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A lectin histochemistry study on the development of rat microglial cells

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INTRODUCTION

Both the origin and the nature of the microglial cell are controversial aspects of this cell type of the central nervous system (for review, see Ling, 1981). Different methods have been applied to the study of these problems, namely autoradiography (Kitamura, Miyake & Fujita, 1984; Kitamura, 1985), cytochemistry (Boya, Calvo & Prado, 1979; Ling, Kaur & Wong, 1982; Tseng, Ling & Wong, 1983*b*; Fujimoto, Miki & Mizoguti, 1987) and electron microscopy (Kaur, Ling & Wong, 1985; Boya, Carbonell, Calvo & Borregon, 1987).

Recently, lectin histochemistry has arisen as a useful tool for the study of the neurobiology of the microglial cell (Mannoji, Yeger & Becker, 1986; Streit & Kreutzberg, 1987; Suzuki *et al.* 1988). However, studies on microglial development using these techniques have been carried out only on the retina (Ashwell, 1989; Ashwell, Holländer, Streit & Stone, 1989). In the present work, we have applied lectin histochemical techniques to study the development of the microglia in the central nervous system.

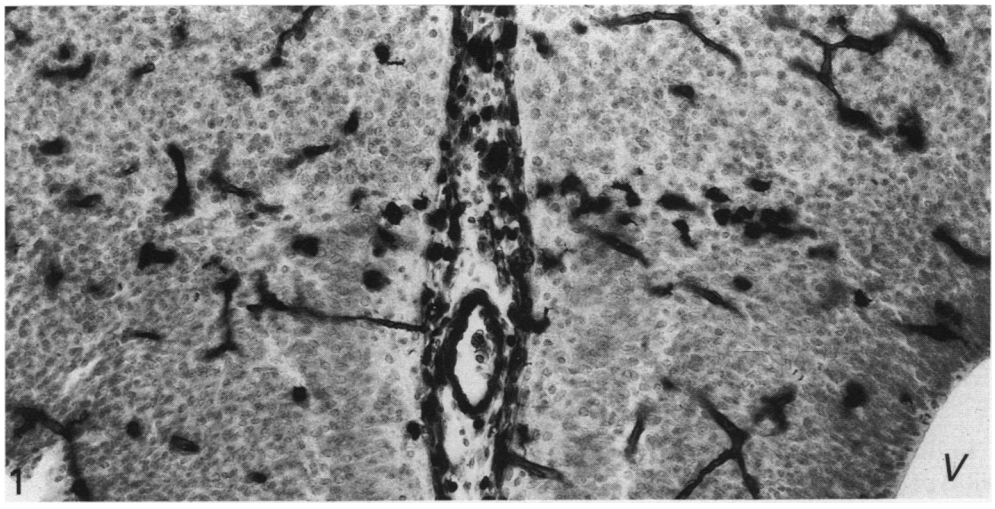
MATERIALS AND METHODS

Tissue preparation

Wistar rat fetuses aged 18 and 21 days (considering the fertilisation day as Day 0, based on vaginal cytology control) and postnatal rats aged 1, 2, 3, 5, 7, 9 and 12 days were used for the study. The embryonic heads were fixed in block by immersion. Postnatal rats were decapitated under ether anaesthesia and the brain quickly removed and fixed. In all cases, Bouin's fluid was used as fixative for 18 hours (75 ml saturated picric acid in distilled water, 25 ml formalin, 5 ml glacial acetic acid). Afterwards, specimens were washed in tap water, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections (7 μm thick) were cut at different levels of the cerebrum and cerebellum and mounted on glass slides.

Lectin histochemistry

Tissue sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (15 minutes). After rinsing in phosphate-buffered saline, (PBS) 0·01 M at pH 7·3 for 15 minutes, sections were incubated overnight with the biotinylated lectins (Griffonia (Bandeiraea) simplicifolia B4 isolectin (GSA I-B_4) and Ricinus communis agglutinin-120 (RCA-1)) diluted in PBS, in a humidity chamber. Both lectins were obtained from Sigma (St Louis, USA), and used at an optimum concentration of 5 μg/ml for GSA I-B_4 and 2·2 μg/ml for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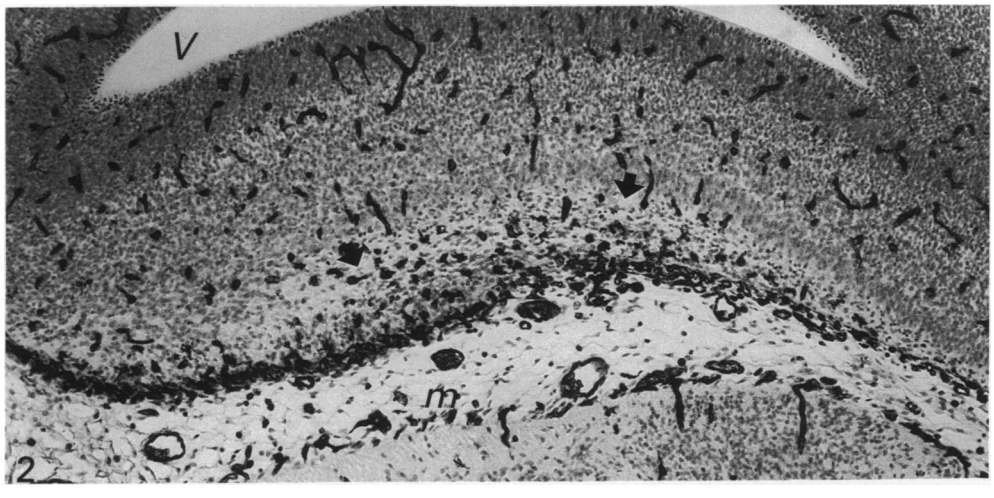
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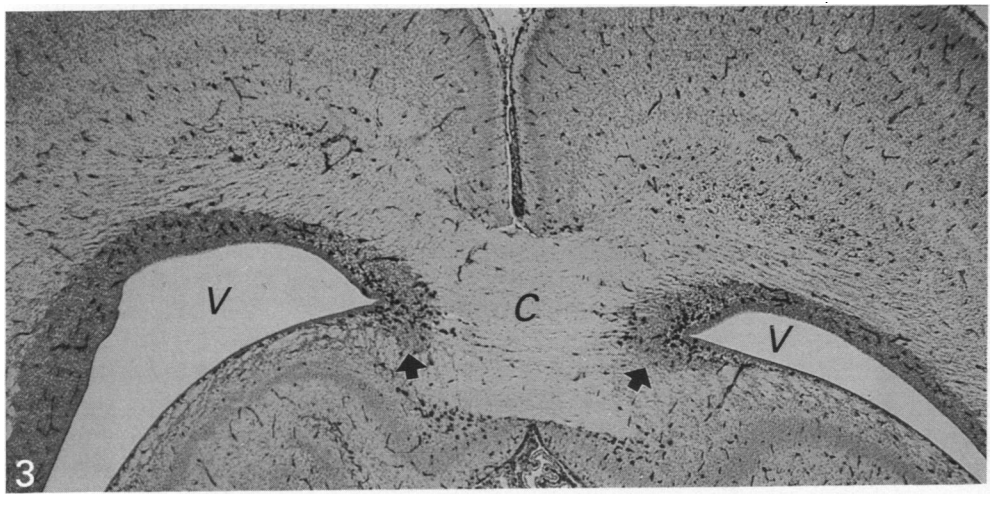
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RCA-1. After the incubation, sections were rinsed in PBS for 15 minutes, and incubated in Extravidin–horseradish peroxidase (Extravidin–HRP, Sigma, St Louis, USA) at a concentration of 5 μg/ml in PBS, for one hour in a humidity chamber. Sections were rinsed in PBS for 15 minutes, and treated with a solution of 3-3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, USA) and hydrogen peroxide (5 mg DAB, 10 ml PBS, 30 μl 0·3 % H_2O_2) under light microscopical control. After a rinse in distilled water, sections were dipped in 1 % osmium tetroxide in distilled water for 20 minutes to intensify the reaction product. Sections were washed in tap water and briefly counterstained with Carazzi's haematoxylin for 20–30 seconds. Finally, they were washed in tap water, dehydrated, cleared and mounted in Eukitt (Kindler, Freiburg, Germany). The histochemical procedure was performed at room temperature.

Controls

Controls were performed by incubating either with GSA I-B_4 in the presence of 0·2 M melibiose (6-*O*-α-D-galactopyranosyl-D-glucose, Sigma, St Louis, USA) or with RCA-1 in the presence of 0·2 M lactose (Sigma, St Louis, USA), to saturate lectin binding sites, thus preventing interaction with sugars of tissue components. In addition, controls not previously treated with the biotinylated lectins were incubated in Extravidin–HRP in order to exclude non-specific tissue binding. All these procedures resulted in a complete absence of any histochemical staining.

RESULTS

Both lectins used – GSA I-B_4 and RCA-1 – labelled developing microglial cells. However, GSA I-B_4 provided, for all phases studied, a more intense and complete staining of this cell type. The following descriptions are therefore based principally on our findings using GSA I-B_4.

At E18, groups of rounded or pseudopodic lectin-positive cells crowded the surface of the nervous parenchyma abutting the meningeal connective tissue infoldings such as the septum medium (Fig. 1) and the meningeal sheath interposed between the mesencephalon and the posterior part of the telencephalon (Fig. 2). Some rounded lectin-positive elements were found also within the meningeal connective tissue. Occasionally, incipient branching lectin-positive cells could be seen in deeper zones of the nervous parenchyma. The vascular bed of both the meningeal envelopes and the nervous tissue was well developed. A marked positive staining could be seen in the endothelial lining of blood vessels (Fig. 1).

Abundant rounded lectin-positive cells appeared at E21 in the subependymal region of the lateral ventricle, near the corpus callosum (Fig. 3). Elements of similar morphology could be traced laterally along the corpus callosum. Cell clusters related to the meningeal infoldings were similar to those found in the previous stage.

Fig. 1. Lectin GSA I-B_4. Eighteenth prenatal day. Frontal brain section through the interhemispheric septum medium. Lectin-positive rounded elements are seen in the meningeal connective tissue of the septum medium and in the adjacent nervous parenchyma. *V*, lumen of the lateral ventricle. × 215.

Fig. 2. Lectin GSA I-B_4. Eighteenth prenatal day. Frontal brain section showing the meningeal septum (*m*) separating the mesencephalon and the posterior telencephalic region. Note the presence of lectin-positive rounded elements in the nervous parenchyma next to the septum (arrows). × 107.

Fig. 3. Lectin GSA I-B_4. Twenty-first prenatal day. Horizontal brain section, showing the corpus callosum (*C*). Rounded lectin-positive cells (arrows) in the subependymal region of the lateral ventricles (*V*), adjacent to the corpus callosum. × 43.

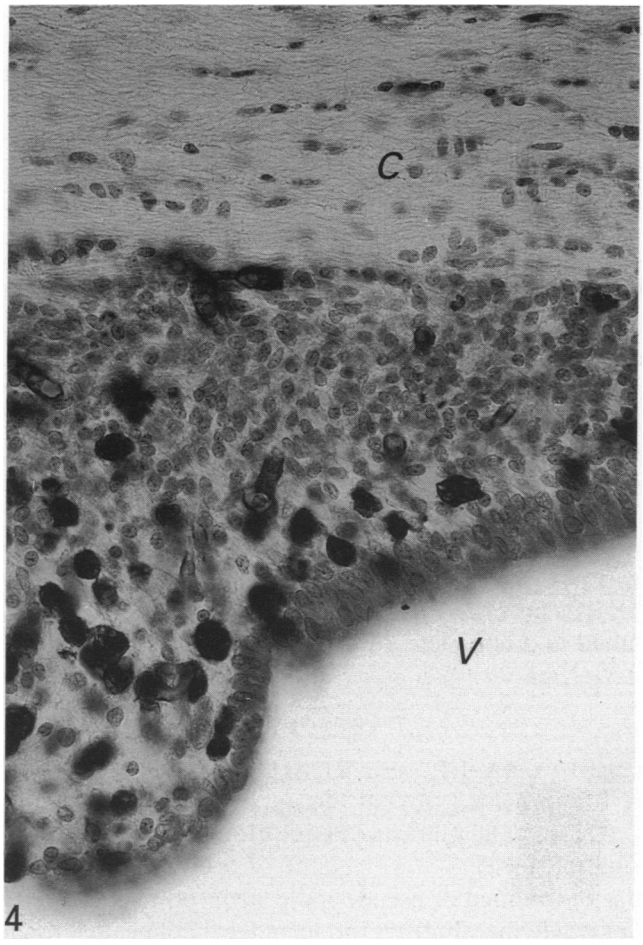


Fig. 4. Lectin GSA I-B_4. One day old rat. Rounded lectin-positive cells in the subependyma of the lateral ventricle. *C*, corpus callosum; *V*, ventricular lumen. ×370.

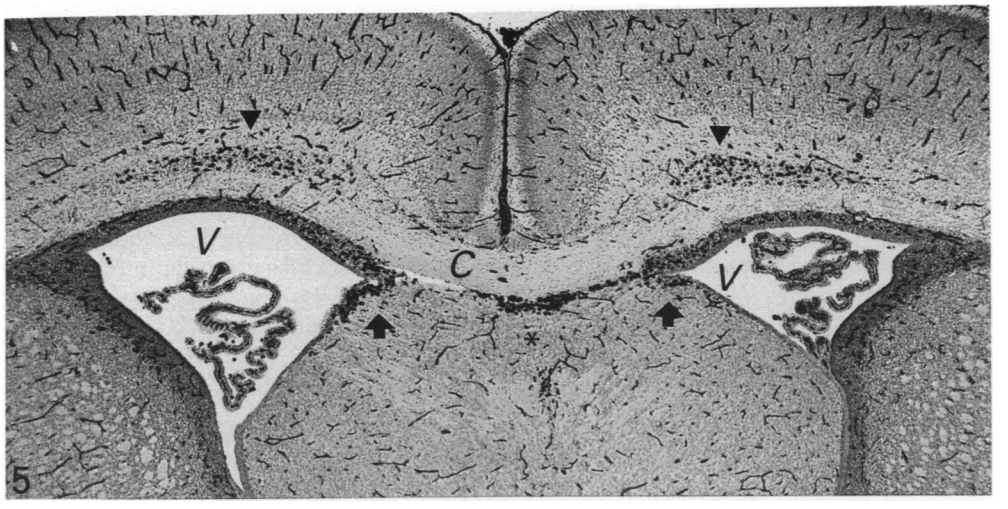
Branching cells found occasionally in the deep parenchymal zones did not yet present morphological features of the fully developed microglial elements.

In postnatal Day 1 (P1), clusters of lectin-positive rounded cells could be found in the most medial subependymal regions of the lateral ventricles (Fig. 4), near the cavum septum pellucidum. Small groups of positive cells similar to those described above were also seen within the lumen of the cavum. Lectin-positive elements were also present in the corpus callosum, particularly above the lateral ventricles. More externally in the corpus callosum, these cells were more scarce and often of an

Fig. 5. Lectin GSA I-B_4. 2 days old rat. Frontal brain section. Rounded lectin-positive cells near the cavum septum pellucidum (asterisk), subependyma of the lateral ventricles (arrows) and supraventricular corpus callosum (arrowheads). *C*, corpus callosum; *V*, lateral ventricles. ×43.

Fig. 6. Lectin GSA I-B_4. 7 days old rat. Frontal brain section. Numerous rounded and pseudopodic lectin-positive elements in the region of the supraventicular corpus callosum. Note transition forms towards branching microglia (arrows). *V*, lumen of the lateral ventricle. ×107.

Fig. 7. Lectin GSA I-B_4. 9 days old rat. Section of a cerebellar folium. Rounded and pseudopodic lectin-positive elements (asterisk) accumulate in the white matter. Branching microglia (arrows) extend into the grey matter. ×215.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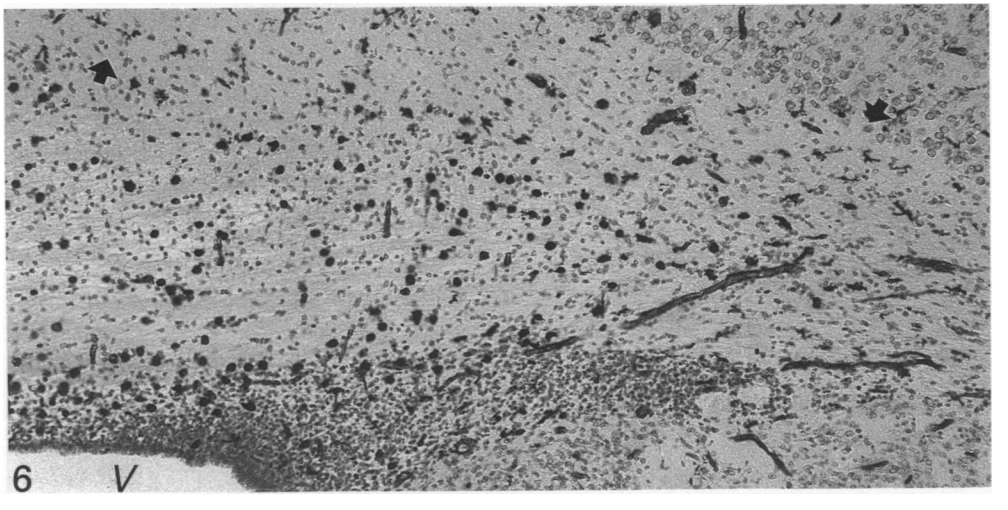
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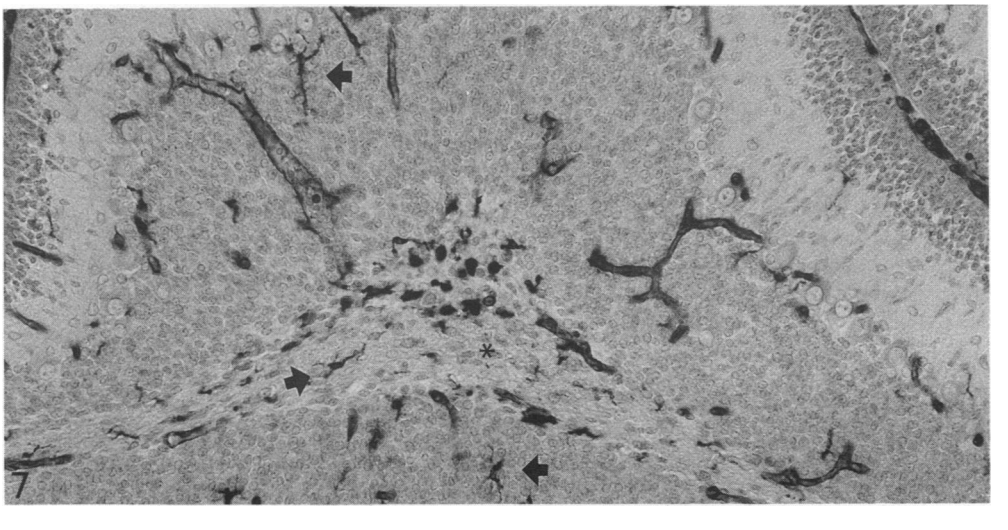
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elongated shape. Next to the medial meningeal septum and the base of the brain, there were superficial groups of lectin-positive rounded cells. Deep in the nervous parenchyma, positive branching elements were still scarce.

The number of lectin-positive rounded elements related to the region of the cavum septum pellucidum, subependyma of the lateral ventricles and the supraventricular corpus callosum (Fig. 5) increased considerably at P2. The superficial nervous parenchyma related to the meninx covering the base of the brain, and that interposed between the telencephalon and the mesencephalon, also showed abundant lectin-positive rounded or pseudopodic cells. Branching cells localised deeply in the nervous parenchyma were more abundant and showed more ramifications than those in previous stages. Furthermore, lectin-positive cells were detected within the ventricular lumen, closely related to the choroid plexuses.

The general picture at P3 is very similar to that described for P2. Numerous clusters of lectin-positive cells crowded the region of the cerebellar peduncles.

At P5 lectin-positive branching elements were easily found throughout the nervous parenchyma. In the cerebellum, lectin-positive microglial cells, mostly showing pseudopodia, were seen in the white matter but were seldom encountered in the grey matter.

At P7 and P9, numerous lectin-positive cells bearing pseudopodia remained in the supraventricular corpus callosum (Fig. 6), the nervous parenchyma abutting the external region of the lateral ventricle and the internal capsule. Throughout the nervous tissue, branching forms of microglial cells clearly predominated. In the cerebellum, some branching microglial cells appeared in the grey matter, though never within the external granular layer (Fig. 7).

Rounded and pseudopodic lectin-positive cells virtually disappeared from the nervous parenchyma around P12. Most microglial elements, very similar to those of the adult brain, showed thin cell processes. In the cerebellum, microglial cells clearly predominated in the white matter as opposed to the grey matter. Some ramified microglial cells could be found in the external granular layer.

DISCUSSION

The present investigation demonstrates the usefulness of lectin-histochemical techniques in the study of the early stages of development of microglial cells in the rat central nervous system. Rounded microglial cells were labelled with both lectins used (GSA I-B_4 and RCA-1). However, pseudopodic forms and branching forms, in particular, were better defined using GSA I-B_4. This data confirms previous studies of our group related to the lectin-histochemical demonstration of microglia in the adult brain of two species of mammals (Boya, Carbonell, Calvo & Borregon, 1990).

Hitherto, there have been only two reports devoted to the study of the origin of the microglia using lectin histochemistry; both with GSA I-B_4 and both in the retina of two species, rabbit (Ashwell, 1989) and rat (Ashwell *et al.* 1989). However, these techniques have not yet been applied to the study of microglial origins in other localities.

Lectin histochemistry confirms once more the interstitial and infiltrating character of microglial cells. As first pointed out by Río-Hortega (1921) in his light microscopical descriptions, immature microglial cells undergo a morphological transformation from rounded or pseudopodic cells to the typical branching appearance of the adult resting microglia as they progress towards the inner regions of the nervous parenchyma.

Immature microglial elements (rounded and pseudopodic) have been termed generally 'amoeboid microglia' (Ling, 1981).

The morphological changes undergone by the microglial cells while penetrating the nervous parenchyma can be easily explained if one takes into account the immaturity of the nervous tissue that is being invaded. In a previous ultrastructural study (Boya *et al.* 1987) we have already confirmed that the immature nervous parenchyma is loosely organised in these early developmental stages, with large extracellular spaces, particularly in the external regions. The ongoing maturation of the nervous tissue presumably forces the microglial cells to emit cell processes to adapt to narrowing interstitial spaces.

According to our results, the meningeal source for microglial cells, from meningeal connective tissue infoldings which penetrate deeply into the nervous parenchyma, is established at E 18. This source is very active until P5, with a progressive loss of activity thereafter.

In the cerebellum, apparently, there is no meningeal source for microglial cells. Perhaps the high cellularity of the external granular layer halts the flow of microglia from the pial connective tissue. Most cerebellar microglia penetrate the brain after crowding the cerebellar peduncles at P3. In further stages, microglial cells are restricted to the cerebellar white matter. Only after P9 are some branching microglial elements seen within the cerebellar grey matter. These cells do not reach the external granular layer until P12 and always in small numbers.

An interesting finding is the presence of abundant rounded microglial cells in the subependyma of the lateral ventricles and the supraventricular corpus callosum from E21, and particularly between P1 and P2. These microglial cells keep a close spatial relationship with the cavum septum pellucidum, which contains accumulations of lectin-positive rounded cells comparable to microglial precursors. Similar results have been reported by Tseng, Ling & Wong (1983*a*) and Tseng *et al.* (1983*b*).

All our findings favour a mesodermal nature for microglial cells. We confirm that the meningeal envelope is a source of these cells, in agreement with the classical theories of Río-Hortega (1921). This fact was previously confirmed by our group in a study combining silver impregnation and histochemical detection of hydrolytic enzymes (Boya *et al.* 1979). In our opinion, the cavum septum pellucidum can also be a mesenchymal source for microglial elements. This would easily explain the preferential localisation of groups of rounded microglial cells in the corpus callosum and in the subependyma of the lateral ventricles. In a recent ultrastructural study, Alonso *et al.* (1989) demonstrated, in the cavum septum pellucidum of the rat, collagen microfibrils and stellate cells similar to fibroblasts, thus confirming the mesenchymatous character of that cavity.

SUMMARY

A lectin-histochemical study on microglial development has been performed on the rat central nervous system. Isolectin B_4 from the Griffonia simplicifolia (GSA I-B_4) and Ricinus communis agglutinin-120 (RCA-1) were used as labelling lectins. Our results demonstrate the existence of microglial elements in the nervous parenchyma at E18, derived from the meningeal connective tissue layer. Later, another microglial source became evident, namely the cavum septum pellucidum, which serves for entrance of microglia pervading the supraventricular corpus callosum and the

subependyma of the lateral ventricles. From P12 onwards, the microglial sources became inactive.

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