Evaluation of the Toxicity of Intravitreally Injected PLGA Microspheres and Rods in Monkeys and Rabbits: Effects of Depot Size on Inflammatory Response

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Submitted: December 20, 2016
Accepted: July 14, 2017
Citation: Thackaberry EA, Farman C, Zhong F et al. Evaluation of the toxicity of intravitreally injected PLGA microspheres and rods in monkeys and rabbits: effects of depot size on inflammatory response. Invest Ophthalmol Vis Sci. 2017;58:4274–4285. DOI:10.1167/iovs.16-21334

PURPOSE. Poly(lactic-co-glycolic) acid (PLGA) inserts have been successfully developed for the treatment of posterior eye disease as a means of reducing injection frequency of intravitreally administered therapeutics. PLGA microspheres are also of interest for the delivery of intravitreal drugs, since they offer the advantage of being easily injected without surgical procedures or large injectors.

METHODS. In the current study, the toxicity of PLGA microspheres and rods was investigated in nonhuman primates (NHPs) and rabbits. An in vitro assessment of cytokine responses to PLGA in peripheral blood mononuclear cells (PBMCs) and macrophages was also performed.

RESULTS. Intravitreal administration of 3, 10, or 12.5 mg/eye of PLGA microspheres in NHPs resulted in a severe immune response characterized by a foreign body response. Follow-up studies in the rabbit confirmed this finding for PLGA microspheres ranging in size from 20 to 100 μm. In contrast, administration of PLGA rod implants with a similar PLGA mass did not elicit a significant immune response. In vitro assays in PBMCs and macrophages confirmed proinflammatory cytokine release upon treatment with PLGA microspheres but not PLGA rods.

CONCLUSIONS. These data demonstrate a lack of tolerability of PLGA microspheres upon intravitreal injection, and suggest that the size, shape, and/or surface area of PLGA depots are critical attributes in determining ocular toxicity. Keywords: PLGA, ocular toxicology, drug development, foreign body response

The treatment of posterior segment eye diseases such as AMD, diabetic macular edema (DME), and uveitis generally requires intravitreal administration of therapeutics. While intravitreal injection procedures have become routine in clinical practice, a reduction in injection frequency is desirable both from a safety, compliance, and treatment burden perspective. Poly(lactic-co-glycolic) acid (PLGA) is a commonly used bioerodible polymer that is generally synthesized via the ring-opening polymerization of lactide and glycolide monomers.1 The ratio of lactide to glycolide monomers governs the hydrolytic degradation rate of the polymer and thus the release kinetics of any therapeutic loaded in the matrix. PLGA has been used extensively in sustained release delivery systems and can be formulated as microspheres, discs, films, rods, pins, and screws as well as in solvent depots. Several PLGA-based sustained-release formulations have been approved for clinical applications. For example, PLGA microspheres have been approved for administration via the intramuscular or subcutaneous routes.2–6 More recently, PLGA implants (Surodex; Oculex Pharmaceuticals, Inc., Sunnyvale, CA, USA, and Ozurdex; Allergan plc, Parsippany, NJ, USA) have been used to successfully deliver steroids to the eye over the course of weeks to months, thereby reducing the frequency of intravitreal injections.7,8 Importantly, recent advances in protein processing techniques and formulation development have enabled the preparation of protein-loaded PLGA rods9 and PLGA-solvent depots10 for sustained-release applications.

The drug release characteristics of microsphere or nanosphere PLGA formulations for ocular administration have also been of great interest,11,12 since these formulations allow for easy intravitreal or intracameral injection rather than using a large diameter injector or being surgically implanted. To date, most reports indicate that PLGA microspheres are well tolerated within the eye,13–16 consistent with the widely accepted biocompatibility of PLGA-based biomaterials and the established safety of steroid-loaded PLGA implants in the eye.

In the current report, the toxicity of PLGA microspheres was assessed in cynomolgus monkeys. The results revealed an unexpectedly severe immune reaction to placebo, or unloaded (polymer only) microspheres, characterized by a foreign body response within the vitreous. Follow-up studies in rabbits confirmed the lack of tolerability of different PLGA micro-
spheres batches having diameters ranging from 20 to 100 μm. Interestingly, large (0.9 × 3.7 mm) PLGA rods did not produce a similar foreign body response and were well tolerated in rabbit eyes. Finally, in vitro studies confirmed the greater potential of PLGA microspheres to elicit an innate immune response compared to PLGA rods. These data suggest that the biocompatibility of PLGA within the eye may be dependent on particle/implant size and that a detailed assessment of the toxicity of injectable PLGA formulations intended for the eye is warranted prior to advancement into the clinic.

Materials and Methods

**PLGA Microsphere Preparation for Nonhuman Primate (NHP) and Rabbit Studies 1 and 2**

PLGA microspheres (8515 DLG 5A/7525 DLG 5.5 E [50/50] and 8515 DLG 6A; Lakeshore Biomaterials, Birmingham, AL, USA) for the NHP study 1 and rabbit study 2 were supplied by Brookwood Pharmaceuticals (acquired by SurModics Pharmaceuticals, Inc. and now Evonik Industries, Birmingham, AL, USA). Table 1 lists the characteristics of the PLGA microspheres tested in detail. The microspheres were prepared using a continuous double emulsion, solvent-extraction microencapsulation process. The aqueous phase comprised of 0.1% wt/vol poly(vinyl alcohol) (PVA). The polymer solution was prepared by dissolving each polymer excipient in ethyl acetate and filtered using a positive displacement pump. The two streams were each filtered inline separately and mixed in an inline homogenizer to form the primary emulsion, then pumped to a second homogenizer with the introduction of the continuous phase, an aqueous PVA solution to form a water-in-oil-in-water emulsion. The microparticles were formed by extraction of the solvent with water for injection and collected in a tank for hardening. The microparticles were isolated inside a class A glove box on 20-μm stainless steel mesh sieves, rinsed with deionized (DI) water supplemented with 0.5% wt/vol PVA (Sigma-Aldrich Corp., St. Louis, MO, USA), which served as an extraction medium for the ethyl acetate. Following extraction and hardening, the microspheres were lyophilized to a free-flowing powder, stored frozen at −20°C until further use. Microspheres were later imaged with a light microscope (Motic Instruments) to determine their size, and endotoxin content was measured by limulus amebocyte lysate (LAL) assay. Representative optical microscopy images of the precisely sized microspheres are included in the Supplementary Figures.

**Precisely Sized PLGA Microsphere Preparation for Rabbits (Study 3)**

Monodisperse microspheres were made using spraying plus acoustic excitation with carrier stream, a process described previously (Table 1). Briefly, PLGA (7525 DLG; Lakeshore Biomaterials) was added to ethyl acetate at a concentration of 10% wt/vol in vial and then vortexed until dissolved. The polymer solution was loaded into a plastic Luer-lock syringe affixed to a precision syringe pump, and connected to the custom particle fabrication nozzle with small volume PTFE tubing. The polymer solution was released through the nozzle at a fixed rate and the stream excited at various frequencies via vibratory mechanism to fabricate 20-, 50-, and 100-μm particles, respectively. The droplets fell into a glass beaker of deionized (DI) water supplemented with 0.5% wt/vol PVA (Sigma-Aldrich Corp., St. Louis, MO, USA), which served as an extraction medium for the ethyl acetate. Following extraction and hardening, the microspheres were lyophilized to a free-flowing powder, stored frozen at −20°C until further use. Microspheres were later imaged with a light microscope (Motic Instruments) to determine their size, and endotoxin content was measured by limulus amebocyte lysate (LAL) assay. Representative optical microscopy images of the precisely sized microspheres are included in the Supplementary Figures.

**PLGA Rods Preparation for Rabbits (Study 4)**

PLGA polymers (Resomer RG 756 S) were sourced from Evonik Industries (Darmstadt, Germany). PLGA rods were prepared as described in Rajagopal et al. (Table 1). Briefly, hot-melt extrusion was performed using a conical counter rotating twin-screw extruder (HAAKE MiniLab; Thermo Fisher Scientific, Karlsruhe, Germany) in a laminar flow hood. The extruder barrel was preheated to the extrusion temperature of 90°C and the screw speed was set to 30g. About 4.75 g of polymer pellets and 0.25 g of carboxymethylcellulose filler was starved slowly into the extruder and recirculated back to the barrel for microcompounding. The melt-phase polymer blending was continued for 30 minutes and then extruded through a 1.0-mm circular die. The extrudate was cooled and solidified at room temperature, then cut to cylindrical rods of desired length using a razor blade. Endotoxin content was measured by LAL assay. A representative scanning electron microscopy image is included in the Supplementary Figures.

**In Vivo Studies**

Table 2 provides a description of all four in vivo studies performed, including species, number of animals, test articles...
This page discusses the toxicity study of PLGA microspheres in rabbits and NHPs, with a focus on investigating the translatability of NHP findings to rabbits. The study was conducted in accordance with GLP standards and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Table 2: Primary and Investigative Studies of PLGA Microspheres/Rod IVT Injection in NHPs and Rabbits

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Species</th>
<th>Sex</th>
<th>n*</th>
<th>PLGA Dose, mg/Eye</th>
<th>Size (d50)</th>
<th>Duration of Study†, wk</th>
<th>Study Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NHP</td>
<td>M/F</td>
<td>5/sex/group</td>
<td>3, 10, 12.5</td>
<td>71.5 μm</td>
<td>26 + 13†</td>
<td>Safety study for PLGA microspheres on toxicity</td>
</tr>
<tr>
<td>2</td>
<td>Rabbit</td>
<td>M</td>
<td>4 control, 10 treated</td>
<td>10</td>
<td>71.5 μm</td>
<td>5</td>
<td>Determine translatability of NHP findings to rabbits</td>
</tr>
<tr>
<td>3</td>
<td>Rabbit</td>
<td>F</td>
<td>3/group</td>
<td>10</td>
<td>20, 50, 81.8, 100 μm</td>
<td>6</td>
<td>Investigate effect of size of microsphere on toxicity</td>
</tr>
<tr>
<td>4</td>
<td>Rabbit</td>
<td>M</td>
<td>2 control, 3 treated</td>
<td>3</td>
<td>0.9 × 3.7 mm</td>
<td>8</td>
<td>Determine toxicity of PLGA rod</td>
</tr>
</tbody>
</table>

* Number of animals, all animals received unilateral ITV injection/implantation.
† All studies were single dose, duration of observation period is indicated.
‡ Mid- and high-dose monkeys were euthanized on study week 20 post-dose due to intolerability of microspheres.

The initial rabbit study investigating the species translation of the NHP findings was performed at Genentech, Inc. (South San Francisco, CA, USA). Naïve male New Zealand White (NZW) rabbits (supplied by Myrtle’s Rabbitry, Inc., Thompson Station, TN, USA) were housed in the animal facilities at Genentech, Inc., under standard conditions. Prior to the dosing procedures, animals were anesthetized with ketamine and xylazine. After aseptic preparation of the ocular surface, inferior temporal dose administration was performed using 1-mL syringes and 25-gauge needles. Rabbits were administered either control vehicle (n = 2) or PLGA microspheres (n = 5) via a single 50-μL intravitreal injection into the right eye only on day 1. The control vehicle was 1% hyaluronic acid in PBS with 0.1% polysorbate 20, pH 7.4. The left eye was not treated in all dose group animals. A topical anesthetic (proparacaine or equivalent) was then applied to each eye. Slit-lamp biomicroscope exams, indirect ophthalmoscope exams, and IOP measurements (TonoVet rebound tonometer; iCare, Vantaa, Finland) were performed on each eye once using the predose phase; post dosing on study day 1; on study days 2, 4, 8, 15, 29, and 56. The left eye was fixed and stained with hematoxylin and eosin, and examined by a board-certified veterinary pathologist.

### Study 3: Investigative Rabbit Study With PLGA Microspheres

Study 3 was performed at Charles River Laboratories (Montreal, Canada). Anesthesia and dosing procedures were the same as described for the rabbit study above. Naïve female rabbits (n = 3/group) were administered a 50-μL injection of either heterogeneous placebo microspheres (the same material used in the NHP and rabbit studies above) or homogenous microspheres with a diameter of 20, 50, or 100 μm via a single 50-μL intravitreal injection into the right eye. The left eye of all animals received a single 50-μL injection of control buffer (1% hyaluronic acid in PBS with 0.1% polysorbate 20, pH 7.4). Slit-lamp biomicroscope exams; indirect ophthalmoscope exams; and IOP measurements (iCare) were performed.
Intravitreal PLGA Microsphere Toxicity

In each eye once using the predose phase; postdosing on study days 1, 5, 8, 18, 29, and 42. Davidson's fixed eyes were prepared for standard histopathology procedures. Paraffin-embedded tissues were sectioned approximately 5-μm thick, stained with hematoxylin and eosin, and examined by a board-certified veterinary pathologist.

Study 4: Investigative PLGA Rod Study in Rabbits. Study 4 was performed at Covance Laboratories, Inc., using rabbits from Covance Research Products, Inc. Anesthesia and dosing procedures were the same as described for the rabbit studies above. PLGA rods were implanted surgically. Prior to incision, the eyes were stabilized using one or more stay sutures of 6-0 silk on a cutting needle. Naïve female rabbits (n = 3) were implanted with PLGA rods (3 mg) at the superior temporal quadrant position in both eyes on day 1. The conjunctiva was incised, and a scleral incision was made using a blade (No. 65 Beaver) or other suitable surgical blade. The rod was gently grasped with a smooth-tipped forceps and introduced into the vitreous cavity through the scleral incision. Prolapsed vitreous, if any, was removed from the incision with a sterile cellulose sponge and scissors before suturing the sclera closed with one or more sutures of 80 Vicryl (polygalactin 910). At the discretion of the surgeon, the conjunctival incision was closed with the same suture material. A sham surgery cut with a 3-mm incision was made in both eyes of the sham control animals (n = 2) eye in all animals. All dosing procedures were performed under aseptic conditions using equipment approved for use in clinical surgery. A topical antibiotic (Alcon Laboratories, Inc.) was instilled in each eye following surgery on study day 1. Slit-lamp biomicroscope exams (Kowa handheld); indirect ophthal-moscope exams (Volk 2.2 condensing lens) and IOP measurements (iCare) were performed on each eye once during the predose phase; prior to dosing on study day 1; on study days 3, 5, 8, 15, 29, and 59. Davidson's fixed eyes were prepared for standard histopathology procedures. Paraffin-embedded tissues were sectioned approximately 5-μm thick, stained with hematoxylin and eosin, and examined by a board-certified veterinary pathologist.

In Vitro Studies

The same materials used in in vivo study 1 (PLGA microspheres) and study 4 (PLGA rods) were utilized for in vitro assessments (Tables 1, 2). Placebo microspheres and rods do not dissolve in the culture media hence the concentrations tested in vitro are approximate. Treatments with PLGA rods were performed using a single rod with equivalent mass concentration as the PLGA microspheres tested in each well. The length of a PLGA rod to achieve 5.5 and 8 mg/mL was approximately 3 and 5 mm, respectively.

In Vitro Cytokine/Chemokine Release Assay. 0.2 × 10⁶ peripheral blood mononuclear cells (PBMCs), isolated from human whole blood from three donors were incubated with PLGA microspheres or PLGA rods at an in vivo relevant concentration range (5.5 and 8 mg/mL) in 0.2 mL of RPMI media (Gibco Laboratories, Gaithersburg, MD, USA), with 10% heat-inactivated AB serum (H3667; Sigma-Aldrich Corp.); 1X cell culture supplement (GluMAX; Gibco Laboratories); and 1% penicillin-streptomycin mixture (Penstrept; Gibco Laboratories) in a 96-well plate. The supernatant was collected 24 hours postincubation and tested for 12 cytokines/chemokines using a commercial assay (Luminex; EMD Millipore Corp., Billerica, MA, USA). We conducted 1-way ANOVA followed by Tukey's multiple comparisons test to determine statistical significance among treatments (GraphPad Software, Inc.).

RESULTS

Safety Study of Placebo Microspheres in Cynomolgus Monkeys

In order to support rapid clinical development of PLGA microspheres as a long-acting delivery platform, a 26-week toxicology study with a 13-week recovery in cynomolgus monkeys was conducted. Unexpectedly, a progressive inflammatory response was observed, beginning as early as 3 days' postdose, and progressing to a moderate to severe response in most animals within 1 month. This response was the mildest in the low dose group (3 mg/eye), but was of similar severity in the mid and high dose groups (10 and 12.5 mg/eye, respectively). Clinical observations captured via external observations, slit-lamp biomicroscopy, and indirect ophthalmoscopy showed conjunctival hyperemia, aqueous flare, vitreous cells, and iris hyperemia (up to +2 on “0” to +4 scale). Also observed were aqueous and vitreous haze, precipitants/inflammatory cells on the lens, degraded view of the fundus, fibrin clots, dyscoric or miotic pupils, posterior synechia, incomplete pupil dilation, vitreous floaters, iris depigmentation, cataract macular edema, corneal edema, and corneal neovascularization. Due to the severity of the ocular findings, numerous animals were euthanized for animal welfare reasons. This included one low-dose female during week 6, one middose male during week 7, one middose male and three high-dose males during week 16, and one high-dose male during week 17. During weeks 21 through 24, all remaining mid- and high-dose animals were euthanized due to severe clinical signs. Control and low animals were euthanized as scheduled in week 26 (terminal necropsy, n = 3/sex/group) and 39 (recovery necropsy, n = 2/sex for control and low-dose males, n = 1 for low-dose females).

In order to provide a more simplified way of presenting the ocular examination data, an integrated overall inflammation score was determined based on the criteria described in Table 3. Figure 1 shows the mean inflammation scores for each dose group in the cynomolgus monkey study. The inflammation in the mid- and high-dose groups progressed until termination of this dose group prior to the week 25 ocular exams. In contrast, the inflammation in the low dose group was less severe, and
resolved by week 28. In all dose groups, the mean inflammation scores underrepresent the severity of the reactions due to the early euthanasia of the animals with the most severe findings.

Intraocular pressure was generally normal across all dose groups. In cases where low IOP was noted, it was generally considered to be related to inflammation observed or to the use of general anesthesia. OCT data were difficult to interpret due to degraded fundus views (secondary to the inflammation) or cutout caused by the presence of microspheres over the retina, while the microspheres themselves were visible above the retina in the vitreous in many of the scans (Fig. 2A). In several animals, retinal detachment was observed at later time points (study weeks 13–26, Fig. 2B). This finding was considered related to the foreign body response observed (see histopathology results below). The microspheres were visible in the fundus photographs as white clumped masses or diffuse masses (Fig. 2C), and these masses were also noted during fluorescein angiography assessments (Fig. 2D). Fundus photographs revealed optic nerve swelling (Fig. 2E) and vascular membranes on the posterior lens surface, both of which were likely secondary to the ocular inflammation. ERG results were generally within the normal range for all animals on study, though the most severely affected animals had fewer ERG readings due to early termination. In one high-dose female, increased latencies of the scotopic response, suggesting that the inflammatory response was not related to the presence of microbial contamination (data not shown). Overall, the externally visible inflammatory reaction to the microspheres was less than that seen in cynomolgus monkeys, though the short duration of the study makes it difficult to make a quantitative comparison. However, similar histopathologic lesions (granulomatous/histiocytic inflammation and foreign body response, etc.) was observed in both species, which supports the conclusion that a similar inflammatory mechanism is at work, and therefore the rabbit is a useful model to study the effects IVT microsphere administration.

Translation of Microsphere-Related Findings to Rabbits

In order to study the relative tolerability of intravitreal (IVT) administration of PLGA microspheres in a different species, we characterized the toxicity of this technology in NZW rabbits for 5 weeks after a single 10 mg/eye dose of placebo microspheres. Animals were injected in a single eye only (fellow eye was untreated) and a vehicle control group was included. IVT microsphere administration resulted in mild to moderate prolonged inflammatory response, characterized by vitreous cells (trace to +) as well as conjunctival hyperemia, chemosis, iris hyperemia, and mild aqueous cells/lacr (trace to +). Overall inflammation scores using the criteria form Table 3 are shown in Figure 3A.

Histologic findings consisted of minimal to moderate histiocytic inflammation (macrophages) of the vitreous, often associated with the microspheres (visualized as clear, colorless spherical objects) in the anterior ventral portion of the vitreous, and occasional multinucleated macrophages and fibrosis were present (consistent with a foreign body response). Occasionally, individual macrophages and/or heterophils were adherent to the superficial (inner portion) layers of the retina extending as far posterior as the optic disc (Fig. 4A). In more severe instances of inflammation, transretinal inflammatory cell infiltration and retinal atrophy were present. Testing of both gamma-irradiated and nongamma-irradiated microspheres revealed no difference in the inflammatory or microscopic response, suggesting that the inflammatory response was not related to the presence of microbial contamination (data not shown). Overall, the externally visible inflammatory reaction to the microspheres was less than that seen in cynomolgus monkeys, though the short duration of the study makes it difficult to make a quantitative comparison. However, similar histopathologic lesions (granulomatous/histiocytic inflammation and foreign body response, etc.) was observed in both species, which supports the conclusion that a similar inflammatory mechanism is at work, and therefore the rabbit is a useful model to study the effects IVT microsphere administration.

Impact of Size and its Distribution on Intravitreal PLGA Toxicity

In order to further investigate the ocular inflammatory response, we dosed microspheres of different sizes with low

| Table 3. Integrated Inflammation Scoring System Used on All Studies |
|-------------------------|----------------------|
| Inflammation Grade | Findings |
| 1 | Aqueous or vitreous cell score of 1 (exudates) or 2 (nuclear deposits) |
| 2 | Aqueous or vitreous cell score of 3 (nuclear deposits) |
| 3 | Aqueous or vitreous cell score of 4 (nuclear deposits) |
| 4 | Aqueous or vitreous cell score of 5 (nuclear deposits) |
| 5 | Aqueous or vitreous cell score of 6 (nuclear deposits) |
| 6 | Aqueous or vitreous cell score of 7 (nuclear deposits) |
| 7 | Aqueous or vitreous cell score of 8 (nuclear deposits) |
| 8 | Aqueous or vitreous cell score of 9 (nuclear deposits) |
| 9 | Aqueous or vitreous cell score of 10 (nuclear deposits) |

For more detailed information, please refer to the original publication.
polydispersity in order to determine the effect of size on this response. Homogenous formulations of microspheres with diameters of 20, 50 and 100 μm were tested to evaluate the role of microsphere size. The previously used microspheres with a broad size distribution (d_{10} = 394.6 μm, d_{50} = 81.8 μm, d_{90} = 114.9 μm) were used as a positive control and vehicle buffer as a negative control in the contralateral eye.

Ocular inflammation was observed in all PLGA microsphere–dose groups. This was generally characterized as mild to moderate inflammation and consisted of vitreous cells (trace

**FIGURE 2.** Clinical and histopathological findings in NHPs administered PLGA microspheres. (A) Microspheres visible in the vitreous via OCT with associated retinal image cut out in a high dose (12.5 mg/eye) female at week 21. (B) Inferior retinal detachment, likely secondary to a foreign body response to the test article in a male middose (10 mg/eye) animal at week 17. (C) Fundus photograph of a middose male at week 21 showing marked clumping of microspheres over the macula. (D) Fluorescein angiogram of the same mid dose (10 mg/eye) eye taken on the same day showing a clear shadow due to the clumped microspheres. (E) Fundus photography of a mid-dose (10 mg/eye) animal showing prominent nerve head swelling and blurred disk margins. Note the hazy media and the presence of a single microsphere inferotemporally. (F) Foreign body response within the vitreous cavity in a high dose animal (12.5 mg/eye) at week 22. A mature granuloma (G), comprised of large numbers of macrophages and multinucleate giant cells, surrounding both individual beads and a central focus of necrosis (N), was adherent to the pars plana of the ciliary body (C). Variably-degraded beads were encompassed by mature fibrous connective tissue containing infiltrates of mononuclear inflammatory cells. (G) Higher magnification of the foreign body response showing multinucleate giants cells (M), fibrosis (F) and necrosis (N). (H) High magnification of the foreign body response showing encapsulated test article (TA), fibrosis (F), and multinucleate giant cells (M).
to 3) with some evidence of miosis in several animals (Fig. 3B). Also observed were lens capsule precipitates, conjunctival hyperemia, chemosis, and iris hyperemia across all microsphere-dosed groups. In general, there was no significant difference in the external visible inflammation between the different sizes of microspheres, though the homogenous microsphere preparations tended to produce slightly more inflammation.

Histologic findings were similar for all microsphere sizes and types, and primarily consisted of mononuclear cell (including multinucleated giant cell) infiltrates and fibrosis in the vitreous, associated with the microspheres (described as vacuoles in the vitreous). The infiltrates were usually present in the ventral, anterior portion of the vitreous, consistent with the region of initial microsphere deposition, and were sometimes associated with focal areas of retinal degeneration where the inflammatory cells extended into the retina. Vitreal hemorrhage and/or cataracts were sporadically present. In two animals administered the smallest size (20 μm) microspheres, the microspheres, and their associated macrophage and multinucleated giant cell infiltrates extended through the entire thickness of the retina as well as through the wall of the eye into the tissue external to the globe (Figs. 4B–D). This occurred in the back of the eye, and thus it was highly unlikely to be due to an injection mishap, but rather appeared to represent migration of the microspheres caudally within the globe, as well as penetration of the microspheres (and associated inflammatory response) through the wall of the eye.

Intravitreal Toxicity of PLGA Rods

While our data demonstrated that PLGA microspheres of varying sizes induce ocular toxicity in both rabbits and monkeys, intravitreal PLGA implants have been approved for clinical use. Additionally, placebo (nondrug containing) PLGA rods have been reported to be well tolerated in nonclinical studies. Therefore, we investigated the tolerability of PLGA rod implants in the rabbit eye. Nondrug containing (placebo) 3.7 mm–long rods of PLGA were implanted into both eyes of NZW rabbits, with two rabbits receiving a bilateral sham surgical procedure as a control. As described in Figure 3C, only minimal procedure-related inflammation was observed in both dose groups, which included conjunctival hyperemia and chemosis. These findings completely revolved by the end of the study in all animals. Histologically, minimal to mild inflammation consisted mainly of mononuclear or mixed inflammatory cells in the vitreous. Multinucleated giant cells were observed in a single animal only without any evidence of the fibrosis or a granulomatous inflammation reaction that was seen in any of the microsphere studies (Fig. 4E). No traumatic damage to the eye was seen despite the relatively large size of the administered rods in the rabbit eye (3.7-mm rod in a vitreous volume of 1.5 mL), compared to clinical use of the PLGA rods (6 mm rod in a human vitreous volume of 4 mL; Allergan plc). Therefore, our data is consistent with previous reports that large PLGA rods are well tolerated in this species.
In Vitro Acute Cytokine/Chemokine Responses by PBMCs

Subsequent to the in vivo findings which showed an inflammatory response and formation of foreign body response (FBR) in the eye, we assessed the ability of both PLGA microspheres and PLGA rods to elicit acute inflammatory responses in immune cell populations (PBMCs) in vitro, and determined cytokines and chemokines that may be involved in a FBR. PLGA microspheres showed a significant induction of proinflammatory cytokines/chemokines at both concentrations tested (5.5 and 8 mg/mL) compared to the media control. Out of 12 cytokines/chemokines tested, treatment with PLGA microspheres elicited significant induction of MCP-1, MIP-1α, MIP-1β, IL-8, TNFα, IL-6, and IL-1ra versus media control (Fig. 5 showing four representative analytes). In contrast, treatment with PLGA rod over a period of 24 hours did not result in any significant cytokine/chemokine induction compared to the media control. Neither PLGA microspheres nor PLGA rods significantly induced IFNγ, IL-2, IL-4, IL-1β, and IL-10, relative to media control (data not shown).

In Vitro Macrophage Fusion Potential and Cytokine/Chemokine Profile

Since macrophage activation and fusion is the hallmark of FBR, the ability of PLGA microspheres and PLGA rods to activate macrophages and form multinucleated giant cells was assessed in vitro. Macrophage fusion and multinucleated giant cells were readily detected in the IL-4 condition used as a positive control. Additionally, fusion was detected in macrophages treated with PLGA microspheres but appeared less prevalent with PLGA rod treatment (Figs. 6A, 6B). The observed differences in fusion between PLGA microspheres and PLGA rods were supported by significant differences in cytokine/chemokine production in vitro. The macrophage-specific cytokine/chemokine responses were measured at 5 days posttreatment. Treatment with PLGA microspheres resulted in significant production of MIP-1α, MIP-1β, TNFα, and IL-6 compared to media control (Fig. 6C). At similar mass concentrations, PLGA rod did not show any significant cytokine/chemokine induction compared to the media control. Neither PLGA microspheres nor PLGA rod treatments elicited a significant level of MCP-1, IL-6, IL-1β, IL-2, IL-4 IL-10, and IFNγ relative to media control (data not shown).
DISCUSSION

The biocompatibility of PLGA-based biomaterials has been well established via multiple routes of administration outside of the eye. Grossly, these polymers are well tolerated when injected intramuscularly, subcutaneously, or when used in sutures or as bond repair implants. This biocompatibility has led to development of multiple intramuscular or subcutaneous long-acting PLGA formulations. However, foreign body responses are often observed. A foreign body response, which is typically characterized by the presence of multinucleated giant cells with fibrotic encapsulation, is a normal response to a foreign body structure or substance, and in the context of these extraocular routes of administration, is considered nonadverse as long as significant prolonged inflammation, necrosis, or cellular proliferation is absent.

The eye is considered an immune privileged site, and numerous reports have documented both cellular and soluble factors contributing to an immunosuppressive environment within the aqueous and vitreous chambers. However, the eye is able to mount a robust inflammatory response in both appropriate (infectious uveitis) and inappropriate (idiopathic uveitis) scenarios. Additionally, many common degenerative eye diseases are now believed to have an inflammatory component, including AMD, diabetic retinopathy, and retinitis pigmentosa. Several published reports have also documented the ability of the posterior segment of the eye to mount a foreign body response.  Such responses have been observed in response to perfluoro-n-octane endotamponades and artificial intraocular lenses. While several groups have reported that PLGA microspheres are well tolerated and noninflammatory in the eye, Giordano et al. observed a foreign body response 2 weeks after intravitreal injection of PLGA microspheres (average diameter of 60 μm) in rabbits, and this reaction was present within the eye for up to 6 months (the longest time point evaluated). Foreign body reactions have also been described following the intravitreal administration of micro- and nanospheres of composition other than PLGA.

Our data demonstrate that PLGA microspheres with a diameter of 20 to 100 μm elicit an inflammatory foreign body response following intravitreal administration in both rabbits and NHPs. NHPs were more sensitive to this effect, with severe dose-related ocular inflammation evident within 1 week of administration. In contrast, the externally visible inflammatory response was less severe in the rabbits, and was generally reversible even at doses that led to unscheduled euthanasia in NHPs. Histologically, the response was similar in both species, with infiltrates of individual macrophages and multinucleated giant cells, characteristic of a foreign body response, associated with the injected microspheres. With the smallest microspheres we tested in rabbits (20 μm), we observed penetration of the microspheres and associated inflammation through the retina and sclera, with extension into the extraocular tissues. Given the severity of the response observed in these species, it is possible that longer duration studies would produce other serious sequelae characteristic of a progressive foreign body response in both species, and may lead to damage of intraocular structures (e.g., traction retinal detachment, retinal degeneration, and cataracts related to inflammation adjacent to the lens) over time. Our data in the NHP support this, and suggests that over time, intraocular foreign body reactions are unlikely to resolve and may lead to serious site-threatening damage to ocular structures.

It is important to note that, in general, the foreign body response was localized to the area of the vitreous immediately surrounding the PLGA microsphere depot, with the retina and other ocular structures more distant to the depot appearing...
largely normal. This highlights the importance of careful tissue processing and histopathologic evaluation of the eyes, and the authors took great care to locate the microsphere depot and characterize the inflammatory response in each eye on study. The lack of observed foreign body response in the publications described above might be related to the localized nature of the lesions and the relatively small size of the studies. Additionally, previous studies generally tested drug-loaded microspheres, and some of these drugs, such as steroids and cyclosporine, would be expected to inhibit inflammatory responses. Indeed, the presence of dexamethasone in the approved PLGA-based intraocular implants may contribute to their clinical safety profile.

Characterization of the impact of PLGA depot size on toxicity revealed that all injectable PLGA microspheres tested (20, 50, and 100 μm) induced a similar foreign body response in rabbits. Interestingly, the 20-μm microspheres, which were the smallest tested, showed greater toxicity than the 50- and 100-μm microspheres. This was due to microsphere penetration of the retina and with resulting associated retinal degeneration/disruption. These data suggested that smaller sized PLGA spheres are likely to be less tolerated. Xu et al. have reported a lack of toxicity in rats with dexamethasone-loaded PLGA nanospheres (average diameter of 253 nm), but this study was focused on the inhibition of choroidal neovascularization rather than safety.

While all of the microsphere test articles were 100% PLGA polymer, there were some minor differences in DL-lactide to glycolide ratio (which ranged from 85:15 to 75:25), polymer source, and manufacturing methods (Table 1). While these differences may have impacted the final test articles to some degree, the immunological response to all of the PLGA microspheres was consistent, and did not appear to be affected by these differences across studies.

Remarkably, while PLGA microspheres of all sizes tested produced a foreign body response in rabbits, PLGA rods (0.9 × 3.7 mm) were well tolerated. While multinucleated giant cells were present in 1/3 animals administered PLGA rods, no fibrosis or foreign body response was observed 8 weeks after implantation. This is consistent with the preclinical data supporting the use of Ozurdex, in which placebo (non-dexamethasone-loaded) PLGA rods did not produce a significant inflammatory response in rabbits and monkeys. These data suggest that the inflammatory response to PLGA microspheres is driven by their size, shape, and/or surface area rather than the chemical characteristics of the polymer.
While on a different size scale, this is in agreement with the general principles of nanotoxicology, in which the size and shape of nanoparticles are generally considered the main drivers of toxicity. It is possible that surface area plays a role in this inflammatory response, since microspheres have significantly greater surface area than rods. For a given polymer mass the total surface area of microspheres is 25 to 50 times more than the surface area of a single rod. It is worth noting that low levels of residual ethyl acetate were present in the microspheres but not in the rods, though there is no evidence that low levels of this solvent would impact the immune response to a foreign particulate. Further research is warranted to more clearly define the relationship between PLGA depot size, shape, and surface area and inflammatory responses.

The in vitro data support the in vivo findings and suggest that PLGA microspheres have an increased potential for inducing an acute immune response compared to PLGA rods at a similar mass concentration. The cytokine/chemokine profiles from both PBMCs and macrophages suggest a predominantly innate immune response with lack of lymphocyte activation (no IFNγ and IL-2) and induction of a proinflammatory (IL-8, MCP-1, MIP-1α, TNFα) rather than anti-inflammatory (IL-10) activation of macrophages. The differences in the potential to elicit an acute cytokine response by PLGA microspheres and PLGA rod may be influenced by the surface area of these materials. This in vitro cytokine response is in agreement with those reported for nonocular foreign body responses to biomaterials, suggesting that the PLGA microsphere–directed foreign body response observed in the eye is mechanistically similar to innate foreign body responses observed in extraocular tissues.

In conclusion, we have demonstrated that intravitreally administered PLGA microspheres with average diameters between 20 and 100 μm produce ocular inflammation, and a localized, progressive foreign body response in rabbits and NHPs. Over time, this foreign body response may lead to damage to critical ocular tissues, including the retina and the lens. Given that NHPs are more sensitive to this effect, and are considered to be the most relevant species for human risk assessment, there appears to be a high risk for similar effects in humans. By contrast, PLGA rods were well tolerated in rabbits, suggesting that the size, shape, and/or surface area of the PLGA depots are critical attributes in determining toxicity.

Acknowledgments

The authors thank Kelly Flagella, Joe Beyer, David Calise, and Margaret Collins for their technical assistance with study design and conduct, and Helen Booler and Gary Cain for her help with the histopathology images.

Disclosure: E. A. Thackaberry, Genentech (E); C. Farman, None; F. Zhong, Genentech (E); F. Lorget, Genentech (E); K. Staflin, Genentech (E); A. Cercillieux, Genentech (E); P. E. Miller, None; C. Schuetz, Genentech (E); D. Chang, Genentech (E); A. Famili, Genentech (E); A. L. Daugherty, Genentech (E); K. Rajagopal, Genentech (E); V. Bantsev, Genentech (E)

References

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