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Variation with age in the labelling of amoeboid microglial cells in rats following intraperitoneal or intravenous injection of a fluorescent dye

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ABSTRACT

Amoeboid microglial cells (AMC) in the corpus callosum were selectively labelled following a single intraperitoneal (i.p.) injection of the fluorescent dye, rhodamine isothiocyanate (RhIc) into postnatal rats. The frequency of RhIc-labelled cells varied with age, with the largest number occurring in 7-d-old animals. Thereafter, the labelled cells declined drastically in number and fluorescence and were barely detectable in 12-d-old injected rats. Labelled cells were absent in 13-d or older rats given an RhIc injection. When the injected RhIc was followed over a time course sequence, it was first detected in the cerebral blood vessels and their lining endothelia within 5 min after the injection. A variable number of AMC emitting a weaker fluorescence were closely adherent to the outer walls of the blood vessels. With time, the fluorescence in the AMC was progressively enhanced, but that in the blood vessels showed a concomitant reduction. In the rats that received an intravenous (i.v.) injection of RhIc, the labelling pattern of AMC, both in terms of its variation with age and in temporal sequence, paralleled that in rats given i.p. injections. In 12-d-old rats subjected to a stab wound coupled with an i.p. injection of RhIc, a considerable number of AMC not normally labelled at this age were activated. The cells exhibited an intense fluorescence and expressed MHC surface antigen immunoreactivity. It is concluded from this study that when injected i.p. or i.v., RhIc is readily circulated to the cerebral vessels, where it enters brain tissue by transendothelial transport. The tracer which permeates the corpus callosum is endocytosed by the AMC on surveillance. The entry of RhIc, however, is impeded by the maturation of the blood–brain barrier which occurs between 12 and 13 d of age.

INTRODUCTION

A characteristic feature of the developing brain is the occurrence of foci of macrophagic amoeboid microglial cells (AMC) in the subcortical white matter. In postnatal rats, a major site in which the cells are heavily populated is the loosely organised corpus callosum above the lateral ventricle (Ling & Tan, 1974; Ling, 1976); other depots of AMC include the cavum septum pellucidum (Tseng et al. 1983) and the circumventricular region and its associated subependymal cysts (Kaur et al. 1989). One of the main functions of the cell type, pertaining to its phagocytic nature, is the remodelling of nervous tissue in early development (Innocenti et al. 1983*a*, *b*). Their immunological role, however, is gaining recognition following the recent characterisation of their immunophenotypic features, e.g. the expression of complement type 3 (CR3) receptors and major histocompatibility complex surface antigens (MHC) (Ling et al. 1990, 1991). With these immunological properties, it is justifiable to assume that the cells would have the capacity to interact with T lymphocytes or any foreign antigens that may come into contact with them in a possible immune response, although how this may be effected is uncertain. Relevant to this study was our previous report (Kaur et al. 1986) of the labelling of AMC in postnatal rats following an intravenous (i.v.) injection of the exogenous protein, horseradish peroxidase (HRP). In this connection, a remarkable development was our recent finding (Leong & Ling, 1992) of the labelling of the cell type following an intraperitoneal (i.p.) administration of the fluorescent dye, rhodamine isothiocyanate (RhIC). The mode of labelling of the cells, however, was only speculative, although it seems obvious that the

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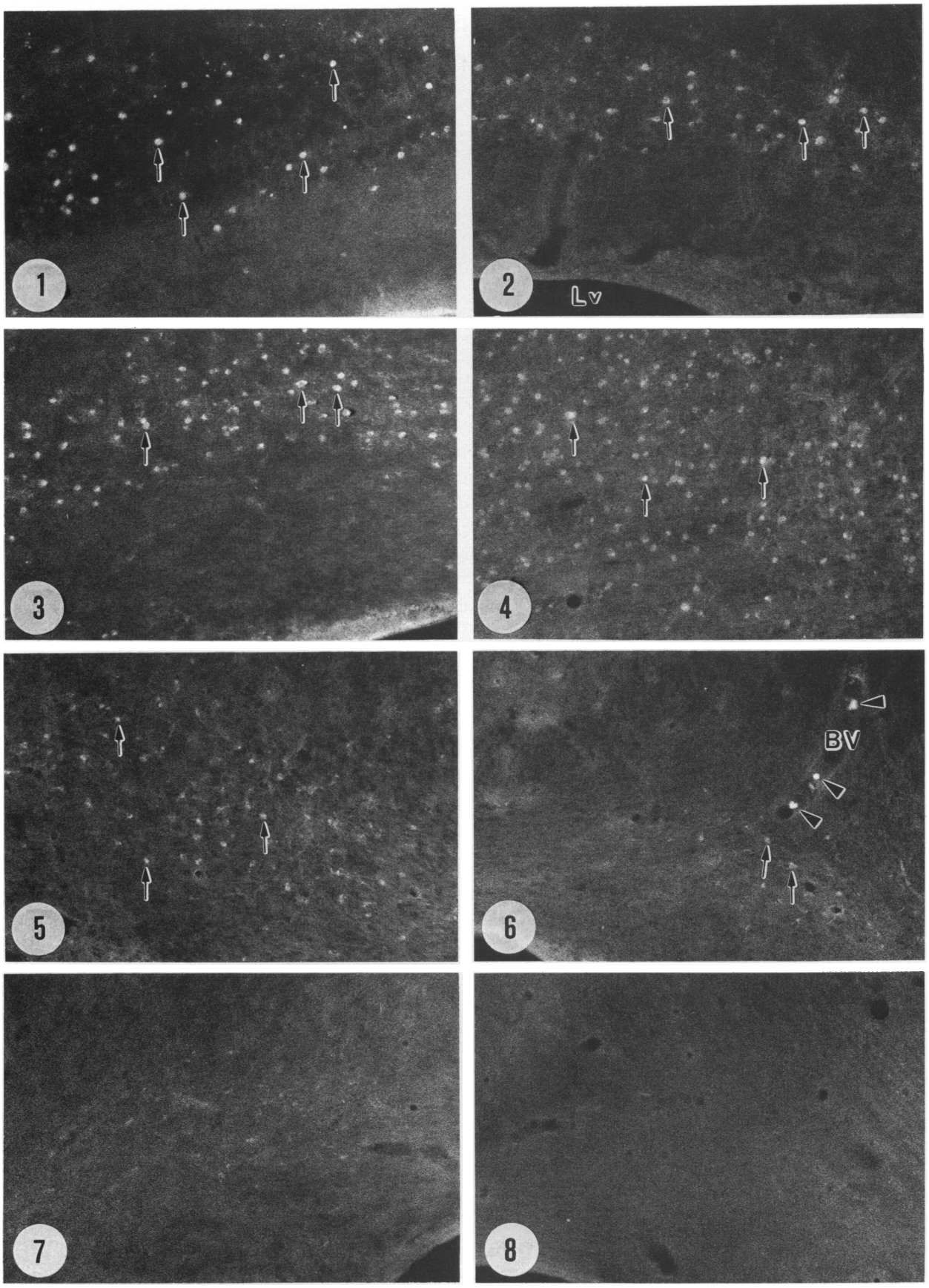


Figs 1, 2. Corpus callosum of a 1-d (Fig. 1) and a 3-d-old rat (Fig. 2) 2 d after an i.p. injection of RhIc. Amoeboid microglial cells (AMC) are brightly labelled (arrows). Note that the labelled AMC are widely scattered in the corpus callosum above the lateral ventricle (Lv). Perfusion fixation. × 140.

exogenous material injected into the animals would consequently gain access into the postnatal brain tissue.

To this end, the present investigation was carried out first to determine the mode of labelling of AMC by i.p. injection of RhIc and secondly to ascertain whether it is age-dependent. We also sought to elucidate if a similar labelling pattern could be obtained when the fluorescent dye was given i.v. as was previously shown with HRP (Kaur et al. 1986). These then provided the basis for the next step, which was to find out whether the labelling pattern may be altered with an experimental lesion. The finding would help to clarify the functional significance of the immunological properties inherent to AMC, which are closely involved in some neurodegenerative disorders, e.g. Alzheimer's disease (McRae et al. 1991).

MATERIALS AND METHODS

Intraperitoneal injection

Postnatal Wistar rats aged 1–15 d were used in this study. They were divided into 9 age groups aged 1, 3, 5, 7, 10, 11, 12, 13 and 15 d, respectively. Each group consisted of at least 10 rats. Under ether anaesthesia, each rat was given a single i.p. injection of 1% rhodamine isothiocyanate (RhIc) in normal saline. For rats between 1 and 10 d of age, each rat received 50 µl of RhIc. The dosage was increased to 100 µl for rats of the older groups. The injected rats were then returned to the mothers and were killed 1, 2, 5 min, 1, 6 h and 2 d after the injection.

Intravenous injection

The postnatal rats were divided into 7 groups aged 2, 5, 7, 10, 12, 13 and 15 d. Under ether anaesthesia, each rat, between 2 and 10 d of age, was given an i.v. injection of 30 µl 1% RhIc via the left external jugular vein. The rats of the older age groups were each given 40 µl of 1% RhIc injection. The rats were killed 5 min, 1, 6 h and 1 d after the injection.

The brains from both the i.p. and i.v. injected rats were fixed either by perfusion or direct immersion. In the first instance, under ether anaesthesia, the rats were perfused with 0.1 M phosphate buffer followed by 6% paraformaldehyde in the same buffer pH 7.4. In the latter, the rats were killed by an overdose of anaesthesia. The brains from these animals were then removed and fixed in toto in 6% paraformaldehyde. In both cases, the brains were stored overnight in sucrose-buffered fixative at 4 °C. Coronal frozen sections of the cerebrum were cut at 40 µm thickness and mounted on gelatinised slides, air dried and coverslipped with the nonfluorescent medium, Entellan (Merck, Darmstadt, Germany). The sections were examined and photographed in a Leitz Aristoplan photomicroscope equipped with a mercury lamp for fluorescence microscopy, using a wide-band ultraviolet excitation filter (excitation range, 515–560 nm).

Experimental lesion and immunohistochemistry

Having successfully established the precise age at which the labelling of amoeboid microglial cells becomes reduced to a negligible level with RhIc either by the i.p. or i.v. route, we attempted to find out whether the cells would respond to a superficial stab wound inflicted at a distant location. Although postnatal rats of various ages were examined in this lesion, for reasons to be described later, rats aged 12 d that were given an i.p. injection of RhIc and coupled with a stab wound were analysed. The following operative procedure was performed on this age group. Following ether anaesthesia, the rat was given an i.p. injection of RhIc. A small incision was then made in the scalp to expose the skull. A superficial stab wound, using a 24 G needle, was then made in the left cerebrum immediately posterior to the coronal suture and to the left of the sagittal suture. The extent of lesion was later verified in sections. All operated rats were re-anaesthetised and perfused 2 days later with 2% paraformaldehyde in 0.1 M phosphate buffer. Serial coronal sections (40 µm) thickness were prepared from the cerebral tissue at and adjacent to the needle wound. The sections were divided into sets consisting of 3 sections each. The 1st section was

Figs 3, 4. Corpus callosum of a 5-d (Fig. 3) and a 7-d-old rat (Fig. 4) 2 d after an i.p. injection of RhIc. Numerous amoeboid microglial cells are intensely labelled. Note that the majority of the labelled AMC are round (arrows). Perfusion fixation. × 140.

Fig. 5. Corpus callosum of a 10-d-old rat 2 d after an i.p. injection of RhIc. Rhodamine-labelled amoeboid microglial cells are recognisable (arrows). Note that the number of AMC is much reduced when compared with that of a 7-d-old rat (compare with Fig. 4). The labelled cells appear smaller and elongated and they exhibit a weaker fluorescence. Perfusion fixation. × 140.

Fig. 6. Corpus callosum of an 11-d-old rat 2 d after an i.p. injection of RhIc. A few small microglial cells are weakly labelled (arrows). Arrowheads indicate 3 strongly labelled leucocytes in a blood vessel (BV). Perfusion fixation. × 140.

Fig. 7. Corpus callosum of a 12-d-old rat 2 d after an i.p. injection of RhIc. Labelled AMC are barely recognisable in the corpus callosum. The cells are small and assume an elongated form. Perfusion fixation. × 140.

Fig. 8. Corpus callosum of a 15-d-old rat 2 d after an i.p. injection of RhIc. None of the microglial cells is labelled. Perfusion fixation. × 140.

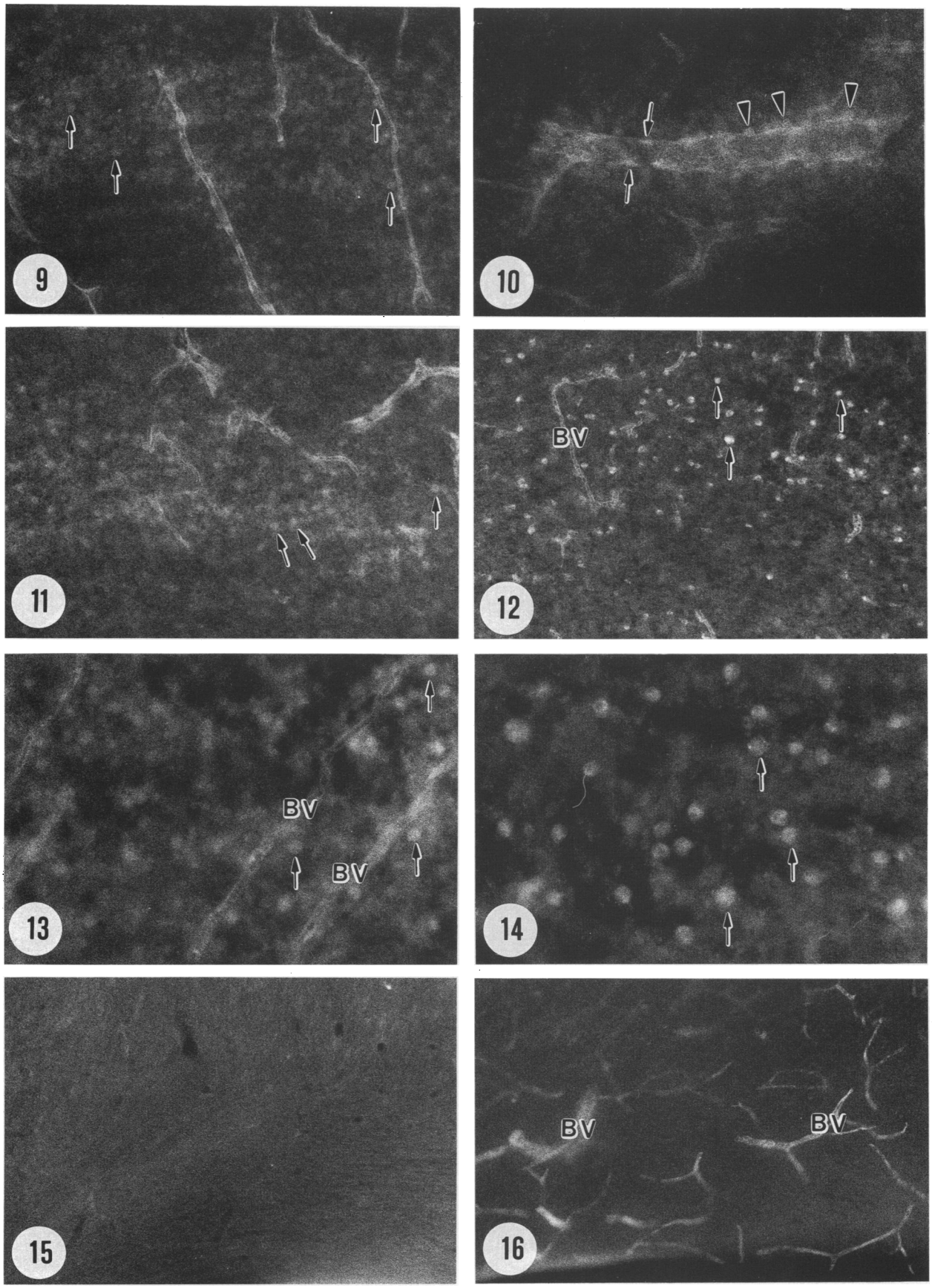


Fig. 9. Corpus callosum of a 7-d-old rat 5 min after an i.p. injection of RhIc. Blood vessels containing the fluorescent dye penetrate the corpus callosum. Numerous amoeboid microglial cells are faintly labelled (arrows). Brain fixed by direct immersion. × 140.

mounted in the usual manner for fluorescence microscopy. The successive 2nd and 3rd sections were incubated with the monoclonal antibodies OX-18 (Sera-Lab MAS 101b) and OX-6 (Sera-Lab MAS 043b), respectively. The immunohistochemical procedures were detailed in a previous report (Ling et al. 1991).

RESULTS

All rats given an i.p. or i.v. injection of RhIc turned pink within a few minutes. The animals appeared healthy with no obvious sign of distress.

Intraperitoneal injection

In the first step of this series all rats receiving an RhIc injection were killed 2 d later. This is because a previous study (Leong & Ling, 1992) had shown that AMC were clearly fluorescent within a few hours to 1 d after the administration of the dye. Thus the time given in the present study is believed to be sufficient to label most, if not all, of the AMC present.

In 1 and 3-d-old rats given an i.p. injection of RhIc, the brightly labelled AMC were widely scattered in the loosely organised corpus callosum above the lateral ventricle (Figs 1, 2). At the level of the optic chiasma, between 40 and 70 cells per section profile were observed in the corpus callosum. The majority of the cells were round.

The number of labelled AMC showed a dramatic upsurge in 5 and 7-d-old rats (Figs 3, 4) ranging from 100 to 300 cells per section profile. In fact, of all the ages of rats examined, the maximal number of labelled AMC was observed in 7-d-old animals. Just as in the rats of the younger group, the majority of the labelled AMC appeared to be round (Figs 3, 4). With age, the number of labelled AMC showed a drastic decline, so that in 10-d-old rats given an RhIc injection, the number of labelled AMC was reduced to a level comparable to that observed in the early postnatal rats (Fig. 5). The labelled cells, however, appeared smaller and/or elongated, emitting a weaker fluorescence.

In 11-d-old rats, only a few small cells were weakly labelled (Fig. 6). They were barely recognisable in a 12-d-old rat given an RhIc injection (Fig. 7). So far, none of the microglial cells have been labelled in 13 and 15-d-old rats (Fig. 8).

Since an i.p. injection of RhIc into 7 d-old-rats resulted in the largest number of labelled AMC, the description of the labelling pattern in temporal sequence will be based on the material from this age group. Within 5 min of the injection, tortuous blood vessels of different calibres became identifiable, penetrating the corpus callosum mainly in a radial fashion (Fig. 9). The lumina of the vessels were well delineated by their content of RhIc (Fig. 9). The AMC in the background displayed a low level of fluorescence (Fig. 9). On closer examination, the endothelial cells of the blood vessels emitted an intense fluorescence (Fig. 10). RhIc-labelled AMC adhered to the outer vascular walls (Fig. 10). The fluorescence intensity of the labelled AMC, which was extremely faint, initially continued to increase with time so that by 1 h after the injection the cells were readily recognisable (Fig. 11). Meanwhile, the interstitial spaces were inundated with a considerable amount of the fluorescent tracer (Fig. 11). In rats killed 2 d after the injection, the labelled AMC became extremely bright, with a concomitant reduction of the background fluorescence (Fig. 12). A variable number of blood vessels were discernible because they still contained a substantial level of injected RhIc.

Intravenous injection

The result for the i.v. injected rats paralleled that for rats given an i.p. injection of RhIc with respect to the correlation between age difference and the labelling of

Fig. 10. Blood vessel in the corpus callosum of a 7-d-old rat 5 min after an i.p. injection of RhIc. The flattened endothelial cells of the blood vessel are intensely labelled (arrows). Closely associated with the wall of the blood vessel are some weakly labelled round AMC (arrowheads). Diffused fluorescent dye is evident in the vicinity of the blood vessel. Brain fixed by immersion. × 200.

Fig. 11. Corpus callosum of a 7-d-old rat 1 h after an i.p. injection of RhIc. Labelled AMC (arrows) are recognisable. A considerable amount of the fluorescent tracer is present in the interstitial spaces. Brain fixed by immersion. × 140.

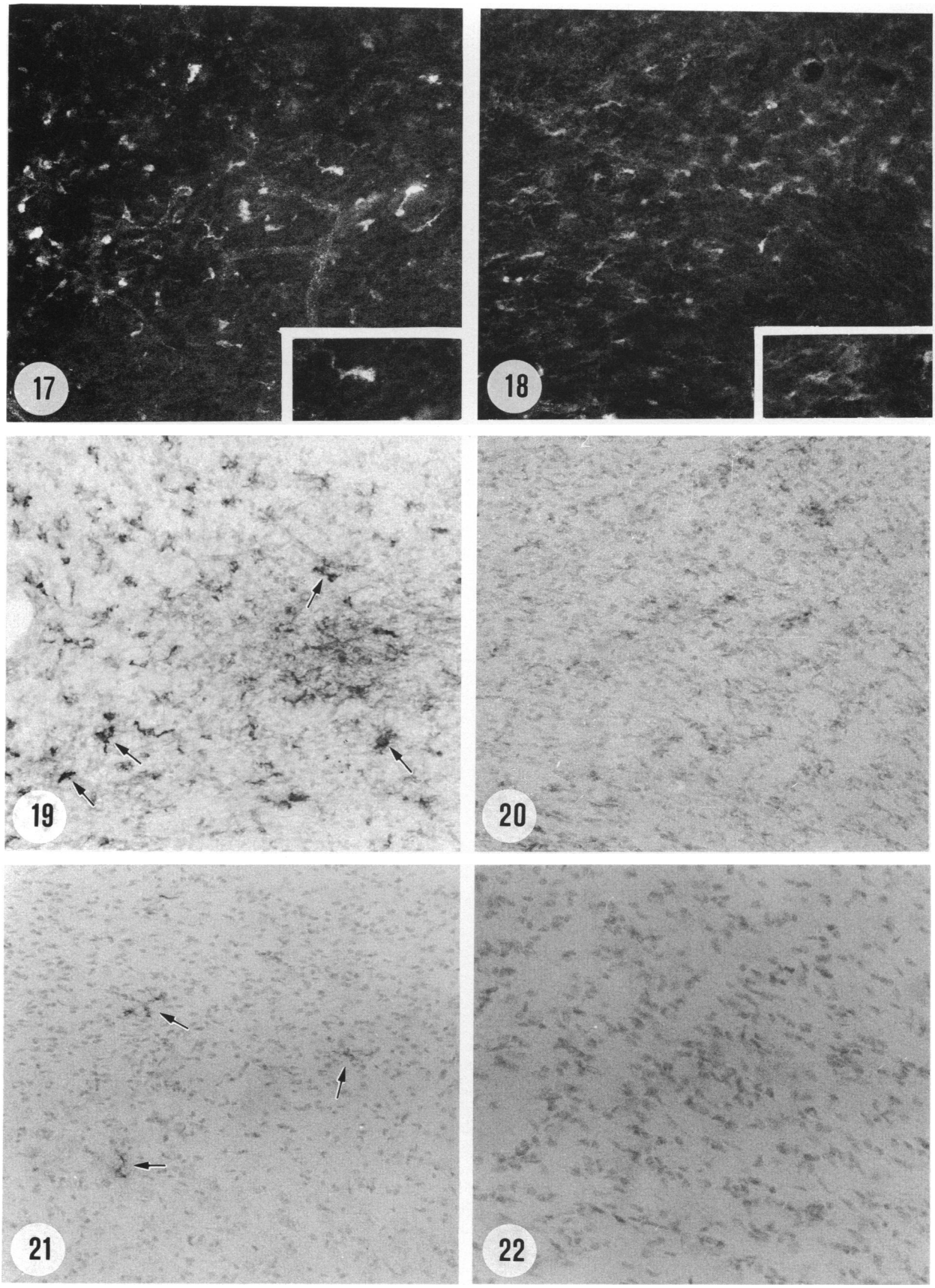
Fig. 12. Corpus callosum of a 7-d-old rat 2 d after an i.p. injection of RhIc. The AMC (arrows) are intensely labelled while the background fluorescence is greatly reduced (compare with Fig. 11). BV, blood vessels. Perfusion fixation × 140.

Fig. 13. Labelling of blood vessels (BV) and AMC (arrows) in the corpus callosum of a 5-d-old rat 20 min after an i.v. injection of RhIc. Perfusion fixation. × 270.

Fig. 14. Intensely labelled round AMC (arrows) in the corpus callosum of a 5-d-old rat 1 d following an i.v. injection of RhIc. Perfusion fixation. × 340.

Fig. 15. Corpus callosum of a 15-d-old rat 1 d after an i.v. injection of a large dose of RhIc (100 µl). Note the absence of RhIc-labelled cells. Perfusion fixation. × 140.

Fig. 16. Corpus callosum of a 15-d-old rat 6 h following an i.v. injection of RhIc. The fluorescent dye is clearly confined to the lumina of the blood vessels (*BV*). The tissue is devoid of labelled microglial cells. Brain fixed by immersion. × 140.



Figs 17, 18. Corpus callosum of a 12-d-old rat injected with RhIc immediately followed by a stab wound and perfused 2 d later. The number of RhIc-labelled microglial cells is significantly increased in the corpus callosum ipsilateral (Fig. 17) as well as contralateral (Fig. 18) to the stab wound (compare with Fig. 7). At the higher magnification, the majority of the labelled cells are seen to be ramified but the fluorescence appears to be brighter in AMC ipsilateral to the stab wound (compare cells in insets in both figures). Perfusion fixation. × 170, insets × 270.

AMC. The labelling pattern of AMC in temporal sequence also concurred in both the i.p. and i.v. injected rats (Figs 13, 14). Again, there was no detectable fluorescent cell beyond 13 d of age given RhIc injection, despite the fact that in some instances the RhIc was increased to 100 µl, which was more than double the usual dosage (Fig. 15). The RhIc injected was clearly confined to the lumina of the cerebral vessels (Fig. 16).

Intraperitoneal injection of RhIc and stab wound

In 12-d-old rats given an i.p. injection of RhIc followed immediately by a stab wound, a significant number (50–100 cells per section profile) of RhIc-labelled amoeboid microglia were observed in the corpus callosum ipsilateral (Fig. 17) as well as contralateral to the stab wound (Fig. 18), although in the latter the intensity of the fluorescence was clearly weaker. The majority of the cells were ramified, but some displayed stout processes (Figs 17, 18). The consecutive section that was immunostained with OX-18 showed that similar cells ipsilateral to the stab wound were strongly immunoreactive (Fig. 19), whereas the cells of the corresponding area on the contralateral side displayed a weaker immunoreactivity (Fig. 20). In the subsequent section that was incubated with OX-6, a few OX-6-positive cells were occasionally detected in the corpus callosum ipsilateral to the lesion, but this was inconsistent (Fig. 21). None of the cells in the corpus callosum on the contralateral side was stained (Fig. 22).

DISCUSSION

Our previous study (Leong & Ling, 1992) demonstrated the labelling of AMC following an i.p. injection of RhIc into neonatal (1–2 d) rats. The RhIc-labelled cells were confirmed as AMC because they showed immunoreactivity with OX-42 (Leong & Ling, 1992), an antibody that recognises CR3 membrane receptors synthesised by macrophages (Robinson et al. 1986), and also specifically marks the microglial cells in the corpus callosum in postnatal rats (Ling et al. 1990). The present investigation corroborates this, and further extends these results in showing that the labelling of AMC is age-related. The number of labelled AMC increased rapidly with age, peaking at the 7th postnatal day. This may have resulted from their vigorous proliferative activity in the early postnatal rats (Imamoto & Leblond, 1978). Thereafter, the number of labelled cells declined drastically, so that they were barely detectable at the 12th postnatal day and disappeared completely by the 13th postnatal day.

This study demonstrated unequivocally that the RhIc injected intraperitoneally was readily absorbed into the blood circulation and reached the cerebral vessels. The temporal sequence of the labelling pattern of AMC in both the i.p. and i.v. injected rats was comparable. Within 5 min of the injection (i.p. or i.v.), the blood vessels in the corpus callosum were clearly evident by their fluorescent dye content. The most remarkable feature, however, was the labelling, albeit with weak fluorescence, of the AMC in the vicinity of the blood vessels as early as 1 min after the injection of RhIc. Some of the labelled AMC that were closely associated with the blood vessels were more brightly labelled. This, taken together with the labelling of endothelial cells and the inundation of the interstitial spaces with the fluorescent dye, indicates that the injected RhIc had earlier entered the brain tissue by transendothelial transport and was readily endocytosed by the phagocytic AMC. It would seem that the endocytotic activity of AMC continued actively for several hours because of their progressive accumulation of RhIc, as shown by the gradual increase in fluorescence intensity with time.

It is possible that some of the labelled cells adherent to the walls of the blood vessels are the fluorescent granular perithelial (FGP) cells as described by Sturrock (1987) and Mato et al. (1980). Although these cells have been shown to take up HRP injected intraventricularly and intravenously (Mato et al. 1980, 1981, 1984) or from cerebral stab wounds (Cancilla et al. 1972), the FGP cells belong to a different type of cell than amoeboid microglia in their developmental sequences (Mato et al. 1985). According to Mato et al. (1985), FGP cells were not clearly defined in 5-d-old rats. Their uptake capacity for HRP did not develop until 2 wk, and reached a high level at 3 months. On the other hand, the present study demonstrated that

Figs 19, 20. Consecutive section to Figures 17 and 18 immunostained with OX-18. The AMC in the corpus callosum ipsilateral to the stab lesion (Fig. 19) show a stronger immunoreactivity when compared with the corresponding area on the contralateral side (Fig. 20). Perfusion fixation. ×170.

Figs 21, 22. Consecutive section to Figures 19 and 20 immunostained with OX-6. A few sporadic AMC (arrows) showing weak immunoreactivity are present in the corpus callosum (Fig. 21) ipsilateral to the stab wound. The cells on the contralateral side remain unstained (Fig. 22). Perfusion fixation. Figure 21, ×170; Figure 22, ×270.

AMC picked up RhIc actively during the 1st week after birth, and this capacity diminished markedly thereafter. We are thus inclined to speculate that the RhIc-labelled cells closely associated with the blood vessels are most likely to be AMC.

The simultaneous diminution in the number as well as the fluorescence of the labelled AMC with age may have one or more of the following explanations. One possibility would be that the full function of the blood–brain barrier (BBB) of the cerebral vessels in the corpus callosum is gradually established and becomes fully developed on the 13th postnatal day. This is supported by the fact that even if a larger dose of RhIc is injected into rats of this age group, it is still retained in the vascular lumina. This view is compatible with the observations by Zanetta et al. (1985) and Vorbrodt et al. (1986) in the endothelial system in the rat cerebellum and mouse frontoparietal cortex respectively that BBB maturation becomes noticeable at approximately the 12th–13th postnatal days. In the present study, it is postulated that the gradual maturation of BBB in the corpus callosum would lead to a reduction of the transvascular transport of RhIc, and this would account for the diminution of labelled cells and their content of the fluorescent dye.

Another possible explanation for the gradual attenuation in the numbers of RhIc-labelled AMC and their fluorescence may be related to their reduced phagocytic activity in the course of their transformation from the round to the regressive ramified form in normal development (Kaur et al. 1985). Indeed, the present study showed that the labelled cells in the early age groups (1 to 7 d postnatal) were round, and exhibited more intense fluorescence when compared with the small and irregular cells in rats of the older age group (10–11 d). The weakly fluorescent and ramified AMC at 12 d of age, however, were readily activated following a stab wound coupled with an i.p. injection of RhIc. The activation of AMC was evidenced by their greater uptake of the tracer. It is possible that the RhIc in circulation might have leaked out of the traumatised blood vessels and consequently have been taken up by the cells. Although the AMC were labelled bilaterally, the labelled cells ipsilateral to the stab wound showed a stronger immunoreactivity with OX-18. The larger amount of RhIc ingested as reflected by their stronger fluorescence may have accounted for a more vigorous expression of their surface antigen. From a speculative point of view, it needs to be emphasised that the RhIc-labelled cells may in fact represent a mixed cell population comprising activated AMC and monocyte-derived macrophages. The latter may have entered the nervous tissue from damaged blood vessels after the stab wound. The techniques we have employed, however, do not allow a clear-cut differentiation of the RhIc-labelled cells from the two sources.

The labelling of AMC by RhIc in the present study leads us to conclude that the early postnatal corpus callosum is readily accessible to exogenous materials, e.g. foreign antigens by way of the vascular walls. The immediate question that arises would be the vulnerability of the early development of neurons and glial elements. It is vital that a protective barrier be provided to safeguard the internal milieu of the brain tissues before the BBB is fully established in the late postnatal period. In this connection, the phagocytic AMC, which are closely associated with the vascular walls, would help to scrutinise or trap any incoming exogenous materials. It is therefore postulated that the preponderant AMC in the early postnatal corpus callosum would serve more than just to remodel the callosal fibres (Ling, 1976; Innocenti et al. 1983*a*, *b*). On the other hand, it remains uncertain whether their close spatial and functional relation with the endothelial system before its maturation would have any direct relevance to their immunophenotypic features (Ling et al. 1990, 1991).

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