The proinflammatory cytokine network: interactions in the CNS and blood of rhesus monkeys

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Reyes, Teresa M., and Christopher L. Coe. The proinflammatory cytokine network: interactions in the CNS and blood of rhesus monkeys. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R139–R144, 1998.—Proinflammatory cytokines [interleukin (IL)-1 and -6 and tumor necrosis factor-α] function within a complex network, stimulating the release of one another, as well as other cytokine agonists and antagonists. These interactions have not been as widely studied in vivo. Therefore, the following studies measured cytokines in blood and cerebrospinal fluid (CSF) from juvenile rhesus monkeys after intravenous administration of cytokines. IL-1α and IL-1β were equally effective in elevating blood levels of IL-6. In contrast, IL-1β was the only cytokine that significantly elevated IL-6 levels in the CSF. Interestingly, both IL-1 and IL-6 increased levels of IL-1 receptor antagonist in the blood and comparably stimulated the release of cortisol. A second study confirmed that the IL-1-induced IL-6 in CSF was brain derived and not a result of diffusion from blood. This research extends studies of the cytokine cascade to the central nervous system (CNS), highlighting the brain response to peripheral activation.

interleukin-1; interleukin-6; cerebrospinal fluid

Cytokine action is complex, involving autocrine self-augmentation and the production and release of related cytokines. This cytokine cascade is a well-known phenomenon within the periphery; for example, during endotoxic challenge there are several sequential waves of cytokines, with tumor necrosis factor-α (TNF-α) appearing first, followed by interleukin (IL)-1 and finally IL-6 (8). In addition to stimulating the release of related cytokines, some cytokines self-regulate through the release of endogenous antagonists [i.e., IL-1 initiates secretion of its own antagonist, IL-1 receptor antagonist (IL-1ra) (21)] and endogenous agonists [i.e., IL-1 fosters release of both IL-6 and its agonist, soluble IL-6 receptor (sIL-6R) (32)]. Recently, cytokines have also been found to act on and be produced within the central nervous system (CNS).

The CNS is often excluded from discussions of immune activation in the periphery. Recent research has changed this perspective by demonstrating that cytokines have numerous CNS effects. One widely studied pyrogenic cytokine, IL-1, mediates certain aspects of sickness behavior, including decreased locomotion (26), decreased food and water intake (27), and increased rapid eye movement sleep (31). IL-1 also affects the acquisition of a cognitive task (2) and can disrupt spatial learning (17). These effects may be mediated by the actions of IL-1 on several neurotransmitter systems, especially the monoamines (36) and γ-aminobutyric acid (5).

However, the means by which peripheral IL-1 is able to exert an influence on the CNS are still controversial. Numerous pathways have been proposed and can be categorized as 1) neural routes or 2) blood-borne processes. The neural hypothesis focuses on the role of vagal afferents (39) communicating information about peripheral immune activation to the brain stem (12). The second category addresses ways through which blood-borne cytokines could gain access to the CNS. IL-1 is a large (17 kDa), hydrophilic protein and is therefore unlikely to cross the blood-brain barrier (BBB) by passive diffusion. Alternative entry pathways have been proposed, including entry at areas where the BBB is “leaky” (e.g., the circumventricular organs) (7) or an active transport system (3). Another important possibility is that IL-1 does not need to gain entry into the CNS. Blood-borne cytokines may bind to endothelial cells on the blood side of the BBB, inducing release of second messengers into the CNS. Prostaglandins are thought to be important in mediating some CNS effects of IL-1, although recent research has found that rat brain microvessels do not release prostaglandins in response to IL-1 (6). Alternatively, cytokines released at the BBB may prove to be important second messengers. Brain endothelial cells both express cytokine receptors (38) and can release cytokines on activation (13), making this an important focus for neuroimmune investigation.

Previously, we have shown that peripheral administration of IL-1β leads to release of IL-6 into the cerebrospinal fluid (CSF) (32). The following studies extended our research on the cytokine cascade within the CNS and were unique for two reasons. First, the assessments were completed in vivo. Immune responses and in turn cytokine action can be impacted by other physiological systems, including both endocrine (10) and neural influences (15). In vivo assessments allow the evaluation of aggregate interactions among systems. The second strength was that this research was conducted in nonhuman primates. Given the extensive species differences in cytokine biology already identified [e.g., expression of the IL-1R in the CNS differs greatly between rats and mice (14, 37)], it was important to evaluate the cytokine cascade in a primate model. Moreover, the size of the monkey allowed us to collect the requisite volumes of CSF. In addition to exploring the cytokine cascade from blood to CNS, the following studies confirmed that IL-6 released into CSF was brain derived. Study 1 showed that CSF IL-6 is not a result of diffusion from blood, and in study 2 the data show that IL-6 is released from a site within the brain and diffuses down the spinal column. Analogous studies comparing neurotransmitter levels in cervical and lumbar taps typically reveal a gradient in concentrations when the brain is the source (35). Together, these two pathways
studies significantly extend what is known about the in vivo cytokine cascade.

METHODS

Subjects. A total of 48 juvenile rhesus monkeys (age 11–24 mo) were used in two separate studies at the Harlow Center for Biological Psychology. Study 1 involved 28 animals (20 females and 8 males), whereas study 2 involved 20 animals (16 males and 4 females). Animals were pair-housed in standard cages (0.9 × 1.8 × 0.9 m) in a room dedicated to this project with a 14:10 light-dark schedule, lights on at 0600. Animals were fed once daily, had water available ad libitum, and received fruit three times weekly. All procedures were approved by the Institutional Animal Use and Care Committee at the University of Wisconsin.

Materials. Recombinant human IL-1β was purchased from Biosource International (Camarillo, CA), recombinant human IL-1α was a generous gift of Dr. Richard Chizzonite (Hoffmann-LaRoche, Nutley, NJ), and rhIL-6 was obtained from Sandoz (East Hanover, NJ). IL-1α was lyophilized and reconstituted in bacteriostatic water. Because of the low working concentration of both IL-1α and IL-1β solutions, final dilutions were prepared using a 0.5% rhesus monkey albumin solution, pH 7.2. Both IL-1α and IL-6 were sterile phosphate-buffered saline with 0.1% bovine serum albumin, pH 7.2. The working concentration of both IL-1α and IL-1β was 0.2 µg/ml.

Study 1. Comparison of IL-1α, IL-1β, and IL-6. There were six animals in each of four conditions: intravenous saphenous injection of 1) IL-1α, 0.5 µg/kg; 2) IL-1β, 0.5 µg/kg; 3) IL-6, 10 µg/kg; or 4) albumin, 0.5% solution. Dose levels of IL-1 were based on previous work in our laboratory (16, 32), and the IL-6 dose was designed to recreate the blood levels of IL-6 observed after IL-1 administration in prepubertal animals. An additional four animals served as undisturbed baseline controls. Animals were given the appropriate injection (0.2 ml), returned to the home cage, and sampled 2 h later. This time point was chosen because there is a robust increase in IL-6 at 1 h post-IL-1 (32), and if IL-6 was going to cross the BBB, we wanted to allow enough time for this response. At 2 h, animals served as undisturbed baseline controls. Animals were given the appropriate injection (0.2 ml), returned to the home cage, and sampled 2 h later. This time point was chosen because there is a robust increase in IL-6 at 1 h post-IL-1 (32), and if IL-6 was going to cross the BBB, we wanted to allow enough time for this response. Under light anesthesia (Ketaset, 15 mg/kg im), both blood and spinal fluid samples were collected. Blood (2 ml) was collected via femoral venipuncture and centrifuged at 2,000 rpm to separate plasma, which was aliquoted and frozen at −70°C. CSF (0.5 ml) was collected into a 25-g needle between C2 and C3, whereas the lumbar tap was collected at the thoracolumbar junction. CSF samples were placed immediately on ice, centrifuged at 2,000 rpm, transferred to a clean tube, and frozen at −70°C.

Study 2. Gradient and time course study. This study utilized a 2 × 2 design, with injection (IL-1β or albumin control) and time point (1 or 3 h) as between-subjects variables. The number of animals in each condition was as follows: IL-1β-1 h, n = 7; IL-1β-3 h, n = 5; albumin-1 h, n = 4; and albumin-3 h, n = 4. After intravenous injections (saphenous 0.2 ml) of either IL-1β or albumin, animals were returned to their home cages. One or 3 h later, animals were sampled under brief ketamine anesthesia (Ketaset, 15 mg/kg im). Blood (2 ml) was collected via femoral venipuncture and centrifuged at 2,000 rpm to separate plasma, which was aliquoted and frozen at −70°C. Both cervical and lumbar taps were collected (0.3 ml at each site). The cervical tap was collected via insertion of a 25-g needle between C2 and C3, whereas the lumbar tap was collected at the thoracolumbar junction. CSF samples were placed immediately on ice, centrifuged at 2,000 rpm, transferred to a clean tube, and frozen at −70°C.

Data analysis. Study 1 used a one-way analysis of variance (ANOVA) with Scheffe’s post hoc comparisons. Study 2 used a 2 × 2 ANOVA, with orthogonal post hoc comparisons where appropriate. Sex was not evaluated as a separate variable because we have shown previously that the effect of sex on the IL-6 response is minor, especially in prepubertal animals.

RESULTS

Study 1. Comparison of IL-1α, IL-1β, and IL-6. Cytokine administration significantly elevated plasma levels of IL-6 [F(4, 23) = 56.64, P < 0.0001]. Scheffe’s post hoc analyses revealed that injections of IL-1α, IL-1β, and IL-6 resulted in blood levels of IL-6 that were significantly elevated over levels seen at baseline or after an albumin injection (Fig. 1A). IL-6 in the CSF
was also significantly elevated \( F(4,23) = 16.09, P < 0.0001 \). In contrast to the blood compartment, however, post hoc analyses revealed that IL-1\( \beta \) was the only cytokine that stimulated a release of IL-6 into CSF (Fig. 1B). Levels of CSF IL-6 found after IL-1\( \alpha \) or IL-6 injections were not significantly different from control conditions. Cortisol levels were also significantly elevated after cytokine administration \( F(4,23) = 25.83, P < 0.0001 \). All three cytokines elevated cortisol above baseline and albumin control levels, with IL-1\( \alpha \) significantly more effective than IL-1\( \beta \) (Fig. 2).

Levels of IL-1ra in the blood were significantly and comparably elevated above control values after injections of IL-1\( \alpha \), IL-1\( \beta \), and IL-6 \( F(4,23) = 1,262.3, P < 0.0001 \); Table 1). There was no IL-1ra detectable in the CSF in any condition (lower limit of sensitivity = 14 pg/ml, data not shown). Levels of TNF-\( \alpha \) in blood and CSF were not different across conditions and similarly rectal temperatures were not different across the five conditions (Table 1).

Study 2. Gradient and time course study. To further localize the source of CSF IL-6, levels of IL-6 were measured in cervical and lumbar taps. IL-1\( \beta \) was used exclusively in study 2 because of its unique ability to stimulate the release of CSF IL-6. There was a significant interaction between treatment and time point for IL-6 levels in CSF collected at a lumbar site \( F(1,16) = 7.22, P < 0.02 \). However, a very different pattern was found with very low levels of IL-6 \( (17.1 \text{pg/ml}) \) at 1 h postinjection of IL-1\( \beta \), which increased to \( 52.6 \text{pg/ml} \) by 3 h. Again, there were undetectable levels of IL-6 in the control conditions (Fig. 3).

Plasma levels of IL-6 were significantly elevated after administration of IL-1\( \beta \) \( (1,16) = 17.47, P < 0.001 \). There was no significant difference between samples collected at 1 h and those collected at 3 h (Table 2). For cortisol, there was a significant interaction between collection time and condition \( F(1,16) = 11.52, P < 0.005 \), indicating that IL-1\( \beta \) significantly elevated plasma cortisol above control conditions and this increase was significantly larger at 1 h (Table 2).

### DISCUSSION

These studies replicate and significantly extend our previous work on the proinflammatory cytokine cascade in monkeys (32). In this report, we have shown that administration of IL-1\( \alpha \) and IL-1\( \beta \) leads to the release of IL-6 into blood, whereas only IL-1\( \beta \) is able to stimulate IL-6 release into CSF. Furthermore, all three cytokines, IL-1\( \alpha \), IL-1\( \beta \), and IL-6, increased levels of IL-1ra in blood and comparably stimulated the release of cortisol. These findings supported our hypothesis.

<table>
<thead>
<tr>
<th>Injection Condition</th>
<th>Baseline</th>
<th>Albumin</th>
<th>IL-1( \alpha )</th>
<th>IL-1( \beta )</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma IL-1ra, pg/ml</td>
<td>9.3 ± 4.7</td>
<td>20.0 ± 5.0</td>
<td>677.8 ± 7.6*</td>
<td>694.1 ± 10.3*</td>
<td>712.5 ± 15.9*</td>
</tr>
<tr>
<td>Plasma TNF-( \alpha ), pg/ml</td>
<td>40.5 ± 5.3</td>
<td>41.2 ± 1.8</td>
<td>39.2 ± 6.6</td>
<td>43.9 ± 4.8</td>
<td>46.3 ± 3.0</td>
</tr>
<tr>
<td>CSF TNF-( \alpha ), pg/ml</td>
<td>15.9 ± 3.4</td>
<td>29.5 ± 0.6</td>
<td>24.6 ± 3.3</td>
<td>22.1 ± 3.0</td>
<td>25.5 ± 3.2</td>
</tr>
<tr>
<td>Temperature, °F</td>
<td>101.5 ± 0.6</td>
<td>101.7 ± 0.4</td>
<td>101.1 ± 0.3</td>
<td>101.4 ± 0.1</td>
<td>101.6 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 6 \) monkeys/group. Interleukin-1 receptor antagonist (IL-1ra) was significantly and comparably elevated in plasma after injections of IL-1\( \alpha \), IL-1\( \beta \), or IL-6. Tumor necrosis factor (TNF)-\( \alpha \) [in plasma or cerebrospinal fluid (CSF)] and temperature did not differ between conditions. *\( P < 0.0001 \).
not cross the BBB in appreciable amounts and then
remain undetectable in CSF, even after marked increases in
the periphery. This difference indicates that within 2 h
after IL-1β injection, IL-6 is brain derived, first showing that IL-6 does not
cross the BBB in appreciable amounts and then that a concentration gradient for IL-6 occurs within the
spinal fluid of the intrathecal compartment.

A significant finding was that the two isoforms of IL-1 have different effects in vivo. IL-1α and IL-1β share only a 26% amino acid homology, yet they bind the same receptor and seem to display nearly identical biological activities. IL-1α is often described as the membrane-bound isoform, whereas IL-1β is typically characterized as the circulating isoform. After being given at the same dosage, IL-1β was significantly more potent in stimulating the release of IL-6 into CSF, in keeping with its blood-borne role. In fact, IL-1α evokes IL-6 in CSF to only 12 pg/ml, a level just minimally above baseline values. It is important to note that IL-1α was not less bioactive in the periphery as IL-1α and IL-1β were equally effective in stimulating the release of IL-6 and IL-1ra into the blood. Therefore, it appears that with regard to initiating IL-6 release into the CSF, IL-1β is a uniquely potent stimulus.

Study 1 provided additional information on the proinflammatory cytokine cascade in vivo. Previously, we had shown that IL-1β induced the release of a cytokine agonist, sIL-6R (32). In the current study, we showed that all three cytokines (IL-1α, IL-1β, and IL-6) can induce the release of a cytokine antagonist, IL-1ra, into the blood. This antagonist is a member of the IL-1 family and blocks binding of both IL-1α and IL-1β to cell surface receptors without inducing a signal of its own (11). It was not surprising that IL-1 should stimulate release of its own antagonist (21), as this is likely involved in modulating the magnitude of the inflammatory response. The fact that IL-6 also induced this antagonist was unexpected, although not without precedent (22), and highlights the extensive interaction between these two cytokines. Interestingly, IL-1ra was undetectable in CSF, even after marked increases in the periphery. This difference indicates that within 2 h there is little diffusion or transport of IL-1ra into the CSF, contrary to what has been reported in the mouse (18). The absence of IL-1ra in CSF may have important implications for inflammatory reactions within the CNS, suggesting that IL-1 may not be present in the monkey CNS at high levels or that different substances may control the cytokine cascade in the CNS.

Another major finding from study 1 was the demonstration that IL-6 does not cross the BBB in appreciable amounts within 2 h. In previous studies, we found elevated IL-6 in both blood and CSF. It was possible that IL-6 in CSF was blood derived and a result of either passive diffusion or an active transport system (4). To address this question, we injected 10 µg/kg of IL-6 intravenously, eliciting elevated blood IL-6 (levels slightly higher than those seen after injections of IL-1β). Yet even in this condition, we found levels of IL-6 in the CSF that were just slightly higher than baseline values. The failure to find IL-6 in CSF after exogenous administration of IL-6 confirms the hypothesis that IL-6 does not readily cross the BBB; i.e., diffusion from plasma to CSF cannot account for elevated levels of IL-6 seen after injections of IL-1β. However, the small increase in CSF IL-6 could be interpreted to suggest a very modest permeability of the BBB to IL-6. The second study was then conducted to confirm that IL-1-stimulated IL-6 in CSF was brain derived. Substances produced within the brain are found at higher concentrations in the ventricles and at much lower concentrations at the lumbar level of the spinal cord (24, 35). We collected spinal fluid at both a cervical site (as close to the brain as possible, while still performing an acute procedure) and contemporaneously at a lumbar site during the same sampling session. At 1 h after IL-1β administration, IL-6 was present at high levels in the cervical CSF samples and was just minimally elevated in lumbar CSF samples. By 3 h, IL-6 was detected at both sites in lower, but comparable, amounts. These data indicate a brain origin for IL-6 found in CSF and suggest that after release from sites within the brain, IL-6 flows down the spinal column, where it is finally detected after 3 h at the lumbar level.

There are several potential cellular sources for this brain-derived IL-6, including astroglia, microglia, endothelial cells, and even some types of neurons (23, 33). Given the time course and levels of IL-6 seen in the CSF (over 100 pg/ml within 1 h after IL-1 injection), it is unlikely that cells deep within the parenchyma are the source of IL-6 we are measuring. For these deep tissue cells (neurons, microglia, or astroglia) to be the source, it would require that 1) cytokines could reach these cells and 2) these cells could release nanogram quantities of IL-6, which would have to rapidly diffuse through tissue to reach the CSF. Instead, a more feasible source is the astroglia or endothelial cells of the BBB. These cells of the brain vasculature readily come in contact with circulating cytokines. Cytokine receptors are expressed on both cell types (9, 28), and both are capable of cytokine production (23). Although these cells are also an important component of the blood-spinal cord barrier, it appears that endothelial cells and/or astrocytes in the brain are unique in their ability to produce IL-6 (as evidenced by the rostral-caudal gradient of IL-6). Therefore, the BBB, already known for its im-

<table>
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<tr>
<th>Time Postinjection</th>
<th>Albumin</th>
<th>IL-1β</th>
<th>Albumin</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>128.3</td>
</tr>
<tr>
<td>3 h</td>
<td>253.3±60.0*</td>
<td>138.8±11.3*</td>
<td>58.8±2.3</td>
<td>39.2±2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Plasma IL-6 levels were elevated after IL-1β injection in study 2, although values at 1 and 3 h were not significantly different. Plasma cortisol levels were also elevated after IL-1β injections, significantly more so after 1 h. Significantly greater than albumin control: *P < 0.0001, †P < 0.001, ‡P < 0.005. Significantly < 1 h time point: §P < 0.05.
tance in regulating access to the brain, may prove to be a critical source of CNS cytokines as well.

These proinflammatory cytokines are also potent activators of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of cortisol into the bloodstream. Cortisol may be one important source of negative feedback during an inflammatory response because glucocorticoids are known to downregulate expression of proinflammatory cytokines [1] and are essential in protecting against cytokine-induced lethality [25]. Cytokine stimulation of the HPA axis may be a critical process that prevents an excessive response to the cytokine cascade in the periphery. In our studies, both IL-1α and IL-1β led to the release of cortisol, and concuring with reports in rats [34], IL-1α was slightly less effective in stimulating the HPA axis. IL-6 has also been reported to stimulate the HPA axis in humans [29], and likewise we found it to be a potent HPA stimulus in monkeys. Similar levels of cortisol were observed after IL-1 or IL-6 administration, although the dose of IL-6 was 20 times that of IL-1, suggesting that IL-1 is a more potent stimulus of the HPA axis. In study 2, we were able to characterize the time course of the cortisol response to IL-1. It appeared to reach maximal levels 1–2 h after IL-1 administration and was beginning to taper off by 3 h post-IL-1.

Extending investigations of the cytokine network into the CNS is an important step in understanding the proinflammatory response. The BBB appears to be a potentially significant source of cytokines, at least IL-6, within the CNS. As these cytokines have both physiological [19] and pathophysiological roles [20, 30], defining the dynamic interplay between peripheral immune activation and the release of cytokines into the CNS is critical.

Perspectives

It has become increasingly apparent that the CNS and immune system are not as separate as once believed and that cytokines provide important communication between these two systems. Conditions such as bacterial infections, sepsis, or tissue trauma, which result in the activation of macrophages and the consequent release of IL-1, likely involve activation of the cytokine cascade at the BBB as well. Therefore, these conditions may have significant CNS sequelae that must be considered. Furthermore, a number of cytokines are being investigated as potential chemotherapies for cancers and in the treatment of immunological disorders. An understanding of their CNS actions will be critical for minimizing unwanted side effects. As evidenced in our studies, peripheral immune activation leads very rapidly (in <1 h) to the release of massive quantities of IL-6 within the brain. The biological function of this cytokine release needs to be determined, in terms of both the potential beneficial and detrimental consequences. Further insight into the actions of cytokines within the CNS will help to elucidate the importance of the neuroimmune axis in health and disease.

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