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Downregulation of endotoxin-induced uveitis by intravitreal injection of polylactic-glycolic acid (PLGA) microspheres loaded with dexamethasone

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ABSTRACT

We tested the short- and long-term ability of polylactic-glycolic acid (PLGA) microspheres loaded with dexamethasone to reduce ocular inflammation in rabbits elicited by intravitreal lipopolysaccharide (LPS) injection. PLGA microspheres loaded with dexamethasone were prepared by the solvent evaporation technique from an oil/water emulsion and sterilized by gamma irradiation (25 kGy). The microsphere fraction selected was 2:10 (dexamethasone:PLGA) and contained 141 $\pm$ 0.38 μg dexamethasone/mg PLGA. Microsphere diameters were 20–53 μm, and the mean encapsulation efficiency was 92.97 $\pm$ 0.75%. Seven days prior to the induction of panuveitis, 10 mg of dexamethasone-free or dexamethasone-loaded microspheres were injected into the vitreous. Control animals received no injection. Panuveitis was induced in male New Zealand rabbits (2.5–3.0 kg) by intravitreal injection of *Escherichia coli* LPS. Clinical evaluation, electroretinography and histopathologic studies were performed in short-term studies of 15 days and in long-term studies of 33 days. Efficacy in reducing inflammation was also studied in vitrectomized eyes. In short-term studies eyes injected with dexamethasone-loaded microspheres had less inflammation than control eyes and eyes injected with blank microspheres. Inflammation reverted in all groups by 15 days after LPS injection. A second LPS dose given on Day 30 provoked a high peak of inflammation in control eyes and in those injected with blank microspheres. In contrast, only slight inflammation occurred in eyes injected with dexamethasone-loaded microspheres. Histopathology and electroretinography supported these results. Dexamethasone-loaded microspheres effectively reduced intraocular inflammation caused by LPS in both short- and long-term studies.

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1. Introduction

Corticosteroids have been used in a broad spectrum of ocular inflammatory conditions over the past 50 years and are among the most frequently prescribed and efficient drugs in ophthalmology (Sherif and Pleyer, 2002; Douglas et al., 2000). Current treatment for non-infectious uveitis usually includes topical, periocular, or systemic corticosteroids. Topical delivery of steroids often fails to provide therapeutic levels in the vitreous cavity or posterior segment for the treatment of vitreoretinal diseases (Velez and Whitcup, 1999). Periocular injections may be used to treat uveitis; however, this route of administration risks penetration of the globe as well as the possibility of scleral melting (Anglade and Whitcup, 1995). Systemically administered corticosteroids are the initial treatment of choice for vision-threatening non-infectious uveitis. The main disadvantages of this route are the numerous adverse reactions that affect nearly every organ of the body.

The administration of steroids by intravitreal injections achieves transitory drug therapeutic levels while avoiding the toxicity associated with systemic treatment. However, elimination of corticosteroids after intravitreal injection is fast (Kwak and D́Amico, 1992), requiring repeated injections to maintain therapeutic levels for a long period of time. Additionally, repeated intravitreal injections sometimes cause complications such as vitreous hemorrhage, retinal detachment, or endophthalmitis, and the risk increases with the number of injections (Tolentino et al., 1989).

Sustained intraocular drug delivery systems prepared from biodegradable or non-biodegradable polymers have been developed to avoid repeated intravitreal injections (Yasukawa et al., 2004). These devices must be implanted through a relatively large surgical incision or through a smaller tissue perforation, depending on the size of the device. Vitrasert® and Retisert™ are non-biodegradable implants (Bausch&Lomb, USA) loaded with ganciclovir

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and fluocinolone acetonide, respectively (Callanan et al., 2008; Del Amo and Urtti, 2008). Vitrasert is effective for eight months in controlling the progression of cytomegalovirus retinitis associated with AIDS. Retisert, when used in chronic non-infectious uveitis of the posterior segment, releases the drug for three years. A non-biodegradable tube-shaped device (Medidur®, Alimera Sciences, USA and pSivida Inc., USA) loaded with fluocinolone acetonide has been developed for the treatment of diabetic macular oedema. However, these non-biodegradable systems require surgical implantation, with all that this implies in terms of health costs. Additionally, implants need to be surgically removed when the active drug becomes depleted.

Biodegradable drug delivery systems, in contrast, release the active substance and then undergo natural degradation at the target site. This avoids the need of a second surgery. These systems can be targeted to specific tissue sites and deliver active ingredients over a long period of time. The most frequently used polymers for intravitreal drug delivery belong to the group of polyesters, mainly polylactic acid and its copolymers with glycolic acid (PLGA). These polymers degrade to metabolic products and are easily eliminated from the body (Anderson and Shieve, 1997; Jain, 2000; Kimura and Ogura, 2001; Okada and Toguchi, 1995). They have been employed to prepare several devices such as implants, scleral plugs, pellets, discs, films, and rods among others (Yasukawa et al., 2004). For instance, micro-sized intraocular drug delivery systems have been prepared from PLGA and dexamethasone (Surodex and Posurdex, Oculex, CA, USA) for inflammation due to cataract surgery and for persistent macular oedema.

Biodegradable micro- and nanoparticles have been investigated for delivering drugs to treat vitreoretinal diseases (Herrero-Vanrell and Refojo, 2001). The inherent advantage over larger devices is that they can be delivered as a conventional injection. Microspheres composed of these polymers have been employed to deliver 5-fluorouracil (Moritera et al., 1991), retinoic acid (Giordano et al., 1993), guanosine (Chowdhury and Mitra, 2000), adriamycin (Moritera et al., 1992), acyclovir (Martínez-Sancho et al., 2004), ganciclovir (Herrero-Vanrell et al., 2000; Veloso et al., 1997), and monoclonal antibody (Mordenti et al., 1999), among others.

Dexamethasone is well known for its anti-inflammatory and anti-angiogenic properties. Because of its potency, this corticosteroid is widely used in the treatment of ocular inflammatory diseases. In rabbits, ocular toxicity can develop, depending upon the dose and volume of injection (Nabih et al., 1991; Kwak and ĎAmico, 1992). The main goal of this work was to develop a new formulation of dexamethasone-containing, biodegradable microspheres capable of sustained release of the drug for treatment of uveitis. A second goal was to study the efficacy of this new delivery system in a rabbit model of panuveitis. Any new drug delivery system should have the ability to treat acute processes and to prevent or diminish recurrences. Treating recurrences is of paramount importance in human uveitis, as it is precisely the recurrence of inflammation that eventually leads to visual loss if inadequately controlled. Thus, in addition to the short-term efficacy studies, we also performed long-term efficacy studies using our rabbit panuveitis model.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (50:50) with an inherent viscosity of 0.2 dl/g was supplied by Boehringer Ingelheim (Resomer RG® 502, Chemicals Division, Ingelheim, Germany). Dexamethasone was supplied by Sigma Chemical Company (Saint Louis, MO, USA). Polyvinyl alcohol (PVA) from Fluka (Steinheim, Germany) had an average molecular weight of 78,000 Da and was 88% hydrolyzed. Methylene chloride and ethanol (analytical grade, Merck, Darmstadt, Germany) were used as received. Other reagents and solvents were of analytical grade and obtained from Merck (Mollet del Vallés, Spain). Distilled and deionized water (Millipore, Billerica, MA, USA) was used in the preparation of solutions and buffers.

2.1.1. Microsphere preparation

Microspheres were formed at room temperature and atmospheric pressure by the solvent evaporation technique from an oil/water emulsion (O/W). Briefly, 400 mg PLGA was dissolved in 1 ml methylene chloride. Dexamethasone was added to this solution in a ratio of 2:10 dexamethasone:PLGA. This ratio was selected from an orthogonal factorial design described elsewhere (Herrero-Vanrell et al., 1999). The resultant mixture was then sonicated for 1 min. Once the dispersion was formed, it was added to 5 ml of 1% PVA aqueous solution and stirred at 5000 rpm for 2 min with a Polytron (RECO Kinematica GmbH PT 3000, Staufen, Germany). Thereafter, the volume was adjusted to 15 ml with an aqueous PVA solution (0.1% wt/vol) and stirred with a Polytron for 1 min. In the process of microencapsulation, 1% PVA was initially used when preparing the first emulsion and, 0.1% was used thereafter to reduce the proportion of PVA incorporated in the final formulation. The resulting O/W emulsion was then added to 100 ml of 0.1% PVA aqueous solution, which was incorporated as a stabilizer of the emulsion, and stirred at room temperature and atmospheric pressure for 3 h until evaporation of the organic solvent was almost complete.

The microspheres were washed three times with distilled water to remove any residual organic solvent. To separate different granulometric fractions, particles were sized mechanically with a series of 5 standard sieves of 20, 53, 106, 212 and 300 μm apertures (CISA, Barcelona, Spain). Finally, the microspheres were freeze-dried and kept in desiccators under vacuum until analysis. The granulometric fraction selected for further studies was that of 20–53 μm.

2.1.2. Morphological characterization of microspheres

Optical (Reichert Optical Microscope, Switzerland) and scanning electron microscopy (SEM, JSM 6400 Scanning Electron Microscope, Tokyo, Japan) were used to study the morphology and surface characteristics of the microspheres. For SEM, the dried microspheres were sputter-coated with a thin layer of gold under an argon atmosphere and then photographed.

2.1.3. Analysis of microsphere dexamethasone content

The dexamethasone content of the microspheres was determined by dissolving the 10 mg of the particles in methylene chloride. The PLGA was then precipitated by addition of 12 ml ethanol. The resulting clear supernatant obtained after centrifugation was filtered through 0.45 μm filters and analyzed by direct spectrophotometry at 240 nm using a Beckman DU-6 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The analytical technique was validated with respect to the linearity, accuracy, and precision of the spectrophotometer and no other components of the microspheres interfered at the wavelength used.

When necessary to confirm the results obtained by spectrophotometry, samples were analyzed by high performance liquid chromatography (HPLC, Gilson HPLC system, Gilson Medical Electronics, Middleton, WI, USA) as described by Hainsworth et al. (1996). A Kromasil 100 column (25 mm × 4 mm, 5 μm, Teknokroma, S. Coop., Barcelona, Spain) was used with a mobile phase consisting of acetonitrile:acetic buffer (35:65) at pH 4. The flow rate was 1.5 ml/min, the injection volume was 20 μl, and the detection wavelength was set at 240 nm. The retention time obtained for dexamethasone was 7 min. The HPLC method was validated by the absence of interference between dexamethasone and PLGA.

The amount of drug incorporated within the microspheres was determined after dissolution in methylene chloride, and extraction with 25 ml ethanol. Microencapsulation efficiency was calculated as the ratio of the actual drug content in the microspheres divided by the amount of initial drug added during the preparation of the microspheres.

2.1.4. Gamma-sterilization of dexamethasone microspheres

Samples were conditioned according to the procedure described elsewhere (Herrero-Vanrell et al., 2000). Several batches of microspheres (10 mg each) were introduced into sealed cap vials, labeled, and packed into a polyurethane container containing dry ice. This assured a low temperature during the irradiation process required for sterility, avoiding possible hydrolytic degradation of PLGA (Hausberger et al., 1995). The samples were treated with Co-60 in a Gamma Sterilization Unit (Aragogamma S.A., Barcelona, Spain). Following USP recommendations, an effective sterilizing dose of 25 kGy was used (USP Pharmacopoeia, 2007).

2.1.5. In vitro release studies

The release rate of dexamethasone from the microspheres was studied *in vitro* in a water bath (37 °C) and with constant agitation (100 strokes/min). The release medium was 0.01 M Sörensen phosphate buffer made isotonic with NaCl and adjusted to pH 7.4. Microspheres (10 mg) were suspended in vials containing 5 ml of release medium to maintain sink conditions. At appropriate time intervals up to 53 days, all of the volume was withdrawn, and the sample was filtered to separate the microspheres from the release medium. The filtrates were subjected to spectrophotometric analysis at 240 nm to determine the amount of dexamethasone released. The recovered microspheres were then resuspended in 5 ml fresh release medium for later sampling. All experiments were carried out in triplicate while protected from direct light exposure. At the end of the release assays some samples were also analyzed by HPLC.

2.2. Animal model of endotoxin-induced uveitis

Male New Zealand white rabbits weighing 2.5–3 kg were used. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. According to these guidelines, only one eye could be used, and we chose the right eye. The rabbit model of panuveitis was developed at IOBA, Valladolid, Spain. The 0111:B_4 strain of *Escherichia coli* lipopolysaccharide (LPS, Sigma, Madrid, Spain) was dissolved in sterile PBS immediately prior to injection. We wanted to develop a panuveitis that was severe enough so that any effect of therapy could be readily observe, but not so severe as to cause irreversible damage that was medically unresponsive. To determine the appropriate stimulus, we performed a preliminary dose-response study. Briefly, 4 groups of rabbits ($n = 5$–10 each) were injected intravitreally with 0.1 ml PBS containing 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, or 0.001 mg/ml of LPS. A fifth group, the controls, received the same volume of PBS. Based on this preliminary study, we selected 0.01 mg/ml as the best dose for producing moderate inflammation in both the anterior chamber and the posterior segment. The panuveitis peaked at Days 1–3 and disappeared at Day 15. No retinal detachments occurred at this dose as it did with the higher doses (data not shown).

2.2.1. Treatment groups

The animals were randomly divided into the following groups: one group left was untreated ($n = 5$). Group A (control, $n = 10$) was injected with LPS only. Group B ($n = 7$) was given an intravitreal injection (described below) of dexamethasone-free PLGA microspheres 7 days before the first LPS injection. Group C ($n = 11$) was given an intravitreal injection of dexamethasone-loaded microspheres 7 days before the first LPS injection.

Human uveitis often recurs, so we determined if the slow-release pharmacologic preparations could inhibit new increments of inflammation. Thus, thirty days after the first intravitreal LPS injection, it was repeated to determine if sufficient active drug remained to mitigate the new stimulus.

Some human panuveitis patients undergo vitrectomy, a procedure that can affect drug clearance. Therefore two additional groups of animals were given gas vitrectomies 2 days before microsphere administration. Vitrectomized eyes were injected with dexamethasone-free microspheres (Subgroup B_1, $n = 10$) or dexamethasone-loaded microspheres (Subgroup C_1, $n = 7$). Because these studies involved a new formulation of dexamethasone in the microsphere preparation, this justifies the need to include all the animal groups indicated. Each animal group originally included 12 animals (Cheng et al., 1995); however, some were discarded due to the development of cataracts that impeded examination of eye fundus.

2.2.2. Intravitreal injection of microspheres

To eliminate animals with abnormalities, all eyes were examined by indirect ophthalmoscopy through dilated pupils. Animals were anesthetized intramuscularly with 1 ml/kg of a mixture composed of xylazine (Rompún® 2%, Bayer AG, Leverkusen, Germany) and ketamine (Ketolar®, 50 mg, Parke Davis, El Prat de Llobregat, Spain) in a ratio of 8.5:1.5. One drop of a mixture (1:1) composed of oxybuprocaine hydrochloride and tetracaine hydrochloride (Colirio Anestésico Doble® 0.05%, Alcon Cusí, Barcelona, Spain) plus povidone iodine (Betadine®, Asta Médica, Mundipharma AG, Basle, Switzerland) was also instilled prior to each injection. Mydriasis was induced with tropicamide (Colircusi Tropicamida, Alcón Cusí, Barcelona, Spain).

In Groups B and C, a 0.1 ml suspension containing 10 mg of dexamethasone-free or dexamethasone-loaded microspheres respectively was injected into the vitreous cavity through a 25G needle. The vehicle used to suspend the particles was a sterile 0.5% aqueous solution of hydroxypropyl-methyl-cellulose, which was incorporated to facilitate injectability of the microsphere suspension. Each eye was proptosed and immobilized. A scleral site 3 mm lateral to the limbus was punctured with the bevel of the needle positioned upward. The suspension was injected into the vitreous chamber with an appropriate angle and depth to reach the mid-vitreous, thus avoiding damage to the lens. Administration was done slowly, and before removing the needle, an occlusion of the scleral hole was maintained for 1 min to avoid reflux. Following the injection, a fundus examination was performed to evaluate potential ocular trauma due to the injection.

For vitrectomy, gas compression of the vitreous body with 0.2 ml of sulfur hexafluoride gas (SF_6) was performed as described elsewhere (Veloso et al., 1997). Two days later the rabbits were re-anesthetized and the fluidized vitreous removed through a 25G needle on a tuberculin syringe, and the vitreous was exchanged with a 100 μl suspension containing 10 mg of dexamethasone-free (Group B) or dexamethasone-loaded (Group C) microspheres suspended in hydroxypropyl-methyl-cellulose.

2.2.3. Clinical evaluation

Clinical evaluation was carried out before LPS injection and on Days 1, 3, 7 after first injection and every 15 days thereafter. After that, clinical evaluation was done twice during the third week and on Day 30. For those animals that received a second injection of LPS, evaluation was performed daily for another 3 days.

Anterior and posterior inflammation signs were evaluated and summed. Anterior segment inspection was done by surgical microscopy (Zeiss OPMI99, Carl Zeiss, Strasse, Germany) before

inducing pharmacological mydriasis. Anterior chamber opacity and posterior synechiae were graded on a scale of 0 (absent) to 1 (present). The values were summed so that the maximum score was 2. Other signs such as conjunctival were not evaluated due to considerable lack of specificity.

Following the same criterion as in human and other models of posterior uveitis, we selected vitreous opacity as the most relevant posterior segment sign of inflammation (Mruthyunjaya et al., 2006). Our dexamethasone-PLGA formulation was specifically intended to address this aspect of uveitis. Indirect ophthalmoscopy and fundus photographs were taken under pharmacological mydriasis on the days described above. A scale of 0–3 was used to represent the gradations of vitreal haze described by other authors (Cheng et al., 1995; Park et al., 1995). In this scheme, 0 = absence of opacity, 1 = opacity that allowed visualization of the main vessels, 2 = vitreous opacity with most of the retina obscured, but the optic nerve still visible, and 3 = severe opacity with visualization of the retina totally obscured, including the optic nerve. A comprehensive score was calculated by summation of the anterior and posterior signs with a maximum value of 5.

2.2.4. Electroretinography (ERG)

On the last day of the study, the rabbits were anesthetized and an ERG was performed in all eyes. The eyes were dilated and dark adapted for 15 min. An infant contact lens-type Henkes electrode was placed on the cornea and reference and mass electrodes (LACE Elettronica, Pisa, Italy) were fixed on the ears. The eyes were positioned 18 cm from a stimulator light source. A one-flash ERG was performed after amplification.

2.2.5. Histopathologic studies

At the end of the assays, all animals were anesthetized and then euthanatized with an injection of air (5 ml) in the marginal ear vein. The eyes were enucleated and fixed in 10% neutral-buffered-formaldehyde until histopathologic examination. The globes were vertically opened and embedded in paraffin. Four-micrometer sections of the bisected globe were cut and stained with hematoxylin-eosin for optical microscopy. Polymorphonuclear leukocyte, lymphocyte, and macrophage infiltrates throughout the entire eyeball were graded (Cheng et al., 1995) as follows: 0 = no inflammation, 1 = mild inflammation; 2 = moderate inflammation and, 3 = severe inflammation.

2.3. Statistical analysis

All data are expressed as means ± standard deviations. Animal groups were compared by ANOVA with statistical significance set at $P < 0.05$. Scheffé's multiple comparison test was used to calculate the type I error. The similarity factor f_2 was used to compare the *in vitro* release profiles obtained for non-sterilized and sterilized formulations (Shah et al., 1998).

3. Results

3.1. In vitro release of dexamethasone

The microspheres prepared by the O/W solvent evaporation technique were spherical and exhibited smooth surfaces (Fig. 1).

After preparation, the granulometric fractions obtained were 20–53 μm, 53–106 μm, 106–212 μm, and 212–300 μm. The overall yield of the microencapsulation process ranged from 48% to 63% with encapsulation efficiencies higher than 70% for all fractions. On average, 10 mg of microspheres contained 1410 μg of dexamethasone. From these, the granulometric fraction selected for further studies was that of 20–53 μm.

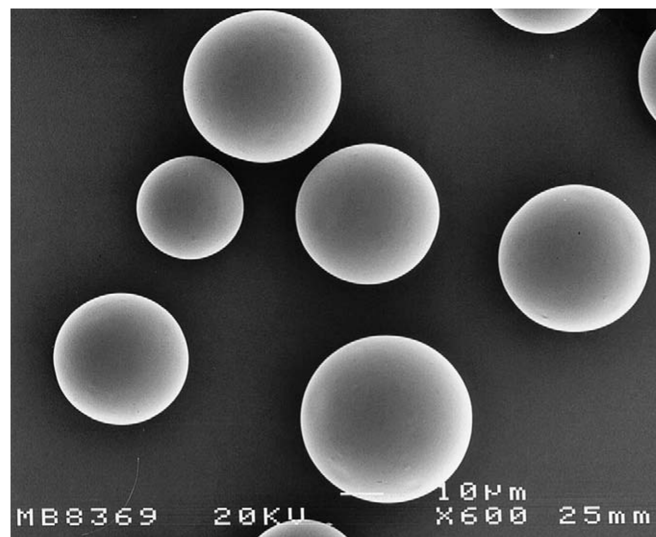

Fig. 1. SEM micrograph of dexamethasone microspheres prepared by the O/W solvent evaporation technique (x600).

The *in vitro* drug release profile of sterilized microspheres was obtained by measuring the mean percentages of dexamethasone released at pH 7.4 over 53 days (Fig. 2). During the first 24 h, 301.20 ± 0.50 μg (19.43 ± 0.32%) dexamethasone/mg was released into the phosphate buffer. After that, drug release occurred in a biphasic manner. Between Days 3 and 21, release followed zero-order kinetics with a mean rate constant of 0.83 μg dexamethasone/day per mg of microspheres. From Day 22 to the end of the release assay at Day 53, a mean release rate of 2.60 μg dexamethasone/day per mg of microspheres occurred. At the end of the release assay, 98.98 ± 0.66% of the dexamethasone content was released from the microspheres.

Non-sterilized and sterilized microspheres were previously assayed by the authors for loading efficiency, differential scanning calorimetry (DCS) and, gel permeation chromatography (GPC) (data not shown). For instance, encapsulation of dexamethasone was not affected by gamma irradiation, with mean values of 92.97 ± 0.75% and 93.96 ± 1.53%, for non-irradiated and gamma-irradiated microspheres, respectively. Regarding GPC analysis, gamma irradiation of the microspheres resulted in a very slight modification of

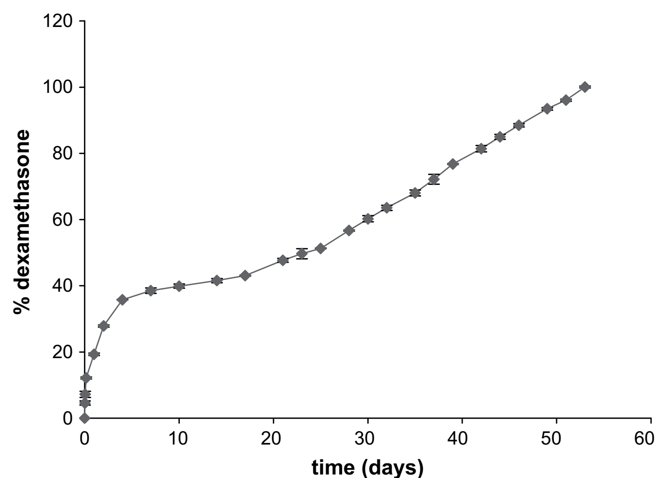

Fig. 2. Mean percentages of dexamethasone released from sterilized dexamethasone-PLGA microspheres in 0.01 M Sörensen phosphate buffer (pH 7.4) at 37 °C.

the molecular weight, which was not statistically significant. Moreover, *in vitro* release tests were also conducted with sterilized microspheres. Comparison of *in vitro* release profiles of non-sterilized and sterilized formulations gave a value of 98.27 for the f_2 parameter (similarity factor), confirming the similarity of the release profiles.

3.2. Short-term study

One day after the first LPS injection, eyes pre-treated with dexamethasone-loaded microspheres had lower signs of inflammation than control eyes and eyes injected with blank microspheres (Fig. 3). By Day 7, inflammation in eyes pre-treated with dexamethasone-loaded microspheres was undetectable. In contrast, inflammation in control eyes and eyes pre-treated with blank microspheres reverted to undetectable levels by 15 days after LPS injection (Fig. 3, Table 1).

After vitrectomy, eyes receiving blank microspheres prior to LPS injection had inflammation signs lower than 0.30 in the short-term studies. After the first LPS injection, vitrectomized eyes injected with dexamethasone-loaded microspheres had similar inflammation grades, 1.29 (Fig. 4), as non-vitrectomized eyes, 1.90 (Fig. 3).

Inflammatory infiltration was present in the form of vitreous condensations behind the crystalline lens. Control eyes (Group A) with no microspheres and eyes with dexamethasone-free microspheres (Group B) had significantly more inflammatory infiltrates ($P < 0.05$, Table 1) than did eyes treated with dexamethasone-loaded microspheres (Group C).

The mean ERG amplitudes and implicit times recorded at the end of the short-term study showed that LPS injection alone (Group A, without microspheres) generated increases in B amplitudes when compared to non-treated eyes ($P < 0.05$, Table 2).

3.3. Long-term study

By means of indirect ophthalmoscopy, microspheres were visible in the vitreous cavity after injection and at the end of the long-term studies (Fig. 5). After injection of the second LPS dose 37 days after microsphere administration, a high inflammatory peak occurred one day later in both control eyes and eyes pre-treated with blank microspheres (Fig. 3). In contrast, only a slight inflammation was detected in eyes that were injected with dexamethasone-loaded microspheres (Fig. 3, Table 1).

LPS-injected eyes pre-treated with microspheres, either without (Group B) or with dexamethasone (Group C), had significantly lower B ERG amplitudes than the control (Groups A) LPS-injected group ($P < 0.05$, Table 1); however the B implicit times were not affected by either LPS or microsphere pre-treatment. For vitrectomized eyes, the B amplitudes values were closer to the amplitudes in the untreated animals. Between vitrectomized and non-vitrectomized eyes injected with dexamethasone-loaded microspheres, there were no significant differences ($P > 0.05$) in B implicit times.

Table 1
Inflammatory cell infiltrates in short-term and long-term efficacy studies.

Study	Animal Group	Inflammatory Cells
S-T	Group A: Control	2.20 ± 0.20
S-T	Group B: M	2.33 ± 0.33
S-T	Group C: M + DXM	0.60 ± 0.40
L-T	Group A: Control	1.80 ± 0.20
L-T	Group B: M	2.00 ± 0.00
L-T	Group C: M + DXM	1.20 ± 0.20
L-T	Group B1: M (SF6)	1.75 ± 0.25
L-T	Group C1: M + DXM (SF6)	0.67 ± 0.33

S-T, Short-term; **L-T**, Long-term; Untreated, Non-treated eyes; **Group A**: Control, Eyes injected with LPSl; Group B: M, blank microspheres; **Group C: M + DXM**, microspheres loaded with dexamethasone; $\mathbf{SF_6}$, gas vitrectomy; DXM, dexamethasone; **Inflammatory** cells: 0 = absence of inflammation; 1 = mild inflammation; 2 = moderate inflammation; 3 = severe inflammation.

4. Discussion

PLGA 50:50 (PLA:PGA) was selected because this ratio of polymer was the most biodegradable (Anderson and Shieve, 1997). Furthermore, the lowest molecular weight of this copolymer was selected. The microsphere fraction selected from the original formulation was composed of a 2:10 dexamethasone:polymer ratio and 20–53 μm size, which was adequate for intravitreal administration through a 25G syringe needle (Herrero-Vanrell and Refojo, 2001). For this formulation, the mean encapsulation efficiency was 92.97 ± 0.75% (Herrero-Vanrell et al., 1999), which corresponds to 141 ± 0.38 μg dexamethasone/mg microsphere.

The theoretical amount of microspheres to be injected into the vitreous was calculated as follows:

$$K_0 = \overline{C_{ss}} \times Cl$$

where K_0 is the zero-order drug release rate from the microspheres to achieve therapeutic levels in the vitreous. $\overline{C_{ss}}$ is the therapeutic drug concentration in the vitreous, and Cl is the drug clearance from the vitreous. The precise concentration $\overline{C_{ss}}$, needed to achieve maximum anti-inflammatory efficacy has not been established.

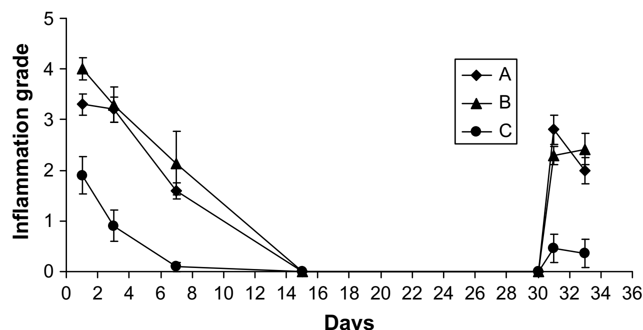

Fig. 3. Time-dependent changes in inflammation grade. Group A: No microsphere injection; Group B: Intravitreal injection of blank microspheres; Group C: Intravitreal injection of dexamethasone-loaded microspheres. All treatments were administered 7 days before the first LPS injection. Day 0 represents the day of LPS injection.

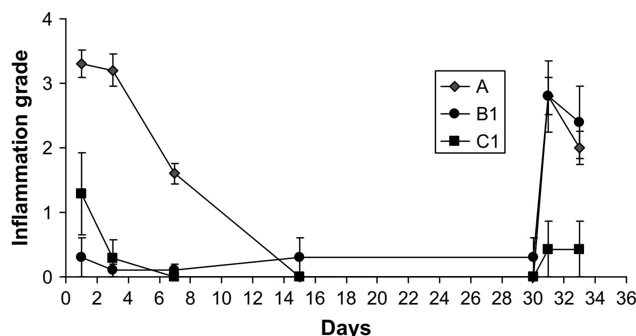

Fig. 4. Time-dependent changes in inflammation grade for vitrectomized eyes. The fluidized vitreous was removed just prior to the injection of control or microsphere suspension. Group A: No microsphere injection; Group B_1: Intravitreal injection of blank microspheres; Group C_1: Intravitreal injection of dexamethasone-loaded microspheres. All treatments were administered 7 days before the first LPS injection. Vitrectomy was performed two days before microspheres administration. Time 0 represents LPS injection.

Table 2
ERG amplitudes and implicit times.

Study	Animal Group	B Amplitude	B Implicit Time
	Untreated	3.97 ± 0.90[a,b,c]	25.54 ± 1.80
S-T	Group A: Control	13.59 ± 0.31[a]	27.00 ± 0.00
S-T	Group B: M	10.06 ± 1.20[b]	28.20 ± 0.00
S-T	Group C: M + DXM	12.68 ± 0.73[c]	30.80 ± 1.40
L-T	Group A: Control	18.11 ± 0.87[d,e]	27.48 ± 0.22
L-T	Group B: M	1.24 ± 0.70[d]	21.60 ± 0.00
L-T	Group C: M + DXM	−1.76 ± 0.78[e]	28.13 ± 1.25
L-T	Group B1: M (SF_6)	2.74 ± 0.72	27.00 ± 0.81
L-T	Group C1: M + DXM (SF_6)	3.46 ± 1.99	29.40 ± 0.27

S-T, Short-term; **L-T**, Long-term; Untreated, Non-treated eyes; **Group A**: Control, Eyes injected with LPSl; **Group B: M**, blank microspheres; **Group C: M + DXM**, microspheres loaded with dexamethasone; **SF_6**, gas vitrectomy; **DXM**, dexamethasone.
Note: Cells with like letters are significantly different ($P < 0.05$) from one another.

However according to Flower and Blackwell (1979), a concentration of 1 μg/ml markedly reduced phospholipase activity in perfused organs as estimated by the release of thromboxane A_2 or by the hydrolysis of radioactive phosphatide.

Dexamethasone clearance from the vitreous can be calculated according to the following equation (Rowland and Tozer, 1995):

$$Cl = Vc \times Ke = Vc \times \frac{0.693}{t_{1/2}}$$

where Vc is the volume of the vitreous, 1.5 ml in rabbits (Leeds et al., 1997), and Ke is the drug intravitreal elimination rate constant that is derived from the half-life of the drug. The half-life of dexamethasone sodium phosphate in the rabbit vitreous is 3.48 h (Kwak and D́Amico, 1992).

According to these calculations, a K_0 value of 7.2 μg dexamethasone/day is obtained. This is the minimum amount of dexamethasone needed to be released from the microspheres to achieve a $\overline{C_{ss}}$ of 1 μg/ml. Based upon the *in vitro* study and applying these calculations, we estimated that 9 mg of dexamethasone-loaded microspheres would potentially achieve therapeutic levels in the vitreous. We chose 10 mg as the final amount of microspheres because conventional treatment of uveitis consists of intravitreal administration of a single dose of 400 μg dexamethasone sodium phosphate injected in 0.1 ml saline. Additionally, 10 mg of our microspheres contained 1410 μg of dexamethasone and gave an initial burst release of 301.20 ± 50 μg during the first day, a value that was equivalent to a clinical injection.

Rabbit eyes are particularly sensitive to LPS from *E. coli*, *Salmonella typhi*, and *Klebsiella pneumonie*, and the inflammatory response is similar among them (Howes et al., 1980). We selected a dose of 0.01 mg/ml *E. coli* LPS because the effects disappeared after 15 days. Other authors have also demonstrated this recovery pattern (Hebort et al., 1988; Kulkarni, 1994). At 30 days after the initial injection, the eyes remained quiet, whereupon we delivered the second LPS injection. This in fact mimics the clinical setting in which uveitic inflammation flares up after the eye has been quiet for a period. We found this model of recurrent uveitis especially useful to address the most critical problem in uveitis treatment, which is how to avoid or minimize inflammation flare-ups.

The sum of anterior and posterior signs reflects the whole inflammatory response and was lower one day after the first LPS injection in eyes pre-treated with dexamethasone-loaded microspheres than control eyes and eyes injected with blank microspheres. The presence of proteins in aqueous humor is not considered a specific sign to evaluate inflammation because proteins readily appear with the most minimal disturbance. For instance, in our study the presence of proteins was similar in both control and non-treated groups (data not shown). On the other hand, the amount of cells present in aqueous humor can be used as a specific measure of inflammation (Cheng et al., 1995). In our case, the amount of aqueous humor cells counted in control animals receiving LPS but not microspheres (data not shown) was highly variable and the mean was considerably greater than that reported by Kulkarni (1994). Due to the high variability, this parameter was not considered useful to account for inflammation.

At the end of the short-term study, LPS injection generated an increase in B amplitude in all groups. Increases in B amplitudes are related to irritability of bipolar cells (Tsuji et al., 2004), whereas increased implicit times could be attributed to the damaging effect of the LPS injection on photoreceptors. Increases observed in eyes injected with microspheres are probably the result of a foreign body reaction that has been described after intramuscular or intravitreal administration of PLGA microspheres (Veloso et al., 1997; Visscher et al., 1985). The reactions gradually decrease with time and are non-toxic for the retina, but they do cause a certain degree of retinal irritation (Giordano et al., 1993; Moritera et al., 1992; Veloso et al., 1997).

The inflammation observed with blank microspheres could be due to the aggregate effect caused by both LPS and PLGA. For instance, Giordano et al. (1995) also reported an inflammatory reaction in the vitreous due to microsphere injection. Eyes receiving the dexamethasone-loaded microspheres had significantly fewer inflammatory cells than either of the other 2 groups.

From a clinical point of view, we conclude that PLGA microspheres-loaded with dexamethasone effectively reduced intraocular inflammation between 1 and 7 days after initiation of inflammation and diminished vitreous opacity when compared to control and blank PLGA microspheres groups. In human uveitis, it is essential to bring down inflammation as soon as possible. Intraocular inflammation can easily and rapidly cause damage in the macular area (i.e. macular edema, choroidal neovascularization, etc), leading to permanent and irreversible profound visual loss if not promptly

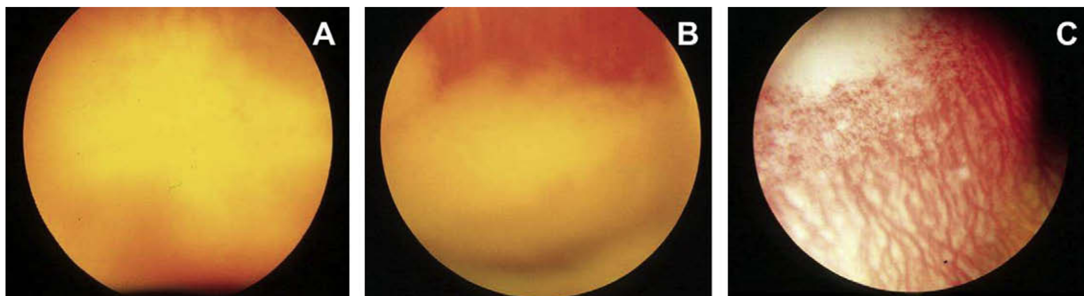

Fig. 5. Eye fundus photographs obtained at the end of the long-term studies (40 days). A: Control group; B: Injected with blank microspheres; C: Injected with dexamethasone-loaded microspheres. Microspheres can be seen as the white material in fundus.

reversed. In fact, macular edema is the most significant cause of visual loss associated with uveitis, and intravitreal injections of triamcinolone are now being used to treat this condition (Sallam et al., 2008; Choudhry and Ghosh, 2007). This further supports our belief that intravitreal injection of longer lasting formulations will add an additional advantage. Transeptal injections or oral formulations of steroids usually take 1–3 weeks to begin to exert their effects. Our microsphere formulation could be clinically advantageous as we saw a decrease of inflammation already on the first day after injection. While intravitreal triamcinolone is effective, a longer lasting treatment needs to be developed (Hogewind et al., 2008). We believe that a formulation that releases drugs for longer period of time would be a large step in achieving that goal. Finally, an additional advantage of our formulation is that it could avoid one, and possibly two, surgical interventions.

Common side effects of all steroids, such as elevation of intraocular pressure and cataract, can also occur with intravitreally injected formulations (Galor et al., 2007). These potential adverse events will need to be studied with our formulation in a future clinical trial setting. However, the reduction was not as great as expected, possibly due to a slower release rate of dexamethasone than expected. Based on *in vitro* release tests performed at pH 7.4, the mean zero-order release rate between Days 3 and 21 was 0.83 μg dexamethasone/day per mg microspheres. Because inflammation usually produces a reduction of the pH (Da-Wen et al., 2001), we suspect that the pH of the vitreous was lower than optimal. In subsequent preliminary *in vitro* studies, we have confirmed that at pH 5, the zero-order release rate is only 0.27 μg dexamethasone/day per mg microsphere during the first 28 days. Thus in future studies, a much higher dose of microspheres, perhaps 30 mg, will be needed to achieve and maintain therapeutic concentrations of dexamethasone in the vitreous. To avoid the injection of high amounts of microspheres, additives can be incorporated at the time of their preparation to increase the release rate of the drug. These additives have already demonstrated an ability to modulate the release of different drugs (Herrero-Vanrell and Refojo, 2001).

Microspheres were visible by indirect ophthalmoscopy of the vitreous cavity after injection at the end of the long-term studies. These findings are in agreement with Veloso et al. (1997) who showed that 5, 10, and 15 mg of gancyclovir PLGA microspheres were still present in the rabbit eyes 8 weeks after injection. Similar results have also been reported by Moritera et al. (1992) who described the presence of PLGA microsphere remnants at the implantation site 8–12 weeks after administration.

One important aspect of a drug delivery system is its ability to treat not only the acute processes but also to diminish recidivism. For this matter, we introduced a modification in the rabbit model of uveitis that mimics recurrent intraocular inflammation. In our study, posterior inflammation subsided in all eyes by 15 days after the LPS injection. When a second LPS dose was administered 30 days after the first one, the inflammatory reaction in the control group and the dexamethasone-free microsphere group was much higher than that in the group pre-treated with dexamethasone-loaded microspheres. These results are in agreement with the behavior of a controlled delivery system (Chen, 2007) in which the drug is gradually released to maintain an effective concentration of dexamethasone in the vitreous, in this case for at least 30 days. Our results are consistent with another study (Cheng et al., 1995). We hypothesize that the first LPS injection originates a local immune sensitization of the inflammatory reaction that becomes manifested after the second LPS injection. In this study, the dexamethasone-loaded microspheres significantly reduced vitreous inflammation in the long-term study when compared to control group and group injected with blank microspheres. Furthermore, dexamethasone-loaded microspheres significantly reduced the presence of inflammatory cells in both non-vitrectomized (Groups C) and vitrectomized (Group C_1) eyes, demonstrating a potent anti-inflammatory response to this formulation. At 33 days after LPS injection, the mild inflammatory reaction that occurred with this formulation was probably due to the foreign body reaction reported for PLGA microspheres.

5. Conclusions

PLGA microspheres loaded with dexamethasone effectively reduced the short- and long-term intraocular inflammation in rabbit eyes caused by injection of LPS into the vitreous. From a clinical point of view, it would be necessary to assure that this new formulation releases sufficient dexamethasone to achieve intravitreal concentrations in humans with maximum anti-inflammatory efficacy in uveitis.

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