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IL-12 inhibits endotoxin-induced inflammation in the eye

Interleukin-12 (IL-12) is a heterodimeric cytokine that induces interferon (IFN)-γ production and an increased generation of Th1 cells. Both IL-12 and IL-12 antagonists are being studied for the treatment of allergic reactions, autoimmune disease and malignancy. The goal of the present experiments was to examine the importance of IL-12 in endotoxin-induced ocular inflammation. The number of inflammatory cells infiltrating eyes with endotoxin-induced uveitis (EIU) was significantly increased in animals treated with intraperitoneal anti-IL-12 antibody when compared to control animals, but there was no difference in infiltrating inflammatory cells in the eyes of animals treated with IL-12 when compared to controls. In contrast, intraocular injection of IL-12 significantly inhibited the development of endotoxin-induced intraocular inflammation. The infiltrating inflammatory cells were reduced in the eyes of animals receiving intraocular IL-12 when compared to controls. Cytokine analysis of the aqueous humor obtained from eyes with EIU showed increased levels of IFN-γ and decreased levels of IL-6 in eyes receiving intraocular IL-12. These data show that IL-12 has an inhibitory effect on endotoxin-induced inflammation in the eye and suggest that IL-12 can have an immunoregulatory function in some forms of inflammatory disease.

1 Introduction

Interleukin 12 (IL-12) is a heterodimeric cytokine produced by macrophages and B cells in response to bacteria, bacterial products and intracellular parasites [1–3]. Recent data has shown that IL-12 stimulates the production of IFN-γ and TNF-α and induces the differentiation of T helper 1 (Th1) cells from uncommitted, precursor T helper lymphocytes [1, 4–6]. Injection of bacterial endotoxin induces intraocular inflammation in susceptible animals with breakdown of the blood-aqueous barrier [7–9], and this endotoxin-induced uveitis (EIU) is an animal model for anterior uveitis in humans. A number of inflammatory mediators have been found in eyes with EIU including leukotrienes, thromboxanes and prostaglandins [10, 11]. However endotoxin induces the release of a number of inflammatory cytokines [12–14] and experiments suggest that these cytokines play an important role in the pathogenesis of EIU. For example, injection of IL-1, IL-6 and TNF into the eye results in acute uveitis [15–19]. Recently, investigators have shown that systemically administered antibodies against IFN-γ or TNF-α can paradoxically exacerbate EIU [20, 21]. Since IL-12 is a potent inducer of IFN-γ and TNF-α production, we decided to investigate the role of IL-12 in the pathogenesis of endotoxin-induced intraocular inflammation.

[I 15172]

Received December 10, 1995; in revised form February 5, 1996; accepted February 14, 1996.

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Abbreviation: EIU: Endotoxin-induced uveitis

Key words: Interleukin-12 / Endotoxin / Eye / Inflammation / Cytokine

2 Materials and methods

2.1 Animals

Female C3H/HeN mice (6–8 weeks old) were obtained from the NCI breeding facility (Frederick, MD) and housed in standard pathogen-free conditions. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2 Endotoxin-induced uveitis

Endotoxin-induced uveitis was produced by injecting 200 μg *Salmonella typhimurium* endotoxin (Difco Laboratories, Detroit, MI) diluted in 0.1 ml sterile saline into one hind footpad.

2.3 Effect of systemic anti-IL-12 antibody or recombinant IL-12 on EIU

Mice received an intraperitoneal injection of 1 mg anti-IL-12 mAb ($n = 21$) [5], 1 μg mouse recombinant IL-12 ($n = 5$) (kindly provided by Dr. Maurice K. Gately, Hoffman-La Roche, Nutley, NJ), or PBS as a control ($n = 18$), at the time of endotoxin injection. Previous studies showed that there was no difference in the effect of an irrelavant mAb or PBS on the development of EIU [22]. In addition, IgG was felt to be an inappropriate control for the IL-12 injections, and PBS has been used in previous studies using this disease model [21]. The endotoxin content of the mAb against IL-12 was tested using an E-toxate detection kit (Sigma, St. Louis, MO) and found to have less than 0.1 EU/ml (the lower limit of detection for this assay).

Mice were killed 24 h after endotoxin injection and the right eye was enucleated and processed for routine histo-

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logy. Infiltrating inflammatory cells were counted blind on coded 4-μm hematoxylin- and eosin-stained sections containing the cornea, pupil and the optic nerve head. Left eyes of animals in the above experiment were enucleated, embedded in optimum cutting-temperature compound (OCT, Miles, Naperville, IL), snap-frozen in a 2-methylbutane/dry ice slurry and stored at −70 °C. Cryostat vertical sections (6 μm thick) containing the pupillary-optic nerve axis were prepared on poly-L-lysine-coated slides and stored at −4 °C for 48 h before routine hematoxylin and eosin and immunohistochemical staining. The immunohistochemical staining was performed using an avidin-biotin-peroxidase complex (ABC) technique [23]. Slides were fixed in acetone for 7 min, transferred to Tris-buffered saline (TBS; 0.05 M Tris-HCl pH 7.6, 0.9% NaCl), and then immersed in 1% normal horse serum in TBS for 10 min. mAb against CD3, CD4, CD8, M1/70.15, and CD20 (Becton Dickinson, Mountain View, CA) were the primary antibodies. Rat IgG (Sigma, St. Louis, MO) was the control primary antibody, and biotin-conjugated goat anti-rat IgG (American Qualex, La Mirada, CA) was the secondary antibody. The avidin-biotin-peroxidase complex was applied and the bound complex revealed using H_2O_2 as substrate and Ni_2SO_4-enhanced 3,3′-diaminobenzidine as chromogen. The number of macrophages, T lymphocytes and B lymphocytes were counted blind on coded immunohistochemically stained sections.

2.4 Intraocular injection of IL-12

Animals received footpad injections of either 200 μg endotoxin (n = 10) or PBS (n = 10). Within each of these two groups, five animals received 3 μg rIL-12 in 3 μl PBS into the anterior chamber of the right eye and five animals received 3 μl PBS alone into the anterior chamber of the right eye. Intraocular injections were performed with a 33-gauge needle on a Hamilton syringe. Mice were killed 24 h after footpad injection, and the injected eye was enucleated and processed for histology and inflammatory cell infiltration was assessed as described. Since injection itself causes some ocular inflammation, the contralateral, uninjected eye is not regarded as an appropriate control.

2.5 Cytokine analysis of aqueous humor

In two separate experiments, EIU was produced in animals with footpad injections of endotoxin as described above. Both eyes of each animal were then injected with either IL-12 (n = 11 for experiment 1 and n = 22 for experiment 2) or PBS (n = 10 for experiment 1 and n = 20 for experiment 2). An anterior chamber paracentesis was performed on the right eye of all animals using a 33-guage needle on a Hamilton syringe 24 h after endotoxin injection. Approximately 5 μl aqueous humor was collected from each eye and stored at −70 °C for cytokine analysis. The left eye was enucleated and processed for routine histology and graded as described above.

The aqueous humor was pooled from the right eyes of animals treated with either IL-12 or PBS and analyzed for the presence of IL-6, IL-10 and IFN-γ. Cytokines were analyzed by a conventional ELISA [24]. Briefly, high-binding sterile 96-well plates (Costar) were coated with the respective anti-cytokine antibody overnight in carbonate-bicarbonate buffer pH 8.2 leaving the border wells empty. The next day, plates were blocked with PBS + 5% BSA, pH 7.2 for 2 h and then washed. Samples were diluted in the blocking buffer and incubated at room temperature for 4 h. The dilution buffer was critical at this step since apparently some unknown constituent of the aqueous humor inhibits the enzymatic activity of horseradish peroxidase. Unbound material was washed, the pertinent biotin-conjugated secondary antibody was added and the plates were incubated at 37 °C for 45 min. After washing the unbound material streptavidin-horseradish peroxidase (Southern Biotechnologies Associates, Birmingham, AL) was added for 30 min. Plates were developed using H_2O_2 and o-phenylenediamine and the absorbance read at 490 nm. Concentrations of the cytokines in each sample were extrapolated from a standard curve constructed with recombinant cytokine. Anti-IL-10 and anti-IFN-γ antibodies were obtained from Pharmingen (La Jolla, CA). Anti-IL-6 antibody was obtained from Endogen (Cambridge, MA). Recombinant IL-10 was a gift from Dr. Kevin Moore and Dr. Maureen Howard (DNAX, Palo Alto, CA), recombinant IFN-γ was a gift from Genentech (South San Francisco, CA) and recombinant IL-6 was a gift from Dr. Gideon Strassmann (Otsuka Pharmaceuticals, Germantown, MD).

2.6 Statistical analysis

When more than two experimental groups were involved, comparisons were made using an analysis of variance using Fisher's protected least significant difference test. In the experiments analyzing cytokine concentration in eyes with EIU, the number of infiltrating inflammatory cells in animals treated with IL-12 or PBS were compared with an unpaired Student's t-test. In all analyses, the null hypothesis, *i.e.* that there is no difference between groups, was rejected at the $p < 0.05$ level.

3 Results

3.1 Effect of systemic anti-IL-12 antibody and recombinant IL-12 on EIU

The number of inflammatory cells (mean ± SEM), predominantly neutrophils and macrophages, infiltrating eyes with EIU was significantly increased in animals treated with anti-IL-12 antibody when compared to control animals (41.6 ± 9.3 *vs.* 17.7 ± 2.1; p = 0.02) (Fig. 1). In contrast, there was no difference in infiltrating inflammatory cells in the eyes of animals treated with 1 μg IL-12 when compared to controls (17.6 ± 3.5 *vs.* 17.7 ± 2.1; p = 0.99).

Immunohistochemical staining using the M1/70.15 revealed increased numbers of macrophages in the eyes of animals treated with anti-IL-12 antibody when compared with animals treated with recombinant IL-12 or PBS that approached statistical significance (p = 0.06 (Fig. 2). Fewer T cells and no B cells were found among the infiltrating inflammatory cells in all three groups.

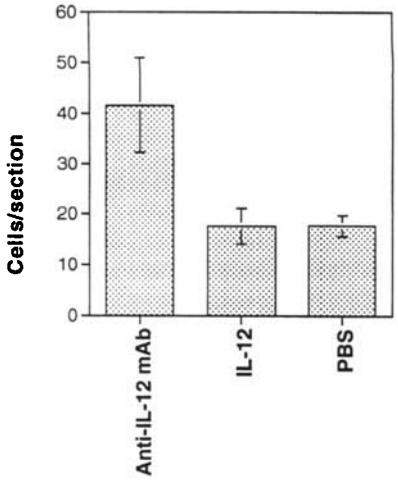

Figure 1. Bar graph showing the effect of systemic anti-IL-12 antibody and recombinant IL-12 on EIU. The number of inflammatory cells (mean ± SEM), predominantly neutrophils and macrophages, infiltrating eyes with EIU was significantly increased in animals treated with an intraperitoneal injection of anti-IL-12 antibody (n = 21) when compared to control animals receiving PBS (n = 18, p = 0.02). There was no statistically significant difference in the number of infiltrating inflammatory cells in the eyes of animals treated with recombinant IL-12 (n = 5) compared to the control animals receiving PBS (p = 0.99).

3.2 Effect of intraocular IL-12 on normal eyes and eyes with EIU

Since the intraperitoneal injection of 1 μg of IL-12 had no significant effect on the development of EIU, we investigated the effect of a higher dose (3 μg) of IL-12 injected locally into the eye. None of the eyes of animals that received footpad injections of PBS developed ocular inflammatory disease (Fig. 3). The number of inflammatory cells (mean ± SEM) infiltrating the eyes was 0.2 ± 0.2

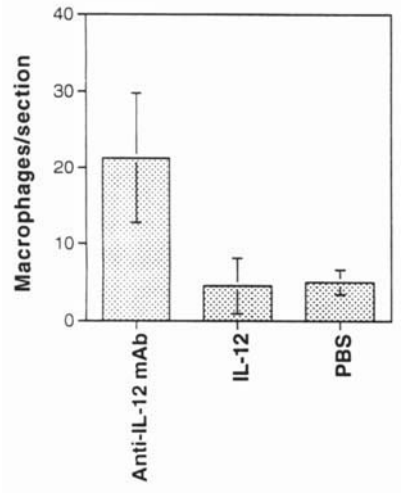

Figure 2. Bar graph showing the effect of systemic anti-IL-12 antibody and recombinant IL-12 on the number of macrophages (mean ± SEM) infiltrating eyes with EIU. Increased numbers of macrophages were noted in eyes of animals treated with intraperitoneal anti-IL-12 antibody (n = 4) when compared to animals treated with recombinant IL-12 (n = 4, p = 0.06) or PBS (n = 4, p = 0.06).

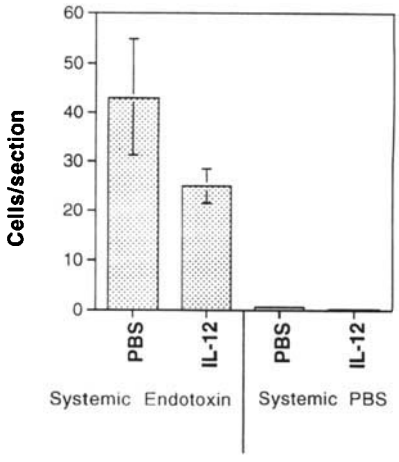

Figure 3. Bar graph showing the effect of intraocular IL-12 or PBS on eyes from animals receiving systemic endotoxin or PBS. In the absence of systemic endotoxin injection, none of the eyes of animals receiving intraocular injections of IL-12 (n = 5) or PBS (n = 5) developed uveitis. In contrast, animals receiving footpad injections of endotoxin developed substantial intraocular inflammatory disease. There was a statistically significant decrease in the number of infiltrating inflammatory cells (mean ± SEM) in the eyes of animals receiving intraocular IL-12 (n = 5) when compared to controls (n = 5, 25.0 ± 3.5 *vs.* 43.0 ± 11.7, p = 0.05).

Table 1. Intraocular cytokine levels in EIU[a)]

Experiment 1			
Treatment	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-6 (pg/ml)
IL-12 (n = 11)	900	3110	8300
PBS (n = 10)	2910	1755	23600
Experiment 2			
Treatment	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-6 (pg/ml)
IL-12 (n = 22)	208	269.8	894
PBS (n = 20)	178	118	4170

a) Twenty-four hours after systemic endotoxin injection, cytokine levels were determinded by ELISA in aqueous humor samples pooled from eyes treated with intraocular injections of either IL-12 or PBS. Results from two separate experiments are presented.

in animals receiving intraocular IL-12 and 0.6 ± 0.2 in animals receiving intraocular PBS (p = 0.96), demonstrating that the intraocular injection of either IL-12 or PBS did not induce uveitis. In contrast, all animals receiving footpad injections of endotoxin developed substantial intraocular inflammatory disease (Fig. 3). Of note, the infiltrating inflammatory cells (mean ± SEM) were reduced in the eyes of animals receiving intraocular IL-12 when compared to controls (25.0 ± 3.5 *vs.* 43.0 ± 11.7; p = 0.05) (Fig. 3).

3.3 Cytokine analysis of aqueous humor from eyes with EIU

The quantity of IL-6, IL-10, TNF-γ in the aqueous humor of eyes with EIU were assessed in two experiments shown in Table 1. As expected, IFN-γ was increased in the aqueous humor of eyes treated with IL-12 when compared to eyes receiving PBS. In contrast, IL-6, a cytokine thought

to be important for the induction of uveitis, was reduced in eyes treated with IL-12 when compared to controls in both experiments. IL-10 was barely detectable in the aqueous humor of mice receiving IL-12 or control injections. TNF-α was diminished in the aqueous humor of mice treated with IL-12 in the first experiment, but unaltered in the second experiment. Similar to the results shown above, the number of inflammatory cells (mean ± SEM) infiltrating the contralateral eyes with EIU was reduced in eyes treated with IL-12 when compared to controls (mean ± SEM; 45.7 ± 5.9 *vs.* 75.8 ± 13.5; $p = 0.04$).

4 Discussion

We have shown that the local administration of IL-12 inhibits ocular inflammation in EIU. This diminished inflammatory response correlated with higher levels of IFN-γ and lower levels of IL-6 in the eye. TNF-α was diminished in the aqueous humor of mice treated with IL-12 in one of the two experiments and unaltered in the second. In contrast, IL-10 was barely detectable in the aqueous of both IL-12-treated and control mice. In addition, IL-4 and IL-5 were not detected in the aqueous humor of eyes treated with IL-12 or PBS (data not shown).

Most studies have demonstrated a pro-inflammatory effect of IL-12. However, a recent study by Garett et al. [25] showed that IL-12 inhibits antigen-induced airway hyperresponsiveness and pulmonary eosinophilia. The authors suggested that the anti-inflammatory effect of IL-12 was mediated by an increase in IFN-γ, and anti-IFN-γ antibody partially reduced the suppressive effect.

This is the first report of IL-12 inhibiting endotoxin-induced inflammation. Again, the anti-inflamatory effect of IL-12 may be linked to the locally elevated levels of IFN-γ. IFN-γ is a potent inhibitor of cell proliferation [26], and the high intraocular levels seen after IL-12 injection could inhibit the recruitment of other leukocytes to the inflammatory site [27]. IFN-γ has had a suppressive effect on the development of other diseases such as experimental autoimmune encephalomyelitis, experimental autoimmune uveitis, and adjuvant arthritis [28, 29]. Recently, Matthys et al. [30] showed that IFN-γ receptor-deficient mice are hypersensitive to anti-CD3-induced cytokine release syndrome. In a previous study, the authors found that pre-treatment with anti-IFN-γ antibody provides protection against the syndrome [31]. The paradoxical effect of IFN-γ may be dose-dependent: high levels of IFN-γ appear to have an inhibitory effect on induced inflammation, while in contrast, lower concentrations of IFN-γ may actually promote the recruitment of inflammatory cells by upregulating MHC class II antigens and several other cell surface molecules involved in cell adhesion and cell activation [32].

Wysocka et al. [6] showed that IL-12 is required for lipopolysaccharide-induced shock in cooperation with TNF-α and IFN-γ. We previously showed that inhibition of TNF-α decreased endotoxin-induced mortality, but exacerbated EIU [21]. These data suggest that IL-12-mediated TNF-α production can also have a suppressive effect on endotoxin-induced ocular inflammation. Other researchers have demonstrated paradoxical effects of cytokines in the EIU model. Rosenbaum et al. [33] recently showed that IL-10 paradoxically inhibits EIU, presumably by decreasing local cytokine production.

In our study, IL-12, a cytokine that usually promotes the inflammatory response, had an anti-inflammatory effect when injected locally into the eye. Similarly, a systemically administered monoclonal antibody that blocked IL-12 exacerbated ocular inflammation induced by endotoxin. In agreement with previous studies of antigen-induced airway hyperresponsiveness, our data suggest that IL-12 can have an immunosuppressive effect on endotoxin-induced inflammation, possibly by modulating the levels of cytokines such as IFN-γ and IL-6.

We are grateful to Dr. Maurice Gately and the Hoffman-LaRoche Research Institute for the gift of recombinant mouse IL-12; to Drs. Kevin Moore and Maureen Howard and the DNAX Research Institute for the gift of recombinant mouse IL-10; to Dr. Giorgio Trinchieri and the Wistar Institute for the gift of the anti-mouse IL-12 monoclonal antibodies; and to Dr. Gideon Strassman and Otsuka Pharmaceuticals for the gift of recombinant IL-6.

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