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Research report

Expression of LFA-1α and ICAM-1 in the developing rat brain: a potential mechanism for the recruitment of microglial cell precursors

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Abstract

Several studies agree that microglial cells derive from monocytes that infiltrate the central nervous system during development, but the precise mechanism by which these cells enter into the nervous tissue is still unknown. In this way, the aim of the present study was to analyze the expression of two cell adhesion molecules involved in the recruitment of blood leukocytes into tissues, the lymphocyte function-associated antigen-1α (LFA-1α) and the intercellular adhesion molecule-1 (ICAM-1) in the developing rat brain (from E16 to P18). By means of immunohistochemistry, our observations showed that LFA-1α and ICAM-1 were expressed in the developing rat brain with a definite distribution pattern and a characteristic time course of appearance. In the embryonic period, LFA-1α immunoreactivity was displayed not only by intravascular blood cells but also by intraparenchymal round cells with a horseshoe-shaped nucleus, showing the typical morphological features of monocytes. Monocyte-like cells present in the embryonic brain parenchyma often displayed mitotic profiles. LFA-1α immunohistochemistry also revealed the presence of some LFA-1α-positive cells belonging to the ameboid microglial population (mostly in the white matter from E18). In the postnatal period, LFA-1α immunoreactivity was displayed by some ameboid microglial cells (P0–P9) and also by some ramified microglia. LFA-1α immunoreactivity observed in ramified microglia was weaker when compared to LFA-1α stained ameboid microglia. In contrast, ICAM-1 immunolabeling during the embryonic period was mainly located in endothelial cells of parenchymal brain blood vessels (principally from day E18). Blood vessels in choroid plexus and meninges also expressed ICAM-1 during the embryonic time. In postnatal animals, ICAM-1 immunoreactivity was found in relation to endothelial cells of blood vessels, but the density of ICAM-1-positive blood vessels was lower than that during the embryonic period. The gradual regulation in the expression of LFA-1α by monocyte-like cells and cells of the microglial lineage, and the expression of ICAM-1 by the brain vasculature strongly suggest that the LFA-1/ICAM-1 system may be a mechanism involved in the entry of microglial cell precursors into the developing rat brain. © 1997 Elsevier Science B.V.

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1. Introduction

The monocytic origin of microglial cells is, at present, a widely accepted theory [32]. Recruitment of circulating blood monocytes and later gradual transformation into microglial cells during the development of the central nervous system (CNS) was demonstrated by Ling and collaborators [29,30] and largely supported by several recent developmental studies [4,10,20,39]. However, the signals and mechanisms mediating the monocyte entry into the developing CNS are still being debated [59].

It is well established that the expression of cell surface adhesion molecules is involved in the leukocyte–endothelium interaction and the subsequent migration of leukocytes into lymphoid, non-lymphoid and inflamed tissues [7,55,56]. An important adhesion molecule receptor–ligand pair participating in the extravasation of circulating leukocytes is the LFA-1/ICAM-1 adhesion system [60]. Lymphocyte function-associated antigen-1 (LFA-1) is a heterodimeric β2-integrin constituted by the alpha chain (LFA-1α; also known as CD11a) and the beta chain (CD18), the expression of which is restricted to leukocytes and their kindred [24,25,54,55]. Intercellular adhesion

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molecule-1 (ICAM-1), a ligand for the LFA-1, is a glycoprotein of the immunoglobulin gene superfamily that is basally expressed on vascular endothelium [33,51,55] and the expression of which is markedly up-regulated by the presence of inflammatory mediators [15,46,47,56].

When compared to other tissues, the endothelial cells of the cerebral blood vessels in the normal adult brain express low levels of adhesion molecules and the leukocyte traffic through the vascular network is very restricted [34,45]. However, in inflammation and some pathological states, the entrance of monocytes into the nervous tissue is enhanced and this mechanism contributes to the increase of the number of brain macrophage crowding CNS lesions [20,22,39]. In these circumstances, the migration of monocytes from the blood to the nervous parenchyma has been associated with the up-regulation in the expression of the LFA-1/ICAM-1 system [5,35], suggesting that these molecules play an important role in the regulation of blood cell recruitment into the damaged tissue [8,16,26,27,52].

On the basis of current knowledge, some of the physiological processes taking place during the inflammatory response may be based on similar mechanisms running in the developing CNS [36,37,40,41]. Therefore, the expression of cell adhesion molecules during the embryonic and early postnatal period could also play an important role in the recruitment of hematogenous precursors of microglial cells. The aim of the present study was to investigate the expression of LFA-1α and ICAM-1 cell adhesion molecules in the developing rat brain and analyze the correlation, if any, between the time course of expression of both cell adhesion molecules and the presence and distribution of microglial cell precursors.

2. Materials and methods

2.1. Tissue collection and fixation

The experimental material consisted of embryos and postnatal rats from the Wistar strain with ages ranging from E16 to P18 and grouped as follows: E16 ($n = 3$), E18 ($n = 3$), E21 ($n = 3$), P0 ($n = 3$), P6 ($n = 3$), P9 ($n = 3$) and P18 ($n = 3$). Fetuses were delivered by cesarean section while the mothers were under anesthesia by intraperitoneal injection of sodium pentobarbital (0.1 ml/100 g b.wt.). Embryonic brains were removed and fixed for 3 h at room temperature with Bouin's fluid (75 ml of saturated picric acid in distilled water, 25 ml of formalin and 5 ml of glacial acetic acid). Postnatal animals, under pentobarbital overdose anesthesia, were perfused through the left ventricle with Bouin's fluid for 5 min and brains dissected out and immersed for an additional 2 h at room temperature in the same fixative solution. Then, embryonic and postnatal brains were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Coronal sections (10 μm thick) were obtained with the aid of a microtome, serially collected, and mounted on gelatine-coated slices. Tissue sections were finally dewaxed, hydrated by sequentially transferring the slides through xylene, a degraded series of ethanol and distilled water, and immunocytochemically processed.

2.2. Immunohistochemical demonstration of LFA-1α and ICAM-1 adhesion molecules

After an initial rinse in 0.05 M Tris-buffered saline at pH 7.4 (TBS), endogenous peroxidase activity was abrogated by incubation of the sections in 2% H_2O_2 in Trizma base saline (TBS) for 10 min at room temperature. After rinsing in TBS (3×10 min), care was taken to prevent non-specific binding by pre-incubation of sections at room temperature for 30 min in 10% fetal calf serum (FCS) diluted in TBS. Then, sections were incubated overnight at 4°C either with the primary monoclonal antibody to rat LFA-1α (Serotec, UK) diluted 1:40 in TBS with 10% FCS, or with the primary mouse antibody against rat ICAM-1 (Serotec, UK) diluted 1:30 in TBS with 10% FCS. After rinsing in TBS (3×10 min), the sections were incubated at room temperature for 60 min with the biotinylated anti-mouse antibody (Amersham, UK) diluted 1:300 in TBS with 10% FCS, and rinsed again in TBS (3×10 min). The samples were then incubated for 60 min at room temperature with avidin-peroxidase (Sigma, USA) diluted 1:650 in TBS with 10% FCS and rinsed in TBS (3×10 min). The peroxidase reaction was visualized by using 3-3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen following either the standard method or the intensified glucose oxidase method. Briefly, for the standard DAB method, the peroxidase labeling was carried out by incubating sections for 3 min in 50 mg DAB diluted in 100 ml of 0.05 M Trizma base (TB), pH 7.4, containing 0.033 ml of H_2O_2. For the glucose oxidase method, sections were rinsed in 0.1 M acetate buffer at pH 6 (2×10 min) and immersed for 7 min at room temperature in fresh incubation medium. The incubation medium was prepared by adding 4 mg glucose oxidase (Boehringer Mannheim) to a mixture composed of a solution containing 3.6 g ammonium nickel sulphate (Fluka), 0.3 g D-glucose and 0.06 g ammonium chloride (Probus) in 75 ml of 0.2 M acetate buffer at pH 6, and a solution with 30 mg DAB in 75 ml of distilled water. Some sections were counterstained with Harris' haematoxylin. Finally, sections were dehydrated in alcohol, cleared in xylene, and coverslipped with DPX synthetic resin. Control sections were processed with the omission of the primary antibodies.

2.3. Double-labeling technique

Simultaneous demonstration of LFA-1α and cells of the microglial lineage was achieved through the sequential combination of LFA-1α immunostaining and tomato lectin histochemistry, a selective microglial cell marker [1,2].

2.3.1. Histochemical reaction for tomato lectin binding

LFA-1α immunoreacted sections from each age were rinsed in TBS (3 × 10 min) and peroxidase activity was blocked with 2% H_2O_2 in 100% methanol for 10 min at room temperature. Following a rinse in TBS (3 × 10 min) containing 0.1% Triton X-100, the sections were incubated overnight at 4°C with the biotinylated tomato lectin (*Lycopersicon esculentum*, Sigma) diluted to 15 μg/ml in TBS. Sections were then rinsed in TBS (3 × 10 min) and incubated for 60 min at room temperature with avidin–FITC conjugate (Sigma, USA) in a 1:150 dilution in TBS with 10% FCS. Finally, sections were mounted in Glycergel (Dako). Some of sections were lightly counterstained with haematoxylin. As a control for the histochemical staining, lectin was omitted.

3. Results

Microscopic examination of sections revealed that the expression of LFA-1α and ICAM-1 in the developing rat brain follows a definite cell distribution pattern and a characteristic time course of appearance. Developing brain areas analyzed in this study were cerebral cortex, subcortical white matter (corpus callosum and internal and external capsule) and hippocampus at the central/mid-posterior level. Double staining combining LFA-1α immunohistochemistry and tomato lectin histochemistry enabled us to demonstrate LFA-1α immunoreactivity related to cells of the microglial lineage. Both LFA-1α and ICAM-1 immunohistochemistry in control sections did not show any labeling. Results are summarized in Fig. 1.

3.1. Expression of LFA-1α adhesion molecule

During the embryonic period (E16–E21), LFA-1α immunoreactivity was displayed by some intravascular blood cells present within blood vessels in brain, meninges and choroid plexus. In the nervous parenchyma, LFA-1α immunolabeling was observed in relation to intraparenchymal round cells which were observed from E16 to P0, but mostly encountered at days E18 and E21. Intraparenchymal LFA-1α-positive cells were characterized by a round shape, were devoid of processes and, when counterstained with haematoxylin, often displayed a horseshoe- or kidney-shaped nucleus (Fig. 2A). These LFA-1α-positive intraparenchymal cells resemble monocytes and were referred to in this study as monocyte-like cells. LFA-1α-labeled monocyte-like cells were often observed in white matter areas: subcortical white matter and white matter of the hippocampus. In addition, these cells often showed mitotic profiles, preferentially at days E18 and E21. Fi-

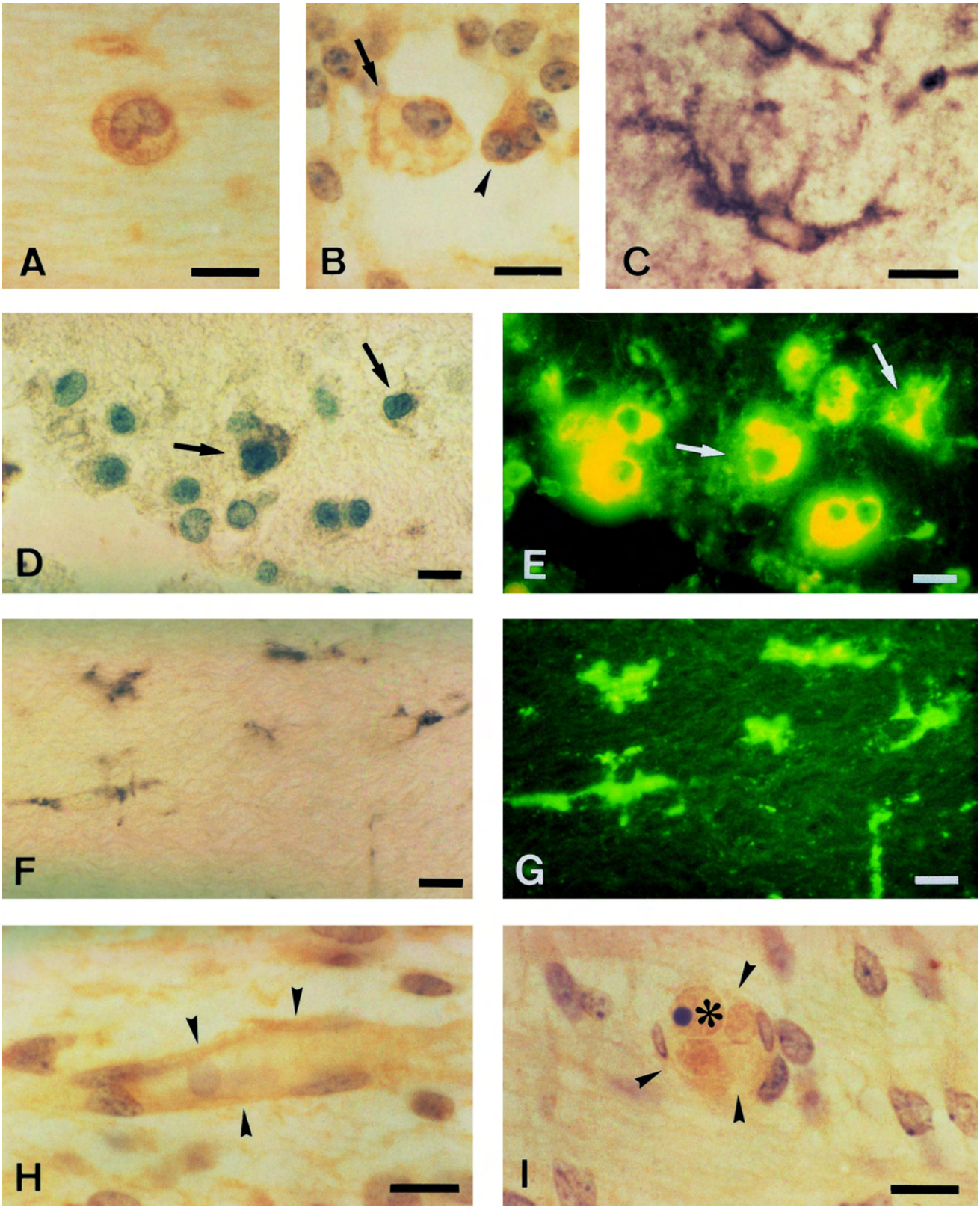
			E16	E18	E21	P0	P6	P9	P18
LFA 1α	Extravasated monocyte-like cells		+	++	+	+/-	-	-	-
	Ameboid microglia		+/-	++	++	+/-	+/-	+/-	-
	Ramified microglia	Primitive ramified microglia	-	-	+/-	+	+	+	-
		Resting microglia	-	-	-	-	-	-	+
ICAM 1	Endothelium of blood vessels in brain parenchyma		+	++	++	+/-	+/-	+/-	+/-

- Cells that are not found at the corresponding age or show negative staining.
+ Cells displaying the adhesion molecule.
++ High density of cells displaying the adhesion molecule.
+/- Low density of cells displaying the adhesion molecule.

Fig. 1. Summary of results related to LFA-1α expression in cells of the microglial lineage and ICAM-1 expression in brain parenchymal blood vessels from E16 to P18.

nally, LFA-1α-positive cells of round shape were also found in lateral ventricles.

During the embryonic period (E16–E21) and the postnatal ages from day of birth to P9, the double staining combining tomato lectin histochemistry and LFA-1α immunohistochemistry enabled us to demonstrate a subpopulation of tomato lectin-positive ameboid microglia expressing LFA-1α (Fig. 2D,E). These LFA-1α-positive ameboid



microglial cells were often distributed in white matter areas and mostly demonstrated on days E18, E21 and P0. They had a pleomorphic shape, often somehow roundish with a characteristic vacuolated cytoplasm, and devoid of well-defined cell processes (Fig. 2B).

In the postnatal period (P0–P18), LFA-1α immunoreactivity in the parenchyma was also associated with some cells of the primitive ramified (Fig. 2F,G) and resting (Fig. 2C) microglial population in the developing gray and white matter when double-labeling technique combining LFA-1α-immunohistochemistry and tomato lectin histochemistry was carried out. Primitive ramified microglia were poorly ramified microglia mostly observed during the first 2 postnatal weeks [11]. Resting microglia were cells showing the characteristic shape of adult microglia and mostly observed from the third postnatal week [32]. Primitive ramified microglia and resting microglia were generally termed in this study as ramified microglia. LFA-1α-positive ramified microglial cells showed different levels of immunoreactivity, but generally weaker than that found in ameboid microglial cells. This was especially the case for resting microglia, which displayed the most weaker LFA-1α immunoreactivity, often diffuse and frequently restricted to their processes, being these cells for that difficult to visualize in the brain parenchyma.

3.2. Expression of ICAM-1 adhesion molecule

ICAM-1 immunoreactivity was found in relation to endothelial cells of blood vessels belonging to the vascular network of the brain parenchyma, choroid plexus and meninges. Parenchymal ICAM-1-positive blood vessels (Fig. 2H) were seen in both gray and white matter during both prenatal and postnatal periods, but were more abundant and displayed higher levels of immunoreactivity in the developing white matter during the prenatal period, particularly at days E18 and E21. ICAM-1-negative blood vessels were also demonstrated during all embryonic periods (Fig. 2I) and specially during the postnatal period. In the choroid plexus of lateral ventricles, blood vessels displaying ICAM-1 immunoreactivity were preferentially observed from day E16 until day P0, the labeling being less significant in the later postnatal days (P6–P18). Immunoreactive blood vessels in meninges were observed from E16 to P18.

4. Discussion

The active migration of leukocytes into tissues is regulated by the expression of cell surface adhesion molecules on both circulating and vascular endothelial cells [56]. In particular, several adhesion proteins of the selectin, super immunoglobulin and integrin family have been demonstrated in the physiologic process of blood-borne monocyte recruitment [17,23,58]. In the normal brain, the extravasation of monocytes was first shown by Roessman and Friede [44] by injecting radioactively labeled cells from the bone marrow of donor animals into recipient animals. Later, Ling and collaborators [31] confirmed the entry of monocytes by the appearance of labeled cells in the CNS parenchyma either by intravascular administration of a carbon suspension to mark circulating monocytes or after direct injection of carbon-labeled monocytes. In these studies, Ling and collaborators reported that the entrance of these blood cells into the developing CNS is a common phenomenon occurring during the perinatal period. Subsequently, these extravasated monocytes differentiate into ameboid microglia and then into ramified microglia. In concordance with these observations, we recently reported that microglial cell precursors in the developing rat hippocampus may originate from different mesodermal sources, for example, from monocyte-like cells that gradually transform into ameboid microglia [11].

Reporting further insights into the above results, the present work shows that LFA-1 and ICAM-1 are expressed in the developing rat brain and follow a definite distribution pattern and a characteristic time course of appearance. LFA-1α immunoreactivity was displayed by intravascular and extravasated monocyte-like cells during the fetal period, mostly, as well as in the early postnatal days. ICAM-1 immunoreactivity was observed in endothelial cells of cerebral blood vessels and was particularly manifest during the embryonic period. Thus, potentially, blood monocytes

Fig. 2. Immunostaining for LFA-1α (A–G) and ICAM-1 (H–I) adhesion molecules. All sections except (C) and (F,G) are counterstained with haematoxylin. A,B,H,I: immunoreactivity was visualized by standard peroxidase labeling using DAB as chromogen (brownish colour). C,D,F: the peroxidase reaction was intensified by the glucose oxidase method (blackish colour). E,G: tomato lectin-labeled cells of the microglial lineage were visualized by means of fluorescence (yellowish colour). A: developing internal capsule on day E18 showing an intraparenchymal LFA-1α-labeled monocyte-like cell with a horseshoe-shaped nucleus. Note that LFA-1α immunoreactivity in LFA-1α-labeled monocyte-like cell is observed in the cell membrane and the cytoplasm. B: Developing subcortical white matter at day E18. LFA-1α-stained ameboid microglia (arrow) and LFA-1α-positive monocyte-like cell (arrowhead) showing a kidney-shaped nucleus. C: Molecular layer of the cerebral cortex on P18. Resting microglia displaying high levels of LFA-1α immunoreactivity are occasionally encountered in the brain parenchyma. Resting microglia are ramified shape with a roundish to slightly elongated cell body bearing long, fine, tortuous, crenulated cell processes. D: LFA-1α-positive amoeboid microglial cells (arrows) in the developing subcortical white matter at E18. E: notice that these ameboid microglial cells clearly LFA-1α-labeled in (D) are also stained with tomato lectin (arrows). F: LFA-1α-labeled primitive ramified microglial cells in corpus callosum at day of birth. G: same LFA-1α-immunolabeled microglial cells showed in (F) are tomato lectin positive. H: blood vessel (arrowheads) in the hippocampal fissure at day E18 displaying ICAM-1 immunoreactivity. I: ICAM-1 non-labeled blood vessel (arrowheads) in the developing corpus callosum on day E18. Note the presence of some red blood cell types (asterisk) ICAM-1 immunolabeled within this blood vessel. Scale bars: 10 μm.

expressing LFA-1 on their surface may interact with ICAM-1-positive endothelial cells and enter into the nervous tissue during the perinatal period. In particular, this entry could be mainly made in developing white matter areas, since we often observed intraparenchymal monocyte-like cells expressing LFA-1α in the subcortical white matter. In the same way, ICAM-1-positive blood vessels were abundant in white matter. Our observations also revealed the presence of proliferating LFA-1α-positive monocyte-like cells, preferentially in the embryonic parenchyma. This may indicate that not only the recruitment of blood monocytes but also the proliferation of extravasated cells contribute to the increase of microglial cell precursors in the developing CNS. In addition, it should be noted that we visualized LFA-1α-positive monocyte-like cells within meninges and LFA-1α-labeled roundish cells in lateral ventricles as well as ICAM-1 immunoreactivity associated with blood vessels in meninges and choroid plexus. Therefore, potentially, cell precursors of microglia may also enter into the developing nervous parenchyma from the cerebral ventricles and/or the meningeal connective tissue layer, as it has been previously suggested by other authors [4,6,9,12,13,21,28,39].

The expression of the LFA-1/ICAM-1 adhesion system is fundamental to the firm adhesion between the leukocyte and the endothelium and it seems to be critical for the process of extravasation/diapedesis of leukocytes. However, to initiate the recruitment of leukocytes, the endothelium must be activated by appropriate signals inducing ICAM-1 expression. A wide number of factors such as inflammatory mediators (histamine, thrombin), cytokines (interleukin (IL)-1, IL-4, IL-6, tumor necrosis factor-alpha (TNFα), and interferon-gamma, chemoattractants (IL-8 and monocyte chemoattractant protein-1 or platelet-activating factor) and complement proteins have been reported to induce or up-regulate the expression of adhesion receptors in leukocytes and endothelium in response to inflammation [53]. Two of these factors, IL-1 and TNFα, which regulate and induce ICAM-1 expression in the vascular network [15,42,46,56,61], are found during normal development of the CNS [36,37,41]. In particular, Giulian et al. [19] reported that the highest concentrations of IL-1 in the normal developing rat cerebral cortex were found during the perinatal period from E18 to the time of birth. This peak in the expression of IL-1 during the perinatal period closely correlates with the peak of LFA-1α-positive monocyte-like cells and ICAM-1-positive blood vessels observed by us and correlates well with the increase in microglial cell numbers reported in other studies during the late embryonic period [4,39]. Therefore, although some other signals may be involved in the regulation of the expression of the LFA-1/ICAM-1 pair, the presence of molecules like cytokines in the early brain development may be of importance for monocyte migration into the brain parenchyma.

In addition to LFA-1α-positive monocyte-like cells, we

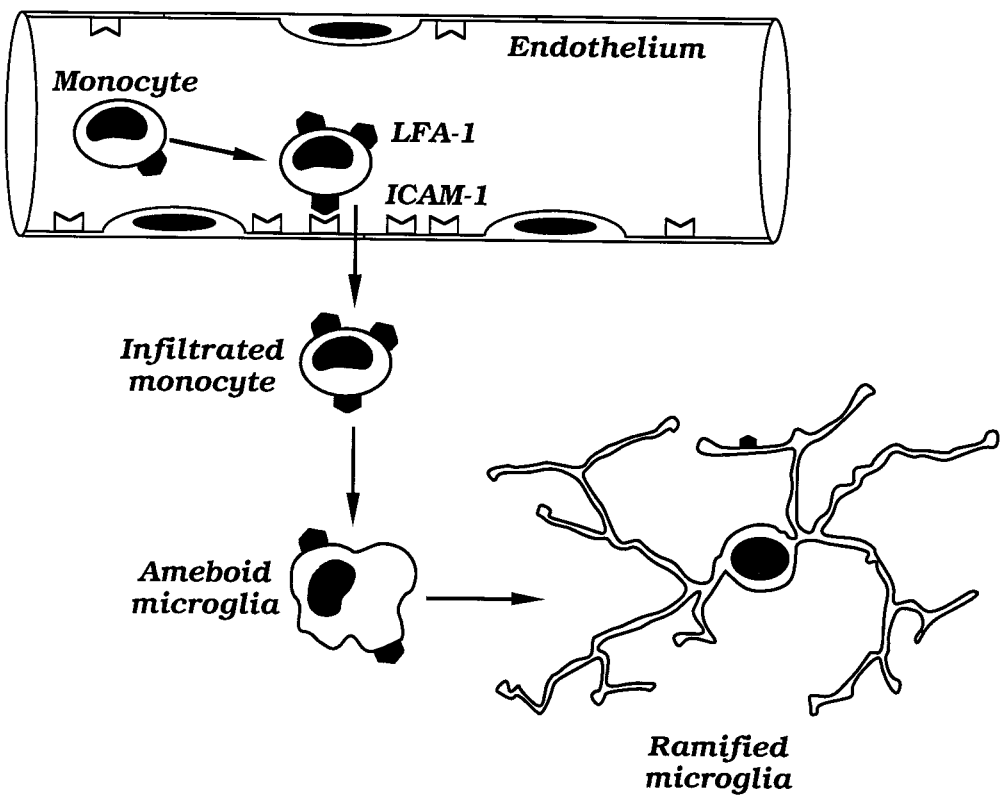


Fig. 3. Possible implication of the LFA-1/ICAM-1 system as mechanism for the recruitment of microglial cell precursors into the developing brain.

also found LFA-1α expression in some cells of the ameboid and ramified microglial population, suggesting that LFA-1α parenchymal monocyte-like cells may differentiate into LFA-1α-positive ameboid microglial cells and later into LFA-1α-positive ramified microglial cells. Thus, there may be a subpopulation of microglial cells derived from extravasated blood monocytes that migrate into the brain from parenchymal blood vessels. The maturation process from LFA-1α-positive monocyte to LFA-1α-labeled ramified microglia is, in addition, accompanied by a gradual down-regulation in the LFA-1α expression, so that monocyte-like cells express higher LFA-1α levels than ameboid microglial cells and the expression of LFA-1α is higher in ameboid than in ramified microglia. In fact, mature resting microglial cells observed at day P18 normally express low levels of LFA-1α immunoreactivity or even lack this cell adhesion molecule. In this way, in vitro LFA-1α expression in differentiated macrophages has been shown to be lower than in monocytes [43], and it is known that resident differentiated macrophages in some tissues lack this cell adhesion molecule [57]. In agreement with these results, it has been reported that in the normal adult rodent brain resting microglial cells are occasional [14] or partially [38] LFA-1-immunostained. Under certain pathological conditions, however, the expression of LFA-1 is induced in reactive microglia [3,14,18,38,48].

In summary, our results indicate that the LFA-1 and ICAM-1 adhesion molecules may participate in the mechanism of recruitment of monocytes as microglial cell precursors in the developing brain (Fig. 3). However, other cell adhesion molecules like the very late-antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1) pathway may also contribute with this recruitment because it has been demonstrated as an integral system for the monocyte recruitment [58] in the brain inflammatory reaction [16] and in neurological CNS disorders [5,49]. Moreover, this may be supported by the description of VLA-4 expression by microglial cells [50]. Therefore, further studies are needed for the accurate identification of the factor or factors implicated in the mechanism that regulates monocyte entry into the developing brain.

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