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Research report

Early events of the inflammatory reaction induced in rat brain by lipopolysaccharide intracerebral injection: relative contribution of peripheral monocytes and activated microglia

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Abstract

We have previously demonstrated that lipopolysaccharide (LPS) intracerebral injection induced only minimal inflammatory reaction in rat brain, apart from an increased number of 'brain macrophages' observed 24 h after LPS administration [Montero-Menei et al., *Brain Res.*, 653 (1994) 101–111]. However, the nature of these 'brain macrophages' in the inflammatory response is still unclear. The present study focused on the early time-points (from 5 h to 24 h) after LPS injection or stab-lesion, and was aimed at the identification of the peripheral (monocytes) or parenchymal (microglia) origin of these 'brain macrophages'. OX42- and ED1-labeling did not clearly discriminate between monocytes/macrophages and reactive microglia, both cell types being immunoreactive. In other experiments, rats were made aplasic by irradiation prior to lesioning. These experiments clearly demonstrated that LPS induces an intense monocyte recruitment and, to a lesser extent, microglial activation since about 80% of the cells present 24 h after LPS injection consisted of recruited monocytes not observed in aplasic rats. Interestingly, our data show that LPS exerts a sequential dual action by first inhibiting the monocyte recruitment observed 5 h after stab lesion and then enhancing it at 15 h and 24 h after injection. A possible involvement of cytokines, chemokines and adhesion molecules in the mechanisms occurring in the early events of brain inflammatory reaction is discussed.

Keywords: Lipopolysaccharide, intracerebral injection; Brain inflammation; Microglial activation; Monocyte recruitment; Rat; Irradiation

1. Introduction

In a previous paper, we used an experimental paradigm consisting in the direct injection of lipopolysaccharide (LPS) into the rat brain, in order to investigate the central nervous system (CNS) response to inflammation. The data showed that the inflammatory response was far less intense than in peripheral organs and was only restricted to the lesioned area; in particular, only some neutrophils were present in the brain parenchyma [27]. It is now well established from various types of CNS injuries (ischemia, traumas, infections, experimental autoimmune encephalitis, etc.) that the proinflammatory cytokines TNF-α and IL-1 are rapidly produced in the brain (for review, see [7]). Our previous results support the hypothesis of the presence of immunosuppressive agents in the brain itself that counteract the effect of proinflammatory cytokines and reduce the extent of brain inflammation in order to avoid irreversible tissue damage. However, 24 h after LPS injection, an increased number of 'brain macrophages' in the lesion area was observed compared to a stab lesion, which leads to a similar disruption of the blood–brain barrier.

The question then arose whether these 'brain macrophages' consisted of monocytes recruited from the bloodstream or of microglia activated in situ. Many attempts to solve this problem have been based on different animal models and on various types of lesions or pathologies (e.g. [10,21,26,32]). The answer is however not quite clear and the early events crucial for the understanding have in particular not been explored since the kinetics of reactions concentrated on later time-points (one day of injury and often even later). It thus seemed of interest to try to answer this question using the same inflammatory model of LPS intracerebral injection where the number of

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'brain macrophages' is remarkably high. Moreover, we took a special interest in the early kinetics of the reaction, from 5 h after injury onwards, when this recruitment must take place in our model.

In an attempt to better characterize the monocytic or microglial phenotype of these 'brain macrophages', immunohistochemical experiments were carried out using the OX42 antibody, which labels both monocytes/macrophages and microglial cells, and the ED1 antibody, originally described as a specific marker of rat monocytes/macrophages [5]. This study has evidenced the high degree of plasticity of microglia, now recognized as being the main immune effector cells of the brain parenchyma (for reviews, see [1,35]). Since the various morphological and functional stages of activation have been described under different terms [3,8,9,28,36], we should first define the terminology used throughout this study for the different stages of microglia present in adult rat brain: resting microglia, with a typical ramified morphology consisting of a small cell body and long thin processes; activated (non phagocytic) microglia, with a hypertrophied soma and shortened and thickened processes; reactive (phagocytic) microglia, with a hypertrophic rounded body where very few short processes can sometimes be observed. As it appeared that ED1 antibody also labels reactive microglial cells in addition to monocytes/macrophages, we used a second approach consisting of the irradiation of rats before LPS injection or stab injury, in order to deplete the pool of circulating monocytes.

Two points of interest concerning the kinetics of the inflammatory reaction and the origin of 'brain macrophages' issued from the present study. First, an important number of monocytes/macrophages was observed 5 h after stab lesion but not 5 h after LPS injection, whereas 15 h and 24 h after injury, the number of these mononuclear phagocytes was far more important after LPS administration. Second, 24 h after LPS injection to rats which had been previously irradiated, the number of 'brain macrophages' was far reduced and was similar to that observed in the case of stab lesion to these aplasic rats, allowing us to clearly demonstrate that LPS induces a massive recruitment of blood monocytes into the brain parenchyma in immunocompetent rats.

2. Materials and methods

2.1. Rats

Sixty six Lewis female rats (200–225 g) from Charles River (Cléon, France) were used throughout this study. An inbred strain was chosen in order to minimize eventual differences due to individual responses.

Some of the animals received a stab lesion in the hippocampus, followed by the injection of 10 μg of LPS from *Escherichia coli* serotype 055: B5 (Sigma, St. Quentin Fallavier, France) in 10 μl of phosphate buffered saline (PBS), while others were submitted to the same lesion with the vehicle fluid only. This lesion will be hereafter referred to as stab lesion. Additional controls consisted of the injection of a suspension of charcoal particles together with LPS to verify the site of injection.

2.2. Stereotaxic injections

Stereotaxic injections were carried out as previously described [27]. Briefly, a hippocampal lesion was inflicted to anaesthetized rats with an 18 gauge needle at the following coordinates: 4 mm posterior from bregma, 3.7 mm lateral from the sagittal suture and 3.5 mm below dura. The LPS (10 μg in 10 μl PBS) or PBS injection was performed at the same coordinates at a rate of 1 μl/min with a 10 μl Hamilton syringe. All surgery was done under aseptic conditions.

2.3. Rat irradiation

To determine whether increases in OX42-positive cell number in rat brains were due to local division of microglia, recruitment of peripheral monocytes or both, we examined the effects of the depletion of circulating monocyte pools by irradiation prior to experimentation.

After a light anaesthesia with 2 ml/kg of Vetranquil (Sanofi, Aulnay-sous-Bois, France), rats were immobilized in Plexiglas boxes. In the first experiment, animals received a single 7 Gy dose by whole body-irradiation in a Saturne 41 linear accelerator (General Electric Co., Milwaukee, WI). The ionizing radiations (photons of 15 MV energy) were delivered at a rate of 3.5 Gy/min. The build-up was moved up to the skin by a bolus of plexiglas, so as to irradiate homogeneously the whole brain and the body. Since immunolabeling with OX42 antibody evidenced an intense microglial activation in the hippocampus and cortex of whole body-irradiated rats, the animals used for the present study received the same 7 Gy dose on the body only, their heads being maintained outside the radiation field. In these conditions, the number of OX42-positive microglial cells was far less important in the brain parenchyma. In addition, immunolabeling with OX6 and W3/25 antibodies demonstrated that the microglial cells of these animals did not express MHC class II and CD4 antigens. The weak activation of microglial cells in animals irradiated on the body only was indeed a requirement for the present study.

Peripheral blood cell counts carried out on a Max-M Coulter (Coultronics, Margency, France) revealed a strong decrease in leukocyte number, and in particular the depletion of 95% of circulating monocytes, 48 h post irradiation.

Two days after irradiation, animals were injected with LPS or with the vehicle fluid alone, then processed for

immunohistochemistry studies 5 h, 24 h or 3 days after the hippocampal lesion.

2.4. Immunohistochemistry

For immunohistochemical analysis, the animals were killed and their brains dissected at 5, 15, 24 h and 3, 7 and 15 days after surgery.

The brains were frozen in isopentane cooled by liquid nitrogen and 10 μm frontal sections were cut throughout the lesion site on a freezing microtome (Cryocut 3000, Leica, Rueil-Malmaison, France). The sections were fixed either in cold ethanol for 5 min (ED1, ED2 and OX42 antibodies), or in a solution of 2% paraformaldehyde in PBS for 10 min, and subsequently in cold acetone for about 10 s (W3/25 and OX6 labelling). They were then washed in PBS and incubated in a solution of 10% normal goat serum in PBS containing 4% bovine serum albumin (BSA) to block the eventual Fc receptor sites. The sections were incubated overnight at 4°C with mouse monoclonal primary antibodies directed against specific cell markers and MHC class II antigens (see Table 1). For ED1 labelling, sections were incubated with the first antibody in the presence of 0.1% Triton X-100. After rinsing in PBS, sections were incubated for 40 min with a rat-adsorbed biotinylated horse anti-mouse antibody (Vector, Burlingame, CA), then washed again.

Endogenous peroxidase activity was blocked by immersing the sections for 15 min in PBS supplemented with 0.3% H_2O_2. After washing, sections were incubated for 1 h at room temperature with an avidin-biotinylated peroxidase complex (Vectastain ABC kit, Vector) followed by washing in PBS. Peroxidase activity was finally revealed by incubating the sections in a solution of 0.04% diaminobenzidine (Sigma) in PBS containing 0.03% H_2O_2. The reaction was stopped by washing with PBS. Sections were counterstained by a 10 to 20% Toluidine Blue solution, then quickly rinsed with distilled water.

2.5. Semi-quantitative counts

For each animal, 6 to 8 sections in the area containing the lesion (about 50 μm in diameter) were examined. For ED1 and OX42 antibodies, counting was carried out in 3 to 4 sections in the region where the reaction was more important and that corresponded to the center of the lesion. The field used for counting comprised the hippocampus on the lesioned side as well as the area of the cortex and that of the corpus callosum directly over the hippocampus. All the ED1- and OX42-positive cells were counted except for the OX42-positive ramified resting microglia of the corpus callosum.

In irradiated rats, the number of OX42-positive cells in the contralateral hemisphere was subtracted from that in the lesioned hemisphere.

3. Results

3.1. Time-course of the cell reaction following a stab lesion or LPS intracerebral injection

The semi-quantitative counts of the number of cells labeled by ED1 and OX42 antibodies 5 h, 15 h and 24 h after stab lesion or LPS injection are presented in Fig. 1.

3.1.1. Five hours

Five hours after stab lesion, the presence of ED1-positive cells was exceptional, and the corresponding labeling was very weak in the brain parenchyma; only some monocytes present in brain microvessels and in ventricles were labeled (not shown). In contrast, an important number (around 700) of OX42-positive rounded cells, morphologically similar to monocytes (illustrated in Fig. 3C), were observed in the brain parenchyma, mostly in the lesioned hippocampus near the third ventricle and in the lesion site (Figs. 1 and 2A). In addition, a great number of these cells (about 300) were present in the third ventricle and in blood vessels and capillaries in the hippocampus and cortex surrounding the lesioned area (Fig. 2A,D). This seems to indicate that the OX42-positive cells observed in the brain parenchyma 5 h after stab lesion were issued from the bloodstream and ventricles. In support of this assumption is the observation that in previously irradiated rats, very few monocyte-like OX42-positive cells could be detected 5 h after stab lesion both in microvessels and in the third ventricle; these cells were moreover totally absent from the parenchyma (Fig. 2B). It is worth mentioning that, as shown in Fig. 2B, the OX42 antibody stained activated microglial cells. Indeed these cells were preactivated by irradiation (see Section 2).

Surprisingly, at the same time-point following intracere-

Table 1
Monoclonal antibodies used for immunohistochemistry

Antibody	Molecule identified	Dilution	Source
MRC-OX42	Complement receptor type 3: present on monocytes/macrophages and microglia	1/100	Serotec (Kidlington, UK)
ED1	CD68 – lysosomal antigen: monocytes/macrophages [5,6]	1/100	Serotec
ED2	Membrane glycoprotein: subpopulation of resident macrophages [5] and perivascular cells [13]	1/500	Serotec
OX6	MHC class II molecules	1/100	Cedarlane (Ontario, Canada)
W3/25	CD4: present on macrophages and activated microglia	1/200	Serotec

bral LPS injection into non-irradiated rats, almost no OX42-positive monocyte-like rounded cells were observed both in parenchyma and in microvessels, whereas some were detected in the third ventricle. In contrast, OX42-positive activated microglial cells, characterized by a hypertrophied body with shortened and thickened processes (Fig. 3H), were present mostly throughout the lesioned hippocampus and also in the corpus callosum (Figs. 1 and 2C). These cells were not labeled by ED1 antibody. In the contralateral hippocampus, no OX42-labeling was observed, either after stab lesion or LPS injection.

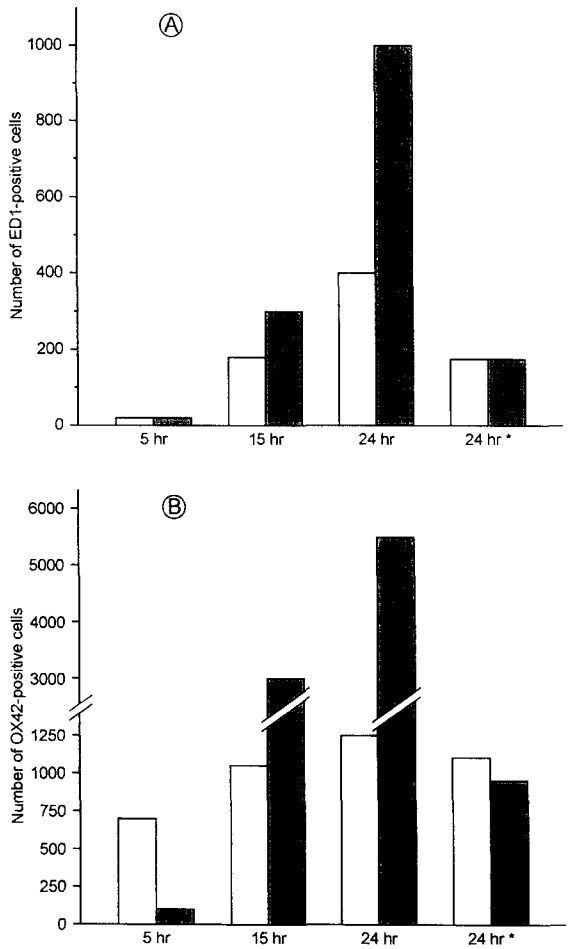


Fig. 1. Semi-quantitative counts of the number of cells labeled by ED1 (A) and OX42 (B) antibodies. The number of ED1- and OX42-positive cells was estimated 5 h, 15 h and 24 h after stab lesion (□) or LPS injection (▨) of non-irradiated rats. The last columns (24 hr*) represent the number of ED1- and OX42-positive cells observed 24 h after stab lesion (□) or LPS injection (▨) of previously irradiated rats. Concerning the OX42-positive cells (Panel B) all cells were counted except for the ramified resting microglial cells, mostly present in corpus callosum, since they do not correspond to activated cells and are present in control brains. Five hours after stab lesion (first column) only the $OX42^+$ cells present in the brain parenchyma were counted. Positive cells observed in microvessels or in the third ventricle were not taken into account.

When compared to control brains, the number of ED2-positive perivascular cells (Fig. 2E), observed in close association with small blood vessels and capillaries, was not modified by stab lesion or LPS injection.

3.1.2. Fifteen hours

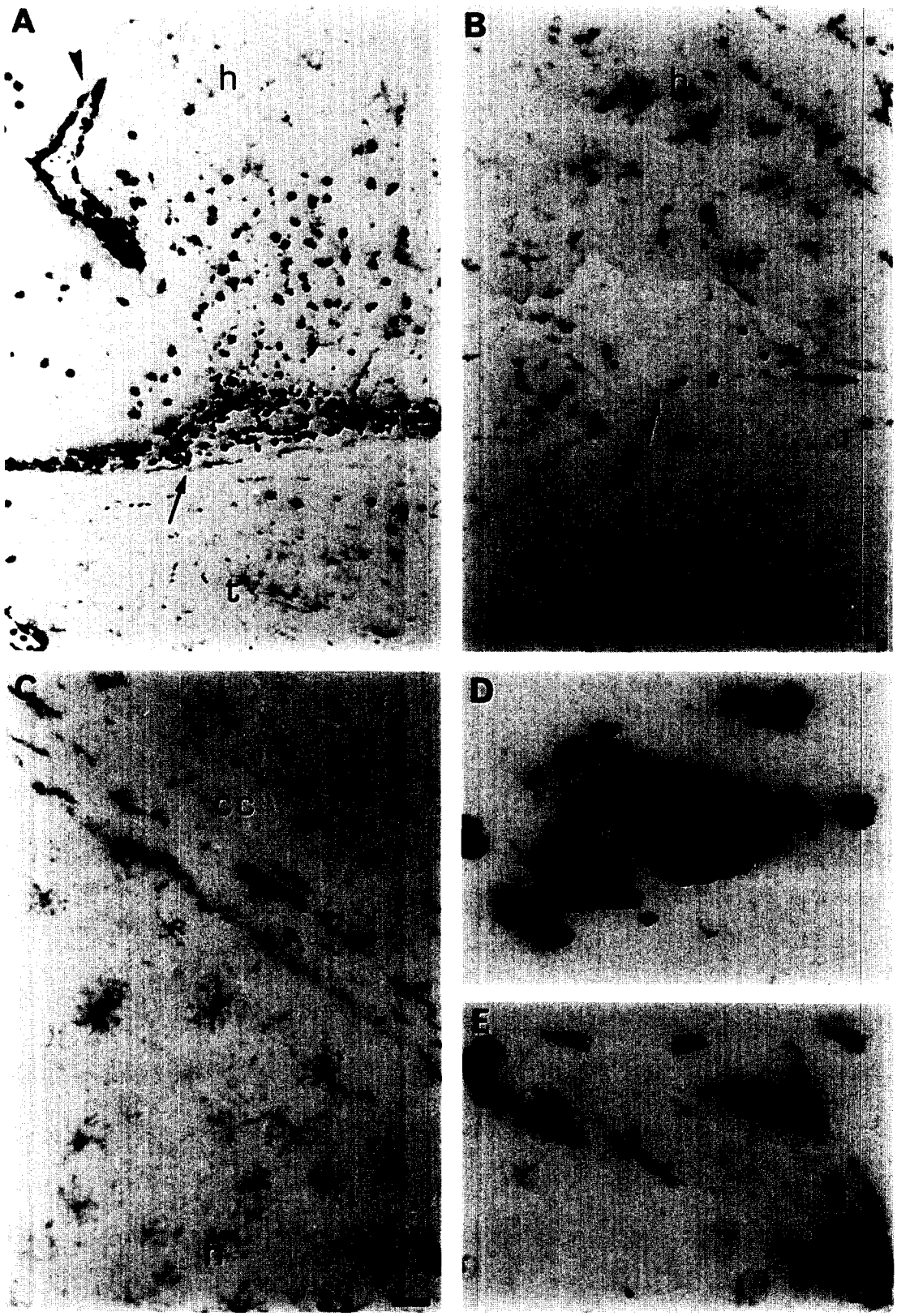
Fifteen hours after LPS injection, the number of total OX42-positive cells had increased considerably (Figs. 1 and 3A). The morphological aspect of these cells, scattered throughout the lesioned area, suggests that the majority of them (more than 2000) were monocytes. In addition, about 400 cells were activated microglia, and about 100 were reminiscent of reactive microglia as they displayed a rounded amoeboid shape with sometimes very short processes (Fig. 3I). Some areas of the corpus callosum near the lesion site were totally devoid of ramified resting microglia (this type of cells is illustrated in Fig. 3F), whereas activated microglia could be observed in this region. Among all these OX42-positive cells, only about one out of ten were labeled with ED1 antibody in adjacent sections (Fig. 3B). As constantly observed in the brain at early stages after injury, this labeling was always rather weak. The aspect of these cells, small and rounded, was mainly characteristic of monocytes (Fig. 3C,E) but quite a few reactive microglial cells with short processes were also observed throughout the lesioned hippocampus (Fig. 3I,J). However, ED1-labeling is cytoplasmic and punctiform, which makes it rather difficult to clearly distinguish reactive microglia. At the same time-point (15 h) after stab lesion, the cells labeled by each marker were far less numerous (Fig. 1) and they were mostly gathered around the lesion site (not shown). However, all the different cell types described above were also seen, thus indicating a delayed microglial activation.

3.1.3. Twenty four hours

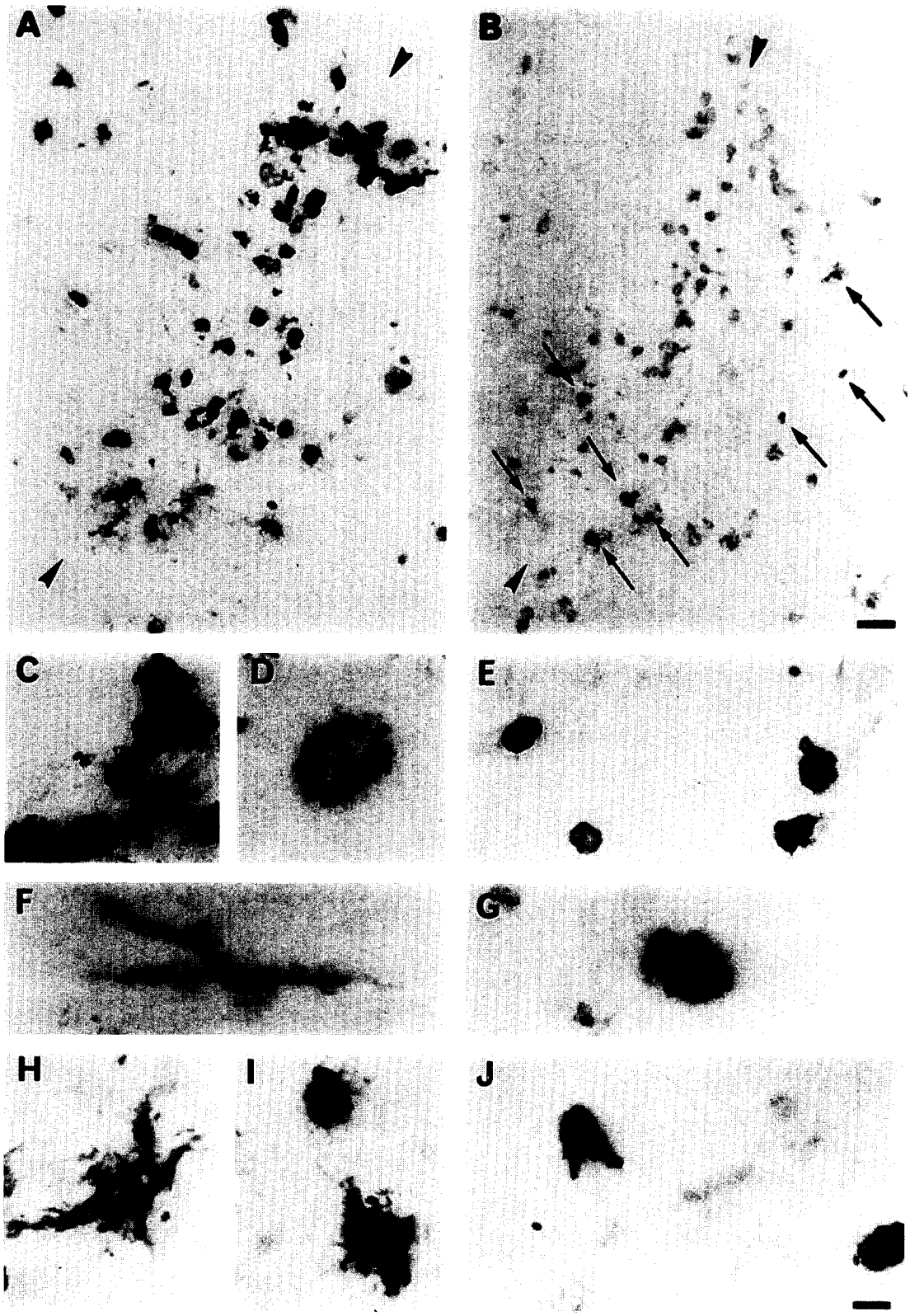
One day after LPS injection, the number of OX42-positive rounded cells showed a further increase since more than 5000 could be observed both around the needle tract and scattered throughout the hippocampus and the cortex

Fig. 2. Cellular response 5 h after a stab lesion or LPS injection (A to D: OX42-labeling, E: ED2-labeling). A: 5 h after a stab lesion, OX42-positive monocytes filled up the third ventricle (out of this field), interventricular junction (we refer to the area located between the thalamus and the hippocampus, which extends from the third ventricle towards the lateral ventricles, as an 'interventricular junction') (arrow) ipsilateral to the lesion and the blood vessels in the hippocampus (arrowhead). OX42-positive cells can also be seen invading the brain parenchyma. B: 5 h after a stab lesion into previously irradiated rats, almost no monocytes were observed in the ventricles and interventricular junction (arrow); a few activated microglia were found in the brain parenchyma in response to irradiation. C: after LPS injection into non-irradiated rats, activated microglia were observed in the hippocampus and cortex ipsilateral to the lesion. A to C: bar = 40 μm. D and E: photomicrographs at higher magnification of blood vessels 5 h after stab lesion illustrating the great number of OX42-positive monocytes present inside blood vessels (D) and the presence of perivascular cells stained with ED2 antibody (E). Bar = 8 μm. h: hippocampus, t: thalamus, c: cortex, cc: corpus callosum.

(Fig. 1, Fig. 4A,C). The majority of these cells seemed to be monocytes whereas about 1000 cells resembled reactive microglial cells, although this is difficult to ascertain on morphological basis only. The observation of adjacent sections presented in Fig. 4A,B and Fig. 4C,D shows that a small number of OX42-positive cells are labeled by ED1 antibody in the same region (Fig. 1). In rat brains studied one day after stab lesion, the number of OX42- and



ED1-positive cells had increased, but to a lesser degree than after LPS injection (Fig. 1). In irradiated rats where peripheral blood monocytes were almost abolished, the number of OX42- or ED1-positive cells was quite similar one day after LPS injection or stab lesion and far lower than in non-irradiated rats (Fig. 1, Fig. 4E,F). Whereas the inflammatory cells present in irradiated rats consist of activated and reactive microglia, the difference in the number of positive cells observed in non-irradiated and irradiated rats is likely to represent the cells of hematoge-



nous origin (about 80% of the cell population in LPS-treated rats).

3.1.4. From three days onwards

Three days after LPS injection, a high number of OX42-positive cells accumulated on the border of the lesion; these cells were no longer scattered throughout the brain parenchyma (Fig. 5A). Their large size and foamy aspect suggest that they have become tissue macrophages (Fig. 3D and Fig. 5C). Interestingly, at this later time-point after LPS injection, almost all these cells were ED1-positive and the ED1-labeling was intense (Fig. 3G, Fig. 5B,D).

As previously described [27], 7 and 15 days after LPS injection, OX42-positive cells accumulated along the needle tract and were more numerous than after a stab lesion, but the difference was not as striking as at 24 h. The majority of these cells were intensely labeled with ED1 antibody (not shown).

Our observation in all sections of the ED2-labeled perivascular cells did not reveal any change in number or other morphological modifications of these cells at all time-points studied following either stab lesion or LPS injection.

4. Discussion

The aim of the present work was to investigate whether 'brain macrophages' present 24 h after LPS intracerebral injection [27] were blood-borne monocytes/macrophages or in situ activated microglial cells as well as to investigate the early events of this inflammatory reaction which seems crucial to the understanding of this phenomenon.

4.1. ED1-immunolabeling

Our first approach was carried out by immunolabeling of brain sections collected 5 h, 15 h, 24 h and at later times following LPS intracerebral injection or stab lesion. Two monoclonal antibodies were used: OX42 which labels both monocytes/macrophages and microglia, and ED1, originally described as a specific marker of rat monocytes/macrophages [5]. Concerning ED1 staining, this model of rat brain inflammation allowed us to observe that resting and activated (including the grades II and III described in [3]) microglia are never labeled. In contrast, reactive microglia display a faint staining at the earlier time-points (15 h and one day) which becomes more and more intense from three days after LPS injection onwards. Since ED1 seems to be a lysosomal antigen [6], the intensity of ED1 labeling should reflect the phagocytic activity of reactive microglia [26]. Our data are in agreement with previous observations that ED1 antibody only stains reactive microglia at late times of injury [26,28,36]. As ED1 is mainly detected in monocytes/macrophages, the distinction between these cells and reactive microglia based on both phenotypic and morphological criteria is thus difficult to determine. In line with this problem, Flaris et al. [8] who used a large panel of monoclonal antibodies developed against rat microglial cells concluded that no single antibody could discriminate between these two populations of 'brain macrophages'.

The comparison between OX42- and ED1-labeling of adjacent sections, demonstrated that at early time-points after lesioning, only a small moiety of OX42-positive monocyte-like cells were labeled by ED1-antibody, and the staining was always very weak in the brain parenchyma; in contrast, the monocytes present in ventricles and in microvessels were more intensely labeled, in agreement with the report that monocytes in peripheral blood are ED1-positive [5]. This finding can be correlated with the down-regulation of CD4 expression on these cells, present in the brain 24 h after stab lesion or LPS injection [27]. The negative regulation of CD4 expression on macrophages and microglia in rat brain was formerly described by Perry and Gordon [30]. It thus seems likely that the particular immunological status of the CNS should lead to the down-regulation of the expression of some molecules, including CD4 and ED1, in immune cells located in the brain parenchyma.

4.2. Brain tissue response to irradiation

Since it turned out that immunohistochemical approaches could not clearly solve the problem of the origin of the 'brain macrophages' observed after injury, we applied the same protocols of intracerebral LPS injection or stab lesion to rats that were previously depleted in circulating leukocytes by irradiation. However, brain irradiation is

Fig. 3. A and B: cellular reaction 15 h after LPS injection. Adjacent hippocampal sections ipsilateral to the lesion (located between the two arrowheads) showing the increase in number of OX42-positive cells (A) and a few cells in the same region stained by ED1 antibody (B). Since the faintly labeled ED1-positive cells are difficult to distinguish from the counterstained cells, they are indicated by arrows. Bar = 20 μm. C to J: illustration of the different cell types observed throughout this study and labeled with OX42 (C, D, F, H, I) and ED1 (E, G, J) antibodies. OX42-positive (C) and ED1-positive (E) monocytes characterized by their rounded morphology and smaller size compared to tissue macrophages observed at later time-points (D and G). The latter shows an increase staining intensity with ED1. F: OX42-positive resting microglia, with a small body and long processes are mostly observed in the corpus callosum and fimbria. H: activated microglial cell with a hypertrophic cell body and thick shortened processes stained with OX42 antibody. I and J: activated microglia may evolve to reactive microglia intensely stained with OX42 (I) and ED1 (J) antibodies. These two photomicrographs illustrate the difficulty to clearly distinguish between reactive microglia with some visible processes (I, bottom and J, left) and monocytes in the case of rounded cells where eventual processes may be located outside the section plane (I, top and J, right). Bar = 8 μm.

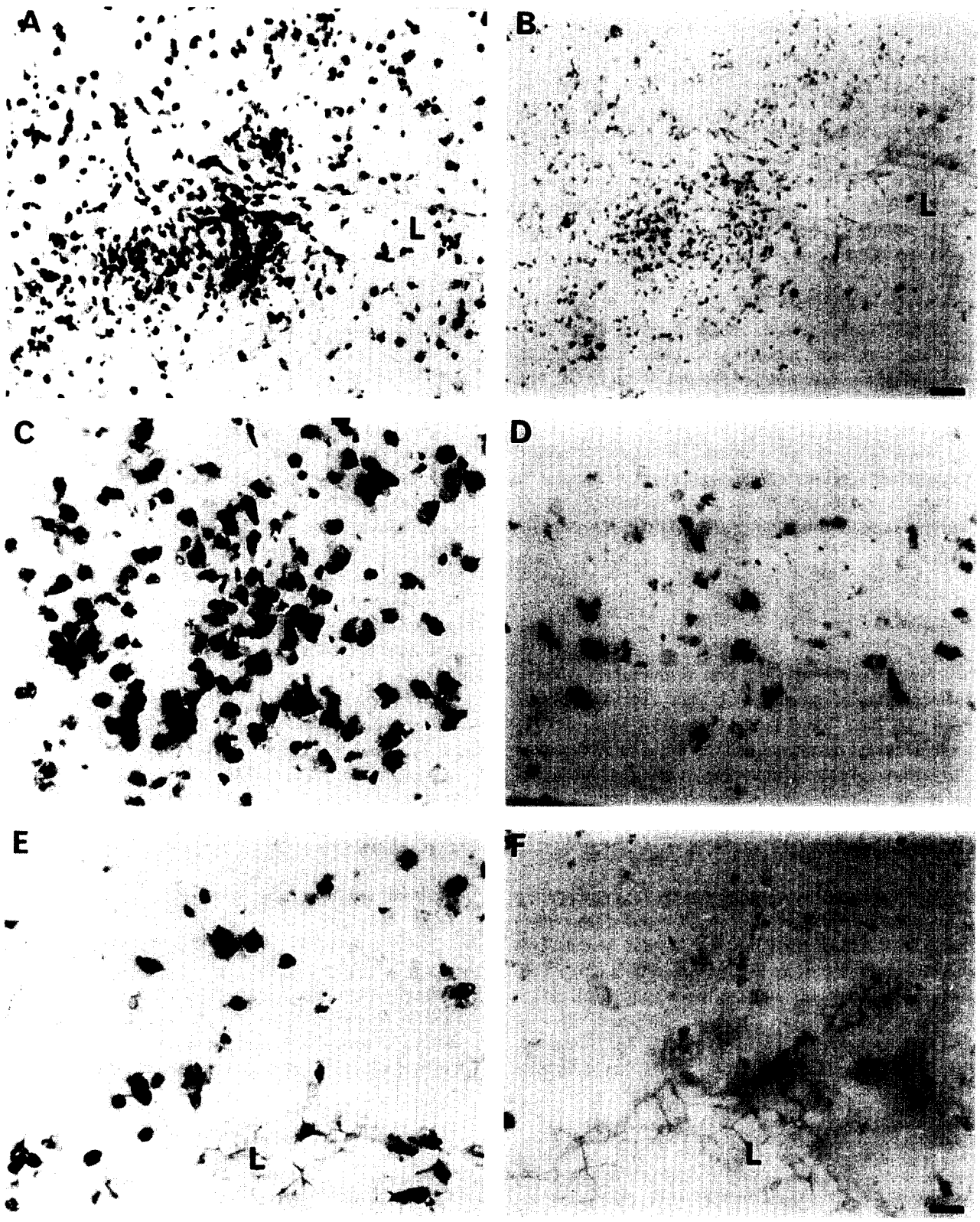


Fig. 4. Photomicrographs of adjacent sections in the lesion area stained for OX42 (A, C, E) and ED1 (B, D, F) 24 h after LPS injection to non-irradiated (A to D) and to previously irradiated (E, F) rats. A: a great number of OX42-positive monocytes accumulate around the lesion tract (L) and throughout the lesioned hippocampus and cortex. B: this adjacent section shows that some cells in the same area are also labeled by ED1 antibody. A and B: bar = 40 μm. C and D: higher magnification of the same area showing the OX42-positive monocytes (C) and the ED1-labeled monocytes (D) in adjacent sections. E: in previously irradiated rats, the number of OX42-positive cells observed throughout the lesioned area is far less important than in non-irradiated rats. F: in this adjacent section, very few cells are labeled with ED1 antibody. C to F: bar = 20 μm. L: lesion tract.

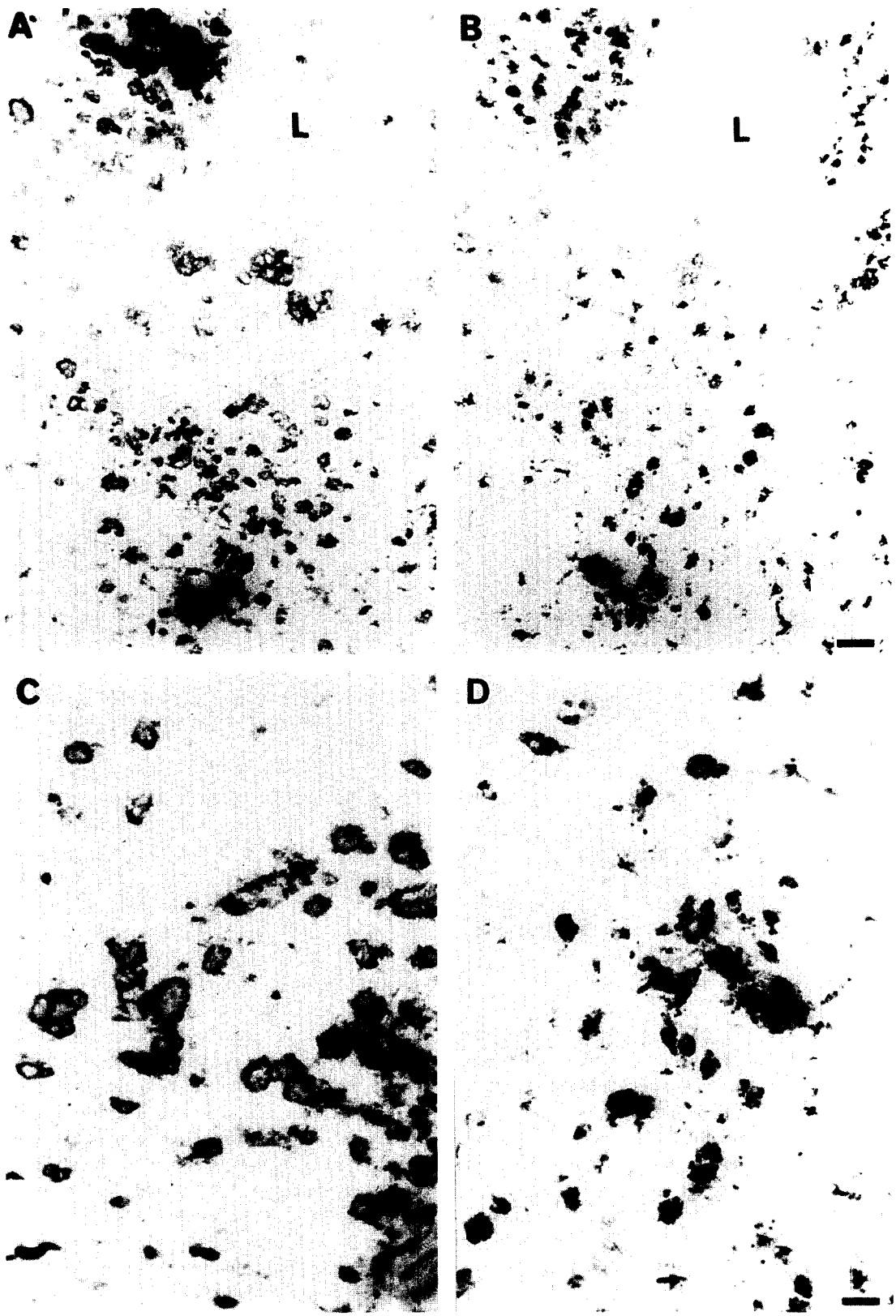


Fig. 5. Cell reaction three days after LPS injection (A and C: OX42-labeling; B and D: ED1-labeling). At this late time-point, the inflammatory cells are gathered around the lesion site (A and B). They display the morphology of tissue macrophages, due to their large size and foamy aspect, more visible at higher magnification (C and D). These cells exhibit a peripheral labeling with OX42 antibody (C) and are intensely stained with ED1 antibody, but in this case the labeling is cytoplasmic and punctiform (D). A, B: bar = 40 μm; C, D: bar = 20 μm. L: lesion site.

reported to induce in mice a dose-dependent increase in the number and staining intensity of Mac I-positive microglial cells [4] and in rats an increased expression of MHC class II antigen [25], that may modify the outcome of postirradiation injury in the brain. In preliminary experiments, rats were whole-body irradiated with a 7 Gy dose, which is not lethal for animals and low enough to avoid blood–brain barrier damage, but sufficient to induce the depletion of peripheral leukocytes. The immunohistochemical labeling of brains collected 48 h postirradiation evidenced an increase in the number and staining of microglial cells. This observation led us to carry out the 7 Gy irradiation experiments on the rat body only, the heads being maintained outside the irradiation field. In these conditions, microglial activation was far less intense; in particular, CD4 and MHC class II antigen were not expressed, as required for the present study. In addition, the 95% depletion of circulating monocytes was also quite suitable.

4.3. Characteristics of the cell reaction induced by injury and inflammation

Concerning the characteristics of the cell reaction occurring 5 h after injury, striking differences between stab lesion and LPS injection were observed. After stab lesion, a great number of OX-42 positive rounded cells with a small size corresponding to that of monocytes were observed inside the third ventricle, the interventricular junction (as defined in legend to Fig. 2) and the blood vessels ipsilateral to the lesion, whereas none of these cells was present in the contralateral hemisphere. This is in agreement with the work of Giordana et al. [10] who observed a large number of small round cells 6 h after a 25 gauge needle wound in the rat cerebral hemisphere; these cells which likely consisted of monocytes, since they bound the lectin *Griffonia simplicifolia* B_4, were also found scattered in the wound area but were more numerous inside and around blood vessels.

In our experimental model, when the stab lesion was inflicted to irradiated aplasic rats, only very few OX42-positive monocytes were present 5 h post lesioning in ventricles and blood vessels and none were found in brain parenchyma. This observation clearly demonstrates the hematogenous origin of the monocytes present in the brain of immunocompetent rats 5 h after stab lesion, and points out an early and intense recruitment of monocytes towards the lesion area. This result likely implies the presence of different factors induced by the lesion, which both attract inflammatory cells to migrate along concentration gradients and enhance leukocyte–endothelial cell interaction. In particular, TNF-α and IL-1 promote the expression of adhesion molecules on endothelial cells, monocytes/macrophages and microglia [7,33], that results in leukocyte and microglial cells migration to the lesion area. Other candidates could be some chemokines of the β (or C-C) family which act mainly upon monocytes, such as MCP-1 (monocyte chemoattractant protein-1). Indeed, MCP-1 mRNA accumulation occurs 3 h after mechanical injury of the rat brain; this chemokine can be produced in particular by macrophages, endothelial cells and astrocytes (for review see [12]). Since no microglial or astrocytic activation was observed in our experiments 5 h after stab lesion, it seems likely that MCP-1 could be expressed by the injured endothelial cells of the blood–brain barrier. In agreement with this hypothesis, Marmur et al. [24] have reported that in rat aorta subjected to balloon dilatation, dilated blood vessels exhibited high levels of MCP-1 (formerly termed JE) 4 h after injury.

In striking contrast to stab lesion, 5 h following LPS injection, OX42-positive monocyte-like cells were only exceptionally observed in blood vessels and brain tissue, whereas microglial cells became activated. This lack of monocyte recruitment was constantly recorded in all brains of LPS-injected rats. This inhibitory action of LPS early after injection is very intriguing. Hirschberg and Schwarz [17] have recently evidenced that in mammalian CNS there is a soluble factor that actively inhibits monocyte migration. In our experimental model, LPS could then act by enhancing the production of this still unknown inhibitory factor or by down-regulating the expression of β-chemokines or of adhesion molecules. Concerning this point, it is worth mentioning the work of Kang et al. [19] who recently reported that LPS can decrease the expression of certain integrins, molecules that are involved in the recruitment of monocytes/macrophages during infection and inflammation.

At 15 h and 24 h after LPS injection, the number of total OX42-positive cells had largely increased (3000 and more than 5000 respectively). Whereas the majority of these cells displayed the morphology of typical monocytes, about one out of five or six consisted of activated or reactive microglia. When rats were previously irradiated, only about 1000 OX42-positive cells were observed 24 h after LPS injection and they likely represent in situ activated microglial cells. These differences between irradiated and non-irradiated rats demonstrate that LPS intracerebral injection induces an intense recruitment of blood-borne monocytes in immunocompetent rats. Our data also show that in stab-lesioned rats, the recruitment of monocytes is far less important at 15 h and 24 h than after LPS injection. This increased monocyte recruitment by LPS which seems contradictory with the inhibition of the monocyte recruitment 5 h after stab lesion, must be due to molecular signals occurring between 5 h and 15 h after LPS injection. Interestingly, LPS but not stab lesion, induced microglial activation as soon as 5 h after injection. This result is in agreement with in vitro and in vivo studies which show that LPS is a potent stimulus for microglia [22,36,38]. These microglial cells activated by LPS intracerebral injection could secrete factors such as TNF-α and IL-1 and also the macrophage inflammatory protein MIP-

1α, a member of the C-C family chemokines which is induced in microglia by LPS [15], and TGF β-1 which is produced by microglia after brain injury [20,23,29]. Indeed, the up-regulation of adhesion molecules can be induced by TNF-α and IL-1, and monocyte chemotaxis by MIP-1α [34,37] and TGFβ [39]. The exact role of the molecules involved in these complex interactions are up to now not elucidated. However, it seems clear that the initial response to LPS is elicited in microglia which, once activated, could lead to the intense monocyte recruitment observed from 15 h onwards after intracerebral injection.

From three days onwards after LPS injection, cells labeled by both ED1- and OX42-antibodies accumulated around the lesion site and displayed the morphological characteristics of tissue macrophages. Our previous observations demonstrated that at later time-points these cells started to disappear and activated microglia again dominated the scene. In addition, astrocyte activation started only several days after either stab lesion or LPS injection [27]. This delayed astrocytic reaction indicates that astrocytes are not involved in the initial events of the inflammatory reaction; astroglial activation is likely induced by different cytokines secreted by activated microglia [22].

It is, however, surprising that ED2-labeling did not evidence changes in perivascular 'microglial' cells reported to belong to the monocyte/macrophage lineage and to present antigen in vivo [16,18]. Since the number of ED2-positive cells increases during the evolution of brain abscess [8], the same phenomenon could be expected during brain inflammatory reaction. The absence of a change in these cells can be explained by other reports suggesting that ED2-positive perivascular cells are not microglial cells, but are likely to represent a specialized type of pericytes [13,14].

To summarize our data, the most important features issuing from the present work consist in the unequivocal demonstration of an early and intense recruitment of blood-borne monocytes to the brain parenchyma induced by stab lesion, but mostly by LPS intracerebral injection. Many authors have reported such a monocyte recruitment after brain injuries, but only after a delay of several days [1,11,31,32,35]. Moreover, our data demonstrate that LPS exerts a dual action on this phenomenon, first by inhibiting the monocyte chemotaxis 5 h after injection, and second by highly enhancing it at 15 h and 24 h. Whereas the mechanisms of LPS inhibitory action are still unclear, the LPS secondary effect on the increase in the monocyte recruitment is easier to understand since this inflammatory molecule induces an early activation (5 h) of microglial cells which are able to secrete monocyte chemoattractants.

Besides the numerous monocytes of peripheral origin, parenchymal microglia also participate, although to a lesser extent, in the inflammatory reaction. Throughout this work, the transition between all functional states of microglia could be observed: ramified resting microglia, in a state of immune alertness which may induce local inflammatory reaction, activated microglia involved in immune intervention, and reactive microglia which exerts cytotoxicity towards cell debris [2].

A better understanding of the initial events occurring during the course of brain inflammatory reactions further requires the molecular identification and the kinetics of expression at the cell level of various signals likely to be involved, such as cytokines, chemokines, and also adhesion molecules.

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