The Paradoxical Effect of Tumor Necrosis Factor Alpha (TNF-α) in Endotoxin-Induced Uveitis

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**Purpose.** To investigate the role of TNF-α in endotoxin-induced uveitis (EIU) in mice.

**Methods.** To neutralize TNF-α activity, mice were pretreated with either repeated injections of this cytokine or a single injection of antibody against it. The mice were then injected intraperitoneally with 500 μg endotoxin, to induce lethal septic shock, or into the footpad with 200 μg to induce EIU.

**Results.** Although both pretreatments conferred protection against the systemic toxic effects of LPS, TNF-resistant mice and mice treated with anti-TNF-α antibody demonstrated an exacerbation of EIU when compared to control animals.

**Conclusions.** Unlike its apparent participation in the systemic effect of endotoxin, TNF-α is not directly involved in the pathogenesis of EIU and may even protect against the inflammatory processes of this disease. Invest Ophthalmol Vis Sci 1993;34:2911–2917.

Tumor necrosis factor alpha (TNF-α), a 17-kD peptide cytokine, plays a role in a variety of inflammatory processes. Much work has been reported recently in attempts to define the function of TNF-α in the process of bacterial sepsis, in which it seems to be prominently involved. Infusion of TNF-α in vivo will cause systemic symptoms mimicking septic shock. Experimental models of gram-negative septic shock, including infusion of live bacteria or bacterial lipopolysaccharide (LPS), have been shown to produce sharp elevations in serum TNF-α levels, and antibodies directed against TNF-α are protective against the mortality induced by many, but not all, of these models. Animals can be made resistant to TNF-α by administration of multiple low doses of the cytokine over a 5- to 6-day period. These animals are then likewise protected against septic shock, although this TNF-resistant state does not last beyond 16 days after the last TNF-α dose.

Endotoxin-induced uveitis (EIU) is an acute, transient, inflammatory response localized to the eye that peaks 24 hours after the administration of a single sublethal dose of LPS to an experimental animal. Although first produced in the Lewis rat, EIU can, in varying intensities, be induced in other rat strains, as well as in mice. While in the Lewis rat, EIU is characterized histologically by acute inflammation localized to the anterior chamber; in the mouse, the anterior inflammation is usually less severe, and a prominent collection of neutrophils develops in the vitreous around the retinal vessels at the optic nerve head. EIU is a model for acute anterior uveitis and is used in studies examining both the pathophysiology and potential treatment of this disease. Despite numerous studies, however, the pathogenic mechanism of EIU remains unclear.

Because TNF-α has been clearly implicated as a mediator in LPS-related processes, its role in EIU has come under question. Recently, we were able to reduce significantly the inflammatory response in EIU through pretreatment of rats with chlorpromazine, a phenothiazine with a number of immunomodulatory effects, including the inhibition of TNF-α release from macrophages. A rise in both aqueous and serum TNF-α levels has been measured during EIU induction; however, in another study, attempts to block the development of EIU with anti-TNF-α antibodies proved unsuccessful (R. Hoekzema, personal communication). Although TNF-α is capable of inducing an inflammatory reaction when injected directly into the vitreous of rabbit eyes, the character of the inflammation is primarily monocytic and lymphocytic, in con-
Animals

ARVO Statement for the Use of Animals in Ophthalmology. All animal experiments adhered to the NIH Guiding Principles in the Care and Use of Animals and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Materials and Methods

Animals

Female C3H/HeN mice, 6 to 8 weeks of age (18 to 22 g), were housed in a 12-hour light/dark cycle environment and allowed free access to autoclaved NH-31 rodent chow (Zeigler Bros., Gardners, PA) and water. All animal experiments adhered to the NIH Guiding Principles in the Care and Use of Animals and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of TNF Resistance

Recombinant murine TNF-α was the generous gift of Genentech, Inc. (San Francisco, CA). It had a specific activity of $1.2 \times 10^7$ U/mg and an endotoxin concentration of 0.511 EU/mg as assessed by the standard limulus assay. Treated animals were injected intraperitoneally twice daily for 6 days with TNF-α at a dosage of 135 μg (1.62 × 10^6 U)/kg, diluted in 0.2 ml of sterile pyrogen-free saline (Mediatech, Herndon, VA). Control animals received injections of saline alone on the same schedule. Daily records were kept of the total weight of the animals in each group, and the establishment of TNF resistance was confirmed by the typical weight loss and the acquired resistance of treated mice to mortality induced by injection of a large dose of LPS, as described below. Injections with LPS to induce mortality or EIU were given 24 hours after the last TNF-α injection.

Anti-TNF-α Antibody

Anti-TNF-α antibody was the generous gift of Dr. R. Gazzinelli (NIAID, NIH, Bethesda, MD). The antibody is a rat monoclonal IgG1 antibody, XT1122, which was originally developed by J. Abrams (DNAX, Palo Alto, CA). The antibody preparation was purified from ascites, did not contain any detectable endotoxin, and was found to neutralize TNF-α activity. The antibody was administered intraperitoneally, 250 μg per mouse, 6 hours before the challenge with LPS. Control mice were similarly injected with 250 μg of rat IgG (Sigma, St Louis, MO).

LPS-Induced Mortality

In two separate experiments, groups of mice pretreated with TNF-α or with antibody to TNF-α and their corresponding controls were given a single intraperitoneal injection of 500 μg Salmonella typhimurium LPS (Difco, Detroit, MI). The mice were examined daily; mortality was assessed for 3 days after LPS injection.

Induction of EIU

EIU was induced by injecting 200 μg LPS dissolved in 0.1 ml of sterile pyrogen-free saline into one hind footpad. EIU induction was tested in two separate experiments with TNF-resistant mice and in two other experiments in mice receiving antibodies to TNF-α.

All animals were sacrificed by CO2 inhalation 24 hours after endotoxin injection, and the right eye was immediately enucleated for histopathologic examination. Eyes were placed in 4% glutaraldehyde for 30 to 45 minutes and then transferred to 4% neutral buffered formaldehyde solution for overnight fixation. The eyes were embedded in methacrylate, and 3-μm thick sections were prepared through the vertical plane of the pupil and the center of the optic nerve and stained with hematoxylin and eosin. Ocular inflammation was graded by two masked independent investigators on a scale of 0 (no inflammation) to 4 (severe inflammation). The grading scale is based on the number of inflammatory cells in the anterior chamber and posterior vitreous, and the presence of protein in the anterior chamber, as detailed in Table 1. Grades given by the two investigators differed in approximately 10% of the eyes, but only by 0.5. In these cases, slides were re-reviewed by both pathologists, and a consensus score was reached.

Statistical Analysis

The effect of pretreatment with TNF-α and anti-TNF antibody on mortality was compared to separate control groups using chi-square analysis in a 2 × 2 contingency table. However, the calculated P values must be interpreted in the context of the small sample size used in these two experiments. The scores of ocular inflammation in mice pretreated with TNF-α and anti-TNF antibody were compared to separate control groups using the Mann-Whitney rank sum test for two independent samples. Because these data are not normally distributed, means were calculated only for descriptive purposes, and statistical tests comparing the
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TABLE 1. The Scoring System for EIU in Mice

<table>
<thead>
<tr>
<th>Anterior Chamber Score</th>
<th>Vitreous Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells in Chamber</td>
<td>Cells in Vitreous</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-2</td>
<td>1-5</td>
</tr>
<tr>
<td>3-5</td>
<td>6-15</td>
</tr>
<tr>
<td>6-10</td>
<td>16-30</td>
</tr>
<tr>
<td>&gt;10</td>
<td>31-50</td>
</tr>
<tr>
<td>Protein in chamber: add</td>
<td>&gt;50</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

Total score: Anterior chamber score + vitreous score

Sections from the right eye of tested mice were used for the scoring.

calculated means were not performed. The null hypothesis was rejected in all statistical analyses when the calculated P value was less than 0.05.

RESULTS

Pretreatments with TNF-α or anti-TNF-α antibody protected against systemic effects of LPS

Animals receiving TNF-α treatment had an average weight loss of 4% to 6% during the first 24 hours and then gradually recovered toward baseline during the subsequent 5 days of treatment (Fig. 1). This pattern of weight change is similar to that reported previously in Lewis rats undergoing TNF treatment.

Pretreatment with TNF-α protected against the mortality induced by intraperitoneal LPS injection (Fig. 2A). Only one of the five pretreated mice died within 36 hours, whereas all five untreated controls succumbed to the LPS effect. Despite the small sample size, these differences approached statistical significance (P = 0.053). An even more potent protective effect against LPS was demonstrated in the animals treated with antibodies to TNF-α (Fig. 2B). In this experiment, all five control mice were dead within 24 hours, whereas all mice pretreated with the antibodies survived the LPS injection. This difference was statistically significant (P = 0.011).

Pretreatments with TNF-α or anti-TNF-α antibody exacerbated EIU

Injection of LPS in all untreated control animals induced the characteristic findings of EIU, including acute inflammatory infiltration localized to the poste-
rior vitreous surrounding the optic nerve head, milder cellular infiltration, and occasional protein in the anterior chamber (Fig. 3). The LPS-induced ocular changes were more severe in mice pretreated with TNF-α or with the antibody against this cytokine. The increased inflammation was noted by more intense cell infiltration of the anterior chamber and by vitreous and proteinaceous exudate in the anterior chamber (Fig. 4). Analysis of the inflammatory scores of individual mice of the different groups (Fig. 5) showed that the scores were significantly higher in both the TNF-resistant mice ($P < 0.05$) and the antibody-treated mice ($P < 0.02$) than in their controls.

**DISCUSSION**

In light of the apparent involvement of TNF-α in LPS-associated processes that has been demonstrated by a number of studies in recent years, it was somewhat surprising that both acquired resistance to this cytokine, and blocking antibodies directed against it actually exacerbated the EIU disease process. A similar effect of anti-TNF-α antibodies has recently been observed by De Vos et al. The induction of EIU has been shown to be accompanied by elevations in TNF-α levels. Increased levels of the cytokine were observed in the serum at 2 and 20 hours and in the aqueous humor at 4 and 22 hours after injection of LPS. The association of TNF-α levels with EIU or other inflammatory conditions does not necessarily imply a cause-and-effect relationship. The data on the effect of anti-TNF-α antibodies in inflammatory processes have been mixed, depending on the type of antibody and the model system used. Although some studies report that blocking the effect of TNF-α protects animals in experimental models of sepsis, others find no such effect. Most recently, for example, Eskandari et al failed to demonstrate protection against mortality.
by a polyclonal rabbit antibody against murine TNF-α in CD1 mice after either LPS injection or cecal ligation and puncture, despite significant reduction in serum TNF-α levels in the antiserum-treated animals. Bagby and associates\textsuperscript{19} reported a dichotomy between septic shock models involving parenteral administration of LPS or bacteria where anti-TNF-α was protective, and equally lethal models of peritonitis where the antibody had no effect on mortality.\textsuperscript{19} Other studies have suggested that TNF-α actually confers beneficial effects, with protection against morbidity and mortality in some circumstances.\textsuperscript{50-53} The apparent inconsistencies among these various studies underscore the complex nature of the inflammatory process and the interaction of the many cytokines involved in its induction and modulation. It is likely that there can be compartmentalization of an inflammatory process that creates a distinct local milieu of cytokines and inflammatory mediators with different dynamics from a concurrent systemic inflammatory process. In line with this notion, Otsuka et al\textsuperscript{24} have reported that TNF-α inhibits neutrophil migration to local inflammatory sites. Thus, the ability of TNF-α antibody to block completely the lethal effects of an LD\textsubscript{100} dose of LPS while increasing inflammation in the EIU model could be explained through such a compartmentalization.

The significance of our finding that TNF resistance exacerbates the inflammation in EIU while conferring protection against LPS mortality is more difficult to interpret. TNF resistance is probably a complex phenomenon involving an entire network of cytokines, and its precise mechanism is not known. It has been determined, however, that TNF resistance cannot be explained on the basis of an increased rate of elimination of the cytokine from the circulation or the endogenous production of anti-TNF-α antibodies.\textsuperscript{7} There is some evidence to suggest that the induction of a hepatic superoxide dismutase may be involved, which would enhance protection against toxic superoxide radicals.\textsuperscript{25,26} An analogous resistant state to LPS can be induced through multiple injections, a finding that has been known for years,\textsuperscript{27} and both endotoxin-resistant and TNF-resistant animals are mutually resistant to the lethal effects of LPS and TNF-α.\textsuperscript{7} Despite this apparent similarity between the two phenomena, their mechanisms are different: peritoneal macrophages from mice rendered tolerant by prior injections with LPS will not secrete TNF-α, among a variety of other cytokines, in response to subsequent LPS challenge in vitro.\textsuperscript{28} On the other hand, inducing TNF resistance in mice actually augments circulating TNF-α levels in vivo and TNF-α production in vitro after LPS challenge.\textsuperscript{29} Thus, because the resistance to TNF-α operates by principles different from those of resistance to LPS, it is not surprising that, unlike the exacerbation of EIU in mice resistant to TNF-α, recorded here, the resistance to LPS was reported to diminish the ocular inflammatory response in EIU.\textsuperscript{30}

Initial exposure to TNF-α stimulates mutual adherence factors on neutrophils and vascular endothelium,\textsuperscript{31,32} which may contribute to the massive influx of neutrophils into the organs of animals challenged with a single high dose of TNF-α or LPS. Part of the mechanism of TNF resistance may be downregulation of
these adhesion factors, resulting in protection from the lethal inflammation and necrosis. On the other hand, it may be that in the case of the ocular tissues, the initial high levels of adhesion factors are maintained in the face of repeated TNF-α treatments, leading to the observed increase in acute inflammation on subsequent LPS challenge. Alternatively, TNF-α may normally play a counterregulatory role in EIU through the induction of antiinflammatory mechanisms, such as the one described by Otsuka et al. The constantly elevated levels of the cytokine during the induction of TNF resistance may serve to desensitize these effects of TNF, perhaps through receptor downregulation, thus allowing the exacerbated ocular inflammation in pretreated eyes.

A high level of circulating neutrophils could also facilitate an increase in ocular tissue inflammation during EIU induction. However, although a massive leukocytosis is induced 1 to 2 hours after initial TNF-α injection in rats, this response disappears with the acquisition of TNF resistance, making it an unlikely contributing factor in our experiments.

Although the mechanisms behind the seemingly paradoxical role of TNF-α in EIU remain unclear, our findings on TNF resistance, coupled with the data on anti-TNF-α antibody, do suggest that TNF-α does not play a proinflammatory role in this experimental disease. The inflammatory mechanisms operative in EIU appear to be different from those involved in systemic sepsis, although they are both initiated by LPS.

**Key Words**
edotoxin-induced uveitis, tumor necrosis factor alpha, inflammation, cytokine, septic shock

**Acknowledgments**
The authors thank Genentech, Inc., for a generous supply of TNF-α; Dr. Robert Schreiber, Washington University, and Dr. Ricardo Gazzinelli, LPD, NIAID, NIH, for providing the anti-TNF-α antibodies; and Dr. Ruth Neta, AFRRI, for the anti-TNF-a antibody, do suggest that TNF-a does not

**References**

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serum against tumor necrosis factor enhances lipo-