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Labelling of retinal microglial cells following an intravenous injection of a fluorescent dye into rats of different ages

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ABSTRACT

Retinal microglia were selectively and sequentially labelled in different layers of the retina of postnatal rats following a single intravenous injection of the fluorescent dye, rhodamine isothiocyanate (RhIc). The fluorescent cells were doubly immunostained with OX-42 and ED-1 antibodies that recognise complement type 3 (CR3) receptors and macrophage antigen, respectively. RhIc was first detected in the retinal blood vessels 5 min after injection. At 1 h, a variable number of microglia in the inner layers of the retina, namely, the nerve fibre and ganglion cell layers appeared to emit weak fluorescence. Labelled microglial cells in the inner nuclear and outer plexiform layers were not detected until 1 and 2 d had elapsed following RhIc injection. The number of labelled retinal microglia was progressively increased with time, peaking at 4 d after RhIc injection. The frequency of RhIc labelled cells also increased with age, with the largest number of cells occurring in 7-d-old rats but declined thereafter. In 11 d or older rats, RhIc was confined to the retinal blood vessels. It is concluded that when injected into the circulation, RhIc could readily gain access into the retina tissues due to an inefficient blood-retina barrier in early postnatal stages. It became impeded with maturation of the blood-retina barrier, which was established between 11 and 13 d of age. RhIc that inundated the retinal tissues was thoroughly sequestered by the resident microglial cells. It is therefore suggested that the latter could play a protective role against serum-derived substances that may be deleterious to the developing retina.

Key words: Microglia; blood-retinal barrier; RhIc; OX-42; ED-1.

INTRODUCTION

The distribution of retinal microglial cells was first studied by the silver carbonate method (Ling, 1982) and subsequently by enzyme histochemistry using peroxidase-conjugated lectin, TPPase and NDPase (Ashwell, 1989; Schnitzer, 1989), immunohistochemistry using antibodies F4/80 (Hume et al. 1983), CD-45, MHC-I and MHC-II (Diaz-Araya et al. 1995; Provis et al. 1996). During development and following optic nerve crush and transection, the cells function as phagocytes (Schnitzer & Scherer, 1990; Pearson et al. 1993; Egensperger et al.1996; Kacza & Seeger, 1997). The phagocytic nature of microglia in the brain has been demonstrated by their uptake of exogenous materials such as carbon particles (Ling, 1979), horseradish peroxidase (Kaur et al. 1986; Xu & Ling, 1994). This is also evidenced by the avid uptake of the fluorescent dye, rhodamine isothiocynate (RhIc) administrated intraperitonally or intravascularly (Xu et al. 1993; Li et al. 1997). This study was aimed to ascertain if retinal microglia could also be labelled by RhIc when administered into the blood circulation. The information obtained would help to improve understanding of the functional roles of microglia in the retina.

MATERIALS AND METHODS

Injection of RhIc

A total of 126 Wistar rats ranging from 1 to 14 d of age were used in this study. Under 4% chloral hydrate anaesthesia, each rat was given a single intravenous (i.v.) injection of 0.5% rhodamine isothiocynate (RhIc) (Sigma, R-1755) in normal saline through the left external jugular vein as described previously (Xu

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et al. 1993). For rats between 1–10 d of age, each rat received 30 μl of 0.5% RhIc injection; rats in older age groups (11–13 d) were given 40 μl of 0.5% RhIc injection. The dosage was increased to 80 μl for 14 d old rats. After RhIc injection, the rats were returned to the mothers. Rat puppies were killed by perfusion at 5 min, 1 h, and 1, 2 and 4 d after RhIc injection. Under deep anaesthesia, the rats were perfused with normal saline followed by 4% paraformaldyhyde in 0.1 M phosphate buffer (pH 7.4). Both eyeballs were removed and postfixed in the same fixative for another 2–4 h before storing in 10% sucrose-buffer overnight at 4 °C. Horizontal frozen sections of the eyeball were cut at 20 μm and mounted on gelatinised slides, air-dried and coverslipped with Dako fluorescent mounting medium (Dako, S3023). The sections were viewed and photographed in a Carl Ziess LSM410 confocal microscope (excitation wavelength 543 nm, emission wavelength 590 nm).

Semiquantitative analysis of RhIc-labelled cells

Since a large number of RhIc-labelled microglial cells was observed in 7-d-old rats, cell counts were also carried out in this experimental age group. Tissue sections prepared from rats killed at 5 min, 1 h, and 1, 2 and 4 d after RhIc injection ($n = 3$ at each time interval) were scrutinised and the number of labelled cells estimated. Every fifth section was used whereby a total area of 0.16 mm^2 across the different layers of the retina was randomly selected for semiquantitative analysis at 12.5×16 times under the fluorescence microscope. Since the nerve fibre layer (NFL), ganglion cell layer (GCL) and inner plexiform layer (IPL) at this age group were not well differentiated and defined, they were considered collectively as one layer.

Immunohistochemistry

After fluorescence photomicrography, tissue sections prepared from age groups of 5 and 7 d receiving RhIc injection and killed 4 d later, were further processed for immnohistochemistry. Following the removal of the coverslips, the tissue sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, and incubated at room temperature for 16–20 h using the monoclonal antibodies OX-42 (Harlan Sera-Lab, MAS 370b) and ED-1 (Harlan Sera-Lab, MCA 341), at dilutions of 1:500 and 1:400 in PBS, respectively. Subsequent antibody detection was carried out using biotinylated antimouse IgG (rat absorbed) and Vectastain ABC kit (Vector Laboratory, Pk-4000) with 3,3′-diaminobenzidine (DAB, Sigma, D-5637) as a peroxidase substrate, and intensified with nickel ammonium sulphate. The sections were counterstained with 1% methyl green, dehydrated and mounted in Permount.

RESULTS

All rats given an i.v. injection of RhIc turned pink within a few minutes. They appeared physically healthy with no obvious signs of distress.

External morphology and distribution of RhIc-labelled cells

The description of the external morphology and distribution of RhIc-labelled cells is based on materials prepared from 5 or 7-d-old rats receiving RhIc injection and killed 4 d later, since the largest number of labelled cells occurred at these 2 age groups. The majority of the RhIc-labelled cells were located in the NFL, GCL and IPL, with a moderate number of them localised in the inner nuclear layer (INL), but rarely in the outer plexiform layer (OPL). Labelled cells were absent in the outer nuclear layer (ONL) and photoreceptor layer (PRL). Although the microglial morphology and size appeared to vary in different layers of the retina, most of the labelled cells were oval or round. The labelled cells in the NFL and GCL tended to be smaller in size, and they appeared to be round and brightly labelled, especially those that were closely

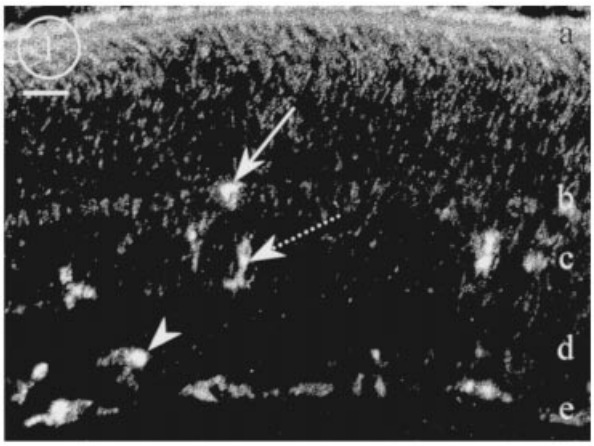

Fig. 1. Confocal photomicrograph of the retina of a 5-d-old rat killed 4 d after RhIc injection. The majority of RhIc-labelled microglial cells are located in the nerve fibre layer (NFL), ganglion cell layer (GCL) and inner plexiform layer (IPL); moderate numbers are in the inner nuclear layer (INL) and only sporadic cells occur in the outer plexiform layer (OPL) (arrow). The broken arrow indicates a larger microglial cell bearing a sturdy process in the INL. The arrowhead indicates a small and oval microglial cell in the GCL. a, choroid layer; b, OPL; c, INL; d, IPL; e, GCL (this and other figures). Bar, 25 μm.

adherent to the blood vessels. In the INL, the labelled cells were generally larger and oval bearing a few short sturdy processes. Occasional RhIc-labelled cells were observed in the OPL (Fig. 1).

RhIc labelling at different time intervals

Previous studies (Leong & Ling, 1992; Xu et al. 1993) have shown that the majority of microglial cells in the brain were brightly labelled by RhIc at 2 d after an intravenous injection of the dye into postnatal rats. Furthermore, Ashwell et al. (1989) had reported that microglial population in the retina reached its peak between the fifth and seventh postnatal day. On the basis of these findings, 5-d-old rats were used in our preliminary examination in which and the rats were killed at 5 min, 1, 6, 12 h, 1 and 2 d following RhIc injection. Since there was no noticeable difference in RhIc labelling in rats killed between 1 h and 1 d after RhIc injection, the time points between the two, i.e. 6 and 12 h, were omitted in subsequent studies. At 2 d after RhIc injection, all labelled cells in the retina emitted intense fluorescence, but only occasional labelled cells were detected in the OPL. The survival interval was then extended to 4 and 7 d. Although the intensity and frequency of fluorescent labelled cells were further augmented at 4 d, they remained unchanged thereafter. In view of the above, present results were primarily based on materials prepared from rats killed at 5 min, 1 h, 1, 2 and 4 d after RhIc injection.

In rats killed at 5 min after RhIc injection, the retinal tissues exhibited moderate RhIc fluorescence. On closer examination, the retinal blood vessels located in the NFL displayed a brighter RhIc fluorescence when compared with the background retinal tissues. On the other hand, strong RhIc fluorescence was detected in the cornea, iris, ciliary body, the outer layer of lens, vitreous body, choroid and sclera. Labelled cells were absent across the different layers of the retina (Fig. 2). At 1 h after RhIc injection, some cells in the inner layers of retina including the NFL, GCL and IPL appeared to emit weak fluorescence (Fig. 3). At 1 d, labelled cells in the INL were clearly labelled. Meanwhile, the fluorescence intensity of labelled cells in the NFL, GCL and IPL were further enhanced (Fig. 4). Occasional labelled cells were observed in the OPL at 2 d after RhIc injection (Fig. 5). Four days after RhIc injection, all cells across the retinal layers were brightly fluorescent. With the progressive increase in numbers of labelled cells in different layers of the retina, the background retinal tissues were clear of the tracer. In longer surviving rats, more microglial cells in the outer layers such as the INL and OPL were labelled with RhIc. The sequential RhIC labelling of microglia in different layers of the retina is reflected in semiquantitive analysis summarised in the Table.

RhIc labelling in different age groups

The frequency of labelled cells in the retina increased steadily from 1 to 7 d of age (Figs 6–9). An upsurge of labelled cells occurred in 5 and 7-d-old rats receiving RhIc injection and killed 4 d later, peaking in the latter age group. The number of labelled cells then declined drastically at 10 d, when only a few labelled cells were observed. The retinal blood vessels in the NFL and OPL retained weak RhIc fluorescence. In 11 and 13-d-old rats receiving RhIc injection, the blood vessels in the NFL and OPL were strongly fluorescent after 1 h injection and remained so with the passage of time. No detectable fluorescent cells were found in these 3 age groups. The fluorescent dye was clearly confined to the lumina of the retinal blood vessels. Similar features were observed in 14 d rats given a larger dose (80 μl) of RhIc injection, in which the dye was clearly contained in the retinal blood vessels (Fig. 10).

Immunohistochemistry study

Tissue sections from rats aged 5 and 7 d and killed 4 d after RhIc injection were immunoreacted with OX-42 and ED-1 antibodies after selected RhIc-labelled cells were photographed. The majority of the RhIc-positive cells were double labelled by OX-42 or ED-1 antibodies, but not all the OX-42 and ED-1 immunoreactive cells were RhIc positive (Figs 11*a*, *b*; 12*a*, *b*).

DISCUSSION

We previously demonstrated that microglial cells (amoeboid form) in different areas of the brain of postnatal rats are selectively labelled by RhIc injected systemically (Leong & Ling, 1992; Xu et al. 1993). This study has further shown that the same fluorescent tracer administered intravascularly also labels the microglial cells in the postnatal rat retina. The RhIc-labelled cells were confirmed to be microglia since they were double labelled by OX-42 and ED-1 antibodies. This showed that most of the retinal microglial cells in early postnatal rats have a phagocytic function as shown by their content of ingested

Table. *RhIc-labelled microglia in the retina of 7-d-old rats killed at different time intervals after RhIc injection*

Retinal layers	5 min	1 h	1 d	2 d	4 d
NFL, GCL, IPL	−	±	+ +	+ + +	+ + + +
INL	−	−	+ +	+ + +	+ + +
OPL	−	−	−	±	+ +

RhIc-labelled cells in an area 0.16 mm² outlined by a graticule: −, none; ±, 0–1 cell; +, 1–3 cells; + +, 4–6 cells; + + +, 7–9 cells; + + + +, > 10 cells.

fluorescent dye injected intravascularly. On the other hand, some OX-42 and ED-1 immunoreactive microglial cells did not exhibit RhIc fluorescence, suggesting that not all the microglial cells are active phagocytes. It is also possible that some of the RhIc-labelled microglial cells might have lost their contents of ingested tracer during confocal microscopy. Furthermore, the technical procedures in immunohistochemical processing could also have diminished the RhIc fluorescence of microglial cells in some sections. The possibility that some RhIc-labelled cells were endothelial cells of the retinal blood vessel cannot be excluded.

The present study has shown that RhIc injected intravenously could gain access into the retinal tissues through the blood circulation in the developing retina. Five minutes after RhIc injection, the retinal tissues exhibited moderate fluorescence, but the retinal blood vessels were not strongly labelled by RhIc, especially in rats younger than 11 d of age. This suggests that RhIc in circulation could readily cross the retinal blood vessels into the subjacent tissues in early age groups. This is in contrast to the blood vessels in the brain which became clearly fluorescent 5 min after RhIc injection (Xu et al. 1993). It is speculated that RhIc had readily gained access into the retinal tissues by direct and massive diffusion rather than active transendothelial transportation as was reported in the brain (Xu et al. 1993). This is supported by the study of Stewart & Tuor (1994), who demonstrated that the blood-retina barrier (BRB) was 4 times more permeable than the blood-brain barrier (BBB) by measuring the transfer of a vascular tracer, ^{14}C-AIB from blood to tissue; a similar phenomenon was also reported by other authors (Alm & Tornquist, 1981; Ennis & Betz, 1986). It is suggested that the extravasated RhIc in the retinal tissues was readily endocytosed by the resident microglia. The phagocytic process appeared to continue actively for a few days because the accumulation of RhIc by microglia was progressive, as reflected by the gradual increase in fluorescence with time.

The RhIc-labelled cells were distributed mainly in the NFL, GCL and IPL, with a moderate number of them in the INL, but rarely in the OPL. The distribution of RhIc-labelled retinal microglia paralleled that described previously using other staining methods (Ling, 1982; Schnitzer, 1989). Microglial cells in various layers of the retina were also found to be sequentially labelled by RhIc. The cells in the NFL, GCL and IPL, albeit with weak fluorescence labelling, were first detected in rats killed at 1 h after RhIc injection. The intensity and number of RhIc-labelled microglial cells increased in longer survival rats. Cells in the INL and OPL were only observed in rats killed 1 or 2 d after injection. One explanation for the sequential labelling of the microglia is the migration of labelled cells from the inner layers of the retina to the INL and OPL during development. This observation corroborates the finding of Hume et al. (1983) who reported the migration of retinal microglial cells from the GCL to INL and OPL in different age groups of mice. The study by Vinores (1995) had shown that the retinal blood vessels could be grouped into 2 vascular plexuses: the inner vascular plexus residing in the NFL and GCL, and the outer in the OPL. The vascular inner plexus appeared to develop earlier than the outer plexus (Zeng et al. unpublished observations). Furthermore, the inner plexus appeared to ramify in retinal tissues which had a loose texture with few cellular components. It is possible that this may facilitate the rapid diffusion of RhIc into the NFL, GCL and IPL. Thus the early labelling of microglial cells in these layers maybe attributable to their rapid phagocytosis of the dye from the ambient environment. The INL, on the other hand, contains densely packed neurons which would impede the diffusion of RhIc from the inner layers into the outer layers of the retina. This would then explain the delayed labelling of microglial cells in the INL and OPL following RhIc injection.

Like the amoeboid microglial cells (AMC) in the brain, the labelling of retinal microglia by RhIc is age-related (Xu et al. 1993). The maximal number of

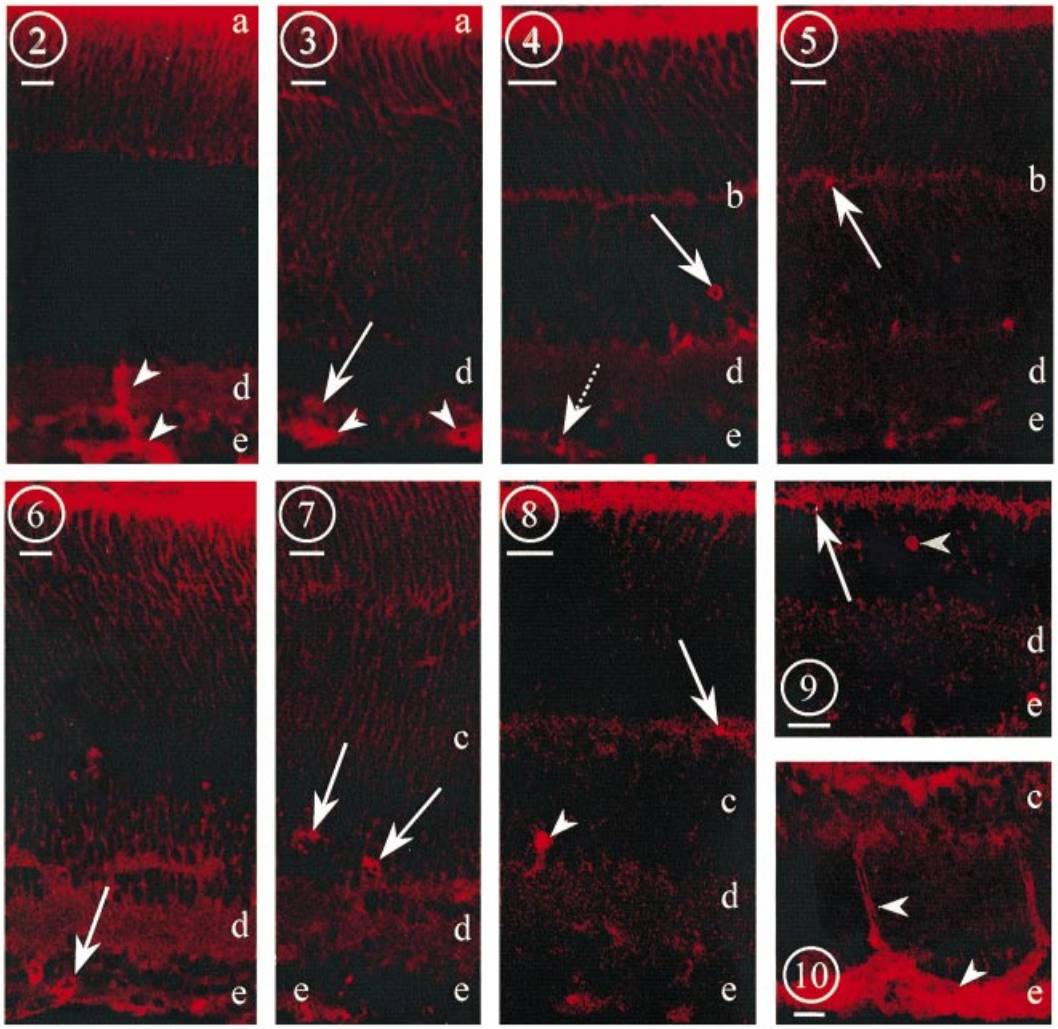

Fig. 2. Confocal photomicrograph of the retina of a 5-d-old rat killed 5 min after RhIc injection. Note the intense RhIc fluorescence in the blood vessel (arrowheads) in the NFL, GCL and IPL against the background retinal tissues. Labelled cells are absent in all layers of the retina. Bar, 25 μm.

Fig. 3. Confocal photomicrograph of the retina of a 5-d-old rat killed 1 h after RhIc injection. Arrowheads indicate intensely labelled blood vessels in the NFL. Some microglia (arrow) in the GCL appear to emit weak fluorescence. Bar, 25 μm.

Fig. 4. Confocal photomicrograph of the retina of a 5-d-old rat killed 1 d after RhIc injection. Note the labelling of microglia in the INL (arrow). Microglia in the NFL and GCL are intensely fluorescent (broken arrow). Bar, 25 μm.

Fig. 5. Confocal photomicrograph of the retina of a 5-d-old rat killed 2 d after RhIc injection. All labelled microglia are brightly fluorescent. Microglial cells are now detected in the OPL (arrow). Bar, 25 μm.

Fig. 6. Figs 6–9. Confocal photomicrographs of the retinae of 1 (Fig. 6), 3 (Fig.7), 5 (Fig. 8) and 7-d-old (Fig. 9) rats killed 4 d after RhIc injection. All RhIc-labelled microglial cells are brightly fluorescent. Note the increase in number of labelled cells with advancing age. In Fig. 6, most of the RhIc-labelled microglia (arrow) are located in the NFL, GCL. In Fig. 7, more labelled cells are observed in the INL (arrows). In Figs 8, 9, microglial cells are detected in the OPL (arrows) and in the INL (arrowheads). Bar, 25 μm.

Fig. 10. Confocal photomicrograph of the retina of a 14-d-old rat killed at 4 d after RhIc injection. The retinal blood vessels (arrowheads) located in the NFL and IPL are strongly labelled by RhIc. RhIc-positive microglia are absent. Bar, 25 μm.

labelled retinal microglia was observed in 7-d-old rats given RhIc injection and killed 4 d later. Labelling, however, was diminished in 10 d or older rats. The occurrence of large numbers of labelled cells at 7 d may be attributed to active microglial proliferation. Using peroxidase-conjugated lectin, it has been reported that the number and density of microglia in the developing retina of rats increased gradually reaching a peak at 7 d but declining thereafter (Ashwell et al. 1989). The diminution in number and fluorescence of labelled microglial cells in 11 d and older rats may have one or more of the following explanations. First, this maybe attributed to the reduced phagocytic capability of microglial cells with age because of their transformation from the amoeboid form into ramified cells (Ling, 1982; Hume et al. 1983). Secondly, the

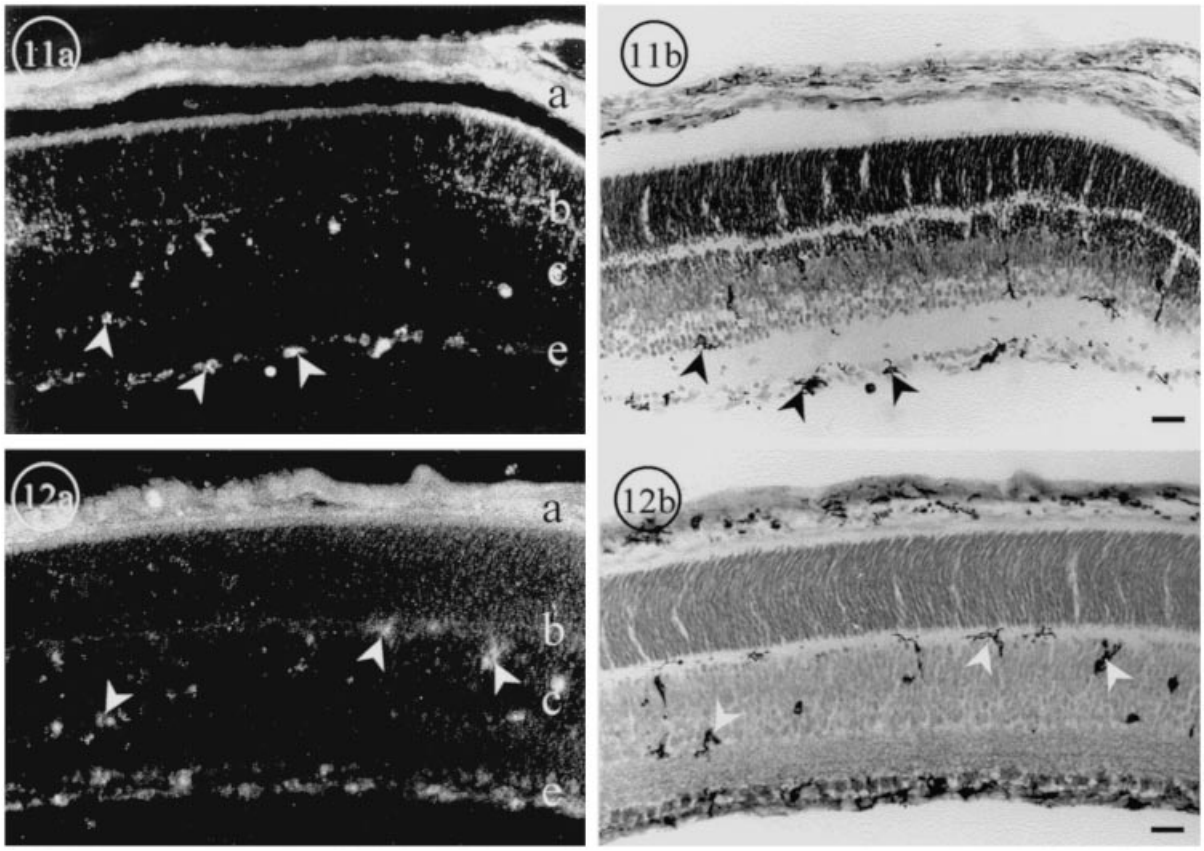

Fig. 11. Fluorescence photomicrograph (*a*) and corresponding OX-42 immunostained photomicrograph (*b*) of the retina of a 7-d-old rat killed 4 d after RhIc injection. Note the majority of RhIc-labelled microglial cells (arrowheads) are OX-42 positive. Bar, 25 μm.

Fig. 12. Fluorescence photomicrograph (*a*) and corresponding ED-1 immunostained photomicrograph (*b*) of the retina of a 5-d-old rat killed 4 d after RhIc injection. Arrowheads indicate RhIC-labelled microglia (*a*) and corresponding ED-1 positive cells (*b*). Bar, 25 μm.

maturation of the BRB is gradually established between 11 and 13 d postnatally. The BRB has similar features to the BBB (Cunha-Vaz et al. 1966; Stewart & Tuor, 1994). It has been reported that the BRB in young rats has a simpler configuration than in adult rats from electron microscopic observations (Cunha-Vaz et al. 1966), and the BBB was fully developed at 11 or 13 d postnatal day (Xu & Ling, 1994). It is speculated that RhIc could readily cross the blood-retina barrier and was phagocytosed by retinal microglia in the younger rats. The more complex organisation of the blood-retinal barrier in 11 d or older rats may exclude the RhIc from entering the retinal tissues. This is shown by the fact that RhIc was retained in the retinal blood vessels when RhIc was injected into these age groups. Such, in fact, remained so even when a larger dose of RhIc was administered.

This study has shown that retinal microglial cells can be selectively labelled by RhIc when administered intravascularly. This facilitates a rapid and accurate localisation of microglial cells in different layers of the retina. The method is also useful for assessment of the integrity of the blood-retina barrier. The present results suggest that the retinal microglial cells may have a protective role in the developing retina by phagocytosing serum-derived substances that may be harmful to the early developing retinal neurons and photoreceptors.

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