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Regional induction of tumor necrosis factor α expression in the mouse brain after systemic lipopolysaccharide administration

(cachectin/*in situ* hybridization/acute-phase response/circumventricular organs/central autonomic regulation)

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ABSTRACT **Tumor necrosis factor α (TNF-α) is a cytokine that is responsible, in part, for several aspects of the acute-phase response to inflammation, including the generation of fever. TNF-α has direct effects on central nervous system neurons deep within the hypothalamus that are involved in producing the febrile response, but the blood–brain barrier prevents circulating TNF-α from having access to these sites. We therefore have hypothesized that TNF-α may be produced in the brain and used as a mediator in the cerebral components of the acute-phase response. We used *in situ* hybridization to determine the distribution of production of TNF-α mRNA in the mouse brain after systemic administration of lipopolysaccharide. During the initial phase of fever, hybridization was observed in perivascular cells and neurons in circumventricular organs, including the vascular organ of the lamina terminalis, median eminence, and area postrema, as well as along the ventral surface of the medulla; hybridization was also prominent over many cell in the meninges. During the late phase of the response, hybridization was observed over neurons in the pericircumventricular nuclei such as the anteroventral periventricular and arcuate nuclei of the hypothalamus and the nucleus of the solitary tract. TNF-α produced by a cascade of neurons within the brain may participate in the complex autonomic, neuroendocrine, metabolic, and behavioral responses to infection and inflammation.**

Tumor necrosis factor α (TNF-α) or cachectin is a cytokine that is responsible for many of the systemic effects of infection including septic shock and for the wasting syndrome cachexia that is often associated with chronic infection and cancer (1). Systemic infusion of TNF-α results in fever, nausea, fatigue, and an elevation of plasma glucocorticoids and catecholamines, all of which are mediated by the central nervous system (CNS) (2–6). Intracerebral infusion of TNF-α increases δ, or slow wave, electroencephalographic activity and peripheral sympathetic nerve activity and alters feeding and macronutrient metabolism in a pattern similar to that observed during systemic infection (7–9). Although circulating TNF-α does not penetrate the blood–brain barrier in biologically significant amounts (10, 11), direct application of TNF-α modulates the firing rate of glucose-responsive and thermosensitive neurons (12) that are located deep in the hypothalamus, suggesting that TNF-α may be synthesized in the brain and used as a mediator in neural pathways involved in the regulation of the acute-phase response. Furthermore, systemic injection of bacterial lipopolysaccharide (LPS), which produces a febrile response, induces an elevation of TNF-α activity in the cerebrospinal fluid (13), which would suggest a locus of synthesis within the blood–brain barrier.

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We have recently demonstrated a discrete population of TNF-α-immunoreactive (IR) neurons and widespread distribution of TNF-α-IR nerve fiber pathways in the normal mouse brain (14). Although the extensive pattern of TNF-α-IR innervation is typical for a neuronally synthesized modulator, it is possible that the TNF-α-IR neurons in the brain may merely take up and store TNF-α, rather than synthesize it. In addition, it is not known whether the TNF-α-IR neurons in the brain play a role in the CNS response to immune challenge.

In this study, we address these issues by examining the expression of TNF-α mRNA in the intact normal mouse brain to determine the possible sources of TNF-α within the CNS and their response to the systemic administration of LPS. To determine the pattern of cells expressing TNF-α, we have used *in situ* hybridization with a complementary RNA probe transcribed from the murine TNF-α cDNA encoding amino acids 1–120 (15). In addition, we have investigated the identity of the mRNA species in the brain that hybridized with this probe by Northern blot analysis, RNase protection assay, and reverse transcriptase–polymerase chain reaction (RT–PCR).

MATERIALS AND METHODS

LPS Treatment. Forty BALB/c mice (Sprague Dawley) were injected intraperitoneally with 50 μg of *Salmonella typhimurium* LPS (lot RIR-305; Ribi Immunochem) in 200 μl of pyrogen-free saline (PFS) and 5 mice were sacrificed at each time point of 1.5, 3, 6, 9, 12, 15, 18, or 24 h after injection. Ten control mice received an injection of PFS.

Probe Preparation. The mouse TNF-α probe was prepared using the PCR to amplify 353 bases of the mouse TNF-α cDNA. The upstream oligonucleotide (5′-CCCAAGCTTAG-ATCATCTTCTCAAAATTCG) was designed with a *Hin*dIII sequence and the downstream oligonucleotide (5′-CCCG-GATCCCAGGTATATGGGCTCATACCA) was designed with a *Bam*HI site to facilitate subcloning into the vector pGEM-3Z(+) (Promega). These markers flank the third intron of the TNF-α gene and, hence, allow the distinction of amplified genomic DNA (which should include the intron) from reverse-transcribed mRNA (which should not and is, therefore, only 353 bp long). Antisense probes were prepared as described (16).

***In Situ* Hybridization.** *In situ* hybridization was performed as described (16). Sections were analyzed by both bright-field and dark-field microscopy; neuroanatomical terminology is as in ref. 14.

Abbreviations: LPS, lipopolysaccharide; OVLT, vascular organ of the lamina terminalis; TNF-α, tumor necrosis factor α; CNS, central nervous system; IR, immunoreactive; TS-M, thioglycolate-stimulated macrophage; RT, reverse transcriptase.

Control Studies. The specificity of *in situ* hybridization was determined by comparing the results of hybridization with antisense and sense probes to adjacent tissue sections. Northern blot analysis was also performed on mouse brain RNA by using the inserts from the same DNA constructs used for *in situ* hybridization.

RNA Isolation. RNA was prepared with standard techniques (17) from whole brain homogenates and polyadenylylated RNA was purified with a Poly(A) Quik kit (Stratagene) from the control and 1.5-, 6- and 18-h time points. Total RNA was isolated from thioglycolate-stimulated macrophages (TS-Ms) from C3H/HeN mice and used as controls in each RNA analysis.

Northern Blot Analysis. Northern blot analysis of polyadenylylated RNA (3.5 μg) from the control, 1.5-h, 6-h, and 18-h time points was performed as described (16).

Ribonuclease Protection Assay. The mouse TNF-α cDNA template used for RNase protection assay was prepared in the same manner as that used for *in situ* hybridization (16). RNase protection assay was performed by the method of Miller *et al.* (18). The TNF-α probe (50,000 cpm) was added to 3.5 μg of polyadenylated RNA from control, 1.5-h, 6-h, and 18-h time points and total TS-Ms. Yeast tRNA was included as an RNase digestion control. To control for equivalent recovery of protected product, an actin RNase protection assay was done using a *Sac* I-linearized plasmid pAct-BB that was transcribed to generate a 154-base RNA probe that protected a 64-nt actin fragment. Gels were fixed, dried, and exposed to autoradiographic film for 30 min (actin and TS-M RNA) or 5 days.

RT–PCR. The cDNA from 200 ng of polyadenylylated RNA derived from whole-brain homogenates from animals at the unstimulated, 1.5-h, 6-h, and 18-h time points or from TS-Ms was prepared by reverse transcription primed with random hexamers using the GeneAmp RNA PCR kit (Perkin–Elmer/Cetus) according to manufacturer's instructions. One complete series of RNA was amplified using *Taq* DNA polymerase without the RT as a control. The cDNA was amplified using the 5′ oligonucleotide encoding nt 404–426 (GAGTGACAAGCCTGTAGCCCAC) and the 3′ oligonucleotide complimentary to nt 687–708 (CTCAGCCCCCT-CAGGGGTGTC) of the mouse TNF-α cDNA. PCR conditions were as follows for 45 cycles; denaturation, 95°C, 1 min; annealing and extension, 65°C, 2 min. After PCR amplification, the reaction mixture was extracted with $CHCl_3$ and 20 μl of each reaction mixture and 500 ng of pGEM DNA markers (Promega) were electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide and an R_f curve was generated using the DNA size markers to determine the size of the PCR product.

RESULTS

***In Situ* Hybridization.** The principle cell types labeled in these hybridization studies were perivascular cells and neurons (Fig. 1). These cells were identified on the basis of the morphology of thionin-stained cell bodies underlying the clusters of silver grains. The somata of perivascular cells were thin and ellipsoid and they seemed to be wrapped around blood vessels. It was not possible with the resolution of our staining technique to identify these cells further, although their distribution and morphology are consistent with perivascular microglia described by Hickey and colleagues (19, 20). Neurons were identified based on their relative size (>10 μm) and the neuronal morphology of thionin-stained cell bodies. No labeling was observed in material hybridized with the sense-strand complementary RNA.

In unstimulated mice, we observed only a small number of clusters of autoradiographic silver grains representing TNF-α mRNA hybridization over perikarya in regions where we have observed TNF-α-like IR neurons (14), including the periventricular preoptic and suprachiasmatic nuclei in the hypothalamus and in the preoptic portion of the bed nucleus of the stria terminalis and the ventrolateral medulla. In comparison, we observed robust hybridization in the brains of mice after systemic LPS administration. At 1.5 h after LPS, intense hybridization was present in the OVLT, a circumventricular organ at the anteroventral tip of the third ventricle (Figs. 1*A* and 2*B*). In the body of the OVLT, hybridization was located over thin ellipsoid-shaped cells that wrapped around the vasculature. Very intense hybridization was also observed over medium-sized (12–15 μm) round neurons in the ventral portion of the OVLT and in the surrounding anteroventral periventricular nucleus. A similar pattern of expression was noted in the median eminence, a circumventricular organ constituting the floor of the third ventricle (Fig. 3*D*). Most of the hybridization was present over perivascular cells in the median eminence, although a few neurons in the internal lamina also expressed TNF-α. Intense hybridization was also observed over neurons and perivascular cells in the area postrema, a circumventricular

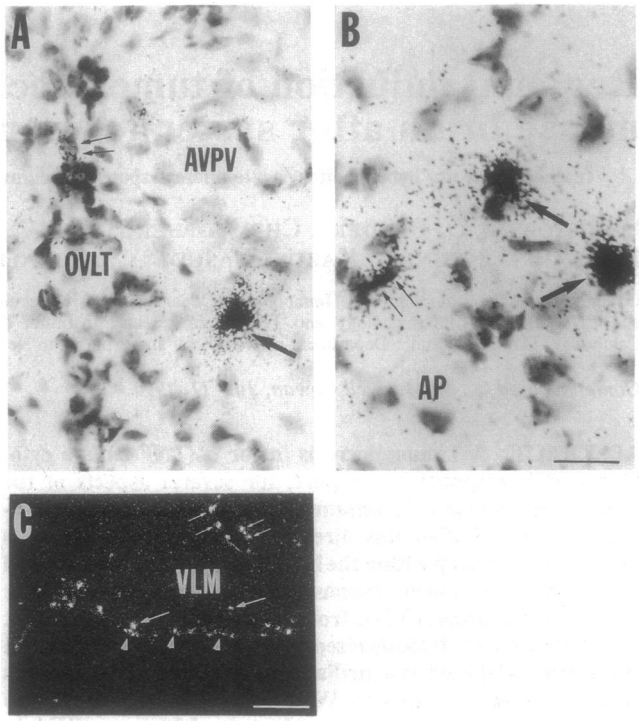

FIG. 1. TNF-α expression in the mouse brain 1.5 h after systemic LPS administration. (*A*) A bright-field photomicrograph of hybridization in the region surrounding the anteroventral tip of the third ventricle. At this time, we observed expression in perivascular cells (small arrows) in circumventricular organs such as the vascular organ of the lamina terminalis (OVLT). We also observed expression by neurons (large arrows) in the OVLT and in the surrounding portions of the anteroventral periventricular nucleus (AVPV) at this time point. (*B*) A bright-field photomicrograph of TNF-α expression in the area postrema (AP), a circumventricular organ at the caudal end of the fourth ventricle. Dense levels of hybridization were observed over neurons (large arrows) and perivascular cells (small arrows) at this time point. (*C*) A dark-field photomicrograph of TNF-α expression in the ventrolateral medulla (VLM). Hybridization over cells in the meninges (arrowheads) was only observed at 1.5 h. Intense clusters of hybridization were often observed over cells associated with larger penetrating vessels in the parenchyma of the brain (small arrows). Neurons (larger arrows) along the ventral surface of the medulla also expressed TNF-α. A few cells from this population were observed at each time point after systemic LPS and in the unstimulated controls. (Bars: *A* and *B*, 30 μm; *C*, 100 μm).

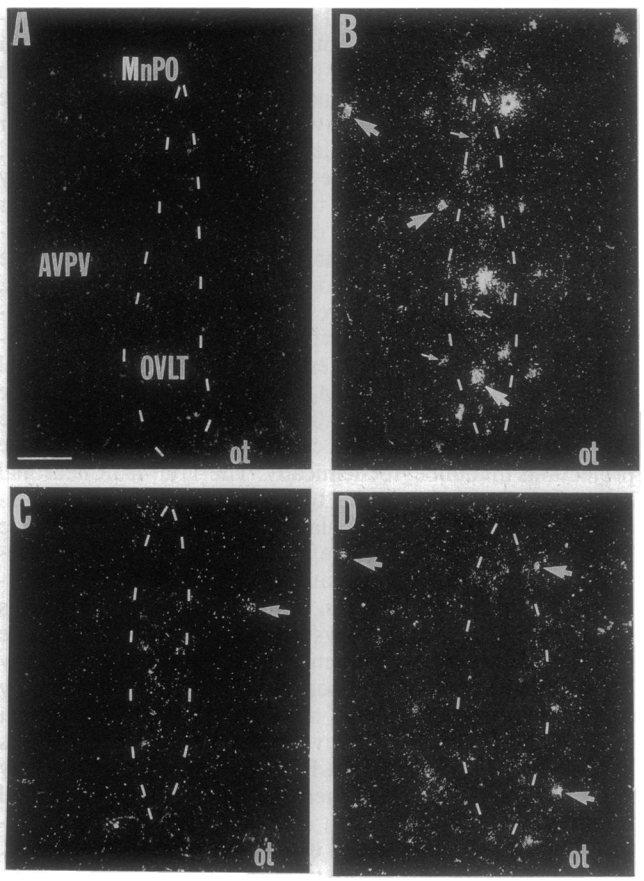

FIG. 2. Effect of systemic administration of endotoxin on the expression of TNF-α in the region surrounding the anteroventral third ventricle shown in dark-field photomicrographs. (*A*) No hybridization was observed in this region in the brains of mice infused with PFS. (*B*) In contrast, intense hybridization was seen over small cells that were associated with the vasculature (small arrows) and over neurons (large arrows) of the OVLT 1.5 h after systemic LPS administration. A few neurons in the anteroventral periventricular nucleus (AVPV) and median preoptic nucleus (MnPO) also expressed TNF-α mRNA at this time point. (*C*) At 6 h after LPS treatment, only a few neurons were labeled in the AVPV. (*D*) A second wave of expression of TNF-α mRNA was seen in neurons in the AVPV and MnPO (arrows) but not in the OVLT at 18 h after systemic LPS. ot, Optic tract. (Bar = 100 μm).

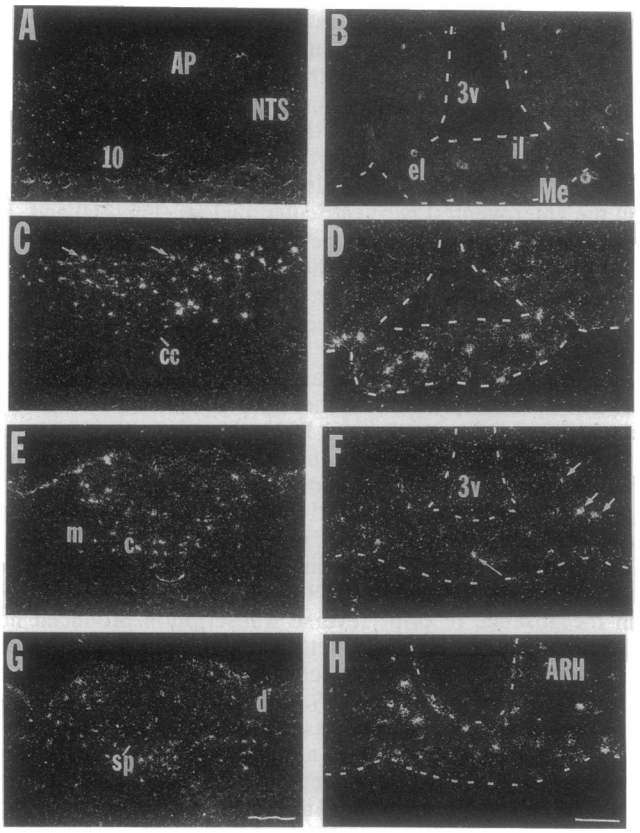

FIG. 3. Effect of systemic LPS on the expression of TNF-α in the dorsal vagal complex (*A*, *C*, *E*, and *G*) and in the mediobasal hypothalamus (*B*, *D*, *F*, and *H*) shown in dark-field photomicrographs. Dashed lines mark the boundaries of the third ventricle (3v) and ventral surface of the diencephalon. (*A* and *B*) No expression was observed in the dorsal vagal complex or mediobasal hypothalamus in the unstimulated mouse. (*C* and *D*) At 1.5 h after LPS, dense clusters of hybridization were observed (*C*) over neurons and perivascular cells in the area postrema (AP) and cells in the meninges (arrows) on the dorsal surface of this circumventricular organ. (*D*) Intense hybridization was also seen over perivascular cells and a few neurons in the internal lamina (il) of the median eminence (Me) at this time point. (*E* and *F*) At 6 h after LPS, a few clusters of silver grains were observed over neurons in the pericircumventricular nuclei. (*E*) In the dorsal vagal complex, hybridization was primarily observed over neurons in the subpostremal zone (sp) and in the commissural (c) and parvicellular subnuclei of the nucleus of the solitary tract. (*F*) In the mediobasal hypothalamus, we observed expression over only a few neurons in the arcuate nucleus of the hypothalamus. (*G* and *H*) At 18 h after LPS, the densest levels of hybridization were observed. (*G*) Dense clusters were noted over neurons in the AP, subpostremal zone, and in the commissural, dorsomedial (d), parvicellular, and medial (m) subnuclei of the nucleus of the solitary tract. A few clusters of silver grains were also observed over neurons in the dorsal motor vagal nucleus. (*H*) Hybridization was observed over neurons in the internal lamina of the median eminence and in the arcuate nucleus of the hypothalamus (ARH). (Bars: *A–D*, 100 μm; *E–H*, 75 μm.) 10, Dorsal motor vagal nucleus; NTS, nucleus of the solitary tract; cc, central canal; el, external lamina of the median eminence.

organ at the caudal end of the fourth ventricle, and over a few neurons in the subpostremal zone and the parvicellular and commissural parts of the nucleus of the solitary tract (Figs. 1*B* and 3*C*). A few neuronal cell bodies along the ventrolateral surface of the medulla demonstrated intense hybridization, as did perivascular cells associated with the penetrating arteries in this region (Fig. 1*C*). TNF-α expression was also observed over cells in the meninges at 1.5 h (Fig. 1*C*) but not at later times. These thin cells were uniformly oriented parallel to the surface of the brain.

By 3 h after LPS, the pattern of TNF-α expression was similar, but the intensity of expression in the circumventricular organs had decreased. At 6 h after LPS, a moderate level of hybridization was observed over neurons in the anteroventral periventricular nucleus (Fig. 2*C*) and internal lamina of the median eminence (Fig. 3*F*). This expression was increased at 9 and 12 h after LPS and small numbers of intensely labeled neurons were also seen in the arcuate nucleus and lateral hypothalamic area. Moderate labeling was also located over neurons in the commissural, medial, and parvicellular subnuclei of the nucleus of the solitary tract but labeling was relatively sparse in the area postrema 6 h after LPS (Fig. 3*E*). The density of hybridization in the regions adjacent to the area postrema increased progressively at 9 and 12 h after LPs, whereas the hybridization signal from the area postrema had decreased further by the end of this period.

The most intense TNF-α expression in the mouse brain was noted 15 and 18 h after systemic LPS. Dense silver grain clusters were seen over neurons in the arcuate nucleus of the hypothalamus and in the internal lamina of the median eminence (Fig. 3*H*). In two of the five mice at 18 h, this group of labeled neurons extended laterally into the dorsomedial portion of the ventromedial nucleus of the hypothalamus. Nu-

merous clusters of silver grains were observed over neurons in the area postrema and the parvicellular, dorsomedial, commissural, and medial parts of the nucleus of the solitary tract. A few clusters were seen over neurons in the ventrolateral subnucleus of the nucleus of the solitary tract, dorsal motor vagal nucleus, and ventral portion of the hypoglossal nucleus (Fig. 3G). Hybridization was also observed over a few neurons in the anteroventral periventricular nucleus (Fig. 2D) and, in two cases out of five, over the OVLT at this time point.

RNA Analysis. Several methods were used to characterize the hybridized transcript in the mouse brain. We were unable to detect a cross-reactive species in Northern blot analysis using polyadenylylated RNA from the brains of normal or LPS-stimulated mice, presumably reflecting a low abundance of this mRNA species. We therefore performed a RT–PCR analysis of polyadenylylated RNA from brains of mice at each of the time points used in our study. The PCR product we generated was ≈300 bp long, corresponding to the expected size for the segment of TNF-α mRNA bracketed by our primers. As our primers flanked the third intron of the TNF-α gene, this result eliminated the possibility that we might have cloned genomic DNA. Furthermore, control PCR in the absence of prior reverse transcription produced no DNA bands. The PCR product obtained from brain migrated at the same location on gel electrophoresis as the mRNA band from TS-Ms (Fig. 4B). In addition, to determine whether the brain mRNA was identical to that for macrophage TNF-α, we performed an RNase protection assay. A single protection fragment, identical in size to that identified in TS-M RNA, was visualized in each of the lanes containing brain RNA (Fig. 4A).

DISCUSSION

Our data indicate that there is a cascade of TNF-α expression in the brain during the acute-phase response to infection. During the initial response to LPS, perivascular cells and neurons in the circumventricular organs produce TNF-α.

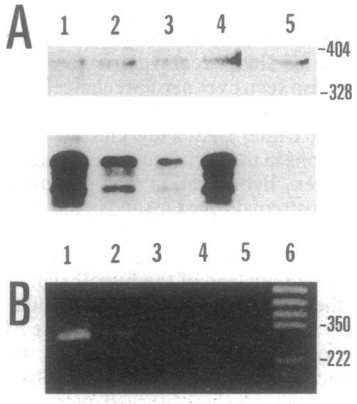

FIG. 4. (*A Upper*) RNase protection assay using the probe with which *in situ* hybridization was performed. Protection fragments from polyadenylylated brain RNA from unstimulated mice (lane 1) and from mice 1.5 h (lane 2), 6 h (lane 3), and 18 h (lane 4) after LPS. Samples were electrophoresed on a 6% polyacrylamide gel and visualized after a 5-day exposure to autoradiographic film. Lane 5 contains the TS-M protection fragment visualized after a 30-min exposure. Numbers on the right side mark the position of DNA size markers in bases. Note that only a single mRNA, identical with macrophage TNF-α mRNA, was observed in the brain. (*A Lower*) Actin RNase reference fragments, a control demonstrating that the protection assay procedure also left the actin mRNA intact. (*B*) RT–PCR analysis of the transcripts amplified from 200 ng of RNA from the brains of mice that were not stimulated (lane 5) and 1.5 h (lane 4), 6 h (lane 3), and 18 h after LPS (lane 2) vs. TS-M RNA (lane 1). DNA size markers in bases are in lane 6. Note that our primers identified in brain a single mRNA that was identical in length to macrophage TNF-α mRNA.

Interestingly, this rapid initial peak of TNF-α mRNA expression follows the same time course as the elevation of TNF-α activity in the plasma after systemic LPS administration in the rat (21). It would be of considerable interest to determine whether these cells are directly responsive to LPS or whether some other intermediate mediator stimulates this expression.

The TNF-α produced in the circumventricular organs may be released and act locally to induce expression of other cytokines or prostaglandins, or it may be transported axonally by neurons with efferent projections to regions of the brain involved in the generation of fever and other endocrine, autonomic, and behavioral responses to infection. We previously identified an extensive population of TNF-α-IR axons in the mouse brain, innervating key sites in the basal forebrain, brainstem, and spinal cord that are involved in the CNS response to immune stimulation (14). However, we found it difficult to stain the cell bodies giving rise to these projections immunocytochemically. The TNF-α-IR neuronal cell bodies that we were able to stain after colchicine administration, in the periventricular hypothalamic and preoptic areas, the bed nucleus of the stria terminalis, and the ventrolateral medulla, demonstrated only modest and inconsistent amounts of hybridization in the current study, and this signal was not amplified by systemic endotoxin. It is possible that the colchicine pretreatment used in our earlier study may have caused TNF-α expression in cells that do not normally do so. Alternatively, these neurons may express TNF-α continuously at a very low level, whereas cells that express TNF-α mRNA briskly after LPS treatment might do so only after immune stimulation. There is precedent for differential regulation of constitutive vs. inducible forms of cyclooxygenase (22), another product of LPS stimulation. However, the two isoforms of cyclooxygenase derive from different genes, whereas our nuclease protection and RT–PCR experiments indicate that there is only one TNF-α mRNA transcript produced in the CNS. Hence, differential control of TNF-α expression might be accomplished by activation of different promoters or by a posttranscriptional form of regulation.

The presence of two distinct populations of CNS neurons expressing TNF-α under different conditions raises the issue of the neurons of origin of the rather extensive TNF-α-IR axonal network in the normal mouse brain. It is possible that many TNF-α-IR terminals may derive from neurons whose cell bodies in the circumventricular organs and adjacent nuclei only contain TNF-α mRNA at detectable levels when stimulated by a systemic immune response. If the resultant TNF-α protein is rapidly exported to the terminals, it may never reach levels in the cell body that are detectable with immunocytochemistry. Hence, the source of TNF-α-IR innervation of different terminal fields in the brain will have to be established experimentally—e.g., by studies combining *in situ* hybridization with retrograde transport.

Although we could visualize *in situ* hybridization signal over meningeal and perivascular elements, we could not establish the cell types involved with any certainty. Hickey *et al.* (19, 20) have described a population of OX-42$^+$ perivascular microglia of similar morphology and localization in the rat brain. It would be of interest to combine *in situ* hybridization and OX-42 immunohistochemistry to determine whether these are identical populations. Our work supports the concept that a system of a meningeal and perivascular cells is capable of responding to inflammation with production of cytokine mediators. However, it is unclear whether the role of these cells is limited to antigen presention during parenchymal injury or whether they are involved in the febrile response.

A second or "late-phase" of TNF-α expression begins about 6 h after LPS administration. TNF-α expression was particularly prominent in neurons of the pericircumventric-

ular nuclei, the neuronal cell groups adjacent to the circumventricular organs. This expression was quite pronounced at 9 h after LPS and appeared to be maximal by 18 h. It is interesting that neurons in these regions may project dendrites beyond the blood–brain barrier into the circumventricular organs (23, 24). Alternatively, cells that express TNF-α in the second wave may receive local projections from neurons in the circumventricular organs that express TNF-α during the initial central response. The possibility that TNF-α may induce cells in the circumventricular and pericircumventricular nuclei to express TNF-α is consistent with the presence of a TNF-α-responsive element in the TNF-α gene (25).

Many studies on the cerebral components of the acute-phase response have focused on those events that occur during the first 3 h after administration of LPS, although several reports have demonstrated marked effects after 6 h or even for days after such treatment. There is typically a second rise of body temperature at about 4 h after systemic injection of LPS (26). In the rat, intravenous LPS causes a steady increase in plasma norepinephrine that persists after 6 h (27). In the rabbit, systemic LPS reduces arterial pressure by 25% between 125 and 500 min after injection (28). Appetitive changes may occur over the course of days after systemic or central administration of TNF-α (29). Interestingly, TNF-α mRNA is expressed at 9–18 h after LPS in the regions of the dorsal vagal complex that receive gastrointestinal visceral afferent innervation (30), closely paralleling the time course of serum hypertriglyceridemia in infected rabbits (31). TNF-α expression by neurons inside the blood–brain barrier during the late phase of the cerebral response to LPS may play an important role in the complex long-term autonomic, metabolic, and appetitive adaptations to infection.

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