GLIA 54:376–393 (2006)

# Behavior of In Vitro Cultured Ameboid Microglial Cells Migrating on Müller Cell End-Feet in the Quail Embryo Retina

**MOHAMED TASSI, RUTH CALVENTE, JOSÉ L. MARÍN-TEVA, MIGUEL A. CUADROS, ANA M. SANTOS, MARIA-CARMEN CARRASCO, ANA M. SÁNCHEZ-LÓPEZ, AND JULIO NAVASCUÉS***

*Departamento de Biología Celular, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain*

*KEY WORDS*

β1 integrin subunit; cell death; cell migration; phagocytosis

*ABSTRACT*

Ameboid microglial cells migrate tangentially on the vitreal part of quail embryo retinas by crawling on Müller cell end-feet (MCEF) to which they adhere. These microglial cells can be cultured immediately after dissection of the eye and isolation of sheets containing the inner limiting membrane (ILM) covered by a carpet of MCEF (ILM/MCEF sheets), to which the cells remain adhered. Morphological changes of microglial cells cultured on ILM/MCEF sheets for 4 days were characterized in this study. During the first minutes in vitro, lamellipodia-bearing bipolar microglial cells became rounded in shape. From 1 to 24 h in vitro (hiv), microglial cells swept and phagocytosed the MCEF on which they were initially adhered, becoming directly adhered on the ILM. MCEF sweep was dependent on active cell motility, as shown by inhibition of sweep after cytochalasin D treatment. From 24 hiv on, after MCEF phagocytosis, microglial cells became more flattened, increasing the surface area of their adhesion to substrate, and expressed the β1 subunit of integrins on their membrane. Morphological evidence suggested that microglial cells migrated for short distances on ILM/MCEF sheets, leaving tracks produced by their strong adhesion to the substrate. The simplicity of the isolation method, the immediate availability of cultured microglial cells, and the presence of multiple functional processes (phagocytosis, migration, upregulation of surface molecules, etc.) make cultures of microglial cells on ILM/MCEF sheets a valuable model system for in vitro experimental investigation of microglial cell functions. 

## INTRODUCTION

Microglial cells in the central nervous system (CNS) acquire various morphological appearances that have been correlated with distinct functional states derived from different stimuli (Stoll and Jander, 1999; Streit et al., 1999). Factors controlling changes between different functional states of microglia are more readily studied in vitro than in the extremely complex in vivo CNS. In fact, purified microglial cell cultures have been widely used to study factors participating in the change of microglia from the ameboid to the ramified state (Chamak and Mallat, 1991; Fujita et al., 1996; Ilschner and Brandt, 1996; Schilling et al., 2001; Wilms et al., 1997; Wollmer et al., 2001; Yagi et al., 1999), a process that apparently mimics microglia differentiation. Factors involved in the transformation of ramified microglia into ameboid phagocytes, which would be similar to microglia activation, were also studied by using microglia cultures (Kalla et al., 2003; Kloss et al., 2001). These cultures also proved to be a powerful model to analyze other aspects of microglia biology, including motility (Calvo et al., 1998; Cross and Woodroofe, 1999; Nolte et al., 1996; Rezaie et al., 2002), proliferation (Brown et al., 1996; Dobbertin et al., 1997; Flanary and Streit, 2006), and phagocytosis (Chamak and Mallat, 1991; Chan et al., 2001; Smith, 2001; Thery et al., 1991; Zhang and Fedoroff, 1996).

Most methods to obtain microglial cell enriched cultures are based on differences in in vitro adhesion properties among cell types of the nervous tissue (Dobrenis, 1998; Giulian and Baker, 1986; Nakajima et al., 1989; Suzumura et al., 1987). These techniques take advantage of the weak adherence of microglial cells to the culture substrate in order to isolate them from primary mixed brain cell cultures and have also been used to isolate and culture microglia from the retina (Matsubara et al., 1999; Roque and Caldwell, 1993; Wang et al., 2005). Nevertheless, there is a relatively long delay between the in vivo state of microglial cells and their availability for experimental testing. In the present study, ameboid microglia were isolated from embryonic quail retinas by using a rapid method that avoids the complex procedures used in their isolation from primary mixed cell cultures and allows microglial cells to be cultured only a few minutes after retinal dissection.

Previous studies by our group (Marín-Teva et al., 1998; Navascués et al., 1995; Sánchez-López et al., 2004) showed that ameboid microglial cells enter the retina of quail embryos at the site of the insertion of the pecten into the optic nerve head. Then, they migrate tangentially in a central-to-peripheral direction on the vitreal

Grant sponsor: Spanish Ministry of Education and Science; Grant number: BFU2004-01209.

*Correspondence to: Dr. Julio Navascués, Departamento de Biología Celular, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain. E-mail: navascue@ugr.es

Received 31 March 2006; Revised 2 June 2006; Accepted 28 June 2006

DOI 10.1002/glia.20393

Published online 2 August 2006 in Wiley InterScience (www.interscience.wiley.com).

part of the embryonic retina. Subsequently, these cells migrate radially in a vitreal-to-scleral direction to reach their final location at the inner and outer plexiform layers, where they ramify. Microglial cells migrating tangentially on the vitreal part of the retina crawl on Müller cell end-feet (MCEF), to which they adhere. In the present study, we took advantage of this adhesion to isolate and in vitro culture ameboid microglial cells adhered on thin sheets of the vitreal part of embryonic retinas containing the inner limiting membrane (ILM, basal lamina) covered by a carpet of MCEF (ILM/MCEF sheets). In these cultures, microglial cells were available for in vitro experimental study within a few minutes after the eye dissection. We characterized changes undergone by microglial cells isolated on ILM/MCEF sheets and cultured in a serum-containing medium for 4 days.

## MATERIALS AND METHODS

Retinas from embryonic quails (*Coturnix coturnix japonica*) at day 10 of incubation (E10) were used to obtain ILM/MCEF sheets with adhered microglial cells. These sheets were subsequently incubated in vitro as described below to obtain microglial cell enriched cultures.

### Isolation and In Vitro Culture of ILM/MCEF Sheets

The technical procedure to isolate ILM/MCEF sheets was based on mechanical disruption of the retina mounted between two adhesive substrates (Halfter et al., 1987; Marín-Teva et al., 1998). E10 embryonic retinas were dissected in Hank's saline solution with calcium and magnesium (Sigma, St. Louis, MO) supplemented with 0.35 g/L sodium bicarbonate (Sigma) and 1% antibiotic/antimycotic solution (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin, Sigma) and pieces of their central part were excised and mounted vitreal side up on a nitrocellulose filter. A poly-L-lysine (PLL, Sigma) coated glass coverslip (0.4 mg/mL for 2 h) was placed on the flat-mounted retina-filter assembly, with the vitreal side of the retina facing the PLL-coated surface. The PLL-coated glass coverslip was gently weighted to press the vitreal retinal surface against the PLL coating. Excess fluid was then removed with filter paper and the retina–filter assembly was swiftly lifted away from the PLL-coated glass. An ILM/MCEF sheet containing a carpet of MCEF on the ILM, with adhered ameboid microglial cells, remained attached to the PLL-coated surface, whereas the rest of the retina was removed with the filter.

Each glass coverslip with an attached ILM/MCEF sheet was placed on a well in a 24-well plate and incubated with culture medium for up to 96 h at 37°C in a 5% $CO_2$ humidified atmosphere. The culture medium was serum-containing Dulbecco's modified Eagle's medium (SC-DMEM) consisting of DMEM (Sigma) supplemented with 4 mM L-glutamine (Sigma), 10% fetal calf serum (Sigma), 6% chick serum (Sigma), and 1% antibiotic/antimycotic solution. Sera were used after heat deactivation. Cultures incubated for more than 96 h were not included in this study because the microglial cell growth gave rise to a cell monolayer, hampering observations of single cells. There were no medium changes during the incubation period. Morphological features of microglial cells adhered on ILM/MCEF sheets were studied throughout 96 h of in vitro incubation (hiv) by specimen fixation at the following culture times: at the very beginning of in vitro incubation (0 hiv) and at ¼, ½, 1, 3, 6, 12, 24, 48, 72, and 96 hiv. Microglial cells were then observed under a phase contrast microscope before being treated by immunocytochemistry (see below) and examined under either a traditional fluorescence microscope or a Leitz DMRB microscope equipped with a Leica TCS-SP scanning laser confocal imaging system (Leica, Heidelberg, Germany). In confocal microscopy studies, stacks of horizontal (*xy*) optical sections (parallel to the ILM/MCEF sheet surface) were collected at 0.5–1.5 μm intervals throughout the *z*-dimension of microglial cells. Leica Confocal Software was used to superimpose optical sections from each microscopic field onto projection images of microglial cells contained in the field. Vertical (*xz*) optical sections (perpendicular to the ILM/MCEF sheet surface) were generated from selected microglial cells of which projection images had previously been obtained.

### Immunofluorescence Labeling of ILM/MCEF Sheet Cultures

Monoclonal antibodies (mAbs) QH1, 39.4D5, H5, 3H11, 6D2, JG22, and C4 [all from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA] were used to label different components of ILM/MCEF sheet cultures by immunofluorescence. Microglial cells were identified with the QH1 mAb, which labels ameboid, ramified, and reactive microglia of quail (Cuadros et al., 1992). The 39.4D5 and H5 mAbs, which recognize antigens islet-1 and vimentin, respectively, were used to reveal retinal ganglion cells (Fischer et al., 2002) and Müller cells (Fischer et al., 2004), which were present in low numbers in some ILM/MCEF sheets. Laminin and agrin, two components of the ILM, were recognized with 3H11 and 6D2 mAbs (Halfter et al., 1997), respectively. MCEF were labeled with JG22 mAb, which was raised against the β1 subunit of avian integrins (Hofer et al., 1990; Hynes, 2004); in the retina, this mAb specifically labels the surface of developing and mature Müller cells and is highly concentrated at their end-feet (Hering et al., 2000). C4 mAb recognizes s-laminin, which is expressed in the retina by Müller cells (Libby et al., 1997; Sánchez-López et al., 2004); it was used to label broken ends of Müller cell radial processes connecting with MCEF.

*TABLE 1. Primary and Secondary Antibodies Used in This Study, with Their Sources, Working Dilutions, and Specificities*

| | Antibody | Source | Working dilution | Specificity | Secondary antibodies used with each primary antibody |
|---|---|---|---|---|---|
| Primary antibodies | QH1 | Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA | 1:4 | Quail microglia | (1), (2), (4) |
| | 39.4D5 | DSHB | 1:100 | Islet-1 (ganglion cells) | (2) |
| | H5 | DSHB | 1:10 | Vimentin (Müller cells) | (2), (3) |
| | 3H11 | DSHB | 1:100 | Laminin (Inner limiting membrane) | (2) |
| | 6D2 | DSHB | 1:20 | Agrin (Inner limiting membrane) | (2) |
| | JG22 | DSHB | 1:20 | Avian integrin β1 subunit | (2), (3) |
| | C4 | DSHB | 1:2 | s-laminin (Müller cells) | (1), (4) |
| Secondary antibodies | 1. Alexa Fluor 488-conjugated goat anti-mouse IgG | Molecular Probes, Eugene, OR | 1:1,000 | Mouse IgG | — |
| | 2. Alexa Fluor 594-conjugated goat anti-mouse IgG | Molecular Probes | 1:1,000 | Mouse IgG | — |
| | 3. Cy3-conjugated Fab fragments of goat anti-mouse IgG | Jackson Immuno Research, West Grove, PA | 1:800 | Mouse IgG | — |
| | 4. FITC-conjugated goat anti-mouse IgG | Sigma, St. Louis, MO | 1:50 | Mouse IgG | — |

For immunofluorescence, ILM/MCEF sheets were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature. After several washes in 0.1 M PBS, they were incubated with normal goat serum (NGS) diluted 1:30 in 1% BSA in 0.1 M PBS (BSA-PBS) for 45 min at room temperature. ILM/MCEF sheets were then incubated overnight with primary mAb supernatant diluted in BSA-PBS at 4°C. Working dilutions of the different primary mAbs are specified in Table 1. After washing with 0.1 M PBS, ILM/MCEF sheets were incubated with secondary antibodies diluted in BSA-PBS at different concentrations (Table 1) for 2 h at room temperature. ILM/MCEF sheets were then washed with 0.1 M PBS and incubated with Hoechst 33342 (Sigma) diluted in 0.1 M PBS (5 μg/mL) for 15 min to reveal cell nuclei. Sheets were washed again with 0.1 M PBS and coverslipped with Fluoromount (Southern Biotech, Birmingham, AL).

All of the above mAbs were used for single immunolabeling. Additionally, double immunofluorescence was carried out by successive QH1 immunolabeling and either JG22 or C4 immunolabeling to simultaneously reveal both microglial cells and underlying MCEF. Care was taken to ensure that the two labelings obtained with double immunofluorescence were equivalent to those seen with single immunofluorescence.

## Mitotic Index

The mitotic index of proliferating microglial cells was determined in ILM/MCEF sheet cultures at the following incubation times: 0, 1, 3, 6, 12, 24, 48, and 96 hiv. Four sheets for each incubation time were processed for QH1 immunolabeling and Hoechst staining as described above. All interphasic and mitotic QH1-positive cells were then counted in each sheet. The mitotic index of microglial cells at each incubation time was considered to be the percentage of mitotic QH1-positive cells with respect to the total number of QH1-positive cells counted in the four ILM/MCEF sheets analyzed for each incubation time.

## Cell Viability

Cell viability in cultured ILM/MCEF sheets was analyzed by double labeling with Hoechst 33342 and propidium iodide (Sigma) as previously described (Courtney et al., 1997). Hoechst 33342 and propidium iodide (both at 5 μg/mL) were added to the culture medium at 25 min before the end of the incubation time. Immediately after their incubation, ILM/MCEF sheets were fixed and QH1-immunolabeled as described above. Nuclei from dead or dying cells were labeled by both Hoechst 33342 and propidium iodide, whereas healthy cells excluded propidium iodide and showed only the Hoechst 33342-label.

### Experimental Treatments of ILM/MCEF Sheet Cultures

One aim of this study was to analyze interactions between cultured microglial cells and the substrate. Information on the mechanisms of these interactions was obtained by experimental treatments of cultured ILM/MCEF sheets with either GM6001 (Calbiochem, La Jolla, CA) or cytochalasin D (Sigma). GM6001 is a broad-spectrum metalloproteinase inhibitor (Webber et al., 2002) and was used to test the possibility that interactions between microglial cells and substrate were caused by metalloproteinase secretion from microglial cells. Cytochalasin D is a cell-permeable fungal toxin that inhibits cell motility by actin filament depolymerization. Therefore, cytochalasin treatment was used to examine the effects of microglial cell motility inhibition on the ILM/MCEF substrate.

In experiments with GM6001, ILM/MCEF sheets were attached on PLL-coated glass coverslips as described above and then incubated with either 20 or 100 μM GM6001 in SC-DMEM for variable time periods. To obtain these final concentrations, solutions of either 10 or 50 mM GM6001 in dimethylsulfoxide (DMSO, Sigma) were diluted in SC-DMEM at 1:500. GM6001-treated ILM/MCEF sheets were fixed at 0, 1, 3, 6, 12, 24, 48, and 96 hiv. Control ILM/MCEF sheets were incubated for the same times in SC-DMEM containing DMSO (dilution 1:500).

In experiments with cytochalasin D, a final concentration of 2 μM in SC-DMEM was used. This concentration was obtained from a solution of 2 mM cytochalasin D in DMSO that was diluted in SC-DMEM at 1:1,000. Experimental ILM/MCEF sheets were incubated with cytochalasin-containing medium for 6 or 24 hiv and then fixed. ILM/MCEF sheets incubated for the same times in SC-DMEM containing DMSO (dilution 1:1,000) were used as controls.

In both experiments, control and experimental sheets were examined after immunolabeling with the mAbs JG22 and QH1, as described above, to reveal the MCEF substrate and microglial cells, respectively.

## RESULTS

Incubation of ILM/MCEF sheets with SC-DMEM yielded cell cultures highly enriched in microglial cells. Although virtually all cells in most ILM/MCEF sheets were QH1-positive microglial cells, some QH1-negative nonmicroglial cells (ganglion cells or Müller cells) were occasionally present. Ganglion cells were recognized by their small size and islet-1 labeling and Müller cells by their large size, flattened shape, large lobed nucleus, and well-developed vimentin cytoskeleton (not shown). The propidium iodide cell viability technique showed that ganglion cells were nonviable in cultured ILM/MCEF sheets after 48 hiv. By contrast, the Müller cells observed in cultured sheets grew, forming a monolayer in small areas of the culture.

Only ILM/MCEF sheet cultures containing negligible numbers of nonmicroglial cells were selected for observations and experiments in the present study. Microglial cells in these cultures were not present outside ILM/MCEF sheet boundaries and were heterogeneously distributed on the sheet surface, with some areas showing numerous microglial cells and others none.

### Morphological Changes of Cultured Microglial Cells

ILM/MCEF sheets fixed at 0 hiv showed microglial cells adhered on their surface, most of which were bipolar in shape with thick processes emerging from their poles (Figs. 1A and 2A). They lacked the lamellipodia possessed by ameboid microglial cells in the intact retina (Marín-Teva et al., 1998), which apparently retracted during the explantation of ILM/MCEF sheets.

Microglial cells on ILM/MCEF sheets cultured for 4 days in SC-DMEM showed two phases of morphological change, from 0 to 24 hiv and from 24 to 96 hiv. During the first phase, cells very rapidly rounded, losing their bipolar shape (Fig. 1B), with no further modifications during the first 24 hiv (Figs. 1C–F), when they were generally seen as single cells, without aggregation. During the second phase, microglial cells significantly increased in size and progressively aggregated from 48 hiv on (Figs. 1G–I). Observations with phase contrast microscopy confirmed the size increase after 24 hiv and also showed a major increase during this phase in the number and size of cytoplasm vesicles, probably derived from phagocytic activity (compare J with K in Fig. 1).

Cells were already rounded within 15 min of culture (Fig. 1B), frequently bearing thin projections of variable length (Fig. 2B) reminiscent of retracting cell processes. At the end of the rounding process, microglial cells contained an ovoid nucleus displaced to the cell periphery. During the first phase of culture (0–24 hiv), two morphological types of microglial cells were seen: small erect cells and large flattened cells (Figs. 2C–E). These two microglial cell phenotypes were already evident at ¼ hiv (Fig. 2C). The small erect cells were rounded, with both diameter and height of 10–16 μm; they were strongly QH1-immunoreactive and eventually bore short, thin filopodia (Fig. 2D, asterisk). The large flattened cells were round (Fig. 2D, arrowhead) or moderately elongated (Figs. 2C,E, arrowheads) with diameter of 20–30 μm and height of 6–8 μm. Their QH1 labeling was less intense than that of the small erect cells. The observation of intermediate forms between small erect and large flattened cells suggested that they belong to the same cell type with changing morphological features.

Large flattened (Fig. 2F) and smaller erect (Fig. 2G) cells continued to be seen during the second phase of culturing ILM/MCEF sheets but their size was considerably increased (compare large flattened cells in Figs. 2D,E with those in Figs. 2F,I or small erect cells in Figs. 2D,E with those in Fig. 2G). In fact, small erect cell diameters were now 15–20 μm and large flattened cell diameters were 30–50 μm. Occasionally, giant cells with diameter of $\geq$70 μm were also seen.

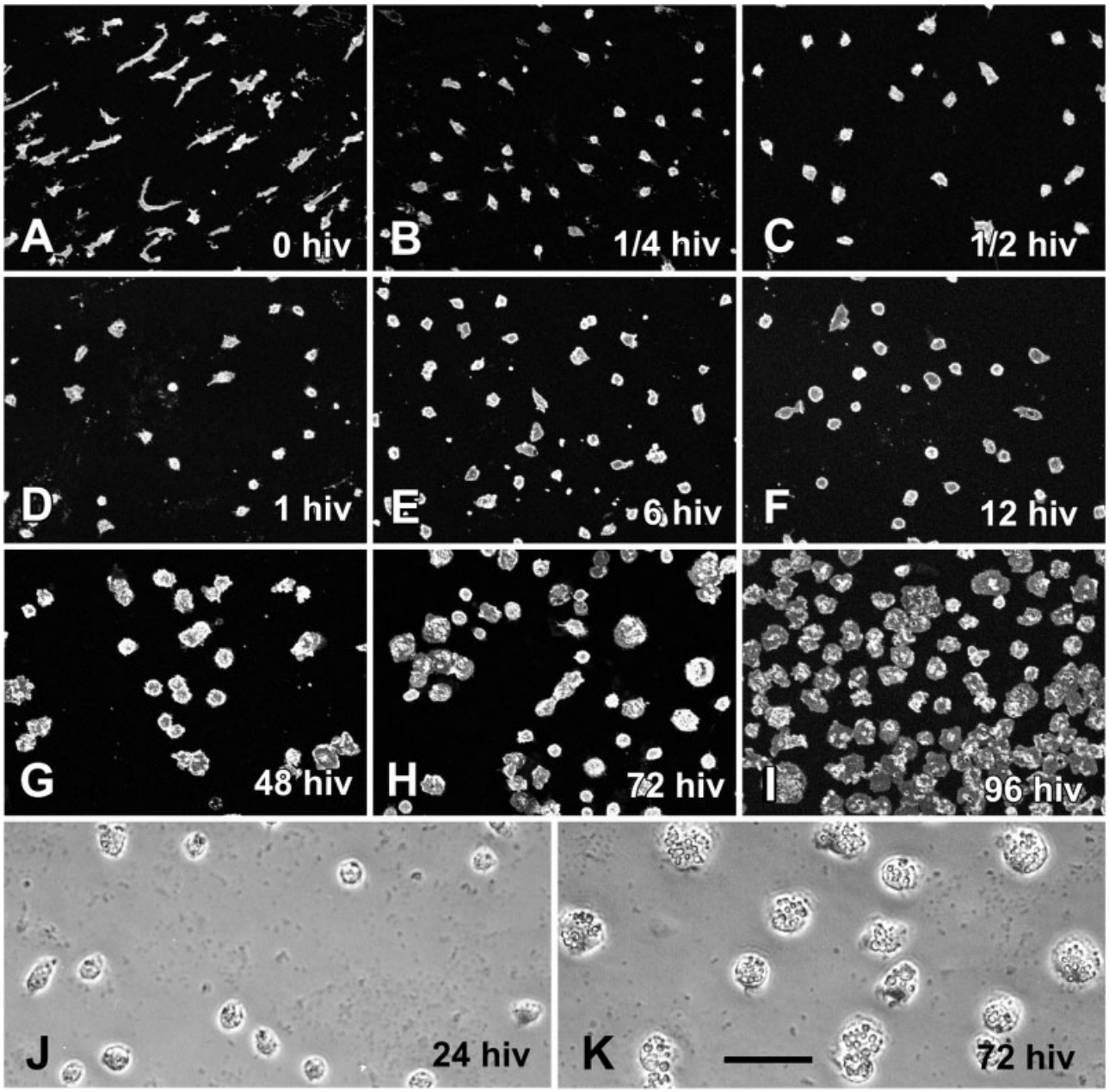


Fig. 1. Morphological features of microglial cells cultured on ILM/MCEF sheets at different incubation times from the beginning of in vitro incubation (0 hiv) to 96 hiv. Microglial cells are labeled with QH1 immunofluorescence (**A–I**) or seen with phase contrast microscopy (**J–K**). They are bipolar with thick processes at 0 hiv (A) and become rounded from ¼ hiv onward (B–I). Microglial cells are small, single and have scarce vesicles in their cytoplasm at incubation times before 24 hiv (B–F and J) whereas they are larger with a tendency to aggregate and accumulate numerous cytoplasmic vesicles from 24 hiv onward (G–I and K). Scale bar: 100 μm for A-I; 46 μm for J and K.

The above results were confirmed in a quantitative study (see Fig. 3), in which the mean area of microglial cell profiles was estimated at incubation times during the first (6, 12, and 24 hiv) and second (48, 72, and 96 hiv) phases of culture. The cell profile area, i.e., the surface area of adhesion of microglial cells to the substrate, was significantly larger in the second culture phase than in the first one (see Fig. 3).

Both small erect and large flattened cells could be either spinous or smooth. Spinous cells emitted numerous substrate-adhered thin spines from their surface (Fig. 2H) whereas smooth cells had a more or less ruffled surface without spines (Figs. 2F,I).

### Changes in the Substrate: Phagocytosis of MCEF

At 0 hiv, the substrate to which ameboid microglial cells remained adhered was similar to that in the intact retina, as revealed with phase contrast microscopy. It was apparently composed of the ILM (basal lamina of the retina) covered by a continuous carpet of MCEF. The short vitreal ends of Müller cell radial processes, which were broken

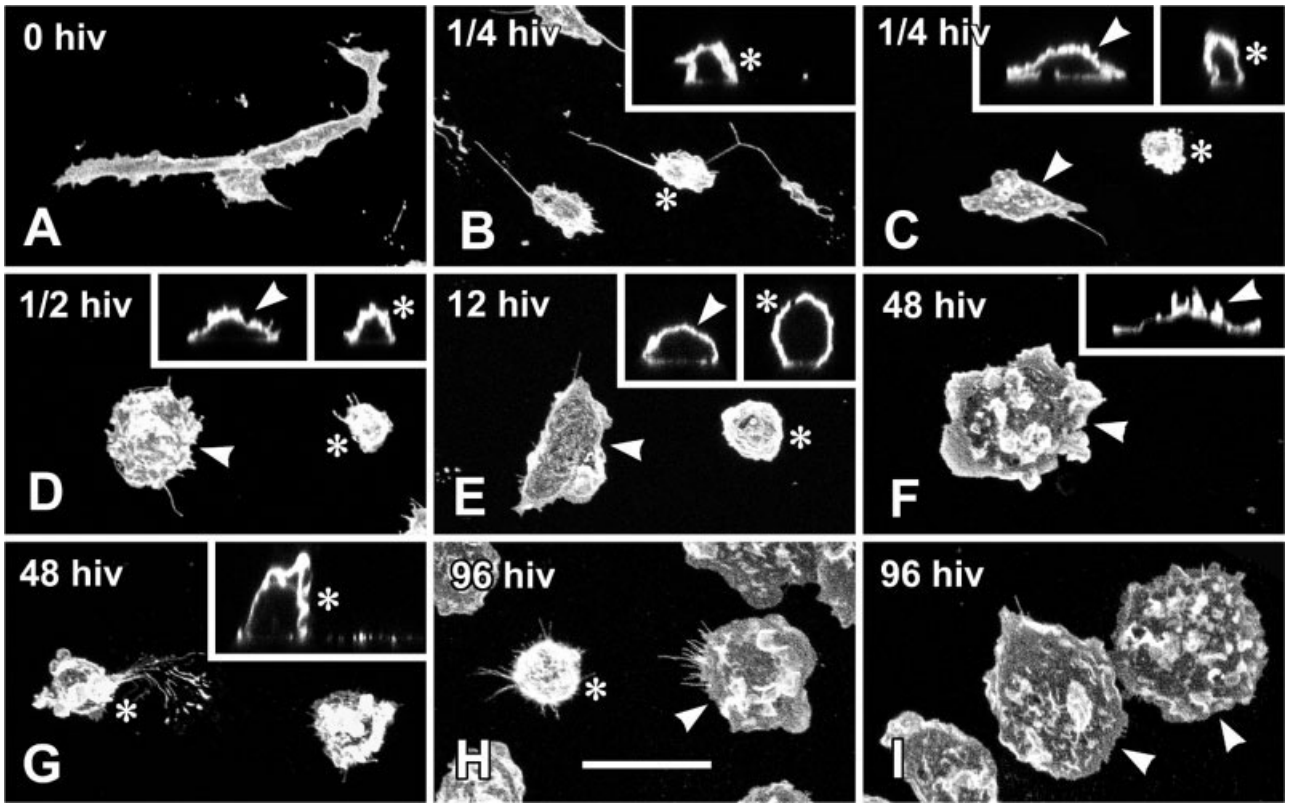


Fig. 2. Morphological types of QH1 immunostained microglial cells cultured on ILM/MCEF sheets as seen in projection images generated from stacks of horizontal optical sections obtained with confocal microscopy. At the beginning of in vitro incubation (0 hiv), they show a bipolar shape with thick processes (**A**). At ¼ hiv, microglial cells show a rounded shape (**B**, **C**), frequently with thin filaments (B) that are remains of thick processes. Similar morphological features are maintained from ¼ hiv to 24 hiv (**C**–**E**) and larger cells are seen after 24 hiv (**F**–**I**). Two general types of cultured microglial cells are seen at both incubation times: small erect (asterisks in C–E, G, and H) and large flattened (arrowheads in C–F, H, and I) cells. Inserts in B–G show vertical confocal optical sections of the cells indicated by asterisks and arrowheads. Note that large flattened cells have a ruffled surface and a much larger area of adhesion to the substrate versus small erect cells. Thin spines radiate from the cell surface in some microglial cells irrespective of their morphological type (H). Scale bar: 30 μm.

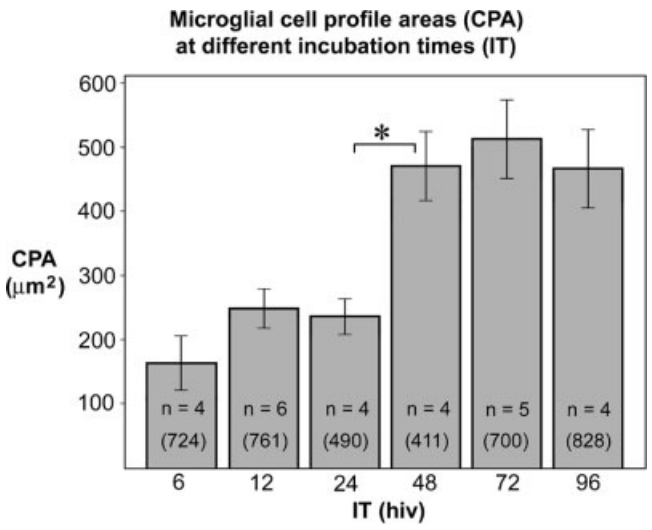


Fig. 3. Cell profile areas (CPA) of cultured microglial cells at different incubation times (IT). Data are presented as the mean ± SEM of values obtained in at least four ILM/MCEF sheets at each IT. Numbers of ILM/MCEF sheets used (n) and total numbers of measured cells (in parentheses) at each IT are shown within each bar. Asterisk depicts statistically significant differences ($P < 0.01$) of data between 24 and 48 hiv. No significant differences were found between 6, 12, and 24 hiv or between 48, 72, and 96 hiv.

during the isolation of ILM/MCEF sheets, were arranged in rows (Fig. 4A). At 1 hiv, the vitreal ends of Müller cell radial processes continued to be observed, but they had lost their original arrangement and were irregularly distributed in zones of ILM/MCEF sheets containing microglial cells (Fig. 4B). As the in vitro incubation advanced, Müller cell radial processes progressively disappeared in the areas around microglial cells (Fig. 4C). These observations suggested that the underlying substrate of the microglial cells underwent major changes during incubation. Different components of this substrate, including basal lamina molecules and MCEF, were then studied with immunocytochemistry to reveal their possible changes during the incubation period.

Components of the ILM, such as laminin (Figs. 4D,E) and agrin (Figs. 4F,G), remained unaltered on the entire ILM/MCEF sheet surface throughout the incubation time, including regions occupied by microglial cells. By contrast, the MCEF carpet underwent evident changes (see Fig. 5), apparently caused by the action of microglial cells. These changes were only seen in areas around microglial cells, and MCEF were unchanged in microglia-devoid zones. At 0 hiv, cultured ILM/MCEF sheets were covered by a continuous carpet of JG22-positive MCEF (Fig. 5A). At 1 hiv,

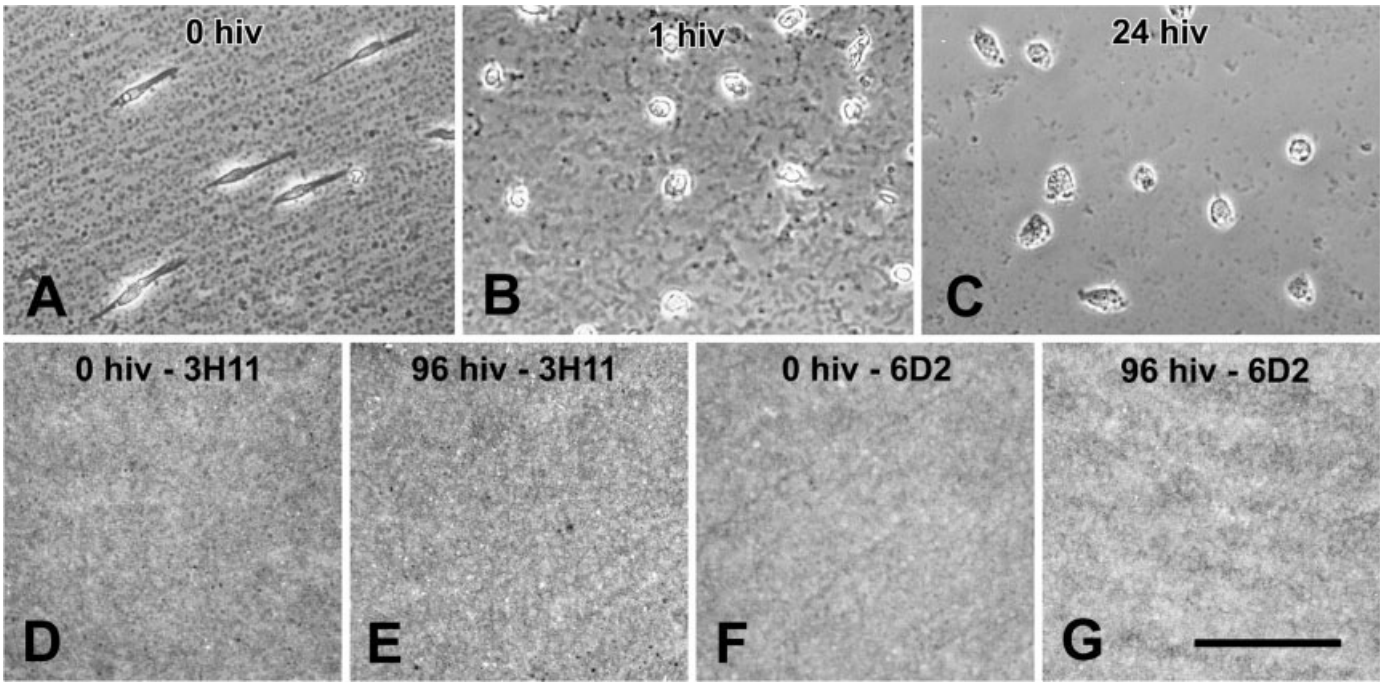


Fig. 4. Appearance of the substrate in cultured ILM/MCEF sheets as seen with phase contrast microscopy (**A**–**C**) and with either anti-laminin (3H11, **D**, and **E**) or anti-agrin (6D2, **F** and **G**) immunofluorescence. At 0 hiv, vitreal ends of broken Müller cell processes are arranged in rows between microglial cells (A). These vitreal ends of Müller cell processes are irregularly distributed at 1 hiv (B) and have disappeared at 24 hiv (C). Components of the ILM laminin and agrin remain unaltered throughout the incubation time, showing the same appearance at 0 hiv (D and F) and 96 hiv (E and G). Scale bar: 78 μm for A–C; 50 μm for D–G.

small JG22-negative areas began to be seen on the MCEF carpet coinciding with sites occupied by microglial cells (Fig. 5B). This indicated that these cells swept the MCEF located beneath them. Small accumulations of JG22-positive material (arrows in Fig. 5B) were seen next to each microglial cell, apparently as a consequence of the MCEF sweep. With the advance of the culture, JG22-negative areas around each microglial cell progressively increased in size (Figs. 5C–F). Between 3 and 12 hiv, increasingly large accumulations of apparently phagocytosed JG22-positive material were often observed next to or on microglial cells (arrows in Figs. 5C,D). Confocal microscopy revealed that microglial cells spread beneath accumulations of JG22-positive material (Figs. 5G,G′,G″) before their cytoplasm was filled with numerous JG22-positive phagosomes (Figs. 5H,H′,H″). From 24 hiv on, the cell surface became JG22-positive, allowing microglial cell profiles to be clearly distinguished in JG22 immunostained ILM/MCEF sheets (Figs. 5E,I,I′,I″,J,J′,J″). JG22 labeling of microglial cells persisted from 24 hiv to the end of the incubation time (96 hiv), although flattened cells showed a discontinuous immunostaining on their cell surface with intensely labeled focal spots (Figs. 5F,K,K′,K″).

Alterations of the substrate around microglial cells were not caused by metalloproteinase secretion, which was demonstrated by adding different concentrations of GM6001, an inhibitor of metalloproteinase activity, to the incubation medium of cultured ILM/MCEF sheets (see Fig. 6). MCEF-free areas around microglial cells were not present in ILM/MCEF sheets cultured for 6 and 24 hiv in presence of 2 μM cytochalasin D (see Fig. 7). Since cytochalasin D alters the organization of the actin cytoskeleton and consequently inhibits cell movement, these results corroborated the hypothesis that microglial cells swept the MCEF located

Fig. 5. Non-confocal (**A**–**F**) and confocal (**G**–**K**) fluorescence images showing changes in the carpet of JG22 immunostained MCEF (red) during incubation time in microglial cell-containing areas of cultured ILM/MCEF sheets. Cell nuclei (blue) are stained with Hoechst. A: A continuous carpet of JG22-positive MCEF is seen at 0 hiv. B: At 1 hiv, sites occupied by cultured microglial cells show small MCEF-free areas bordered by accumulations of JG22-positive material (arrows). C and D: At 6 and 12 hiv, MCEF-free areas are larger than at 1 hiv (B) and accumulations of JG22-positive material (arrows) are located next to or on cell nuclei, apparently in the process of being incorporated within microglial cells. E: At 24 hiv, extensive MCEF-free areas continue to be present around microglial cells, whose surfaces are now JG22-positive. F: At 96 hiv, flattened microglial cells (arrowheads) show a weaker JG22 labeling than at 24 hiv (see E) except for some intensely labeled spots. Extensive MCEF-free areas continue to be seen around microglial cells. G/G″–K/K′/K″: Confocal fluorescence images showing the proposed sequence of events before, during, and after phagocytosis of JG22 material by microglial cells. G–K are projection images from stacks of optical sections in the horizontal plane (*xy*) throughout the z-dimension of cells, G′–H′ are single horizontal optical sections of cells, and G″–H″ are single optical sections in the vertical (*xz*) plane at the location indicated by broken lines in G–K. G/G′/G″: an accumulation of JG22-positive MCEF on a JG22-negative microglial cell, as identified by its Hoechst-labeled nucleus. H/H′/H″: microglial cell with a weakly JG22-positive cytoplasm containing several strongly JG22-positive phagosomes. I/I′/I″–J/J′/J″: interphasic (I/I′/I″) and mitotic (J/J′/J″) JG22-positive microglial cells showing strong immunolabeling on their surface and some immunolabeled vesicles in their cytoplasm, as revealed in *xy* and *xz* optical sections. K/K′/K″: flattened microglial cell showing JG22-labeled spots on its surface and JG22-labeled cytoplasmic vesicles. Scale bar: 50 μm for A-F; 30 μm for G/G′/G″-K/K′/K″.

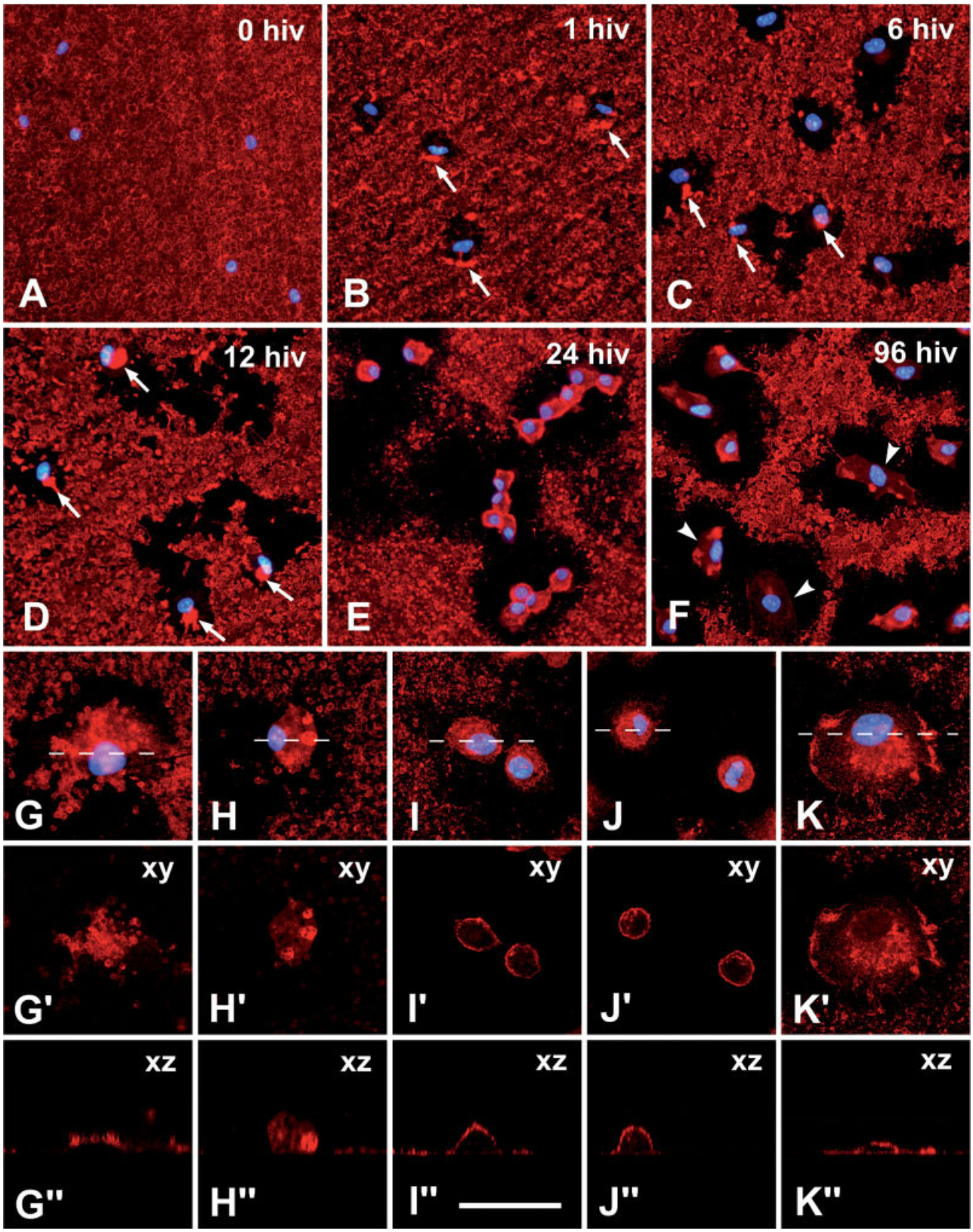
0 hiv
1 hiv
6 hiv
A
B
C
12 hiv
24 hiv
96 hiv
D
E
F
G
H
I
J
K
xy
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Figure 5.

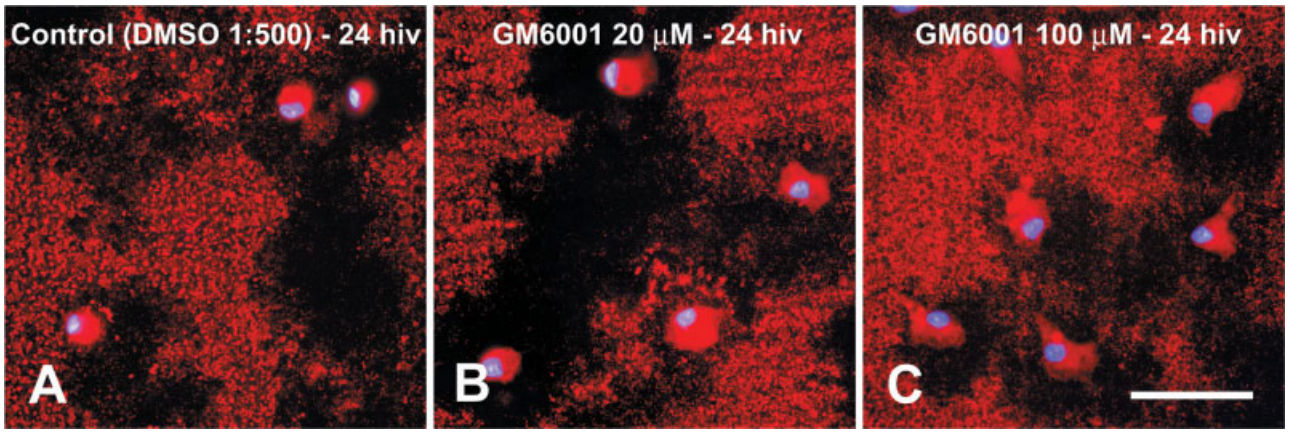


Fig. 6. Treatment of cultured ILM/MCEF sheets with GM6001, an inhibitor of metalloproteinase activity. The MCEF carpet (red) and microglial cell nuclei (blue) are labeled by JG22 immunofluorescence and Hoechst staining, respectively. At 24 hiv, no differences are seen between control and experimental specimens, with similar MCEF-free areas around microglial cells in both control (**A**) and GM6001-treated ILM/MCEF sheets at concentrations of either 20 (**B**) or 100 μM (**C**). Scale bar: 40 μm.

beneath them in the course of their movement. Microglial cells in cytochalasin-treated cultures (Figs. 7C,G) were smaller and more rounded than those in control cultures (Figs. 7D,H).

Phagocytosis of Müller cell materials by microglial cells from 3 hiv on was demonstrated by double immunostaining of ILM/MCEF sheets with QH1 and C4 mAbs at different incubation times. The C4 mAb labels Müller cell radial processes but not MCEF (Sánchez-López et al., 2004). At 0 hiv, vitreal ends of these processes were seen in ILM/MECF sheets as C4-positive dots with diameter of 1–2 μm (Fig. 8A). Scarce C4-labeling was seen within microglial cells at this culture time. At 6 hiv, C4-positive dots were smaller and less numerous on the substrate around microglial cells, while C4-labeling began to be clearly seen within microglial cells (Fig. 8B). At 12 hiv, very few C4-positive dots were present in the substrate surrounding microglial cells, which were themselves filled with C4-positive material (Fig. 8C). These results showed that the disappearing vitreal ends of C4-positive Müller cell radial processes were phagocytosed by microglial cells.

## Proliferation and Aggregation of Cultured Microglial Cells

Microglial cells were generally observed as single cells until 24 hiv, i.e., during the first culture phase (Figs. 1A–F). By contrast, pairs of cells were frequently seen at 48 hiv (Figs. 1G and 9A), apparently as a consequence of the nonseparation of the two daughter cells after mitosis of proliferating cells. There was a progressive aggregation of microglial cells in cultured ILM/MCEF sheets from 48 hiv on (Figs. 1G–I), and cell clusters were observed at 72 (Figs. 1H and 9B) and 96 hiv (Fig. 1I). A few binucleated microglial cells of both small erect and large flattened type were also present from 48 hiv on (Fig. 9C), suggesting that no cytokinesis had taken place in some dividing cells.

Aggregation apparently resulted from an intense proliferation of microglial cells. In fact, mitotic cells were seen in cultured ILM/MCEF sheets throughout the incubation time. They were scarce between 0 and 3 hiv, as revealed by mitotic indexes of $<4$, but were more abundant (mitotic indexes of around 8) between 6 and 72 hiv (see Fig. 10). At 96 hiv, when strong cell aggregation was present in microglial cell cultures (Fig. 1I), the mitotic index returned to the values observed at 3 hiv (see Fig. 10).

Most mitotic cells were of the small erect type (Figs. 9D, E), although mitotic cells of the large flattened type were also occasionally seen (Fig. 9F). Small erect mitotic cells were generally larger during the second phase of culture (from 24 hiv on) than during the first day of culture (compare D with E in Fig. 9). This matched the general increase in cell size from 24 hiv on, as described above.

## Death of Damaged Microglial Cells on Cultured ILM/MCEF Sheets

In the course of the preparation of ILM/MCEF sheets, migrating microglial cells were subjected to mechanical trauma and some of them underwent amputation of cell portions. Sometimes, a considerable portion of the microglial cell was removed, and only a small nucleus-free cell fragment remained on the cultured ILM/MCEF sheet (arrow in Figs. 11A,A′). These fragments survived transiently in culture, undergoing similar morphological changes to those observed in whole microglial cells (arrows in Figs. 11B,B′,C,C′). They were not longer observable at 48 hiv, suggesting that they degenerated.

According to propidium-iodide labeling results, amputation of a considerable proportion of cytoplasm appeared to induce the death of some nucleus-retaining microglial cells. In contrast to healthy cells, dying microglial cells incorporated propidium iodide into their nucleus (Figs. 11D, D′,D″) and were seen between 3 and 24 hiv but not at 48 hiv.

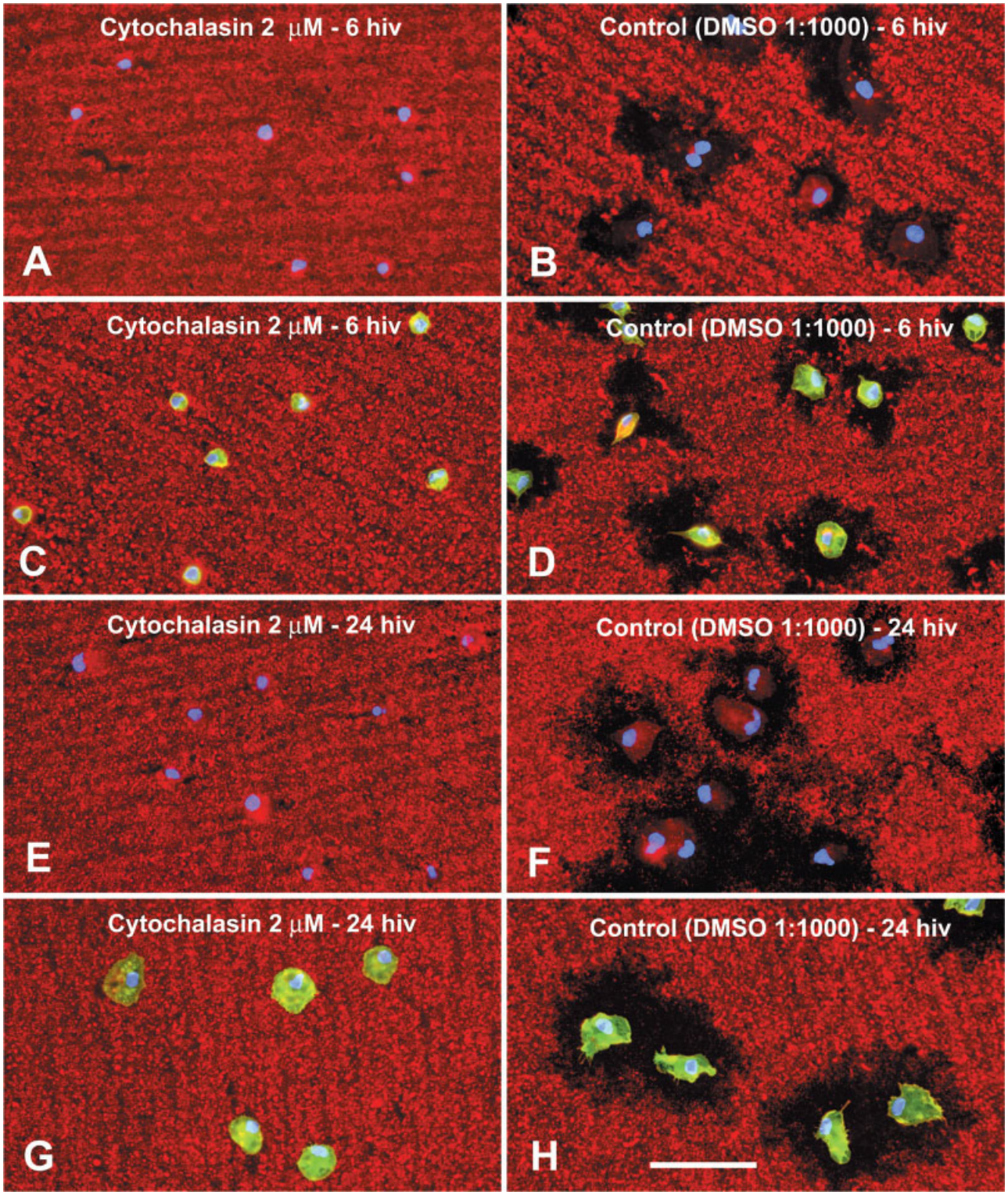


Fig. 7. Treatment of cultured ILM/MCEF sheets with 2 μM cytochalasin D. Microglial cell nuclei are stained with Hoechst (blue). Simple JG22 (**A**, **B**, **E**, and **F**) or double JG22/QH1 (**C**, **D**, **G**, and **H**) immunofluorescence reveals MCEF (red) only or both MCEF (red) and microglial cell profiles (green), respectively. MCEF-free areas are seen around microglial cells in control specimens at 6 hiv (B and D) and 24 hiv (F and H) that are absent in cytochalasin-treated specimens at these incubation times (A, C, E, and G). Scale bar: 50 μm.

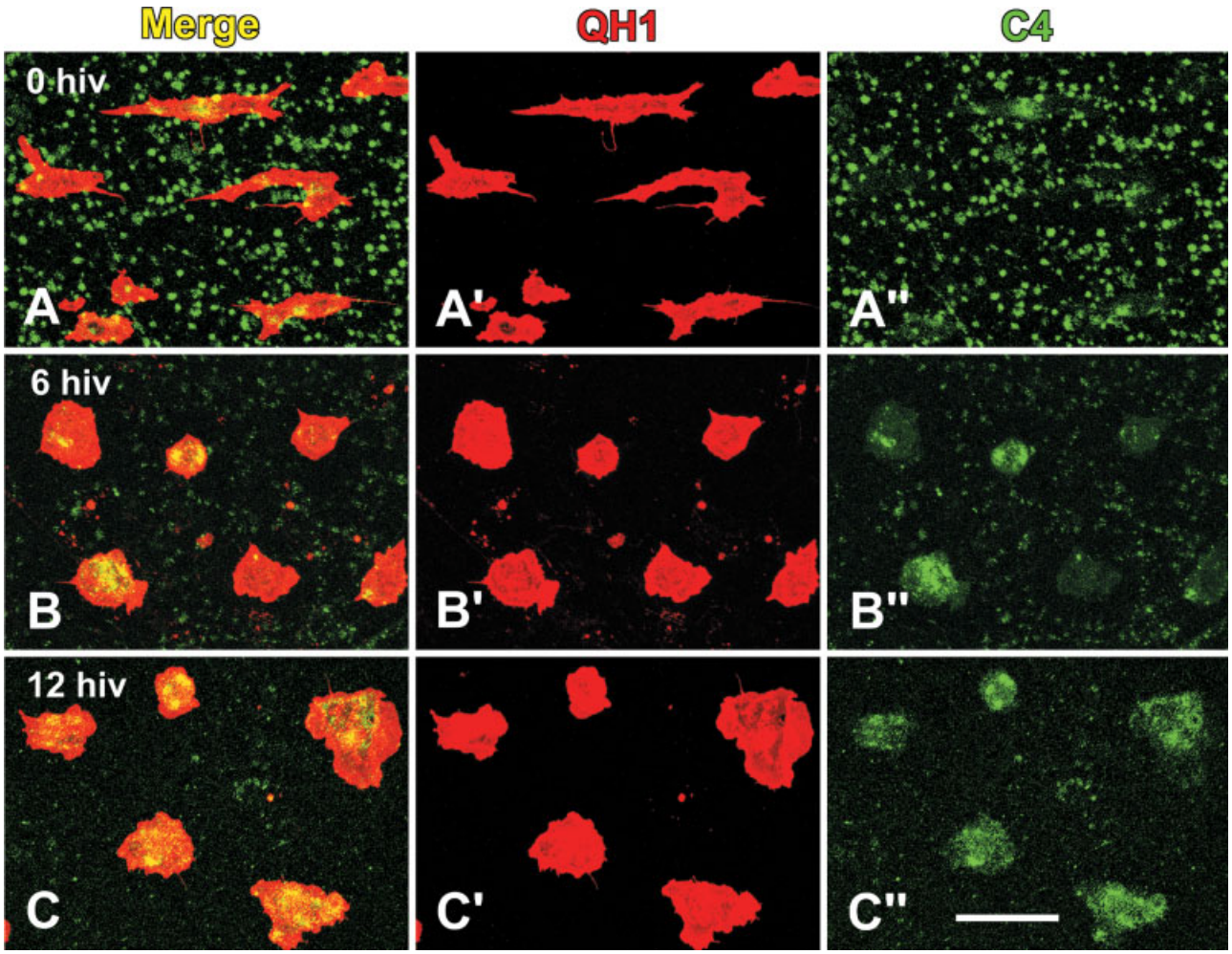


Fig. 8. Phagocytosis of Müller cell material by microglial cells as revealed by double QH1/C4 immunofluorescence of cultured ILM/MCEF sheets labeling microglial cell profiles (red, **A′**–**C′**) and Müller cell processes (green, **A″**–**C″**). The corresponding merged images are seen in A–C. C4-positive Müller cell processes are uniformly distributed at 0 hiv (A) and progressively disappear at 6 (B) and 12 (C) hiv. In parallel, microglial cells are progressively filled with C4-positive material, suggesting that they phagocytose Müller cell processes. Scale bar: 30 μm.

### Migration of Cultured Microglial Cells

Between 24 and 96 hiv, a trail of QH1-positive thin filaments was seen behind some round microglial cells cultured on ILM/MCEF sheets (Fig. 12A). These morphological observations suggested the migration of some microglial cells, since the presence of a trail behind the cell is a characteristic of cells migrating on ILM/MCEF sheets (Halfter et al., 1988). In addition, some microglial cells had a cell portion containing the nucleus that was joined by a thin process to a second portion bearing spine-like projections (Figs. 12B,B′). These cells were apparently caught in the act of retraction of the rear cell portion. Sometimes, pairs of microglial cells appeared to be migrating in opposite directions according to the position of their trails (Fig. 12C), suggesting that they were daughter cells separating after mitosis. QH1-positive trails could be seen behind both small erect (Figs. 12A,C) and large flattened (Figs. 12D,E) cells, suggesting that both cell phenotypes were able to migrate. Irrespective of the cell phenotype, all migrating microglial cells that left a trail showed spine-like filaments projecting from their surface at the cell pole facing the trail (Figs. 12A,C–E).

It is noteworthy that cultured microglial cells never moved from the ILM/MCEF sheet to the PLL-coated surface on which it was attached, despite their migratory ability. Accumulations of microglial cells in rows at the edge of the sheet were frequently observed at advanced incubation times (Fig. 12F), suggesting their ability to detect the change of substrate and stop their migration in order to remain on the ILM/MCEF sheet.

## DISCUSSION

Four major conclusions can be drawn from our results. First, ameboid microglial cells can be cultured immediately after the isolation of ILM/MCEF sheets from dissected quail embryonic retinas. Second, micro-

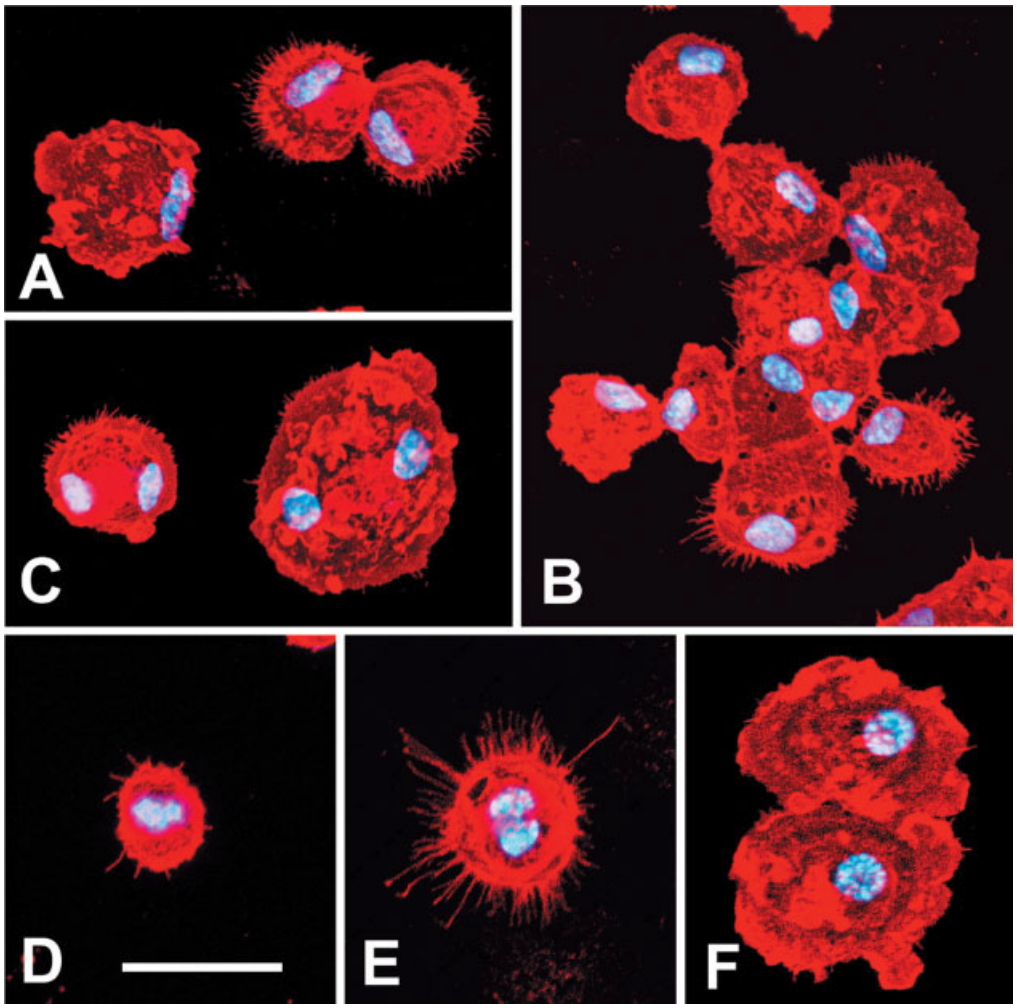


Fig. 9. Proliferation and aggregation of microglial cells cultured on ILM/MCEF sheets for 72 (**A–C**), 6 (**D**), and 96 (**E** and **F**) hiv. Microglial cell profiles (red) and cell nuclei (blue) are revealed by QH1 immunofluorescence and Hoechst staining, respectively. A: A pair of microglial cells in close contact one another. B: Cluster of microglial cells. C: Two binucleated microglial cells, one small and erect (on the left) and the other large and flattened (on the right). D and E: Small erect microglial cells in mitosis showing thin spine-like projections radiating from their cell surface. Note that the mitotic cell in D (6 hiv) is smaller than that in E (96 hiv), a fact frequently observed when comparing mitotic cells before and after 24 hiv. F: Large flattened microglial cell in telophase. Scale bar: 30 μm for A-C; 25 μm for D–F.

glial cells in these cultures phagocytose the MCEF on which they were initially attached and become adhered to the unchanged ILM. Third, the β1 integrin subunit begins to be expressed on the surface of microglial cells after they phagocytose the MCEF. Finally, cultured microglial cells move on ILM/MCEF retinal sheets by a different mechanism to that observed in situ in the embryonic retina.

### Culture of Microglial Cells on ILM/MCEF Sheets

To date, most methods of microglial cell culture have obtained primary mixed cell cultures after tissue dissociation, generally from neonatal rodent brain or retina (Dobrenis, 1998; Giulian and Baker, 1986; Roque and Caldwell, 1993; Santambrogio et al., 2001; Wang et al., 2005). These primary mixed cell cultures are maintained for 7–14 days and microglia are then isolated by shaking because of their weak adherence to the cell substrate. Repeated steps of cell resuspension and adhesion are required to obtain a pure microglial cell culture. In this study, a novel approach was adopted for the rapid isolation and in vitro culture of ameboid microglial cells. The isolation of these cells was based on the method of Halfter et al. (1987) as adapted by our group to study morphological features of ameboid microglial cells in fixed quail embryonic retinas (Marín-Teva et al., 1998, 1999). Mechanical disruption of the fixed retina mounted between two adhesive substrates allows the isolation of ILM/MCEF sheets on which numerous migrating ameboid microglial cells are adhered. In the present study, this isolation method was applied to fresh retinas in order to culture living microglial cells adhered on the ILM/MCEF sheets. These sheets were previously isolated from nonfixed avian retinas by Halfter et al. (1988, 1990) in order to study the migratory behavior of different cell types that were subsequently seeded onto the sheets. In Halfter's studies, ILM/

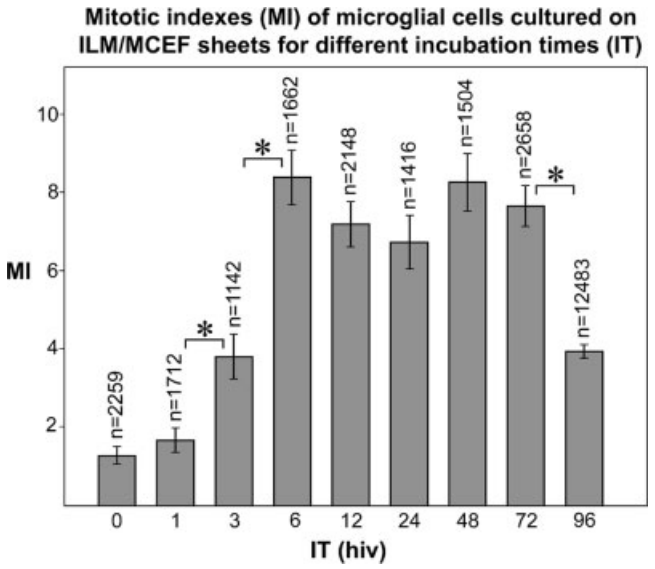


Fig. 10. Mitotic indexes (MI) of cultured microglial cells at different incubation times (IT) obtained from cell counts on four ILM/MCEF sheets at each IT. Vertical lines represents SEM; numbers of counted cells (n) are shown above each bar. Asterisks depict statistically significant differences ($P < 0.01$) of data between 1 and 3 hiv, 3 and 6 hiv, and 72 and 96 hiv. Mitotic indexes are less than 4 from 0 to 3 hiv, double this value from 6 to 72 hiv, and return to less than 4 at 96 hiv.

MCEF sheets were devoid of microglial cells because they were isolated from quails at 6–7 days of incubation, when microglial cells are not yet present within the retina.

Our technique for isolating ameboid microglial cells is based on their strong adhesion to MCEF, whereas the traditional approach relies on their weak adhesion to the cell substrate. This apparent contradiction can be explained by the difference in the functional state of microglia at the time of their isolation. Thus, our group previously demonstrated (Sánchez-López et al., 2005) that ameboid microglial cells migrating tangentially on MCEF in the developing retina are in a nonactivated state and are only activated when subjected to appropriate stimuli (e.g., retinal injury). In contrast, microglial cells in primary mixed cell cultures incubated in a serum-containing medium are in an activated state (Hurley et al., 1999; Lee et al., 2002; Slepko and Levi, 1996). The adhesion ability of microglia might be modified by changes in their functional state. In our method, the nonactivated ameboid microglia would be strongly adhered to the substrate at the time of isolation and remain on the isolated ILM/MCEF sheet. In primary mixed cell cultures, the activated microglia would be weakly adhered, allowing their detachment by shaking.

Our microglial cell culture system has several advantages over methods based on the isolation of microglia

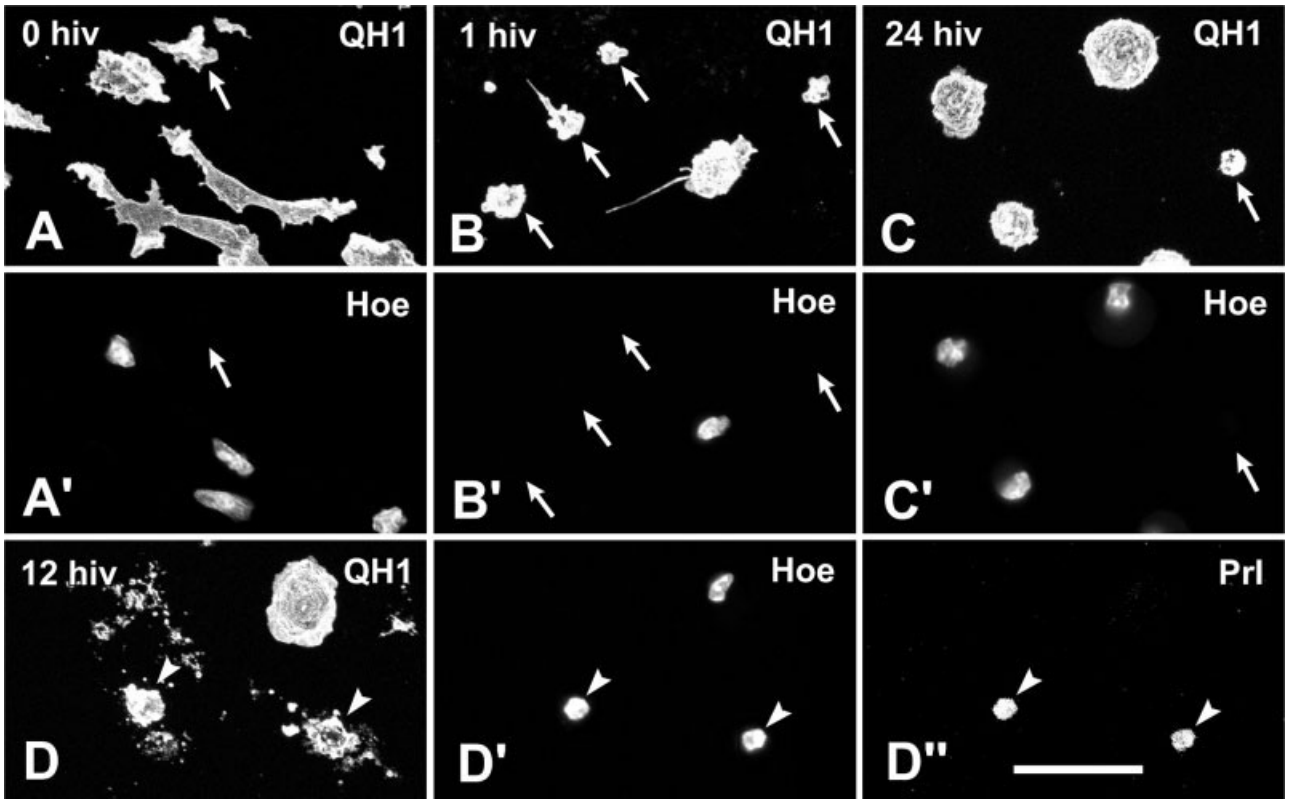


Fig. 11. Mechanical trauma and death of microglial cells cultured on ILM/MCEF sheets. Each micrograph series (**A**/**A**′–**D**/**D**′/**D**″) shows the same microscopic field as seen with QH1 immunocytochemistry (QH1, A–D), Hoechst staining (Hoe, A′–D′), and propidium iodide labeling (PrI, D″). A/A′–C/C′: Nucleus-free microglial cell fragments (arrows) in ILM/MCEF sheet cultures at 0 (A), 1 (B), and 24 (C) hiv. Arrows in Hoechst staining micrographs (A′–C′) indicate the sites occupied by nucleus-free cell fragments. D/D′/D″: Dying microglial cells (arrowheads in D) at 12 hiv as revealed by double labeling of their nuclei with Hoechst (arrowheads in D′) and propidium iodide (arrowheads in D″).

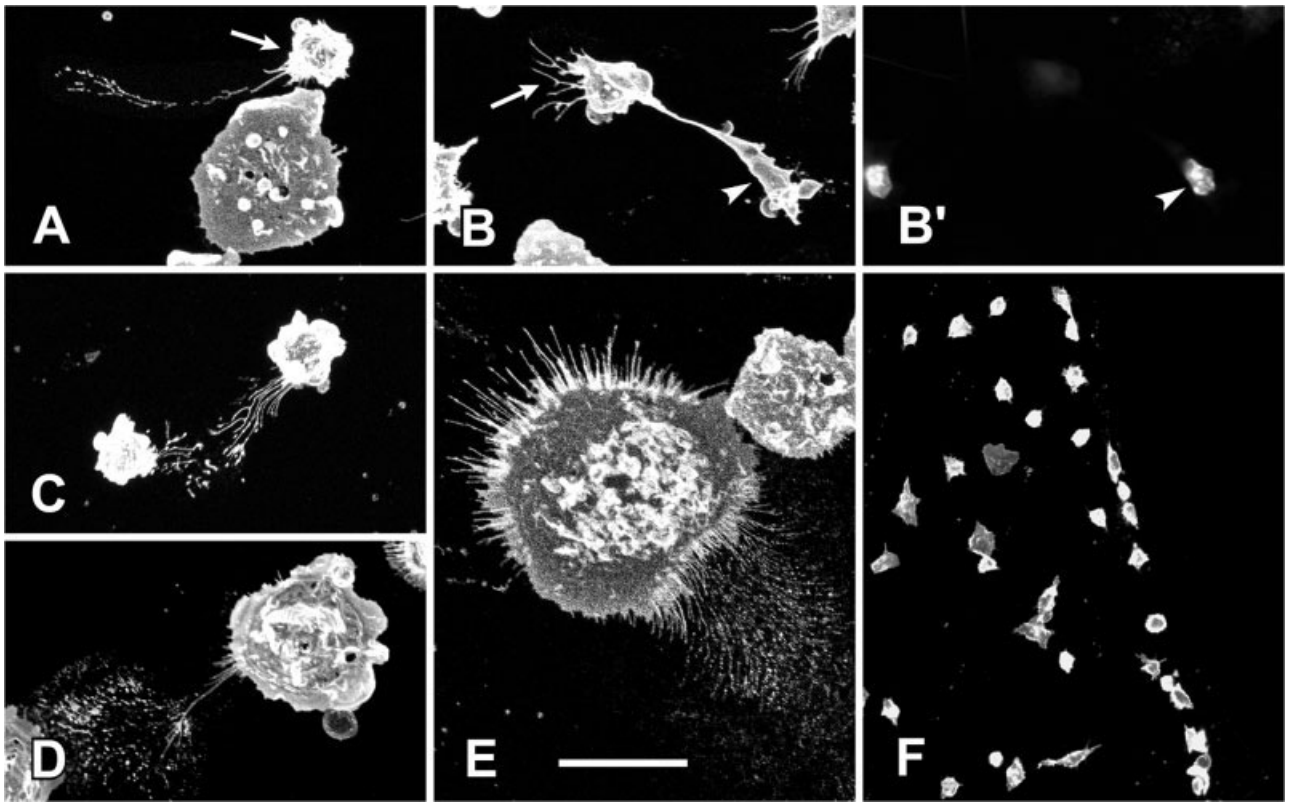


Fig. 12. Morphological evidence of the migratory activity of microglial cells cultured on ILM/MCEF sheets. QH1 immunofluorescence. **A**: Trail of QH1-positive thin filaments behind a small erect microglial cell (arrow). Spine-like filaments are present at the cell pole facing the trail. **B**/**B′**: Elongated microglial cell with two portions joined by a thin process. One cell portion (arrowhead in B) contains a Hoechst-stained nucleus (arrowhead in B′) whereas the other is nucleus-free and bears thin projections (arrow in B). **C**: A pair of small erect microglial cells with trails of QH1-positive filaments facing each other. **D** and **E**: Large flattened microglial cells showing trails of QH1-positive filaments behind them. Spine-like filaments project from the cell pole facing the trail; the cell in E also shows spinous projections at the opposite pole. **F**: Row of microglial cells at the border of an ILM/MCEF sheet. No microglial cells are present outside the sheet (to the right of the cell row). Scale bar: 30 μm for A-E; 130 μm for F.

from primary mixed cell cultures. Importantly, microglial cells are isolated only a few minutes after being in the intact retina. A much longer time (generally more than a week) passes before microglia are isolated from primary mixed cell cultures and the cells are subjected to influences from treatments with different reagents; therefore, the functional state of the microglial cells may be profoundly affected before the start of their incubation. This problem is avoided by our cell isolation method, which yields microglial cells in a physiological state similar to that in the intact retina when they begin to be cultured. Evidently, these physiological microglial cells will undergo subsequent changes throughout incubation in our culture system. An additional advantage of our method is the major saving in time and material.

Microglial cells isolated and cultured on ILM/MCEF sheets change their shape during the first few minutes of incubation from a bipolar elongated shape with thick branches emerging from their poles (typical of tangentially-migrating ameboid microglia) to a rounded shape with no branches. These changes appear similar to those observed in these cells when activated in response to retinal injury (Sánchez-López et al., 2005). Therefore, our microglial cell culture system provides a potentially useful model system to examine cell mechanisms of ameboid microglial cell activation.

An interesting finding of our study was the presence of two morphological types of cultured microglial cell, here designated small erect cells and large flattened cells. They appear to belong to a single cell type with changing morphology, since intermediate forms were observed. The changing morphology of cultured microglial cells appears to be related to their cell cycle phase, since most mitotic cells were small and erect. Nevertheless, many small erect cells contained an interphasic nucleus, although it was not known whether they were close to mitosis. Irrespective of their morphological type, some microglial cells cultured on ILM/MCEF sheets showed a ruffled surface without spines whereas others had a spine-bearing surface. This conflicts with the claim by Giulian et al. (1995) that a spine-bearing surface is a specific marker for cultured microglia. This disagreement may be explained by differences in both substrate and culture medium. Giulian and colleagues grew rat microglial cells on glass in chemically defined N2 medium, whereas we cultured quail microglial cells on

ILM/MCEF sheets in SC-DMEM. Alternatively, spine-lacking and spine-bearing microglia might correspond to two different types of microglial cells in the retina, as previously proposed (Chen et al., 2002; Dick et al., 1995; Provis et al., 1996; Zhang et al., 1997, 2005).

Some microglial cells die in our culture system as a consequence of mechanical cell trauma in the course of the ILM/MCEF sheet isolation. Various studies have shown death of neurons and glial cells after mechanical trauma in organotypic brain slice culture (Ellis et al., 1995; Lusardi et al., 2004; Slemmer and Weber, 2005). Mechanical trauma of microglial cells also produces small nucleus-free cytoplasm fragments that survive transiently in culture and become round during the first hour in culture, i.e., they undergo morphological changes similar to those of whole microglial cells. Nucleus-free cell fragments retaining motile properties have also been obtained from polymorphonuclear leukocytes in culture (Malawista and van Blaricom, 1987; Mizuno et al., 1996).

### Phagocytosis of MCEF by Microglial Cells

The present study showed that microglial cells cultured on ILM/MCEF sheets phagocytose MCEF but not the underlying basal lamina (i.e. the ILM), to which they therefore become adhered. Phagocytosis of MCEF was demonstrated by the presence of JG22-positive phagosomes (see Fig. 5) and C4-positive material (see Fig. 8) in the microglial cell cytoplasm. JG22 (anti-β1 integrin subunit) and C4 (anti-s-laminin) are mAbs that specifically label Müller cells in the retina. However, the pattern of ILM/MCEF sheet labeling differs between these mAbs. JG22-labeling reveals a continuous carpet of MCEF (Fig. 5A), whereas C4-labeling shows many dots at regular intervals (Fig. 8A). Therefore, JG22 labels the entire surface of MCEF whereas C4 only reveals the broken radial process at the center of each MCEF. However, both mAbs specifically recognize Müller cell material present in the ILM/MCEF sheets at the beginning of the incubation. Thus, the presence of labeled phagosomes within microglial cells at later incubation times shows that their content derives from Müller cells.

MCEF phagocytosis by microglial cells takes place in our culture system from 3 hiv onwards. Before and during phagocytosis, microglial cells sweep the MCEF around each microglial cell to produce accumulations of JG22-positive material that are subsequently internalized into phagosomes within microglial cells. The sweep of the MCEF carpet gives rise to MCEF-free pericellular areas in which the underlying basal lamina remain unaltered (see Fig. 4). These MCEF-free areas are similar to the migratory trails behind different cell types cultured on ILM/MCEF retinal sheets (Halfter et al., 1988, 1990) and to the so-called phagokinetic tracks of cultured cells moving on a glass substrate covered with particles (Albrecht-Buehler, 1977; Chen et al., 1994; Scott et al., 2000). Experiments showed no effects on MCEF-free pericellular areas after culture of ILM/MCEF sheets in presence of the broad-spectrum metalloproteinase inhibitor GM6001 (see Fig. 6), demonstrating that the disappearance of MCEF around microglial cells is not due to proteolysis by metalloproteinases. In fact, our experiments with cytochalasin D (see Fig. 7) indicate that the sweep of MCEF is a consequence of the actin-dependent motility of microglial cells.

The phagocytosis ability of microglial cells has been extensively demonstrated in vitro by using phagocytosis assays on cultured microglial cells purified from brain primary mixed cell cultures (reviewed in Smith, 2001). In these assays, different materials to be phagocytosed, such as latex beads (Dijkstra et al., 2001; Giulian and Baker, 1986), erythrocytes (Stangel and Compston, 2001), zymosan (Chamak and Mallat, 1991; Ohsawa et al., 2000), bacteria (Giulian and Baker, 1986), myelin (Dijkstra et al., 2001; Smith et al., 1998), β-amyloid (Koenigsknecht and Landreth, 2004), and apoptotic cells (Chan et al., 2001; Magnus et al., 2002) were added to the culture system to test the phagocytic ability of microglial cells. In our culture system, phagocytosed MCEF are inherent materials, therefore phagocytosis by microglial cells can be demonstrated without the addition of other materials.

### Expression of β1 Integrin Subunit in Microglial Cells After MCEF Phagocytosis

We show in the present study that the surface of cultured microglial cells becomes strongly JG22-positive, thereby revealing expression of the β1 integrin subunit on the cell membrane. Ameboid microglial cells in the in situ retina are not immunoreactive for β1 integrin subunit (results not shown), and this phenotypic feature is maintained at the beginning of the culture. Strong upregulation of β1 integrin subunit expression takes place at 24 hiv (see Fig. 5), after microglial cells have carried out MCEF phagocytosis and their cytoplasm is filled with phagosomes. Expression of β1 integrin subunit in activated microglia was previously shown in situ (Hailer et al., 1997; Kloss et al., 1999) and in vitro (Hailer et al., 1996; Kloss et al., 2001; Milner and Campbell, 2002, 2003; Nasu-Tada et al., 2005; Zuckerman et al., 1998). β1 integrins are known to be involved in functions of cell adhesion on extracellular matrix (Danen and Sonnenberg, 2003; Elangbam et al., 1997), and the close relationship between its expression in microglial cells and adhesion of these cells to the substrate has been documented (Kloss et al., 1999, 2001; Milner and Campbell, 2002, 2003). Therefore, strong β1 integrin subunit expression on microglial cells at 24 hiv in our culture system might increase their adhesion on the underlying basal lamina after removal of MCEF by phagocytosis. In support of this view, we found that microglial cells change their morphology after 24 hiv to become larger and flatter cells (see Fig. 2) that appear to be strongly attached to the basal lamina. Integrin expression in some cell types has been shown to be upregulated by the extracellular matrix, which contains integrin ligands (Delcommenne

and Streuli, 1995; Milner and Campbell, 2003). Therefore, our results suggest the interesting possibility that microglial cells, which do not express the β1 integrin subunit at the beginning of the culture, may become immunoreactive for it due to their contact with the basal lamina after removal of underlying MCEF, thereby increasing their adhesion on the substrate. This is compatible with previous observations that laminin induces an adherent ameboid phenotype in cultured microglial cells (Chamak and Mallat, 1991; Giulian et al., 1995).

In addition to its relationship with cell-to-substrate adhesion, β1 integrin subunit expression may be related to phagocytosis. In fact, Koenigsknecht and Landreth (2004) reported the presence in microglial cells of a β1 integrin-mediated mechanism of phagocytosis in addition to the classical mechanism involving Fc immunoglobulin receptors. Furthermore, Hailer et al. (1997) suggested that some molecules in the substrate incorporated by phagocytosis would induce second-messenger pathways that lead to the expression of adhesion molecules. Since microglial cells in our culture system phagocytose MCEF during the first 24 hiv and strong β1 integrin subunit expression takes place at 24 hiv, it is possible that phagocytosis of MCEF may induce upregulation of the β1 integrin subunit expression on cultured microglial cells. Further studies are warranted to test this hypothesis.

A striking finding of our study is that immunoreactivity for β1 integrin subunit persists in microglial cells at 96 hiv, although the immunostaining is discontinuous with intensely labeled focal spots. This suggests a redistribution of β1 integrins on the surface of microglial cells at more advanced culture stages, when these cells spread on the basal lamina and increase their size. A similar redistribution of β1 integrins was reported in microglia cultured on fibronectin-coated coverslips in response to ADP stimulation (Nasu-Tada et al., 2005). Translocation of β1 integrins might contribute to the formation of focal contacts, thereby improving cell spreading and adhesion. Further research is required to test this proposition.

### Short-Distance Migration of Cultured Microglial Cells on ILM/MCEF Sheets

As stated above, MCEF-free areas that develop around each microglial cell as a consequence of phagocytic activity are similar to phagokinetic tracks of cultured cells moving on a substrate covered with particles (Albrecht-Buehler, 1977). The shape of each phagokinetic track around a microglial cell reveals the previous trajectory of this cell. Our results show that most phagokinetic tracks around microglial cells are more or less ovoid in shape, although some of them are relatively long (Fig. 5D). We conclude from these findings that most microglial cells in our culture system only move for short distances on ILM/MCEF sheets. This pattern of cell migration would result from frequent changes in the direction of cell movement, as reported in cultures of other cell types (Halfter et al., 1988). The presence of QH1-positive thin filaments behind some microglial cells, similar to trails of extracellular matrix molecules behind migrating fibroblasts (Halfter et al., 1988, 1990), also supports the view that cultured microglial cells actively migrate on ILM/MCEF sheets.

The migration mechanism of microglial cells cultured on ILM/MCEF sheets is clearly different from that of the same cells in the in situ retina (Marín-Teva et al., 1998; Navascués et al., 1995). Thus, microglial cells migrating tangentially on MCEF in the embryonic retina have a clearly polarized shape with a small soma from which emerge thick processes bearing broad lamellipodia. By contrast, in the culture system used in the present study, they show a lamellipodia-free nonpolarized ameboid shape with spine-like filaments projecting from the cell surface (Figs. 12D,E). It has been suggested that lamellipodia of microglial cells function as devices to recognize signals in the retinal environment that would indicate the direction of their subsequent movement (Marín-Teva et al., 1998). This view is compatible with the absence of lamellipodia in microglial cells migrating in our culture system, which lacks the complex signals present in the in situ retina. Spine-like projections could be produced by the strong adhesion of microglial cells to the basal lamina, which resists the movement of the cell. This strong cell-to-substrate adhesion would also be responsible for detaching material from the spine-like projections to produce the QH1-positive trail behind migrating microglial cells, which is similar to that observed behind cultured fibroblasts migrating on retinal basal lamina (Halfter et al., 1990). Deposition of QH1-positive material along migration trails of microglial cells also appears to take place during the normal development of the in situ retina. Thus, previous studies in our laboratory described long and thin QH1-positive filaments behind ameboid microglial cells migrating on the vitreal side of the quail embryonic retina (Marín-Teva et al., 1998).

## CONCLUDING REMARKS

The method for culturing microglial cells described in the present study is a valuable model system to carry out in vitro experimental studies on microglial cell functions because of the simplicity of the isolation method and the immediate availability of cultured microglial cells. The morphofunctional characterization of microglia in our cultures revealed a diversity of functional processes, including phagocytosis, migration, upregulation of surface molecules, proliferation, and changes in cell shape. Therefore, this cell culture model system would be useful to design assays for testing the influence of different factors on these functional activities.

## ACKNOWLEDGMENTS

The mAbs QH1 (developed by F. Dieterlen-Lièvre), 39.4D5 (developed by T.M. Jessell), H5, C4 (both devel-

oped by J.R. Sanes), JG22 (developed by D.I. Gottlieb), 3H11, and 6D2 (both developed by W. Halfter) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences of the University of Iowa. The authors thank Richard Davies for improving the English and Dr. David Martín-Oliva for assistance with confocal microscopy studies at the Mixed Medical Research Unit of San Cecilio University Hospital, Granada.

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