

## Aqueous Humor Levels of Different Interleukins 1- $\beta$ , 2, 6 and 10, Tumor Necrosis Factor- $\alpha$ and Vascular Endothelial Growth Factor in Uveitis Treated with Adalimumab

María Luisa Hernández Garfella<sup>1</sup>, MD; Paula Palomares Fort<sup>1</sup>, MD; José Andrés Román Ivorra<sup>2</sup>, MD, PhD  
Enrique Cervera Taulet<sup>1</sup>, MD, PhD

<sup>1</sup>Service of Ophthalmology, University General Hospital of Valencia, Valencia, Spain

<sup>2</sup>Service of Rheumatology, University and Polytechnic Hospital La Fe, Valencia, Spain

### Abstract

**Purpose:** To assess changes in aqueous humor levels of different interleukins (IL), tumor necrosis factor (TNF)- $\alpha$  and vascular endothelial growth factor (VEGF) in patients with uveitis treated with adalimumab.

**Methods:** In this study, 24 aqueous humor samples including 12 pre- and post-treatment samples from 6 patients with uveitis treated with subcutaneous adalimumab and 12 samples from patients with cataracts (serving as controls) were evaluated. The levels of IL-1 $\beta$ , IL-2, IL-6, IL-10, TNF- $\alpha$  and VEGF were measured using a Luminex<sup>®</sup> 200<sup>™</sup> flow cytometer (Merckmillipore, Merck KGaA, Darmstadt, Alemania) and a highly sensitive ELISA system.

**Results:** The levels of IL-1 $\beta$ , IL-2, IL-6 and IL-10 in the aqueous humor before and after treatment with adalimumab did not show significant differences. Aqueous VEGF levels significantly reduced after treatment with adalimumab ( $P = 0.028$ ). Aqueous TNF- $\alpha$  levels did not significantly change after treatment with adalimumab, however the post-treatment level was significantly higher in patients as compared to control subjects ( $P = 0.032$ ). IL-2 showed significantly higher levels in uveitis patients before treatment as compared to controls ( $P = 0.024$ ), while its post-treatment levels were almost normalized.

**Conclusion:** Decrease in the aqueous humor levels of VEGF and IL-2 after treatment with systemic adalimumab indicates that anti-TNF- $\alpha$  therapy induces modifications of some inflammatory mediators involved in the pathogenesis of uveitis. Aqueous humor samples may be useful to assess the effect of adalimumab on intraocular inflammation through measurement of cytokines.

**Keywords:** Adalimumab; Aqueous Humor; Interleukins; Tumor Necrosis Factor Alpha; Uveitis; Vascular Endothelial Growth Factor

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### INTRODUCTION

Autoimmune uveitis comprises an extensive array of inflammatory eye diseases and often results in irreversible visual loss. Uveitis may be primarily restricted to the eye or occur in association with systemic

inflammatory conditions. The disease is particularly prevalent in young people and can be difficult to manage due to frequent relapses and complications.<sup>[1,2]</sup> Uveitis represents the fifth leading cause of visual loss in Europe with a prevalence of approximately 35-80 per 100,000<sup>[3]</sup>

#### Correspondence to:

María Luisa Hernández Garfella, MD. Service of Ophthalmology, University General Hospital of Valencia, Avda. Tres Cruces s/n, E-46014 Valencia, Spain.  
E-mail: marisahergar@gmail.com

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and responsible for 5-20% of blindness.<sup>[3]</sup> The severe morbidity of uveitis provides a stimulus for research on its pathogenetic mechanisms and the development of effective therapies.

Although the dysregulation of the immune system in uveitis is not thoroughly characterized, experimental models of autoimmune uveitis revealed a pivotal role for antigen-specific CD4 + T-helper (Th) lymphocytes with activation of Th-1 and Th-2 mediated intraocular inflammatory pathways and overexpression of chemokines related to the Th1 and Th2 systems. High levels of interleukin (IL)-2, IL-6, IL-10, IL-13, IL-18, tumor necrosis factor (TNF)- $\alpha$ , interferon- $\gamma$  and vascular endothelial growth factor (VEGF) were detected in the aqueous humor of patients with uveitis<sup>[4-6]</sup> and in endotoxin-induced models of uveitis.<sup>[7,8]</sup> It has been shown that TNF- $\alpha$  actively participates in the pathogenesis of clinical uveitis<sup>[5]</sup> and elevated serum levels of TNF- $\alpha$  seem to be associated with recurrent uveitis.<sup>[9]</sup>

Therapeutic options for non-infectious uveitis have largely improved with the introduction of immunosuppressant agents, such as cyclosporine, azathioprine, methotrexate and mycophenolate mofetil. However, despite successful immunosuppression of ocular inflammation, some cases of uveitis cannot be controlled. The availability of biologic agents has markedly improved the therapy of uveitis and considerably increased the possibility of long-term remissions, in particular, for patients with refractory and sight threatening inflammatory disorders.<sup>[10,11]</sup> Adalimumab, a recombinant IgG1 monoclonal antibody targeting TNF- $\alpha$ , showed efficacy in approved targeted indications for a number of systemic autoimmune disorders. Administering a single 40 mg dose of adalimumab subcutaneously is absorbed and distributed slowly, reaching peak serum concentrations within almost 5 days.<sup>[12]</sup> After single intravenous doses ranging from 0.25-10 mg/kg, concentrations are dose-proportional. For a dose of 0.5 mg/kg (~40 mg), clearance range from 11 to 15 mL/hour, the distribution volume ranges from 5 to 6 litres and the mean terminal phase half-life is approximately 2 weeks.<sup>[12]</sup> Adalimumab concentrations in the synovial fluid in patients with rheumatoid arthritis range from 31 to 96% of those in the serum.<sup>[12]</sup> No data is available on the ocular penetration of adalimumab but clinical trials are currently being conducted for uveitis. However, adalimumab has been used off-label for ocular inflammatory diseases, such as uveitis, with satisfactory results.<sup>[13-15]</sup> Moreover, in a recent evidence-based interdisciplinary guideline for anti-inflammatory treatment of uveitis associated with juvenile idiopathic arthritis, adalimumab was recommended as the preferred TNF- $\alpha$  inhibitor agent.<sup>[16]</sup>

The present study was designed to assess changes in the aqueous humor levels of four different interleukins,

TNF- $\alpha$  and VEGF in patients with uveitis before and after treatment with adalimumab and in patients with cataract as a control group.

## METHODS

The study was conducted at the Uveitis Unit in the Ophthalmology Service at General University Hospital, Valencia, Spain in daily practice conditions. In this study, the levels of IL-1 $\beta$ , IL-2, IL-6, IL-10, TNF- $\alpha$  and VEGF in 24 aqueous humor samples including 12 pre-and post-treatment samples from 6 non-consecutive patients with chronic, non-infectious, refractory uveitis treated with subcutaneous adalimumab and 12 samples from patients with cataract were measured. Six eligible patients were selected for the study according to criteria proposed by the Standardization of Uveitis Nomenclature (SUN) Working Group.<sup>[17]</sup> The inclusion criteria comprised age  $\geq$  18 years, patients with bilateral, chronic, non-infectious, refractory uveitis in whom treatment with at least one additional immunosuppressant drug (cyclosporine, methotrexate, azathioprine or mycophenolate mofetil) as well as oral corticosteroids was ineffective, Inability to maintain the disease without relapse for more than one year before enrolment. The patients were treated with 40 mg adalimumab (Humira, Abbott Laboratories, Chicago, IL, USA) subcutaneously every other week for 6 months (12 episodes of treatment with a total dose of 480 mg adalimumab). Treatment was administered on a compassionate-use basis.

Aqueous humor samples were obtained before initiating adalimumab and at the day after administration of the last dose of adalimumab at month six. During this period, the underlying immunosuppressive therapy was not modified and if a patient required additional topical or systemic treatment owing to disease relapse, the subject was excluded. All patients underwent a protein-purified derivative skin test (PPD) and chest radiography before enrolment because of the risk of tuberculosis associated with TNF inhibition. The control group included non-selected patients of any age routinely scheduled for cataract surgery. All subjects underwent complete physical and ocular examination as well as a detailed medical history. Inclusion criteria for the control group were absence of systemic diseases, inflammatory diseases, infection, neoplasms, diabetes mellitus, hypertension, cardiovascular disease, osteoporosis or other rheumatic diseases, as well as eye diseases including age-related macular degeneration and diabetic retinopathy. Cases and controls were not matched for age.

The study protocol was approved by the Ethics Committee of the University General Hospital of Valencia, and written informed consent was obtained from all participants.

Samples of aqueous humor were obtained by anterior chamber paracentesis. Pre- and post-procedure antibiotic

prophylaxis was used before and after sampling, the eyes were rinsed with 5% povidone-iodine solution. Anterior chamber paracentesis was performed at the slit lamp using a 30G needle in patients with uveitis, or using an operation microscope before starting cataract surgery in the control subjects. Samples of aqueous humor (0.1-0.2 mL) were collected in sterile tubes and immediately frozen to -80°C until further analysis, when samples were thawed just prior to the assays.

Measurement of IL-1 $\beta$ , IL-2, IL-6, IL-10 and TNF- $\alpha$  was performed employing a Luminex<sup>®</sup> 200™ flow cytometer (Merck Millipore, Merck KGaA, Darmstadt, Germany) multiple array assay system using Multiplex kits (Millipore) based on the Luminex xMAP technology designed to simultaneously measure multiple protein targets in a single sample according to the manufacturer's instructions.<sup>[18]</sup> The MILLIPLEX MAP Human Cytokine/Chemokine panel (EMD Millipore Corporation, Billerica, MA, USA) incubating 25  $\mu$ L of the sample with antibodies at - 4°C was used. Values were expressed as pg/mL, standard curve range from 3.2-10,000 pg/mL. Aqueous humor levels of VEGF were measured with a high sensitive ELISA kit (RandD Systems, Minneapolis, MN, USA) using 25  $\mu$ L of the sample and values were expressed as ng/mL.

## Statistical Analysis

Results were expressed using median and range (10<sup>th</sup>-90<sup>th</sup> percentile). Data in the control group and pre- and post-treatment data in patients with uveitis were compared employing the Mann-Whitney U test. Pre- and post-treatment data in patients with uveitis were compared using the Wilcoxon signed-rank test. *P* values less than 0.05 were considered as statistically significant.

## RESULTS

A total of 24 aqueous humor samples were analysed, comprised of 12 samples from 6 patients with bilateral uveitis including 2 male and 4 female subjects aged 33-68 years, and 12 samples from the control group. Out of six uveitis cases, two patients had idiopathic panuveitis, two were affected with Vogt-Koyanagi-Harada (VKH) syndrome, one subject had idiopathic vasculitis and the last one was affected with Behçet disease. Individual aqueous humor levels of cytokines in patients and controls are shown in Table 1.

Aqueous humor levels of VEGF in patients with uveitis after 6 months of treatment with adalimumab were

**Table 1. Individual aqueous humor levels of cytokines in patients with uveitis and in control eyes**

Sex, age (years)	Diagnosis	Immunosuppressive regimen	Treatment with adalimumab	Aqueous humor levels of cytokines					
				VEGF ng/mL	TNF- $\alpha$ pg/mL	IL-1 $\beta$ pg/mL	IL-2 pg/mL	IL-6 pg/mL	IL-10 pg/mL
Female, 49	VKH	Prednisone, cyclosporine	Before	47.5	0.62	0.34	1.04	57.4	4.7
			After 6 months	4.8	0.46	0	0.32	66.0	10
Female, 46	VKH	Prednisone, cyclosporine	Before	24.7	2.42	0.32	1.04	116.1	46.7
			After 6 months	6.6	4.68	0	2.92	881.7	81
Male, 68	Idiopathic panuveitis	Prednisone, methotrexate	Before	248.4	2.12	0	1.36	930.9	49.4
			After 6 months	93.2	0.48	0	0	20.7	7.7
Female, 44	Idiopathic panuveitis	Prednisone, mycophenolate mofetil	Before	79.5	0	0.9	0	27.0	2.1
			After 6 months	11.8	0	0	0	2	0.4
Female, 33	Idiopathic vasculitis	Prednisone, methotrexate	Before	63	0	0	3.25	1.18	0.4
			After 6 months	0	0.34	0	0	1.62	0.4
Male, 47	Behçet syndrome	Prednisone, cyclosporine	Before	53	0	0	0.98	1.92	0.9
			After 6 months	6.1	0.26	1.32	0	2.2	0.9
Female, 63	Cataract surgery	NA	NA	52.1	0	0	0	15	3.4
Male, 76	Cataract surgery	NA	NA	16.3	0	0	0	35.48	1.7
Female, 68	Cataract surgery	NA	NA	184.5	0	0	0.22	259.56	10.2
Female, 72	Cataract surgery	NA	NA	1.8	0	0	0	1348.54	26.2
Male, 81	Cataract surgery	NA	NA	56.6	0	0	0	42.88	0.9
Male, 65	Cataract surgery	NA	NA	1.3	0	0.26	0.48	8.32	2
Female, 75	Cataract surgery	NA	NA	0	1	1.96	1.98	43.7	3
Male, 77	Cataract surgery	NA	NA	162.3	0.36	0.24	0.32	3.04	1.3
Female, 80	Cataract surgery	NA	NA	0	0	0	0	532.94	13.2
Male, 69	Cataract surgery	NA	NA	271.2	0	0	0	424.04	14.1
Male, 72	Cataract surgery	NA	NA	6.4	0	0	0	181.36	4
Female, 68	Cataract surgery	NA	NA	29.2	0	0	0	259.8	5.1

VEGF, vascular endothelial growth factor; TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; NA, not applicable; pg, picogram; ng, nanogram; mL, microliter; VKH, Vogt-Koyanagi-Harada

significantly decreased (median [10<sup>th</sup>-90<sup>th</sup> percentile], 6.4; range, 0.0-32.2 ng/mL) as compared to pre-treatment levels (mean, 58.0; range, 24.7-216.5 ng/mL) ( $P = 0.028$ ). This reduction was correlated with the clinical response observed in all patients after 6 months of treatment with adalimumab. All patients remained without inflammatory activity both in the anterior and posterior segments of the eye and maintained stable visual acuity. Moreover, in one patient with Behçet disease and another one with idiopathic vasculitis, visual acuity was improved by 5 and 7 letters, respectively.

There was a non-significant reduction in the aqueous humor levels of IL-1 $\beta$ , IL-2, IL-6 and IL-10 after treatment. TNF- $\alpha$  level after treatment (mean, 0.40; range 0.0-2.27 mg/mL) remained comparable to pre-treatment levels (mean, 0.31; range 0.0-2.45 pg/mL).

Comparing uveitis patients with the control group revealed that levels of TNF- $\alpha$  significantly increased after treatment in patients as compared to controls ( $P$  value = 0.032). In addition, IL-2 showed significantly higher levels in uveitis patients before treatment (mean 1.04; range, 0.0-2.45 pg/mL) as compared to controls ( $P$  value = 0.024), while its post-treatment levels were almost normalized [Table 2]. With regard to IL-6, although there were no significant differences between the study groups, high levels were found in the uveitis group (mean, 42.2; range, 1.2-353.6 pg/mL) [Table 2]. All comparisons between control groups and patients with uveitis before and after 6 months of treatment with adalimumab are detailed in Table 2.

## DISCUSSION

Adalimumab is a first fully human (100% human peptide sequences) anti-TNF- $\alpha$  monoclonal antibody which also inhibits the production of some pro-inflammatory cytokines, such as IL-1 and IL-6.<sup>[19]</sup> Secondary effects reported in association with the use of adalimumab include reactivation of tuberculosis, a higher incidence of infections (severe infections, sepsis and other opportunistic infections) and deterioration of demyelinating neurological

diseases. Therefore, patients should be closely monitored to minimize the risk of adverse reactions.

TNF- $\alpha$  is a very potent pro-inflammatory cytokine playing a key role in the regulation of ocular inflammation,<sup>[20]</sup> therefore agents inhibiting TNF- $\alpha$  might be effective in the control of ocular inflammatory processes.<sup>[13,21,22]</sup> The mechanisms of action of cytokines and TNF- $\alpha$  in intraocular inflammation have been studied in experimental autoimmune uveitis,<sup>[23]</sup> however, the type of effector and regulatory responses to one or more retinal antigens have remained obscure.<sup>[24]</sup>

In the present study, the effects of TNF- $\alpha$  blockade on intraocular inflammation were investigated via measurement of cytokines in the aqueous humor. Lower levels of IL-1 $\beta$ , IL-2 and IL-6 in eyes group treated with adalimumab were observed but differences were not significant. However, significantly lower VEGF levels were found in adalimumab-treated patients. VEGF is a glycoprotein which can induce angiogenesis and enhance microvascular permeability, and there are increasing experimental and clinical data regarding its role in inflammation and neovascularization. TNF- $\alpha$  is known to modulate angiogenesis resulting in the promotion of angiogenesis,<sup>[25]</sup> with an increase in the expression of angiogenic factor VEGF. Thus, biologic therapy targeting inflammatory cytokines such as TNF- $\alpha$  is able to significantly reduce VEGF levels as shown in current study by measurement of aqueous humor levels of VEGF before and after treatment with adalimumab. On the other hand, aqueous humor levels of TNF- $\alpha$  did not show any modification.

Aqueous humor levels of IL-6 were higher than expected both in patients with uveitis and in the controls, consistent with other studies.<sup>[26]</sup> It seems that IL-6 is an acute-phase reactant increasing in patients with uveitis and also in other inflammatory processes. The high level of IL-6 in the control subjects may be related to older age as compared with patients with uveitis. An increase in IL-6 due to other diseases (e.g., osteoporosis, arthritis, type 2 diabetes, cancers, etc.) seems to be unlikely given our inclusion criteria for selection of control subjects.

**Table 2. Comparison of aqueous humor levels of cytokines in the control group and uveitis patients before and after treatment with adalimumab**

Cytokines	Controls median (10-90 <sup>th</sup> percentile) (n=12)	Adalimumab treatment		P		
		Before median (10-90 <sup>th</sup> percentile) (n=6)	After 6 months median (10-90 <sup>th</sup> percentile) (n=6)	Controls versus before adalimumab	Controls versus after 6 months adalimumab	Before versus after 6 months adalimumab
VEGF (ng/mL)	22.8 (0.0-245.2)	58.0 (24.7-216.5)	6.4 (0.0-32.2)	0.213	0.494	0.028
TNF- $\alpha$ (pg/mL)	0.0 (0.0-0.81)	0.31 (0.0-2.45)	0.40 (0.0-2.27)	0.213	0.032	0.500
IL-1 $\beta$ (pg/mL)	0.0 (0.0-1.45)	0.16 (0.0-0.48)	0.0 (0.0-0.33)	0.335	0.820	0.715
IL-2 (pg/mL)	0.0 (0.0-1.53)	1.04 (0.0-2.45)	0.0 (0.0-0.97)	0.024	0.892	0.345
IL-6 (pg/mL)	112.5 (4.6-1103.9)	42.2 (1.2-353.6)	11.4 (1.6-269.9)	0.385	0.151	0.917
IL-10 (pg/mL)	4.0 (1.0-22.6)	3.4 (0.4-47.4)	4.3 (0.4-48.1)	0.892	0.494	0.999

VEGF, vascular endothelial growth factor; TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; pg, picogram; ng, nanogram; mL, microliter

Studies on other biologic drugs such as tocilizumab, a new humanized monoclonal antibody against the IL-6 receptor, have confirmed the efficacy of this agent in refractory uveitis,<sup>[27]</sup> supporting the important role of IL-6 in intraocular inflammation.

The use of an anti-TNF- $\alpha$  agent (adalimumab) is a novel aspect of the study as compared to previous reports on the measurement of cytokines in the aqueous humor. Although our patients were previously treated with immunosuppressant agents, we attempted to analyze changes following the administering adalimumab. However, the level of cytokines already altered due to previous immunosuppressive therapy, and thus the altered immune response, is an important limitation in the current study. Ideally, naïve patients for immunosuppressive therapy should be selected but in routine daily practice, this is hardly applicable because patients with uveitis usually have been previously treated with corticosteroids and other immunosuppressive agents.

On the other hand, in order to increase the statistical power of the study, a larger sample size was necessary but recruitment of more patients was difficult due to the selection criteria set for patients with uveitis treated with adalimumab. Patients with uveitis and control subjects were not matched by age given that the control group consisted of patients undergoing cataract surgery, which are usually older people, and aqueous humor samples were obtained at the time of surgery. The selection of younger healthy patients was difficult considering the interventional nature of aqueous humor extraction.

In summary, modifications of some inflammatory cytokines involved in the pathogenesis of uveitis after TNF- $\alpha$  blocking treatment, herein adalimumab, were observed in the current series. Aqueous humor samples may be useful to assess the immune response to biologic therapies<sup>[28-30]</sup> but further studies with large sample size are needed. Immune responses in the eye have complex mechanisms and many interacting antigen-presenting cells, T and B lymphocytes, antibodies and other inflammatory mediators are still unclear. More homogeneous studies with regard to uveitis subtypes with a higher number of patients may clarify differences in the response to immunosuppressant agents among patients with certain types of uveitis, and may be beneficial to define appropriate therapy for those with refractory or relapsing chronic uveitis.

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