Anti-Tumor Necrosis Factor Alpha Therapy Suppresses the Induction of Experimental Autoimmune Uveoretinitis in Mice by Inhibiting Antigen Priming

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Purpose. Experimental autoimmune uveoretinitis (EAU) serves as a model for several immune-mediated diseases that affect the eye in humans. Previous studies indicated that tumor necrosis factor alpha (TNF-α) has an important proinflammatory role in EAU and possibly in human uveitis. In this study, the authors investigated the effect of anti-TNF-α therapy on EAU in mice.

Methods. Experimental autoimmune uveoretinitis was induced in B10.A mice by immunization with interphotoreceptor retinoid-binding protein (IRBP). The mice were treated with 100 or 300 μl rabbit antiserum or polyclonal antibodies to human TNF-α. The treatment spanned either the afferent or the efferent stage of EAU (days −1, 1, 3, 5, 7, or days 8, 10, 12, 14, 16, respectively). Control animals were injected with preimmune rabbit serum at the corresponding times or were not treated. Three weeks after immunization, EAU was assessed by clinical evaluation and by histopathology. Immunologic responses were assessed by delayed-type hypersensitivity (DTH), lymphocyte proliferation to IRBP, and relative abundance of IRBP-primed splenocytes.

Results. The treatment with rabbit anti-TNF-α serum significantly ameliorated disease when given during the afferent stage but had no effect when given during the efferent stage of EAU. The effect on DTH, lymphocyte proliferation, and abundance of antigen-reactive cells roughly paralleled the effect on disease.

Conclusions. Neutralization of systemic TNF ameliorates EAU. The effectiveness of afferent treatment in comparison to the treatment during the efferent stage, together with the reduced proliferation and the reduced abundance of IRBP-responsive cells, suggest that interference with afferent-acting processes such as antigen priming is important to achieve protection from EAU by anti-TNF treatment. Invest Ophthalmol Vis Sci. 1996;37:2211-2218.

Experimental autoimmune uveoretinitis (EAU), induced in mice by immunization with interphotoreceptor retinoid-binding protein (IRBP), serves as a model for several immune-mediated diseases that affect the posterior segment of the eye in humans1,2. Some of these diseases are part of a generalized systemic syndrome (e.g., Sarcoidosis, Behçet’s disease, and Vogt–Koyanagi–Harada syndrome), whereas others are confined primarily to the eye (e.g., sympathetic ophthalmia and birdshot retinochoroidopathy).3 The EAU model in animals has been useful in elucidating basic mechanisms of uveitic disease and has served as a template for the development of novel therapeutic modalities.

The proinflammatory cytokine tumor necrosis factor alpha (TNF-α) originally was identified as a serum factor causing hemorrhagic necrosis of tumors and inducing cachexia4,5 and is now known to possess many cell-activating and proinflammatory activities6,7. Tumor necrosis factor is produced by many cell types, among them macrophages, T cells, and natural killer cells.8,9–12 It is a costimulator of T cells, B cells, cyto-
toxic T cells, and polymorphonuclear leukocytes. Proinflammatory effects include induction of expression or upregulation of major histocompatibility complex molecules (class I and class II), IL-2 receptors, and adhesion molecules. Furthermore, TNF induces the production of IL-1 and promotes the synthesis of prostaglandins.

Tumor necrosis factor was postulated to have a central role in experimental and clinical autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), multiple sclerosis, and rheumatoid arthritis. Experimental evidence indicates that TNFα may have a similar proinflammatory role in EAU and possibly in human uveitis. Intravitreal injection of TNF induces ocular inflammation and elevated levels of intraocular IL-6. Nakamura et al suggested that the ability to produce TNFα contributes to genetic susceptibility to EAU: Susceptible B10.A mice are high producers of TNF (in response to endotoxin challenge), whereas EAU-resistant B10.D2 mice are low producers of TNF. Furthermore, EAU was enhanced in B10.A mice that received an injection of TNF at the time of uveitogenic challenge. TNFα also was found in human eyes with inflammatory conditions.

This central role for TNF in inflammation makes it an attractive target for immunotherapy. In this article, we evaluate the ability of systemic treatment with a neutralizing antiserum to TNFα to suppress EAU induced in mice by immunization with IRBP. We present evidence that anti-TNFα treatment, instituted at the time of immunization but not 7 days after immunization, effectively protects mice from EAU: Susceptible B10.A mice are high producers of TNF in response to endotoxin challenge, whereas EAU-resistant B10.D2 mice are low producers of TNF. Furthermore, EAU was enhanced in B10.A mice that received an injection of TNF at the time of uveitogenic challenge. TNFα also was found in human eyes with inflammatory conditions.

**MATERIALS AND METHODS**

**Animals**

Female B10.A mice were purchased from Frederick Cancer Research (Frederick, MD). The animals were housed under specific pathogen-free conditions, given water and laboratory chow (Purina, St Louis, MO), and were used at 6 to 8 weeks of age. Animals were treated in accordance with institutional guidelines and the with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Antigens and Adjuvants**

Interphotoreceptor retinoid-binding protein was prepared from bovine retinas as reported previously by Concanavalin A (Con A) Sepharose affinity chromatography and fast-performance liquid chromatography. Antigen preparations were aliquoted and stored at −20°C until use. Purified Bordetella pertussis toxin was purchased from Sigma (St. Louis, MO). Complete Freund’s adjuvant was purchased from Difco (Detroit, MI) and was supplemented with additional Mycobacterium tuberculosis strain H37RA to a final concentration of 2.5 mg/ml.

**Experimental Autoimmune Uveoretinitis Induction**

Groups of five to eight mice were immunized on day 0 with 50 μg of IRBP in 0.2 ml emulsion in complete Freund’s adjuvant (1:1 vol/vol) divided among three subcutaneous sites, 100 μl in the base of the tail and 50 μl in each thigh. All mice received 0.5 or 1 μg of Bordetella pertussis toxin intraperitoneally. Mice were killed 21 days after immunization (approximately 7 days after the onset of EAU in control animals).

**Anti-Tumor Necrosis Factor Antibody**

Polyclonal antiserum against TNFα was produced in rabbits and characterized as previously described, except for using recombinant human TNFα instead of corticotropin-releasing hormone. Briefly, rabbits were immunized with rhTNFα conjugated to bovine serum albumin by the carbodiimide reaction. Characterization of the antiserum showed that neutralization titer was 1:80,000 (the concentration that causes 10% displacement of radiolabeled tracer (ED₅₀) was 25 pg/ml, and the concentration that causes 50% displacement of radiolabeled tracer (ED₅₀) was 200 pg/ml). No significant crossreactivities (0.001%) were found for tumor necrosis factor-β, interleukin 1 (α and β), and interleukin-6. For several experiments, gamma globulins were purified by two-step precipitation in ammonium sulfate: Primary precipitation occurred by 25% saturation, then gamma globulins were precipitated from the supernatant at 40% saturation. Normal rabbit serum was used as a control treatment and was processed in the same way.

**Treatment**

Anti-serum or purified gamma globulins were injected intraperitoneally on alternate days from day 1 to day 7 (afferent treatment) or on alternate days from day 8 to day 16 (effferent treatment). Each dose consisted of 100 or 300 μl antiserum or its equivalent in purified gamma globulin fraction, as specified. Controls received an equivalent dose of preimmune serum or gamma globulin fraction. The dose was chosen according to Karalis et al, who showed that a single injection of 1 ml polyclonal rabbit anti-TNF serum inhibits carrageenin-induced inflammation in rats. Because mice are approximately one tenth the body weight of rats, we calculated the lower dose to match...
Anti-TNF Therapy Suppresses Experimental Autoimmune Uveoretinitis

Karalis et al.\(^{28}\) and tripled that amount for the higher dose. We found that (normal) rabbit serum doses in excess of 300 µl tended to have adverse effects on the mice after prolonged treatment.

**Experimental Autoimmune Uveoretinitis Grading and Presentation of Results**

Eyes were collected for histopathology on day 21, approximately 7 days after onset of disease. Freshly enucleated eyes were fixed for 1 hour in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde until processing. Fixed and dehydrated tissue was embedded in methacrylate, and 4- to 6-µm sections, cut through the pupillary–optic nerve plane, were stained by standard hematoxylin and eosin. Six to eight sections cut at different levels were examined for each eye in a masked fashion by one of the authors, an ophthalmic pathologist, and the presence and extent of lesions were determined. Incidence and severity of EAU were scored on a scale of 0 to 4 in half-point increments, according to the semiquantitative grading systems described previously for mice and for rats.\(^{29,30}\) Briefly, the minimal criterion for scoring an animal as positive by histopathology was inflammatory cell infiltration of the uvea and the retina. Progressively higher scores were assigned for the presence of discrete lesions in the tissue (such as choroiditis, retinal vasculitis, granuloma formation, retinal folding and/or detachment, photoreceptor damage), taking into account lesion type, size, and number. Severity of disease is calculated as mean grading of all eyes in the group. Incidence is shown as the number of positive out of total animals in each group.

**Delayed-Type Hypersensitivity**

Animals were anesthetized, and 10 µg of IRBP was injected into the ear pinna in a volume of 10 µl. The other ear received an equal volume of phosphate-buffered saline. Ear thickness was measured 48 hours later with a spring-loaded micrometer. Specific delayed-type hypersensitivity (DTH) was calculated as the difference between the antigen and the phosphate-buffered saline-injected ear.

**Lymphocyte Proliferation Assay**

Draining lymph nodes were collected from mice 21 days after immunization. Triplicate 0.2 ml cultures of 5 × 10^5 cells were cultured in 96-well, round-bottom plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10 × 10^-5 M 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 µg/ml gentamycin, and 1.0% mouse serum. Alpha-methyl-D-mannopyranoside (α-MM, 20 µg/ml; Sigma) was added to the medium to neutralize any possible traces of Con A, which is used in the initial stages of IRBP purification. This concentration of α-MM had been determined not to affect cell proliferation. Stimulants were added to the wells to the following final concentrations: IRBP, 50 µg/ml; purified protein derivative of tuberculin (Connaught Laboratories, Toronto, Canada), 20 µg/ml; and phytohemagglutinin (PHA; Murex, Research Triangle Park, NC), 1 µg/ml. Cultures were incubated for 66 hours and were pulsed with 1 µCi/well of ^3^H-thymidine (New England Nuclear, Wilmington, DE) during the last 18 hours. The cells were harvested with a PHD harvester (Cambridge Technology, Watertown, MA), and ^3^H-thymidine uptake was determined by standard liquid scintillation. Stimulation index (SI) is calculated as the counts per minute (cpm) in the antigen-stimulated wells divided by cpm in wells containing medium only. SI ≥ 2 was considered a positive response.

**Estimation of the Abundance of Interphotoreceptor Retinoid-Binding Protein-Primed Cells by Limiting Dilution**

Splenocytes were pooled within each group on day 21 after immunization and were cultured in 96-well, round-bottom plates (Costar) in serial 10-fold dilutions: 5 × 10^5, 5 × 10^4, and 5 × 10^3 cells/well. The medium was supplemented as described in the lymphocyte proliferation assay, and syngeneic irradiated antigen-presenting cells were added (1 × 10^5/well). At each cell dilution, 60 replicate wells were cultured with IRBP (30 µg/ml) for 5 days, and 60 wells were cultured without it. All cultures were pulsed with ^3^H-thymidine (1 µCi/well) for the last 18 hours. Cells were harvested as in the proliferation assay. IRBP-stimulated cultures were considered positive if the cpm were more than twice the mean cpm of control wells. Data are presented as percent of positive wells.

**Statistical Analysis**

Parametric data (DTH) were analyzed by independent t-test. Nonparametric data (EAU scores) were analyzed by frequency distribution using linear trend in proportions.\(^{31}\) Each mouse, not each eye, was treated as one statistical event (both eyes were averaged for analysis). P ≤ 0.05 was considered statistically significant.

**RESULTS**

Anti-Tumor Necrosis Factor Antibodies Can Suppress the Induction of Experimental Autoimmune Uveoretinitis

Mice were immunized with IRBP as described in Materials and Methods. Anti-TNF treatment was started the day before immunization until day 7 to include the efferent (priming) stage of disease induction, or it was conducted from day 8 to day 16 to cover only the efferent phase of disease induction. For the purpose...
No treatment -1
Rabbit serum eff. -1
Rabbit serum aff. -1
Anti-TNF eff. -13/13
Anti-TNF aff. -1/11

Average EAU score ± SE

FIGURE 1. Effect of anti-tumor necrosis factor (TNF) antibodies during the afferent and efferent phases of experimental autoimmune uveitis in mice. B10.A mice were immunized with a uveitogenic regimen of interphotoreceptor retinoid-binding protein on day 0, and were given 100 μg of anti-TNF on days -1, 0, 1, 5, 7 (afferent) or 8, 10, 12, 14, 16 (efferent). Shown are average EAU scores as analyzed by histopathology 21 days after immunization. Incidence is shown within the columns as positive-total animals. Statistically significant difference from control is denoted by an asterisk. Data are a composite of two experiments.

of these experiments, the afferent phase of EAU is defined as the priming stage, and the efferent phase is considered the stage after antigen-specific effector cells have been generated. On the basis of previous studies, day 7 is considered to represent a boundary between these two stages.

Protection by treatment with anti-TNF antibodies was significant in mice treated during the afferent stage of disease, compared to control or to treatment during the efferent stage of disease (Fig. 1). Fundoscopic examination of the mice, performed 14 days after immunization, showed that the animals that did develop disease were already positive by day 14, whereas the animals that were negative remained so until day 21, when the eyes were evaluated by histopathology. Thus, protection did not result simply from a delay in the onset of disease in the afferent treatment group. Representative histopathology in these treatment groups is shown in Figure 2.

Delayed-Type Hypersensitivity to Interphotoreceptor Retinoid-Binding Protein Is Reduced in Mice Treated With Anti-Tumor Necrosis Factor Antibodies

The DTH response is considered an in vivo measure of cell-mediated immunity. Mice that received treatment during the afferent stage of EAU had significantly reduced DTH responses to the uveitogen. The disease developed by these animals is shown in Figure 1. Delayed-type hypersensitivity responses of mice that received efferent treatment and were not significantly

FIGURE 2. Histopathology of experimental autoimmune uveitis in mice treated with anti-tumor necrosis factor α. (A) Control mouse given preimmune serum. Note total absence of photoreceptors and scattered inflammatory cells in the subretinal space and vitreous (arrows). The retina is detached from the choroid, indicating active inflammation. (B) Mouse given afferent anti-TNF treatment. Note well-preserved retinal architecture with only a few infiltrating leukocytes (arrows). (C) Mouse treated with anti-TNF during the efferent phase. Note marked loss of photoreceptors with chorioretinal adhesion (asterisk) and numerous infiltrating inflammatory cells in the vitreous (arrows). Hematoxylin and eosin, X400.
Anti-TNF Therapy Suppresses Experimental Autoimmune Uveoretinitis

TABLE 1. Effect of Anti-TNF Treatment on Delayed-Type Hypersensitivity to IRBP

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>DTH ± SE (mm × 10⁻³)</th>
<th>Statistics (versus control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TNF, afferent</td>
<td>8 ± 2</td>
<td>(* P &lt; 0.006)</td>
</tr>
<tr>
<td>Anti-TNF, efferent</td>
<td>15 ± 2</td>
<td>(* P &lt; 0.049)</td>
</tr>
<tr>
<td>Preimmune serum, afferent</td>
<td>17 ± 2</td>
<td></td>
</tr>
<tr>
<td>Preimmune serum, efferent</td>
<td>19 ± 1</td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>-1 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were immunized on day 0 with IRBP and were given either afferent or efferent treatment with anti-TNF antibodies or with preimmune rabbit serum (100 μl). DTH to IRBP was tested by ear assay. Shown is the average difference between the IRBP-challenged and the control ear in micrometers on day 21, 48 hours after challenge. EAU developed by these mice is shown in Figure 1.

TNF = tumor necrosis factor; IRBP = interphotoreceptor retinoid-binding protein; DTH = delayed-type hypersensitivity; EAU = experimental autoimmune uveoretinitis.

Protected from disease achieved a smaller difference from the untreated control (Table 1). Thus, cellular immunity as measured by DTH paralleled the severity of disease as measured by histopathology.

Depressed Lymphocyte Proliferation Suggests Interference With Antigen Priming

Draining lymph nodes of mice whose EAU scores are shown in Figure 1 were collected 21 days after immunization and were stimulated with IRBP as described in Materials and Methods. The level of proliferation in vitro is considered to reflect the prior in vivo effects of the treatment. Results showed that afferent treatment with anti-TNF antibodies strongly suppressed lymphocyte proliferation to IRBP in comparison to control and to efferent treatment (Fig. 3). In contrast, proliferation to the nonspecific mitogen PHA was not affected by anti-TNF therapy (not shown). The unreduced response to PHA indicates that the cells are healthy and capable of proliferation to nonspecific stimuli. We thus interpret the depressed proliferation in vitro to IRBP as indicative of a reduced number of in vivo primed antigen-specific lymphocytes in the lymph node cell population.

Anti-Tumor Necrosis Factor Therapy Reduces the Abundance of Interphotoreceptor Retinoid-Binding Protein-Responsive Cells

To address the hypothesis that the suppressed proliferative response to IRBP was caused by a reduced number of IRBP-primed lymphocytes, a modified frequency analysis of IRBP-responsive cells was performed. Spleens of mice that received afferent anti-TNF treatment and had reduced proliferative responses to IRBP were compared to controls treated with preimmune serum and to controls left untreated. Sixty replicate cultures containing graded numbers of splenocytes were cultured with or without IRBP in the presence of a constant number of antigen-presenting cells. Wells with counts greater than twice the mean of the background without antigen were considered positive. The percentage of positive wells was calculated from replicates containing 5 × 10⁵ cells/well because, in some experiments, 5 × 10⁵ cells/well resulted in 100% positivity for all groups whereas 5 × 10⁴ presented 0% positivity for animals treated with anti-TNF-α. Actual counts in positive wells at this cell concentration ranged from 2x to 5x over background (200 to 400 cpm in different experiments) in a continuous distribution.

It should be noted that this method yields only a relative estimation and not a true frequency of antigen-responsive cells because a minimal number of proliferating cells that may be greater than 1 is required to be measurable above background counts and because the actual number of precursors in a positive well cannot be calculated directly from the cpm, even though it is clear that some wells have more proliferation than others. For this reason, the results are expressed as a percent of positive wells and not as a calculated cell frequency.

As shown in Table 2, comparison of the number of positive cultures between the groups revealed that afferent anti-TNF treatment decreased the abundance
TABLE 2. Anti-TNF-α Treatment During the Afferent Phase of the Immune Response Decreases the Abundance of Cells Responding to IRBP

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Anti-TNF-α</th>
<th>Preimmune Serum</th>
<th>No Treatment</th>
<th>Naive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>63</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>50</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Animals were immunized on day 0. Anti TNF-α or preimmune serum was given intraperitoneally from day −1 to day 7, every other day. Untreated mice were given immunization only.

Twenty-one days later, proliferation of splenocytes to 50 μg/ml IRBP was assayed under conditions of limiting dilution. Shown is the percent of positive cultures (≥2X over background) out of 60 replicates containing 5 x 10⁴ splenocytes/well and 1 x 10² irradiated APC/well. Background counts in experiment 1 were 194 cpm and in experiment 2 were 401 cpm. ND = not done.

**DISCUSSION**

In this study, we examined the suppressive effect of anti-TNF therapy on EAU. Results indicated that treatment administered during the afferent stage of EAU significantly suppressed the severity of disease (P < 0.04), as well as the DTH response and lymph node cell proliferation to IRBP. In contrast, treatment instituted during the efferent stage of the disease had only marginal effects.

These results are in line with reports that documented more pronounced effects of afferent than of efferent anti-TNF treatment in experimental arthritis, diabetes, myocarditis, and contact sensitivity. Nevertheless, the complete inability of efferent treatment to reduce the histopathology of EAU was surprising in view of the reports that encephalitogenicity of T-cell clones in EAE correlates with their ability to produce TNF and that treatment with anti-TNF serum prevents adoptively transferred EAE. A number of hypotheses can be advanced to explain this unexpected result; the most prosaic is that a higher dose of antiserum might have had an effect on the efferent stage of EAU as well. However, increased doses of treatment serum (or the purified immunoglobulin fraction) were toxic to the mice, and this possibility could not be confirmed. Another possibility that should be considered is the ability of TNF to cause apoptosis of activated T lymphocytes. Thus, in contradiction to its well-known proinflammatory role, TNF could, at the same time, act to limit inflammation by triggering programmed death of the uveitogenic effector T cells. Indeed, in a separate study, we observed that a uveitogenic mouse T-cell line was strongly suppressed by recombinant TNF-α in culture (Xu et al, manuscript in preparation). Under this scenario, neutralization of TNF during the expression stage of EAU could cause opposing effects that might counterbalance each other.

Alternatively, the involvement of TNF in the pathogenesis of EAU might have different aspects than in other autoimmune disease models. Some ocular resident cells can produce TNF after an inflammatory stimulus, and such locally produced cytokine might not be easily accessible to peripherally injected antibodies. This hypothesis is also supported by the observation that although EAE and adjuvant arthritis are suppressed by treatment with recombinant soluble TNF receptor type I, such treatment was completely ineffective in suppressing EAU, either during the afferent or the efferent phase of disease (Sartani et al, unpublished data). Finally, Otsuka et al reported that systemic TNF inhibits migration of neutrophils to inflammatory sites. It is conceivable that neutralization of systemic TNF during the efferent phase of EAU might have facilitated migration of neutrophils to the eye, counterbalancing any inhibitory effect against lymphocytes and macrophages. This last possibility is supported by the observation that efferent-treated animals had a higher percentage of neutrophils in their ocular inflammatory infiltrate than did the preimmune serum controls (13.6% [mean of 11 animals] versus 6.5% [mean of 13 animals]), although, because of the large variability among individual eyes, this difference did not achieve statistical significance at P = 0.05 (unpaired t-test) (unpublished observations).

The reduced proliferative response to IRBP in treated mice suggested that the mechanism of protection from EAU by anti-TNF treatment involves, at least in part, interference with the priming of antigen-specific cells. This hypothesis was confirmed by the limiting dilution analysis, showing that the abundance of IRBP-responsive cells was reduced in the spleens of mice that received afferent treatment with anti-TNF. This interpretation is supported by the observation that nonspecific proliferation to the mitogen PHA remained unaffected, indicating that the treatment did not cause a general defect in lymphocyte proliferation. An inhibitory effect on antigen priming through systemic neutralization of TNF is compatible with the
known ability of TNF to upregulate the production of IL-1 and the expression of IL-2 receptors. In addition, IL-12 production by macrophages may depend in part on TNF so that removal of TNF could, in theory, inhibit the maturation of Th1-like lymphocytes. As recent data from our laboratory have suggested, uveitis may be mediated by Th1-like cells, and such an effect on IL-12 and Th1-like cells would be compatible with the observed suppressive effects on disease and on the immune response.

In summary, anti-TNF therapy has a suppressive effect on EAU, and this effect is caused by the inhibition of priming of IRBP-specific lymphocytes rather than by the suppression of effector mechanisms. Because chronic uveitis is thought to involve continuous recruitment and priming of antigen-specific cells, systemic neutralization of TNF could be useful as a clinical approach to treatment of uveitis, either by itself or as an adjunct to existing therapies.

Key Words
antibodies, autoimmune disease, experimental autoimmune uveitis (EAU), mouse, tumor necrosis factor (TNF), uveitis

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