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Activation of Immature Microglia in Response to Stab Wound in Embryonic Quail Retina

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ABSTRACT

Activation of mature (ramified) microglia in response to injury in the adult central nervous system (CNS) is well documented. However, the response of immature (ameboid) microglia to injury in the developing CNS has received little attention. In this study, a stab wound was made in embryonic quail retinas at incubation days 7 and 9, and the response of retinal microglial cells was analyzed at different times between days 1 and 37 postinjury. The appearance of microglial cells within the wound occurred at the same time as the arrival of the first migrating ameboid microglial cells at an equivalent area in control retinas. Therefore, no specific attraction of microglia toward the wound was observed. Microglial cells in the wound had phenotypic features similar to those of activated microglia in the adult CNS. Thus, their shape was more compact compared with microglial cells outside the wound, expression of the molecule recognized by the QH1 antibody was up-regulated, and their lysosomal compartment was markedly increased. Transitional forms between normal ameboid and activated-like microglial cells were seen at the wound edge, supporting the view that ameboid microglia become activated when they contact the wound during the normal course of their migration in the retina. The microglial reaction was maintained within the wound at 37 days postinjury. In addition to the stab wound, secondary damage areas were found in experimental retinas. Activated cells could still be observed in these areas at 37 days postinjury. J. Comp. Neurol. 492:20–33, 2005.

Indexing terms: activated microglia; ameboid microglia; retinal injury; cell migration; development

Several types of microglial cells are observed during the life span of the central nervous system (CNS; Streit, 1995). Ameboid microglia predominate in the developing CNS and consist of immature microglial cells of round or irregular shape, with relatively thick lamellipodia-bearing processes (Navascués et al., 1995; Marín-Teva et al., 1998). These microglia actively migrate through the developing nervous parenchyma, proliferating and phagocytozing remnants of dead cells (Navascués et al., 2002). In normal adult brain, however, the most abundant form of microglia is fully ramified cells, also designated *quiescent microglia*. Although the relationship between these two functional states has been debated, it is now largely accepted that ameboid microglia are the precursors of ramified microglia (Ling, 1981; Ling and Wong, 1993; Cuadros and Navascués, 1998).

Activated microglia, a third type of microglial cell, appear in response to injury in the mature CNS (Amat et al., 1996; Streit et al., 1999; Raivich et al., 1999; Dihné et al., 2001). Activated microglial cells originate from ramified cells that retract their processes and become round, extending lamellipodia and migrating to injury sites (Stence

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et al., 2001); they change their ability to secrete cytotoxic substances and proinflammatory cytokines, increase their proliferative activity (Raivich et al., 1999; Streit et al., 1999; Cross and Woodroofe, 2001; Nakamura, 2002), and phagocytose cellular debris (Becher et al., 2000). Therefore, the activation of microglia modifies their morphological and functional features, and some of the new characteristics are similar to those shown by ameboid microglia in the developing brain. However, the concept of microglial activation was developed to explain the response of ramified microglia in the mature CNS. Therefore, a number of questions arise. Is the response to injury of immature or ameboid microglia similar to that of mature microglia? Do the phenotypic characteristics of ameboid microglia, which appear similar to those of activated microglia, enable them to respond directly to injury in the embryonic CNS, or, on the contrary, must ameboid microglia also be activated to respond to injury in the immature CNS?

Numerous studies have demonstrated that the macrophage/microglia response to injury differs between the developing and the adult CNS (Milligan et al., 1991; Morioka and Streit, 1991; Lawson and Perry, 1995; Acarin et al., 1996; Wu et al., 1997; Graeber et al., 1998; Cuadros et al., 2000). These differences may result from the particular features of the immature CNS and the intrinsic properties of developing microglial cells.

The embryonic retina of birds is a useful model for studying the microglial cell response to injury in the developing CNS. First, experimental procedures can be performed in the retina of one eye of each animal while the other is preserved as a control. Second, the chronotopographic pattern of microglial development in the quail retina is known (Navascués et al., 1995; Marín-Teva et al., 1998, 1999b; Sánchez-López et al., 2004), allowing ready identification of alterations in the microglia of the affected retina by using the QH1 antibody, which recognizes all phases of development of microglial cells (Cuadros et al., 1992). Finally, the complete absence of blood vessels in the avian retina prevents a rapid invasion of the retina by blood-derived macrophages; this is crucial for study of the microglial reaction, because macrophages from outside the nervous parenchyma are also involved in the response to injury (Stoll and Jander, 1999; Jones and Tuszynski, 2002; Schilling et al., 2003).

In the present study, we inserted a needle into the developing retina of quail embryos, without injecting any substance, and studied the microglial reaction after the injury. This injury induced major phenotypic changes in ameboid microglial cells that arrived at the damaged zone during their normal migration across the retina. These changes are compatible with the activation of immature microglial cells in a process similar to that undergone by mature microglia in the injured adult CNS.

MATERIALS AND METHODS

The right eye of quail (*Coturnix coturnix japonica*) embryos was stabbed through the sclera, choroid, and retina with a Hamilton needle (Aldrich 710N), simulating the action of an injection but without injecting any substance. The stab wound was made on the dorsal region of the peripheral retina at either the seventh or ninth day of incubation (E7 or E9; see Fig. 1). These ages were chosen because migrating ameboid microglia remain located at the central retina at E7, far from the wound on the peripheral retina, whereas microglial cells are beginning to arriving at the peripheral region by E9 (Marín-Teva et al., 1999b), adjacent to the wound. Therefore, the retinal injury produced at E7 was not in contact with retinal microglial cells for at least 2 days, whereas the injury produced at E9 was in immediate contact with microglial cells. The injured embryos were allowed to survive for different times postinjury (Table 1). The noninjured, left eye of each animal was used as a control.

TABLE 1. Ages at Injury and Survival of Experimental Animals

Embryonic age at which the wound was performed	Survival days after the wound	Age at time of sacrifice (wound day + survival days)	Numbers of animals analyzed
E7	1	E8 (E7 + 1)	3
	2	E9 (E7 + 2)	3
	3	E10 (E7 + 3)	5
	5	E12 (E7 + 5)	4
	7	E14 (E7 + 7)	3
E9	1	E10 (E9 + 1)	3
	2	E11 (E9 + 2)	4
	3	E12 (E9 + 3)	5
	5	E14 (E9 + 5)	15
	7	E16 (E9 + 7)	3
	11	P4 (E9 + 11)	2
	17	P10 (E9 + 17)	3
	37	P30 (E9 + 37)	3

E, embryonic day; P, posthatching day.

Histological preparation

The quails were killed at different embryonic and posthatching ages with euthanasia methods approved by the Research Ethics Committee of the University of Granada. The injured and control retinas from each animal were quickly dissected and isolated in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) and processed in one of three different ways to obtain whole mounts, histological sections, or sheets of the vitreal part of the retina.

Whole-mounted retinas were obtained by making radial incisions in isolated retinas that permitted their flattening on slides. Care was taken that the incisions did not affect the injured area of the retina. The whole-mounted retinas were then additionally fixed in 4% paraformaldehyde in 0.1 M PBS for 24 hours.

To obtain retinal sections, isolated retinas were fixed in 4% paraformaldehyde in 0.1 M PBS for 2 hours, washed in 0.1M PBS, and cryoprotected in 30% sucrose in PBS for 24 hours. They were then soaked in OCT cryocompound (Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen, and stored at −40°C before sectioning on a Leica CM1850 cryostat. Retinal sections 20 μm in thickness were captured onto Superfrost slides (Menzel-Glaser, Braunschweig, Germany) and immunocytochemically treated as described below.

Sheets of the vitreal part of selected areas, including the wound, were obtained from some retinas by a procedure described elsewhere (Marín-Teva et al., 1998). In normal areas of the retina, these "vitreal sheets" contained the inner limiting membrane and a carpet of Müller cell end-feet on which ameboid microglial cells were migrating. In the injured area, where the inner limiting membrane and Müller cell end-feet were destroyed, the sheets contained microglial cells that were invading the vitreal part of the wound.

Immunocytochemistry

QH1, LEP100, and M1-B4 antibodies (Developmental Study Hybridoma Bank, University of Iowa, Iowa City, IA) were used. QH1 and LEP100 antibodies recognize microglial cells (Cuadros et al., 1992) and a lysosomal membrane glycoprotein (Lippincot-Schwartz and Fambrough, 1986), respectively, whereas M1-B4 antibody labels tenascin, which is present in the plexiform layers of the developing retina (Sánchez-López et al., 2004).

Whole-mounted retinas were subjected to QH1 immunocytochemistry. The QH1 antibody was usually revealed by immunofluorescence with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma, St. Louis, MO) as previously described (Sánchez-López et al., 2004). Sometimes, however, the QH1 antibody was revealed by incubation in biotinylated anti-mouse IgG (Sigma) and then in avidin-biotin-peroxidase complex (Extravidin; Sigma) as described elsewhere (Navascués et al., 1995; Marín-Teva et al., 1998).

Retinal sections were immunostained by either single immunofluorescence for QH1 or double immunofluorescence for M1-B4 and QH1. In the latter case, sections were first subjected to immunocytochemistry for M1-B4 by using a tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody and then treated for QH1 immunofluorescence by using a FITC-conjugated secondary antibody as previously described (Sánchez-López et al., 2004). Nuclei were labeled in single- or double-immunostained sections with bisbenzimide (Hoechst 33324; Sigma; 20 μg/ml) to recognize the nuclear layers of the retina.

Retinal vitreal sheets were subjected to double immunofluorescence for LEP100 and QH1. Sheets were permeabilized with methanol at –20°C for 5 minutes, rehydrated in 0.1 M PBS, and incubated in normal goat serum (NGS) diluted 1:30 in 1% bovine serum albumin in 0.1 M PBS (BSA-PBS) for 45 minutes. They were then incubated in LEP100 antibody (dilution 1:1 in BSA-PBS) for 24 hours at 4°C, washed in 0.1M PBS, and subsequently incubated in the secondary antibody (FITC-conjugated anti-mouse IgG diluted 1:50 in BSA-PBS) for 4 hours. After careful washing in 0.1M PBS and NGS blocking, sheets were incubated in QH1 antibody (dilution 1:4 in BSA-PBS) for 24 hours at 4°C, washed again, and incubated in TRITC-conjugated anti-mouse IgG (Sigma) diluted 1:60 in BSA-PBS. After washing, sheets were mounted on slides and coverslipped with the antifading mounting medium Vectashield (Vector Laboratories, Burlingame, CA).

Microscopy

Observations of immunofluorescent specimens were made with a Leitz DMRB microscope equipped with a Leica TCS-SP scanning laser confocal imaging system. Nonfluorescent specimens were studied with a Zeiss Axiophot microscope.

For confocal microscopic observations in whole-mounted retinas and vitreal sheets, stacks of optical sections in planes parallel to the retinal surface were collected at 0.5–1.5-μm intervals throughout the thickness of the specimen. Leica confocal software (Leica, Heidelberg, Germany) was used to superimpose optical sections from each series onto two-dimensional (2D) projection images of all microglial cells contained throughout the specimen thickness. Sometimes, optical sections from stacks in each microscopic field were classified in different series according to their location at different depths of the retinal thickness. Therefore, 2D projection images of microglial cells at different retinal depths could be obtained separately.

Confocal microscopic observations on double-immunolabeled retinal sections were made by obtaining 2D projection images from stacks of optical sections through variable portions of the section thickness. Hoechst 33342-stained cell nuclei were imaged by nonconfocal microscopy with a Leica DC200 digital camera adapted to the Leitz DMRB microscope described above. Photoshop 5.5 (Adobe Systems) was used to merge the Hoechst-labeled nonconfocal image with FITC- and TRITC-labeled confocal images of each microscopic field.

RESULTS

In response to the stab wound in the retina, microglial cells in the injured area showed alterations in their morphology and distribution that varied according to the stage at which the injury was performed and the survival time.

Microglial cells in stab wound of retinas injured at E7

At developmental stages between E7 and E9, as previously reported (Marín-Teva et al., 1999b), microglial cells migrated tangentially on the vitreal surface of the quail retina without reaching the peripheral region, the site of the stab wound (Fig. 1). In experimental retinas injured at E7, no microglial cells were detected within the wound during day 1 or 2 postinjury (E7 + 1 and E7 + 2; Fig. 2A). At E7 + 3, when microglial cells first arrived at the injured retinal region during their normal tangential migration (Fig. 1), some microglial cells were observed at the edge of the wound (Fig. 2B).

Microglial cells in stab wound of retinas injured at E9

In experimental retinas injured at E9, i.e., just before the arrival at the injured area of ameboid microglial cells in the course of their normal migration (Fig. 1), the distribution of microglial cells in the stab wound of E9 + 1 retinas was similar to that in the wound of E7 + 3 retinas: microglial cells accumulated at the wound edge nearest the central retina (Fig. 2C,D), from which migrating microglia came. Therefore, the presence of microglia in the wound appeared to depend on the developmental stage of the retina (note that both E9 + 1 and E7 + 3 retinas are at developmental stage E10), irrespective of the timing of the retinal injury. Because similar results were obtained in retinas injured at E7 or E9 that survived until E11 or longer, we shall refer only to results obtained in retinas injured at E9.

The phenotype of microglial cells accumulating at the wound edge clearly differed from that of ameboid microglial cells migrating in noninjured areas of the retina. Normal ameboid microglial cells were QH1 positive and had a small soma from which emerged thick processes (generally one or two, although sometimes more) of variable length bearing broad lamellipodia, as previously reported (Navascués et al., 1995; Marín-Teva et al., 1998). In contrast, microglial cells contacting the wound showed much more intense expression of QH1 than that of normal ameboid microglial cells, and they were rounded, with a

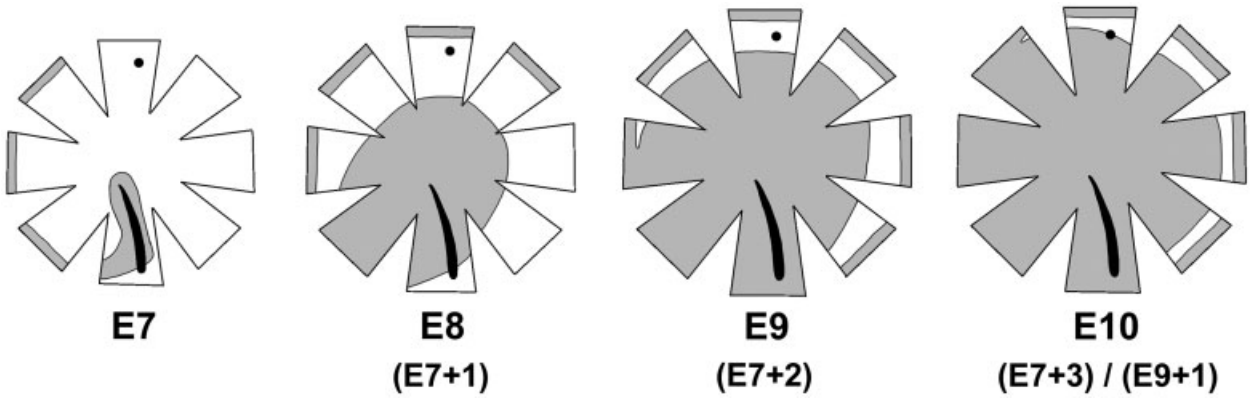

Fig. 1. Schematic drawings of whole-mounted retinas of quail embryos at different developmental stages showing the area occupied by microglial cells (gray areas) in the course of their central-to-peripheral migration and the position of the stab wound (black circles). At day 7 of incubation (E7), the area occupied by microglial cells migrating on the vitreal surface of the retina is far from the lesion site, and the first microglial cells do not arrive there until 3 days later (E7 + 3). When the traumatic injury is performed at E9, the front line of microglial migration is near the lesion site, and, 1 day later (E9 + 1, also equivalent to E7 + 3), the first migrating microglial cells have reached the wound. The elongated black area in each retina represents the optic nerve head.

large soma bearing very short or no processes. These morphological features are similar to those described for activated microglia (Streit et al., 1999; Stence et al., 2001). These results suggested that ameboid microglial cells were activated only when they contacted the wound in the course of their normal tangential migration on the vitreal part of the retina.

By E11–E12, ameboid microglial cells in normal retinas had migrated to cover their entire vitreal surface (Marín-Teva et al., 1999b). In E9 + 2 and E9 + 3 experimental retinas, the wound was filled with activated-like microglial cells and was readily identified as a strongly QH1-labeled area under low magnification (Fig. 3A). Higher magnification revealed that microglial cells within the wound had an activated-like appearance, i.e., predominantly round (sometimes bearing short processes) and strongly QH1 labeled (Fig. 3B). In contrast, microglial cells outside the wound showed typical morphological features of migrating ameboid microglial cells, i.e., weaker QH1 labeling and elongated shape, with thick lamellipodia-bearing processes (Fig. 3B).

E9 + 5 (Fig. 3C) and E9 + 7 experimental retinas showed an evident increase in the number of activated-like microglial cells in the wound. The strongly QH1-labeled microglial cells formed numerous aggregates in some areas of the wound, hampering identification of single cell profiles (central areas of the wound in Fig. 3C). Outside the wound, ameboid microglial cells appeared to undergo normal tangential migration (Fig. 3D).

The distribution of activated-like microglial cells through the retinal thickness at the injured area was investigated by using histological sections of retinas subjected to double immunofluorescence for M1-B4 (which labels plexiform layers in developing retinas) and QH1 and stained with Hoechst 33342 to reveal nuclear layers. Activated-like microglial cells appeared through the entire thickness of the retina (Fig. 3E–G), whose normal layering was profoundly altered. In central areas of the wound, microglial cells were round throughout the retinal thickness (Fig. 3F). At the wound periphery, they were round at scleral levels and showed short and broad processes in the vitreal part (Fig. 3G). Our observations did not rule out entry into the wound of some QH1-positive cells from nonretinal tissues.

The wound remained evident in experimental retinas during posthatching development. Observations in retinas of hatched quails injured at E9 and sacrificed after 11 (E9 + 11) and 17 (E9 + 17) days, i.e., at posthatching days 4 (P4) and 10 (P10), revealed that the wound area was occupied by numerous activated-like microglial cells showing more intense QH1 labeling than that of normal microglia in neighboring areas (Fig. 4A).

Microglial cells were heterogeneously distributed in the wound, with some areas containing more QH1-positive cells than others (Fig. 4A). Differences were also observed in the distribution and morphology of activated-like microglial cells through the retinal thickness at the injured area. At vitreal levels, numerous QH1-positive cells were seen, either rounded or bearing short and broad processes (Fig. 4B). At scleral levels, microglial cells had variably ramified morphological features (Fig. 4C) but showed a certain degree of activation, with more intense QH1 immunoreactivity and shorter and broader processes compared with ramified microglial cells at scleral levels in normal areas of the retina.

The wound remained present in experimental retinas of P30 quails that had been injured at E9 and sacrificed after 37 days (E9 + 37; Fig. 5). The retinal layers were considerably distorted (Fig. 5D), and numerous activated-like microglial cells with intense QH1 immunoreactivity and variable morphological features were still located within the wound. Most of them were ramified to a variable degree with short and broad processes, and only a few were round (Fig. 5B). Histological sections showed that rounded cells were generally located in the scleralmost part of the wound (Fig. 5E), whereas activated-like cells with ramified appearance were seen in portions of plexiform layers irregularly interspersed between discontinuous portions of nuclear layers (Fig. 5E).

In E9 + 37 retinas, as in earlier posthatching developmental stages, microglial cells located in plexiform layers at some distance from the wound had a normal ramified

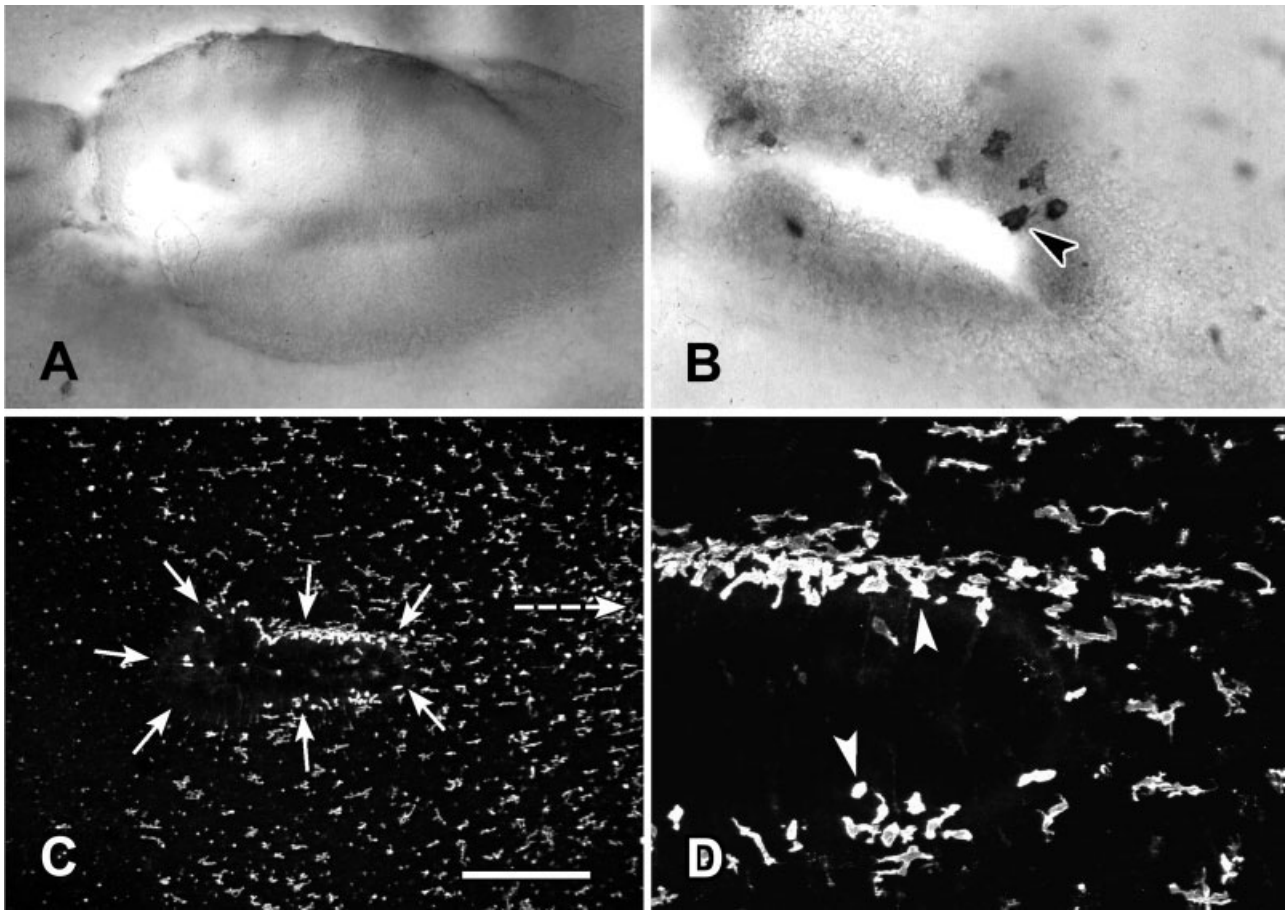

Fig. 2. Area containing the wound in QH1-immunostained whole-mounted retinas stabbed at E7 and examined after 1 (E7 + 1, A) and 3 (E7 + 3, B) survival days or stabbed at E9 and examined after 1 survival day (E9 + 1, C,D). Immunocytochemistry with peroxidase was used for A and B, whereas immunofluorescence with an FITC-conjugated secondary antibody was used for C and D. **A:** No QH1-labeled cells are seen in the wound at E7 + 1. **B:** Some labeled cells (arrowhead) are present at the wound edge at E7 + 3. **C:** At E9 + 1, QH1-positive cells accumulate at the wound edge nearest the central retina. The solid arrows demarcate the area occupied by the wound. The dashed arrow points toward the central retina. **D:** Higher magnification of the immunoreactive cells at the wound edge. They show strong QH1 immunolabeling, and many of them are round (arrowheads). Scale bar = 390 μm in C; 100 μm for A,B,D.

morphology. However, the closer they were located to the wound, the larger they were, with shorter processes and more intense QH1 immunoreactivity (Fig. 5C,F). In young adulthood, therefore, microglial activation remained observable in the wound, whereas microglial cells in neighboring noninjured areas were nonactivated and had differentiated normally to become ramified microglia.

Lysosomal compartment in activated-like microglia vs. normal ameboid microglia

Microglial activation within the wound was shown based on the aforementioned morphological features of QH1-positive cells. To elucidate this point, vitreal sheets were obtained from the injured area of experimental retinas and subjected to double immunofluorescence for QH1 and LEP100 to reveal lysosomes in microglial cells, because an increased lysosomal compartment is considered to indicate microglial activation. Activated-like, round microglial cells within the wound showed a considerably enlarged lysosomal compartment (Fig. 6A–C) compared with that of normal ameboid microglial cells outside the wound (Fig. 6D–F). Microglial cells at the edge of the wound showed a lysosomal compartment of intermediate size between that observed in normal ameboid microglia and that in activated-like round microglia (Fig. 6G–I). These results further supported the observation that migrating ameboid microglia were activated only when they arrived at the wound.

Secondary damage areas

In addition to the direct or primary damage produced by the needle's insertion, small areas of the experimental retinas showed different changes in their layering and microglial content, designated *secondary damage areas* by our group. These areas were small and of variable location, remaining present in experimental retinas until at least young adulthood (E9 + 37). There appeared to be no topographical relationship between the primary injury site and secondary damage areas.

In embryonic experimental retinas (up to E9 + 7), some small, circular areas irregularly distributed in the retina showed alterations in their structural organization, with

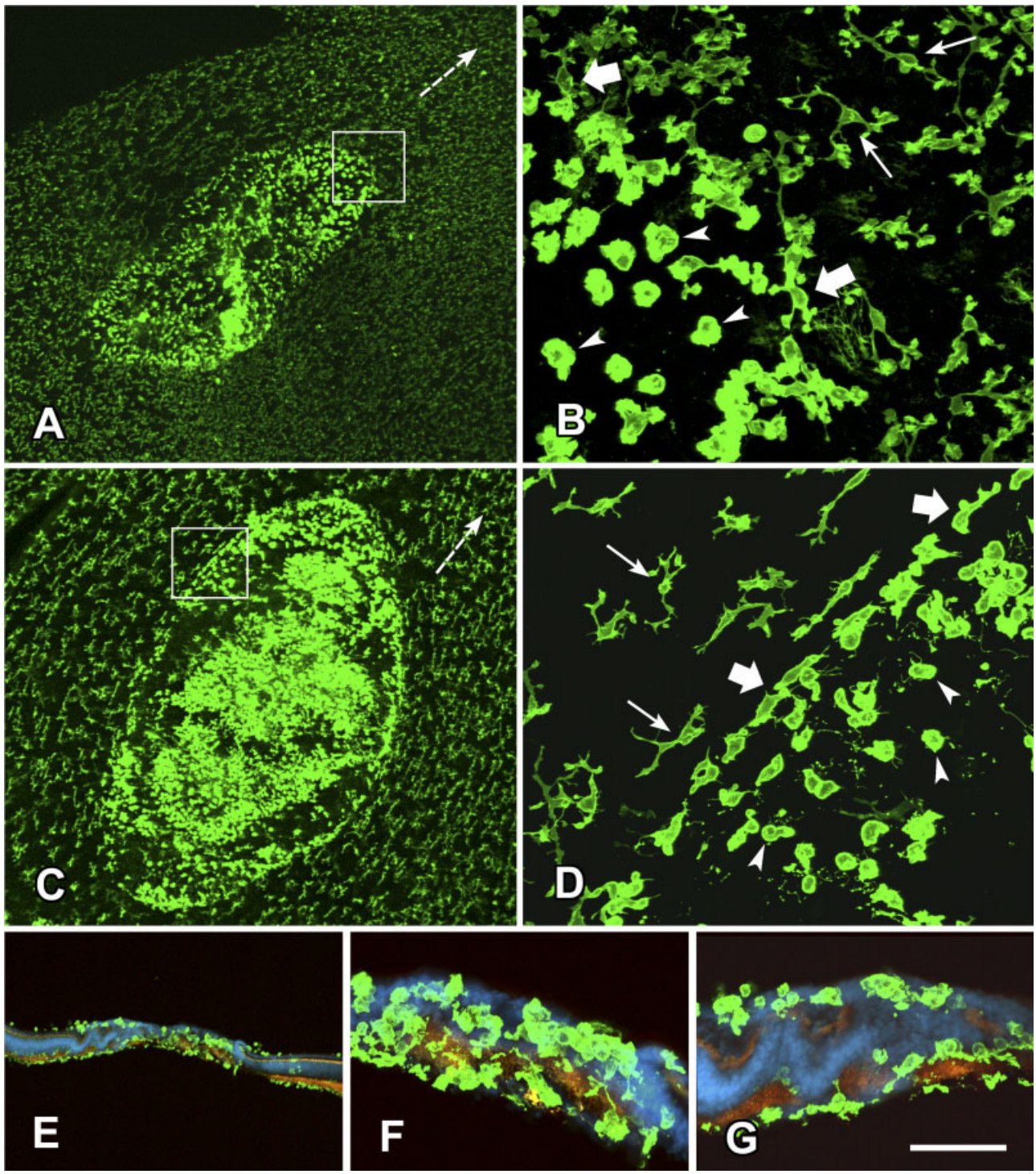

Fig. 3. Activated-like microglial cells in the wound of retinas stabbed at E9 and examined after 3 (E9 + 3, A,B) and 5 (E9 + 5, C–G) survival days. **A–D:** QH1-immunostained whole-mounted retinas. The wounds are clearly distinguished in A and C as bright areas because of the presence of numerous microglial cells showing a stronger QH1 immunoreactivity than that of microglial cells in noninjured areas. The dashed arrows point toward the central retina. Selected areas boxed in A and C are seen at higher magnification in B and D, respectively, showing the presence within the wound of round activated-like microglial cells with intense QH1 labeling (arrowheads), whereas microglial cells outside the wound (solid thin arrows) have weaker QH1 immunoreactivity and are elongated with lamellipodia-bearing processes. Microglial cells with intermediate features (solid thick arrows) are seen at the wound edge. **E:** Histological section of an E9 + 5 retina with double immunostaining for QH1, revealing microglial cells (green), and M1-B4, revealing tenascin in plexiform layers (red). Hoechst staining reveals nuclear layers of the retina (blue). Disruption of normal layering of the retina is seen at the wound. **F,G:** Magnifications of E showing the distribution of activated-like round microglial cells within the wound throughout the retinal thickness. Scale bar = 60 μm in G (applies to F,G); 475 μm for A,C; 70 μm for B,D; 240 μm for E.

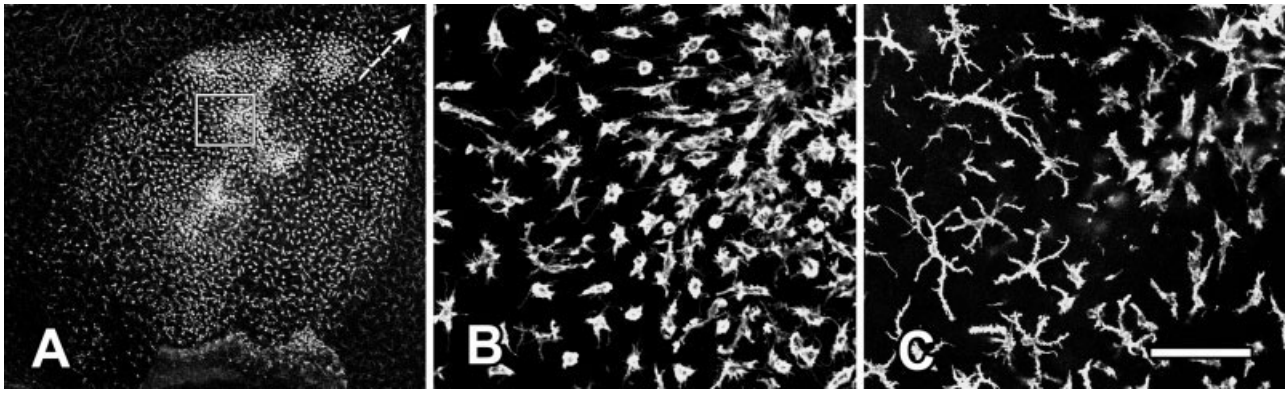

Fig. 4. Activated-like microglial cells in the wound of a QH1-immunostained whole-mounted retina stabbed at E9 and examined after 17 survival days (E9 + 17). **A:** General view of the wound showing numerous activated-like microglial cells with strong QH1 immunoreactivity. The arrow points toward the central retina. **B,C:** Magnification of the boxed area in A at different focal planes taken at vitreal (B) and scleral (C) levels of the retina. Microglial cells are round or bear short thick processes on the vitreal side (B), whereas they are ramified with thick processes at the scleral level (C). Scale bar = 60 μm in C (applies to B,C); 460 μm for A.

interruption of the inner plexiform layer, whose place was taken by inner nuclear and outer plexiform layers that were raised toward the vitreal surface (Fig. 7A). Migrating ameboid microglial cells appeared to avoid entry into these rosette-like structures, which they surrounded (Fig. 7B). Thus, no microglial cells were seen within these altered areas, and ameboid microglial cells surrounding them had a normal appearance.

Secondary damage areas were more extensive in experimental retinas of young adult quails (E9 + 37), and several types of secondary damage could be distinguished. In one type, the retinal organization was reminiscent of the rosette-like structures in prehatching retinas, with the disappearance of portions of the inner plexiform layer and their replacement with a projection of the inner nuclear layer (Fig. 7C). These areas, unlike secondary damage areas in embryonic stages, were colonized by microglial cells that had a ramified appearance, although they also showed signs of activation (Fig. 7C,D).

A second type of secondary damage area in E9 + 37 experimental retinas showed a large accumulation of strongly QH1-labeled microglial cells. These activated-like cells were rounded at the vitreal surface of secondary damage areas (Fig. 8A) and showed an irregular morphology, with short, thick cell processes at intermediate retinal depths (Fig. 8B).

In E9 + 37 experimental retinas, no round microglial cells were seen in areas other than the primary (wound) or secondary damage areas. However, some areas with apparently normal structural layering showed higher numbers of microglial cells and changes in their ramified appearance, with fewer and thicker processes compared with microglial cells in equivalent areas of control retinas (compare A–C with D–F, respectively, in Fig. 9).

DISCUSSION

Four conclusions can be drawn from findings in the present study: 1) immature microglial cells can be activated after traumatic injury in the retina, as occurs with mature microglia; 2) they are activated only when they contact the injured area; 3) activation of microglia in a stab wound made in the embryonic retina persists until adulthood; and 4) sustained microglial activation also occurs in secondary damage areas.

Terminological remarks

Demonstration of the ability of immature microglia to be activated introduces some confusion in the terminology used for different functional states of microglia. In fact, the term "activated" was previously used to refer to the functional state of the mature (ramified) microglia after responding to CNS injury (Streit et al., 1999). The present findings impose the need for a distinction between activated immature and activated mature microglia.

These findings may also increase confusion about the term *ameboid*. Properly speaking, ameboid microglia are immature microglial cells that are present in the developing CNS and are the precursors of ramified microglia (Ling and Wong, 1993). However, the term *ameboid* is descriptive and refers to the rounded morphology of these cells (Streit, 2001), and it has been also used to refer to phagocytic rounded activated microglial cells (Streit, 2004). We propose that the traditional term *ameboid microglia* be solely used for nonactivated immature microglia and that the term *activated ameboid microglia* be applied to the rounded microglial cells resulting from activation of immature microglia. We also recommend that the term *ameboid* not be used to refer to rounded activated mature microglia.

Activation of immature microglial cells

Our results demonstrate that immature (ameboid) microglial cells in the retina are activated in response to a traumatic injury. Thus, QH1-positive cells within the stab wound showed morphological features typical of activated microglia; 1) they underwent evident changes in shape to a more compact morphology with respect to their counterparts in noninjured areas of the retina; 2) their QH1 immunoreactivity was far more intense than that of cells outside the wound; and 3) they showed an evident increase in their lysosomal compartment. The changes in shape of immature microglia in the embryonic quail retina after activation by injury culminated in a rounded appear-

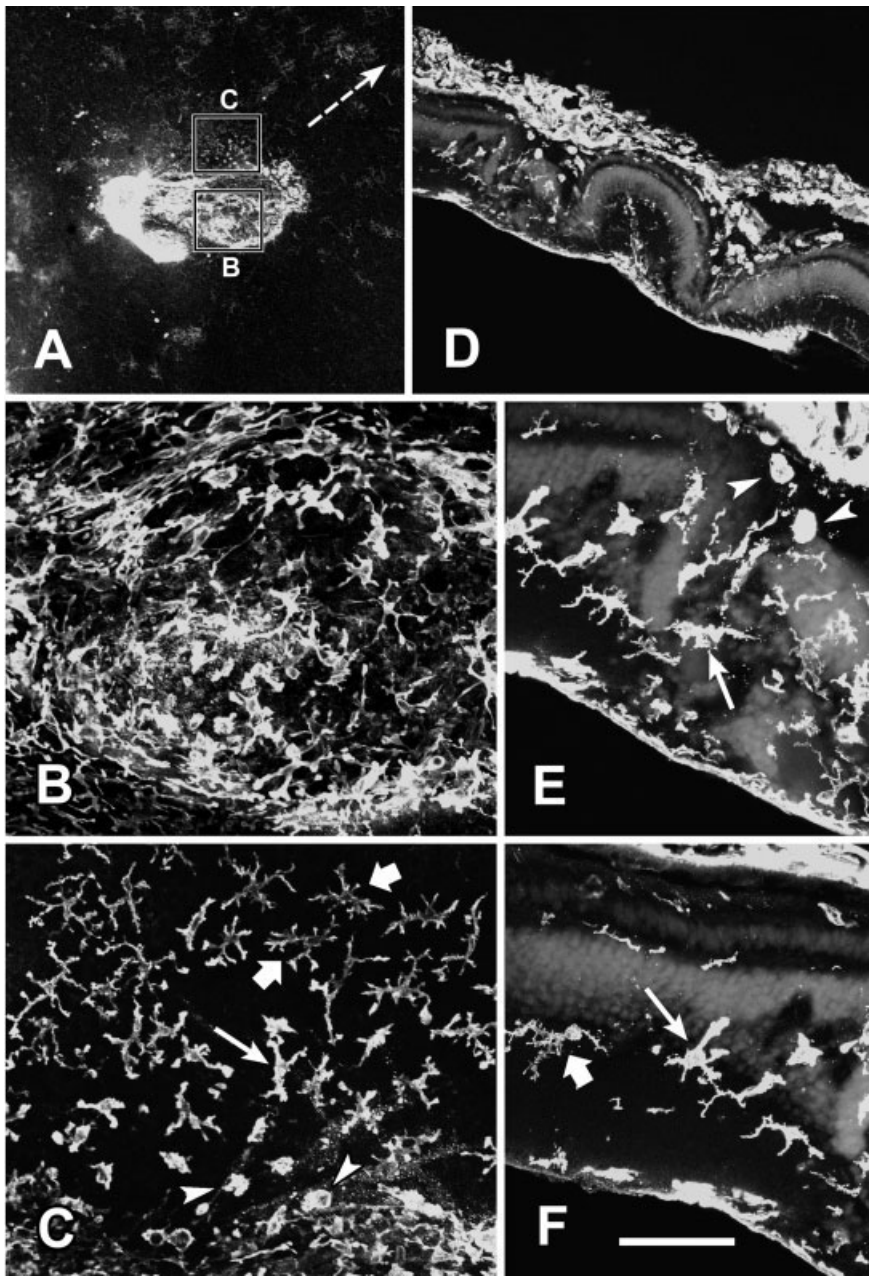

Fig. 5. Activated-like microglial cells in the wound of retinas stabbed at E9 and examined after 37 survival days (E9 + 37). **A–C:** QH1-immunostained whole-mounted retinas. The wound is distinguished in A as a bright area. The dashed arrow points toward the central retina. Selected areas boxed in A are seen at higher magnification in B and C. A dense net of activated microglial cells showing strong QH1 immunoreactivity and variable morphological features is evident in central parts of the wound (B). Transitional forms (solid thin arrows) between intense QH1-positive round (arrowheads) and weaker QH1-positive ramified (solid thick arrows) microglial cells are seen at the edge of the wound (C). **D–F:** QH1-immunostained histological section of an E9 + 37 retina that has been additionally stained with Hoechst to reveal nuclear layers (gray areas). Disruption of the normal arrangement of the retinal layers is evident in the wound. E and F are magnifications of selected areas in D showing that round activated microglial cells (arrowheads) are present in the scleral part of the wound, whereas activated microglial cells bearing short thick processes (arrows) are seen in plexiform layers. Scale bar = 55 μm in F (applies to E,F); 575 μm for A; 75 μm for B,C; 160 μm for D.

ance similar to that described for activated microglia derived from ramified microglia after injury in the mature nervous parenchyma (Raivich et al., 1999; Streit et al., 1999; Stence et al., 2001). The increased QH1 immunoreactivity in the activated immature microglia was similar to that reported for mature microglia of adult quail retina after optic nerve transection (Jeon et al., 2004). It is not unreasonable to assume that up-regulation of other molecules may occur during activation of immature microglia in the same way as during activation of mature microglia

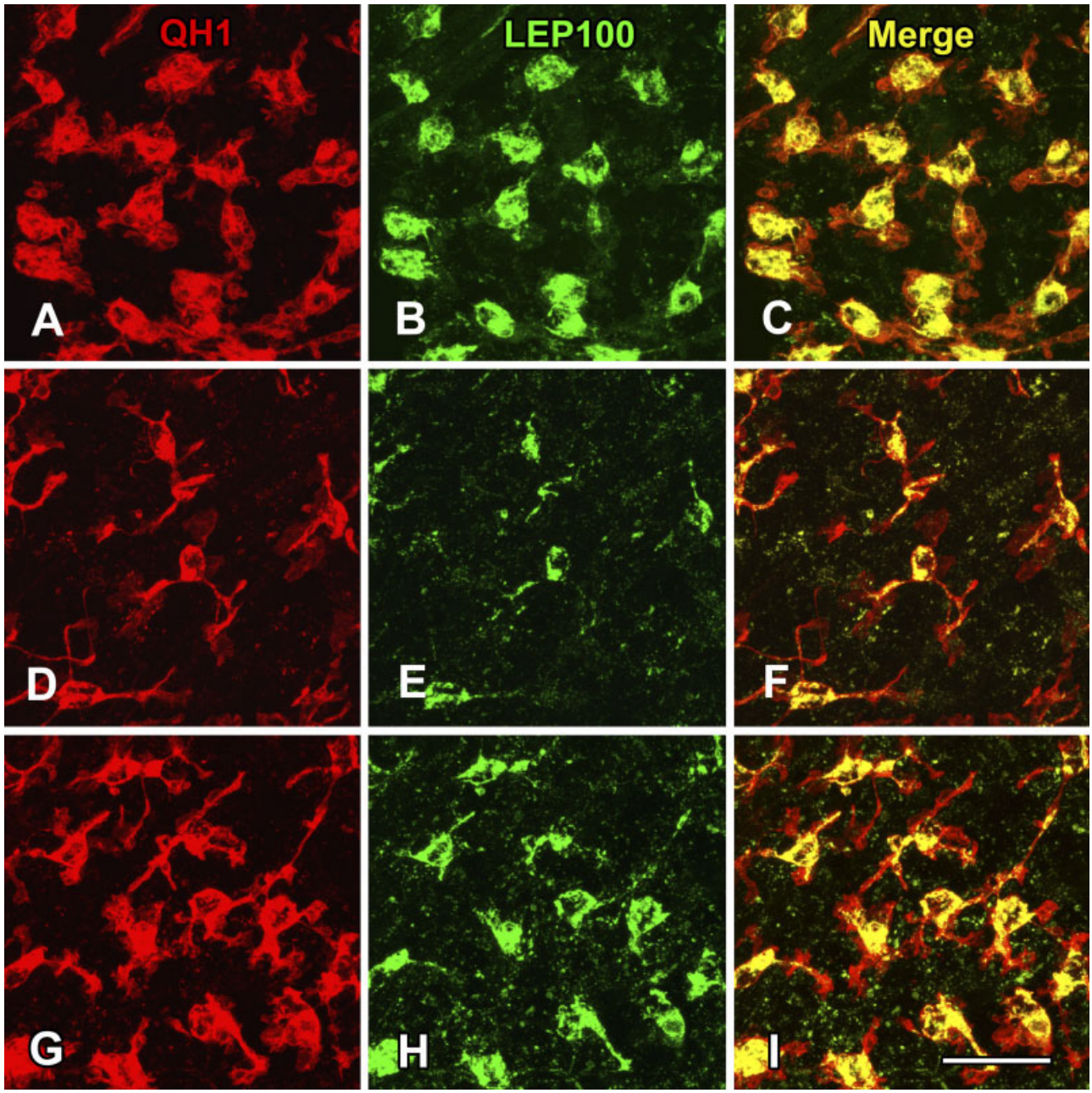

Fig. 6. Microglial cells within the wound (A–C), in a noninjured area (D–F), and at the wound edge (G–I) as seen in a sheet of the vitreal part of an E9 + 5 retina with double immunostaining for QH1, revealing microglial cells (red, A,D,G), and LEP100, labeling the lysosomal compartment (green, B,E,H). C, F, I: Illustrate the merged images. **A–C:** Activated microglial cells within the wound showing a rounded shape and a well-developed lysosomal compartment. **D–F:** Nonactivated microglial cells outside the wound with lamellipodia-bearing thick processes emerging from a small cell body. They contain a much less well-developed lysosomal compartment. **G–I:** Microglial cells at the edge of the wound with transitional shapes between those of microglial cells in A–C and D–F. The lysosomal compartment also shows an intermediate development. Scale bar = 35 μm in I (applies to A–I).

(Raivich et al., 1999; Nakajima and Kohsaka, 2001; Hanisch, 2002); nevertheless, this issue was not investigated in the present study. The larger lysosomal compartment in the immature microglia after activation also appears to be equivalent to findings in activated mature microglia of the adult CNS (Bauer et al., 1994; Frank and Wolburg, 1996; Origasa et al., 2001; Brauer et al., 2004; Pearse et al., 2004), including the retina (Engelsberg et al., 2004). In conclusion, comparison of our results in the embryonic retina with previous findings in the adult CNS shows essentially similar morphological changes of ameboid immature and ramified mature microglia in response to injury.

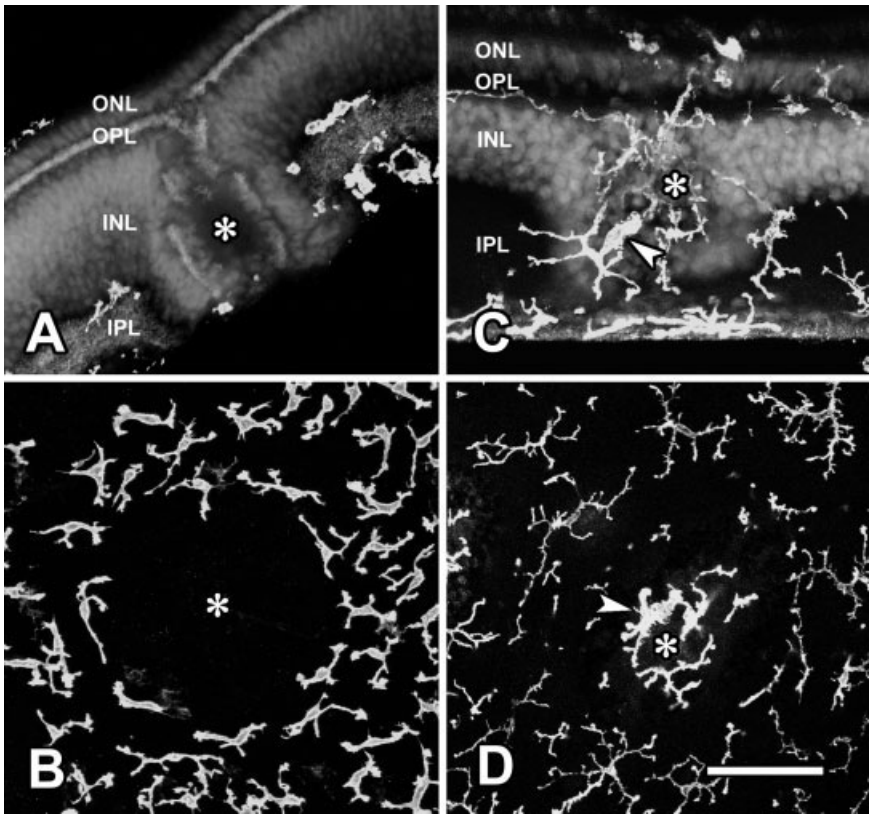

Fig. 7. Microglial cells in rosette-like areas (asterisks) of secondary damage in injured retinas of embryonic (E9 + 5, **A,B**) and young adult (E9 + 37, **C,D**) quails. Immunocytochemistry for the QH1 antibody in retinal sections (A,C) and whole-mounted retinas (B,D) reveals the distribution of microglial cells. Counterstaining with Hoechst in histological sections shows inner (INL) and outer (ONL) nuclear layers, whereas immunocytochemistry for the M1-B4 antibody in A labels inner (IPL) and outer (OPL) plexiform layers. Alterations of normal retinal layering are seen in embryonic (A) and adult (C) retinas with interruption of the IPL by bulging of the INL and OPL toward the vitreal surface. In E9 + 5 retinas, migrating ameboid microglial cells surround the rosette-like area (asterisk) without entering it (A,B), whereas ramified microglial cells showing signs of activation (arrowheads) are present within the rosette-like area (asterisk) in E9 + 37 retinas (C,D). Scale bar = 50 μm in D (applies to A,D); 65 μm for B; 40 μm for C.

The idea that immature microglia are activated after injury in the developing CNS was not supported by the study of Milligan et al. (1991), who found that developing microglia did not respond to retrograde degeneration of neurons in the dorsal lateral geniculate nucleus of neonatal rats after visual cortex lesions. In addition, Lawson and Perry (1995) found that lipopolysaccharide injected into the brain of newborn mice produced a minimal microglial alteration until several days after the injection, also suggesting that immature microglia cannot react to injury. By contrast, various studies showed the presence of activated-like microglial cells in the nervous parenchyma of different parts of the embryonic or neonatal CNS in response to different insults (Morioka and Streit, 1991; Acarin et al., 1996; Bobryshev and Ashwell, 1996; Wu et al., 1997; Graeber et al., 1998; Cuadros et al., 2000; Fiske and Brunjes, 2000; Hao et al., 2001). Nevertheless, most of these studies did not unequivocally establish that the activated-like cells derived from immature microglia, because they could not rule out their derivation from either blood-borne monocytes or partially ramified microglial cells that had already acquired activation capacity during their differentiation process. The present study clearly demonstrates that many of the activated microglial cells observed in the wound derive from ameboid immature microglia, based on the following observations. First, no microglial cells were present within the wound during the time period that adjacent retinal areas were also devoid of migrating ameboid microglial cells. Second, activated microglial cells first appeared within the wound at the same time as ameboid microglial cells first arrived at the site of the lesion. Third, the first microglial cells arriving at the wound accumulated at the edge nearest to the central retina from which they arrive during their central-to-peripheral migration (Navascués et al., 1995). Finally, transitional forms between normal ameboid and activated-like microglial cells were observed on the vitreal side of the wound edge (see Figs. 3B, 6).

The ability to detect morphological changes taking place during the activation of immature microglia was favored by the use of the embryonic quail retina. In this system, migrating ameboid microglial cells show a flattened shape, including an elongated soma bearing thick processes with extensive lamellipodia (Navascués et al., 2002). These morphological features are very different from the lamellipodia-lacking rounded shape of activated ameboid microglial cells, facilitating the distinction between the two functional states. Furthermore, the quail

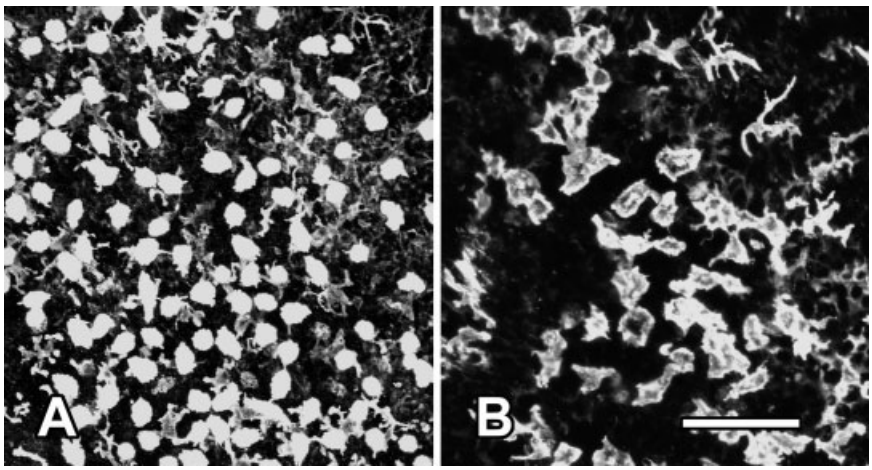

Fig. 8. Activated microglial cells in areas of secondary damage in an E9 + 37 injured retina that has been whole mounted and QH1 immunostained. Both micrographs represent the same microscopic field at different focal planes. An accumulation of round activated microglial cells is seen on the vitreal surface of the retina (**A**), whereas activated microglial cells with thick short cell processes are present at more scleral retinal levels (**B**). Scale bar = 60 μm in B (applies to A,B).

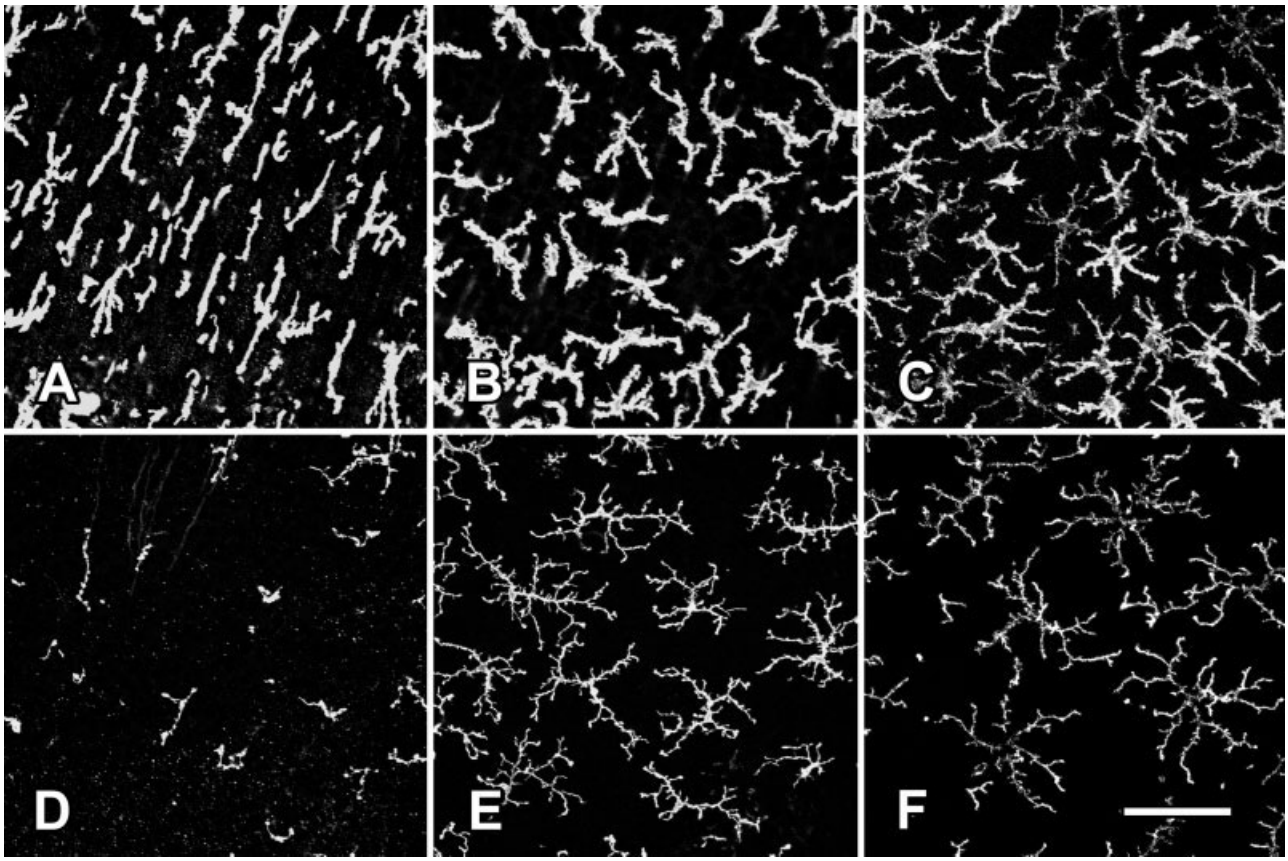

Fig. 9. Comparison between ramified microglia showing signs of activation in an apparently noninjured area of an E9 + 37 stabbed retina (**A–C**) and nonactivated ramified microglia in an equivalent area of the control retina (**D–F**). Micrographs are from QH1-immunostained whole-mounted retinas and represent two microscopic fields (A–C and D–F) at different focal planes located at the nerve fiber layer (A,D), the vitreal part of the inner plexiform layer (B,E), and the scleral part of this layer (C,F). Scale bar = 60 μm in F (applies to D–F); 50 μm for A–C.

retina used in the present study is completely avascular, which appears to rule out the blood origin of microglial cells that arrived at the wound. Nevertheless, the injury penetrated the eyeball wall, including the sclera, choroid, and retina, so a proportion of the activated QH1-positive cells observed in the wound might derive from the choroid.

Because microglia commonly divide when activated by neuronal injury in adults (Raivich et al., 1999; Nakajima and Kohsaka, 2001), it could be argued that conspicuous accumulation of activated microglia at the injury site is due primarily to cell proliferation rather than cell migration. However, very scarce QH1-positive mitotic cells were seen at the injury site in E9 + 3 and E9 + 5 retinas with Hoechst staining (results not shown), suggesting that proliferation of activated immature microglia was not greater than that of the nonactivated microglia migrating in the normal developing quail retina (Marín-Teva et al., 1999a). Therefore, proliferation does not appear to have made a major contribution to the accumulation of activated microglia. A similar finding was reported for osteopetrotic mice, in which microglial cells were activated with no noticeable change in their proliferation ability (Rogove et al., 2002). Nevertheless, further research using specific methods for the analysis of cell proliferation is required to give a clear-cut answer on this issue.

Apoptosis of neurons is taking place in the normal developing quail retina at E7 and E9 (Marín-Teva et al., 1999b), the times when the stab wound was made in this study, and ameboid microglial cells engaged in phagocytosis of cell debris during normal development show a nonactivated appearance (Marín-Teva et al., 1999b). Similar findings have been described for the retina of mammals (Hume et al., 1983; Ashwell et al., 1989; Pearson et al., 1993; Thanos et al., 1996; Egensperger et al., 1996). This is in accordance with the idea that apoptosis does not trigger widespread microglial activation during CNS development (Streit, 2001). Cell death during development is limited to a relatively low proportion of neurons in contrast to the massive cell death that could be expected from the acute injury produced by the stab wound. Therefore, we cannot rule out the possibility that this massive cell death triggers activation of ameboid microglia to make them more effective in the phagocytosis of cell debris, as occurs in the adult CNS after acute injury (Streit et al., 1988, 1999; Raivich et al., 1999).

Immature microglial cells are activated only when in contact with the injured area

Ramified microglia in the injured CNS can be activated at a certain distance from the cell degeneration focus to which they are subsequently recruited (Giulian, 1995; Rogove et al., 2002; Carbonell et al., 2005). According to the present results, retinal ameboid microglia do not have this ability and are activated only when they make contact with the injured area. In fact, no microglial cells invaded the wound in injured retinas before E9, which is the time when they arrive at this injured area as a consequence of the tangential migration that takes place during normal development of the retina. Therefore, it seems that migrating ameboid microglial cells in E7–E9 quail retinas are unable to respond to signals emanating from an injured focal area in close proximity. This agrees with a report by Leong and Ling (1992) that ameboid microglial cells in the corpus callosum of 1-day-old rats did not migrate to a nearby wound. This behavior contrasts with that of ramified microglia in mammalian retinas with inherited photoreceptor degeneration (Thanos, 1992; Gupta et al., 2003; Hughes et al., 2003; Rao et al., 2003) or in aging avian retinas (Kunert et al., 1999), where ramified microglial cells in the ganglion cell and inner plexiform layers are apparently activated in response to photoreceptor death and then recruited to the outer nuclear layer. In addition, massive recruitment of microglia to the retina in response to diffusible factors has also been described soon after intraocular prion infection in adult mice (Marella and Chabry, 2004).

In the present study, when the retina was stabbed at E9 (i.e., when migrating immature microglial cells were already present in the injured area), an accumulation of activated microglial cells began to be seen within the wound shortly after the injury. This suggests that ameboid microglia can react to injury immediately after making contact with the injured area. It could be argued that activated microglial cells accumulate in the wound not only as a result of the normal migration of microglia in the retina but also because of an effective attraction toward the focal lesion. If this were the case, there would likely be an increase in microglial cell density around the wound area and signs of activation of ameboid microglial cells at a certain proximity to the wound, neither of which was observed in the present study. In fact, the general migratory pattern of microglial cells across the retina did not appear to be affected by the injury, and microglial cells located outside the wound showed no signs of activation. Therefore, it seems that only microglial cells that encounter the focal lesion during their normal migration are activated.

These observations of behavioral differences between immature and mature microglial cells after injury are in line with the fact that ameboid microglia do not express the same cytokines as mature microglia (Hurley et al., 1999). The apparent inability of developing microglia to migrate toward the focal lesion may be related to their immaturity; they might not yet have acquired the ability to detect chemotactic factors released from the wound. This agrees with the conclusion by Lawson and Perry (1995) that the injury response ability of microglia in the mammalian CNS is acquired during postnatal development. An alternative explanation would be that inadequate chemotactic factors are released in embryonic stages to attract microglial cells.

Sustained microglial activation in the wound and in secondary damage areas

A striking finding in our study was the persistence of abundant activated microglia within the wound at 37 days postinjury. This prolonged duration of the microglial reaction contrasts with observations in the CNS of adult mammals subjected to traumatic injury, in which microglia return to their normal nonactivated state at 3–4 weeks postinjury (Fujita et al., 1998; Penkowa et al., 1999; Streit et al., 1999; Schwab et al., 2001; Garcia-Ovejero et al., 2002; Mueller et al., 2003; Sugama et al., 2003). The persistence of activated cells in the wound may be because they derive from immature microglia or because they are favored by the particular environment of the retina. In support of the latter hypothesis, a microglial reaction for more than 3 months after transection of the optic nerve was described for the adult retina of birds (Jeon et al., 2004) and mammals (Schnitzer and Scherer, 1990), in contrast to the much shorter reaction after axotomy in other parts of the CNS (Koshinaga and Whittemore, 1995; Sugama et al., 2003).

Activated microglia present at the injury site in the adult retina could be considered immature because they were activated at an embryonic stage and did not appear

to have changed their state during development. Nevertheless, most activated microglial cells in the wound of E9 + 37 were more or less ramified with short and broad processes; therefore, they had undergone a degree of differentiation despite remaining activated. Presumably, two types of factors act simultaneously on these cells, one type favoring ramification and the other favoring activation.

In addition to the stab wound caused by the needle, focal secondary damage areas such as rosette-like formations were seen in the injured retinas. They may be produced by a local perturbation in the normal arrangement of the layers as a consequence of the sudden decrease of intravitreal pressure after puncture of the eyeball wall. Activated microglial cells were not observed in these secondary damage areas immediately after retinal injury but rather after a longer period, and they remained present at 37 days postinjury. Therefore, microglial activation in secondary damage areas also persisted for a long time after injury. Moreover, some apparently noninjured areas of experimental retinas also showed weak microglial activation in adulthood (Fig. 9) that was not seen a few days after the injury. It appears that microglia in areas that are not directly injured tend to be activated after the passage of time postinjury. This suggests that microglia activated for a long time may release diffusible factors that act on retinal areas at a distance from the wound, contributing to the slight activation of the microglia within them. In support of this view, activated microglia were implicated in the secondary pathology that accompanies traumatic injury to the CNS (Popovich et al., 2002), and this action appears to be mediated by a chronic increase in the production of different factors, including cytokines, that may cause secondary damage to nervous tissue (Streit et al., 1999).

It was interesting that activated microglia in secondary damage areas tended to show a nonramified morphology at vitreal levels, whereas they were partially ramified with thick processes at more scleral levels (Figs. 8, 9). This appears to be related to the location of activated microglia in the nerve fiber layer at vitreal level and in the plexiform layers at more scleral levels. Factors in the plexiform layers stimulate microglial ramification (Sánchez-López et al., 2004), thereby partially counteracting the effects of factors that favor microglial activation.

Sustained microglial activation in either secondary damage areas or apparently noninjured areas of the retina after traumatic injury should be taken into account in studies on the long-term response of retinal microglia after intraocular injection of different factors. Any presence of activated microglia in the retina could wrongly be attributed to the action of the injected substance instead of to the injection injury itself. Therefore, care must be taken in interpreting the results of this type of study.

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