Kinetics of intraocular cytokines in the suppression of experimental autoimmune uveoretinitis by type I IFN

Annabelle A. Okada1,2, Hiroshi Keino1, Jun Suzuki1, Jun-ichi Sakai1, Masahiko Usui1 and Junichiro Mizuguchi2

1Department of Ophthalmology, Tokyo Medical College Hospital, Tokyo, Japan
2Department of Immunology, Tokyo Medical College, Tokyo, Japan

Keywords: IFN-α, IFN-β, IL, interphotoreceptor retinoid-binding protein

Abstract

The systemic administration of IFN-α/β was previously found to suppress inflammation in rats with experimental autoimmune uveoretinitis (EAU); however, an effect on the systemic immune response was not identified. In order to investigate an immunological basis for suppression at the intraocular level, rats immunized with interphotoreceptor retinoid-binding protein (IRBP) were administered daily intramuscular injections of 10^5 IU IFN-α/β and cytokines were measured by ELISA in intraocular extracts prepared by ultrasonification at various timepoints throughout the course of EAU. In control EAU, intraocular concentrations of IFN-γ were found to be non-detectable on day 8 before the onset of inflammation, significantly elevated on day 12 at peak inflammation (182 ± 106 pg/ml), then non-detectable again on day 16 after inflammation had begun to subside. In contrast, intraocular IFN-γ in IFN-α/β-treated rats remained non-detectable or low at all timepoints. Measurement of intraocular IL-2 revealed no difference between the two groups of rats. Intraocular IL-4 concentrations were elevated in rats treated with IFN-α/β, although this cytokine was also detected in the same range in controls as well as normal rats. Finally, intraocular IL-10 was non-detectable on day 8, significantly elevated at peak inflammation on day 12 (588 ± 139 pg/ml), then decreased to low levels on day 16 in control EAU rats, while remaining non-detectable or low in IFN-α/β-treated rats. These results suggest that acute inflammation in IRBP-induced EAU in rats involves both IFN-γ and IL-10 at the local intraocular level, and that systemic administration of IFN-α/β inhibits EAU via a mechanism that involves suppression of both cytokines.

Introduction

The anti-viral and anti-proliferative effects of various type I IFN have been well studied, and these agents have been used for the treatment of a variety of viral and malignant diseases for over a decade (1,2). More recently, the potential for various type I IFN to also act as immunomodulatory agents has led to its clinical use in several diseases of presumed autoimmune etiology. For example, IFN-β has been used in clinical trials for patients with multiple sclerosis with improvement in clinical disease parameters reported (3,4), and IFN-α has been used to treat mucocutaneous lesions and arthritis in patients with Behc¸et’s disease (5,6). IFN-α therapy for the intraocular inflammation (uveitis) associated with Behc¸et’s disease has also been reported (7–9), although the mechanism of such effect has yet to be identified.

Experimental autoimmune uveoretinitis (EAU) is an organ-specific, Th cell-mediated disease that can be induced by immunization of retinal antigens in susceptible strains of rats or mice (10–14). EAU is studied as an animal model for uveitis in humans of autoimmune nature. We previously reported that systemic administration of a preparation of IFN-α/β suppresses the development of EAU in rats (15,16), although analysis of cytokine production by splenocytes in vitro did not reveal a clear trend with respect to cytokines associated with a Th1 response versus a Th2 response. In order to delineate an effect on cytokines at the local intraocular level, this current study examines the effect of systemic administration of IFN-α/β on the kinetics of intraocular cytokines over the course of EAU.
Methods

Purification of mouse natural IFN-α/β

Natural murine IFN-α/β was produced and purified using an in vivo cell proliferation method previously described (17). Briefly, murine L202 cells were transplanted s.c. to newborn hamsters that were subsequently treated with i.p. injections of anti-hamster thymocyte serum. Four weeks later, a solid tumor mass of L202 cells was excised and processed into single-cell suspensions. The cells were then primed with murine IFN-α/β and stimulated to produce IFN by the addition of hemagglutinating virus of Japan. Approximately 10^{10} IU murine IFN-α/β was purified by CPC-10 absorption and ion-exchange chromatography with cellulose phosphate. Endotoxin was removed by Sephadex gel filtration.

IFN activity was assessed by ability to inhibit the cytopathic effect of vesicular stomatitis virus on L202 cells and activity was expressed in IU. The final product was found to contain 2.2×10^{10} IU IFN produced from 1.6×10^{11} cells. The endotoxin content was determined to be <1 ng/2×10^{10} IU of IFN by the Limulus test method. Neutralization using specific mAb against murine IFN-α and murine IFN-β (Yamasu Shoyu, Tokyo, Japan) showed that the preparation contained a ratio of IFN-α to IFN-β of ~1:1. Simultaneous addition of both anti-murine IFN-α and anti-murine IFN-β antibodies completely neutralized IFN activity. Murine IFN-α has been shown to have ~90% DNA sequence homology to rat IFN-α (18).

Induction of EAU and administration of IFN-α/β

Female Lewis rats, 6–8 weeks of age and weighing ~150 g each (Charles River Japan, Atsugi, Japan), were immunized with 50 μg bovine interphotoreceptor retinoid-binding protein (IRBP) emulsified 1:1 with complete Freund’s adjuvant (Difco, Detroit, MI) supplemented with 2.5 mg/ml Mycobacterium tuberculosis (Difco) in a total volume of 0.1 ml in one hind footpad on day 0. Rats were housed in a specific pathogen-free facility. Bovine IRBP was purified using a method previously described (19).

Rats were treated with daily intramuscular (hind leg) injections of 10^5 IU IFN-α/β suspended in 0.9% NaCl solution containing 0.01% rat albumin (volume 0.1 ml) from day 0 (day of IRBP immunization) through 1 day prior to sacrifice. The dose of IFN-α/β used in this study was determined as previously reported (16). Control rats were administered 0.1 ml injections of 0.01% rat albumin alone. Daily observation for the onset of intraocular inflammation was performed by slitlamp biomicroscopy (Kowa, Tokyo, Japan) and severity of inflammation was assessed using the clinical criteria shown in Table 1. Rats were sacrificed using lethal doses of ether and exsanguinated prior to the removal of whole eyes by careful dissection. Eyes were immediately preserved at –80°C until assay.

Measurement of intraocular cytokine levels

Intraocular levels of cytokines were measured by a specific ELISA kit (Biosource, Camarillo, CA) as previously reported (20). Briefly, specimens for each cytokine determination were allowed to thaw. Using a rounded surgical blade, each whole eye was incised at the equator and intraocular contents were scraped into a 0.5 ml Eppendorf tube containing 100 µl RPMI 1640 medium on ice. Samples were homogenized using an ultrasonic disruptor for 20 1-s pulses (UD-201; Tomy, Tokyo, Japan) then centrifuged at 15,000 r.p.m. for 30 min at 4°C. Supermatants were used immediately to assay for IFN-γ, IL-2, IL-4 and IL-10. All assays were specific for rat cytokines and detection limits are noted in the figure legends. Since only one cytokine could be measured per intraocular extract sample derived from each eye, measurement of the four cytokines included in this study was divided into two separate EAU experiments. Intraocular cytokine levels determined for control and IFN-α/β-treated EAU rats were compared to those for healthy, normal rats. For confirmation, each experiment was repeated twice, with similar results to those shown here obtained in each case.

Statistical analysis

Each EAU assessment and cytokine measurement point represents the average of five eyes from five different rats, assigned to treatment or no treatment groups at random following IRBP immunization. Data are expressed as mean ± SD. Comparisons between parametric data were made using the two-tailed Student’s t-test. Differences were considered significant for P < 0.05.

Results

Figure 1 shows the results for IFN-γ and IL-4 measured in intraocular extracts prepared from rats immunized with IRBP in the first experiment. Mean biomicroscopic scores for days 8–16 for control rats and rats that received daily intramuscular injections of 10^5 IU IFN-α/β are shown in Fig. 1(a). Onset of inflammation occurred for both groups on day 9. Administration of IFN-α/β was associated with significantly reduced biomicroscopic scores for days 9 and 10, although the scores for days 11–16 were not significantly different. Peak EAU severity scores were 4.2 ± 1.7 for control rats and 2.9 ± 2.2 for IFN-α/β-treated rats on day 11.

The concentrations of IFN-γ and IL-10 in intraocular extracts prepared from immunized rats sacrificed on days 8, 12 and 16 are shown in Fig. 1(b and c). The results of measurements performed at the same time in intraocular extracts prepared

Table 1. Scoring of biomicroscopic severity of EAU in rats

<table>
<thead>
<tr>
<th>Biomicroscopic criteria</th>
<th>Maximum score</th>
</tr>
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<tbody>
<tr>
<td>Mydriasis after tropicamide instillation</td>
<td>2</td>
</tr>
<tr>
<td>0 = complete</td>
<td></td>
</tr>
<tr>
<td>1 = partial</td>
<td></td>
</tr>
<tr>
<td>2 = absent</td>
<td></td>
</tr>
<tr>
<td>Fibrinous exudate in the anterior chamber</td>
<td>2</td>
</tr>
<tr>
<td>0 = none</td>
<td></td>
</tr>
<tr>
<td>1 = limited to the pupil</td>
<td></td>
</tr>
<tr>
<td>2 = covering the iris and pupil</td>
<td></td>
</tr>
<tr>
<td>Hypopyon</td>
<td>3</td>
</tr>
<tr>
<td>0 = none</td>
<td></td>
</tr>
<tr>
<td>1 = mild</td>
<td></td>
</tr>
<tr>
<td>2 = moderate</td>
<td></td>
</tr>
<tr>
<td>3 = severe</td>
<td></td>
</tr>
<tr>
<td>Maximum total score</td>
<td>7</td>
</tr>
</tbody>
</table>
Cytokines in EAU suppression by type I IFN

Fig. 1. (Top panel) Biomicroscopic score of anterior segment intraocular inflammation in rats that received daily intramuscular injections from day 0 (day of IRBP immunization) to 1 day prior to sacrifice of either control or 10^5 IU IFN-α/β. Number of rats examined at each timepoint was 15 for day 8, 10 for days 9–12 and 5 for days 13–16. (Middle panel) Concentrations of IFN-γ in intraocular extracts from normal healthy rats and from control and IFN-α/β-treated rats on days 8, 12 and 16 (right eyes). IFN-γ detection limit = 13 pg/ml. (Lower panel) Concentrations of IL-4 for same groups of rats (left eyes). IL-4 detection limit = 15 pg/ml.

from non-immunized, healthy rats (normal) are shown for comparison. IFN-γ was non-detectable in normal eyes, in eyes obtained on day 8 (prior to onset of inflammation) and in eyes obtained on day 16 (after inflammation had already started to resolve). However, IFN-γ was detected in eyes obtained on day 12, near the time of peak inflammation, with the intraocular IFN-γ concentration being significantly increased in control rats (182 ± 106 pg/ml) compared to IFN-α/β-treated rats (33 ± 49 pg/ml).

In contrast, IL-4 was detected in intraocular extracts from normal rats as well as from both groups of immunized rats. However, the IL-4 concentration was consistently higher in rats treated with IFN-α/β when compared to controls on all days examined, with the difference being statistically significant for days 8 and 16. The intraocular IL-4 concentration peaked for both control rats (437 ± 119 pg/ml) and IFN-α/β-treated rats (550 ± 91 pg/ml) on day 12.

Figure 2 shows the results of the second experiment in which IL-2 and IL-10 were measured. Mean biomicroscopic scores for control rats and rats that received daily intramuscular injections of 10^5 IU IFN-α/β are shown in Fig. 2(a). Similar to the first experiment, onset of EAU inflammation occurred on day 9 for both groups. Administration of IFN-α/β was associated with significantly reduced biomicroscopic scores for days 10, 11 and 12, while the scores for day 9 and 13–16

Fig. 2. (Top panel) Biomicroscopic score of anterior segment intraocular inflammation in rats that received daily intramuscular injections from day 0 (day of IRBP immunization) to 1 day prior to sacrifice of either control or 10^5 IU IFN-α/β. Number of rats examined at each timepoint was 15 for day 8, 10 for days 9–12 and 5 for days 13–16. (Middle panel) Concentrations of IL-2 in intraocular extracts from normal healthy rats and from control and IFN-α/β-treated rats on days 8, 12 and 16 (right eyes). IL-2 detection limit = 10 pg/ml. (Lower panel) Concentrations of IL-10 for same groups of rats (left eyes). IL-10 detection limit = 15.6 pg/ml.
were not significantly different. Peak severity scores were 5.8 ± 1.7 for control rats and 4.3 ± 1.4 for IFN-α/β-treated rats on day 12.

Intraocular IL-2 concentrations are shown in Fig. 2(b). IL-2 appeared to gradually increase through the course of EAU in control rats, with the highest concentration of 125 ± 108 pg/ml detected on day 16. In contrast, rats treated with IFN-α/β appeared to exhibit a rise in intraocular IL-2 from non-detectable to a small peak coinciding with the time of maximum inflammation near day 12 (49 ± 42 pg/ml), followed by a decrease in concentration at day 16 (21 ± 73 pg/ml). For comparison, IL-2 was also measured in normal rats and found to be detectable at a level of 19 ± 43 pg/ml. None of the differences in IL-2 were statistically significant.

The results for measurements of IL-10 are shown in Fig. 2(c). IL-10 levels in intraocular extracts were non-detectable in normal rats, and in both IFN-α/β-treated rats and control rats on day 8. On day 12 at near peak inflammation, IL-10 was significantly elevated in control rats (588 ± 139 pg/ml) compared to IFN-α/β-treated rats (23 ± 113 pg/ml), followed by a decreased to a low level in control rats (27 ± 265 pg/ml) and a non-detectable level in IFN-α/β-treated rats on day 16.

Discussion

Recombinant IFN-α has been used in small clinical trials with reported success in controlling inflammation in patients with ocular Behc¸ et’s disease (7–9). However, the scientific basis of such use had not been investigated in experimental models of intraocular inflammation. We recently reported that the systemic administration of a mouse natural IFN-α/β preparation significantly suppressed EAU inflammation in Lewis rats by both clinical and histopathological criteria (16). However, when the systemic immune response of these IFN-α/β-treated rats was investigated, we were unable to identify any difference from controls with respect to cellular proliferation response nor anti-IRBP antibodies. In addition, there was no difference in the production of IFN-γ, IL-2, IL-4 and IL-10 by splenocytes in vitro between IFN-α/β-treated rats and control rats. Given the degree of suppression of EAU we observed, these results were unexpected. This current study therefore attempts to identify a difference in intraocular cytokine production patterns in vivo with IFN-α/β treatment of EAU.

Our results show that in eyes of rats immunized with IRBP that developed EAU, IFN-γ increased abruptly with acute inflammation, then decreased just as rapidly as inflammation subsided. In addition, when EAU was suppressed in rats by daily injections of 10^5 IU IFN-α/β, this IFN-γ response was significantly inhibited. Interestingly, the same abrupt increase and decline in intraocular levels in control EAU rats, as well as suppression by IFN-α/β treatment, was observed for IL-10 as well. Such a clear cut difference in intraocular cytokine levels of IFN-γ and IL-10 appears somewhat out of proportion to the ~25–30% suppression of inflammation we observed clinically with IFN-α/β in the current series of experiments. However, degree of inflammation (cellular infiltration) may be reflected in the amounts of other cytokines as well, such as IL-2, which was no different between IFN-α/β-treated rats and controls.

Although differential cytokine production patterns of T₈ cells were originally defined in murine T cell clones (21), the development of EAU in rats is widely believed to be associated with activation of a T₈₁-like response, with its suppression associated with activation of a T₈₂-like response. This view has been supported by the demonstration of IFN-γ by immunostaining techniques and RT-PCR in EAU eyes in vivo (11,22), the ability to transfer EAU by T cell lines that produce IFN-γ but not IL-4 in vitro (14) and the observation that cells shown to exhibit IL-4 expression in vitro could prevent the onset of EAU (23). However, none of these studies examined actual levels of cytokines in vivo through the course of EAU and therefore could not assess the relative contributions of purported T₈₁- or T₈₂-like responses in the intraocular environment.

While our results support a role for T₈₁-type cytokines such as IFN-γ at the local level in acute EAU inflammation, they cast into doubt the true role of IL-10 and other T₈₂-type cytokines produced within the eye in EAU. Based on studies of peripheral immune cells, IL-10 and other T₈₂-type cytokines are believed to down-regulate T₈₁ development and activity, with T₈₁-type cytokines down-regulating T₈₂ development and activity, in a cross-regulatory manner (24,25). However, the results of our study suggest that a T₈₂-like immune response by infiltrating lymphocytes may be activated in EAU, in addition to a T₈₁-like response. One possible explanation is that the presence of a T₈₂ response contributes to the self-limiting nature of EAU inflammation. It has previously been suggested that both T₈₁ and T₈₂ responses are activated in many normal immune responses, and that the immune response is more likely to polarize to either a T₈₁ or T₈₂ response in chronic or relapsing diseases (21,25). Thus, the non-relapsing, non-chronic nature of the EAU model may similarly involve both types of T₈ immune responses simultaneously within the intraocular environment.

The possible role of IL-10 in limiting EAU inflammation in control rats likely depends greatly upon the amount of IL-10 present (or dose administered) and whether the IL-10 is present (or is administered) intraocularly or systemically. For example, it has been shown that endotoxin-induced uveitis, another experimental model for human uveitis, was suppressed when IL-10 was injected directly into the eye (26). However, it has also been shown that systemic administration of IL-10 at doses of 10^4 and 10^5 U failed to prevent EAU, while a lower dose of 10^2 U exacerbated EAU inflammation in association with increased IFN-γ, IL-2 and nitric oxide (27). Even the supposedly ‘proinflammatory’ cytokine IFN-γ, in its endogenous systemic form, has been found to play a protective role in EAU in mice (28), although when injected directly into rat eyes it is well known to induce MHC class II expression and an inflammatory response (29,30). Therefore, extreme care must be taken when interpreting the results of cytokines measured or cytokines administered in any experimental model, since dose and site of action of the cytokine can contribute to seemingly paradoxical results.

The mechanism of EAU suppression by IFN-α/β is difficult to determine using the intraocular cytokine data of this study alone. However, the possibility of local activation of a T₈₂ response appears to be unlikely, given the profound suppression of IL-10 with IFN-α/β treatment. If anything, it appears
that both intraocular T_{h}1 and T_{h}2 responses may be co-suppressed with IFN-α/β. This is in agreement with studies in multiple sclerosis patients, in which IFN-β has been found to have no influence on numbers of IL-10-secreting T_{h}2 cells in peripheral blood mononuclear cell cultures, and it has been tentatively suggested that IFN-β does not activate T_{h}2 cells in these patients (4). Recently, the existence of a third type of T_{h} cell has been proposed; this ‘regulatory’ T cell has neither T_{h}1- nor T_{h}2-type profile, is associated with transforming growth factor-β production, and may be capable of suppressing both T_{h}1 and T_{h}2 responses (25,31). The fact that IL-4 was elevated (and not suppressed) in eyes of rats treated with IFN-α/β in the present study is not necessarily inconsistent with the effect of such ‘regulatory’ T cells, since IL-4 can originate from numerous sources other than infiltrating T_{h}2-type cells, such as mast cells which reside in large numbers in the choroid (25,32,33). Indeed, IL-4 was detected previously documented a rise in intraocular IL-4 in rats immunized with complete Freund’s adjuvant alone, without IL-10, suggesting that the Th2-type cytokine IFN-γ in the intraocular environment may be capable of suppressing both T_{h}1 and T_{h}2 immune responses in the intraocular environment.

In summary, the present study confirms a role for the T_{h}1-type cytokine IFN-γ in the intraocular environment in the development of IRBP-induced EAU in Lewis rats and suggests that the T_{h}2-type cytokine IL-10 is also involved in the acute inflammatory process at the intraocular level. Furthermore, IFN-α/β at a daily systemically administered dose of 10^{5} IU was found to inhibit EAU in association with suppression of both intraocular IFN-γ and IL-10, suggesting that the mechanism of suppression by type I IFN may involve co-inhibition of both T_{h}1 and T_{h}2 immune responses in the intraocular environment.

Acknowledgements
This work was supported in part by a research grant from Otsuka Pharmaceutical Corp.

Abbreviations

EAU  experimental autoimmune uveoretinitis
IRBP  interphotoreceptor retinoid-binding protein

References


Cytokines in EAU suppression by type I IFN