A Pilot Study on Ocular Safety of Intravitreal Infliximab in a Rabbit Model

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Purpose. To determine whether infliximab may be used safely as an intraocular drug, the ocular safety of intravitreal infliximab in rabbits was studied by clinical examination, electroretinography (ERG), and histology in rabbits.

Methods. Twelve New Zealand albino rabbits were selected for this study. Different infliximab doses, namely 1.0 mg, 1.7 mg, and 3.3 mg in 0.1 mL, were injected intravitreally into one eye each of three rabbits. As a control, the vehicle solution was injected into the fellow eye of each animal. Eye clinical examination and ERG recordings were made before and 2, 6, and 12 weeks after injection. Eventually, the rabbits were humanely killed, and the retinas were examined by light microscopy. In addition, the elimination half-life of the drug in the vitreous was assessed.

Results. Slit lamp biomicroscopy, indirect funduscopy, and ERG evidenced no significant differences between control and infliximab-injected eyes in this rabbit model, at any of the tested doses. Histologic examination revealed no retinal abnormality in the rabbits injected with 1 mg and 1.7 mg intravitreal infliximab. In two of three eyes injected with 3.3 mg infliximab, significant edema of the nerve fibers was detected compared with the control group. The half-life of the drug was estimated to be 8.5 days.

Conclusions. These results indicate that infliximab may be a safe intravitreal drug in the rabbit model at a dose of up to 1.7 mg. If proven safe and efficacious in further studies, intravitreal injection of infliximab could be considered an alternative to systemic administration in selected patients. (*Invest Ophthalmol Vis Sci.* 2008;49:1151–1156) DOI:10.1167/iovs.07-0932

Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine primarily released by macrophages, T lymphocytes, and natural killer cells; it plays a key role in apoptosis and cell survival and in inflammation and immunity.[1] Although originally named for its antitumor properties, TNF-α is involved in a wide spectrum of inflammatory diseases, and its pharmacologic inhibition has proven to be an effective therapeutic modality in rheumatoid arthritis,[2] Crohn disease,[3] Behçet disease,[4] and ankylosing spondylitis.[5] In addition, evidence from animal and human studies suggests that TNF-α is a potentially important mediator in uveitis.[6–8] During inflammation, TNF-α mediates the start of leukocyte infiltration through the upregulation of adhesion molecules, dendritic cell maturation and survival, macrophage activation, and T-helper type 1 cell responses in experimental autoimmune uveitis.[9] Interestingly, diabetic retinopathy may have an inflammatory basis manifested by the involvement and adhesion of leukocytes to the retinal vessels and the upregulation of inflammatory genes.[10,11] In an animal model of diabetic retinopathy, the administration of TNF-α inhibitors was found to reduce leukocyte adhesion and to inhibit blood-retinal barrier breakdown.[12] TNF-α involvement in retinal angiogenesis is under investigation. Experiments in rabbit[13] and murine[14] models evidenced that TNF-α is able to induce the formation of new vessels in vivo through its proinflammatory properties. In animal models, regression of choroidal neovascularization after treatment with anti–TNF-α drugs has been demonstrated.[15] Furthermore, TNF-α was beneficial in patients with age-related macular degeneration.[16]

In patients affected with ocular inflammation, a drug that specifically targets TNF-α might be a helpful therapeutic agent.[17] Infliximab (Remicade; Roche, Mannheim, Germany) is a human-mouse chimeric IgG1 antibody that, when administered systemically, inhibits the active soluble TNF-α. Its efficacy for the treatment of uveitis has been evaluated in a number of clinical studies.[18–20]

Nevertheless, serious adverse effects have been described, including autoantibody formation,[21] autoimmune disease,[22] demyelinating disease,[23] propensity for infections (such as tuberculosis,[24] toxoplasmosis,[25] and listeriosis[26]), infusion reaction,[27] neutropenia-thrombocytopenia,[28] bilateral anterior optic neuropathy,[29] and increased risk for malignancy.[30]

Considering the serious potential adverse effects of this immunomodulatory agent, intravitreal administration might be of benefit in selected patients affected by ocular inflammatory diseases while possibly improving efficacy. In the present pilot study, the safety of intravitreal administration of infliximab was assessed.

Retinal toxicity is a primary concern when using intravitreal drugs. We used a rabbit model to evaluate the ocular toxicity of infliximab injected at various doses. Retinal function and morphology were examined using electroretinography (ERG) and histology studies. Furthermore, the elimination half-life of infliximab in the vitreous was investigated.

Methods

Animals

Twelve New Zealand albino rabbits weighing between 2.3 and 3 kg were selected for this study. The rabbits were divided into four groups of three animals. Groups A, B, and C were used to evaluate ocular toxicity after intravitreal infliximab injection. Group D was used to evaluate the elimination half-life of the drug in the vitreous. The animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Slit lamp examination and

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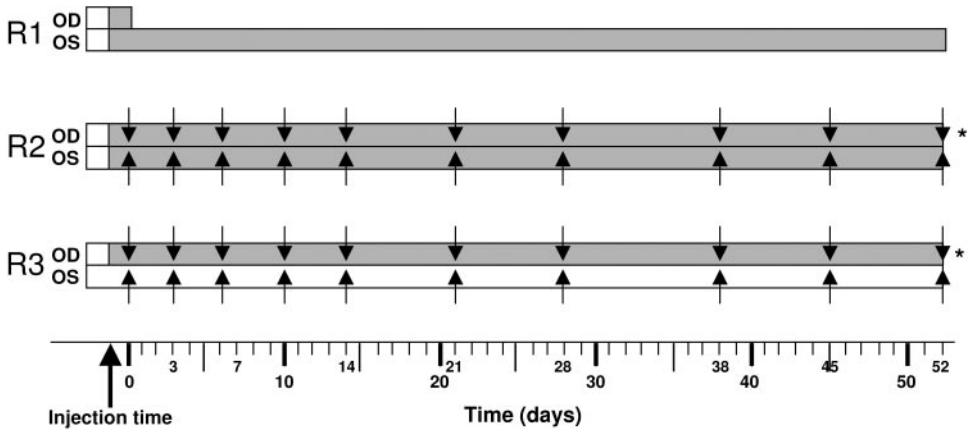

FIGURE 1. Diagram of microsampling strategy used for the half-life determination (rabbit group D). Each double bar represents a rabbit (R1, R2, R3). Horizontal bars represent left and right eyes of each rabbits, and their lengths take into account the enucleation times for whole-vitreous measurement. *Gray*: presence of the drug in the eye. *Arrows*: microsampling points. The *injection arrow* is placed closely before time 0 because 30 minutes were allowed to elapse for the drug to distribute in the eyes. In the end, all rabbits were humanely killed, and their eyes were enucleated. The numbers close to the time axis indicate the exact microsampling time. Eyes marked with *asterisks* are those for which the pharmacokinetic curves are reported in Figure 5.

indirect ophthalmoscopy were performed on all eyes before the study. Animals with corneal or lens opacity or retinal damage before the study were excluded.

Rabbits were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). Benoxinate hydrochloride (0.4%) was applied for topical anesthesia. Eyes were dilated with topical tropicamide (1%) applications. Fluoroquinolone antibiotic was used topically before and after all surgical procedures.

Intravitreal Injection

Infliximab (Remicade; Roche) was reconstituted with sterile saline solution. Three different infliximab doses were tested, namely 1.0 mg (group A), 1.7 mg (group B), and 3.3 mg (group C), injected in the right eyes. Left eyes were treated with sterile saline solution. Each rabbit in group D received a 1.7-mg dose in the right eye, with the exception of one rabbit injected in both eyes for whole-vitreous controls. In all cases, the injection volume was 0.1 mL. Intravitreal injection was performed approximately 2 mm posterior to the limbus with a 30-gauge needle attached to a tuberculin syringe. The rabbits were kept for 12 weeks in ambient light on a 12-hour light/12-hour dark schedule.

Clinical Examination

Slit lamp examination and indirect ophthalmoscopy were used to evaluate changes in cornea, lens, vitreous, retina, and optic nerve. Such observations were performed on all eyes immediately after the injections and at weeks 2, 6, and 12.

Electroretinography

The eyes of group A, B, and C were evaluated by ERG before injection and 2, 6, and 12 weeks after injection. Pupils were dilated with tropicamide, and then the animals were dark adapted for at least 2 hours. Standard ERGs were recorded in both eyes using corneal electrodes (CSO Strumenti Oftalmici, Florence, Italy). Reference and ground electrodes were made of stainless steel surgical needles and were inserted into the ears. ERG signals were recorded using Retimax (CSO Strumenti Oftalmici). Dark-adapted scotopic responses (rod response) and scotopic flash responses (maximal response, cone, and rod) were recorded simultaneously in both eyes. Flashes varied in intensity from -2.50 to $+0.5$ log scot cd $\cdot$ s/m^2. At each scheduled examination, ERGs were repeated three times for each rabbit. The amplitudes of a- and b- waves were calculated for each recording.

Histologic Examination

Nine rabbits were humanely killed with an overdose of pentobarbital 12 weeks after injection. The eyes were immediately enucleated, and the sclera were incised 2 mm posterior to the limbus and fixed in a solution of 10% formalin. Eyes were sectioned, and the tissues were processed, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin-eosin. Histologic examination was performed with light microscopy.

Microsampling from Vitreous

Group D rabbits were used to evaluate the elimination half-life of the drug in the vitreous. To allow for homogeneous drug distribution in the vitreous, the first samples (time 0) were delayed 30 minutes after infliximab injection. The sampling procedure consisted of aspirating approximately 10 to 20 μL vitreous with short-needled, no-empty-volume 100 μL insulin-type syringes, inserted approximately 2 mm posterior to the limbus. Ten samples were taken at times ranging from 0 to 50 days after infliximab injection (Fig. 1). At each scheduled time, sampling was performed in different eye quadrants. Samples were measured both freshly and after -20°C storage (after centrifugation at 14,000*g* for 5 minutes at room temperature).

To check for the absence of a microsampling effect on pharmacokinetics, infliximab was injected in both eyes of a control rabbit: one eye was enucleated shortly after infliximab injection and the other at day 50, when the eyes from all rabbits were enucleated and concentration values were measured. Values obtained from full eyes and from microsamples were compared.

Infliximab Concentration Assessment

To quantitate vitreous infliximab concentration, a competitive ELISA with anti–human IgG was optimized for the small vitreous samples. After overnight coating at room temperature of modules (Maxisorp Nunc-Immmuno; Nunc, Wiesbaden, Germany) with 100 μL of 1 μg/mL infliximab, wells were saturated with 200 μL phosphate-buffered saline plus 1% bovine serum albumin (PBSA) for 1 hour at room temperature. A standard calibration curve was obtained by mixing horseradish peroxidase (HRP)-conjugated goat anti–human IgG 1:50,000 with increasing amounts of infliximab, ranging from 1.7×10^{-5} μg to 3 μg in PBSA

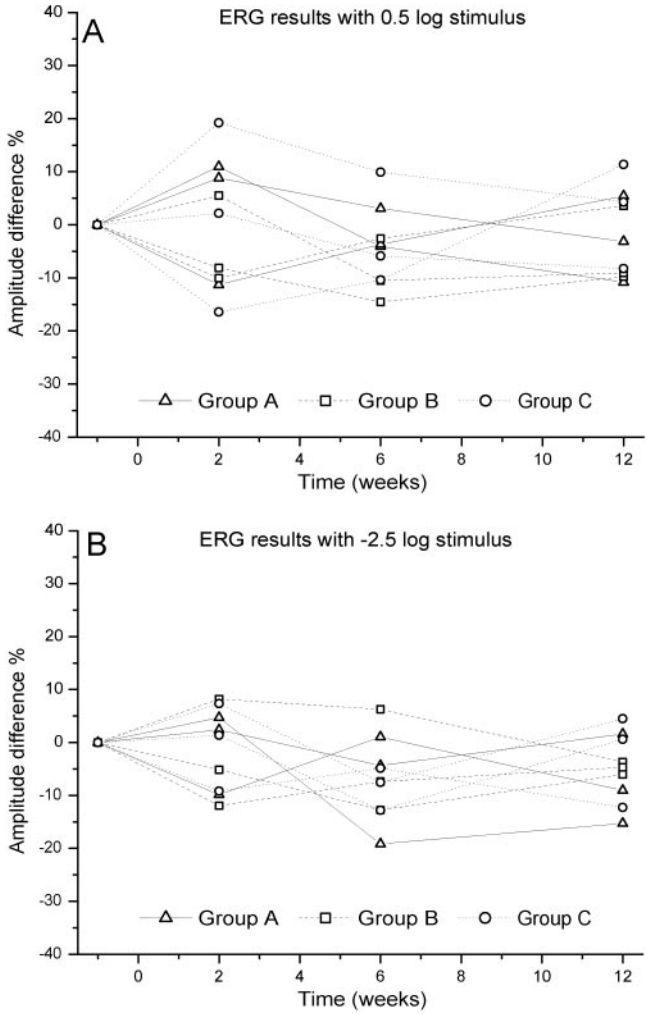

FIGURE 2. ERG amplitude variation in the experimental eyes with respect to pretreatment with a stimulus of +0.5 log units of cd · s/m^2 (**A**) and −2.5 log units of cd · s/m^2 (**B**). Each group consisted of three rabbits. Individual rabbit ERG amplitudes are plotted as *triangles* (group A), *boxes* (group B), and *circles* (group C). Group A, treated with 3.3-mg dose. Group B, treated with 1.7-mg dose. Group C, treated with 1-mg dose. The *y*-axis reports the percentage of a- and b-amplitude difference of posttreatment recordings with respect to pretreatment. The first point (before zero) is the pretreatment measure.

(final total volume, 100 μL), sufficient to cover the entire competition range (0%–100% competition). The same incubation was performed with 0.5- to 1-μL aliquots taken from vitreous samples at different time intervals. After 2-hour incubation at room temperature, wells were washed three times with 400 μL TPBS (PBSA + 0.1% Tween 20). Chromogen mixture (100 μL; *o*-phenylenediamine 0.4 mg/mL dissolved in phosphate-citrate buffer 50 mM with sodium perborate 0.03%) was added to each well, and the color was developed for 10 minutes at room temperature. The reaction was blocked by adding 50 μL of 3 M sulfuric acid, and the absorbance values were read with a microplate reader (Bio-Rad, Hercules, CA) at 495 nm against blank chromogen.

A nonlinear curve-fitting procedure was performed using the sigmoid logistic equation $y = a/\{1 + \exp[-k \times (x - x_c)]\}$, where a, x_c, and k were amplitude, curve center, and a fitting coefficient, respectively. The resultant fitted equation was then used to interpolate vitreous sample values and to obtain the infliximab concentration. To correct for the inherent variability of test measurement, each data point was measured in quadruplicate, and each measurement was repeated at least five times.

RESULTS

Clinical Observation

To detect clinical signs of toxicity, treated and control rabbit eyes were carefully evaluated by slit lamp examination and indirect ophthalmoscopy. No inflammation was observed. Optical media remained clear. Retina and optic nerve appeared unaltered. In summary, there was no clinical evidence of ocular changes in any group of rabbits.

Electroretinography

ERG changes were considered significant if the follow-up differences in amplitude (a- and b-waves) were lower than 30% from the preinjection values.[31] In no group was significant decrease in amplitude observed. Figure 2 displays the percentage of variation of a- and b-amplitude differences in all experimental eyes with respect to preinjection. In all cases, signal variations were not higher than 30%. As an additional control,

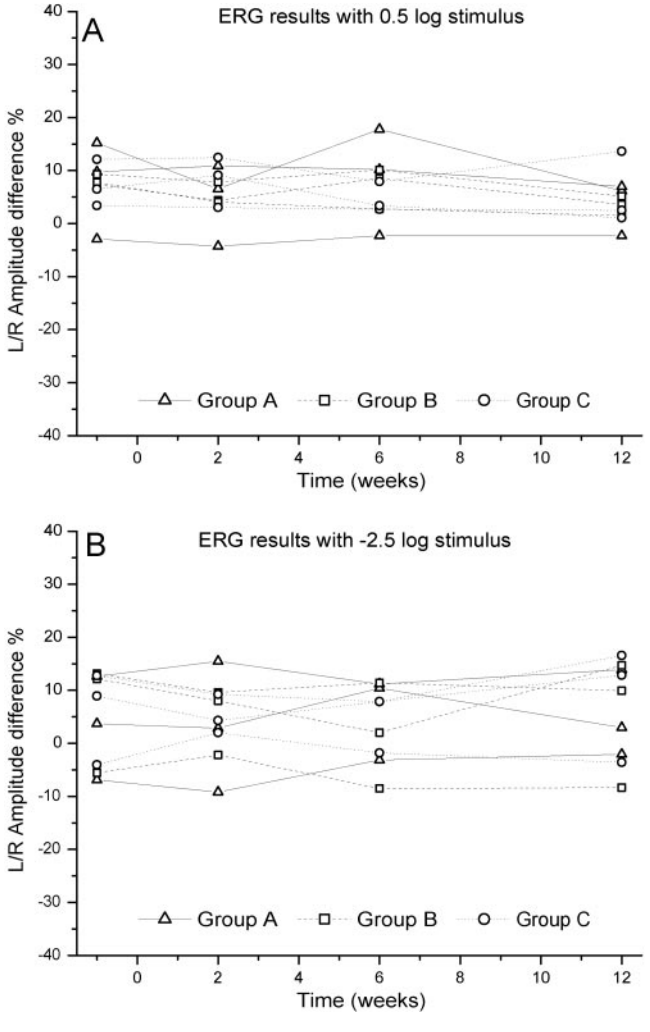

FIGURE 3. ERG amplitude variation between experimental and control eyes of each rabbit stimulated with (**A**) +0.5 log units of cd · s/m^2 and (**B**) −2.5 log units of cd · s/m^2. Each group consisted of three rabbits. Individual rabbit ERG amplitudes are plotted as *triangles* (group A), *boxes* (group B), and *circles* (group C). Group A, treated with 3.3-mg dose. Group B, treated with 1.7-mg dose. Group C, treated with 1-mg dose. The *y*-axis reports the percentage of amplitude difference (a- and b-amplitude difference) between the left (L, control) and the right (R, experimental) eye at each measurement time. The first point (before zero) is the pretreatment measure.

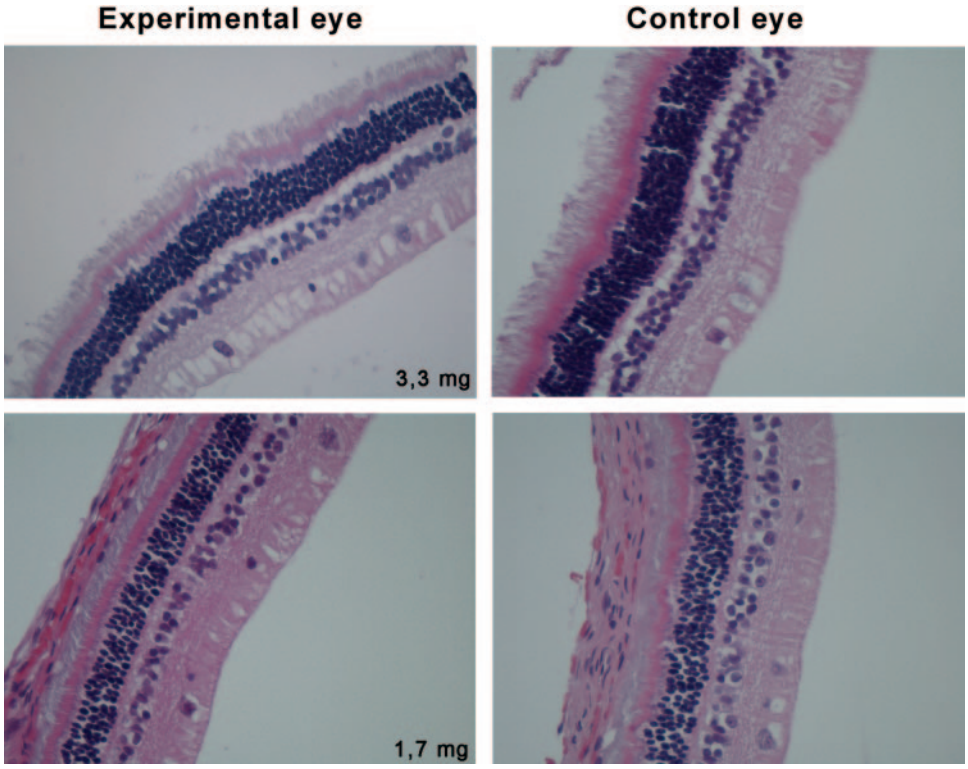

FIGURE 4. Photomicrographs of hematoxylin-eosin–stained sections of experimental and control retinas from two rabbits 12 weeks after injection (original magnification, ×40). Micrographs of experimental eye (*top left*) injected with 3.3 mg infliximab shows diffuse signs of edema of the nerve fibers compared with the control eye (*top right*) of the same rabbit treated with balanced saline solution. Micrographs of experimental eye (*bottom left*) and control eye (*bottom right*) of a rabbit injected with 1.7 mg infliximab and sterile saline solution, respectively. No significant differences were noted between two eyes on histologic examination.

Figure 3 shows the percentage of variation of a- and b-amplitude difference of experimental eyes with respect to control eyes at each time point. Again, few differences were revealed, confirming the absence of toxicity in our rabbits. The b-waves measured in infliximab-injected eyes demonstrated morphology and amplitude similar to that of control eyes. ERG studies, therefore, concluded that there was no evidence of functional changes in the retinas of any rabbit group.

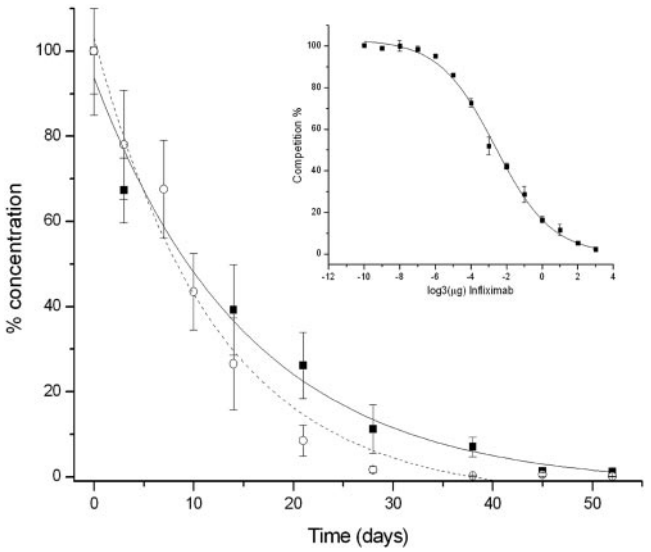

FIGURE 5. Infliximab concentration in vitreous, assessed by competitive ELISA on vitreous microsamples over time. *Full symbols* and *empty symbols* describe two different rabbits, and *solid lines* and *dashed lines* are the corresponding curves resulting from fitting, used to extrapolate half-life. *Inset*: typical calibration curve of each ELISA assay showing the high sensitivity of the measurement.

Histologic Findings

Retinal histology was examined by light microscopy. There were no differences in histologic appearance between the eyes injected with infliximab and those injected with sterile saline solution in groups A and B (Fig. 4, bottom). However, in two treated eyes of group C, we found more extensive signs of edema than in the control eyes (Fig. 4, top), suggesting morphologic retinal damage of the inner layers. Nevertheless, no pathologic changes were observed in the outer retinal layers or the retinal pigment epithelium.

Elimination Half-Life of the Drug

Infliximab levels in vitreous were determined by competitive ELISA. Figure 5 shows the decrease curve as measured in two rabbits over time. At time 0, the microsamples and the control whole vitreous samples agreed in indicating an infliximab concentration of approximately 1 mg/mL, as expected considering the volume of the rabbit vitreous. During the following 50 days, the microsampling technique allowed us to accumulate a good number of measurements that offered a consistent mathematical fitting. With these results, we were able to estimate an average infliximab half-life of 8.5 days, reaching a zero value at approximately 40 days. At day 50, all rabbits were both microsampled and killed, including the control rabbit (which did not undergo microsampling), to extract whole vitreous. Endpoint concentration measurements evidenced that the infliximab amount in the eye vitreous was almost zero, confirming the values obtained with microsampling.

DISCUSSION

This study indicates that up to 1.7 mg infliximab administered intravitreally in rabbit eyes did not display ocular toxicity when examined by ERG or histology, slit lamp biomicroscopy, and indirect funduscopy during a 3-month follow-up. To our

knowledge, the safety of intraocular injection of infliximab has never been reported.

According to a recent study by Lee et al. (*IOVS* 2007;48: ARVO E-Abstract 5171), intravitreal infliximab injection proved to be useful for the treatment of experimental uveitis in rabbits. In their study, the minimal effective dose was estimated to be 0.1 mg, whereas a 10-fold higher dose was suspected to be toxic. The results of our study suggest that such toxicity at a 1-mg dose might not be related to infliximab injection but to the concomitant presence of induced uveitis. Further studies will be required to clearly determine the effective dose of intravitreal infliximab. Although we did not find histologic differences between experimental and control eyes in the group of rabbits injected with 1 or 1.7 mg infliximab, two of three eyes injected with 3.3 mg infliximab revealed significant edema of the nerve fibers compared with controls, suggesting possible drug toxicity when administered at this high dose. In all the tested doses, ERG recordings in treated and control eyes were comparable during the follow-up time. This did not necessarily conflict with histologic findings because electroretinography does not assess the function of the ganglion cells or the nerve fiber layer. The scotopic b-wave is a measurement of the extracellular field potential that mainly arises from rod bipolar cells in response to flashes of light.[32] In addition, the absence of alterations by light microscopy observed with 1- and 1.7-mg doses does not exclude possible changes at the submicroscopic level. Follow-up studies might benefit from the use of additional techniques, such as electron microscopy, to better characterize retinal cellular damages or from visual-evoked potentials to assess functional alterations of ganglion cells and nerve fiber layers.

It is important to underscore that the number of rabbits used in this pilot study was not sufficient to draw final conclusions regarding intravitreal infliximab safety or toxicity. Moreover, our rabbit model was based on eyes with vitreous volume, retinal anatomy, and vascularization different from those of human eyes. These are important caveats when extrapolating our studies to safety in human subjects.

The intravitreal half-life of infliximab was determined by a microsampling technique coupled to ELISA. As a first step, we optimized a very sensitive competitive ELISA procedure for small vitreous samples. Given that the method was based on the interaction of infliximab (a human–mouse chimeric antibody) with an anti– human secondary antibody, we tested this secondary antibody for cross-reaction with rabbit antibodies that might be present in vitreous specimens. Results indicate that little interference, and then only in the presence of high amounts of purified rabbit IgG (data not shown), was present. This validated the ELISA technique as a suitable method for our purposes. A similar approach was used in a recent work on bevacizumab conducted by Bakri et al.[33]

Infliximab concentration in our rabbits was found to decrease in a monoexponential fashion with a half-life of approximately 8.5 days. The half-life of chimeric IgG1 antibodies was ascertained in rabbits by three studies on bevacizumab,[33] rituximab,[34] and herceptin,[35] with lower values. We were unable to establish a connection between higher half-life value and possible interactions with some ocular tissues, considering the lack of pigmentation in our rabbits, which was not taken into account in this work. On the other hand, the high experimental error associated with half-life determinations caused by interindividual variability and other technical issues such as mathematical fitting procedures could smooth such differences. In contrast to previous works,[33,34] to determine the half-life of infliximab in the vitreous, we decided to increase the number of measured points (and thus improve the curve-fitting process) by using a minimally invasive technique with the aspiration of multiple, very small vitreous samplings with microsyringes.

Several possible limitations of this technique should be considered. First, the number of vitreous samplings could lead to vitreous syneresis or some inflammatory response that could alter the distribution of the drug. Our clinical examination did not demonstrate any inflammation. The problem of vitreous syneresis was not assessed satisfactorily and therefore should be considered a possible limitation of the technique. We used samplings of much reduced volume changing the sampling position in different eye quadrants. The infliximab concentration at time 0 (including microsamples and whole-vitreous) was similar in all eyes tested, thus indicating an initial homogeneous distribution of the drug in the vitreous. Similar considerations can be applied for end point measurements. Apart from this, we cannot exclude local different concentrations of the drug because of vitreous syneresis during follow-up.

Finally, a reduction of the drug because of the multiple samplings might have occurred. However, the sample volume was carefully measured and led us to estimate a drug reduction caused by sampling of less than 1%, a very low, likely negligible, value compared with experimental error. To clarify whether this technique could be used as a valuable alternative to current standard techniques consisting in the rabbit kill and eye enucleation, full validation is still required, such as by measuring, at each time point, whole vitreous and microsamples. However, the exponential shape of the kinetic that we observed with this technique was similar to that obtained with classical methods.

Intravitreal injection is commonly practiced in ophthalmology to treat retinal and choroidal diseases, and the procedure has been associated with a relatively low incidence of complications.[36] The clinical usefulness of anti–TNF-α therapy is under investigation for a variety of ocular diseases. If its efficacy were confirmed, selective blockade of intraocular TNF-α would be of benefit, minimizing the risks of adverse systemic events in selected patients in whom systemic treatment is not strictly required or contraindicated.

In conclusion, our preliminary study suggests that a 1.7-mg dose of intravitreally injected infliximab may be safe for the rabbit eye. Therefore, if proven to be safe by further studies, intraocular infliximab might be considered a possible treatment for retinal diseases such as uveitis, cystoid macular edema, diabetic macular edema, and age-related macular degeneration.

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