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Rapid induction of tumor necrosis factor α in the cerebrospinal fluid after intracerebroventricular injection of lipopolysaccharide revealed by a sensitive capture immuno-PCR assay

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ABSTRACT **Tumor necrosis factor α (TNF-α) is an important mediator in many pathophysiologic processes, both in the central nervous system (CNS) and in the periphery. For this study, we have designed a very sensitive immuno-PCR detection system to investigate the time course of TNF-α induction in the rat cerebrospinal fluid after intracerebroventricular administration of bacterial lipopolysaccharide (LPS). Immuno-PCR combines antibody specificity with PCR signal amplification and provides a sensitivity in the picomolar range. The enhanced sensitivity of this assay allowed the detection of TNF-α in the cerebrospinal fluid as early as 15 min after intracerebroventricular administration of LPS. The present results suggest that the ventricular compartment of the CNS, although confined within the blood–brain barrier, is highly responsive to proinflammatory stimuli such as LPS administration. Insight into the molecular mechanisms underlying this compartmentalization could be key to the pathology and treatment of many CNS diseases, especially the meningitides.**

Tumor necrosis factor α (TNF-α) is a crucial regulatory polypeptide abundantly released by activated cells of the monocytic lineage and, to a lesser extent, by lymphocytes and other cell types (reviewed in ref. 1). Bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria (also known as endotoxin), is the most potent known inducer of TNF-α (1). TNF-α and other cytokines are believed to play a role in the pathogenesis of many diseases affecting the central nervous system (CNS), including human immunodeficiency virus-induced neuropathology, multiple sclerosis, head trauma, Alzheimer disease, bacterial meningitis, and other conditions (2–7). The study of inflammatory events in the ventricular system of the CNS is crucial to the understanding of CNS pathophysiology and is particularly relevant to the pathophysiology of meningitis. Bacterial meningitis is still a leading cause of morbidity and mortality in the United States (8, 9). The recent introduction of a polysaccharide vaccine against *Haemophilus influenzae* type b (Hib), the most common cause of bacterial meningitis in the United States in the 1980s, substantially decreased deaths and hospitalization from Hib meningitis (8, 9). However, the vaccine had little impact on the incidence of bacterial meningitis at large (8, 9). TNF-α has been detected in the cerebrospinal fluid (CSF) of the majority of patients with bacterial meningitis and in some patients with viral forms (10–12). Furthermore, CNS damage in the course of bacterial meningitis correlates with the extent of the inflammatory response in the subarachnoid space (13, 14). These considerations have led to the experimental administration of steroid therapy in human bacterial meningitis in combination with antibiotics (15, 16). Thus a better understanding of the molecular events responsible for inflammatory mechanisms in the ventricular system may contribute to more effective therapeutic approaches to CNS inflammatory and infectious diseases.

The availability of sensitive detection and quantification methods is crucial in the study of potent mediators, such as cytokines, which often function at concentrations at or below the thresholds of detection of currently available assays (17). In the present study, we have applied a modified immuno-PCR (I-PCR) assay to the quantification of TNF-α in the rat CSF after central administration of bacterial LPS. The I-PCR is a modification of the ELISA (18, 19), in which the enzymes used for detection are replaced with a biotinylated reporter DNA bound to the antigen–antibody complex through a streptavidin-protein A fusion protein (20). Antigen quantification is attained through PCR amplification of the reporter DNA (20). We have developed a modified I-PCR, which utilizes an avidin bridge to link a biotinylated antibody to a biotinylated reporter DNA (Fig. 1). A preliminary account of this work has been published in abstract form (21). Similar modifications have been reported independently by others (22, 23).

MATERIALS AND METHODS

Animals and LPS Treatment. Ten Wistar rats (350 g) were chronically implanted with a guide cannula (27 gauge) aimed at the lateral ventricle (stereotaxic coordinates: A, −0.8; L, 1.2; V, 4.6 mm from bregma). After surgery rats were housed three per cage under standard conditions for 2–3 weeks. On the day of the experiment, rats were placed on a stereotaxic apparatus (Kopf) under halothane anesthesia (1.5%). The dorsal skin and muscles of the neck were dissected, and the needle of a Hamilton syringe was gently inserted into the atlanto-occipital foramen, entering the cisterna magna by using a micromanipulator. The needle was kept in position for about 4 hr. A thermostatically controlled heating pad was used to stabilize the rat core temperature. Samples of CSF (70 μl) were collected from the cisterna magna 15 min before and 0, 15, 30, 60, 90, 120, and 180 min after LPS or vehicle administration. LPS (Sigma) was dissolved in water and diluted to a final concentration of 1.5 mg/ml in artificial CSF (130 mM NaCl/3.5 mM KCl/1.25 mM NaH_2PO_4/1.5 mM $MgSO_4$/2.0 mM $CaCl_2$/24 mM $NaHCO_3$/10 mM glucose). By using an injection unit (30 gauge) connected to a 10-μl Hamilton syringe, 7.5 μg of LPS in 5 μl of artificial CSF was injected into the lateral ventricle. Half of the animals received a vehicle injection. Each sample was split into two aliquots and processed for ELISA or I-PCR to compare the two methods. Because some of the samples from two rats were contaminated with blood, results from these animals were discarded.

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Abbreviations: BSA, bovine serum albumin; CSF, cerebrospinal fluid; I-PCR, immuno-PCR; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; CNS, central nervous system.

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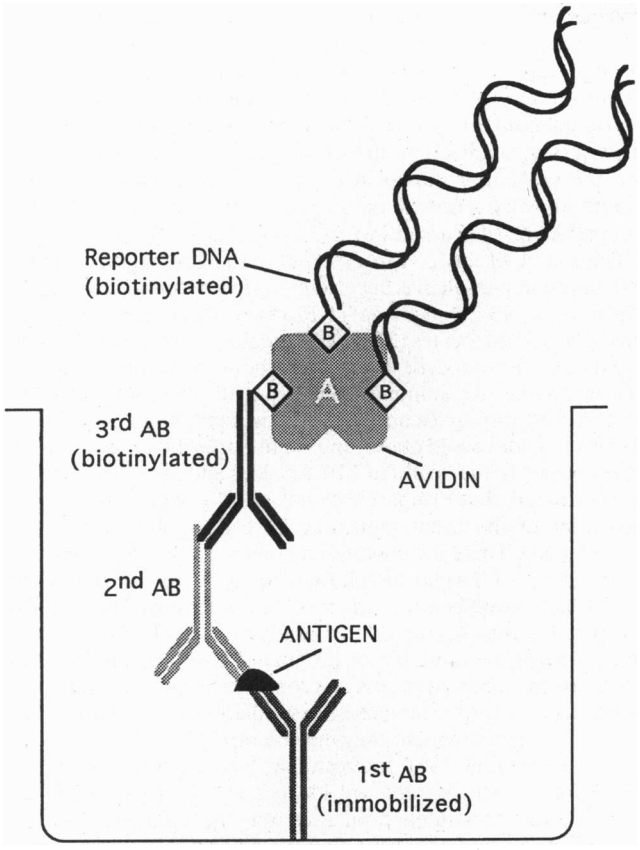

FIG. 1. Schematic representation of the I-PCR approach employed in the study. A mouse monoclonal primary antibody (AB) immobilized on ELISA microtiter plates was used to "capture" the antigen. A "sandwich" is then obtained by incubating with a secondary antibody that selectively binds other epitopes of the same antigen. Finally, a biotinylated tertiary antibody is used to built an avidin bridge linking the antigen–antibody complex to a biotinylated reporter DNA. Avidin has four biotin binding sites and binds biotin with an affinity many orders of magnitude higher than antigen–antibody complexes. The amount of antigen present in the sample is then quantified by PCR amplification of the reporter DNA.

I-PCR. High-binding, flat-bottom ELISA plates (Costar) were coated overnight with a mouse monoclonal anti-TNF-α antibody (Genzyme) in 0.1 M $NaHCO_3$ (pH 8.6). On day two, the plate was blocked with 3% (wt/vol) bovine serum albumin (BSA) in PBS for 2 hr at 37°C. The blocking buffer was discarded, and CSF samples and TNF-α standard curve dilutions were applied to the wells in 1% BSA in PBS and incubated for 2 hr at 37°C. The plates were then washed with PBS containing 0.05% Tween 20. A secondary polyclonal goat anti-TNF-α antibody (Genzyme) was then applied in 1% BSA in PBS and incubated for 1.5 hr at 37°C. After washing with PBS/Tween, a biotinylated polyclonal rabbit anti-goat third antibody was applied for 45 min at room temperature. A monobiotinylated reporter DNA of 324 bp had previously been generated by PCR amplification with a 5′-biotinylated M13 sequencing primer and a nonbiotinylated M13 reverse-sequencing primer of pBlueScript (Stratagene), containing a 110-bp spacer insert. An avidin–biotinylated DNA complex was obtained by incubating a 1:33 dilution of Vector avidin reagent (ABC kit; Vector Laboratories) in 1% BSA in PBS with 200 ng of the reporter DNA for 30 min at room temperature. After washing, the avidin–biotinylated DNA complex was added to the wells and incubated for 30 min at room temperature. The wells were washed repeatedly and drained. Fifty microliters of water was then dispensed per well. The plates were heated to denature the reporter DNA. The supernatants, containing the denatured reporter DNA, were collected, and 5 μl of each was used as a template for PCR with the following parameters: 20 sec at 94°C, 60 sec at 42°C, and 60 sec at 72°C for 25 cycles in standard PCR conditions {10 mM Tris, pH 8.3/1.5 mM $MgCl_2$/50 mM KCl/0.1% (wt/vol) gelatin/200 mM each dNTP, 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim)/12.5 mCi (1 Ci = 37 GBq) of [^{32}P]dCTP/100 ng of each primer} in a 50-μl reaction volume. PCR was carried out using T7 and T3 sequencing primers, which are nested to M13 and M13 reverse, respectively. Ten microliters of the PCR products was then separated on a 5% acrylamide gel in 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3, and exposed to autoradiography film. The bands were cut, and radioactivity was determined with a scintillation β counter. All samples were assayed in duplicate. A three-standard deviation confidence interval defined as the mean values of 24 determinations of CSF samples of control animals was used for comparison with the measurements performed in rats injected with LPS or vehicle. Two-way ANOVA was also used to directly evaluate differences between the two treatment groups, LPS or vehicle.

ELISA. A conventional ELISA assay for TNF-α was carried out with the same steps described for the I-PCR except that the biotinylated polyclonal rabbit anti-goat third antibody was replaced by a horseradish peroxidase-conjugated polyclonal rabbit anti-goat antibody (Genzyme). After washing, a horseradish peroxidase-mediated color reaction was carried out utilizing hydrogen peroxide and the chromogen *o*-phenylenediamine dihydrochloride. The optical density of the samples was determined at 492 nm.

RESULTS AND DISCUSSION

The standard curves obtained with the same TNF-α serial dilution using ELISA and I-PCR are shown in Fig. 2. Under the conditions employed here, I-PCR sensitivity was 6.25 pg/ml (0.625 pg/well), a 16-fold improvement with respect to the very sensitive ELISA run in parallel (100 pg/ml). Interestingly, the two methods shared all the same products and reagents for sandwich antigen capture and recognition but differed only at the step of the tertiary antibody (horseradish peroxidase-conjugated rabbit anti-goat for the ELISA and biotinylated rabbit anti-goat for the I-PCR). Therefore, the

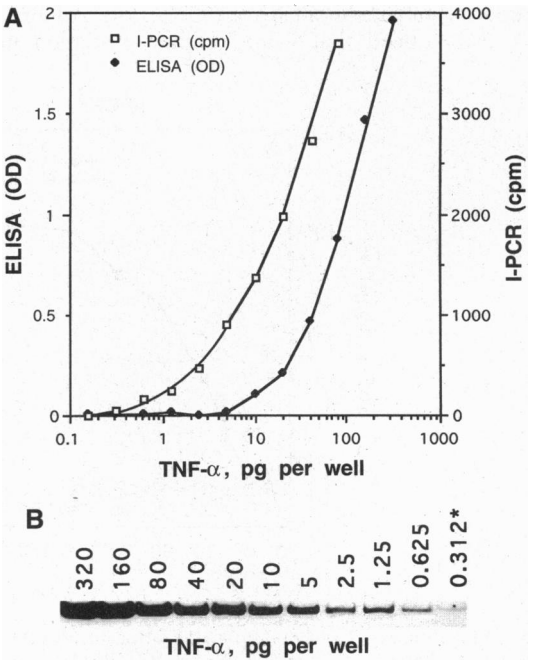

FIG. 2. (*A*) Comparison of I-PCR and ELISA for the detection of TNF-α (standard curves). Under the conditions employed, I-PCR sensitivity was 0.625 pg/well (6.25 pg/ml) and the sensitivity of this ELISA was 100 pg/ml. (*B*) Example of autoradiogram of I-PCR standard curve (*, background level).

difference in sensitivity between the two methods is ascribable to the use of PCR amplification in the I-PCR rather than the chromogenic enzymatic reaction used in the ELISA.

CSF samples were collected from the cisterna magna of anesthetized rats after LPS administration into the lateral ventricle. With this sampling design, TNF-α measurements were not biased by the local effect of LPS and are likely to represent the entire ventricular compartment after stimulation. The samples were divided in two aliquots so that the same samples could be assayed with each of the two methods. The effects of LPS injection on TNF-α levels in the CSF are shown in Fig. 3. The enhanced sensitivity of I-PCR allowed us to detect TNF-α elevations in the CSF by 15 min after intracerebroventricular administration of LPS ($P < 0.05$), whereas the ELISA, on the same serial CSF samples, revealed no detectable TNF-α elevations until 90 min after LPS injection (Fig. 3).

No previous description of the time course of TNF-α induction in the rat CSF after LPS injection could be found in the literature. Our data suggest that the ventricular compartment of the rat CNS responds very rapidly to LPS administration. The kinetics of TNF-α induction in the rat CSF observed in this study are reminiscent of serum TNF-α induction after i.v. injections of LPS (24) and contrast with the relative anergy of the brain parenchyma to the action of LPS (25). Although the ventricular system and the brain parenchyma are both confined within the blood–brain barrier, morphologic observations suggest these compartments are immunologically independent (25, 26). Thus, the recruitment and activation into the brain parenchyma of cells of microglial morphology, morphologically characterized by their shorter cell processes, and the expression of the F4/80 surface marker takes place over days and only with extremely high doses of LPS (25, 26). The microglia are relatively down-regulated macrophages with respect to endocytic and secretory functions (27, 28). In contrast, cytokine and LPS doses too low to induce morphologic changes in the brain parenchyma nevertheless provoke very rapid and vigorous recruitment of polymorphonuclear cells and monocytes in the ventricular compartment (CSF leukocytosis), as well as inflammation of the choroid plexus and the subarachnoid space (25, 26, 29). Andersson *et al.* (26) also noticed that while cellular exudation in the parenchyma tends to be limited and delayed, margination in CNS blood vessels occurs quite rapidly. Taken together, these findings support the notion that while there is a tight control on inflammatory responses in the brain parenchyma, a less restricted control governs the cerebroventricular compartment. The specialized nature of the parenchymal endothelium and the molecular milieu in the parenchyma itself may contribute to such control on inflammatory responses in this compartment (discussed in refs. 25–28, and 30).

The source of the TNF-α detected in the CSF within minutes in the present paradigm is uncertain. *In vitro* observations suggest that in the CNS parenchyma TNF-α is synthesized primarily by microglia and astrocytes (31–33). Microglia, the resident CNS cell population of monocytic lineage, are believed to play a pivotal role in linking the immune system and the nervous system in concert with astrocytes and the endothelium (7, 25, 27, 28, 31). However, as discussed above, the brain parenchyma is relatively unresponsive to the action of LPS (25, 26), and therefore it could be speculated that nonparenchymal cellular elements may be important in the rapid induction of TNF-α observed in the present study. These include resident epiplexus macrophages, the macrophages of the choroid plexus stroma, as well as ependymal cells [which have been recently reported to express TNF-α (34)] and possibly the choroid epithelium [which has been shown to play a role in the initiation of inflammatory events in the CNS (35)]. Stromal choroid plexus macrophages display morphologic and surface receptor characteristics similar to tissue macrophages elsewhere, suggesting that they may be rapidly reactive to proinflammatory stimuli (36). Interestingly, because macrophages in the choroid plexus stroma are located at the crucial interface between the fenestrated capillaries and the epithelium of the choroid plexus and are regularly arrayed similarly to the Langerhans cells in the skin, these cells have been proposed to be functional equivalents of Langerhans cells in eliciting inflammatory responses (35, 36). Cells in the myelomonocytic exudate may contribute to the amplification of the TNF-α response, although it is unlikely that they can initiate the response, since their own recruitment is attributed to the induction of TNF-α and other cytokines in the ventricular system (29).

From a technical point of view, the present results also demonstrate that I-PCR methodology is a feasible means to detect and quantify antigens present at very low concentra-

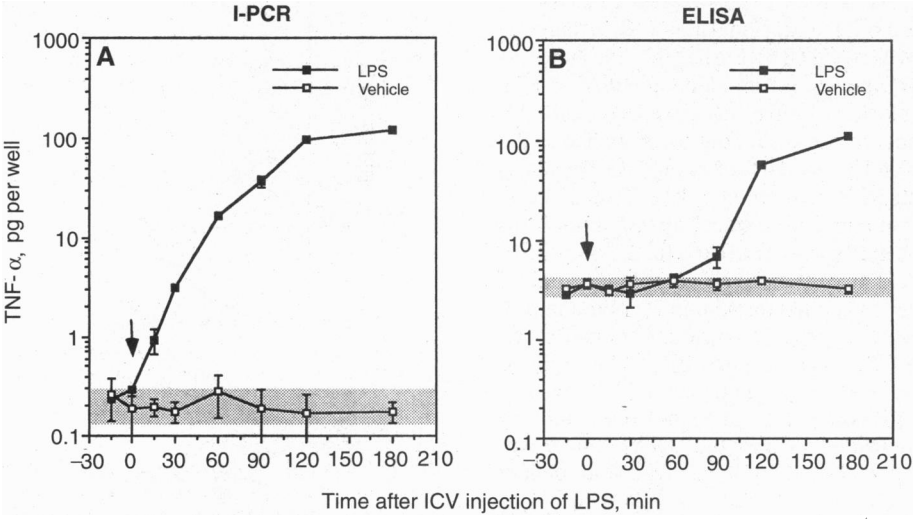

FIG. 3. Induction of TNF-α in the rat CSF after intracerebroventricular injection of LPS. (*A*) I-PCR was used to measure TNF-α levels in the CNS after central administration of LPS (arrow). (*B*) Comparison of I-PCR and ELISA for the detection of TNF-α in the rat CSF after central administration of LPS (arrow). The enhanced sensitivity of I-PCR allowed the detection of TNF-α in the CSF at 15 min postinjection, whereas with the ELISA a significant elevation of TNF-α was detected at 90 min. Data are represented as mean ± SEM. A three-standard deviation confidence interval defined as the mean values of 24 determinations of CSF samples of control animals was used for comparison with the measurements performed in rats injected with LPS or vehicle (shaded areas). Two-way ANOVA was also used to directly evaluate differences between the two treatment groups, LPS or vehicle.

tions such as cytokines and, presumably, other neuroactive peptides in the CSF. TNF-α levels are most often measured by bioassays based on the cytoxic action of TNF-α on the cell line L929 or other cell lines (17, 37, 38). The sensitivity of TNF-α bioassays has been reported to be around 125 pg/ml in the presence of actinomycin D, but the detection threshold can vary considerably across different sublines of L929 (37, 38). A clone of the fibrosarcoma cell line WEHI 164 has been claimed by some authors to be more sensitive to the action of TNF-α than L929 cells and to allow for higher sensitivity when employed in TNF-α bioassays (39). However, others have reported that this subclone displays progressively lower sensitivity to TNF-α with serial passage, resulting in both diminished sensitivity and reduced reproducibility of the bioassay (17). Bioassays may also be covertly affected by synergistic actions of other cytokines, such as interferon γ (38). Therefore, immunological detection methods such as those used in the present study have the potential to provide higher specificity and reproducibility. I-PCR may be utilized when ELISA sensitivity is insufficient, such as the experimental situation described in this report, and for the measurement of TNF-α and other peptides present at very low concentrations in the CSF. In particular, this method could find practical application in the detection of peptides with intracranial microdialysis (40). In addition, because of the flexibility of PCR-based detection and quantification, the sensitivity of I-PCR can potentially be made considerably higher to meet the needs of other experimental paradigms.

In the present paper, we have adopted the recently developed I-PCR approach to demonstrate that TNF-α accumulates rapidly in the rat CSF after intracerebroventricular injection of LPS. CSF samples were collected from the cisterna magna of anesthetized rats after LPS administration into the lateral ventricle. This rapid response was not detected using a very sensitive capture ELISA run in parallel. These results, in concert with recent morphological evidence (25, 26), support the hypothesis that the ventricular system and the brain parenchyma are distinct immunological compartments and that the ventricular compartment is highly responsive to the proinflammatory action of LPS. The study of inflammatory events in the ventricular system of the CNS is relevant to the understanding of many pathologic processes in the CNS and especially the meningitides (13, 34). Host inflammatory responses are believed to contribute significantly to CNS damage in the course of bacterial meningitis (13–16). Presumably, future studies will define the molecular basis of the different characteristics of the ventricular, vascular, and parenchymal compartments of the CNS and their contributions to brain pathology.

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