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# Macrophage populations and expressions of regulatory proinflammatory factors in the rat meninx under lipopolysaccharide treatment *in vivo* and *in vitro*

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**Summary.** Macrophages play important roles in host defense mechanisms. In the brain, besides microglial cells, meningeal macrophages are present. However, the pathobiological characteristics of meningeal macrophages in rats remain to be investigated. In normal meninx, immunohistochemically, macrophages reacting to CD163 (macrophage scavenger receptor) and major histocompatibility complex (MHC) class II-expressing cells (involving activated macrophages or dendritic cells) were sporadically seen without age-dependent changes. Injection of lipoplysaccharide (LPS) (5 μg; *Escherichia coli*) into the cerebrum increased the number of anti-CD68-positive macrophages (with greater phagocytic activity) in the meninx, with a peak at 12 h during observation period until 48 h; MHC class II-expressing cells showed a gradual increase in number from 3 h after injection; however, anti-CD163-positive macrophages did not show significant change. In in vitro studies, LPS (0, 0.02, 0.05, 0.5, 5, 50 and 100 μg/ml) was added to KMY-1 or KMY-2 cells, both of which had been established from a rat malignant meningioma. KMY-1 originally reacted to CD163, but LPS addition at 0.5 μg/ml and greater concentrations decreased the anti-CD163-positive cell number and instead increased the anti-CD68-positive cell number. LPS-treated KMY-2 increased the anti-CD163-positive cell number at 0.05 and 0.5 μg/ml. By RT-PCR methods, LPS (0, 0.5, 5, 50, and 100 μg/ml)-treated KMY-1 and KMY-2 showed an increase in mRNA of monocyte chemoattractant protein-1 (MCP-1, a chemokine), and LPS-treated KMY-2 increased mRNA of nerve growth factor (NGF, an immunological effecter). Collectively, under LPS treatment, macrophages with heterogeneous functions appear in rat meninx; rat meninx-forming cells may be involved in pathogenesis of meningeal inflammation by expressing different immunophenotypes and by producing regulatory proinflammatory factors such as MCP-1 and NGF.

**Key words:** Lipoplysaccharide, MCP-1, Meningeal macrophage, NGF, Rat

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## Introduction

Macrophages play crucial roles in host defense mechanisms, such as phagocytosis, antigen presentation, and inhibition or killing of pathogens and tumor cells (Takahashi et al., 1996; Yamate et al., 2000; O'Mahony et al., 2008). The brain contains as many macrophages as the liver (Perry et al., 1985; Lawson et al., 1990; McMenamin, 1999; McMenamin et al., 2003); the liver contains a lot of Kupffer cells, the resident tissue macrophages (histiocytes), which have critical roles in the development of hepatic lesions (Hines et al., 1993; Ide et al., 2005). The majority of brain macrophages are microglial cells that reside as a down-regulated form in the brain parenchyma (Perry et al., 1985; Andersson et al., 1992; Bell et al., 1994; McMenamin et al., 2003). Activation of microglial cells is regarded as a histopathological hallmark of neurodegenerative diseases, including Parkinson's disease, Alzheimer disease, multiple sclerosis and dementia complexes (Kim et al., 2000). The functional roles of microglial cells have been intensively investigated (Andersson et al., 1992; Bell et al., 1994). In brain tissues, besides microglial cells, the presence of macrophage-like cells has been reported in the meninx (so-called meningeal macrophages) (Kida et al., 1993; Polfliet et al., 2002; McMenamin 1999; McMenamin et al., 2003). These macrophage populations in the brain tissues may differ

from each other in derivation and functions (Polfliet et al., 2002; Newman et al., 2005). Rats have been used as an experimental animal in neuropathological studies. In contrast to microglial cells, however, the pathobiological characteristics of meningeal macrophages in rats remain to be investigated.

A panel of monoclonal antibodies, applicable for identification of macrophages with different functions, has been extensively used in pathological settings of liver and kidneys of rats, in order to investigate the pathobiological characteristics of these macrophages (Hines et al., 1993; Yamate et al., 2002, 2005; Haralanova-Ilieva et al., 2005; Ide et al., 2005). In this study, we examined the appearance of functionally-different macrophages in the meninx of normal and lipoplysaccharide (LPS)-treated rat brains by immunohistochemistry, with different monoclonal antibodies recognizing varied rat macrophages. LPS is a well-known, gram-negative bacteria-derived factor capable of activating macrophage functions (Iwabuchi et al., 1997). Such a microbial product is recognized by macrophages through the germline-encoded pattern recognition receptors, particularly Toll-like receptors (TLRs) (Peiser et al., 2002; O'Mahony et al., 2008). Thus, LPS has been experimentally injected into brains to cause acute inflammation in mice or rats (Andersson et al., 1992; Bell et al., 1994; Kim et al., 2000). The *in vivo* studies revealed that resident tissue macrophages constitutively exist in the normal meninx, and that functionally up-regulated macrophages appeared in inflamed meninx.

The meninx is a series of tissues covering the central nervous system, consisting of the dura mater, arachnoid, and pia mater from the outermost layer. The meninx is developed from complicated embryogenic tissues; thus, it is well known that human meningiomas have a broad histological spectrum representing stromal, epithelial or histiocytic characteristics (Kepes, 1986, 1994; Scheithauer, 1990). Previously, we established cell lines (KMY-1 and KMY-2) from a rat spontaneous malignant meningioma (Tsujino et al, 1997). KMY-1 and KMY-2 may have multipotential differentiations. To further explore the pathobiological features of the meninx-constituting cells, possible expressions of macrophage immunophenotypes, and mRNAs of monocyte chemoattractant protein-1 (MCP-1) and nerve growth factor (NGF) were investigated by treating these cell lines with LPS. The *in vitro* studies showed that LPS-treated meningeal cell lines were capable of expressing macrophage immunophenotypes and producing regulatory proinflammatory factors, such as MCP-1 and NGF.

## Materials and methods

### *Animals and injection of lipopolysaccharide (LPS)*

Specific-pathogen free male F344 rats were obtained from Charles River Japan, Inc. (CRJ, Hino, Japan). These animals were housed in animal rooms at a controlled temperature of 21±3°C and with 12-h light dark-cycle and fed a standard commercial laboratory diet for rats *ad libitum*. For observations of normal brains, three to six rats were sacrificed at the age of 3, 6-10, 20-24, 72 and 96 weeks; these rats had been kept to establish the base-line data of aging lesions. For LPS injection, twenty-four rats at 7 weeks old were used. These rats were anesthetized by intraperitoneal injection of sodium pentbarbital (50 mg/kg). A tiny hole was made by an electric drill for dental surgery on the coronal suture of the right side of the skull, 3 mm lateral to the bregma. A needle was inserted into the right cerebral hemisphere at a depth of 3 mm from the skull (Fig. 1). Twenty µl of phosphate buffered solution (PBS) containing 5 µg LPS (*Escherichis coli*, 0127: Bacto, Detroit, MI, USA) was injected by a Hamilton micro-syringe (50 µl in volume; Osaka Chemical Inc., Osaka, Japan) over a period of 30 sec. The hole was closed with bone wax after withdrawal of the needle and the skin was sutured. All animals were allowed to recover from the anesthetic and four rats were sacrificed at each examination point of 0 (control; PBS only), 3, 6, 12, 24 and 48 h. During the observation period, none showed any neurological deficit.

These experiments were in compliance with our institutional guidelines for animal care. All rats used in this study were euthanatized by exsanguinations under ether anesthesia.

### *In vivo observations*

The brains of all rats were carefully removed. The brains were fixed in 10% neutral buffered formalin or Zamboni's solution (0.21% picric acid, 2% paraformaldehyde and 130 mM phosphate buffer, pH 7.4) (Yamate et al., 2005) for 1-3 days, embedded in paraffin, and sectioned at 3-5 µm in thickness. Formalin-fixed, deparaffinized tissue sections were stained with hematoxylin and eosin (HE) for histopathology.

The immunohistochemical analyses were performed using the avidin-biotin complex (ABC) method with the labeled streptavidin biotin (LSAB) kit (Dako, Santa Barbara, CA, USA). Primary monoclonal antibodies described below were used to identify macrophages with different functions. As described previously (Kawashima et al., 2003; Yamate et al., 2005), Zamboni's solution-fixed, deparaffinized tissue sections were available for ED1 (Chemicon International Inc., Temecule, CA, USA; x 500), ED2 (Serotec Ltd., Tokyo, Japan; monoclonal antibody; x 200) and OX6 (Serotec; monoclonal antibody; x 200). After deparaffinization, sections were treated with 0.1% trypsin for 10-15 min, and then incubated with 3% $H_2O_2$ for 10 min to quench endogenous peroxidase. The sections were incubated with 1.5% non-fat milk for 45 min, and then incubated with each primary antibody for 14 h at 4°C. Next, 30 min of incubation with biotinylated goat anti-mouse antibody for monoclonal antibodies was performed.

Final incubation was carried out for 30 min with an avidin-biotinylated peroxidase complex, and positive reactions were visualized with 3,3'-diaminobenzidine (DAB). Non-immunized mouse serum in place of the primary antibody served as negative controls. Rat splenic macrophages were used as positive controls. The ED1 antigen is CD68 sited on lysosomal membranes; the expression implies enhanced phagocytosis of macrophages or dendritic cells (Damoiseaux et al., 1994; Haralanova-Ilieva et al., 2005). The ED2 antigen was identified as rat CD163; CD163 is a member of the macrophage scavenger receptor family. Anti-CD163 antibody usually labels with resident macrophages (e.g, Kupffer cells in liver) in rats (Dijkstra et al., 1985; Hines et al., 1993; Forrester et al., 1998; Polfliet et al., 2006). OX6 recognizes major histocompatibility complex (MHC)-class II molecules (rat Ia) on mature dendritic cells or activated macrophages (Andersson et al., 1992; Zhang et al., 1993; Yamashiro et al., 1994; Newman et al., 2005).

The dura mater is the external meninx, made of dense connective tissue continuous with the periosteum of the skull bone. Thus, it was difficult to completely remove the dura mater from the skull. Therefore, we evaluated the immunoreactive cells appearing in the leptomeninx. Cells labeled with anti-CD68, anti-CD163 or anti-MHC class II antibody in normal and LPS-injected brains were counted per linear (250 µm at 400x) of the meninx in 5 different areas (vertexes and bases of the right or left cerebral hemispheres, and the longitudinal cerebral fissure) shown in Fig. 1. Blood vessels penetrate the central nervous system through tunnels covered by the pia mater, and the pia mater appears before the blood vessels are transformed into capillaries (Kida et al., 1993). In addition to meningeal macrophages, thus, perivascular cells labeled with anti-CD68, anti-CD163 or anti-MHC class II antibody were also counted in 10 different blood vessels (greater than 50 µm in major axis at 400x) within the cortex of left and right cerebral vertexes. Means±SD of the positive cell numbers were calculated.

### *In vitro observations*

Two cloned cell lines (KMY-1 and KMY-2) established from a rat spontaneous malignant meningioma were used. KMY-1 and KMY-2 cell lines are useful for studies to explore the pathobiological characteristics of rat meningeal cells. The derivation and cellular characteristics of these cell lines have been described (Tsujino et al., 1997). Eagle's minimum essential medium (E-MEM; Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Bioserum, UBL, Japan), 0.03% L-glutamine (Nissui), penicillin (100 U/ml), and streptomycin (100 µg/ml) was used as the growth medium. These cell lines were cultured in a humidified 5% $CO_2$ atmosphere at 37°C as described previously (Tsujino et al., 1997).

Cells of KMY-1 ($1x10^4$/ml) or KMY-2 ($2x10^4$/ml) were grown on chamber slides for 24 h, and then washed twice with PBS. Thereafter, these cells were incubated in one ml of E-MEM containing LPS (0, 0.02, 0.05, 0.5, 5, 50, and 100 µg/ml diluted in PBS). Twenty-four h after the incubation, cells were fixed in cold acetone for 10 min, and stained immunocytochemically with anti-CD68, anti-CD163 or anti-MHC class II antibody by the ABC methods described above. The number of cells reacting to these antibodies was counted 4 times in 300 cells in different fields at 400x (Yamate et al., 2001).

For reverse transcription-polymerase chain reaction (RT-PCR), cells of KMY-1 or KMY-2 cells were incubated for 72 h in E-MEM containing LPS (0, 0.5, 5, 50 and 100 µm/ml). Total RNAs were isolated from KMY-1 or KMY-2 cells by Trizol™ reagent (GIBCO BRL, Gland Island, NY, USA) (Yamate et al., 2001). RT-PCR was conducted as described previously (Tsujino et al., 1997). From 0.5 µg/µl of total RNA, the single-strand cDNA was synthesized with Super Script™ Preamplification System (GIBCO BRL). cDNAs were amplified by PCR with Taq DNA polymerase (Takara Shuzou, Kyoto, Japan) and each of the specific primers (sense and antisense) for MCP-1, NGF or ß-actin (control). The following conditions were used for the amplification: for MCP-1, 27 cycles of 15 sec of denaturation at 94°C 30 sec of annealing at 55°C, and 1 min of synthesis at 72°C (Kawashima et al., 2003); for NGF, 25 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, and 1 min of synthesis at 72°C (Tsujino et al., 1997). The oligonucleotides used for

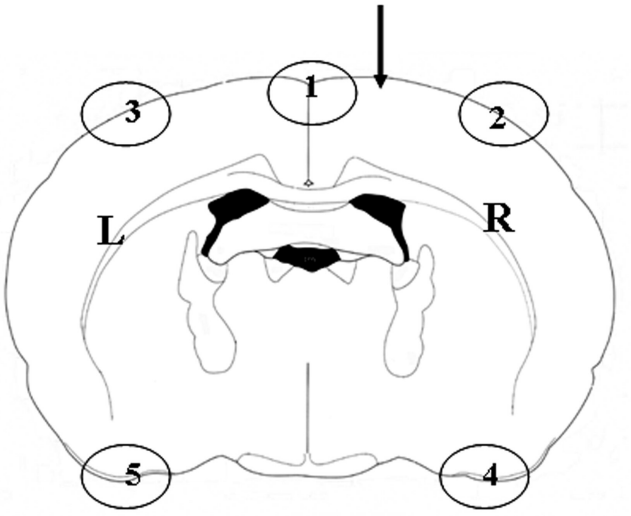


**Fig. 1.** Drawing of cross-cut at the optic chiasm of rat cerebrum. For lipopolysaccharide (LPS) injection, an arrow shows a portion (around the leptomeninx) of needle inserted into the right cerebral hemisphere. Cells reacting to monoclonal antibodies useful for detecting rat macrophages were counted in 5 different areas of the meninx: the longitudinal cerebral fissure (1), vertexes (2, 3) and bases (4, 5) of the right (R) or left (L) cerebral hemisphere.

PCR were as follows: MCP-1 sense primer 5'-ATGCAGGTCTCTGTCACG-3' and antisense primer 5'-CTAGTTCTCTGTCATACT-3' (Kawashima et al., 2003): NGF sense primer 5'-AAGCGCTCATCCACCCACCCAGTC-3' and antisense primer 5'-TCTTCTTGTAGCCTTCCTGCTGAGC-3' (Tsujino et al., 1997): ß-actin sense primer 5'-TAAAGACCTCTATGCCAACAC-3' and antisense primer 5'-CTCCTGCTTGCTGATCCACAT-3' (Tsujino et al., 1997). The PCR products were electrophoresed (100 V, 20min) in 1% agarose gels. DNA was stained with ethidium bromide on the gel. The size of the amplification bands of MCP-1, NGF and ß-actin is 447, 435 and 214 bp, respectively. The intensity of the band of MCP-1 and NGF was evaluated semiquantitatively using image analysis software (NIH Image J), and compared to that of ß-actin. Measurement was performed three times.

### *Statistical evaluation*

Paired samples were evaluated using Student's t-test. Significance was accepted at $P<0.05$.

## Results

### *Macrophage populations in the meninx of normal brains*

Immunoreactive cells in the leptomeninx consisting of the arachnoid, subarachnoidal space and pia mater, as

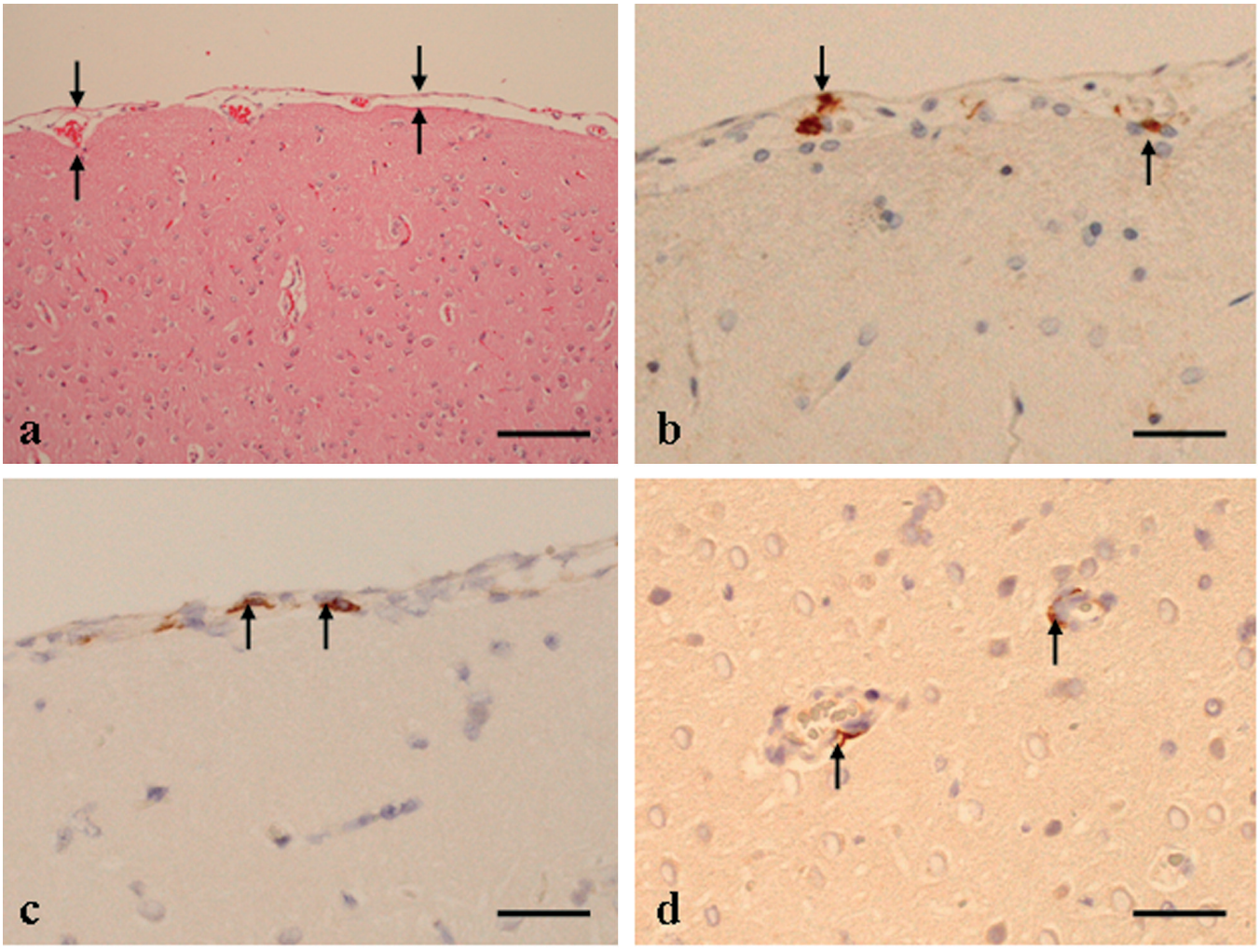


**Fig. 2.** The leptomeninx consists of the arachnoid (down arrows), subarachnoidal space and pia mater (upper arrows) **(a)**. In normal brains of rats examined at the age of 72 weeks, MHC class II-expressing cells (activated macrophages or dendritic cells) (arrows), labeled with OX6, are seen in the meninx **(b)**; there are anti-CD163-positive resident tissue macrophages in the meninx (arrows) **(c)** and around blood vessels (arrows) **(d)**. These positive cells are round, spindle-shaped or stellate in shape **(b-d)**. a, HE stain; b, c and d, immunohistochemistry counterstained with hematoxylin. Bars: a, 120 µm; b, c, 20 µm; d, 40 µm.

well as around blood vessels in the brain parenchyma were evaluated (Fig. 2a). In normal brains of rats examined at the age of 3, 6-10, 20-24, 72 and 96 weeks, ant-CD68-positive cells were not seen in the meninx or around blood vessels. A small number of anti-MHC class II-positive cells were present in the meninx (less than 3 cells/250 µm linear in the number) (Fig. 2b), but the positive cells were not seen around blood vessels. Among the anti-CD68-, anti-CD163- and anti-MHC class II-positive cells, anti-CD163-positive cells were most frequently seen; the anti-CD163-positive cell number in the meninx was less than 7 cells/250 µm linear (Fig. 2c), and that around blood vessels (Fig. 2d) was less than 3 cells/blood vessel. There was no age-dependent change in the anti-CD163- and anti-MHC class II-positive cell numbers. The anti-CD163-positive cell number tended to be greater in the longitudinal cerebral fissure as compared with that in the vertices or bases of the cerebrum. In the meninx, anti-CD163-positive cells were along the pia mater (Fig. 2c), whereas anti-MHC class II-positive cells appeared to be present in the subarchnoidal space (Fig. 2b). These positive cells were round, spindle-shaped or stellate in shape (Figs. 2b-d).

### *Macrophage populations of the meninx in LPS-injected brains*

In HE-stained sections at 3, 6 and 12 h after LPS injection, edema and hemorrhage were seen in the

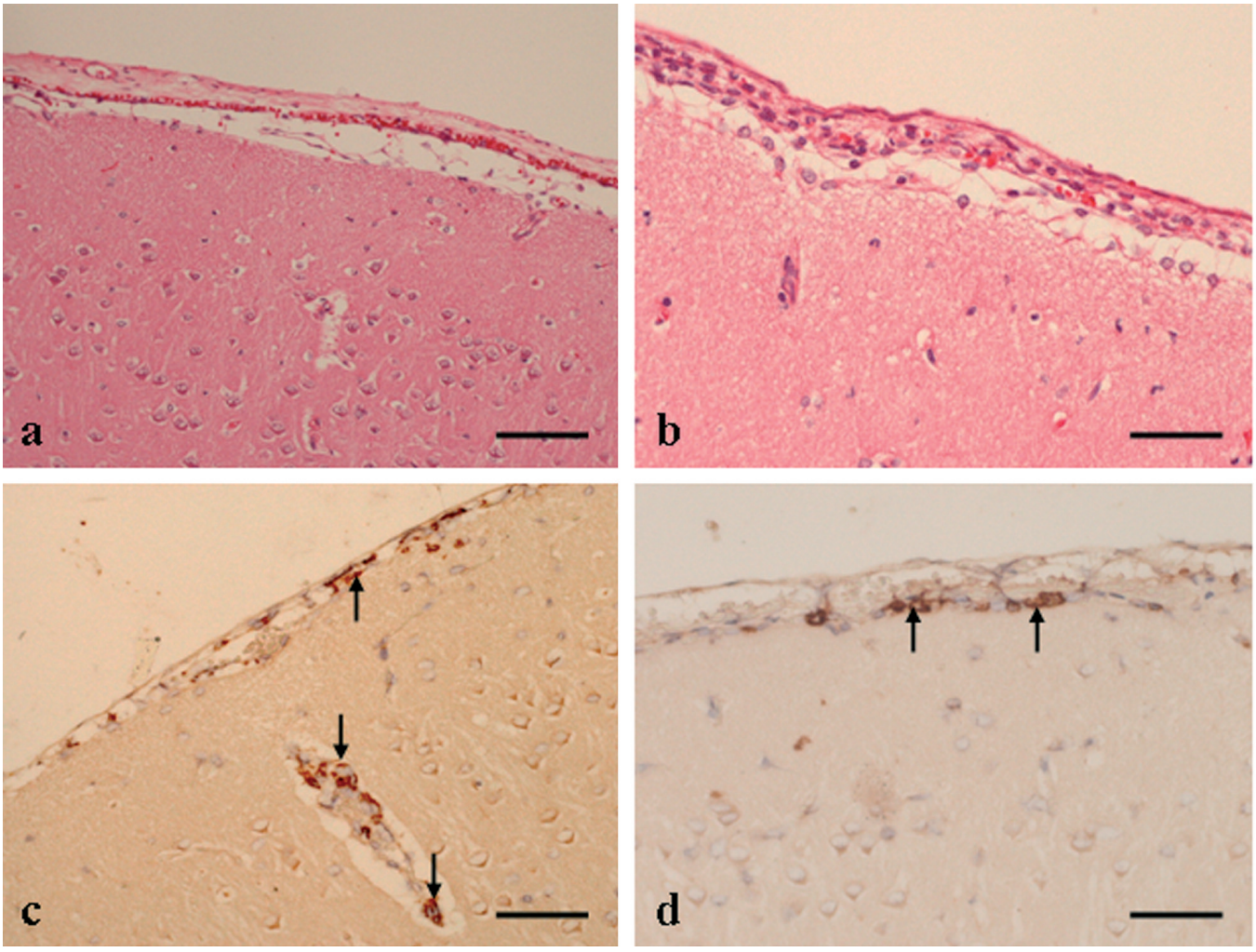


**Fig. 3.** Histopathological **(a, b)** and immunohistochemical **(c, d)** findings of LPS-treated rats. At 3 h after LPS injection, edema and hemorrhage, as well as a small number of inflammatory cells are seen in the meninx, particularly in the dilated subarachnoidal spaces **(a)**. Many inflammatory cells are seen in the menix at 12 h after LPS injection **(b)**. Anti-CD68-positive macrophages with activated phagocytosis are observed in the meninx and around blood vessels (arrows) at 12 h after LPS injection **(c)**. MHC class II-expressing cells are present in the meninx at 24 h after LPS injection **(d)**. a and b, HE stain; c and d, immunohistochemistry counterstained with hematoxylin. Bars: a, 100 µm; b, 50 µm; c, 40 µm; d, 30 µm.

leptomeninx, particularly in the slightly dilated subarachnoidal spaces (Fig. 3a). Inflammatory cells began to be seen at 6 h and then the number gradually increased at 12 h (Fig. 3b), and thereafter, decreased at 24 and 48 h. At the injection site of the parenchymal tissue of the cerebrum, slight hemorrhage and a small number of inflammatory cells were seen at 12 and 24 h; however, the degree was very slight.

There was no significant change in the anti-CD68-, anti-CD163- and anti-MHC class II-positive cell numbers between the meninx of 5 different cerebral portions examined (Fig. 1). At 3, 6 and 12 h, the number of anti-CD68-positive cells was significantly greater than that at 0 h, showing a maximum at 12 h; thereafter, the number gradually decreased at 24 and 48 h, but it remained significantly elevated up to 48 h (Fig. 4a). Anti-CD68-positive cells were round or spindle-shaped (Fig. 3c), and the immunolabelling appeared in a granular pattern in the cytoplasm. The anti-MHC class II-positive cell number showed a significant increase at 3 h, and thereafter the number gradually increased until 48 h, although the number was lower than that of anti-CD68-positive cells at 6-48 h (Fig. 4a). The anti-MHC class II-positive cells appeared exclusively along the pia matter (Fig. 3d). No significant change was seen in the anti-CD163-positive cell number during the 48 h-observation period following LPS injection; the cell number was around 4-7 cells per linear of 250 μm at each examination point (Fig. 4a).

In perivascular areas of brain parenchyma, no significant changes in anti-CD163- and anti-MHC class II-positive cell numbers were seen following LPS injection. At 12 h, cells reacting to CD68 appeared around blood vessels (Fig. 3c), and the number showed a significant increase at 12, 24, and 48 h, with a peak at 24 h (Fig. 4b).

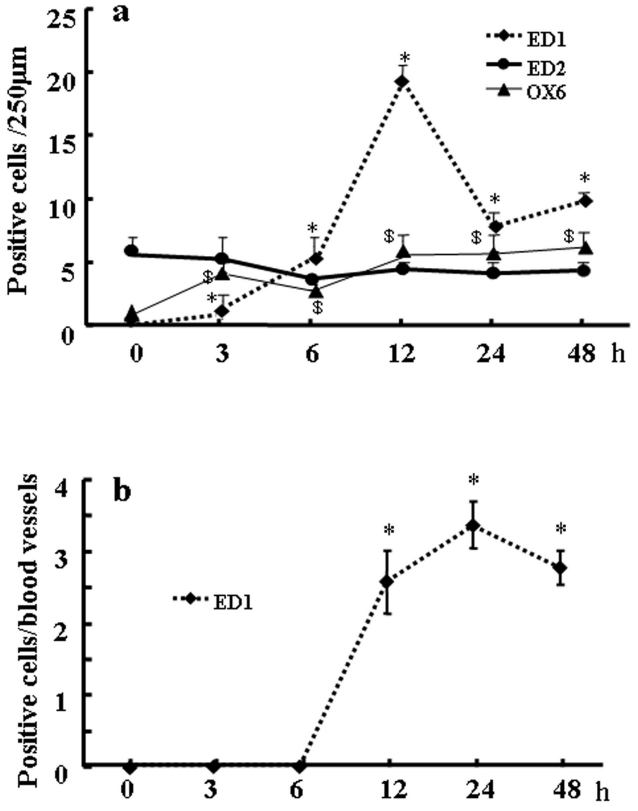


**Fig. 4.** The number of cells labeled with anti-CD68 (ED1), anti-CD163 (ED2) or anti-MHC class II (OX6) antibody at 0 (control), 3, 6, 12, 24, and 48 h after LPS injection. **a**, in the menix (250 μm in linear); b, around blood vessels in the brain parenchyma; each value represents the mean±SD; *, $, significantly different from controls at P<0.05.

### *Macrophage immunophenotypes of KMY-1 and KMY-2 cells under LPS treatment*

At confluency in the absence of LPS, monolayer cultures of KMY-1 (Fig. 5a) and KMY-2 (Fig. 5b) consisted of large round or polygonal cells with a round nucleus; in KMY-2 culture, additionally, multinucleated giant cells were occasionally present (Fig. 5b). Immunocytochemically, a small number of KMY-1 cells (less than 2%) reacted to CD68, whereas 15-30% of KMY-1 cells gave a positive reaction to CD163 (Fig. 5c). Occasional cells of KMY-2 cells (less than 3%) gave a positive reaction to CD163, but cells reacting to CD68 were not seen in KMY-2 culture (Fig. 5d). Neither KMY-1 nor KMY-2 cells reacted with anti- MHC class II antibody.

In KMY-1, no significant change was seen in the anti-CD68- and anti-CD163-positice cell number at 0.02 and 0.05 μg/ml of LPS; at these concentrations, the percentage of anti-CD163-positive cells (20-30%) was similar to that of 0 μg/ml, and anti-CD68-positive cells were rarely detected (Fig. 6a). At 0.5, 5, 50 and 100 μg/ml of LPS, however, the anti-CD68-positive cell number significantly increased (Figs. 5e and 6a), whereas the anti-CD163-positive cell number significantly decreased (Fig. 6a).

In KMY-2 cells, a reaction to CD68 did not appear at any concentration of LPS; on the contrary, at 0.05 and 0.5 μg/ml of LPS, the anti-CD163-positive cell number significantly increased, peaking at 0.5 μg/ml (Figs. 5f, 6b), and thereafter, the number decreased at 5, 50 and 100 μg/ml of LPS, the level being similar to that at 0 μg/ml (Fig. 6b).

Anti-MHC class II-positive cells were not developed in LPS-treated KMY-1 and KMY-2 cells.

### *mRNA expressions of MCP-1 and NGF in LPS-treated KMY-1 and KMY-2 cells*

In both KMY-1 (Fig. 7a) and KMY-2 (Fig. 7b), LPS addition (0.5, 5, 50 and 100 μg/ml) significantly increased mRNA levels of MCP-1. While mRNA of NGF was significantly increased in LPS-treated KMY-2

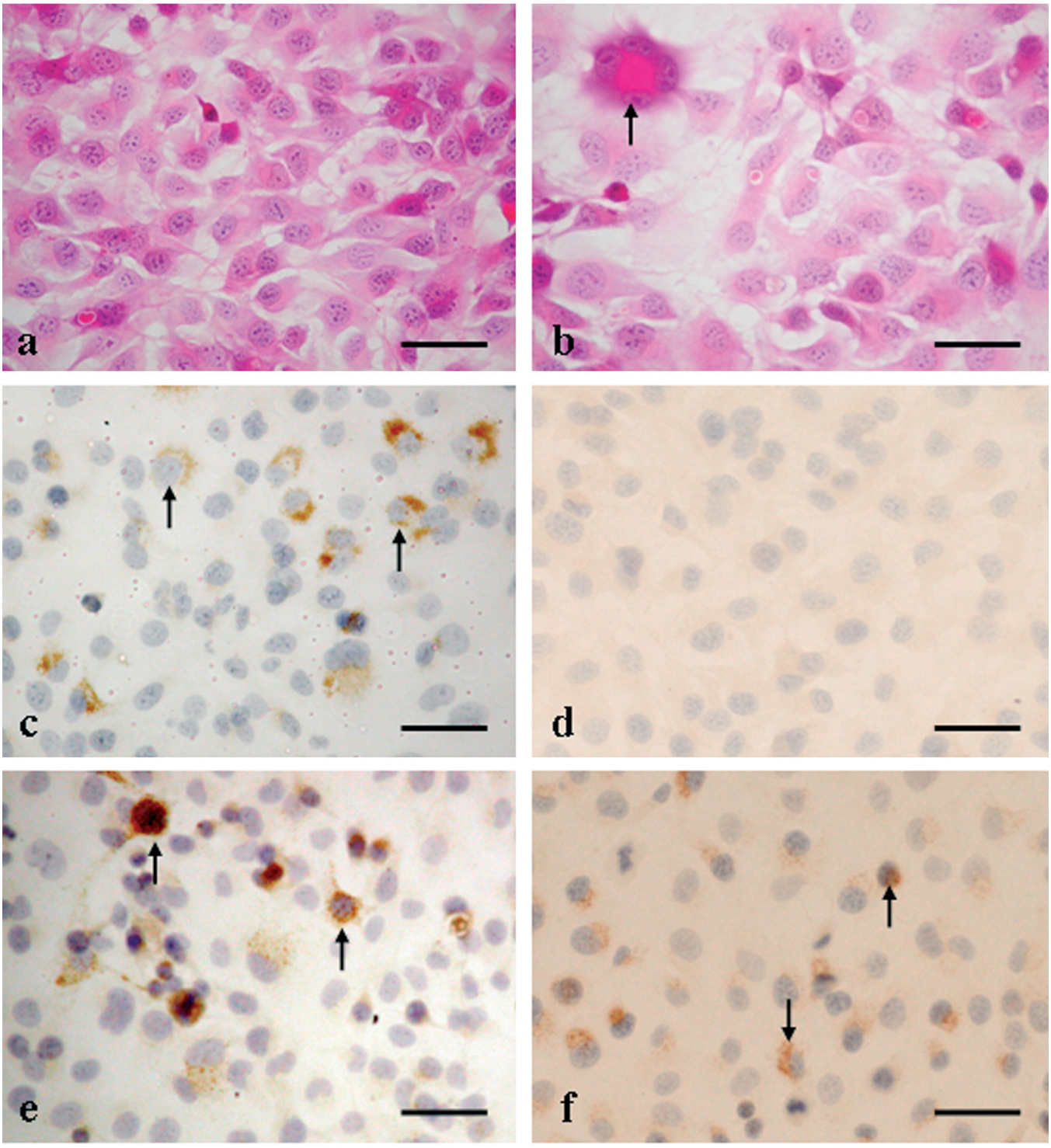


**Fig. 5.** Cloned cell lines, KMY-1 **(a, c, e)** and KMY-2 **(b, d, f)**, established by a rat malignant meningioma. Monolayer culture of KMY-1 **(a)** and KMY-2 **(b)** consists of large round or polygonal cells with a round nucleus; additionally, one multinucleated giant cell (arrow) is present in KMY-2 culture **(b)**. In the absence of LPS, some KMY-1 cells react to CD163 **(c)** (arrows), but KMY-2 cells do not give a positive reaction to CD68 **(d)**. LPS-treated KMY-1 cells at 0.5 μg/ml increased the number of anti-CD68-positive cells **(e)** (arrows), and LPS-treated KMY-2 cells at 0.5 μg/ml increased the number of anti-CD163-positive cells **(f)** (arrows). a and b, HE stain; c, d, e and f, immunocytochemistry counterstained with hematoxylin. Bars: 30 μm.

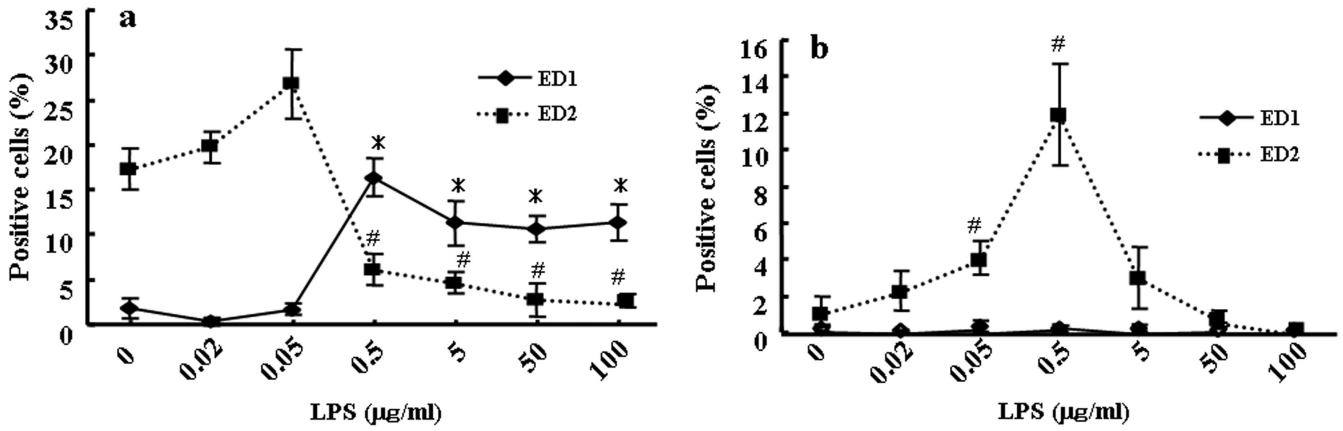


**Fig. 6.** The percentage of anti-CD68(ED1)- or anti-CD163(ED2)-positive cells in KMY-1 **(a)** and KMY-2 **(b)** cells in the absence (0 μg/ml, control) or presence of LPS at concentrations of 0.02, 0.05, 0.5, 5, 50 and 100 μg/ml. Each value represents the mean±SD; *, #, significantly different from controls at P<0.05.

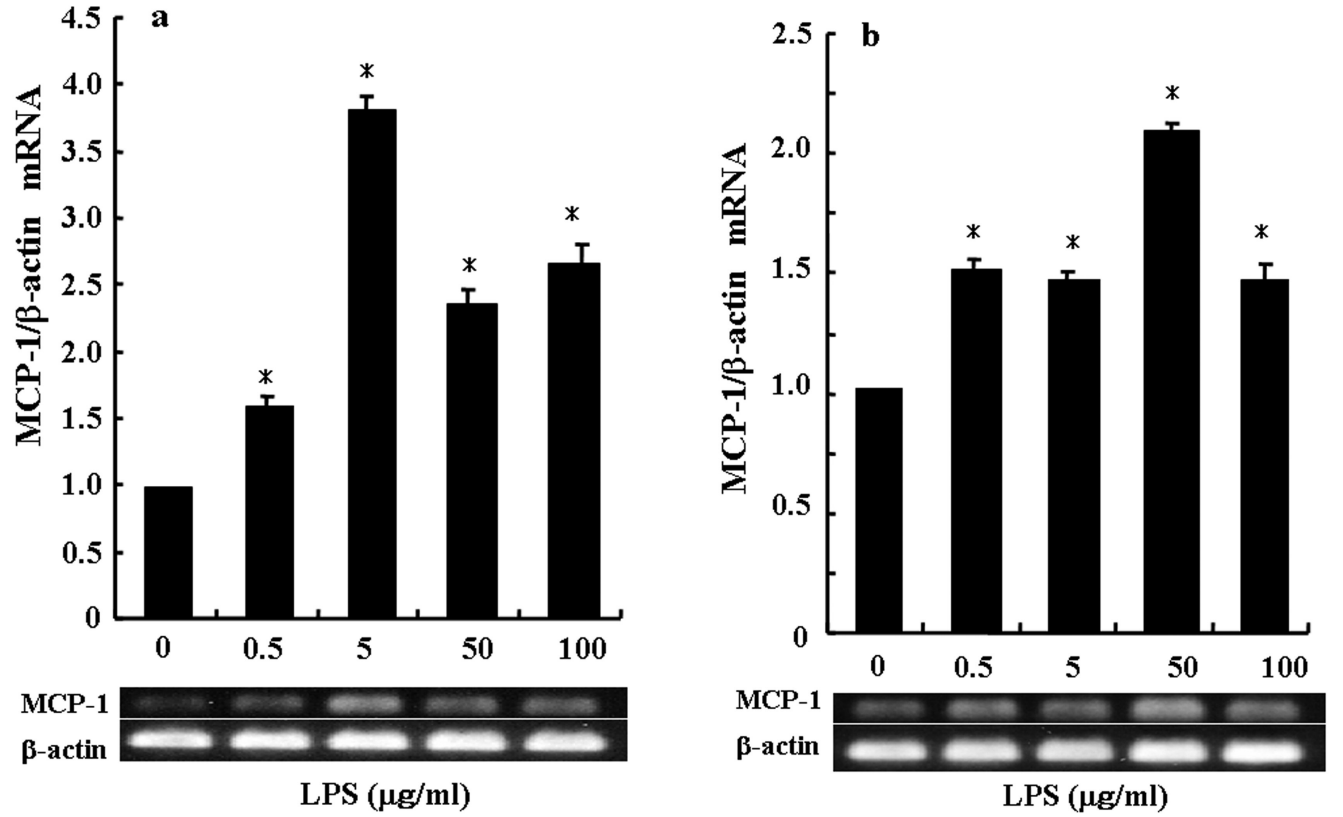


**Fig. 7.** Expression of mRNA of monocyte chemoattractant protein-1 (MCP-1) in KMY-1 **(a)** and KMY-2 **(b)** in the absence (0 μg/ml, control) or presence of LPS at a concentration of 0.5, 5, 50 or 100 μg/ml. Representative bands of MCP-1 and ß-actin by the RT-PCR method are shown. Each band is normalized to that of ß-actin and evaluated relative values (mean±SD) in terms of the control, which is assigned a value of 1; *, significantly different from controls at P<0.05.

cells in a dose-dependent manner (Fig. 8), LPS treatment did not influence NGF mRNA expression in KMY-1 cells.

## Discussion

Macrophages are not homogeneous cell populations; they differ in ontogeny, morphology, immunophenotypes and functions (Takahashi et al., 1996; Valledore et al., 1998; Yamate et al., 2000; Mueller et al., 2001). In this study, monoclonal antibodies (anti-CD68, anti-CD163 and anti-MHC class II antibodies) were used to identify macrophages with different functions. These antibodies have also been utilized in experimentally-induced rat hepatic and renal injury models (Hines et al., 1993; Ide et al., 2005; Haralanova-Ilieva et al., 2005; Yamate et al., 2005).

In normal rat brains, it has been reported that there are cells reacting to CD163 or MHC class II in the meninx and perivascular areas (Kida et al., 1993; McMenamin, 1999; Polfliet et al., 2002). Such cells around blood vessels in the brain parenchyma have been identified as perivascular macrophages (Kida et al., 1993; Polfliet et al., 2002; McMenamin et al., 2003). The current studies also confirmed the present of anti-CD163- and anti-MHC class II-positive cells in the meninx and perivascular space, although the number of anti-MHC class II-positive cells was smaller than that of anti-CD163-positive cells. Anti-CD68-positive macrophages, which are considered to be cells with enhanced phagocytosis (Damoiseaux et al., 1994; Haralanova-Ilieva et al., 2005), were rarely seen in normal rat brains. These findings indicate that meningeal and perivascular macrophages existing as resident tissue macrophages constitutively express macrophage scavenger receptors (CD163) and MHC class II molecules. It was reported that dendritic cells, demonstrable with OX62 (a marker specific for rat dendritic cells), were present in the meninx, forming networks (McMenamin, 1999). The present MHC class II-expressing cells might involve dendritic cells. The present study further investigated age-related change in the appearance of meningeal and perivascular macrophages, although no age-dependent change was found until 96 weeks old. The meningeal and perivascular macrophages seem to be constitutively present in rat brains for life. In mice, similarly, it has been reported that the pattern of macrophage scavenger receptor expression was not different from that in the young adult brain (Bell et al., 1994). Microglial cells, which are diffusely distributed in brain parenchyma, did not give a positive reaction to CD68, CD163 or MHC class II, indicating a difference in immunophenotypes between microglial cells and meningeal or perivascular macrophages in normal rat brains. This implies that microglial cells in normal brain are functionally down-regulated.

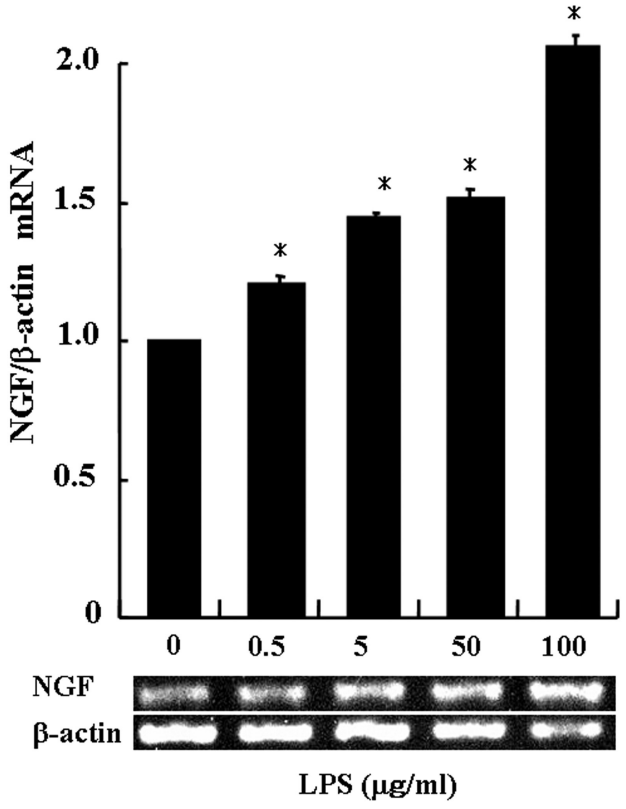


**Fig. 8.** Expression of mRNA of nerve growth factor (NGF) in KMY-2 in the absence (0 μg/ml, control) or presence of LPS at a concentration of 0.5, 5, 50 or 100 μg/ml. Representative bands of NGF and ß-actin by the RT-PCR method are shown. Each band is normalized to that of ß-actin and evaluated relative values (mean±SD) in terms of the control, which is assigned a value of 1; *, significantly different from controls at $P<0.05$.

LPS activates the phagocytosis and production of proinflammatory cytokines of macrophages (Andersson et al., 1992; Bell et al., 1994; Jungi et al., 1996; Baker et al., 1999). To investigate the kinetics of reactive macrophages in the meninx, we injected LPS into rat brains. The most frequent appearance of macrophage populations had been expected at the injection point. However, no significant difference was seen in the macrophage numbers between five different cerebral portions examined (Fig. 1). This might be because the injection point (depth of 3 mm from the cranial bone at 3 mm right side to the bregma) used in this study was around the superficial or leptomeninx of the cerebrum, and thus, LPS solution might spread into the whole brain through the cerebrospinal fluid in the subarachnoid cavity. Following LPS injection, the number of anti-CD68-positive cells significantly increased from 3 h with a peak at 12 h in the meninx and from 12 h with a peak at 24 h in perivascular areas. At 48 h after LPS injection, the number of anti-CD68-positive cells still remained elevated in both portions. On the other hand, there was no significant change in the anti-CD163-

positive cell number during the 48 h-observation period. In rat models of experimental allergic encephalomyelitis (EAE), it has been shown that meningeal and perivascular macrophages expressing CD163 began to increase at the late stage (from day 9 post-EAE induction) (Polfliet et al., 2002). Additionally, in isoproterenol-induced rat myocardial injury, anti-CD163-positive cells appeared 3 days after injection (Nakatsuji et al., 1997). It is considered that in the early phase of meningeal inflammation, macrophages expressing CD68 are quickly recruited, whereas macrophages expressing CD163 may increase in the late inflammation phase. The amount of CD68 expression depends on phagocytic activity (Damoiseaux et al., 1994; Haralanova-Ilieva et al., 2005). It is likely that anti-CD68-positive macrophages seen at the early stages of this *in vivo* study appeared for the sake of clearance via phagocytosis of tissue debris. In peripheral nerve injury rat models, macrophages phagocytosing myelin have been found at very early stages (Mueller et al., 2001).

The number of cells labeled with anti-MHC class II antibody gradually increased from 3 h up to 48 h, although the number was much smaller than that of anti-CD68-positive cells. The expression of MHC class II molecules implies the increased ability of antigen presentation of macrophages or dendritic cells (Yamashiro et al., 1994; McMenamin, 1999; Zhao et al., 2006). Generally, MHC class II-expressing cells present antigens to unprimed T cells (McMenamin, 1999), and subsequently Th1/Th2 lymphocyte commitment is determined; cytokines produced mainly by Th2 lymphocytes stimulate B cell proliferation, and Th1-derived cytokines further activate macrophage populations (Banchereau et al., 2003; Gao et al., 2007; Zimmerli and Hauser, 2007). In the present study, lymphocytes were not detected. Anti-MHC class II-positive cells have been observed in thioacetamide (TAA)-induced acute hepatocyte injury of rats (Ide et al., 2005) without lymphocyte reactions. The expression of MHC class II molecules might only have reflected a capacity for non-specific antigen presentation, rather than actual antigen presentation (Kida et al., 1993). However, in renal or myocardial injury of rat models, anti-MHC class II-positive cells persisted longer, and then lymphocyte proliferation was developed in the late phases (Zhang et al., 1993; Yamate et al., 2002). Recently, it was reported that in rat excitotoxic brains, dendritic cells expressing MHC class II molecules were recruited mainly in the subarachnoidal space from a blood cell population at the late stages between 3 and 5 days (Newman et al., 2005). To understand the significance of activated macrophages or dendritic cells expressing MHC class II molecules in rat meninx, longer period studies following LPS injection are needed.

KMY-1 and KMY-2 cells are cloned cell lines established from a rat spontaneous malignant meningioma; the original tumor showed cellular and nuclear pleomorphism, indicating anaplastic type of the meningioma (Tsujino et al., 1997); tumors induced in syngeneic F344 rats by inoculating KMY-1 cells had histopathological features of meningeal fibrosarcomas, whereas KMY-2 cells gave a morphological appearance of arachnoid trabecular cells. In humans, lipid-filled cells forming xanthomatous meningioma reacted to an antibody (HAM-56) typical of human macrophages/histiocytes (Kepes, 1994). The possible source of the meninx has been considered to be the prechordal plate, unsegmented and segmented mesoderms, mesectoderm, neurilemmal cells and neural tube (Kepes, 1986; O'Rahilly and Müller, 1986). Because of the complicated embryogenesis, meningioma cells may have multidirectional differentiations. In the absence of LPS, although positive reactions to CD68 or MHC class II were barely seen, cultured KMY-1 and KMY-2 cells reacted to CD163 in varying degrees, suggesting that these cell lines may have the capacity to differentiate towards macrophage-like cells. Anti-CD163-positive cells present along the pia matter of normal rat brains might be derived partly from the meninx-constituting cells.

More interestingly, LPS addition (0.5 to 100 μg/ml shown in Fig. 6a) to KMY-1 cells decreased the anti-CD163-positive cell number, whereas the anti-CD68-positive cell number increased at these LPS concentrations. The dual immunohistochemsitry has revealed that there were resident meningeal macrophages which reacted to both CD68 and CD163 (McMenamin, 1999); KMY-1 cells may be comparable to such macrophages in cellular nature. On the contrary, KMY-2 cells transiently increased only the anti-CD163-positive cell number at 0.05 and 0.5 μg /ml of LPS. These findings indicated that KMY-1 and KMY-2 cells differed in cellular nature from each other, and LPS treatment altered macrophage immunophenotypes in KMY-1 and KMY-2 cells.

As described above, increased expression of CD68 is related to phagocytosis (Damoiseaux et al., 1994; Haralanova-Ilieva et al., 2005). Therefore, particularly KMY-1 cells, which showed an increased number of anti-CD68-positive cells, might come to have phagocytic activity under LPS stimulation. Anti-CD68-positive cells developed in the meninx of LPS-injected rats might have reflected the rapid response of the meninx-forming cells. CD163 functions as a scavenger receptor for hemoglobin-haptoglobin complexes, and the engagement of CD163 may induce the production of proinflammatory mediators (Polfliet et al., 2006). An increased number of anti-CD163-positive cells in KMY-2 cells following LPS addition suggests that meningeal cells are able to produce proinflammatory factors leading to subsequent complicated inflammation.

In the present study, therefore, we investigated possible productions of regulatory proinflammatory factors by treating KMY-1 or KMY-2 cells with LPS. MCP-1 is an important chemokine of monocytes and macrophages in injured tissues (Schecter et al., 1997). LPS addition to KMY-1 and KMY-2 cells significantly

increased mRNA expression of MCP-1. Meningeal cells in LPS-injected rat brains might be responsible for macrophage recruitment, particularly anti-CD68-positive macrophages and anti-MHC class II-positive cells, by producing MCP-1. The present study also demonstrated that NGF mRNA expression was dose-dependently increased in LPS-added KMY-2 cells. NGF is a neurotrophin having central roles, such as growth, differentiation and preservation of neurons, and has also been reported as an immunological effecter including modulation of macrophage functions (Santambrogio et al., 1994; Samah et al., 2008; Sivillia et al., 2008). It is likely that in meningeal inflammation, the meningeal cells play important roles not only in the preservation for brain, but also in macrophage activation via production of NGF.

In conclusion, the present study showed that anti-CD163-positive macrophages with scavenger receptors constitutively exist in the normal rat meninx, and that under LPS treatment, anti-CD68-positive macrophages with enhanced phagocytosis and MHC class II-expressing cells (activated macrophages or dendritic cells) appeared in the meninx; these findings indicate that macrophages with heterogeneous functions participate in the pathology of the rat meninx. Additionally, it was found that rat malignant meningioma-derived cell lines (KMY-1 and KMY-2) exhibited macrophage immunophenotypes (in particular, CD68 and CD163) of which the expression degrees were altered by LPS treatment. Furthermore, LPS treatment showed increased expressions of MCP-1 mRNA in KMY-1 and KMY-2 cells, and NGF mRNA in KMY-2 cells. These in vitro studies reveal that the meninx-constituting cells could attribute to recruitment or activation of macrophages via production of MCP-1 or NGF, respectively. Recently, it has been shown that macrophage functions are regulated by pattern recognition molecules, such as TLRs (particularly TLR4), CD14, macrophage scavenger receptors, ß2-integrins, and C-type lections (Peiser et al., 2002; O'Mahony et al., 2008). The functional roles of meningeal macrophages in inflammation should be further investigated in terms of these surface molecules.

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