Intravitreal Infliximab Clearance in a Rabbit Model: Different Sampling Methods and Assay Techniques

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PURPOSE. To investigate the clearance of intravitreal infliximab with the use of different sampling techniques and immunoassay methods in rabbits.

METHODS. Infliximab (1.6 mg) was intravitreally injected into both eyes of 47 rabbits. Two approaches were used to collect the vitreous: the classic method and a microsampling technique. Whereas the classic method consists of collection of the whole vitreous after enucleation, the microsampling technique consisted of the aspiration of small (10–15 μL) samples with a 200-μL syringe. Samples were taken from 30 minutes to 40 days using both methods and were then compared. Infliximab concentration was estimated with competitive ELISA, dot blot analysis, and Western blot analysis.

RESULTS. The vitreous half-life of infliximab was estimated to be 6.5 ± 0.6 days. The data indicated monoexponential decay reaching its conclusion after approximately 40 days. This decay was preceded by 4-day-long diffusion in the vitreous. Microsampling proved to be effective in the vitreous collection, giving statistically comparable signals (± 4%, $P = 0.68$) with respect to the classic procedure. ELISA proved to be the best analytical technique—especially if coupled with microsamplings—because of its lower detection limit, precision, and reduced amount of sample needed. No differences were observed between half-life values obtained by ELISA and dot blot analysis ($P = 0.081$) and Western blot analysis ($P = 0.614$).

CONCLUSIONS. The findings of this study added to the knowledge of infliximab clearance in the vitreous and confirmed the validity of a microsampling technique that was compared with the classic one. ELISA was found to be the best analytical technique when using microsampling. (*Invest Ophthalmol Vis Sci.* 2009;50:5328–5335) DOI:10.1167/iovs.09-3569

Tumor necrosis factor alpha (TNF-α) is one of the major cytokines involved in inflammation and immunity and plays key roles in apoptosis and cell survival.[1] Much evidence from animal and human studies indicates that TNF-α is an important mediator in uveitis.[2–4] Moreover, TNF-α is expressed in the retina of humans with proliferative eye disease[5–7] and in animal models of retinal neovascularization.[8–10]

Infliximab (Remicade; Centocor Ortho Biotech, Inc., Malvern, PA) is a human-mouse chimeric IgG1 antibody specifically designed to inhibit active soluble TNF-α, thus blocking or strongly reducing its physiological effects. When systemically administered, infliximab proved to be of benefit for the treatment of uveitis, as evaluated in a number of clinical studies.[11–13] Nevertheless, the systemic route showed serious side effects, mainly because of the intrinsic antigenicity and nonspecific immunodepression of the molecule at the whole body level.[14–23]

The safety of intravitreal administration of infliximab was recently assessed in a rabbit model.[24,25] In the pilot study conducted by our group, partial characterization of vitreous pharmacokinetics of infliximab was also proposed using a vitreous microsampling method. The half-life of the vitreous was estimated to be approximately 8.5 days. The major drawback at that time was the limited number of animals used.

In the present work, we extended the kinetic study on infliximab clearance from the vitreous by increasing the number of animals and, more important, by parallelizing two different sampling techniques and three different assay methods.

MATERIALS AND METHODS

Animals and Drugs

For the whole work, 47 New Zealand albino rabbits weighing between 2.3 and 3 kg were used and divided into four groups containing 18 (G1), 10 (G2), 14 (G3), and 5 (G4) rabbits (see experimental setup below). To avoid alterations in drug kinetics, care was taken to exclude nonhealthy animals. All surgical treatments were performed on animals anesthetized with intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). Before microsampling, the eyes were subjected to topical anesthesia with benoxinate hydrochloride (0.4%). Fluoroquinolone antibiotics were applied before and immediately after the samplings. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Intravitreal Injection

Taking into consideration previous studies on the ocular safety of infliximab up to 2 mg/mL[24,25] and given that the eye of a rabbit generally contains approximately 1.6 mL vitreous,[26] 80 μL of a 20 mg/mL infliximab solution was intravitreally injected to achieve a vitreous concentration of approximately 1 mg/mL. For the injection, a tuberculin syringe with a 30-gauge, 0.8-cm needle (Microfine U-100 syringe; BD Biosciences, San Jose, CA) was inserted approximately 2 mm posterior to the surgical limbus, in the superonasal quadrant, to reach the midvitreous (Fig. 1). To avoid drug reflux from the injection site, the syringe was extracted with a cotton swab a few seconds after injection.

Vitreous Sampling

Two different approaches were used to collect vitreous samples, a classic method and a microsampling technique recently described by our group.[24] In the classic procedure, the rabbits were enucleated and the eyes were stored at −20°C for at least 2 days. The frozen eyes were
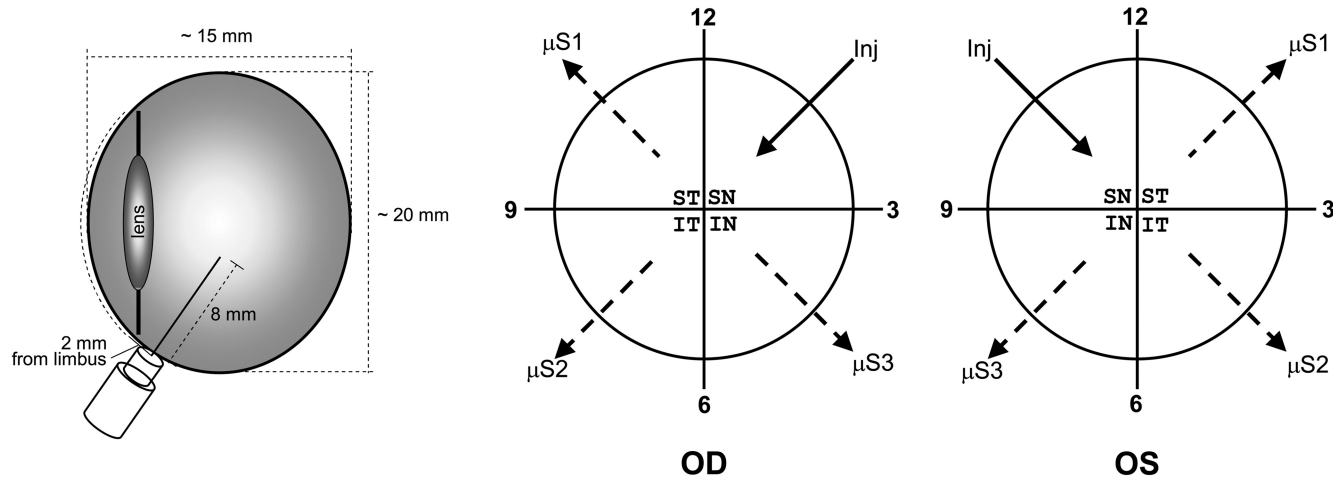
FIGURE 1. Schematic representation of the injection site and microsampling locations. Both the injection and the samplings were in the midvitreous (*left*). Microsamplings were taken from three different eye quadrants (*middle, right*). SN, superonasal; ST, superotemporal; IN, inferonasal; IT, inferotemporal; μS, microsample; Inj, injection.

then dissected so all ocular tissue could be removed while the vitreous was maintained in its frozen form. After thawing, the vitreous was centrifuged for 10 minutes at 3000*g* so that retinal tissue and other cellular material could be removed. The supernatant was vortexed and centrifuged again to collect the liquid part of the vitreous, which was used for the assays, and the aliquots were stocked at $-20°C$ for supplementary measurements. The microsampling technique consisted of the aspiration, directly from the living anesthetized rabbit, of small samples of approximately 10 to 15 μL with the same syringe used for infliximab injection.[24] The stick was made 2 mm posterior to the limbus in different eye quadrants (Fig. 1) to collect portions of the midvitreous. The samples were used for the assays and subsequently stored at $-20°C$ for further assays.

Liquefaction Techniques

In one set of experiments, before centrifugation, we introduced two different modifications after isolation of the frozen vitreous: the gel-like vitreous samples were heated at 60°C for 30 minutes or sonicated for 10 seconds at medium intensity. These two procedures permitted the complete liquefaction of the vitreous before assay.[27] Treated and untreated aqueous solutions of 1.0 mg/mL infliximab were also assayed to check for possible adverse effects on the drug.

Experimental Setup

The experiment was initially divided into three groups. G1 estimated the vitreous half-life of infliximab with both sampling techniques and verified the effectiveness of the microsampling technique. G2 assessed the effect of early microsampling at different times. G3 measured to what extent multiple microsamplings from the same eye could affect the assays. G4 was added to investigate the early phase of vitreous clearance. The experimental scheme is depicted in Figure 2. Each group, apart from G4, was duplicated.

Vitreous ELISA

The concentration of infliximab was estimated with the use of a competitive ELISA recently set up by our group.[24] Briefly, polystyrene modules (ImmunoModules; Nunc GmbH, Wiesbaden, Germany) were
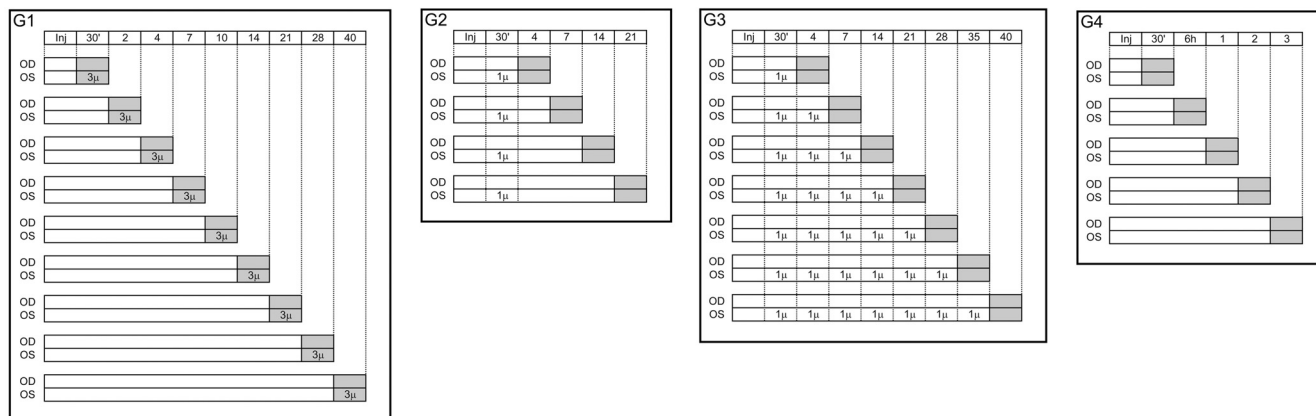

FIGURE 2. Experimental scheme of the four groups. G1: After drug injection (time 0), the rabbits were killed at various times (from 30 minutes to 40 days). The vitreous was extracted from the right eye, and the left eye was microsampled three times from different eye quadrants before vitreous collection. G2: After drug injection (time 0), a single microsample was taken from the left eye of each rabbit at 30 minutes, after which the rabbits were killed and the vitreous was extracted from both eyes. G3: The procedure was the same as for G2, but multiple microsamples were taken from the left eye at times varying from 30 minutes to 28 days. G4: After injection, the vitreous was collected by the classical method at 30 minutes, 6 hours, 1 day, 2 days, and 3 days and was subjected to thermal treatment or sonication to achieve complete liquefaction. *First row*: sampling time (days, where not otherwise specified); *double horizontal bars*: rabbits (two animals per bar); *gray boxes*: enucleation and vitreous extraction. μ, Microsampling (with number of microsamplings); OD, right eye; OS, left eye.

coated with infliximab and saturated with PBS plus bovine serum albumin. Samples of the vitreous (and of calibrator infliximab solution) were incubated for 2 hours with horseradish peroxidase–conjugated goat anti–human IgG 1:50,000 (Dako, Carpinteria, CA) in the pre-coated ELISA wells. After washes, signals were developed using an *o*-phenylenediamine solution for 10 minutes and then were stopped with sulfuric acid. Absorbance was recorded at 495 nm with a plate reader (Microplate Reader; Bio-Rad, Hercules, CA). Signals from calibrator solutions were used to extrapolate the concentration of infliximab in the vitreous samples (Fig. 3). The ELISA lower limit of detection was approximately 0.01 ng.

Vitreous Western Blot Analysis

Aliquots from vitreous samples and from the infliximab standard were diluted 1:10 in Laemmli sample buffer, and 5 μL of each was loaded

onto a 12% SDS-PAGE gel. After electrophoretic separation for 40 minutes at 200 V, the gel was electroblotted onto 0.45 μm supported nitrocellulose membranes (Bio-Rad) for 1 hour at 100 V. The blotting membrane was then saturated for 1 hour in PBS plus 1% bovine serum albumin and incubated with HRP-conjugated goat anti–human IgG 1:10,000 for 2 hours. After three 15-minute washes, HRP signals were revealed by HRP substrate (Immun-Star; Bio-Rad) and detected with the use of a chemiluminescent detection system (ChemiDoc; Bio-Rad). Spots were quantified with Bio-Rad software (Quantity One), and the infliximab standard was used as a calibrator (Fig. 3). Western blot analysis lower limit of detection was approximately 0.1 ng.

Vitreous Dot Blot

Aliquots from vitreous samples were diluted 1:2 four times in PBS, and 0.5 μL of each dilution was spotted onto 0.45 μm supported nitrocellulose membranes (Bio-Rad). Different concentrations of infliximab standard were also spotted onto the same membrane. The membrane was then dried and treated as described for Western blot analysis, including signal acquisition and band quantification (Fig. 3). The dot blot lower limit of detection was approximately 1 ng.

Statistical Analysis

Measurements were repeated several times for each technique used. The number of technical replicates for each measurement type was five for ELISA, three for dot blots, and two for Western blot analysis.

Each signal was quantified and calibrated according to internal infliximab controls, with procedures depending on the specific analytical technique. As depicted in Figure 3, Western blot analysis and dot blots were calibrated with a linear function, whereas ELISA signals were calibrated using the sigmoidal logistic function in equation 1 where *a*, x_c, and *k* represent amplitude, curve center, and a fitting coefficient, respectively. Fitting parameters were extrapolated by non-linear curve fitting

$$y = \frac{a}{1 + e^{-k(x - x_c)}} \qquad (1)$$

Infliximab concentration decay parameters were extrapolated using the standard exponential decay function reported in equation 2 or its log-transformed linear counterpart equation 3:

$$y = C_0 e^{-kt} \qquad (2)$$

$$\log(y) = \log C_0 - kt \qquad (3)$$

where C_0 represents the initial drug concentration, *t* is the measurement time, and *k* is the rate constant of elimination, based on which a $t_{1/2}$ value was calculated with $t_{1/2} = 0.693/k$. Nonlinear fitting was performed with technical graphics and data analysis software (Origin 6.0; Microcal Software, Northampton, MA).

Statistics based on general linear mixed models were used to investigate the effect of sampling methods and assay techniques on measurements, with repeated measures in rabbits considered a random effect. Calculations were conducted using $\log_{10}$-transformed concentrations (Stata 10.2 software; StataCorp, College Station, TX).

RESULTS

Elimination Half-life of the Drug

G1 rabbits were used to estimate the half-life of infliximab. From day 4 onward, the concentration was found to decrease in a monoexponential fashion, as reported for other monoclonal antibodies.[28,29] Data were fitted to an exponential decay curve, resulting in a half-life value of 6.5 ± 0.6. Differences in
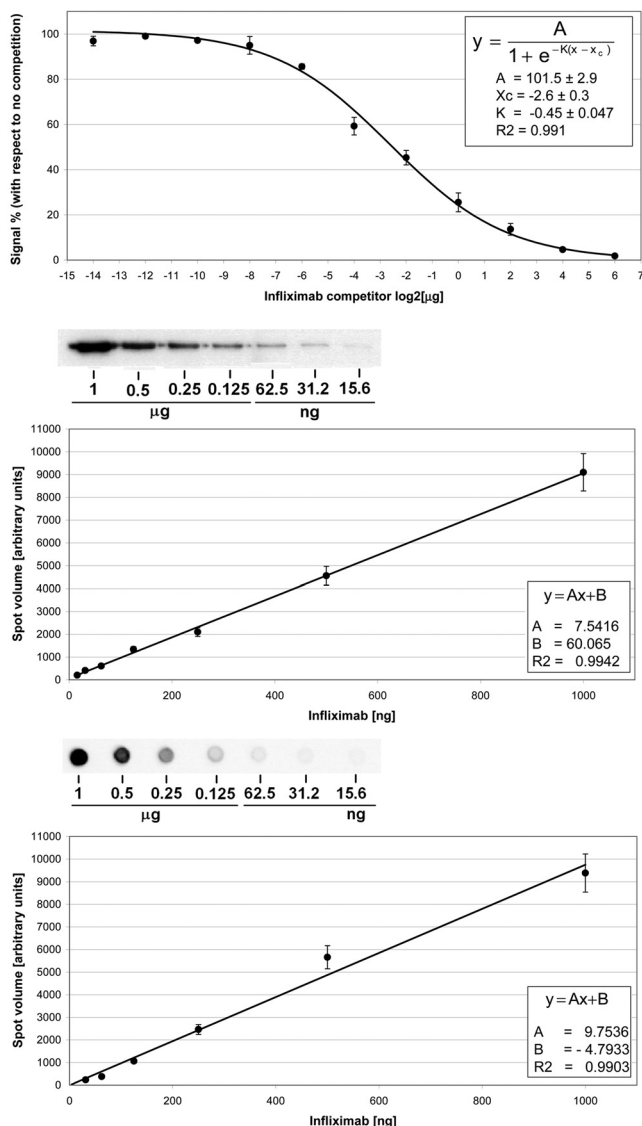
FIGURE 3. Calibration curves and sample standard runs. *Top*: typical calibration fitting for ELISA experiments. Anti–human IgG was run against fixed scalar amounts of infliximab, determining a displacement of the antibody from the infliximab coated in ELISA wells. *Middle*: typical Western blot calibration, with the corresponding densitometric analysis and fitting. *Bottom*: typical dot blot calibration, with the corresponding densitometric analysis and fitting.
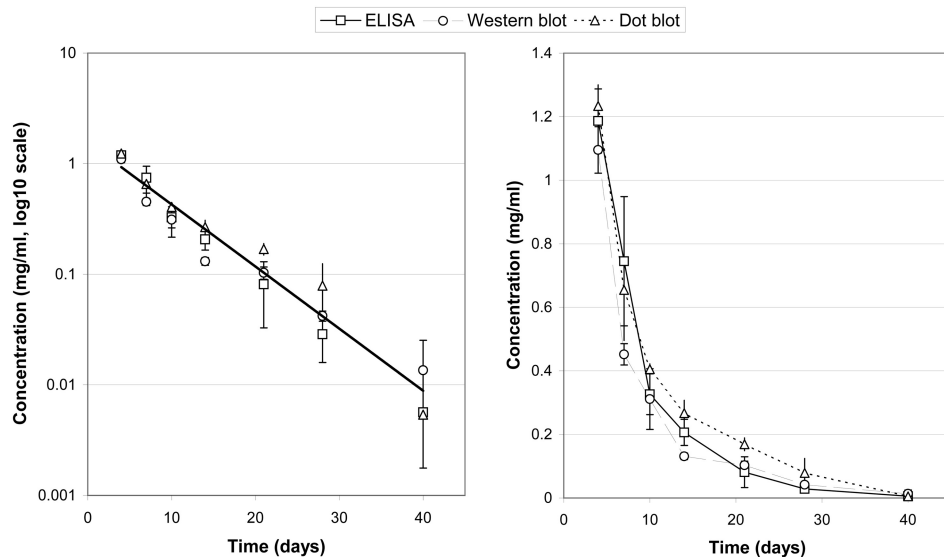
FIGURE 4. Infliximab kinetics assayed in the vitreous using three techniques: ELISA, Western blot analysis, and dot blot analysis. Samples taken at 30 minutes and 2 days were excluded because of their poor consistency. *Left*: data plotted on a $\log_{10}$ scale and showing a shared exponential fitting function (*black line*). *Right*: the same data plotted on a linear scale. Data represent the average of OD and OS vitreous samples of two rabbits per time point and are displayed as mean $\pm$ SD, with $n = 10$ (ELISA), $n = 6$ (dot blot analysis), and $n = 4$ (Western blot analysis). OD, right eye; OS, left eye.

half-lives obtained with different assay techniques were modeled as an interaction term of linear time and methods in a linear mixed model in which $\log_{10}$-concentration was the response variable. Our analyses indicated that there was no difference between half-life values of ELISA and those obtained by dot blot ($P = 0.081$) and Western blot ($P = 0.614$). The concentration reached a quasi-zero value after approximately 40 days (Fig. 4). As a result of microsampling, it was also possible to follow infliximab decreases in the eyes of single rabbits (Fig. 5). The last rabbits in G3 had sufficient numbers of measured points to estimate half-life values of 6.72 ± 0.28 (rabbit 1) and 6.68 ± 0.13 (rabbit 2), in agreement with values from G1.

Microsampling versus Whole Vitreous Sampling

To assess the reproducibility and consistency of the microsampling procedure, rabbits from G1 were microsampled just before enucleation, and the vitreous was collected from both eyes. The samples were then analyzed using all the available techniques. As shown in Figure 6, the values obtained using

different techniques were similar to each other and usually within experimental error. Statistical analyses indicated that the sampling method had no effect on measurements ($P = 0.680$). The width of 95% confidence intervals (95% CIs) was able to exclude measurement differences exceeding $\pm 4\%$ between whole vitreous sampling and microsampling. This effect did not vary among different assay techniques ($P = 0.222$).

We also evaluated the effect of vitreous aspiration by microsampling shortly after the injection (G2; Fig. 7) and repeating microsamplings on the whole vitreous at different sampling times (G3; Fig. 8). Statistical analysis showed that a single microsample had no effect on final whole vitreous measurements ($P = 0.756$; differences within $\pm 6\%$), with no dependence on the assay technique used ($P = 0.152$). The same consideration could be drawn for repeated microsamplings: the infliximab level in both eyes was consistently similar ($P = 0.866$; differences within $\pm 6\%$), again with no dependence on the assay technique used ($P = 0.877$). In all cases, no decreasing trend was seen, as could have been expected in a repeated subtraction of vitreous material.

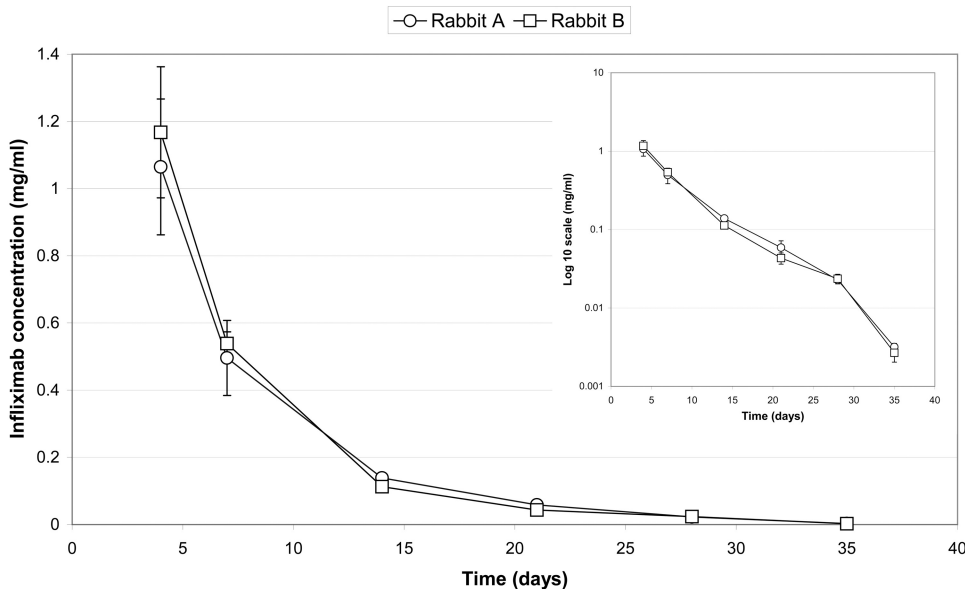**FIGURE 5.** Infliximab clearance curve measured by ELISA and by multiple microsamplings from the same eye of two different animals (last rabbits of G3). Data are displayed as mean $\pm$ SD of different measurements, with $n = 5$ for the first 20 days and $n = 3$ for the subsequent days. Half-lives estimated from the two curves are 6.7 ± 0.28 ($n = 6$; **A**) and 6.68 ± 0.13 ($n = 6$; **B**).
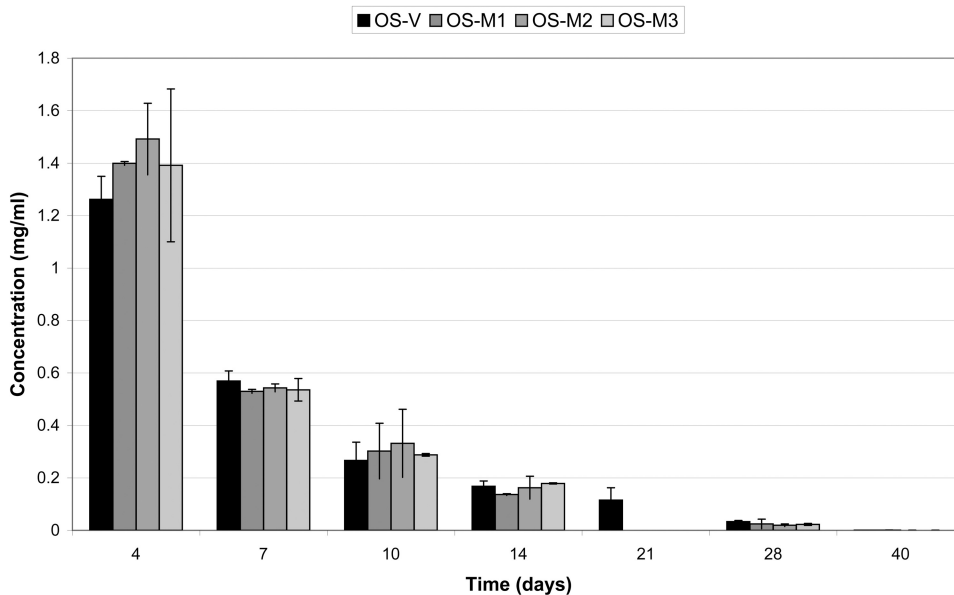
FIGURE 6. Comparison of micro-sampling and vitreous sampling measurements at different times (G1) by ELISA. OS (left eye): determined using the whole vitreous. OS-M1, OS-M2, OS-M3: determined using three microsamplings from different eye quadrants. Data represent the average of samples from two rabbits per time point and are displayed as mean ± SD, with $n = 10$.

Infliximab Dispersion in the First Four Days

To characterize the early stages of infliximab clearance, we investigated samples from the vitreous taken 30 minutes and 2 days after injection (G1; data not shown). In contrast with samples taken at later times, we obtained inconsistent measurements. In fact, when the whole vitreous was assayed 30 minutes after injection, we obtained values that were lower than expected (approximately 1.0–1.2 mg/mL) and that were statistically inconsistent. The samples taken at day 2 showed a drug concentration at least two times lower than expected. At day 4, the concentration was approximately 1.1 mg/mL (i.e., the value expected at time 0). This initial dispersion of data and general inaccuracy in the measurements were also observed in samples obtained using the microsampling technique; the most important difference was that values were spiked (i.e., both very high or very low). To clarify these early-stage kinetics, an extra group of rabbits (G4) was used. The vitreous, collected using the classic technique, was subjected to two treatments aimed at obtaining complete liquefaction, namely thermal treatment and mild sonication.

We verified that the signals emerging from untreated and treated aqueous solutions of infliximab were not statistically different ($P = 0.45$ for ultrasound treatment, $P = 0.63$ for thermal treatment), allowing us to compare corresponding samples in the vitreous.

Monitoring was performed at 30 minutes, 6 hours, 1 day, 2 days, and 3 days after injection. We found that the initial increase in drug concentration and the general inconsistency of the results were completely abolished, resulting in a drug concentration corresponding to that expected at time 0 during the first 4 days (Fig. 9). Statistical analysis indicated that horizontal trends (i.e., stationary drug concentration) were present in samples after liquefaction (slopes approximately 0.02, with $P = 0.950$ and $P = 0.945$ for thermal-treated and sonicated samples, respectively), whereas untreated samples showed a significant ($P = 0.023$) linear increase during the 3 days of measurements.

FIGURE 7. Effect of a single micro-sample on the final vitreous assay (see G2). Bar chart reports the concentration ratio between the right eye (OD) and the left eye (OS), performed using three different techniques. Values inside the bars represent the concentration values of the right (D) and left (S) eyes. Data represent the average of samples from two rabbits per time point and are displayed as mean ± SD, with $n = 10$ (ELISA), $n = 6$ (dot blot analysis), and $n = 4$ (Western blot analysis).
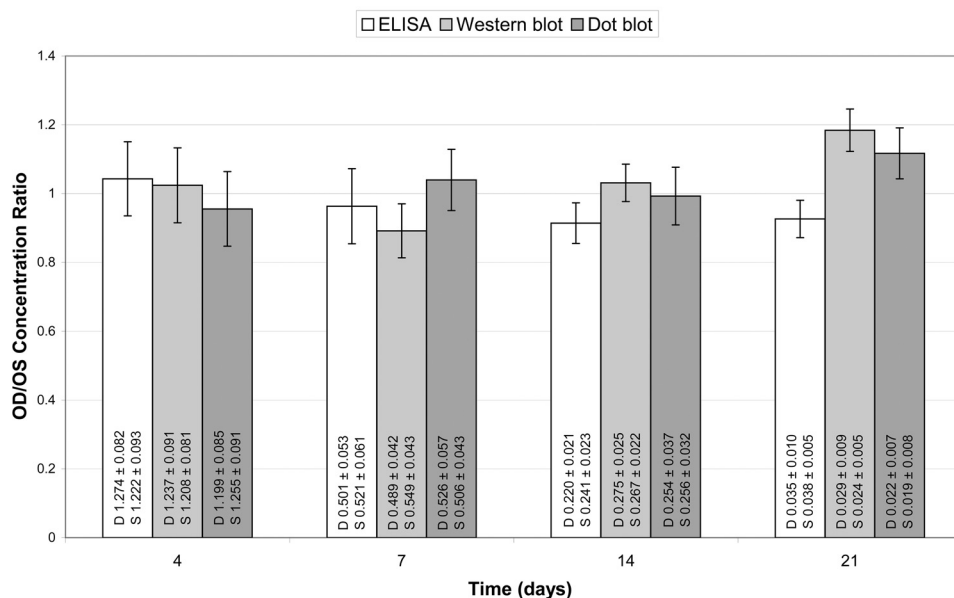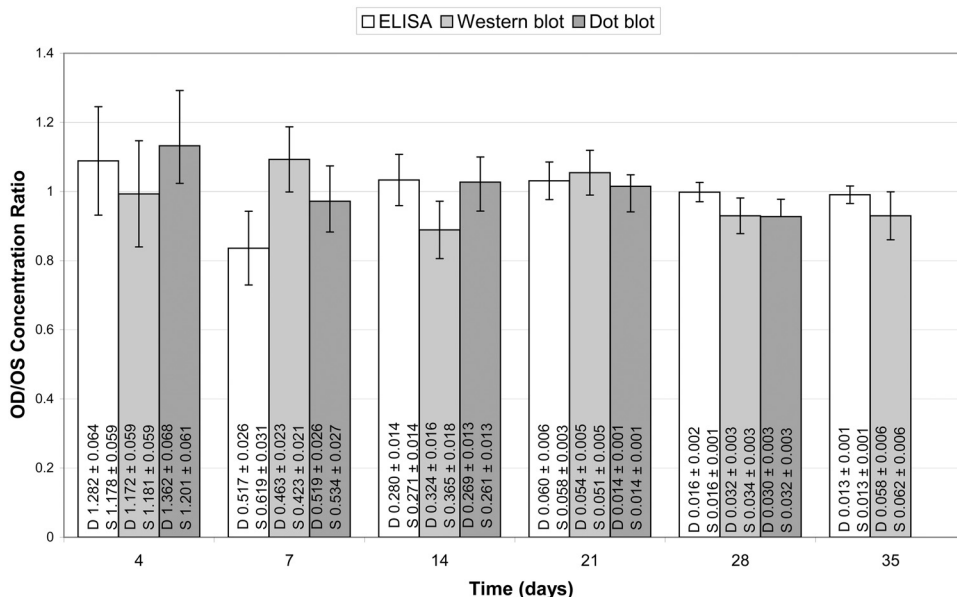
Figure 8. Effect of multiple microsampling on the final vitreous assay (G3). Bar chart reports the concentration ratio between the right and the left eyes (OD/OS), performed using three different techniques. Inside the bars the concentration values for the right and left eyes are reported. Data represent the average of samples from two rabbits per time point and are displayed as mean ± SD, with $n = 10$ (ELISA), $n = 6$ (dot blot analysis), and $n = 4$ (Western blot analysis).

DISCUSSION

Half-life Estimation

One of the goals of this work was to investigate the vitreous half-life of the monoclonal anti–TNF-α antibody infliximab in rabbits.

Our results indicate a half-life of 6.5 ± 0.6 days, a value close to but relatively shorter than that observed in the pilot study conducted by our group.[24] This half-life is higher than that observed in studies carried out with trastuzumab (5.6 ± 0.6 days)[28] and rituximab (4.7 days)[29] in the same rabbit model and higher than that obtained with bevacizumab (4.32 days)[30] in smaller Dutch-belted rabbits. Although the half-life we measured is higher than that previously observed by other groups, it should be noted that a relatively high error is inherent in these types of measurements primarily because of the intrinsic biological variability of the rabbits. Furthermore, it should be pointed out that different collection and analysis methods have been used, making it difficult to directly compare half-life measurements made by other groups.

The elimination half-life of a drug in a vitreal cavity depends on two pathways, the anterior route passage into the aqueous and the posterior route, by active transport, across the retina. Araie et al.[31] showed that, after their intravitreal injection, molecules form a concentration gradient dependent on the efflux route. Maurice et al.[32] measured the rate constant of substances leaving the vitreous by diffusion into the anterior chamber and showed a correlation with the molecular weight of the substances. Bakri et al.[30] determined the intercompartmental transport clearance of the monoclonal antibody bevacizumab in Dutch-belted rabbits, highlighting a preferential efflux route from the posterior chamber. Heiduschka et al.[33] and Shahar et al.[34] showed in monkeys and rabbits, respectively, that bevacizumab can be actively transported across the retina and retinal pigment epithelium into the blood, reinforcing the idea of a posterior efflux route. In contrast, Mordenti et al.[35] showed that trastuzumab (another full-length monoclonal antibody) could not penetrate the retina. Taken together, previous studies do not clarify whether general rules can be applied to monoclonal antibodies, also considering that their specific

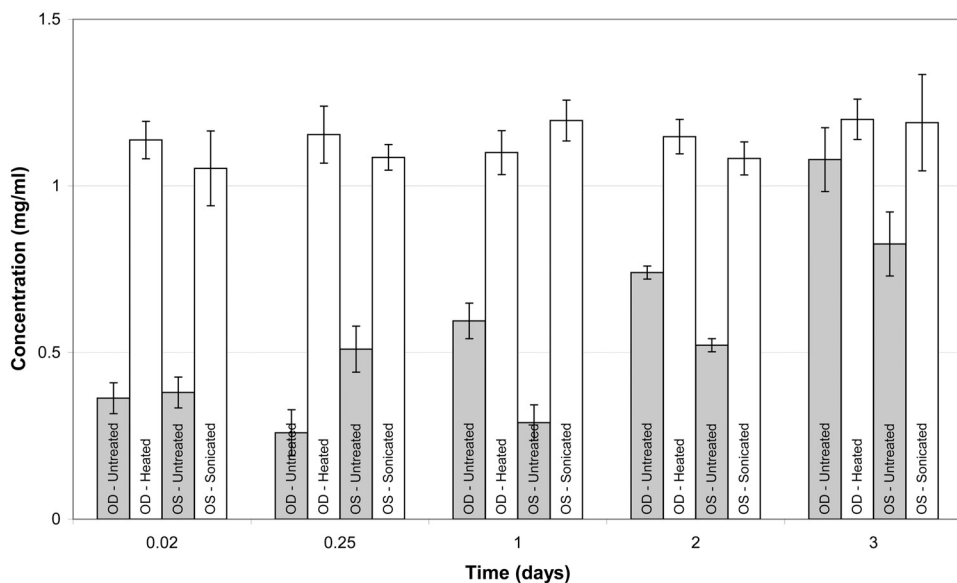**Figure 9.** Early stage of clearance in the liquefied vitreous measured by ELISA. Vitreous samples from one rabbit per time were collected using the classic technique at times ranging from 30 minutes to 3 days after injection. Right eye (OD) was measured before and after thermal treatment. Left eye (OS) was measured before and after ultrasound treatment. Values are displayed as mean ± SD ($n = 5$).

molecular targets can affect their clearance mechanisms. The likelihood of cells actively transporting injected proteins is still to be determined, and passive diffusion also remains to be investigated. The antibody of interest in that study, infliximab, has not previously been investigated in terms of retinal penetration or intercompartmental analysis; therefore, at this time we cannot comment on its preferential efflux route. In this study we have focused our attention on vitreous clearance, leaving out retinal penetration and serum and aqueous compartment analyses.

Although the rabbit is a well-established model for studying the intravitreal pharmacokinetics of a drug,[28,30] this model has some limitations. The rabbit retina is less vascular than the human retina, the volume of distribution is different from the human model, and the lens is larger than that in humans. These factors may result in altered pharmacokinetics compared with the human eye. Humans also have a larger vitreous cavity than rabbits. Moreover, because humans have a larger serum compartment than rabbits, systemic exposure may be reduced.

In addition, aphakia, vitrectomy, and inflamed eyes have major effects on drug clearance from the vitreous cavities. Active mechanisms are involved in transport across ocular barriers, and, given that these are frequently compromised in inflammatory conditions, clearance in such cases could be drastically altered.

Previous studies reported that intravitreal infliximab may be safely administered up to a dose of 2 mg in rabbit eyes[24,25]; such doses may be used for future clinical studies evaluating the efficacy of the drug. We analyzed the vitreous clearance of 1.6 mg infliximab, a dose within the safety range. Future clinical applications should take into account the half-life of the drug, considering the fact that some pathologic conditions require frequent reinjection.

Validation of the Microsampling Technique

A distinctive aspect of this work was the validation of an innovative sampling technique previously proposed by our group.[24] The importance of such a noninvasive technique is twofold. First, the microsampling method could result in fewer animals killed in drug kinetics studies. Second, the microsampling method makes it possible to carry out multiple tests in the same eye, drastically reducing the natural variability of different animals in terms of drug kinetics. In fact, we showed that neither one sampling nor multiple samplings in the same eye significantly affected the concentration or kinetics of the drug.

In making this conclusion, we took into account the possibility that measurements made from a microsample could be affected by the location at which the sample was taken. We verified that multiple samplings in different eye quadrants do not lead to different results. According to this validation, we propose that microsampling is a useful technique in vitreous studies because it drastically reduces the number of animals to be killed and has benefits in both ethical and economic terms.

Drug Diffusion in the First Few Days

We experienced difficulties in determining the vitreous clearance of infliximab in the first days after injection. According to Araie and Maurice,[31] 15 hours should be sufficient for a 66-kDa molecule (e.g., fluorescein-dextran) to diffuse through the vitreous and form a concentration gradient with maximal and homogeneous values in the midvitreous region. Given that we collected microsamples from that region 30 minutes after injection (G1), we clearly had data resembling irregular drug distribution. In our measurements, we also experienced inconsistent results with microsamples at day 2. Furthermore, we also had whole vitreous measurements reflecting the uneven distribution highlighted by microsampling, probably attributed

to technical problems encountered when disrupting the gel-like part of the vitreous. To improve drug dispersion, we repeated measurements in the first 3 days by using liquefaction methods (heating or sonication) after vitreous isolation (G4). As a result, we observed that the variability of measurements was abolished and that concentration values were constant during the first few days. Accordingly, we suggest that initially the drug is distributed irregularly in the vitreous and then starts to diffuse slowly toward the efflux routes, possibly because of the high molecular weight of the antibody, causing the observed initial lag phase.

Vitreous liquefaction was carried out using two techniques, thermal treatment and sonication, neither of which had an effect on infliximab stability or on its antigenic properties. On the other hand, the collagen network responsible for the gel-like nature of the vitreous completely lost its initial consistency. Other techniques for vitreous liquefaction were tested.[36] Collagenase and hyaluronidase treatments were found to be inconsistent (data not shown); we preferred to avoid the use of microplasmin because of possible interactions between this nonspecific proteolytic enzyme and our protein-based target, infliximab.

Complementary Assay Techniques

We used three techniques to detect and measure the concentration of infliximab. ELISA offered the greatest sensitivity and precision and gave statistically valid results with lower amounts of sample (as low as 0.2 μL). This makes it the perfect analysis system for the microsampling technique, which collects as little specimen as possible. However, its major drawbacks are the possibility of error when samples are not in liquid form (e.g., the vitreous) and the possibility of nonspecificity of the detecting antibody, which could give rise to signals independent of those generated by infliximab itself. To clarify such ambiguities, we resolved vitreous proteins on a denaturing and reducing SDS-PAGE and followed this with Western blot analysis with the same antibody used in ELISA. In preparing the sample for electrophoretic separation, complete disruption of the gel-like matrix occurs, generating a uniform distribution of the cross-reacting materials. The fact that the concentration measurements obtained by Western blot analysis agree with those obtained by ELISA proves that the results are consistent and that dispersion of the sample in the assay reaction is not a problem in ELISA. More important, the Western blot confirmed the absence of cross-reactions between the detecting antibody (anti–human IgG) and components extracted from the vitreous. In fact, nonspecific staining of the SDS-PAGE with a Coomassie solution generated a number of bands, possibly derived from blood and other cellular material (data not shown), whose proteins could have interacted with the anti–IgG antibody, which is supposed to be specific for infliximab.

Moreover, the fact that Western blot analysis did not generate any bands apart from the one corresponding to the heavy chain of infliximab clearly proves that the drug remained intact during the entire 40-day follow-up, without traces of nicking or other phenomena that could impair infliximab functionality. Once we were able to verify the specificity of the antibody and that only one immunogenic determinant was present and was giving rise to signals, we also evaluated the simplest assay technique using an antibody detection system (the dot blot), which consists of spotting small amounts of sample onto a nitrocellulose membrane and then probing it with the same antibody used in ELISA and Western blot analysis. In this case, the major drawback could have been related to the gel-like component of the vitreous matrix or other proteins therein dispersed, impairing correct detection of infliximab by the anti–human antibody. Furthermore, the gel-like matrix could also have altered the drying process required to complete the capillarity-based adsorption of the protein onto the

nitrocellulose membrane, possibly leading to the loss of samples during the washing steps. In this case we also obtained concentration measurements similar to those obtained using other techniques, further confirming the consistency of our results. However, low doses assessed by the dot blot technique showed low accuracy.

CONCLUSION

This study characterizes the clearance of infliximab from the rabbit vitreous, describing an initial lag phase that precedes an exponential decrease with a half-life value of 6.5 ± 0.6 days and an end-point of approximately 40 days. These data should be taken into account for future clinical applications of intravitreal infliximab. Furthermore, a multitechnique approach confirms that ELISA results are consistent and may be confidently applied to the microsampling technique that, in this work, was validated by comparison with the classical procedure using the whole vitreous. It is our belief that this technique may offer an interesting tool for future pharmacokinetic studies on intravitreally administered protein-based drugs and that it may have great ethical and economic impact.

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