>
<td>1</td>
</tr>
<tr>
<td>Large</td>
<td>2</td>
</tr>
<tr>
<td>Hypopyon</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td>Maximum possible score</td>
<td>7</td>
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Tombed culture plates (Nunc, Roskilde, Denmark) in triplicate. Washed B9 cells (5 x 10^3) were added per well in 100 μl of RPMI-1640 medium supplemented with 25 mM HEPES, 5% (v/v) fetal calf serum, 50 μM 2-mercaptoethanol, and penicillin and streptomycin. After 72 hr of culturing in a humidified incubator at 37°C and 5% CO₂, the cells were labeled by a 4-hr pulse with 0.5 μCi ³H-dThd (Amersham, Amersham, UK), the nuclei were harvested, and the incorporated radioactivity was counted. In each experiment the values were related to a diluted standard preparation of HrIL-6; 1 U/ml of HrIL-6 is the concentration that leads to half-maximal dThd incorporation in the assay.

Results

Measurement of IL-6 After LPS Injection

Before LPS administration, the serum level of IL-6 was <10 U/ml. The initial rise in serum IL-6 (29 U/ml) began 2 hr after LPS injection (Fig. 1A). A biphasic rise in serum IL-6 levels was seen over the course of the experiment, the peaks being at 4 and 16 hr (480 and 500 U/ml, respectively). At 139 hr post-LPS injection, the level of IL-6 had returned to the preinjection level (<10 U/ml).

In the aqueous humor IL-6 was not detected until 16 hr after LPS injection (700 U/ml). The aqueous humor also showed a biphasic response with peaks at 20 (1940 U/ml) and 40 hr (850 U/ml). Aqueous-humor IL-6 values were found to be higher than their corresponding serum values at 16, 20, 24, 40, and 48 hr. Similar to serum, the aqueous-humor IL-6 level returned to <10 U/ml at 139 hr. All control serum and aqueous humor samples showed <10 U/ml of IL-6.

The total aqueous humor protein concentration showed a noticeable increase at 8 hr and reached its highest level at 24 hr (Fig. 1B). It then gradually fell to almost the pre-LPS level by 139 hr. No appreciable alteration in protein concentration could be seen in the control samples. Clinically, an almost symmetric bilateral uveitis was observed in response to the footpad injection of LPS. Figures 2A–C are representative photographs of these eyes, examined clinically by...
slit lamp and scored as Grades 2, 4, and 6 uveitis, respectively. The scoring system used is shown in Table 1. The degree of uveitis paralleled the total aqueous-humor protein levels. The first signs of uveitis (iris hyperemia) were seen at 8 hr (Fig. 1C). The degree of inflammatory activity then increased until it reached a maximum at 24 hr with severe iris hyperemia and exudation into the anterior chamber; the inflammation then gradually declined, and no signs could be seen at 139 hr. None of the control animals showed signs of uveitis.

Measurement of IL-6 After Repeated LPS Injection

Before LPS administration, serum and aqueous-humor IL-6 levels were <10 U/ml. Raised levels of serum and aqueous-humor IL-6 were seen 1 day post-LPS, 208 and 1300 U/ml, respectively (Fig. 3A). On day 8, 1 day after the second LPS injection, only the serum IL-6 value was raised, but to a much lesser extent than after the first injection, at 44 U/ml. The aqueous-humor IL-6 level was <10 U/ml. On day 68, before the third LPS injection, both serum and aqueous humor showed IL-6 values of <10 U/ml. The following day, similar to that seen after the second injection, there was a small rise in serum IL-6 (52 U/ml) but no change in the aqueous-humor level of <10 U/ml. All control serum and aqueous-humor samples showed <10 U/ml of IL-6.

Total aqueous-humor protein increased after the first injection (Fig. 3B). A smaller increase was seen after the second injection, and by day 68, the protein level was almost back to the value seen preinjection. An even smaller increase in protein was seen on day 69, after the third injection. No appreciable alteration in protein content could be seen in control samples.

A large degree of uveitis was seen the day after the first injection, and by day 7, the eyes were quiet. After the animals had been reinjected a slight degree of inflammation was seen on day 8 (Fig. 3C). On day 68 the eyes were quiet and remained so even after the third injection. Control animals showed no signs of uveitis.

Fig. 2. Different grades of uveitis, observed by slit-lamp examination in Lewis rats after footpad injection of LPS. The scoring system of Table 1 was applied. (A) Grade 2 uveitis. Note the mild peripheral iris hyperemia (score 1) and the beginning flare in the anterior chamber (score 1). (B) Grade 4 uveitis. The anterior chamber exudate is more profound, with a cloudy appearance, due to influx of inflammatory cells (score 2). The moderate iris hyperemia (score 2) is partly obscured by the exudate. (C) Grade 6 uveitis. The dilated iris vessels (score 3) are hardly visible through the large anterior chamber exudate (score 2), which also obscures the missed pupil (score 1) in this photograph. Even the cornea has become involved in the inflammatory process, showing thickening and an irregular surface. Formation of a hypopyon in this situation would yield the maximum possible score of 7.
Intravitreal IL-6 Injection

Intravitreal injection of HrIL-6 induced an acute uveitis in all rats except those injected with the lowest concentration (10 U). The degree of uveitis did not appear to be dose related: the most active uveitis was seen in rats injected with 10^3 U of HrIL-6 (Fig. 4A). The inflammatory response in the saline-injected right eyes was limited to a few cases of mild iris hyperemia. When the protein concentration was determined in the aqueous humors of the eyes injected with HrIL-6, a prozone effect was observed. The greatest amount of protein was found in eyes that had received 10^2 U and increasing amounts of HrIL-6 resulted in corresponding decreasing levels of protein (Fig. 4B). Despite the absence of a clear inflammatory response in the saline-injected right eyes, the protein concentration in the aqueous humors of these eyes was increased, especially in rats that had received 10^2 or 10^3 U of HrIL-6 in their fellow (left) eyes.

Two weeks later 11 rats, that had recovered from the uveitis, were reinjected with 10^3 U of HrIL-6 into their left eyes. The rats that had received 10^3 U in the previous experiment and one rat that had received 10^2 U were not used again due to development of cataract and/or corneal vascularization. As in the previous experiment the right eyes received saline, and uveitis was graded the next day. It was noticed that nine of 11 rats did not have any signs of uveitis after this second challenge with HrIL-6.
The total aqueous-humor protein concentration was 2.4 mg/ml, and the aqueous IL-6 level was <10 U/ml.

**Intravenous IL-6 Injection**

Twenty-four hours after intravenous injection of 10⁶ U of HrIL-6, no signs of uveitis could be seen. The total aqueous-humor protein concentration was 2.4 mg/ml, and the aqueous IL-6 level was <10 U/ml.

**Discussion**

To our knowledge this is the first report of intraocular and systemic measurement of IL-6 in EIU. The appearance of IL-6 in serum after administration of LPS in the footpad was biphasic with peaks at 4 and 16 hr, respectively, but the values were obtained by pooling serum samples of four rats and may not represent the actual serum profiles of IL-6 in individual rats. The peak of IL-6, 4 hr after injection of LPS, agrees with results obtained in LPS-treated human volunteers but differs slightly from results in LPS-treated mice (IL-6 peak at 2 hr) and clearly from results in baboons treated with live bacteria—live bacteria in which plasma IL-6 continued to rise throughout the 8-hr study period.

Influx of IL-6 from plasma into the anterior chamber during the breakdown of the blood–aqueous barrier that accompanies EIU may have contributed to the levels of IL-6 detected in aqueous humor. However, the peak-level in aqueous humor was considerably higher than that in the serum and the initial peak of serum IL-6, although coinciding with the early disruption of the blood–aqueous barrier described by others, was not accompanied by IL-6 in the aqueous humor. These findings indicate intraocular synthesis of IL-6 during EIU rather than leakage from the circulation.

Intravenous injection of 10⁶ U of HrIL-6, which is sufficient to induce an acute-phase response in the rat, did not lead to detectable levels in the aqueous humor and did not induce uveitis. This also argues against circulating plasma IL-6 as the primary mediator eliciting the ocular response in EIU.

The state of ocular unresponsiveness or tolerance to repeated LPS injections was characterized by an absence of IL-6 in the aqueous humor, whereas corresponding sera still showed a small but significant rise in IL-6. Although the data presented here do not explain this phenomenon satisfactorily, we believe there may have been an involvement of TNF. In response to LPS this cytokine is released systemically in laboratory animals and human volunteers. Furthermore, administration of TNF in vivo leads to circulating IL-6. In EIU, trace amounts of TNF could reach the anterior chamber of the eye and induce local synthesis of IL-6 by as yet unidentified cells, expressing TNF receptors.

Several studies indicate that acquired LPS tolerance is associated with an inability of macrophages to release TNF. Extending this to a “TNF-mediated” mechanism of EIU, down-regulation of TNF release would result in low systemic levels of this cytokine in rats rechallenged with LPS. These low levels could still be sufficient to induce some serum IL-6 but inadequate to reach an intraocular “threshold” concentration required for local induction of IL-6 and an accompanying uveitis. To test this hypothesis the uveitogenic properties of systemically administered TNF will have to be studied in LPS-sensitive and tolerant rats and the ability determined of neutralizing antibodies against rat TNF, injected simultaneously with LPS, to prevent or suppress EIU. Others have shown that such anti-TNF antibodies suppress the release of circulating IL-1 and IL-6 during bacteremia, preventing septic shock. With the recent cloning of rat IL-6, neutralizing antibodies against this cytokine will also become available, allowing further studies on the contribution of IL-6 in several experimental models of inflammation in the rat, including EIU.

A recent study in mice indicates that endogenous corticosteroids are also involved in modulating serum levels of TNF after LPS stimulation. Thus, it would be interesting to study the course of EIU in rats treated with dexamethasone and in adrenalectomized or hypophysectomized animals.

Intravitreal injection of HrIL-6 in rats induced an inflammatory response similar to the uveitis observed in EIU. Contaminating LPS is unlikely to have been responsible for this reaction, since others have shown that the amount of LPS required intravitreally for the induction of inflammation is much higher than the trace amounts present in the diluted HrIL-6 used by us.

Interestingly, the rats with IL-6-induced uveitis showed a consensual response in their fellow eyes with regard to increased aqueous protein but did not have major clinical signs of inflammation in these eyes. A consensual response in control eyes has also been reported after intraocular injection of LPS and required the insertion of a needle in these eyes, although injection of saline was not necessary.

The IL-6 tolerance observed in nine of 11 rats after a second intravitreal injection of HrIL-6 was an unexpected finding, and its relation to LPS tolerance is not clear. We observed the same phenomenon in rabbits and noticed that it is not associated with detectable antibodies against HrIL-6 or with other in-
hbitors of HrIL-6 in aqueous humor (Hoekzema et al., manuscript in preparation). Binding studies with labeled HrIL-6 in vitro and in vivo may be helpful in determining whether the number of IL-6 receptors on intraocular cells is down-regulated during this state of unresponsiveness to IL-6.

In conclusion, the data presented here confirm that IL-6 is a potential mediator of intraocular inflammation, as expected from a previous study in patients with uveitis, where IL-6 levels up to 50,000 U/ml were detected in aqueous humor. Our results further suggest that EIU in the rat provides a suitable model to study the kinetics and interactions in the cytokine network. A deeper understanding of the mechanisms underlying the development of ocular tolerance to inflammatory stimuli, as observed with LPS and IL-6, may eventually lead to new therapeutic measures in patients with uveitis.

Key words: endotoxin, LPS, tolerance, interleukin 6, aqueous humor, uveitis

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References


