

Different Ipsi- and Contralateral Glial Responses to Anti-VEGF and Triamcinolone Intravitreal Injections in Rats

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PURPOSE. To investigate the glial response of the rat retina to single or repeated intravitreal injections (IVI).

METHODS. Albino Sprague-Dawley rats received one or three (one every 7 days) IVI of anti-rat VEGF (5 μ L; 0.015 μ g/ μ L), triamcinolone (2.5 or 5 μ L; 40 μ g/ μ L; Trigón Depot), bevacizumab (5 μ L; 25 μ g/ μ L; Avastin), or their vehicles (PBS and balanced salt solution) and were processed 7 days after the last injection. Retinas were dissected as whole mounts and incubated with antibodies against: Iba1 (Ionized Calcium-Binding Adapter Molecule 1) to label retinal microglia, GFAP (Glial Fibrillary Acidic Protein) to label macroglial cells, and vimentin to label Müller cells. The retinas were examined with fluorescence and confocal microscopy, and the numbers of microglial cells in the inner retinal layers were quantified using a semiautomatic method.

RESULTS. All the injected substances caused an important micro- and macroglial response locally at the injection site and all throughout the injected retina that was exacerbated by repeated injections. The microglial response was also observed but was milder in the contralateral noninjected eyes. The IVI of the humanized antibody bevacizumab caused a very strong microglial reaction in the ipsilateral retina. Two types of macroglial response were observed: astrocyte hypertrophy and Müller end-foot hypertrophy. While astrocyte hypertrophy was widespread throughout the injected retina, Müller end-foot hypertrophy was localized and more extensive with triamcinolone use or after repeated injections.

CONCLUSIONS. Intravitreal injections cause micro- and macroglial responses that vary depending on the injected agent but increase with repeated injections. This inflammatory glial response may influence the effects of the injected substances on the retina.

Keywords: intravitreal injections, retina, rat, microglial cells, astrocytes, Müller cells, anti-VEGF, triamcinolone, bevacizumab

Intravitreal injections (IVI) of pharmacologic substances are at present an important therapeutic strategy that is changing the prognosis of many retinal diseases, such as neovascular age-related macular degeneration (AMD), diabetic retinopathy, myopic choroidal neovascularization, posterior uveitis, endophthalmitis, macular edema, vitreoretinal adhesions, intraocular tumors, and many more.^{1–5} They allow ophthalmologists to treat posterior pole diseases directly and at the same time minimize or avoid completely the systemic side effects of the injected drugs (see below).

Intravitreal injections have been used since the beginning of the previous century to introduce space-forming substances in the vitreal space in order to reattach the retina.⁶ Later, IVI were used to treat pharmacologically other diseases, such as uveitis and endophthalmitis. Intravitreal injections maintain sustained therapeutic levels of the substance in contact with the retina, yet their systemic absorption² from the vitreous is limited because of the presence of the inner blood–retinal barrier.^{1,3,7}

The use of IVI was restricted for many years because of the various risks associated with the injections.^{8–12} Recently, however, the advantages of the injections have outweighed

the risks, and their use has become widespread, especially since the introduction of new pharmacologic agents such as anti-vascular endothelial growth factor (anti-VEGF) compounds and corticosteroids.

Vascular endothelial growth factor is a trophic factor that increases endothelial cell survival, promotes proliferation and migration of endothelial cells, and increases vascular permeability. Vascular endothelial growth factor has various isoforms and binds to two receptors, 1 and 2, and the isoform responsible for ocular neovascularization appears to be VEGF₁₆₅.¹³ Anti-VEGF therapy was designed to neutralize the soluble forms of VEGF, especially VEGF₁₆₅, and thus halt the neovascular complications of eye diseases. The first commercialized anti-VEGF drug for IVI was the aptamer pegaptanib (Macugen; Pfizer, New York, NY, USA), a drug that binds the isoform VEGF₁₆₅. Later, the humanized monoclonal antibodies bevacizumab (Avastin; Roche, Basel, Switzerland) and ranibizumab (Lucentis; Novartis, Basel, Switzerland) and the recombinant VEGF-trap aflibercept (Eylea; Bayer, Leverkusen, Germany), all pan-VEGF-binding drugs, were introduced. Soon it was recognized that these latter three drugs have superior effects, and they are the mainstay of the treatment right now.^{13–18}



Anti-VEGF therapy has revolutionized the treatment of many retinal diseases, such as AMD, diabetic retinopathy, and retinal vein obstruction, because it ameliorates the visual prognosis.^{15-17,19} Furthermore, recent investigations have documented that for a better response repeated injections are needed, at least initially, irrespective of the response to therapy.^{13,20-22} However, very little is known about the dosing and the possible local side effects in the retina of repeated IVI.²³ Indeed, bevacizumab and ranibizumab are humanized monoclonal antibodies and thus may trigger even in humans an inflammatory reaction, as has been shown for higher doses of these substances,²⁴ and the trap aflibercept may even be more inflammatory.²⁵ Geographic atrophy may also be the result of anti-VEGF therapy.^{18,26,27} Furthermore, therapeutic²⁸ and adverse effects²⁹⁻³¹ of the intravitreal administration of anti-VEGF or corticosteroids have been documented in the contralateral eye and have been attributed to systemic absorption of the drug.

Our group has wide experience in the study of the rodent retina. During the past years we have documented the number and spatial distribution of different retinal populations in the retina of the adult rodent, both in intact animals³²⁻⁴⁰ or in animals with inherited retinal degeneration⁴¹⁻⁴⁶ and also after different insults such as ocular hypertension,⁴⁷⁻⁵⁰ axotomy,^{37,51-54} or light-induced retinal degeneration.^{40,55,56} In some of these studies, we have shown that manipulations or injuries to one eye or optic nerve, followed or not by IVI of trophic agents, may have molecular and cellular effects on the contralateral retina. For example, topical instillation of saline to one eye resulted in marked upregulation of the mRNA expression levels of fibroblast growth factor 2 (FGF2) in the intact, contralateral fellow retina.⁵⁷ More recently, a detailed study of the macro- and microglia has shown that following elevation of the intraocular pressure in one eye, there are marked changes in the contralateral fellow intact eye,⁵⁸⁻⁶² highlighting the observation that activation of the retinal macro- and microglia in the uninjured eye may result as a consequence of injury to the contralateral fellow eye—thus confirming original observations that unilateral injury to one adult rat optic nerve may cause multiple cellular responses in the contralateral site.⁶³

In this work we studied the retinal glial response (microglia, astrocytes and Müller cells) of the rodent retina after single or repeated IVI of the substances most used at present in ophthalmology, anti-VEGF and corticosteroids, and their respective vehicles.

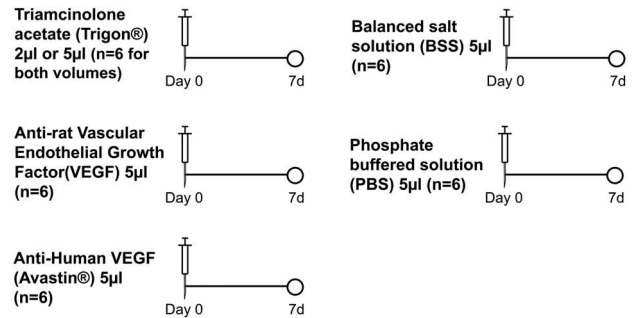
MATERIALS AND METHODS

Animal Handling

A total of 72 adult albino Sprague-Dawley (SD) rats were used. Animals were obtained from the breeding colony of the University of Murcia and were housed in temperature- and light-controlled rooms with a 12-hour light/dark cycle (light from 8 AM to 8 PM) and had food and water ad libitum. Light intensity within the cages ranged from 5 to 30 lux (scotopic to mesopic conditions). Animal manipulations were carried out following the Spanish and European Union regulations for the use of animals in research (Council Directive 86/609/EEC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the ethics committee of the University of Murcia. Adequate measures were taken at all times to minimize pain or discomfort.

General anesthesia for surgical manipulations was induced with an intraperitoneal injection of a mixture of ketamine (70 mg/kg, Ketolar; Parke-Davies, S.L., Barcelona, Spain) and

1) EFFECT OF A SINGLE INTRAVITREAL INJECTION



2) EFFECT OF REPEATED INTRAVITREAL INJECTIONS

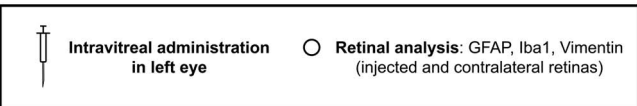
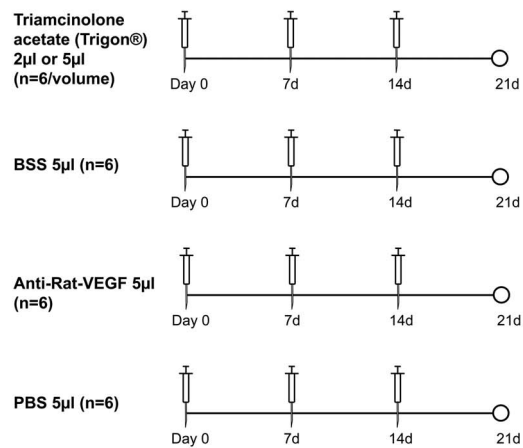


FIGURE 1. Diagram depicting the methods used, the groups, number of IVI, substances injected, and days of processing.

xylazine (10 mg/kg, Rompún; Bayer, S.A., Barcelona, Spain). After surgery, rats were placed in their cages, and an ointment containing tobramycin (Tobrex pomada; Alcon S.A., Barcelona, Spain) was applied on the cornea to prevent corneal desiccation.

Animal Groups

Animals were divided into several experimental groups, each consisting of six animals (Fig. 1). All animals that suffered retinal injury during the IVI or developed cataract were excluded because of the known effects of the crystalline molecule on the retina.⁶⁴⁻⁶⁶

Intravitreal Injections

Intravitreal injections were performed only in the left eye following previously described methods.^{51,67} The injections were made through the superotemporal sclera, where a perforating sclerotomy through the conjunctiva and sclera was made with a sterile 27-gauge (G) needle at approximately 1 to 2 mm from the limbus. Then, the needle of the Hamilton microsyringe (26G, Hamilton 701 N; Esslab, Benfleet, UK) was

introduced first obliquely toward the optic nerve and later perpendicularly toward the center of the globe. Once the needle was visualized retrolenticularly, the injection was made and then the needle was retracted slowly. Two groups of animals were prepared that received a single IVI or three consecutive IVI (at days 0, 7, and 14) of the same substance (Fig. 1). If injury to the retina or the lens was observed at the time of injection or after the injection, the animal was excluded from the study.

The following substances were injected: (1) anti-rat VEGF diluted in PBS (15 $\mu\text{g}/\text{mL}$ = 0.015 $\mu\text{g}/\mu\text{L}$; polyclonal goat anti-rat VEGF; MGC70609; Leinco Technologies, Inc., St. Louis, MO, USA) and vehicle; (2) triamcinolone acetonide (40 $\mu\text{g}/\mu\text{L}$; Trigón Depot; Bristol-Myers Squibb España, Madrid, Spain) and vehicle; (3) bevacizumab (humanized anti-VEGF; 25 $\mu\text{g}/\mu\text{L}$; Avastin; Roche Farma S.A., Madrid, Spain); (4) phosphate-buffered saline (PBS; Sigma Aldrich, Madrid, Spain); or (5) balanced salt solution (BSS; Alcon S.A.). Before administration of triamcinolone, the samples were centrifuged as previously reported⁶⁸ to eliminate the toxic benzyl alcohol present in the formulation and substitute it for BSS. The injection volume was always 5 μL except for triamcinolone, which was administered in a bolus of either 2.5 or 5 μL (Fig. 1). Bevacizumab was administered only as a single IVI (see Results).

Tissue Processing

All animals were processed 7 days after the single or the last injection (Fig. 1). Rats received a lethal dose of sodium pentobarbital (Doletal; Vetoquinol, S.A., Lure, France) and were perfused transcardially through the ascending aorta first with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The eyes were then enucleated and the retinas dissected as whole mounts by making four radial cuts in the superior, inferior, nasal, and temporal retinal quadrants. Retinal orientation was maintained by making the deepest radial cut in the superior retina.^{52,53} The retinas were postfixed flat on a filter paper in 4% paraformaldehyde for 1 hour, washed in PBS, and processed for immunohistochemistry.

Immunohistochemistry

The retinas were first permeated and then incubated overnight at 4°C with a mixture of primary antibodies diluted in blocking buffer (PBS containing 2% Triton X-100 and 5% normal donkey serum [Jackson ImmunoResearch, Inc., Cambridge, UK]). The next morning, the retinas were incubated in a mixture containing the secondary antibodies diluted in PBS-2% Triton X-100 for 1 hour at room temperature. This protocol has been previously described in detail.^{54,43,46} Finally, the retinas were washed in PBS and mounted on subbed slides vitreal side up and covered with antifade mounting medium (M1289; Sigma-Aldrich Química S.A., Madrid, Spain).

Antibodies

Primary Antibodies. Microglial cells were detected by using rabbit anti-Iba1 (Ionized Calcium-Binding Adapter Molecule 1) (1:1000, 019-19741; Wako Chemicals, Neuss, Germany). Both astrocytes and Müller cells were detected by using goat anti-GFAP (Glial Fibrillary Acidic Protein) (1:250, C-19, sc-6170; Santa Cruz Biotechnology, Heidelberg, Germany). To detect Müller cells, goat anti-vimentin (1:100, C-20, sc-7557; Santa Cruz Biotechnology) was used.

Secondary Antibodies. Donkey anti-goat Alexa 594, donkey anti-goat Alexa 488, and donkey anti-rabbit Alexa 488 were all from Molecular Probes (Invitrogen Thermofisher, Madrid, Spain) and diluted at 1:500.

Image Acquisition and Analysis

Retinal whole mounts were examined and photographed under a fluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany) following procedures that have been previously standardized in our laboratory.^{52,34,46,69,70} Our microscope, Axioscop 2 Plus, is equipped with a computer-driven motorized stage (ProScan H128 Series; Prior Scientific Instruments, Cambridge, UK) controlled by the Image Pro Plus software (IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA). To make reconstructions of retinal whole mounts, retinal multiframe acquisitions were taken with a $\times 10$ objective in a raster scan pattern. The 154 frames/retina were combined automatically into a single tiled high-resolution photomontage using IPP for Windows.^{50,71} Magnifications of some retinas were also taken with a confocal microscope (Spectral confocal & multiphoton system Leica TCS SP2; Leica, Jena, Germany). The same settings were always used to acquire GFAP, Iba-1, and vimentin signals.

Quantification of Microglial Cells

To quantify microglial cells in the inner retinal layers (nerve fiber, ganglion cell, and inner plexiform layers) we modified (to adapt it to the rat retina) a semiautomated computerized routine (Image Pro Plus software) previously developed by our group to automatically quantify different retinal cell populations in mouse retinas.⁵⁴ Briefly, first the retinal area was drawn and measured. Then, the optic nerve and the limits of each retinal quadrant were manually pointed, usually at the center of the quadrant. Next, the automatic subroutine selected three standard equidistant areas in line between the optic disc and the marked quadrant limit. Thus, the subroutine selected 12 standard circular samples (0.25 mm^2 each) from each photomontage, and these areas were thus situated 4 in each retinal quadrant and 4 in each retinal region (central, equatorial, and peripheral, Fig. 2). In the superotemporal quadrant, we avoided photographing the region of retinal injections (see Results) by marking the quadrant periphery far from these. Microglial cells were manually dotted on each of the circular samples described above (Fig. 2). Next, the number of cells dotted was automatically quantified and the densities (\pm standard deviation of the mean, SD) of microglial (dotted) cells (cells/mm^2) were calculated in each sample. Finally, because there were no differences in the densities of microglial cells between the central, equatorial, and peripheral retina (see Results), we calculated the total numbers and densities of microglial cells for each retina using the total retinal area.

Statistics

Statistical analysis was carried out using SigmaStat 3.11 for Windows (SigmaStat for Windows Version 3.11; Systat Software, Inc., Richmond, CA, USA). The *t*-test was used to compare two groups, and 1-way ANOVA and Tukey's test were used to compare more than two groups.

RESULTS

Retina Surrounding the Injection Site

At the time of retinal dissection, in some animals it was difficult to dissect the superotemporal quadrant where the injections were made because of the presence of small areas of chorioretinal adhesion. In some experimental retinas, independently of the injected substance or the number of injections carried out, sometimes one or more injection sites could be recognized because at this point the retina was atrophic and sometimes even perforated. Around the injection site(s) the retinas were strongly immunoreactive, with the

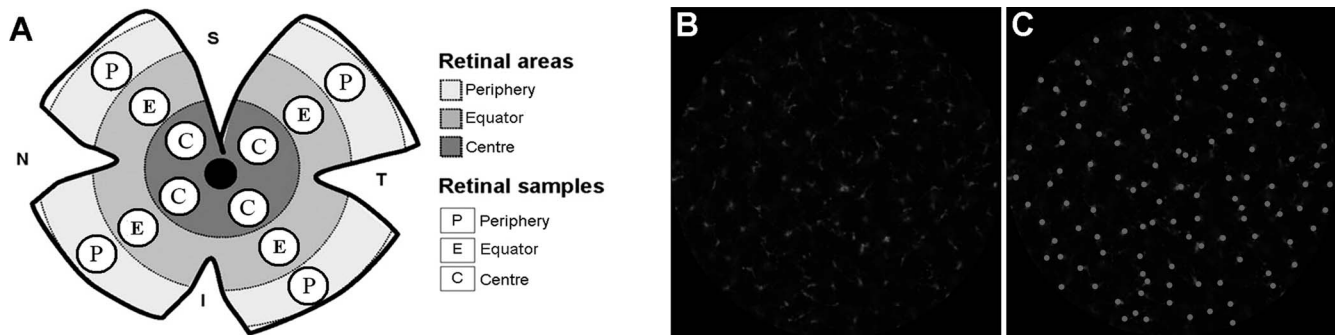


FIGURE 2. Method used to calculate the densities of Iba1-labeled microglial cells present in the inner retinal layers. The 12 areas photographed and counted in each retina are illustrated in the drawing (A) and were situated 3 in each quadrant and 4 in each retinal area (center, equator, and periphery). One of these samples before (B) and after (C) the manual dotting of microglial cells.

presence of a higher density of Iba-1⁺ microglial cells and an intense GFAP immunoreactivity (Fig. 3). In some retinas, we could not observe clearly the injection site(s), while in others we did observe one (in animals that received one IVI) to three (in animals that received three IVI) injection sites.

Microglial Cells

All the left retinas that had received one IVI showed a uniform activation of microglial cells throughout the entire retina, independently of the injected substance. This activation was recognized because the microglial cells had changed their surveying ramified shape to a more amoeboid one with shortened processes^{51,72} (Figs. 4, 5).

The animals that received a single injection of anti-human VEGF (Avastin) showed a very marked activation of the retinal microglial cells in the left retina (Fig. 5). In these retinas, the numbers of microglial cells were largely increased, but it was not possible to quantify them because their processes overlapped and single cells could not be individualized.

In the other groups that received a single IVI, we were able to quantify the numbers of microglial cells in the 12 sampled

areas of each left and right retina. In the fellow right retinas, microglial cell densities had increased but were not significantly different from those found in control naïve animals (Fig. 6). In the injected left retinas, we observed increased numbers of microglial cells when compared to the contralateral retinas (Figs. 4, 6; Table), but these were not significantly different. However, when we compared the number of microglial cells in the left injected eyes with those observed in the eyes from naïve (intact) animals, we found that the cell densities were significantly increased in the injected left eyes, independently of the injected substance (Fig. 6). The fact that there were significant differences between the injected left eyes and the control eyes but not between the left injected eyes and the contralateral right eyes could be explained on the basis of a small response triggered by the injection in the contralateral eye (Table; Fig. 6). Finally, the increased densities of microglial cells were widespread in the retina, since there were no differences between the densities found in the central, equatorial, and peripheral retina (Figs. 2, 6; Table).

Three IVI induced a stronger microglial reaction in both the left and the right contralateral retina (Figs. 4, 6; Table) than a single injection, independently of the substance injected. The

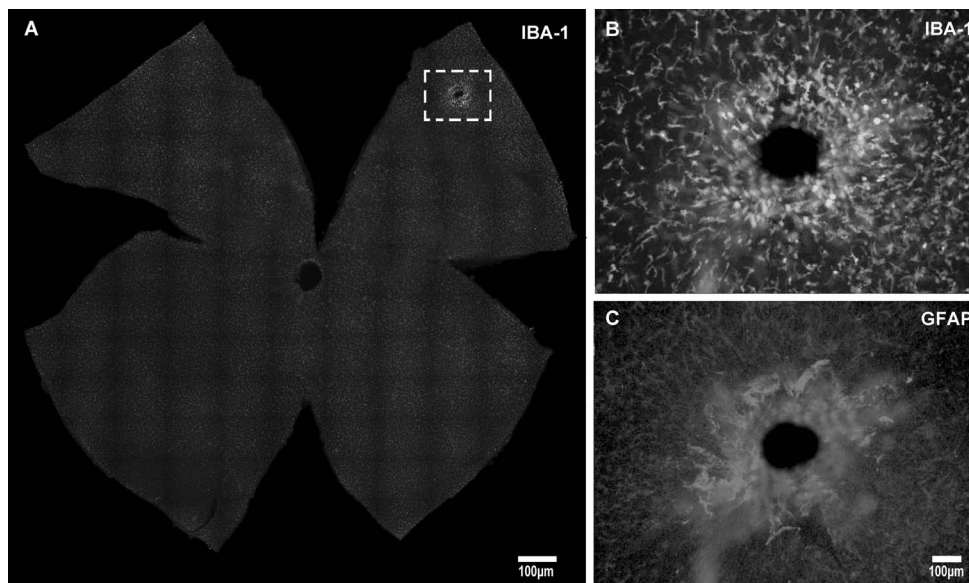


FIGURE 3. (A) Whole-mounted left retina (injected once with 5 μ L triamcinolone) where the microglial cells appear labeled with the anti-Iba1 antibody. In the superotemporal quadrant a hole that corresponds to the sites of the injection is *highlighted* and is shown at higher magnification in (B) and (C) (using two different fluorescence filters for the anti-Iba1 and anti-GFAP antibodies), where it can be observed that the hole is surrounded by an intense cellular micro- and macroglial reaction.

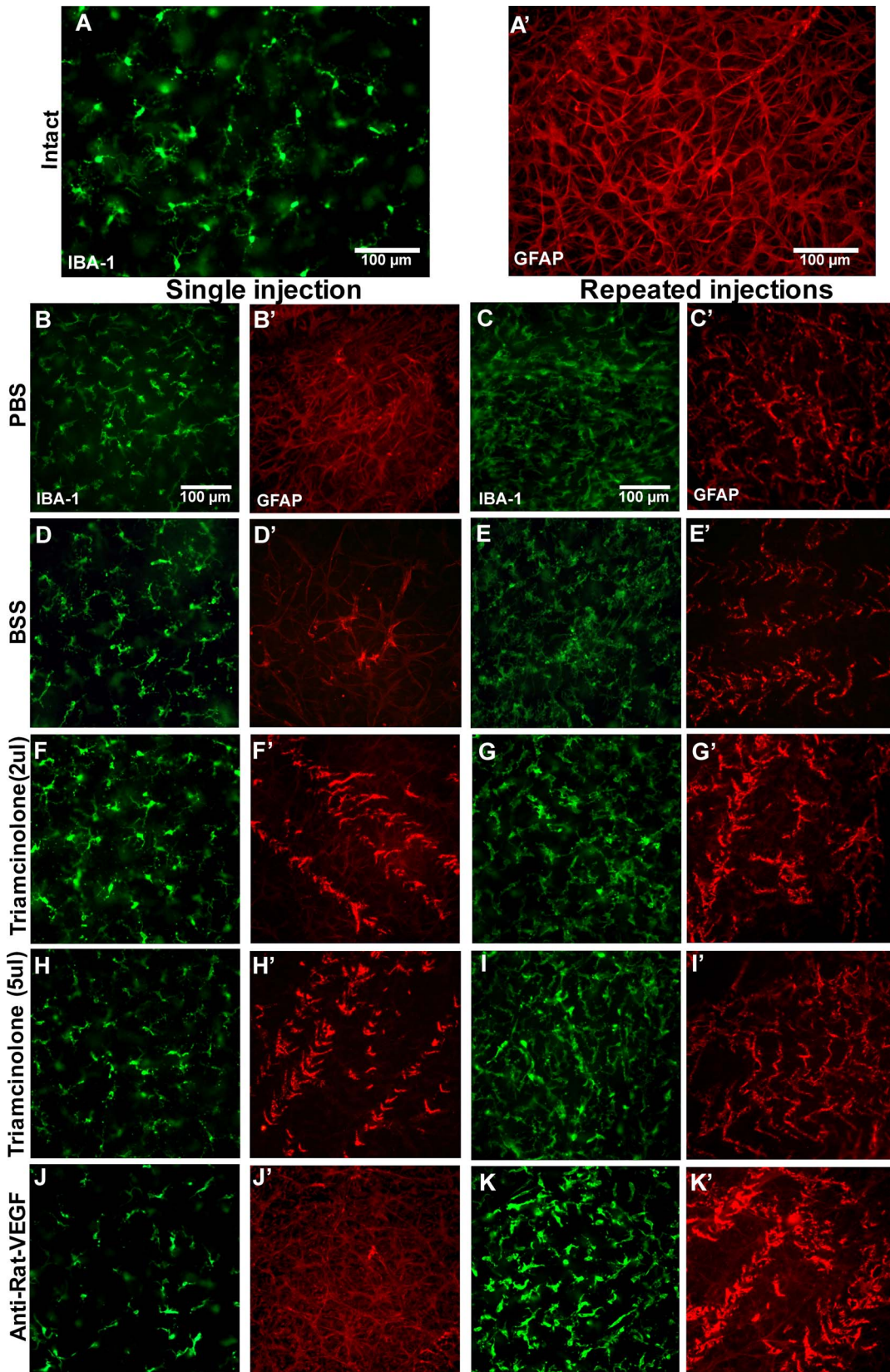


FIGURE 4. Photomicrographs of the same selected representative retinal regions of different retinas immunoreacted with the anti-Iba1 (green) and anti-GFAP (red) antibodies. Control retinas (upper row) did not receive any treatment, and experimental retinas (lower rows) had received one (left two columns) or three (right two columns) injections of different substances depicted on the left-hand side. GFAP immunoreactivity demonstrates astrocyte hypertrophy in all the injected retinas and arrow-shaped areas of strong GFAP immunoreactivity in the animals injected once with triamcinolone (F–H') or three times (C', E', G', I', K') independently of the treatment. Iba1 immunoreactivity documents microglial cell activation that was more marked in the animals that received repeated injections (C, E, G, I, K).

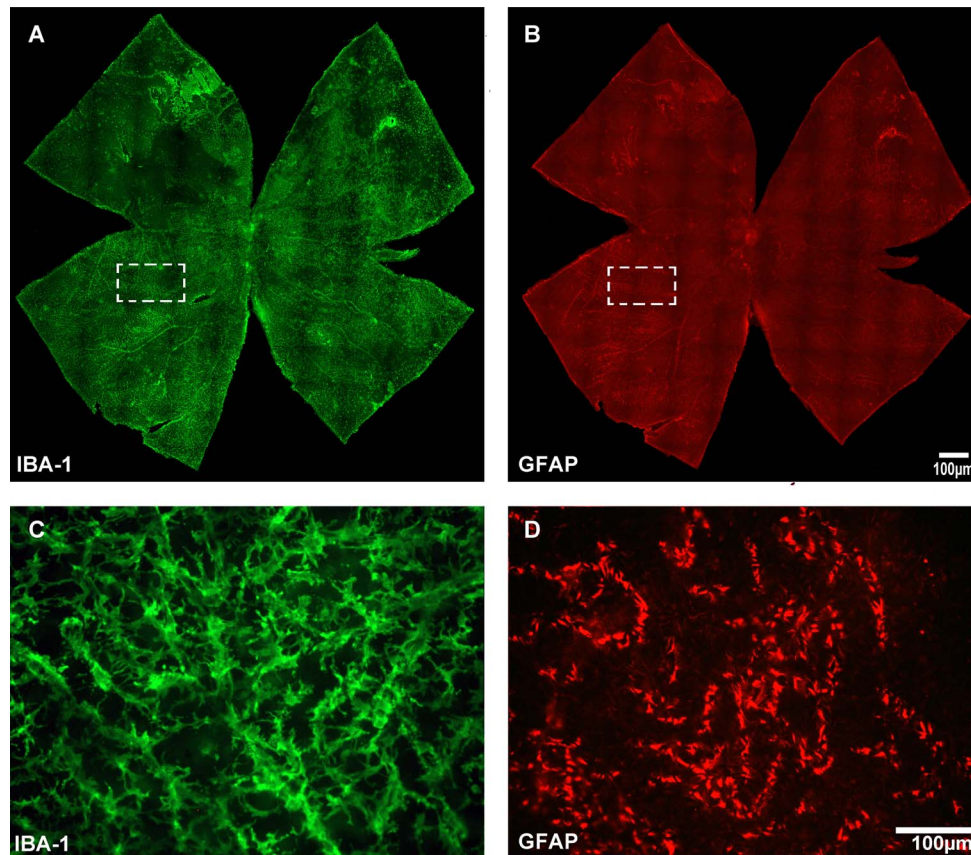


FIGURE 5. Left retina of an animal that had received a single IVI of bevacizumab 7 days before it was immunoreacted with antibodies against Iba1 (A) and GFAP (B). A retinal hole can be observed in the superotemporal retina. Magnifications from the rectangles in (A) and (B) show that intravitreal injection of bevacizumab caused a strong microglial response (C) and arrow-shaped areas of strong GFAP immunoreactivity (D).

numbers of microglial cells in the left and right retinas were increased, but there were no significant differences between the densities found in the contralateral right retinas and the intact retinas (Fig. 6; Table). However, when we compared the microglial cell densities in the left injected eyes with the densities in the right contralateral eyes or in the intact eyes, a significant difference was found (Fig. 6) for all treatments except for 5 μ L triamcinolone. Again, the microglial response was observed in all retinal regions, as no difference was found between retinal areas within a given treatment (Fig. 6; Table).

Macroglial (Astrocytes and Müller) Cells

Changes in astrocytes and the end feet of Müller cells in the ganglion cell layer were studied qualitatively. Neither single nor repeated injections triggered a qualitative response in GFAP or vimentin immunoreactivity in the contralateral right retinas, but in the left injected eyes, two different macroglial responses were identified with anti-GFAP antibodies: cell hypertrophy or increased immunoreactivity.

A single injection of PBS, BSS, or anti-rat VEGF induced a hypertrophy of astrocytes (Figs. 4, 5), but single injections of triamcinolone caused also an increase of GFAP immunoreactivity in localized retinal areas (Figs. 4F', H'). Thus, the macroglial cellular reaction was different depending on the substance injected.

There was some degree of astrocyte hypertrophy in all the injected left retinas. The astrocytes and their cell processes appeared thicker, but this aspect was not quantified. This hypertrophy (Figs. 4, 5) is not evident because the settings used for the microphotographs were always the same and the

normal GFAP immunoreactivity was low (Fig. 4A') and was thus obscured by the increased GFAP immunoreactivity found in the eyes that received a single triamcinolone IVI (Figs. 4F', H') or in the eyes with three IVI (Figs. 4C', E', G', I', K').

The areas of increased GFAP immunoreactivity in the retinas that received a single injection of triamcinolone (Figs. 4F', H') were distributed randomly in all retinal quadrants and did not seem to be related to other retinal structures such as blood vessels. Although they were sometimes linear, many showed curved arrow-shaped trajectories (Figs. 4, 5, 7). These areas showing intense GFAP immunoreactivity were also positive for vimentin, suggesting that they were formed by the end feet of Müller cells (Fig. 7).

Repeated injections produced a stronger macroglial reaction, as observed for microglial cells; all the injected left retinas showed arrow-shaped areas of increased GFAP immunoreactivity, and these areas were more extensive (Figs. 4, 5, 7). We interpreted these arrow-shaped areas of increased GFAP immunoreactivity as areas with more severe structural retinal alterations at the level of the inner limiting membrane and end feet of Müller cells.

DISCUSSION

In this work we have analyzed the effect of single or multiple IVI on the glial cells of the rat retina when administering some of the most commonly used substances in the clinical practice. Glial cells are the first that react to an insult or stress to the retina, and their activation is a good indicator of subtle changes induced by a drug or treatment in the retina.^{51,73-76}

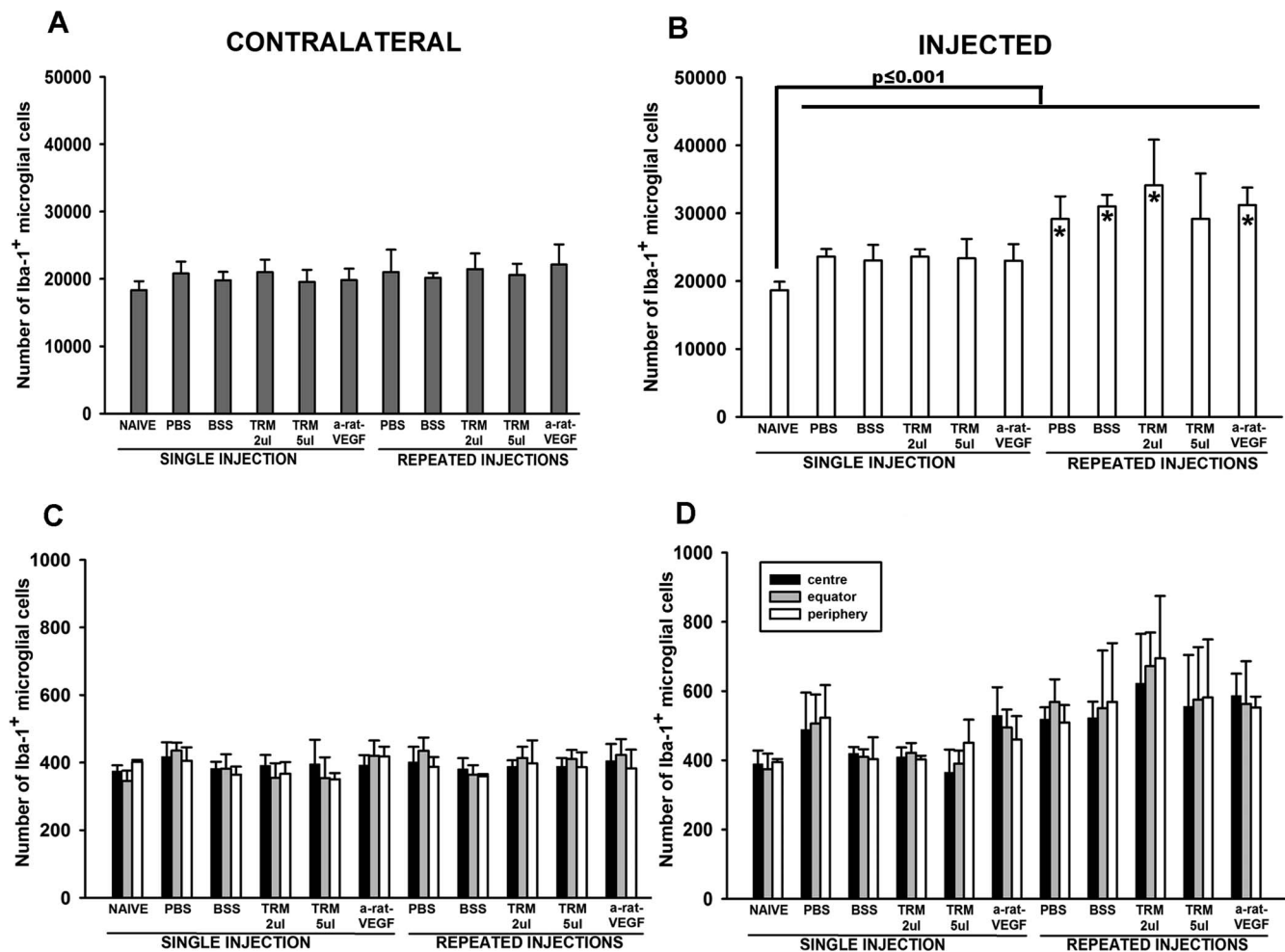


FIGURE 6. Bar diagram showing the calculated mean numbers of Iba1⁺ microglial cells (±SD) per retina in control (naïve) animals and in the right contralateral (left column) and left injected (right column) retinas of experimental animals. In (C, D), the densities were calculated for the center, equator, and periphery, and there were no significant differences between these areas. (B) Statistically significant differences with contralateral fellow eyes, *P ≤ 0.05.

Atrophic Areas or Holes at Injection Site

In some retinas we found atrophic areas or retinal holes in the region of the injection. The number of holes was variable and not always equivalent to the number of injections. In fact, we never observed three atrophic areas, but one or two in the animals that received three injections, indicating that the injection could have been done in the same place or that some injections did not cause a permanent retinal perforation. Because these atrophic areas were not observed in control retinas, were situated in the superotemporal retinal quadrant (the place of the injection), and often had a circular shape, we interpret these areas as the points of eye perforation made at the time of IVI. To our knowledge, this is the first time that atrophic retinal areas and holes have been reported after IVI. The holes could be due at least in part to traction at the moment of the dissection, because we noticed that in these areas it was difficult to dissect the retina free from the choroid, indicating that a chorioretinal scar had formed. However, the increased number and reactivity of micro- and macroglial cells around these areas suggest that there was some inflammation, which we documented at least during the period of the study. Thus, retinal atrophic areas may occur at the place of the injection, and their appearance and size are variable and may

depend on the size of the needle or the retinal structures lesioned by the injection.

We had not observed retinal holes in previous experiments in which we had carried out IVI of different substances,^{40,51,67,77} but this may be related to the different size of the needle used for the present experiments. Thus, for our previous experiments, the perforation was made with a 30G needle and the IVI was made through a 34G needle, while in the present experiments, 27G and 26G needles were used for the same purposes. We increased the size of the needle because these experiments are preliminary experiments for another study in which we inject a cellular suspension.

We also observed in our study some degree of micro- and macroglial reaction not only in the injected retina but also in the contralateral retina; thus it is tempting to suggest that the IVI may itself trigger, at least in part, the micro- and macroglial reaction observed in the ipsi- and contralateral retina and may thus explain some ipsi- and contralateral effects observed after IVI in clinical practice.^{18,26-31} Nevertheless, the technique that we use for IVI in rats is not comparable to that used in clinical practice. In rats, in order to avoid injury to the crystalline lens, we inject at 1 to 2 mm from the limbus and, because in rats the distance between the limbus and the retina is shorter than 1 mm,⁷⁸ when we enter the eye we perforate the retinal periphery. In humans, IVI enter the eye at 3 to 4 mm from

TABLE. Quantification of Microglial Cells After Intravitreal Injections

	Mean Density \pm SD			Inference Total
	Center	Equator	Periphery	
Naïve	1550 \pm 115	1462 \pm 152	1619 \pm 29	18,343 \pm 1305
Single injection				
PBS				
Contralateral	1696 \pm 172	1769 \pm 95	1646 \pm 160	20,805 \pm 1753
Injected	1984 \pm 435	2054 \pm 342	2122 \pm 384	23,597 \pm 1132*†
BSS				
Contralateral	1553 \pm 81	1551 \pm 173	1478 \pm 97	19,813 \pm 1229
Injected	1703 \pm 79	1665 \pm 90	1637 \pm 258	23,042 \pm 2316*‡
Triamcinolone 2 μ L				
Contralateral	1590 \pm 125	1439 \pm 177	1490 \pm 140	20,991 \pm 1861
Injected	1660 \pm 114	1712 \pm 115	1634 \pm 44	23,592 \pm 1092*†
Triamcinolone 5 μ L				
Contralateral	1606 \pm 293	1439 \pm 248	1424 \pm 75	19,555 \pm 1793
Injected	1481 \pm 269	1585 \pm 152	1828 \pm 273	23,347 \pm 2863*
Anti-rat VEGF				
Contralateral	1594 \pm 120	1705 \pm 183	1698 \pm 117	19,826 \pm 1699
Injected	2150 \pm 332	2008 \pm 210	1869 \pm 274	22,993 \pm 2456*†
Repeated injections				
PBS				
Contralateral	1630 \pm 183	1766 \pm 158	1571 \pm 118	21,001 \pm 3335
Injected	2105 \pm 140	2309 \pm 266	2069 \pm 205	29,147 \pm 3337*
BSS				
Contralateral	1546 \pm 130	1477 \pm 115	1461 \pm 25	20,164 \pm 724
Injected	2121 \pm 192	2235 \pm 676	2308 \pm 689	31,008 \pm 1696*
Triamcinolone 2 μ L				
Contralateral	1577 \pm 75	1679 \pm 135	1614 \pm 278	21,460 \pm 2332
Injected	2525 \pm 582	2729 \pm 394	2821 \pm 731	34,104 \pm 6734*§
Triamcinolone 5 μ L				
Contralateral	1576 \pm 103	1666 \pm 110	1568 \pm 179	20,593 \pm 1650
Injected	2256 \pm 603	2335 \pm 618	2360 \pm 682	29,168 \pm 6681*
Anti-rat VEGF				
Contralateral	1644 \pm 204	1714 \pm 192	1556 \pm 224	22,115 \pm 3018
Injected	2380 \pm 260	2287 \pm 499	2243 \pm 128	31,185 \pm 2588*§

Mean density \pm standard deviation of microglial cells (cells/mm²) per retinal region. The rightmost column shows the inferred number of microglial cells/retina.

* Significant difference versus intact animals ($P \leq 0.001$).

† Significant difference between 1 and 3 injections ($P \leq 0.001$).

‡ Significant difference between 1 and 3 injections ($P \leq 0.0001$).

§ Significant difference between injected and contralateral eye ($P \leq 0.001$).

|| Significant difference between injected and contralateral eye ($P \leq 0.0001$).

the limbus¹ through the pars plana and not through the retina, and in humans the recommendation is for needle sizes smaller than 25G.⁵

Microglial Response

When analyzing the retinas after a single IVI we observed different degrees of microglial activation in both retinas and significantly increased densities of microglial cells in the left injected eyes. Other studies have shown similar results after IVI of different substances.^{14,79} These changes were in our study independent of the injected substance, indicating that these represent a general response to the IVI and/or the retinal or eye injury. This is further supported by the fact that for all the

tested substances these changes were stronger after multiple injections than after a single injection.

In this study, a very strong ipsilateral microglial response was triggered by the IVI of bevacizumab. To our knowledge, this is the first time that this reaction has been reported in rats. A previous study comparing the effects of IVI of pegaptanib, ranibizumab, and bevacizumab in rats found toxic effects of the first of these, but not of the others, that was attributed to the carrier solution of pegaptanib.⁸⁰ Bevacizumab is a humanized monoclonal antibody that contains 93% human amino acid sequence and whose safety has been tested extensively both in vivo and in vitro in different animal models.^{18,81} One study documented that higher doses of bevacizumab may cause retinal inflammatory reactions in rabbit eyes,²⁴ and another study has documented that IVI of bevacizumab in monkeys

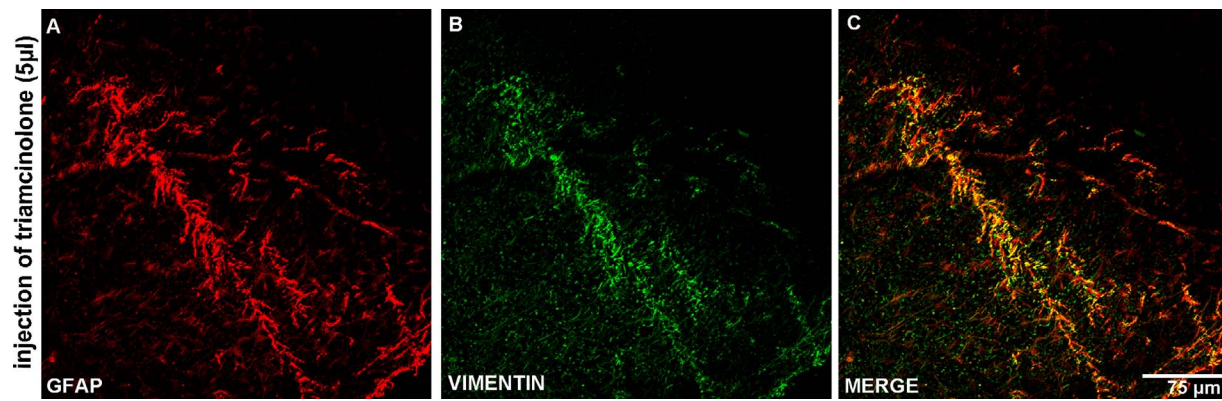


FIGURE 7. Confocal photomicrographs of the same retinal area of one animal that had received an IVI of 5 μ L triamcinolone with different fluorescence filters showing the GFAP immunoreactivity (A), vimentin immunoreactivity (B), or both (C), demonstrating that GFAP and vimentin immunoreactivity were located in the same places and indicating that these immunoreactive areas corresponded to the end feet of Müller cells.

causes microglial activation and even vascular thrombosis.⁸² A recent study has documented that rat retinal microglial cells and macrophages phagocytose bevacizumab and two other anti-rat VEGF antibodies of different origins when administered intravenously or intravitreally and that the immune response is stronger for bevacizumab and when administered intravitreally.⁸³ It is interesting to note that in accordance with the above-mentioned results, we also observed a stronger microglial reaction to bevacizumab than to the other anti-rat VEGF that we used, although this latter was a polyclonal goat anti-rat antibody that could have induced an immune reaction. Therefore, although intravenous or intravitreal injections of bevacizumab may not have apparent toxic local or systemic effects in rats, they trigger a strong retinal microglial response that may have beneficial or adverse consequences in the retina.

We did not observe any diminution in microglial activation when the injected substance was triamcinolone, while other researchers have.^{84,85} Possible explanations for this discrepancy may include the experimental paradigm used and the concentration and dose of the substance used for injection. In fact, the densities of microglial cells that we found in the left injected retinas were smaller in the retinas that had received three IVI of 5 μ L triamcinolone than in the retinas that had received three IVI of 2.5 μ L triamcinolone, suggesting a small anti-inflammatory effect of increased doses of this substance.

Macroglial Response

The macroglial response differed among substances if these were injected once. Phosphate-buffered saline, BSS, or anti-rat VEGF caused astrocyte hypertrophy, while triamcinolone administration triggered an overexpression of GFAP on the end feet of Müller cells (arrow-shaped areas; see next paragraph). Astrocyte hypertrophy and Müller cell reaction have been previously reported in the retina of different animal species after IVI.^{79,82,86,87} Overall, and in accordance with previous studies, our results suggest that macroglial cells are more sensitive to IVI than microglial cells and that the reaction is different depending on the injected substance.

In our study, one injection of triamcinolone or repeated IVI of the various substances produced, independently of the substance injected, a characteristic macroglial response: the appearance of intensely immunoreactive arrow-shaped areas. Because these areas were positive for GFAP and vimentin, we interpreted them as areas of Müller end-foot cell hypertrophy. Although we removed most of the preservative present in the commercial preparation of triamcinolone as previously described,⁸⁸ the substance or the remaining preservative may

have cellular toxic effects,^{88,89} and the retina may react to stress with the initiation of glial blooms or preretinal membranes as has been observed in degenerating retinas.^{87,90,91} On the other hand, treatment with the anti-VEGF aflibercept and ranibizumab has been shown to activate neonatal rat Müller cells in culture, and it has been proposed that it may be responsible for the therapeutic effects of these drugs.⁹²

Effects of IVI on the Contralateral Retina

One of the most striking findings of this study is the documentation that IVI resulted in increased numbers of microglial cells and microglial cell activation in the contralateral eye. Recent studies have shown that certain stimuli applied to one eye may trigger molecular and cellular responses in the contralateral untouched retina. This was the case after optic nerve axotomy,^{37,63} topical instillation of saline drops,⁵⁷ ocular hypertension induced by laser photocoagulation to the limbal tissues,^{58,60-62} or after anti-VEGF injection.²⁹⁻³¹ At present we do not know the mechanism responsible for the response in the contralateral fellow eyes, but one possibility is the limited systemic absorption of the injected substances, and, alternatively, that retinal or eye injury results in systemic inflammatory or paracrine-mediated effects that reach the fellow eye.⁵⁹ In this latter context, the microglial response may be subtle and not reach statistical significance, as in this study, or may be more intense when the insult is clearly damaging, such as after axotomy³⁷ or elevation of the intraocular pressure.⁵⁹⁻⁶²

In summary, this work shows that there are important reactions of the glial cells in the retina to single or repeated IVI of different substances. All the injected substances cause an important micro- and macroglial response locally at the injection site and widely spread throughout the injected retina that is exacerbated by repeated injections. The microglial response in the innermost retinal layers, although stronger in the injected eyes, is also observed in the contralateral noninjected eyes. Injection of the humanized antibody bevacizumab causes a very strong microglial reaction in the injected retina. Two types of macroglial response are observed: astrocyte hypertrophy and Müller end-foot hypertrophy. While astrocyte hypertrophy was widespread throughout the injected retina, Müller end-foot hypertrophy was observed in concrete retinal areas, and these were more extensive when repeated injections were made.

Glial cells and especially microglial cells are the first cells of the central nervous system to react to local and systemic

insults such as intraocular pressure increase, systemic infections, and blood glucose levels,^{57-60,62-65,72-76,93} thus supporting the notion that microglial cells are the sentries of the system and that they react promptly, maybe in order to prepare the system for a further challenge or damage. In this work we observed that unilateral IVI triggers a general glial cell reaction in both eyes that does not fully depend on the substance injected. We do not know if this reaction is secondary to retinal or eye injury and if it is pernicious or beneficial for the retina. Indeed, IVI render successful clinical effects,^{13,15-23} and it is possible that the glial reaction that we observe could in fact be contributing to the therapeutic effects of the IVI, independently of the drugs injected.^{92,94} Thus, our data do not allow us to advise against this route of administration. Further work would be needed to clarify the exact role of IVI in the retina and to assess the possible effects of this subclinical inflammation in the retina.

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