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The expression of tumour necrosis factor in the hypothalamus after treatment with lipopolysaccharide

LAN LIU*, TOSHIRO KITA*, NORIYUKI TANAKA* AND YOSHIMASA KINOSHITA†

*Departments of * Forensic Medicine and †Neurosurgery, School of Medicine, University of Occupational and Environmental Health, Japan*

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Summary. To investigate the effects of tumour necrosis factor (TNF) in the hypothalamus, Wistar rats received an intravenous administration of lipopolysaccharide (LPS) at a dose of 3.0 mg/100 g. Concentrations of TNF-α in the cerebral liquor and blood sera rapidly increased at 30 minutes after administration of LPS, rose to the maximum level at 1 hour, and then gradually decreased. Using horse-radish peroxidase as a tracer, a transient increase in paracellular permeability throughout the tight junctions of the ependymal cell layer covering the third ventricle was observed by electron microscopy at 30 minutes and in that of the capillary endothelium at 1 hour after administration, respectively. Following LPS administration, TNF was preferentially localized by immunoelectron microscopy in the tight junctional area of the ependymal cell layer and the capillary. These data indicate that TNF, synthesized in the ependymal cell layer, induces a deterioration in the cerebrospinal fluid–brain barrier and subsequently in the blood–brain barrier. The present study suggests that oedematous changes in the hypothalamic areas determined by ultrastructural and magnetic resonance analyses were mainly due to TNF conveyed from the ependymal cell layer to the hypothalamus after administration of LPS.

Keywords: endotoxin shock, hypothalamus, tumour necrosis factor, cerebrospinal fluid–brain barrier, blood–brain barrier

Although sepsis shock is thought to be a causative factor for cerebral oedemas (Jeppson *et al.* 1981; Canady *et al.* 1993; Hariri *et al.* 1993), details of the histopathogenesis remain unclear. Experimental sepsis shocks induce an increased microvascular permeability in various organs (Nagano *et al.* 1992; Kita *et al.* 1993; Tanaka *et al.* 1994). Capillaries in certain cerebral areas possess a blood–brain barrier (BBB) due to the extensive tight junctions between the adjacent endothelial cells and the thick basal lamina below the endothelium. Several experiments as to the deterioration of the BBB after administration of lipopolysaccharide (LPS) have been made (Eckman *et al.* 1958; Clawson *et al.* 1966; Schmahl *et al.* 1976; Quagliarello *et al.* 1986; Wispelwey *et al.* 1988; Lustig *et al.* 1992) but ultrastructural analyses, including immunoelectron microscopy, have not been carried out on this endotoxin-induced enhancement in the permeability of the BBB.

Recent reports have demonstrated that the cerebral tissues synthesize certain inflammatory mediators

Correspondence: Toshiro Kita, Department of Forensic Medicine, School of Medicine, University of Occupational and Environmental Health, Yahata Nishiku, Kitakyushu 807, Japan.

induced by LPS (Hynes *et al.* 1991; Lustig *et al* 1992; Tarlow *et al.* 1993; Gatti & Bartfai 1993; Hariri *et al.* 1993). However, immunocytochemical studies of the synthesis and release of these mediators and of how these mediators induce cerebral oedemas have been limited (Tarlow *et al.* 1993). Tumour necrosis factor (TNF) has been implicated as a major pro-inflammatory cytokine (Tracey & Cerami 1992). We know little about how the expression of TNF in cerebral tissues results in various cerebral oedemas and whether TNF affects the deterioration of the BBB.

The aim of the present experiment is to reveal immunolocalizations of TNF after treatment with LPS in the gray matter of the hypothalamus and to determine the effects of TNF on the deterioration of the BBB using horse-radish peroxidase (HRP) as a tracer.

Materials and methods

Animals

Sixty-nine male Wistar rats (Seiwa Experimental Animal Co., Ooita, Japan) weighing 300–350 g were used for the present experiment.

Endotoxin treatment

Under light ether anaesthesia, 48 rats received an intravenous injection of LPS (*Escherichia coli* 0127: B8; Difco, Detroit, Mich., USA) at a dose of 3.0 mg/100 g body weight. LPS-treated animals were divided into three groups (16 rats each) and sacrificed under ether anaesthesia at 30 minutes, 1 and 5 hours after LPS injection, respectively. Sixteen control rats received a solution of sterile saline only and were sacrificed under ether anaesthesia at 5 hours after administration.

TNF assay

Twenty rats were utilized for TNF assay. Cerebral liquor was obtained from the cisterna magna. TNF-α levels of the liquor were determined by the mouse TNF-α ELISA kit (no. 80-2903-00, Genzyme Corporation, Cambridge, MA USA) since the previous investigators described this kind of kit as a useful tool for the quantitation of rat TNF-α (Pizarro *et al.* 1993). The detection limit of the assay was 15 pg/ml. In addition, the serum TNF-α levels were also determined by the same kit. Data were expressed as mean $\pm$ SD for 5 rats in the text and figures. The non-paired *t*-test was used to determine significance by the Welch *t*-test. Significance was accepted if $P < 0.05$.

Magnetic resonance analysis

Five rats utilized for magnetic resonance (MR) analyses were intraperitoneally anaesthetized with pentobarbital (60 mg/kg body weight) and analysed with T2-weighted MR imaging which was performed using a 4.7 Tesla MR system with an 400 mm horizontal bore and proton frequency of 200.0 MHz (SIS 200/400; Spectroscopy Imaging Systems Corp., Fremont, Cal., USA) before and up to 5 hours after injections of LPS. The head of the animal was placed prone in a slotted tube resonator. Spin-echo MR images were obtained in orthogonal planes to confirm desired positioning (acquisition parameters for T2-weighted images: TR, 2500 ms; TE, 80 ms; 256 $\times$ 192 matrix; field of view, 40 $\times$ 40 mm; 1 excitation; and 11 2-mm coronal slices with 0 mm centre-to-centre spacing).

Electron microscopy

For transmission electron microscopy, 12 animals were perfused with a solution of sterile saline (37°C) and then with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PBS) at 4°C from the left ventricle. The hypothalamus was cut into 2 mm thick slices and post-fixed in 2% osmium tetroxide in 0.1 M PBS at 4°C for 1 hour. Specimens were dehydrated in an ascending ethyl alcohol series and embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM 1200 EX electron microscope.

Tracer experiment

Twelve rats received intravenous injections of 250 mg/kg horse-radish peroxidase (HRP; type II peroxidase; Sigma Chemical Co., St Louis, USA) via the tail vein according to the method of Karnovsky (1967) at 30 minutes, 1 and 5 hours after the injection of LPS. At 10 minutes after the HRP injection, a solution of sterile saline, followed by a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PBS, was infused from the left ventricle. The hypothalamus was cut into approximately 2 mm thick slices and post-fixed in the same fixative for 1 hour. Sections of approximately 30–40 μm in thickness were made on a microslicer (Dosaka EM Co, Ltd, Osaka, Japan) and inserted into a sample mesh pack (Shiraimatsu & Co. Ltd., Osaka, Japan). They were treated with a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris buffer containing 0.05% H_2O_2 for 10 minutes at room temperature, thoroughly washed in 0.05 M Tris buffer, post-fixed in 1% osmium tetroxide in 0.1 M PBS for 1 hour at 4°C, dehydrated in an

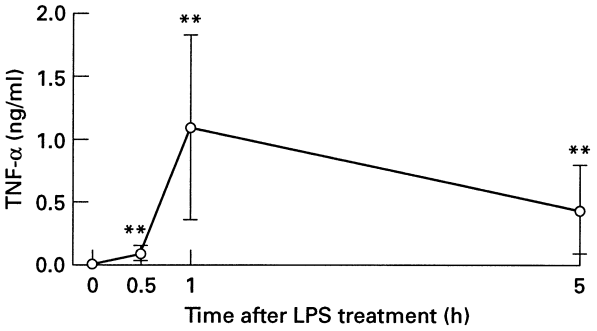


Figure 1. Mean cerebral liquor TNF-α concentrations after treatment with LPS measured with the TNF-α ELISA kit. $^{**}P < 0.01$ (versus 0 hours). Data are expressed as the mean $\pm$ s.d. for 5 rats.

ascending ethyl alcohol series, embedded in Quetol 812, and examined in an electron microscope.

Immunocytochemistry

Antibodies. Hamster anti-murine TNF (α and β) monoclonal antibody (Genzyme Corporation, Cambridge, MA USA) diluted to 1 : 100 in 0.1 M PBS was used. The activity of the antibody was tested by neutralizing the bioactivity of mouse TNF in an L929-cell cytotoxicity assay (Sheehan *et al.* 1989). This antibody is able to totally neutralize 2500–5000 units of mouse TNF bioactivity in the L929 assay and reacts exclusively to both 17-kDa TNF-α and 24.7 kDa TNF-β of mice and rats. It does not exhibit any detectable reactivities to IFN-α, IFN-β and IL-1.

Immunostaining procedures. The streptavidin–biotin technique (Shi *et al.* 1988) was used. Twenty animals were perfused with 0.1 M PBS from the left ventricle, and then with a mixture of 4% paraformaldehyde and 0.5%

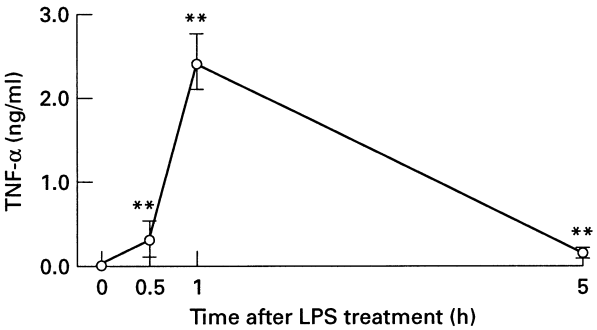


Figure 2. Mean blood TNF-α concentrations after treatment with LPS measured by a use of the TNF-α ELISA kit. $^{**}P < 0.01$ (versus 0 hours). Data are expressed as the mean $\pm$ s.d. for 5 rats.

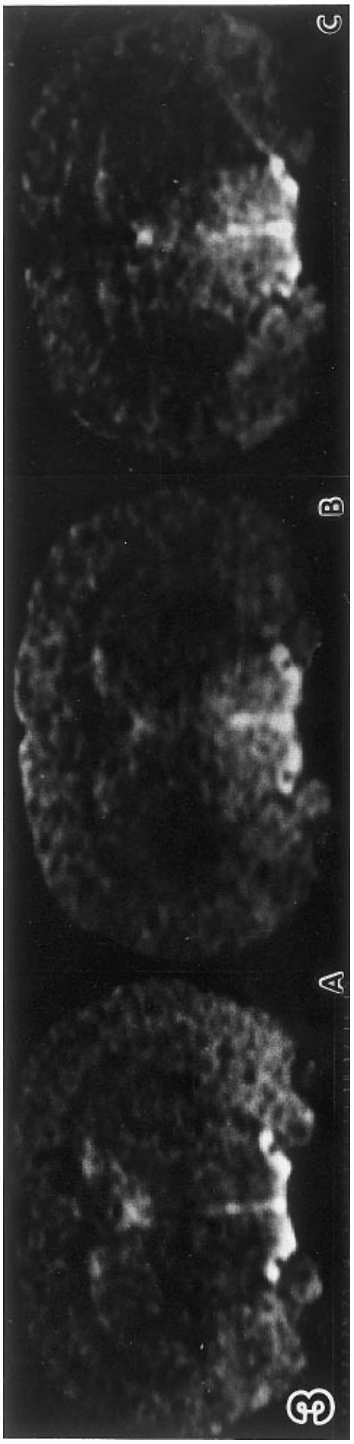


Figure 3. A, Coronal T2-weighted image at the level of the anterior hypothalamus prior to LPS injection. There are no visible signal intensities. B, Coronal T2-weighted image of the same section at 1 hour. The area along the third ventricle characteristically increased signal intensities. C, Coronal T2-weighted image of the same section at 3 hours. The increase of signal intensities became more pronounced when compared to that shown in Fig. 3A.

glutaraldehyde in 0.1 M PBS. The hypothalamus was cut into approximately 2 mm thick slices and post-fixed in the same fixative for 1 hour. Sections of approximately 30 to 40 μm in thickness were made on the microslicer and inserted into the sample mesh pack. The endogenous peroxidase activities were blocked by incubation in a periodic acid solution (Histofine, no. 29271; Nichirei Co. Ltd., Tokyo, Japan) for 45 s. After treatment with normal goat serum at room temperature, sections were incubated overnight with the above-mentioned primary antibody at 4°C. Sections were incubated with a biotinylated secondary multispecies antibody (goat anti-mouse/rabbit immunoglobulin; Histofine, no. 292835; Nichirei Co. Ltd) which was diluted to 1 : 200 with 0.05 M Tris buffer for 1 hour at 37°C, and then with a streptavidin–peroxidase complex (Histofine SAB-PO kits; Nichirei Co. Ltd.) diluted to 1 : 600 with 0.05 M Tris buffer for 1 hour at 37°C. After incubation, sections were treated with DAB in 0.05 M Tris buffer containing 0.05% H_2O_2 at pH 7.6 for 10 minutes at room temperature. They were thoroughly washed in 0.05 M Tris buffer, post-fixed in 1% osmium tetroxide for 1 hour at 4°C, dehydrated in an ascending ethyl alcohol series, embedded in Quetol 812, and examined in the electron microscope.

For controls of each immunostaining, Tris buffer or normal sera was substituted for the primary antibodies.

Results

Figure 1 shows mean TNF concentrations in the cerebral

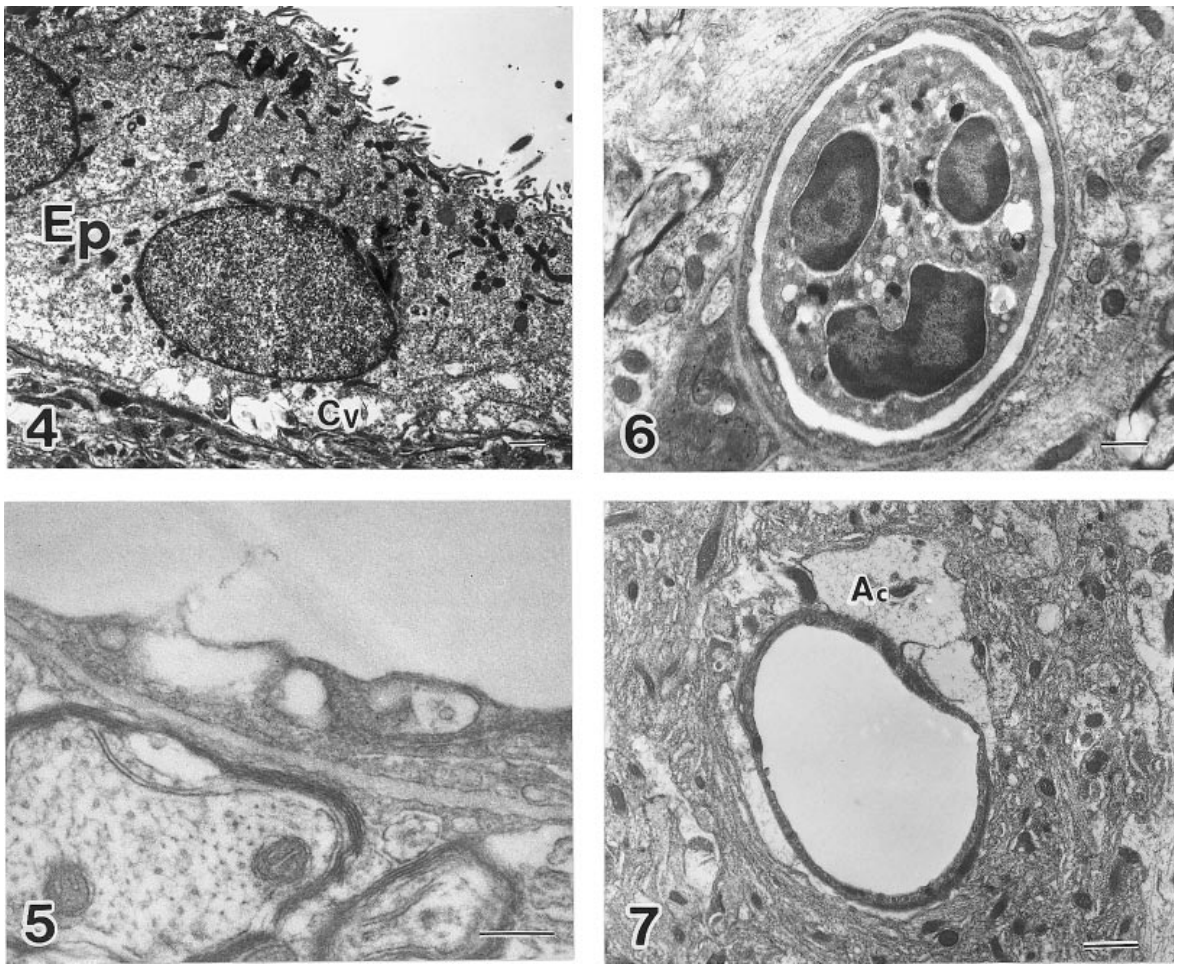
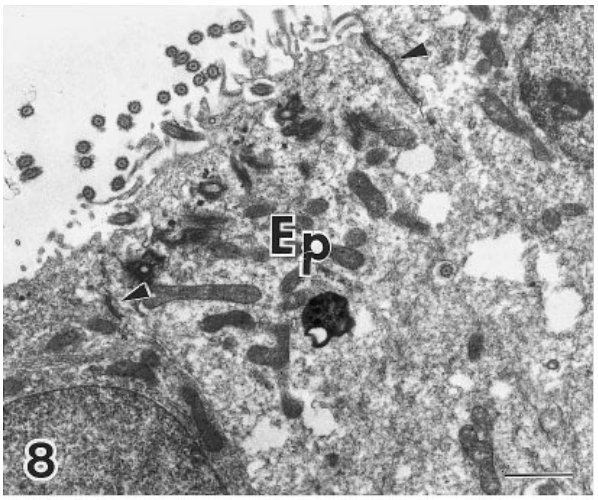


Figure 4. The ventricular ependymal cell layer at 30 minutes after injection of LPS. The oedematous ependymal cells(Ep) have an abundance of intracytoplasmic vacuoles(Cv). Bar 1μm, ×5000. **Figure 5.** Endothelial cells of the capillary contain intracytoplasmic vacuoles at 30 minutes after injection of LPS. Bar 200 nm, ×49000. **Figure 6.** A leucocyte in the lumina capillary at 1 hour after injection of LPS. Bar 500 nm, ×12000. **Figure 7.** The vascular feet of astrocytes(Ac) around the capillary show remarkable oedematous changes at 1 hour after injection of LPS. Bar 1 μm, ×7000.

liquor after administration of LPS. The TNF concentration gradually increased during the first 30 minutes, rose enormously to the maximal level (1.095 ± 0.737 ng/ml) at 1 hour and then decreased to 0.435 ± 0.349 ng/ml at 5 hours. Figure 2 shows mean TNF-α concentrations in the blood sera. As in the case of the TNF concentration in the cerebral liquor, they rose enormously after 30 minutes, reached the maximal level (2.432 ± 0.337 ng/ml) at 1 hour, then decreased to 0.153 ± 0.059 ng/ml at 5 hours.

No visible signal intensities were detected by MRI in the coronal plane of the hypothalamus before LPS administration (Figure 3A). However, a localized area in the hypothalamus along the third ventricle increased signal intensities at 1 hour after LPS injection (Figure 3B). The increase became more pronounced in this area at 3 hours after LPS injection (Figure 3C).



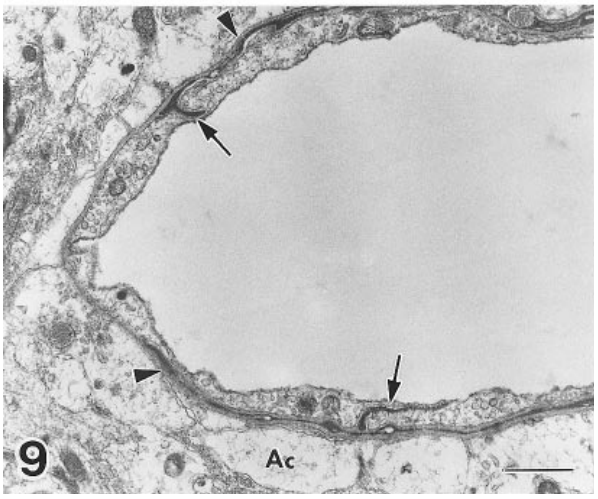


Figure 8. The HRP permeates through the tight junctions (arrowheads) between the ependymal cells (Ep) at 30 minutes after injection of LPS. Unstained. Bar 1μm, $\times$9000. **Figure 9.** The paracellular pathway of the HRP (arrows) through the capillary endothelium at 1 hour after injection of LPS. Electron dense deposits of the tracer exist in the subendothelial layer including the basal lamina (arrowheads). The vascular feet of astrocytes (Ac) show oedematous changes. Unstained. Bar 1 μm, $\times$9900.

At 30 minutes after injection of LPS oedematous changes occurred in the ependymal cells lining the third ventricle. These were evidenced by the presence of an abundance of intracytoplasmic vacuoles in the basal part of the cytoplasm (Figure 4). At the same period, endothelial cells and astrocytes forming the vascular feet around the endothelium in capillaries of the hypothalamus became slightly oedematous including an abundance of cytoplasmic vacuoles (Figure 5). Inflammatory cells such as macrophages and leucocytes were not found in such capillaries. However, inflammatory cells began to appear in the arterioles, capillaries and venules of the hypothalamic area 1 hour after injection of LPS (Figure 6). The oedematous changes of the astrocytes also became more pronounced 1 hour after injection (Figure 7).

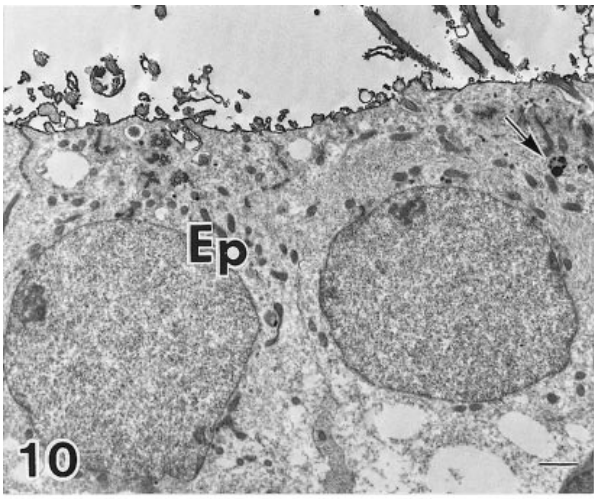
The HRP had permeated throughout the tight junctional areas between the adjacent ependymal cells by 30 minutes after injection of LPS (Figure 8). However, the leakage of the tracer throughout the capillary tight junctions in the hypothalamus was first observed at 1 hour (Figure 9). Electron opaque deposits of the HRP permeated into both the subendothelial space including the basal lamina and the vascular feet of the surrounding oedematous astrocytes. The paracellular permeability of the tracer through the capillary tight junctions was much more enhanced at 5 hours.

At 30 minutes after injecting LPS, immunoreactions of TNF of the ependymal cell layer were preferentially localized along the apical and lateral plasma membrane including the tight junctional areas, in endocytotic vesicles and cytoplasmic vacuoles, and in lysosomes (Figures 10 and 11). The apical plasma membrane of the capillary endothelium and lysosomes in astrocytes forming the vascular feet was also immunoreactive for TNF (Figure 12). These immunoreactivities persisted throughout the stages that were examined. From 1 hour after injection, the extravasation of inflammatory cells through the endothelia of the capillary becomes frequent as described above. Lysosomes of such inflammatory cells, oligodendrocytes and microglia were immunoreactive for TNF.

Discussion

The present experiment revealed a rapid increase in TNF concentrations in the cerebral liquor between 30 minutes

and 1 hour after the LPS injection. Gatti and Bartfai (1993) first demonstrated by the polymerase chain reaction that the hypothalamus is an initial induction site of TNF-α mRNA in the cerebral tissue after an intraperitoneal injection of LPS. Since Tarlow *et al.* (1993) reported that ependymal cells of the choroid plexus begin to produce and release TNF soon after an endotoxin shock, it can be considered that the ependymal cell layer is a production site of TNF which increased in the cerebral liquor measured in our samples. In addition, our immunocytochemical studies revealed immunolocalizations of TNF in the ependymal cell layer including the tight junctions after the injection of LPS. Since Stolpen *et al.* (1986) suggested that TNF induces the deterioration in the molecular arrangement of the tight junctional proteins, the leakage of the HRP throughout the tight junctions of the ependymal cell layer in our experiments may imply a transient increase in the paracellular pathway of the cerebral liquor due to the deterioration of the cerebrospinal fluid–brain barrier (CBB). We consider, at present, that TNF, synthesized in the ependymal cells and released from such cells into the cerebral liquor, permeates throughout the ependymal tight junctions into the hypothalamic areas along the third ventricle. These processes are considered to be the major reason for oedematous changes in the hypothalamus along the third ventricle revealed by MRI. However, we do not exclude the possibility that the transient elevation in TNF concentrations in the blood sera is also related to the deterioration of the BBB and the subsequent hypothalamic oedemas.



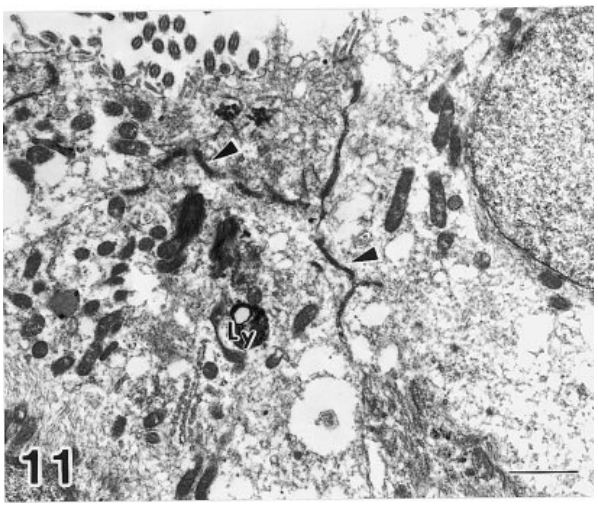


Figure 10. Immunoreactions of TNF along the apical cell membrane and in lysosomes (arrow) of the ependymal cells (Ep) at 30 minutes after injecting LPS. The cells contain an abundance of intracytoplasmic vacuoles. Unstained. Bar 1 μm, $\times$4500. **Figure 11.** Immunoreactions of TNF through the tight junctional areas (arrowheads) of the adjacent ependymal cells layer at 30 minutes after injection of LPS. The immunoreactions also exist in vesicles, vacuoles and lysosomes (Ly). Unstained. Bar 1 μm, $\times$9000.

Our immunoelectron micrographs also demonstrated the immunoreactivities in the pinocytotic vesicles of the ependymal cells. This may mean that TNF in the cerebral liquor is in part taken up by the cells. This process may be a causative factor for earlier oedematous changes in the ependymal cells. Since immunolocalizations of TNF were seen in lysosomes.

The present marker experiment using the HRP demonstrates that an increase in the paracellular permeability through the BBB in the hypothalamic areas is followed by the onset of a remarkable leakage of the tracer through the CBB. Since the deterioration of the BBB occurred prior to the aggregation of the inflammatory cells in the capillary, oedematous changes in the endothelial cells and the vascular feet of the surrounding astrocytes seem mainly to be induced by TNF conveyed from cerebral liquor as described above. This supposition may explain the finding by Mustafa *et al.* (1989) that concentrations of TNF in cerebral liquor significantly increase prior to the appearance of inflammatory cells. According to our immunolectron microscopy, lysosomes of these inflammatory cells were intensely immunoreactive for TNF. Thus TNF, produced and stored in these cells, may also accelerate the cytotoxic changes of the hypothalamic areas as previously described by others (Sprague *et al.* 1989; Claudio *et al.* 1990; Rothstein & Schreiber 1992; Nagano *et al.* 1992; Kita *et al.* 1993).

Gatti and Bartfai (1993) suggested that earlier inductions of TNF in the hypothalamus may contribute to understanding how TNF affects the hypothalamic thermoregulatory centre. Dinarello *et al.* (1986) also suggested

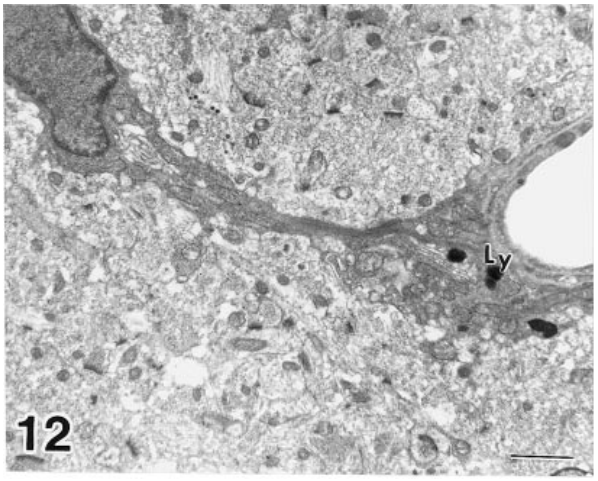


Figure 12. Immunoreactions of TNF in lysosomes (Ly) of an astrocyte forming the vascular feet of an astrocyte at 30 minutes after injecting LPS. Unstained. Bar 1 μm, $\times$7200.

that TNF is one of the endogenous pyrogens which directly affects the hypothalamus. For these reasons, the present data, indicating that TNF induces severe cytotoxic damage in the hypothalamus via cerebral liquor through the ependymal cell layer, give further evidence for the widely accepted concept that this cytokine plays a crucial role in various functions of the hypothalamus.

Acknowledgements

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