Not All Peripheral Immune Stimuli That Activate the HPA Axis Induce Proinflammatory Cytokine Gene Expression in the Hypothalamus

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ABSTRACT: **Administration of low doses of lipopolysaccharide (LPS) that do not disrupt the blood–brain barrier (BBB) results in the expression of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNFα) in the hypothalamus in parallel to stimulation of the hypothalamus–pituitary–adrenal (HPA) axis. This endocrine response is triggered by peripheral cytokines, and we recently obtained evidence that brain-borne IL-1 contributes to its maintenance. LPS preferentially stimulates cells of the macrophage lineage and B lymphocytes. The possibility that primarily stimulation of other types of peripheral immune cells also results in the expression of proinflammatory cytokines in the brain and in the activation of the HPA axis was investigated. Our results showed that, in contrast to LPS, administration of the superantigen staphylococcal enterotoxin B (SEB), which stimulates T cells by binding to appropriate Vβ domains of the T-cell receptor, did not result in induction of IL-1β, IL-6, and TNFα expression in the hypothalamus. Furthermore, although IL-2 transcripts in the spleen were highly increased, expression of this gene was not detected in the brain. However, as with LPS, SEB administration also results in elevated levels of glucocorticoids in blood. Therefore, our data suggest that increased expression of proinflammatory cytokines in the brain is not a necessary step in the stimulation of the HPA axis by SEB.**

INTRODUCTION

Microglial cells, astrocytes, and certain neurons can produce cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNFα).[1] It was initially thought that these cytokines are only induced during inflammatory, infectious, and autoimmune processes in association with a massive entrance of immune cells into the brain. It was later shown that peripheral administration of the bacterial endotoxin lipopolysaccharide (LPS)[2,3] and immobilization stress[4] also trigger the

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expression of cytokines in the CNS. This effect may be due to disruption of the blood-brain barrier (BBB) by LPS,[5] to nonspecific mechanisms such as cardiovascular and respiratory dysfunctions caused by the endotoxin, or to the procedure used to induce stress. However, we have observed that peripheral administration of a low dose of LPS that does not disrupt the BBB[5] and does not cause overt alterations in animals also results in cytokine induction in the CNS, suggesting that stimulation of certain types of peripheral immune cells triggered this effect.[6]

LPS preferentially affects cells of the macrophage lineage and B lymphocytes and induces the production of cytokines that can stimulate the hypothalamus–pituitary–adrenal (HPA) axis. We have now performed similar studies to analyze the possibility that stimulation of other types of peripheral immune cells also results in the expression of proinflammatory cytokines in the CNS in parallel to the activation of the HPA axis. For this purpose, we compared the effect of staphylococcal enterotoxin B (SEB), a superantigen that stimulates a large proportion of T lymphocytes expressing appropriate Vb domains,[7] with that of a low dose of LPS.

MATERIAL AND METHODS

Animals

BALB/c and C57Bl/6J male mice (nine weeks old) were obtained from Harlan-Winkelmann, Germany. Animals were housed individually for 1 week before experiments were started and kept single-caged throughout. Animals were housed in temperature- and light (12-hour cycles)-controlled rooms and were fed *ad libitum.*

Reagents

SEB from *Staphylococcus aureus* was obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, and LPS from *Escherichia coli* 0111:B4 from Difco Laboratories, Detroit, MI, USA.

Experimental Procedure

SEB (10 mg/mouse) was injected i.p. into BALB/c mice. LPS (0.5 μg/mouse) was injected into C57Bl/6J mice. Physiological saline solution (0.9% NaCl) was simultaneously used in both cases to inject control animals. Groups of mice were killed by cervical dislocation two and four hours later, and blood was collected in EDTA-coated tubes. The spleen and hypothalamus were dissected, immediately frozen in liquid nitrogen, and used for determination of mRNA cytokine expression by right RT-PCR. To block IL-1 receptors in the brain, 50 mg of the IL-1 receptor antagonist (IL-1ra) were injected i.c.v. 30 minutes before LPS administration.

Corticosterone Determinations

Corticosterone plasma levels were determined by radioimmunoassay as previously described.[8]

Cytokine Determinations

IL-1β, IL-2, IL-6, TNFα, IL-4, and interferon-gamma (IFNγ) gene expression was determined by semiquantitative RT-PCR as previously described.[6] The values from SEB probes were obtained from nonradioactive RT-PCR. Data acquisition and integration were performed with a BP-MI Charged Couple Device camera using ONE-Dscan (Scananalytics, Billerica, MA, USA) on 1.5% ethydium bromide-stained agarose gels.

Statistical Analysis

Results are expressed as mean ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's exact test for multiple comparisons. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Both LPS and SEB have been used as models of septic shock induced by gram-negative and gram-positive bacteria, respectively. However, for our studies, it was necessary to use doses of these bacterial products that do not cause overt disease, as indicated by the fact that the animals do not lose weight for several days after inoculation. Doses of 10 µg/mouse of SEB and 0.5 µg/mouse of LPS into normal mice fulfill this condition. It was previously reported that the HPA axis is stimulated by administration of a relatively large dose of SEB.[9] Thus, we then established that i.p. administration of SEB at a dose of 10 mg/mouse caused an increase in corticosterone blood levels comparable to those of 0.5 µg of LPS (see FIGURE 1).

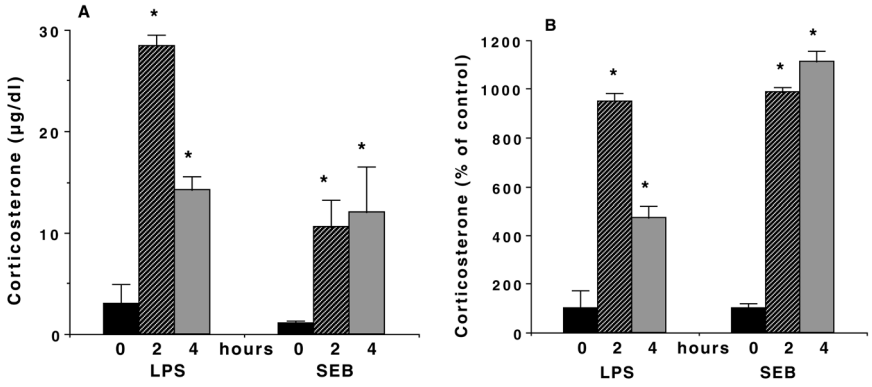

FIGURE 1. LPS and SEB administration results in increased corticosterone blood levels. LPS (0.5 µg) or SEB (10 µg) was injected i.p. into C57Bl/6J and BALB/c mice, respectively. Physiological saline (0.9% NaCl) was used as control in both cases. Groups of animals were killed after 2 and 4 hours. (**A**) Results of corticosterone determinations in plasma. Values obtained for simultaneous controls were pooled and are shown as time 0. For comparison, results are shown in **B** as % of the corresponding controls. Results are expressed as mean ± SE. *$p < 0.05$.

The effect of administration of the aforementioned doses of LPS and SEB on gene expression of different cytokines in the spleen was evaluated. Two hours after LPS administration, IL-1β, IL-6, and TNFα were highly expressed in the spleen. The ratio cDNA/c.f. was: for IL-1β: 0.8 ± 0.2 in controls and 28 ± 3 in LPS-treated mice; for IL-6: 0.1 in controls and 62 ± 12 in LPS-treated mice; for TNFα: 0.5 ± 0.1 in controls and 22 ± 1 in LPS-treated mice. Among the cytokines studied, IL-2, IL-4, and IFNγ showed the highest inducibility in the spleen two hours after SEB administration. The ratio cDNA/c.f. was: for IL-2: 1 ± 0.1 in controls and 23 ± 6 in SEB-treated mice; for IL-4: 0.5 ± 0.2 in controls and 10 ± 3 in SEB-treated mice; for IFNγ: 0.80 ± 0.12 in controls and 9 ± 3 in SEB-treated mice. When compared to the effect of LPS, IL-1β gene expression was only modestly increased two hours following SEB administration (controls: 0.4 ± 0.1; SEB-treated mice: 2.5 ± 0.1).

In the hypothalamus, LPS administration caused a clear increase in IL-1 and IL-6 and, at a lower level, also in TNFα mRNA (see FIGURE 2). By contrast, no changes in expression of the genes for these cytokines were detected two and four hours after SEB administration. Because SEB induced a quick expression of IL-2 in the spleen, we explored the possibility that this superantigen causes a similar effect in the brain. However, in neither control nor SEB-treated mice were mRNA transcripts for this cytokine detected in the hypothalamus.

The semiquantitative RT-PCR used in our experiments is a very sensitive technique with a limit of sensitivity of roughly 10 transcripts/100 cells.[6] Using this technique, we expected to find the IL-2 gene expressed in the brain of SEB-treated animals because IL-2 is produced during the process of activation of T cells and evidence exists that activated T cells can penetrate the brain parenchyma. Indeed, injection of *in vitro* stimulated T lymphocytes to normal animals results in the detection of these cells in the CNS a few hours later.[10,11] Based on these data and on evidence that superantigens stimulate a large proportion of T lymphocytes,[7] some of the cells stimulated *in vivo* by SEB should have crossed the BBB. If they would continue to produce IL-2, the expression of this cytokine should have been detected with the semiquantitative RT-PCR technique used. This apparent contradiction could be explained if it is considered that IL-2 production following SEB stimulation is transient and therefore that expression of this cytokine may not be an appropriate marker for the penetration of SEB-activated T cells in the CNS.

Another series of experiments was designed to study the contribution of IL-1 expressed in the brain for the stimulation of the HPA axis. Mice received LPS injected i.p. and IL-1 receptor antagonist (IL-1ra) or the vehicle alone, injected i.c.v. Two hours after injection, corticosterone blood levels were comparably increased in both groups of LPS-treated mice (time 0: 2.3 ± 0.8 μg/dl; 2 hours: 12.2 ± 0.8 in vehicle-injected and 11.8 ± 1.0 mg/dl in IL-1ra–injected mice). However, four hours after LPS administration, the levels of corticosterone of animals treated with the cytokine receptor antagonist were significantly lower than those of mice that received the vehicle alone (LPS + vehicle: 13.8 ± 1.0; LPS + IL-1ra: 8.2 ± 0.9; $p < 0.01$). These results suggest that IL-1 endogenously produced in the CNS contributes to maintain the stimulation of the HPA axis following LPS administration; although stimulation of this axis still occurred when IL-1ra was administered i.c.v., it was significantly reduced at a later stage. Since this effect coincides temporally with IL-1 gene expression in the hypothalamus, the data obtained indicate a role of brain-borne IL-1 in the

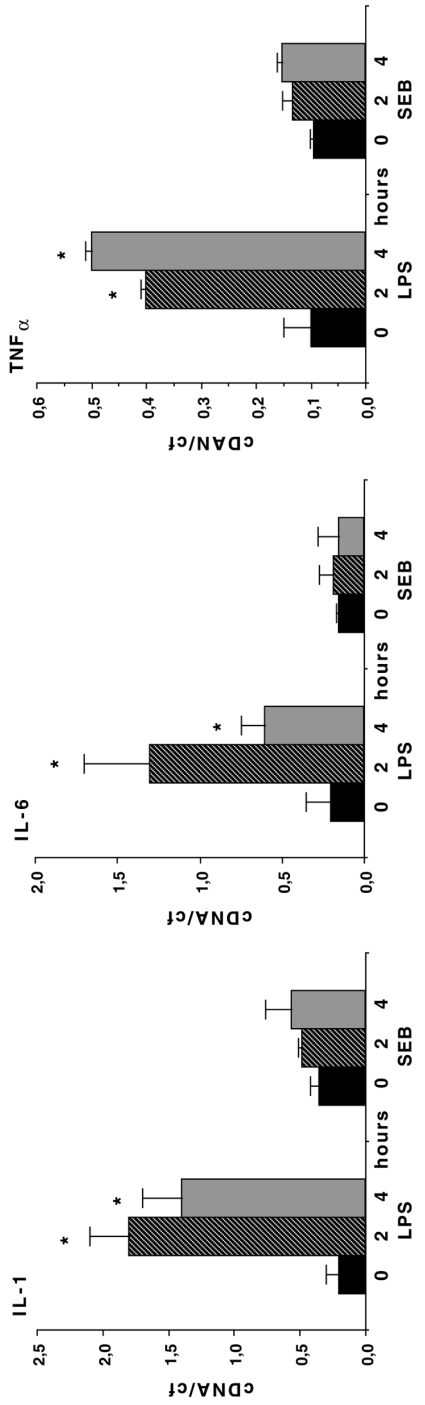

FIGURE 2. LPS, but not SEB, induces increased IL-1β, IL-6, and TNF-α gene expression in the hypothalamus. The hypothalamus of the animals from which the results shown in FIGURE 1 were derived were used to determine cytokine gene expression by semiquantitative RT-PCR. Results are expressed as mean ± SE of the ratio cDNA and the corresponding competitive fragment (cf) for each cytokine. * $p < 0.05$.

maintenance of stimulation of the HPA axis. As shown in FIGURE 1, SEB triggered the stimulation of the HPA axis but, in contrast to the spleen, no induction of IL-1 and of other proinflammatory cytokines was detected in the CNS. This indicates that a mechanism different from the one that maintains the response of the HPA axis to LPS contributes to the sustained increase in corticosterone blood levels induced by SEB. This mechanism seems not to involve centrally produced IL-2, because mRNA transcripts for this cytokine were not detectable in the hypothalamus.

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