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INFLAMMATORY DISORDERS

Evaluation of potential retinal toxicity of adalimumab (Humira)

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Abstract
Purpose The purpose of this study is to evaluate the retinal toxicity of two doses of adalimumab (Humira), a recombinant human IgG1 monoclonal antibody specific for human tumor necrosis factor (TNF), when injected intravitreally in rabbits.
Methods Sixteen male pigmented rabbits (divided into two groups, eight animals per group) were used for this study. Two concentrations of adalimumab were tested: 0.5 mg/0.1 ml and 5 mg/0.1 ml. Each concentration was injected intravitreally randomly in one eye (study group) of each rabbit (group I received 0.5 mg/0.1 ml and group II received 5.0 mg/0.1 ml), while in the other eye (control group) 0.1 ml of sterile balanced saline solution (BSS) was injected. Slit-lamp and funduscopic examinations were performed every second day for 2 weeks for signs of infection, inflammation and toxicity. A baseline electroretinogram (ERG) was performed before the experiment and at the last follow-up day (day 14). ERG examination followed ISCEV standards. At the last follow-up day, the animals were sacrificed and the enucleated eyes were prepared for histological evaluation of retinal toxicity.
Results No differences in ERG responses at photopic and scotopic conditions were observed in eyes injected with either concentration of adalimumab or BSS. Furthermore, histologic examination of the retina in the enucleated eyes (in all groups) did not demonstrate any evidence of drug toxicity.
Conclusions Intravitreal adalimumab did not appear toxic to the retina in this experimental model at concentrations of 0.5 and 5 mg. If found safe in additional studies, intravitreally injected adalimumab could be evaluated for efficacy in the treatment of inflammatory eye conditions.

Keywords Humira · Adalimumab · Intravitreal injection · Retinal toxicity · Anti-TNF

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Introduction

Tumor necrosis factor (TNF) is a cytokine derived mainly from T lymphocytes and macrophages, in response to infection or immunologic injury, which promotes the inflammatory cascade by direct or indirect mechanisms [1]. TNF is present, and plays a significant role in a variety of intraocular conditions [2–4]. Failure to regulate the production of this factor may lead to activation of immune cells and to chronic inflammatory responses leading to tissue damage [5].

The regulation and suppression of TNF with various biological agents has recently emerged as a therapeutic strategy for various inflammatory conditions such as rheumatoid arthritis and psoriasis [6, 7]. The application of such biological anti-TNF strategies has also found ground in ophthalmological inflammatory conditions [8, 9]. Moreover, recent basic and clinical studies implicate TNF in such conditions as diabetic maculopathy [10] and

glaucoma [11], making these conditions possible targets for anti-TNF therapies.

Although the systemic use of anti-TNF agents has shown satisfactory results in rheumatology [6, 12, 13], the downsides of these agents are serious adverse effects such as tuberculosis [14], respiratory diseases [15], infections [16], congestive heart failure [17], neurologic events [18], malignancies [19] and hypersensitivity [20]. To avoid these serious complications, there has been an interest in exploiting the intravitreal administration of TNF antagonists for the treatment of local intraocular conditions [21]. The main concern in such approaches is the interaction of the drug with the ocular tissues and the possible toxicity that it may cause.

The purpose of the present study was to evaluate possible retinal toxicity of two different concentrations of intravitreally-injected adalimumab in a rabbit model. Adalimumab is a recombinant human IgG1 monoclonal antibody specific for human TNF. It is being used in patients with psoriasic and rheumatoid arthritis. Any possible adalimumab toxicity to the retina was tested by clinical ophthalmological inspection, retinal histology, and using the full-field flash electroretinogram (ERG).

Materials and methods

Animals

Sixteen male pigmented rabbits, weighting 2.5 to 3.0 kg each, were included in the study. All the experimental procedures followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional guidelines.

Procedure

The rabbits were initially anesthetized by an intramuscular injection of a mixture containing ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) solution. Topical anaesthesia was applied using proparacaine (0.5%). The pupils were fully dilated by topical application of phenylephrine (2.5%) and tropicamide (0.5%).

The rabbits underwent clinical inspection by slit lamp, indirect ophthalmoscopy and ERG recordings. The ERG was recorded from each rabbit before intravitreal injection (baseline) and 14 days after injection to determine possible damage to the retina. Slit-lamp and funduscopic examinations were performed, and all animals were observed during the 14-day period for signs of infection, inflammation, or toxicity. After the last ERG recording session, the rabbits were sacrificed by intravenous injection of an overdose of pentobarbital sodium (80 mg/kg body weight), and their retinas were prepared for histological examination.

All animals were evaluated prior to the experiment for any media opacities or retinal damage.

Adalimumab

Humira is a recombinant human IgG1 monoclonal antibody specific for TNF; it was created using phage display technology, resulting in an antibody with human derived heavy and light chain variable regions and human IgG1:k constant regions. Adalimumab is produced by recombinant DNA technology in a mammalian cell expression system, and is purified by a process that includes specific viral inactivation and removal steps. It consists of 1330 amino acids. and its molecular weight is approximately 148 kilodaltons.

Two concentrations of adalimumab (Abbott laboratories, Chicago, IL, USA) were prepared: 0.5 mg/0.1 ml and 5 mg/0.1 ml. The dilution was performed using water for injection. The rabbits were divided into two groups (group I and group II) according to the concentration of the injected solution. Rabbits in group I (n=8) were injected with 0.1 ml adalimumab solution, having a concentration of 0.5 mg/ml. Rabbits in group II (n=8) were tested for the effects of 5 mg/ml adalimumab solution (0.1 ml). Each concentration was injected intravitreally randomly in one eye (experimental eye) of each rabbit (group I received 0.5 mg/0.1 ml and group II received 5 mg/0.1 ml), while in the other eye (control eye) 0.1 ml of sterile balanced saline solution (BSS) was injected.

Intravitreal injection

All procedures were performed under sterile conditions using an operating microscope for visualization. A 30-gauge needle attached to a 1.0-ml tuberculin syringe was inserted into the vitreous approximately 1 mm posterior to the limbus. The syringe was directed toward the center of the vitreous. A volume of 0.1 ml was then slowly injected. In order to avoid drug reflux following the injection, the syringe remained in the vitreal cavity for 15 seconds and then was retrieved slowly.

Electroretinogram

The full-field light-evoked electroretinogram (ERG) was recorded from the experimental and control rabbit eyes using the computerized Primus 2.5 system (Tomey, Germany), utilizing Ganzfeld stimulation with a maximum flash intensity of 3.5 cd s /m^2. An active electrode (HK loop electrode by Medelec, Oxford Instruments, UK), placed in the lower palpebral sac was referenced to a 9 mm cup silver-silver chloride electrode (Oxford Instruments, UK)

placed near the orbital rim (rabbit hair was previously shaved to improve conductivity). For ground electrode, an earring clip was placed at the earlobe. Electrical impedance was smaller than 5 kΩ for all electrodes.

Data were sampled at a rate of 1,000 Hz, constrained by online band-pass filtering between 0.3 Hz and 300 Hz. ERG signals were amplified (x 5 K), while artifactual signals (e.g. blinks) were automatically removed. ERG responses were initially recorded in the light-adapted state (background luminance of 25 cd/m^2) and then in dark-adapted state, following 30 minutes of dark adaptation. Three ERG responses were recorded: (a) photopic flash responses (average response of four consecutive events of 1 Hz frequency and 2.5 cd s/m^2 intensity each), (b) photopic steady-state responses (average response of 20 sweeps) with flashes presented at a rate of 30 stimuli per second (30 Hz) and an intensity of 2.5 cd s/m^2, and (c) scotopic flash responses (average response of two consecutive events of 1 Hz frequency and 2.5 cd s/m^2 intensity).

Analysis of the ERG signals was carried out offline using computational scripts written in Matlab mathematical software. ERG analysis for flash responses was based on measurements of the b-wave amplitude, from the trough of a-wave to the peak of b-wave. Recordings to 30 Hz steady-state stimulation were subjected to analysis in the frequency domain using discrete Fourier transformation (DFT) [22]; the magnitude at 30 Hz frequency was taken as response measure.

In all cases, baseline ERG responses were compared to responses 2 weeks after drug administration.

Histology

After enucleation the eyes were prefixed in cold glutaraldehyde 2.5% in 0.1 M cacodylate buffer (pH 7.4). After short prefixation, the retina was removed from all animals and was placed in the same fresh fixative. Tissue samples were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at 4°C, dehydrated in a series of alcohols and propylene oxide, and then imbedded in epoxy resin. For light microscopic examination, 1 to 3 μm sections were prepared and stained with rapid trichzome 5%. For electron microscopic examination, the selected areas were thin-sectioned, stained with uranyl acetate and lead citrate. The examination was performed with a JOEL electron microscope (100 x). Electron microscopy of the retina of all animals was performed in order to study the possible induced-alterations in the retina by the intraocular treatment with Adalimumab.

Statistical analysis

A two-factor ANOVA was performed on both photopic and scotopic flash data, with 'tested eye' (experimental vs control) and 'drug concentration' (0.5 mg/ml, group I vs 5.0 mg/ml, group II) as factors.

Results

Clinical observations

After intravitreal injection, the vitreous of the experimental as well as the control eyes appeared clear. During the follow-up period until the animals were sacrificed, no apparent changes or differences were observed between the two groups. No signs of retinal detachment, media opacity, inflammation, vitreous hemorrhage or optic atrophy were noticed.

Electroretinogram

Waveforms of grand-averaged ERGs for group I are depicted in Fig. 1a and for group II in Fig. 1b. For group I the grand-averaged photopic flash amplitude for the experimental and control eye was 29.9 and 23.5 μV respectively, at the baseline, compared to 28.3 and 26.6 μV 14 days after injection. The grand-averaged scotopic flash amplitude for the experimental and control eye was 52.5 and 53.7 μV respectively, at the baseline, compared to 61.5 and 51.8 μV 14 days after injection.

For group II, the grand-averaged photopic flash amplitude for the experimental and control eye was 21.5 and 21.9 μV respectively, at the baseline, compared to 21.3 and 22.2 μV 14 days after the injection. The grand-averaged scotopic flash amplitude for the experimental and control eye was 42.4 and 44.6 μV respectively, at the baseline, compared to 49.2 and 56.53 μV 14 days after injection. The grand-averaged steady-state amplitude was found increased in all cases (groups I and II) at 14 days after injection.

A two-factor ANOVA was performed on both photopic and scotopic flash data, with "tested eye" (experimental vs control) and "drug concentration" (0.5, group I vs 5.0, group II) as factors. For both photopic and scotopic conditions, no statistically significant difference was revealed between "tested eye" (photopic flash: $F_{1,26}$= 0.143, p=0.62; scotopic flash: $F_{1,26}$=0.446, p=0.52) and "drug concentration" (photopic flash: $F_{1,26}$=1.021, p=0.33; scotopic flash: $F_{1,26}$=0.446, p=0.19). Furthermore, the interaction between "tested eye" and "drug concentration" was not significant (photopic flash: $F_{1,26}$=0.640, p=0.64; scotopic flash: $F_{1,26}$=0.071, p=0.79).

Histology

The histology of both treated and control eyes after intravitreal administration of 0.5 and 5 mgr/0.1 ml adali-

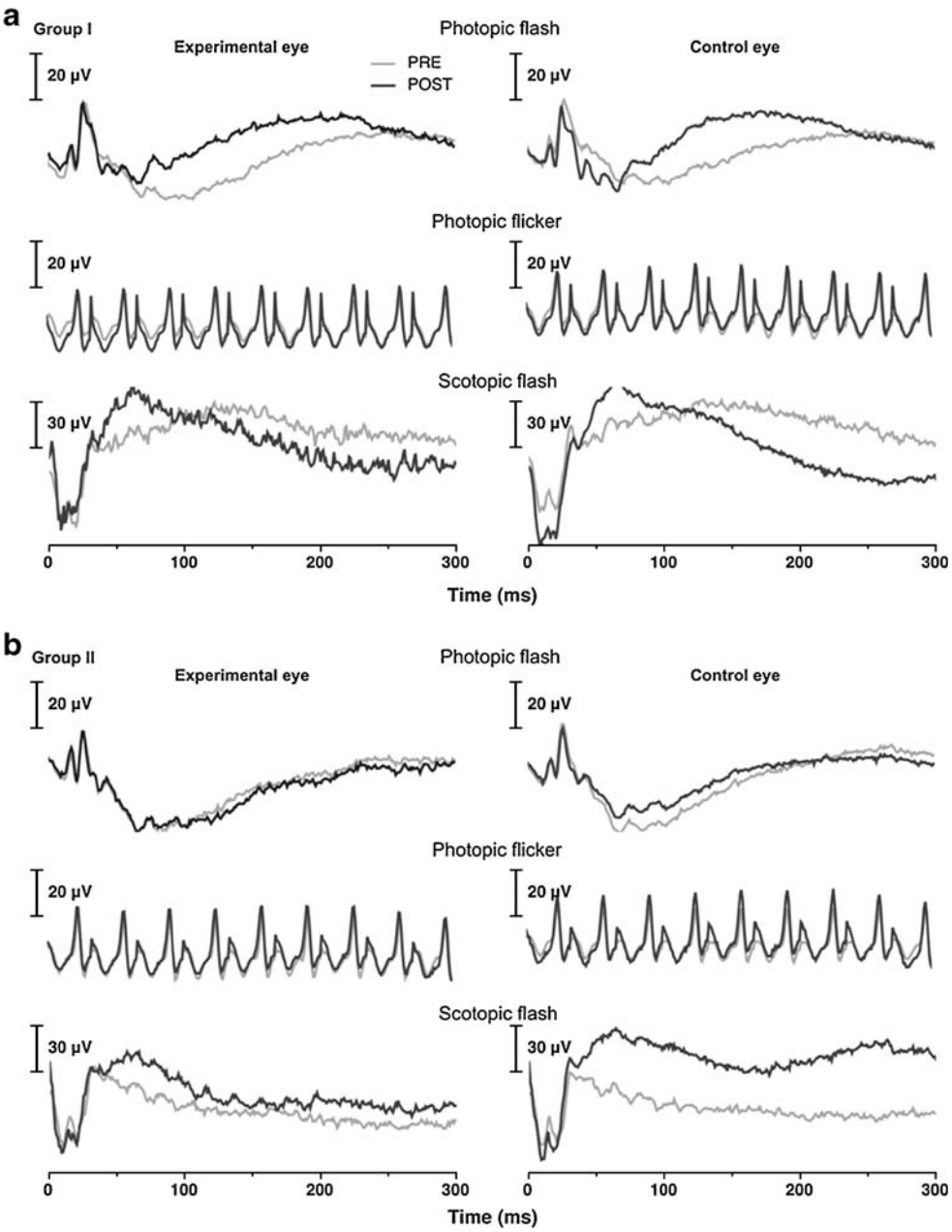

Fig. 1 **a** Grand-averaged (n=8) full-field light-evoked ERG waveforms for experimental (left) and control (right) eyes of group I rabbits at the baseline (*green solid line*) and at 14 days following the injection (*red solid line*). **b** Grand-averaged (n=8) full-field light-evoked ERG waveforms for experimental (*left*) and control (*right*) eyes of group II rabbits at the baseline (*green solid line*) and at 14 days following the injection (*red solid line*)

mumab were not distinguishable, and revealed normal retinal anatomy (Fig. 2). No evidence of retinal toxicity or inflammation was evident in any of the specimens, while no vitreal, retinal or optic nerve abnormalities were seen.

Discussion

Intravitreal injections have emerged during the last years as a new very effective way for delivering therapeutic agents intraocularly [23–25], ameliorating the possible systemic effects of such agents. However, intravitreal administration poses also risks related both to the procedure of administration [26] and to the local toxicity of the various administered agents [27]. In this study, adalimumab, an anti-TNF agent, was tested in rabbits for possible retinal toxicity.

After intravitreal injection of two doses (0.5 and 5 mg/0.1 ml) of adalimumab, no signs of toxicity were found on evaluation of data from clinical examinations, ERG and histology. Adalimumab did not induce any pathological changes up to a 14-day follow-up. These data indicate that adalimumab shows no short-term retinal toxicity, and might be safe to use in clinical studies in order to evaluate its role in various eye conditions where TNF is implicated. It is important to note, though, that ERG outcomes do not provide information for functional changes at the level of ganglion cells or the nerve fiber layer. Furthermore, the lack

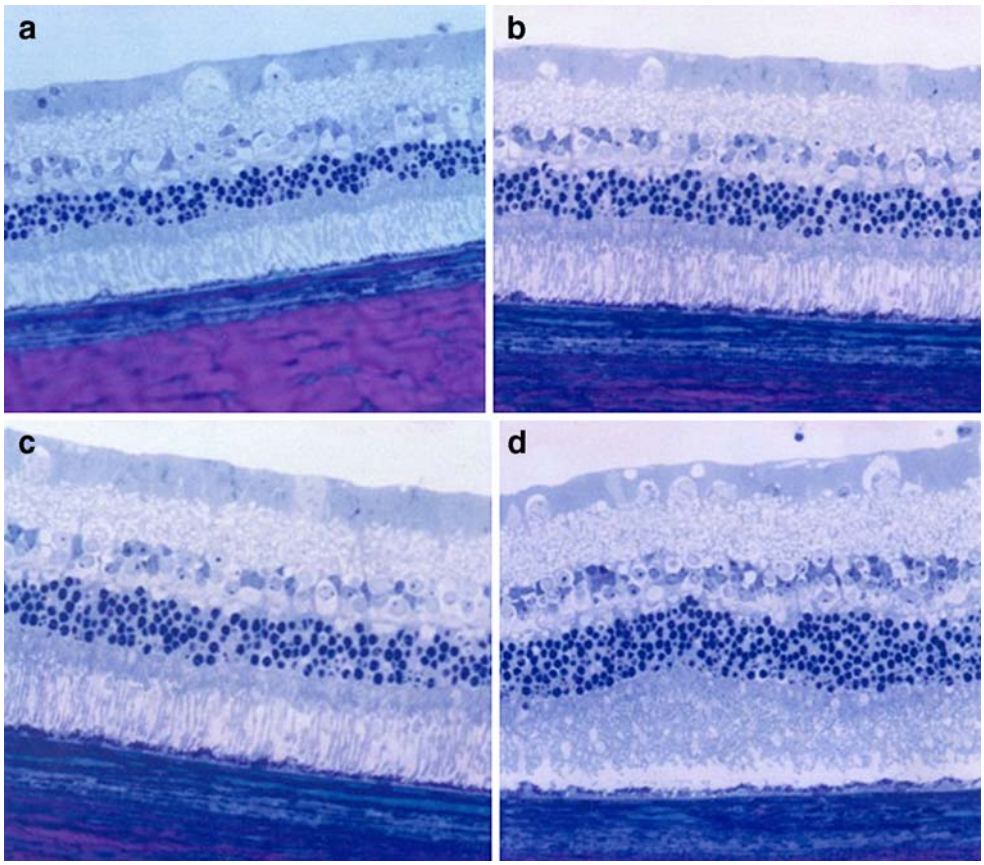

Fig. 2 Histological images. Normal retinal anatomy and no signs of retinal toxicity is evident in both the control eyes (**a**, c) and the experimental eyes (**b**, **d**). **a**,**b** Group I 0.5 mgr/0.1 ml Humira. **c**,**d** Group II 5 mgr/0.1 ml Humira

of changes in the histological evaluation does not exclude possible alterations on a sub-microscopic level.

A recent study in the literature by Manzano et al. [28], investigating intravitreal toxicity of adalimumad in rabbit eyes, concluded that doses 0.2 and 0.5 mg/0.1 ml do not demonstrate any toxic effects; however, administration of 1.0 mg/0.1 ml was associated with an inflammatory reaction in two animals and retinal necrosis in one animal out of the three tested [28]. In the current study we did not find any toxic effect, even though we used a fivefold greater dose (5 mgr/0.1 ml) than the 1.0 mg/0.1 ml used by Manzano et al. [28] which demonstrated toxic effects. Differences in the composition of the administered drug may explain the differences observed in high-dose toxicity. We used a commercially available, sterile, preservative-free preparation of adalimumab in our study [Humira (adalimumab) Abbott Laboratories, Chicago, IL, USA], while no information concerning the source of the drug used and possible mixture with other substances is provided by Manzano et al. Moreover, it must be noted that in the current experiment we used eight animals per group, compared to three animals per group examined by Manzano et al. Despite the fact that the findings of these two studies are contradictory for the high administered doses of adalimumab, both studies revealed that intravitreal administration of a dose up to 0.5 mg/0.1 ml of adalimumab is not associated with retinal toxicity; this finding is significant, since a dose of 0.5 mg/0.1 ml is more than ten times greater than the intravitreal equivalent administration of adalimumab (approximately 0.04 mg) when compared to the systemic (plasma volume ~4 l, ophthalmic volume ~4 ml, systemic administration of Humira=40 mg).

Two recently published articles have demonstrated no retinal toxicity after intravitreally administrated etanercept [29, 30] and infliximab [31] in rabbit eyes in doses as high as 2.5 mg and 1.7 mg respectively. These two anti-TNF agents, along with adalimumab, are approved for clinical use for systemic inflammatory conditions. There are reports, however, indicating that adalimumab, a recombinant human antibody specific for human TNF, is the most potent of the three [32, 33]. The findings of this study provide clinicians with the additional option of evaluating the intravitreal administration of adalimumab for the possible treatment of a variety of intraocular TNF-related conditions.

There are several limitations in this study. The length of the follow-up was selected to be 14 days. Although several studies in the literature follow the same methodology [28, 30, 34–36], there are others that use longer follow-up [37–39]. Since retinal toxicity may some times appear after the 2-week period used in this study [39], toxicity of intra-

vitreal adalimumab after longer follow-up should be tested in future studies. The lack of high adalimumab dose testing represents an additional drawback. In this work, two doses were tested (0.5 mg and 5 mg per 0.1 ml); the 5 mg dose was the highest solution that could be achieved without condensation of the commercially available solution. Condensation was avoided because sterility of the solution could be compromised during the process. Nevertheless, testing higher doses is significant; and should also be studied in the future.

In conclusion, we found no retinal toxicity 14 days following intravitreal administration of two adalimumab doses in rabbits, showing that this biological agent is safe on a short-term basis. However, since toxicity was reported in another publication [28], further studies with longer follow-up are required to test higher concentrations and evaluate the long-term safety of this drug.

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