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Application of lectin and B-lymphocyte-specific monoclonal antibodies for the demonstration of human microglia in formalin-fixed, paraffin-embedded brain tissue

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Summary. To evaluate the usefulness of microglial markers for routine neuropathological material, we studied formalin-fixed, paraffin-embedded human brain tissue with the immunoperoxidase method using the lectin *Ricinus communis* agglutinin (RCA-1) and four monoclonal antibodies (LN-1, LN-2, LN-3, anti-HLA-DR/alpha). RCA-1 stained resting microglia, but the staining intensity was mostly weak. LN-1 also stained resting microglia in paraffin sections first treated with protease. In contrast to LN-1, RCA-1 stained blood vessels heavily. LN-1 stained resting microglia more markedly than RCA-1 in brains fixed for a prolonged period of time. However, LN-1 recognized a small number of astrocytes in routine paraffin sections. LN-3 reactivity was detected on a few resting microglia, but was intensely expressed on large numbers of reactive microglia in many neurological diseases. Both LN-2 and anti-HLA-DR/alpha labelled microglia, but the reactions were inconsistent. This study suggests that the monoclonal antibodies LN-1 and LN-3 are useful for the demonstration of microglia in paraffin sections, and a combination of these antibodies and the antibody to glial fibrillary acidic protein is recommended in attempting to identify microglia.

Key words: Microglia – Immunohistochemistry – Monoclonal antibody – Lectin histochemistry – Human autopsy brain

Introduction

In 1920 del Rio-Hortega introduced the term "microglia" to describe a new central nervous cell type that he considered to be derived from mesodermal elements. The concept of the microglia became widely accepted due to del Rio-Hortega's work (1932), but the origin and nature of microglia have been the subject of much long-standing discussion (Fujita and Kitamura 1975; Ling 1981; Streit et al. 1988) and remains unclear. There is, moreover, much confusion on this cell type, since microglia are difficult to identify reliably in routine paraffin sections.

A number of studies have yielded interesting data concerning functional aspects of microglia. Both in vitro (Sasaki et al. 1989) and in vivo (Hayes et al. 1987; Hickey and Kimura 1988; McGeer et al. 1988) studies have shown that microglia are the major cell type in the central nervous system (CNS) expressing the major histocompatibility complex (MHC) class II antigens and that they may function as antigen-presenting cells. Microglia were also shown to produce interleukin-1 (Giulian et al. 1986) and appeared to promote astrogliosis in developing brains (Giulian et al. 1988). Within the immunological context it has become apparent that microglia might be a target cell for the human immunodeficiency virus, which has been shown to cause an encephalitis or encephalopathy which frequently accompanies acquired immunodeficiency syndrome (Michaels et al. 1988; Price et al. 1988; Vazeux et al. 1987). Microglia, therefore, seem to play an important role in various neuropathological conditions. The reliable identification of microglia in tissue sections will be required as a principal step for further study.

In this study, we applied the lectin *Ricinus communis* agglutinin-1 (RCA-1), two monoclonal antibodies (mAbs) reactive with B-lymphocytes (LN-1, LN-2), a mAb reactive with human leucocyte antigen (HLA)-DR (LN-3) and another mAb against HLA-DR/alpha chain, and compared the reactivity of each reagent to microglia in formalin-fixed, paraffin-embedded tissue sections of normal and diseased human brains.

Materials and methods

Human brains were obtained from 23 autopsy cases with a postmortem interval ranging from 1 to 14 h. The brains were from patients who ranged in age from 2 days to 83 years. They were fixed in 10% formalin for a period of 3 days to 18 months, but mostly for less than 4 weeks. The tissue was routinely processed

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Table 1. Primary reagents used

Lectin/antibody	Molecular specificity	Source	Dilution
Biotinylated RCA-1	β-D-Galactose	Vector, Burlingame, CA, USA	5–20 μg/ml
Mouse mAb LN-1	Sialoglycosubstance (53 kDa?), CDw75	Techniclone, Santa Ana, CA, USA	1:1
Mouse mAb LN-2	Class II associated invariant chain (35 kDa), CD74	Techniclone, Santa Ana, CA, USA	1:1
Mouse mAb LN-3	HLA-DR (29–33 kDa)	Techniclone, Santa Ana, CA, USA	1:1
Mouse mAb HLA-DR/Alpha	HLA-DR, alpha-chain (33 kDa)	Dakopatts, Glostrup, Denmark	1:20

RCA-1, *Ricinus communis* agglutinin-1

and embedded in paraffin. We examined specimens taken from various parts of the brains diagnosed by routine pathological examinations as follows: normal (10), infarction (3), intracerebral haemorrhage (1), necrotizing encephalitis (1), brain abscess (1), glioblastoma (1), metastatic tumour (1), secondary lymphoma (1), ischaemic encephalopathy (1), hepatic encephalopathy (1), Wernicke's encephalopathy (1), and methotrexate (MTX) encephalopathy (1). In the normal brain, some blocks from the same area as the paraffin section were processed for frozen sections with different fixatives: acetone (30 min, 4° C) and periodate-lysine-paraformaldehyde (7 h, 4° C). The paraffin sections were cut 3–4 μm thick and the frozen sections were cut 6–8 μm thick. Both types of sections were attached to albumin-coated glass slides, although some sections for protease treatment were attached to glass slides coated with 0.5% neoprene (Nissin EM, Tokyo) or 0.05% poly-l-lysine (Sigma, St. Louis, Mo., USA).

The source and other details of each of the primary reagents employed are shown in Table 1. A well-characterized rabbit antiserum to glial fibrillary acidic protein (GFAP, prepared by Y.N.) was used as an astrocyte marker (Nakazato et al. 1982a). For the staining with the mAbs and lectin, the biotin-streptavidin amplified system kit (Stravigen; BioGenex, Dublin, CA, USA) was used. For labelling GFAP, both swine anti-rabbit immunoglobulins and rabbit peroxidase-antiperoxidase (PAP) were obtained from Dakopatts (Glostrup, Denmark).

Mouse mAbs were detected with the biotin-streptavidin immunoperoxidase method. The paraffin sections were deparaffinized in xylene. After blocking endogenous peroxidase activity with 0.3%

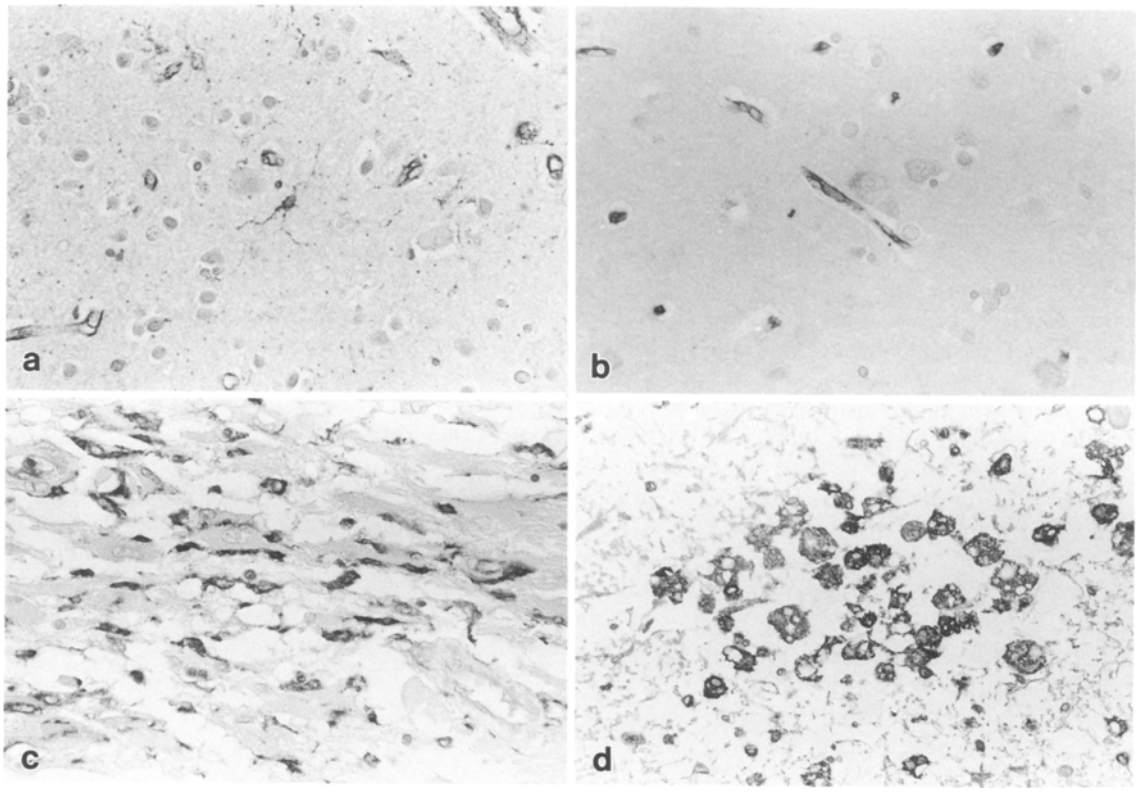

Fig. 1a–d. *Ricinus communis* agglutinin-1 (RCA-1) staining. **a** Anti-RCA-1 stains a few microglial cells with delicate, branching processes and more endothelial cells in normal cerebral cortices. **b** RCA-1 is limited to blood vessels in the grey matter of normal brains. **c** Anti-RCA-1 reacts with reactive microglia but not with reactive astrocytes in the lesion surrounding the glioblastoma. **d** Dense staining of brain macrophages in the necrotic area of the brain infarctions. **a–d** × 280

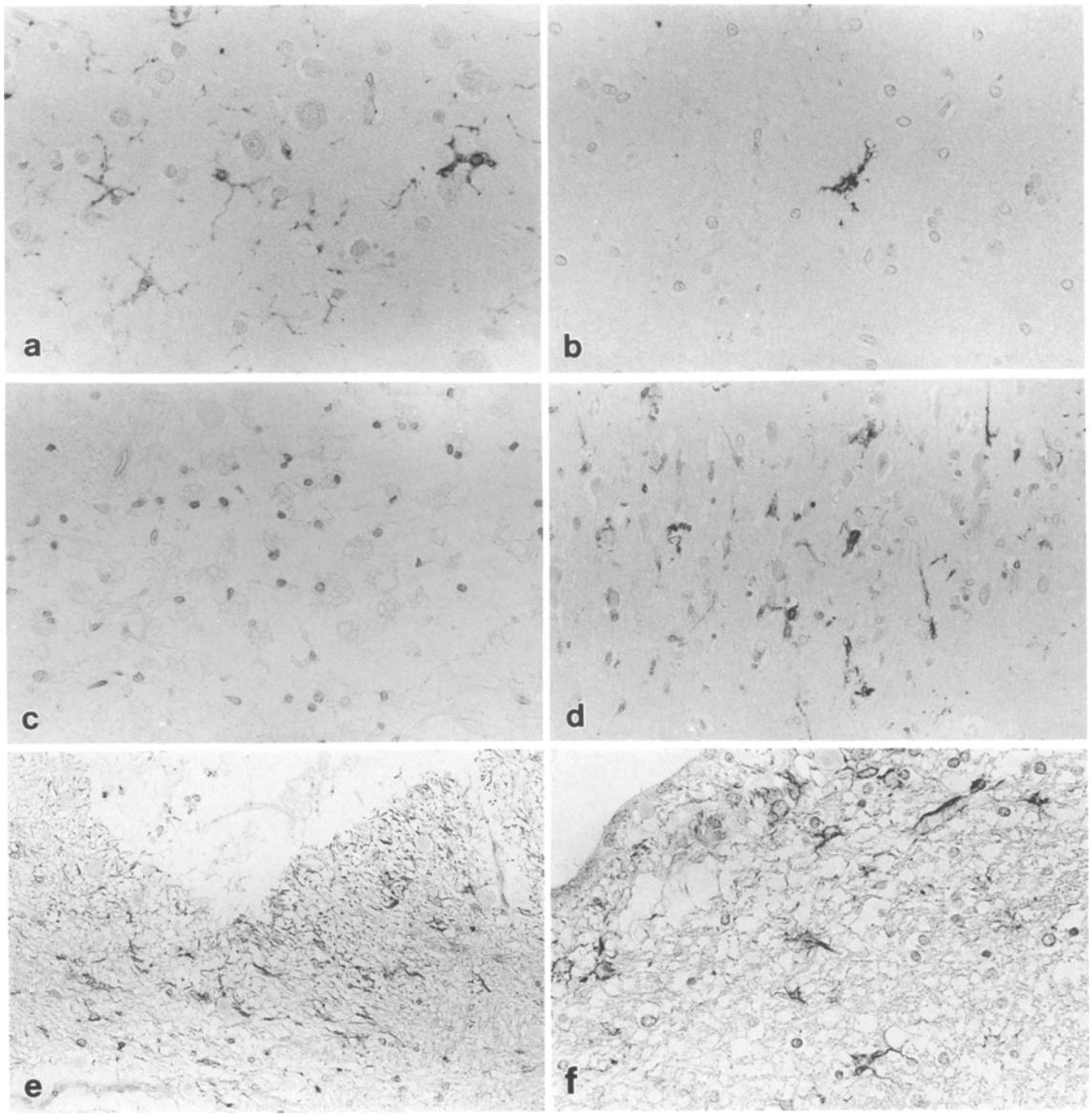

Fig. 2a–f. LN-1 staining on paraffin sections treated with protease. Many resting microglia in the cortical grey matter (**a**) and one microglial cell in the white matter (**b**) are stained with LN-1 in normal brains. **a** is taken from the region corresponding to Fig. 1b. **c** Foamy brain macrophages are negative for LN-1 in the brain from a patient with a brain infarction. **d** LN-1 reacts with reactive microglial cells of various shapes around ischaemic neurones in the hippocampus. LN-1 sometimes reacted with astrocytes in the subpial glial membrane (**e**) and in the subependymal region of the temporal lobe (**f**). **a–c, f**, ×280; **d, e**, ×140

hydrogenperoxide in methanol, the sections were graded in ethanol and transferred to phosphate-buffered saline (PBS). Sections were treated with enzyme digestion using a 0.05% solution of protease XXVII (Sigma, P-4789) for 5 min at room temperature (RT) and washed briefly in PBS. The sections were incubated in 10% normal goat serum for 1 h at RT, followed by primary reagent treatment at 4° C overnight. The sections were sequentially incubated with biotinylated goat anti-mouse immunoglobulin (30 min, RT) and peroxidase-conjugated streptavidin (30 min, RT). Biotinylated RCA-1 was detected as mAbs with the omission of secondary antibody treatment. The peroxidase activity was visualized with 3,3′-diaminobenzidine. The sections were counterstained lightly with haematoxylin, dehydrated and mounted. Paraffin sections of human spleen or lymph nodes were used as a positive control for the primary antibodies. For GFAP staining, the PAP method was used as previously described (Nakazato et al. 1982b). Some sections were double-stained with each of the primary reagents and GFAP. The chromogen 4-chloro-1-naphthol was also used. Immunoperoxidase staining of the frozen sections was similar to that used on paraffin sections.

Results

In this study, "resting microglia" referred to the cells with a morphology fully characterized by del Rio-Hortega (1920, 1932). "Brain macrophages" was used for round phagocytes filled with lipid or iron. We used the term "reactive microglia" for transition forms, intermediates in shape between the resting microglia and brain macrophages.

RCA-1 was observed in the cell bodies and fine processes of resting microglia (Fig. 1a), but the staining in-

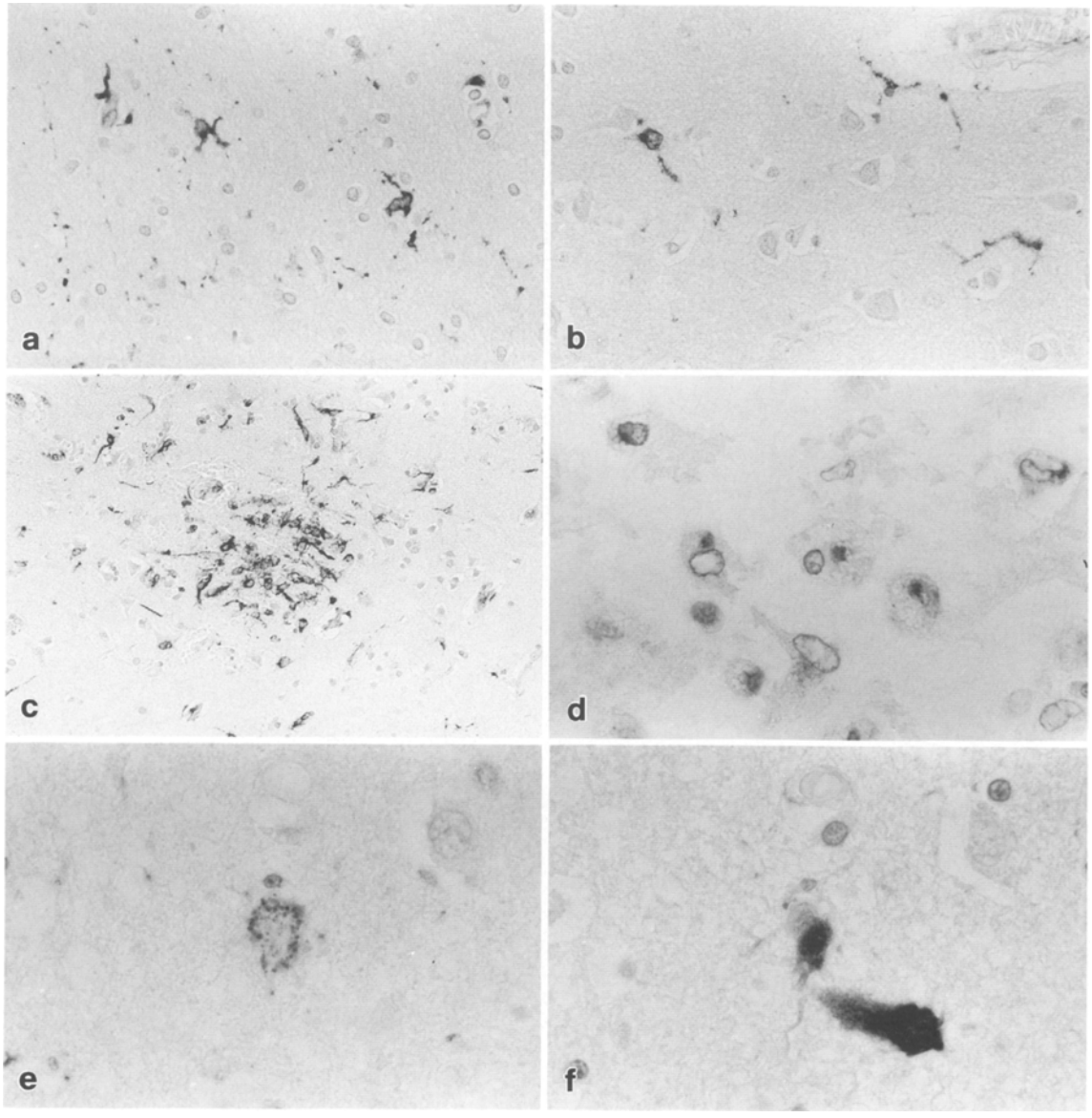

Fig. 3. LN-2 reacts with glial cells with nuclei of various shapes and short processes in the white matter (**a**) and in the cortical grey matter (**b**). **c** LN-2 reacts with reactive microglia forming a glial nodule in the cerebral cortex of a patient with a brain abscess. **d** LN-2 shows nuclear membrane staining and a dense cytoplasmic staining focus in foamy macrophages. One reactive astrocyte shows granular staining with LN-2 (**e**) and intense, cytoplasmic staining with anti-glial fibrillary acidic protein (GFAP) (**f**) in serial sections. **a**, **b** ×280; **c** ×140; **d–f** ×700

tensity was moderate in only 2 of 10 normal brains and weak in the other brains (Fig. 1b). However, reactive microglia were stained more intensely than resting microglia in most of the brains (Fig. 1c). Brain macrophages were stained heavily with RCA-1 (Fig. 1d). RCA-1 always gave a strong reaction in the walls of the microvasculature and sometimes showed high background staining. RCA-1 did not bind to astrocytes including the reactive forms, although the assessment of the reaction was often difficult because of background staining.

LN-1-positive cells were very rare in the brain sections without protease treatment, but the enzyme pretreatment significantly enhanced the immunoreactivity of LN-1 with glial cells. Protease treatment had also another advantage in diminishing non-specific myelin staining by LN-1. LN-1 reactivity was observed in the irregular delicate processes of small glia compatible with resting microglia (Fig. 2a, b). Numerous LN-1-positive microglia were scattered in the white matter as well as in the cortex. In the cortex, they were prominent in the perineuronal space. Both reactive microglia and brain macrophages, present in the lesions of infarctions, were frequently negative for LN-1 (Fig. 2c). However, reactive microglia reacted considerably with LN-1 in the brains from patients with ischaemic encephalopathy (Fig. 2d), brain abscess and necrotizing encephalitis. In the last disorder, some LN-1-positive macrophages were seen. Astrocytic staining by LN-1 was almost undetectable in the entire CNS, but some GFAP-positive cells located in the subpial (Fig. 2e) and subependymal (Fig. 2f) regions were sometimes found to react with

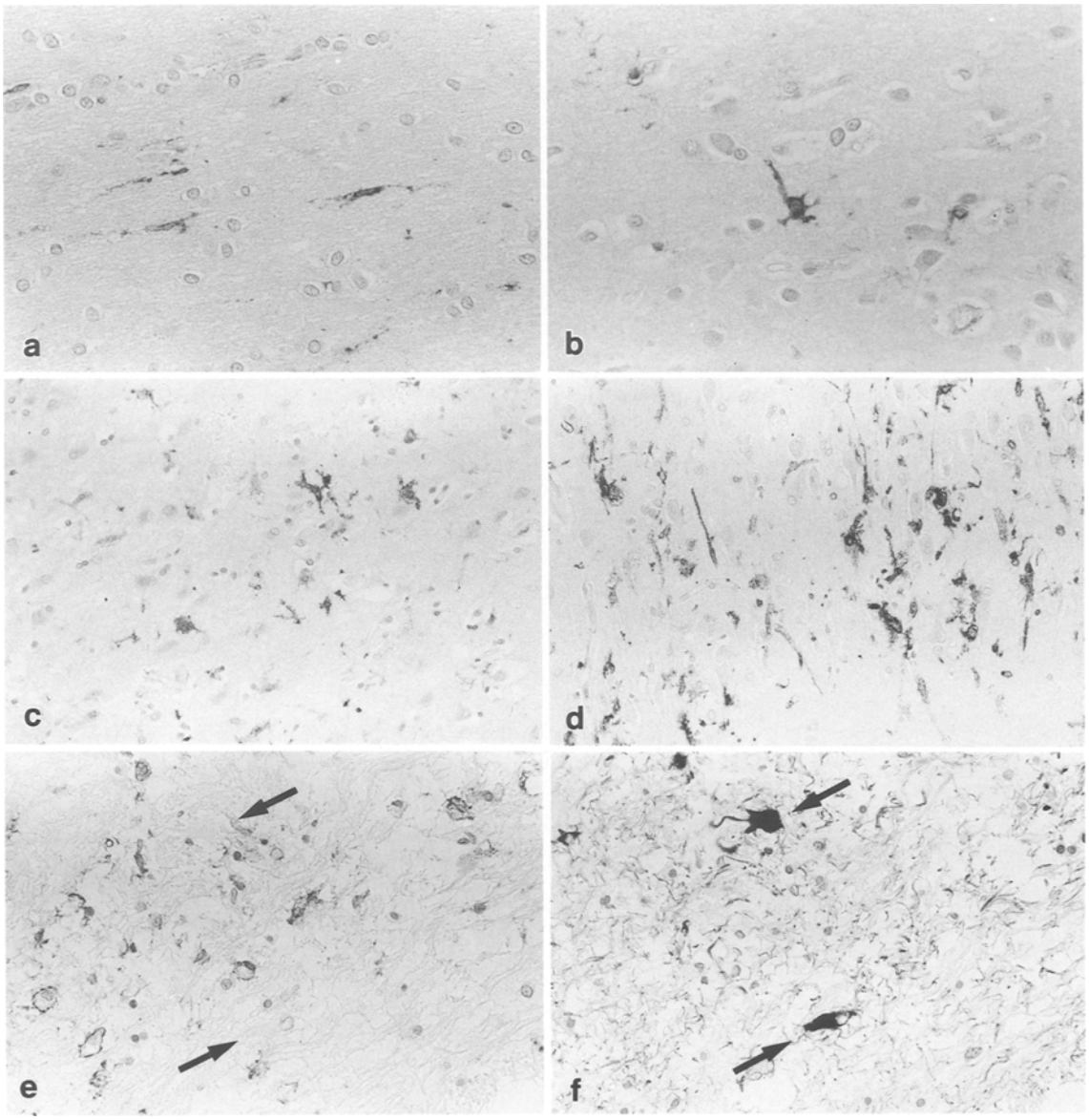

Fig. 4. LN-3 reacts with microglial cells in the white matter (**a**) and in the deep cortex (**b**) of normal brain. An increased number of reactive microglia are LN-3-positive in the lesion near a cerebral haemorrhage (**c**) and in the hippocampus of the brain taken from a patient with ischaemic encephalopathy (**d**). LN-3 reacts with rounded brain macrophages but not with astrocytes (*arrows*), examined using LN-3 (**e**) and anti-GFAP (**f**) in adjacent sections in the brain from a patient with a brain infarction. **a**, **b**, **e**, **f** ×280; **c**, **d** ×140

LN-1. Reactive astrocytes were occasionally labelled with LN-1. Besides glial cells, LN-1 epitope was present in the red blood cells, but not in endothelial cells.

LN-2 was almost completely negative in 3 normal brains, but a small number of LN-2-positive cells were found in 4 normal brains (2 with hepatomas, 1 each with acute lymphocytic laeukemia and malignant lymphoma). LN-2 labelled glial cells often with pale nuclei of various shapes and a few cellular processes extending only a short distance (Fig. 3a, b). They were more numerous in the white matter and favoured the perineuronal space in the cortex. Some of the LN-2-positive cells were morphologically compatible with the resting microglia. LN-2-reactive products were observed in the nuclear membrane and cytoplasm. In the white matter of a normal brain, double-staining using LN-2 and anti-GFAP showed LN-2(+)/GFAP(−), LN-2(−)/GFAP(+) and LN-2(+)/GFAP(+) cells. The reaction of reactive microglia varied in positivity. Numerous glia including reactive microglia were labelled with LN-2 in the brains taken from patients with brain abscess (Fig. 3c), secondary lymphoma and MTX encephalopathy. LN-2 reactivity was present in brain macrophages, where reaction products were often observed in perinuclear areas (Fig. 3d). Reactive astrocytes showed weak, granular staining by LN-2 (Fig. 3e, f).

In normal brains, the positivity of LN-3 was similar to that of LN-2. However, the morphology of the positive glial cells was not identical among the antibodies. LN-3-reactive products were observed in the cell bodies and irregular fine processes of glia cells compatible with the resting microglia more frequently in the white matter

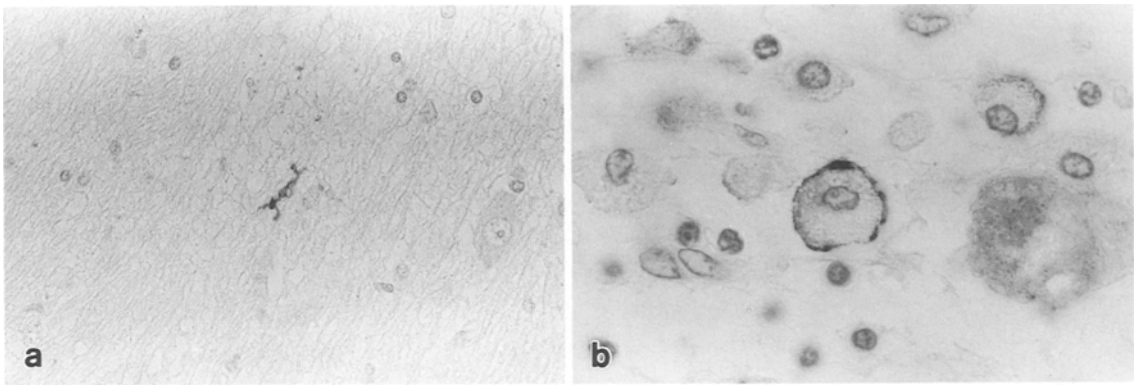

Fig. 5a, b. Anti-HLA-DR/alpha staining. **a** One microglial cell is stained with anti-HLA-DR/alpha in the basal ganglia. **b** HLA-DR/alpha is observed in the cell membrane of brain macrophages in the brain from a patient with necrotizing encephalitis. **a** ×280; **b** ×700

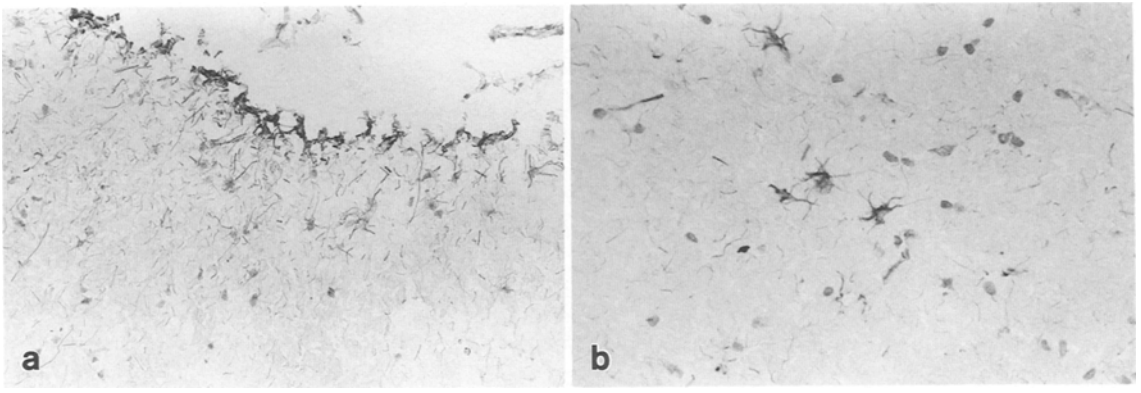

Fig. 6a, b. LN-1 staining on cryostat sections fixed in cold acetone. LN-1 reacts with astrocytes located in the external glial limiting membrane (**a**) and in the white matter (**b**). **a** ×140; **b** ×280

(Fig. 4a, b). In contrast to LN-2, reactive microglia showing various cell morphologies were considerably labelled with LN-3 (Fig. 4c, d). Brain macrophages also were stained heavily with LN-3 (Fig. 4e, f). LN-3 did not bind to the astrocytes in normal brains. However, a small number of reactive astrocytes present around the metastatic tumour and in the necrotic areas of necrotizing encephalitis showed equivocal staining with LN-3. LN-3 labelling was often present in endothelial cells and pericytes.

Anti-HLA-DR/alpha mAb was found to react with B cells and histiocytes both in the control sections and in some brain sections, where the staining pattern was almost identical to that of LN-3. With microglia, however, HLA-DR/alpha staining was far less intense than LN-3 staining. Only a small number of microglia reacted with anti-HLA-DR/alpha in a few brains tested (Fig. 5a), whereas brain macrophages with foamy vacuoles were clearly stained with anti-HLA-DR/alpha (Fig. 5b). HLA-DR/alpha was absent in other types of glia.

Pre-treatment with protease did not enhance the staining intensity of microglia in the reagents other than LN-1. The immunoreactivity of all the primary reagents was independent of the post-mortem interval following the autopsy. All the reagents were found to be capable of reacting with microglia taken from brains fixed in formalin for up to 77 days. However, in brain tissue of prolonged fixation (18 months), microglia were readily demonstrable only with LN-1.

The results of the frozen sections obtained either from acetone fixation or PLP fixation were similar to those of the paraffin sections using RCA-1, LN-2 and LN-3, although more microglia were labelled with all the reagents. However, LN-1 staining in the frozen sections predominantly labelled the astrocytes that were located in the white matter as well as the pial surface (Fig. 6). HLA-DR/alpha staining was almost the same as LN-3 in the frozen sections.

Discussion

Using light microscopy, resting microglia can be identified reliably only by impregnation with the silver carbonate method. This metallic impregnation, however, is difficult and fickle even in skilled hands. A previous study (Perry et al. 1985) using histochemical and immunohistochemical techniques has shown that resting microglia express Fc and complement receptors which are detectable only in frozen sections. However, other authors (Miyake et al. 1984; Oehmichen et al. 1979; Sminia et al.

Table 2. Immunohistochemical staining affinities of microglia, brain macrophages and astrocytes

Marker	Microglia		Brain macrophages	Astrocytes	
	Resting	Reactive		Normal	Reactive
RCA-1	+/−	+	+	−	−
LN-1	+	+/−	+/−	+/− (focal)	+/−
LN-2	+/−	+/−	+	+/−	+
LN-3	+/−	+	+	−	+/− (few)
HLA-DR/ alpha	+/−	+/−	+	−	−

−, Negative; ±, variably positive; +, positive

1987) have been unsuccessful in demonstrating monocyte/macrophage markers in microglia. So far, there is no specific marker for microglia and microglia-derived macrophages.

Recently, some reagents have been shown to be capable of detecting microglia in routine paraffin sections (Chou and Miles 1989; Dickson and Mattiace 1989; Haga et al. 1989; Kaneko et al. 1989; Mannoji et al. 1986; Miles and Chou 1988; Suzuki et al. 1988; Szumanska et al. 1987). Among them, RCA-1 has been used most often in paraffin sections. Other reagents include mAbs – LN-1, LN-2, LN-3 – that react primarily with B cells in paraffin sections. However, LN-1 is questionable both in terms of its specificity and reactivity in formalin-fixed, paraffin-embedded materials (Dickson and Mattiace 1989). Our results showed that any reagent tested may label microglia in routine paraffin sections, but each has its own characteristics with respect to immunoreactivity, specificity, tissue processing and treatment with enzyme digestion (Table 2).

To date, the lectin RCA-1 is the best-known reagent capable of revealing microglia in paraffin sections. We showed that RCA-1 reacted with microglia, but not with other types of glia in the CNS, as described previously (Mannoji et al. 1986; Suzuki et al. 1988). However, this study showed that the reactivity of microglia, especially resting microglia, was often weak in contrast to constant, heavy staining of small blood vessels. Moreover, RCA-1 was sometimes found to yield unfavourable background staining. Thus, this study suggests that RCA-1 is not always suitable for demonstrating microglia, at least in the immunohistochemical staining method applied here.

Resting microglia were best demonstrated by LN-1, of the reagents examined in this study. Previous studies have shown that microglia could be positive for LN-1 in B-5-fixed, paraffin sections treated with pronase (Miles and Chou 1988) and negative in formalin-fixed, paraffin sections without protease (Dickson and Mattiace 1989). Our study showed that LN-1 labelled microglia in formalin-fixed, paraffin-embedded brain tissue and the protease step in immunostaining procedure might be critical. LN-1, moreover, was demonstrated to be useful in tissue fixed for long periods. However, we demonstrated in this study that LN-1 reacted with astrocytes on routinely fixed and processed paraffin sections. Our results from the paraffin sections and cryostat sections indicated that microglia and astrocytes shared an epitope recognized by LN-1 and that the number of LN-1-positive cells was dependent on the type of tissue fixation and processing, results similar to those of Dickson and Mattiace (1989). Therefore, from a practical point of view the sole use of LN-1 as a microglia marker to identify CNS cell types is questionable.

LN-2 was originally reported to react with B lymphocytes and histiocytes, but not with brain cells (Epstein et al. 1984). Recently, however, both LN-2 and LN-3 were shown to react with rod cells in autopsy brain paraffin sections (Chou and Miles 1989). In this study, LN-2 reactivity was often present in glial cells. As with lymphoid cells, LN-2 had a distinctive nuclear staining pattern for glia. Brain macrophages, reacted with LN-2, showed a paranuclear intense focus of staining which has been suggested as an abundance of antigen in the Golgi area in lymphoid cells (Marder et al. 1985). Concerning the glial cell type recognized by LN-2, resting and reactive microglia were found to react with LN-2 in some normal and diseased brains. However, a combination of LN-2 and GFAP staining revealed that LN-2 could react, but not exclusively, with some astrocytes. Moreover, we could not exclude the possibility that LN-2 epitopes are present in oligodendroglia. For these reasons, it is doubtful that LN-2 can be used as a marker for microglia.

LN-3 is a mAb that reacts with the HLA-DR antigen in paraffin sections (Marder et al. 1985). We found a high frequency of LN-3-positive staining of reactive microglia and brain macrophages in injured brains. This result is compatible with a report by McGeer et al. (1988), who used another HLA-DR monoclonal antibody in paraformaldehyde-fixed, brain tissues. Our data demonstrated that the standard formalin fixative, which is used in most laboratories, permits highly consistent HLA-DR staining in microglia by the use of LN-3. Recent in vivo immunohistochemical studies of human brain tissues indicate that HLA-DR-expressing cells in the CNS are microglia (Hayes et al. 1987; McGeer et al. 1988), although very rare expression by astrocytes is reported here and elsewhere (Frank et al. 1986). Thus, LN-3 might be of practical value in demonstration of reactive microglia in routine neuropathological material and simultaneous GFAP staining might be essential as a negative marker for microglia.

Variable positivity of LN-2 and LN-3 with microglia in normal brains was independent of either the length of the post-mortem period or the formalin-fixation interval. The exact cause of the inconsistent reactions of both antibodies was not clarified in this study, but might be related to the molecules recognized by them. LN-2 recognizes a 35 kDa MHC class II associated invariant chain. Class II antigen (HLA-DR) and invariant chain are co-expressed in various tissues, and their expression is found to be co-regulated by interferon-γ or interleukin-4 (Koch et al. 1984; Noelle et al. 1986). In this study,

the brains that reacted with LN-2 were similar to those that reacted with LN-3. However, the cell types that were reactive were not identical. Further study is needed in order to understand the expression of the invariant chain and the relationship between class II and invariant chain in the CNS.

Staining of another HLA-DR antibody which reacts with the alpha-chain monomorphic HLA-DR antigen was less reactive than LN-3 in brain paraffin sections. Our results indicate that an epitope recognized by anti-HLA-DR/alpha might be different from that by LN-3 and less resistant to formalin fixatives.

Our results from LN-1 staining, which showed a shared epitope between microglia and astrocytes, suggest a common precursor for these two glial cells. However, the findings that microglia react with some of the B-cell- or histiocyte-reactive antibodies are likely to be considered as evidence of a bone marrow origin. LN-1 has been reported to be present in various types of cells, including epithelial cells, erythroblasts and smooth muscle cells (Epstein et al. 1984; Okon et al. 1985) and in this study LN-2 was found in astrocytes and HLA-DR antigen is not cell lineage specific (Unanue and Allen 1986). Therefore, it seemed that our results should not be used to speculate about the origin of microglia.

Brain tissues are routinely processed with formalin fixation and paraffin embedding in most hospital laboratories; the use of frozen sections is much less frequent. Reagents which are capable of detecting microglia in routine paraffin sections are important in the field of human pathology. The methods described in this study are of a type typically employed by immunohistochemists, and the reagents have the benefit of being commercially available. Our findings reflect the real benefits and disadvantages of these reagents; thus, our work may assist pathologists and researchers in attempting to identify microglia.

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