The Role of Tumor Necrosis Factor-Alpha in the Induction of Experimental Autoimmune Uveoretinitis in Mice

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Purpose. To examine the role of tumor necrosis factor-alpha (TNF) in the induction of experimental autoimmune uveoretinitis (EAU), the authors compared in vivo TNF production in EAU-susceptible and EAU-resistant strains of congenic mice and attempted to determine whether TNF can enhance the inflammation in EAU by injection of TNF at the time of immunization.

Methods. The production of TNF after stimulation with lipopolysaccharide (LPS) in B10.A and B10.D2 mice was measured by bioassay with L929 cells. The incidence and severity of EAU was compared between the group immunized with conventional methods and the group that alternatively received additional subcutaneous injection of recombinant human TNF (rhTNF) at the time of immunization in both B10.A and B10.D2 mice.

Results. Serum concentration of TNF after stimulation with 50 μg of LPS was significantly higher in B10.A mice than in B10.D2 mice. The incidence of EAU in B10.A mice was 60%, but it was only 10% in B10.D2 mice using the conventional method. Extremely severe chorioretinitis and iridocyclitis occurred in B10.A mice with the injection of rhTNF at the time of immunization for EAU. The incidence of EAU in B10.A and B10.D2 rose to 100% and 40%, respectively. When administered alone, rhTNF did not cause any inflammatory change in the uvea.

Conclusions. The rhTNF was found to enhance the immune response to interphotoreceptor retinoid-binding protein in mice. These results suggest that susceptibility to EAU is in some part mediated by the ability of mice to produce TNF. Invest Ophthalmol Vis Sci. 1994; 35:3884–3889.

Experimental autoimmune uveoretinitis (EAU), an organ-specific autoimmune disease induced by immunization with retinal antigen in rodents and in primates, has been actively investigated in recent years as an animal model of human endogenous uveitis. Although the immunopathogenic mechanisms of EAU are still controversial, it is supposed that T lymphocytes play an important role in the induction of uveitis. Caspi and coworkers studied the genetic background in EAU and reported that not only the major histocompatibility complex (MHC) but also non-MHC genes control the disease susceptibility. In recent years, evidence has been assembled that suggests that tumor necrosis factor-alpha (TNF) may be involved in inflammatory processes, including the activation of T cells; induction of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and human lymphocyte antigens (HLA)-DR in vascular endothelial cells; and activation of polymorphonuclear neutrophils, all of which could mediate the autoimmune organ destruction. We previously reported that TNF plays an important role in the pathogenesis of uveoretinitis in Behçet’s disease. In the present work, we investigated the relationship between susceptibility to EAU and the ability to produce TNF in congenic mice.

MATERIALS AND METHODS

Mice

B10.A and B10.D2 male mice were maintained under specific pathogen-free conditions in the breeding col-
TABLE 1. Histopathologic Grading Chart for EAU in Mice

<table>
<thead>
<tr>
<th>Grading</th>
<th>Retina</th>
<th>Choroid</th>
<th>Vitreous</th>
<th>Iris and Ciliary Body</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No infiltrating cells</td>
<td>No infiltrating cells</td>
<td>No infiltrating cells</td>
<td>No infiltrating cells</td>
</tr>
<tr>
<td>1</td>
<td>Mild cell infiltration with 0–1 granulomas</td>
<td>Mild cell infiltration</td>
<td>Moderate cell infiltration</td>
<td>Mild trace of infiltrating cells</td>
</tr>
<tr>
<td>2</td>
<td>Moderate cell infiltration with 2–3 granulomas</td>
<td>Moderate cell infiltration with granulomas</td>
<td>Moderate cell infiltration</td>
<td>Severe cell infiltration with anterior chamber cell infiltration</td>
</tr>
<tr>
<td>3</td>
<td>Severe cell infiltration with 4 or more granulomas</td>
<td>Severe cell infiltration with granulomas</td>
<td>Severe cell infiltration and exudate</td>
<td>Severe cell infiltration with severe anterior chamber cell infiltration and exudate</td>
</tr>
</tbody>
</table>

Antigen and Adjuvant

Interphotoreceptor retinoid-binding protein (IRBP) was prepared from bovine retinas according to the method of Redmond et al. Complete Freund’s adjuvant (CFA), Mycobacterium tuberculosis H37R, and lipopolysaccharide (LPS) from Escherichia coli 055:B5 were purchased from Difco Laboratories (Detroit, MI). Purified Bordetella pertussis toxin was purchased from Sigma (St. Louis, MO). Recombinant human TNF (rhTNF) was kindly provided by Asahi Chemicals (Tokyo, Japan).

Measurement of TNF Production

We measured the LPS-stimulated TNF production in B10.A and B10.D2 mice in vivo. Ten mice of both strains were injected with 50 μg of lipopolysaccharide (LPS) intravenously. Blood was collected 90 minutes after the injection, when maximum TNF production was observed. After preservation for 24 hours at 4°C, sera were obtained by centrifugation for 10 minutes. The levels of TNF in sera were assayed by using LM cells, a subline of the TNF-sensitive mouse fibroblasts (L929), as target cells. Briefly, 2 × 10^6 LM cells suspended in 0.2 ml of RPMI 1640 supplemented with 5% heat-inactivated bovine serum were cultured in 96-well microculture plates. After confluent cell growth, the medium was replaced with mouse serum diluted 2:1, containing 2 μg/ml of actinomycin D. For neutralization of TNF, the serum was first incubated overnight with rabbit anti-mouse TNF serum. Twenty-four hours later, 100 μl of 0.2% crystal violet was added to each well and left for 3 minutes to stain the cells. After gentle and extensive washing in water, the plates were dried, and optical density at 540 nm was measured with a Titertek Multiskan (Flow Laboratories, McLean, VA) as described by Tanaka et al. Statistical analysis was performed using Student’s t-test.

Immunization

Both B10.A and B10.D2 mice were divided into four groups by different immunization protocols. Each group of both strains consisted of 10 mice, except group 4, which consisted of 5 mice.

Group 1. To compare the incidence and severity of EAU, we immunized both B10.A and B10.D2 mice according to the single-dose induction protocol originally reported by Caspi et al. Mice were immunized with a dose of 100 μg of IRBP in 0.2 ml of emulsified CFA supplemented with additional M. tuberculosis at a final concentration of 2.5 mg/ml. They were additionally given 1 μg of B. pertussis toxin in 0.2 ml of saline intraperitoneally as an adjuvant.

Group 2. To evaluate the role of M. tuberculosis in the immunization, we tried to produce EAU without additional M. tuberculosis in CFA (which contains only Mycobacterium butyricum at a final concentration of 1 mg/ml).

Group 3. To study the influence of TNF in EAU, we injected 3000 U of rhTNF in 0.2 ml of saline twice
TABLE 3. Incidence and Severity of EAU in Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice</th>
<th>Immunization</th>
<th>MT</th>
<th>TNF</th>
<th>Histopathological Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B10.A</td>
<td>IRBP + CFA</td>
<td>+</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>B10.A</td>
<td>IRBP + CFA</td>
<td>–</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>B10.A</td>
<td>IRBP + CFA</td>
<td>–</td>
<td>day 0, 2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>B10.A</td>
<td>IRBP + CFA</td>
<td>–</td>
<td>day 0, 2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>B10.D2</td>
<td>IRBP + CFA</td>
<td>+</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>B10.D2</td>
<td>IRBP + CFA</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>B10.D2</td>
<td>IRBP + CFA</td>
<td>–</td>
<td>day 0, 2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>B10.D2</td>
<td>IRBP + CFA</td>
<td>–</td>
<td>day 0, 2</td>
<td>0</td>
</tr>
</tbody>
</table>

All mice were injected with purified *Bordetella pertussis* toxin intraperitoneally at the time of immunization.

● = One mouse. IRBP = Interphotoreceptor retinoid-binding protein, CFA = complete Freund’s adjuvant, MT = *Mycobacterium tuberculosis*, rhTNF = recombinant human tumor necrosis factor-alpha.

Subcutaneously, at day 0 and day 2. In this group, we did not add any *M. tuberculosis* to CFA.

**Group 4.** To estimate the potential of TNF to induce inflammatory changes in the eyes of mice, mice were immunized with CFA without IRBP and injected with rhTNF as in group 3.

**Evaluation of Disease**

Mice were killed 2 weeks after the immunization. Enucleated eyes were immediately fixed for 24 hours in cacodylic acid formaldehyde and embedded in paraffin. Sections were cut at 4 μm through the pupillary–optic nerve plane and stained with standard hematoxylin and eosin. Severity of EAU was graded from 0 to 3 as described in Table 1.

**RESULTS**

**TNF Production of Mice**

LPS-stimulated TNF production was significantly higher (*P < 0.01*) in B10.A mice (29.8 ± 5.9 U/ml) than in B10.D2 mice (4.4 ± 0.6 U/ml) (Table 2).

**Severity and Incidence of Disease**

The severity and incidence in 70 mice of EAU experiment were shown in Table 3. Six of 10 B10.A mice in group 1 developed mild to moderate EAU (grades 1 and 2), including retinal folds, inflammatory cell infiltration, and granuloma formation in the retina (Fig. 1). There were only traces of iritis and cyclitis. The remainders, however, did not develop any EAU-related changes (grade 0). One of 10 B10.D2 mice in group 1 developed mild EAU, but the other 9 did not. None of 10 mice of either strain immunized without *M. tuberculosis* (group 2) developed EAU (grade 0). All B10.A mice injected with rhTNF (group 3) developed severe EAU (grade 3) 2 weeks after the immunization. Most of them showed not only extensive retinal inflammation but also choroiditis, vitritis, iritis, and cyclitis (Fig. 2). Four of 10 B10.D2 mice developed mild EAU (grades 1 and 2) when injected with 6,000 U of rhTNF (group 3). In contrast, none of 5 mice of either strain injected with 6,000 U of rhTNF and CFA without IRBP (group 4) developed EAU (grade 0).

**DISCUSSION**

The immunopathogenic mechanisms of EAU have been actively investigated in recent years. Caspi showed that S-antigen-specific helper–inducer cells could induce EAU when transferred to naive rats. Interleukin-2 (IL-2) receptors were found on the surface of these cells. Therefore, the participation of...
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FIGURE 2. The histopathology of EAU in B10.A mice injected with rhTNF at the time of immunization. (A) Severe cell infiltration in iris and ciliary body (Original magnification, ×120); (B) granuloma and exudates in retina (Original magnification, ×80); (C) cell infiltration in choroid (Original magnification, ×160); (D) cell infiltration and exudates in vitreous (Original magnification, ×160). Paraffin-embedded tissue stained with hematoxylin and eosin.

IL-2 in the induction of EAU has been suggested. However, the role of other cytokines such as TNF is still obscure. Tumor necrosis factor-alpha is a monocyte-macrophage-derived protein whose major biologic function was presumed to be the mediation of cytotoxicity to tumor cells. In recent years, evidence has been assembled to suggest that TNF may play various regulatory roles in immune responses. Yokota et al5 reported the activation of T cells by TNF, facilitating their capacity to proliferate in response to mitogens and antigens. Induction of surface antigens such as vascular cell adhesion molecule-1,6 intercellular adhesion molecule-1, and HLA-DR7 in vascular endothelial cells and, as a result, enhancement of polymorphonuclear neutrophil adhesion and suppression of migration8 were also reported as functions of TNF. In a previous study, we measured the in vitro production of TNF of peripheral blood monocytes from patients with Behçet's disease and suggested the possible participation of this inflammatory cytokine in the pathogenesis of Behçet's disease.9

In this study, we aimed to elucidate the pathogenic role of TNF in the development of EAU in mice. Adding M. tuberculosis to CFA (or adding excessive M. tuberculosis to incomplete Freund's adjuvant) has been recommended to develop EAU in mice.4,13,14 What is the pathogenic role of M. tuberculosis in the induction of EAU? M. tuberculosis was reported to induce the production of TNF from human T cells.15 Moreno16 showed that purified lipoarabinomannan from M. tuberculosis has the potency to cause the release of TNF in vitro from human blood monocytes and activated mouse peritoneal macrophages, and that it enhances the production of TNF in vivo in mice. Therefore, we hypothesized that the enhancement of TNF production with M. tuberculosis is necessary to raise the disease susceptibility to EAU in mice. Moreover, it is likely that disease susceptibility based on the genetic background may be, in some part, a reflection of the ability to produce TNF in response to immunization.

To confirm this hypothesis, we measured LPS-stimulated TNF production in different strains of B10 congenic mice in vivo. Serum TNF levels after stimulation were significantly higher in B10.A mice...
than in B10.D2 mice. There was also a marked difference in susceptibility to EAU between these two strains. Chung reported that a strain that could not develop experimental allergic encephalomyelitis (EAE) was also a low producer of TNF. Experimental autoimmune uveoretinitis and EAE are both autoimmune disease models and are known to share similar mechanisms of immune responses. It is therefore plausible that TNF might act as an important enhancer in the pathogenesis of autoimmune diseases such as EAE and EAU, and disease susceptibility might be proportional to the production of TNF.

Freund et al reported that certain strains of mice susceptible to toxoplasmic encephalitis expressed elevated levels of TNF mRNA in brain tissue after infection of toxoplasmic encephalitis, whereas resistant strains did not. These data imply that the ability to produce TNF may be strongly correlated with susceptibility to inflammatory diseases. Although we did not perform Northern blot analysis of TNF mRNA in the eyes, it is possible that TNF mRNA is expressed more apparently in B10.A mice than in B10.D2 mice in EAU. Actually, we observed higher levels of TNF mRNA expression in spleen cells from B10.A mice than from other strains of mice without the stimulation of LPS (manuscript in submission, 1994). Furthermore, we could not detect endogenous TNF levels in the serum of fully immunized mice. Therefore, we examined serum TNF levels in EAU after stimulation of LPS (an eliciting agent) and observed the elevation after 3 days (presumably the induction phase) and 10 days (presumably the effector phase) after immunization with IRBP (data not shown).

In this study, some mice immunized with IRBP and CFA supplemented with M. tuberculosis developed mild to moderate EAU, whereas none of the 10 mice immunized with IRBP and CFA, without additional M. tuberculosis, developed EAU. We consider that the insufficient production of TNF caused by the lack of additional M. tuberculosis in the induction phase might be responsible for this phenomenon. From this standpoint, we prepared mice that were immunized with CFA containing IRBP, but not M. tuberculosis, and that received injections of 6000 U of rhTNF (group 3). These mice showed marked increases in the incidence and severity of EAU in both strains examined. All B10.A mice developed severe EAU with extensive retinal inflammation, choroiditis, vitritis, iritis, and cyclitis. These results indicate that rhTNF strongly increased the immune response to IRBP, whereas M. tuberculosis increased it moderately. In addition, mice immunized with CFA without IRBP did not develop EAU, despite injection with 6000 U of rhTNF. Taken together, rhTNF itself does not have any potential to induce EAU. rhTNF seems to enhance the immune responses to IRBP in EAU in mice. Dose-dependent enhancement of rhTNF on EAU induction was also observed in B10.A mice using incomplete Freund's adjuvant in place of CFA (manuscript in preparation, 1993).

We harvested the eyes 14 days after immunization in this study. Although harvesting the eyes after 21 to 28 days of immunization was recommended in previous reports, in our preliminary study, the incidence and severity of EAU were not significantly different in the eyes harvested 14 days and 21 days after immunization. Actually, the article by Caspi et al showed that the incidence of EAU was increased 100% after 14 days of immunization when 100 µg of IRBP was used ("high-dose protocol"). Therefore, in this study, we evaluated the inflammatory changes in EAU 14 days after immunization. Conversely, we observed that the inflammatory changes appear faster in immunized mice that received rhTNF without additional M. tuberculosis.

It is important to consider whether the elevation of TNF production observed in this study was caused by the hyperactivity of immune reaction or by the hypofunction of TNF receptor. However, we observed higher lethality in B10.A mice than in B10.D2 mice when they received 500 µg of LPS, suggesting that the high producer of TNF was not the low responder to TNF in these two strains.

It has been reported that anti-TNF antibody can inhibit effectively the development of murine autoimmune diseases such as autoimmune myocarditis and EAE. In EAU, it is conceivable that anti-TNF treatment reduces the severity of disease, as it does in other autoimmune diseases.

Lane reported that treatment with IL-1 or TNF promoted CB3-induced autoimmune myocarditis in resistant B10.A mice. Jacob suggested that the association between the production of TNF and the MHC class II variation could be involved in the pathogenesis of various autoimmune diseases such as murine lupus models.

It is conceivable that the genetic control of disease susceptibility has relevance to sensitivity to TNF or to production of TNF by macrophages in response to antigens.

Further work on the genetic background of TNF production in the congenic mouse is being performed in our laboratory.

Key Words
experimental autoimmune uveoretinitis (EAU), interphotoreceptor retinoid-binding protein (IRBP), tumor necrosis factor-alpha (TNF), mice

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References