

IN VIVO EVIDENCE THAT THE RISE IN PLASMA IL 6 FOLLOWING INJECTION OF A FEVER-INDUCING DOSE OF LPS IS MEDIATED BY IL 1 β

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Although it has often been speculated that Interleukin (IL) 1 α and IL 1 β are circulating endogenous pyrogens (EP), there are few data demonstrating an elevation of these cytokines in the plasma of febrile animals. We hypothesized that IL 1 is released locally and may act to stimulate the release of another pyrogen, IL 6, which circulates to the brain to cause fever. The major purpose of the present study was to determine whether pretreatment of rats with antiserum to IL 1 β , which attenuates lipopolysaccharide (LPS) induced fever, also results in an attenuation of the rise in plasma and cerebrospinal fluid (CSF) concentrations of IL 6. Our results show that injection of IL 1 β produced dose-dependent rises in temperature and increases in plasma and CSF IL 6 activity, and that pretreatment of rats i.v. with antiserum to IL 1 β produced a 55% decrease in the fever caused by LPS injection, a 68% decrease in plasma IL 6, and a 67% decrease in CSF IL 6. These data confirm the findings of previous studies that IL 1 β is required for a portion of LPS-induced fever and also provide the first in vivo demonstration that the rise of IL 6 in rats injected with a fever-inducing dose of LPS can be significantly blocked by antiserum to IL 1 β . Overall, the data in our study can be interpreted as being consistent with the hypothesis that the pyrogenic effect of IL 1 β is mediated mainly through the release of IL 6, but conclusive confirmation of this hypothesis must await studies with antibodies to IL 6.

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Over the past decade, it has been hypothesized that Interleukin (IL) 1 α and/or IL 1 β are circulating endogenous pyrogens (EP). IL 1 meets several of the criteria for an endogenous pyrogen: (1) recombinant human (rh) and murine (rm) IL 1 α and IL 1 β are capable of inducing fever in rat and rabbit¹⁻⁹; (2) rh IL 1 can stimulate the release of prostaglandin E₂, which is believed to be a neural mediator that increases the temperature set-point¹⁰⁻¹⁴; (3) antiserum to IL 1 β attenuates LPS fever in the rat.¹⁵⁻¹⁶ Since antiserum to IL 1 α did not attenuate the LPS fever,^{15,17} these data are consistent with the hypothesis that IL 1 β , but not IL 1 α , is an endogenous pyrogen. There are, however, few data demonstrating an elevation of either form of IL 1 in the plasma of febrile animals. However, another putative endogenous pyrogen, IL 6,¹⁸⁻¹⁹ not only caused prostaglandin-dependent fevers upon injection into animals,

but also increased markedly in the plasma and cerebrospinal fluid (CSF) of LPS-injected rats.²⁰ In that study, there was a strong correlation between either the plasma or CSF IL 6 activity and fever. Because the release of IL 6 can be stimulated by IL 1 β both in vivo and in vitro,²¹⁻²² we hypothesized that IL 1 is released locally in response to LPS and may act to stimulate the release of another pyrogen, IL 6, which circulates to the brain to cause fever.

The purpose of the present study was to determine in the rat: (A) whether injection of fever-inducing doses of IL 1 β cause a rise in plasma and CSF concentrations of IL 6, (B) whether pretreatment with antiserum to IL 1 β , which attenuates LPS-induced fever, also results in an attenuation of the rise in plasma and CSF concentration of IL 6 in response to injection of LPS, and (C) whether there is a correlation between LPS-induced fever and the plasma and CSF concentration of IL 6 in rats treated with antiserum to IL 1 β .

Experimental Protocols

Protocol 1. Injection of Recombinant Mouse IL 1 β and Saline. Recombinant mouse IL 1 β was injected intraperitoneally (i.p.) in doses of 0.75, 1.5, 3 and 6 μ g/kg dissolved in a total volume of 1 mL

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saline/kg. Control rats were injected with the same volume of saline. Blood and CSF were collected at 4 hr after the injection.

Protocol 2. Injection of LPS into the rats that were previously treated with antiserum to IL 1 β and control serum. The antiserum to mouse IL 1 β or the control serum was injected into anesthetized rats through the penis vein (i.v.) in a dose of 400 μ l/rat 40 hr before the administration of LPS. LPS was injected intraperitoneally (i.p.) at a dose of 10 μ g/kg dissolved in a total volume of 1 mL saline/kg. Blood and CSF were collected at 4 hr after the injection of saline or 10 μ g/kg LPS.

Data Analysis

Data are presented as means \pm SE. Statistical differences among group means were determined for plasma and CSF IL 6 activities in the IL 1 β injection study using ANOVA. Statistical differences between any two group means were determined using Student's t-tests, corrected for multiple comparisons by the method of Bonferroni whenever necessary. The *P* value of the correlations between body temperature and IL 6 was determined by logarithmic regression analysis.

RESULTS

Protocol 1. Injection of Recombinant Mouse IL 1 β and Saline. The thermal responses of rats to i.p. injection of various doses of rm IL 1 β are shown in Fig. 1. The initial temperature increase during the first hour in all groups is a nonspecific transient effect seen following injection of any substance i.p. in the rat. After this first hour, in the groups injected with 0.75 μ g/kg

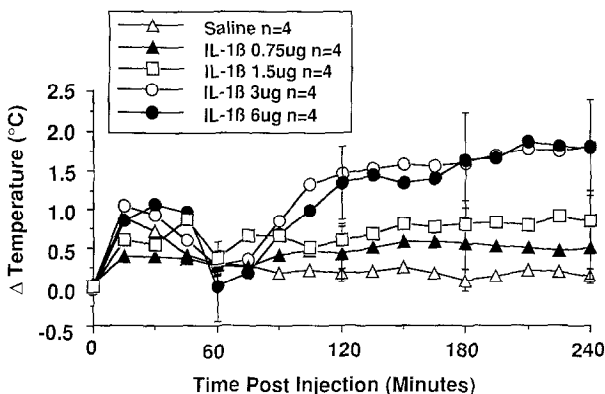


Figure 1. Change in core temperature of rats after i.p. injection of either saline or one of four doses (0.75, 1.25, 3, and 6 μ g/kg in saline) of recombinant mouse IL 1 β .

Injection occurred at time 0. Temperatures at time 0 for the five groups were not significantly different from each other and averaged 37.3°C. *n* = sample size. For clarity, not all Standard Error bars are illustrated.

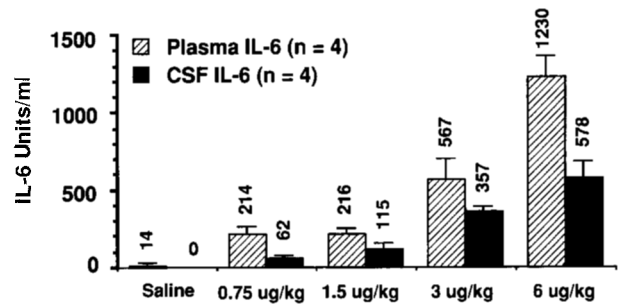


Figure 2. Plasma and CSF IL 6 activity in rats injected with either saline or one of four doses (0.75, 1.25, 3, and 6 μ g/kg in saline) of recombinant mouse IL 1 β .

Blood samples were collected at 4 hr after the injection in the rats whose temperature changes are shown in Fig. 1. Sample size is indicated in parentheses.

and 1.5 μ g/kg of recombinant mouse IL 1 β , a small rise (approximately 0.5°C) in body temperature occurred. In the groups injected with 3 μ g/kg and 6 μ g/kg of recombinant IL 1 β , the temperature started to rise more markedly after 90 min, and reached a peak of about 1.8°C at about 200 min (*P* = 0.0014, ANOVA with repeated measurement).

We also measured the IL 6 activities in plasma and CSF in these same rats 4 hr following i.p. injection with saline or IL 1 β (Fig. 2). In control (saline-injected) animals, plasma and CSF IL-6 were 14 \pm 14 and 0 U/mL respectively. Following injection of 0.75 μ g IL 1 β /kg, IL 6 activity was 214 \pm 53 U/mL in plasma and 62 \pm 19 U/m in CSF. Following injection of 6 μ g IL 1 β /kg, IL 6 activity was 1,230 \pm 138 U/mL in plasma and 578 \pm 109 U/mL in CSF. Both plasma and CSF IL 6 activity increased significantly as the dose of recombinant mouse IL 1 β was increased (*P* = 0.0001, ANOVA).

Protocol 2. Injection of LPS Into the Rats That Were Previously Treated With Antiserum to IL 1 β and Control Serum.

The mean change in core temperature of rats after i.p. injection of saline or 10 μ g LPS/kg in groups of rats treated 40 hr earlier with either control serum or antiserum to IL 1 β is shown in Fig. 3. These temperature data are the mean changes seen from 120 min to 240 min following the injection of saline or LPS. There were no significant differences in the changes of core temperature between the saline-anti-IL 1 β serum and saline-control serum groups (*P* = 0.54). The changes in body temperature, following injection of LPS, were significantly lower in the group receiving antiserum to IL 1 β (0.54 \pm 0.12°C) than in the control-serum treated group (1.19 \pm 0.11°C) (*P* = 0.0008). There was no significant difference in core temperature changes between the saline-anti-IL 1 β serum and LPS-anti-IL 1 β serum groups (*P* = 0.84). Although the changes in body temperature following injection of LPS were significantly lower in the rats receiving antiserum to IL 1 β , neither the rise of body temperature from the injection

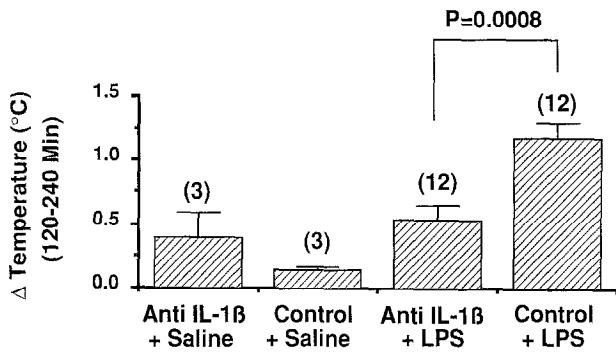


Figure 3. Mean change in core temperature of rats after i.p. injection of saline or LPS in groups of rats previously treated with either control serum or antiserum to IL 1 β .

Sample size is indicated in parentheses. These temperature data are the mean values occurring from 120 min to 240 min following the i.p. injection of saline or LPS.

(stress hyperthermia) nor the circadian night-time rise of body temperature were altered by the antiserum to IL 1 β (data not shown).

Fig. 4 shows the plasma IL 6 data in these animals 4 hr after injection of saline or LPS. After injection of saline, plasma IL 6 activity was 119 \pm 4 U/mL in the group treated with antiserum to IL 1 β and 44 \pm 25 U/mL in the control serum group. Following injection of 10 μ g/kg of LPS, plasma IL 6 activity was 15,964 \pm 8,940 U/mL in the antiserum-treated group and 50,184 \pm 14,771 U/mL in the control-serum group ($P = 0.03$).

Fig. 5 shows the 4 hr CSF IL 6 data for the same rats as in Fig. 4. After injection of saline, CSF IL 6 activity was 30 \pm 28 U/mL in the antiserum-treated group and 17 \pm 9 U/mL in the control serum group. Following injection of 10 μ g/kg LPS, CSF IL 6 activity

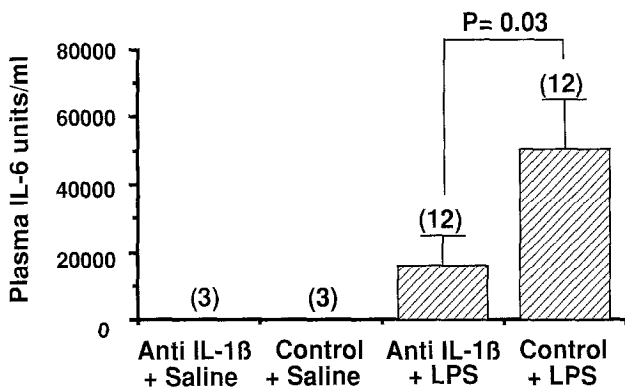


Figure 4. Plasma IL 6 activity in rats injected with (1) antiserum to IL 1 β plus saline, (2) control serum plus saline, (3) antiserum to IL 1 β plus 10 μ g/kg LPS, and (4) control serum plus 10 μ g/kg LPS.

Plasma samples were collected at 44 hr after injection of control serum or antiserum to IL 1 β (400 μ l/rat) and 4 hr after injection of saline or LPS (10 μ g/kg). Sample size is indicated in parentheses. P values were calculated by comparing the group of rats injected with control serum plus 10 μ g/kg LPS to the group of rats injected with antiserum to IL 1 β plus 10 μ g/kg LPS using a Student's t -test.

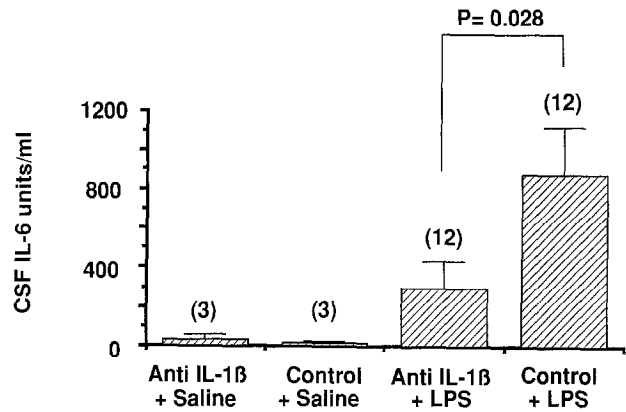


Figure 5. CSF IL 6 activity in rats injected with (1) antiserum to IL 1 β plus saline, (2) control serum plus saline, (3) antiserum to IL 1 β plus 10 μ g/kg LPS, and (4) control serum plus 10 μ g/kg LPS.

CSF samples were collected at 44 hr after injection of control serum or antiserum to IL 1 β (400 μ l/rat) and 4 hr after injection of saline or LPS (10 μ g/kg). Sample size is indicated in parentheses. P values were calculated by comparing the group of rats injected with control serum plus 10 μ g/kg LPS to the group of rats injected with antiserum to IL 1 β plus 10 μ g/kg LPS using a Student's t -test.

was 292 \pm 146 U/mL in the antiserum treated group and 877 \pm 251 U/mL in the control serum group ($P = 0.028$).

The quantitative relationships between IL 6 and changes in body temperature at 120 min to 240 min post-injection for each individual rat are shown in Fig. 6 and Fig. 7. The correlation coefficient relating IL 6 activity and body temperature, based on a logarithmic regression, was 0.74 ($P < 0.0062$) for plasma and 0.75 ($P < 0.0003$) for CSF.

Fig. 8 shows the result of the IL 6 recovery study in plasma from LPS-treated animals. There is no indication of any factors present in the plasma of control or

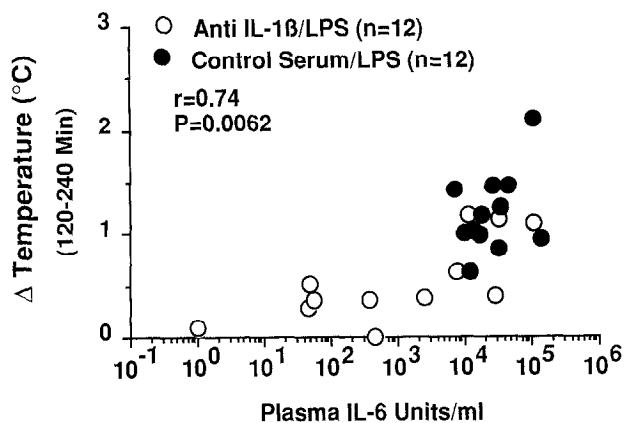


Figure 6. Plasma IL 6 activity vs change in body temperature for rats injected with control serum plus 10 μ g/kg LPS or with antiserum to IL 1 β plus 10 μ g/kg LPS.

The data used are the same as those from Figs. 3 and 4 and are for individual rats at the relevant time. r = correlation coefficient, which was calculated based on best-fit logarithmic regression, and P = level of statistical significance.

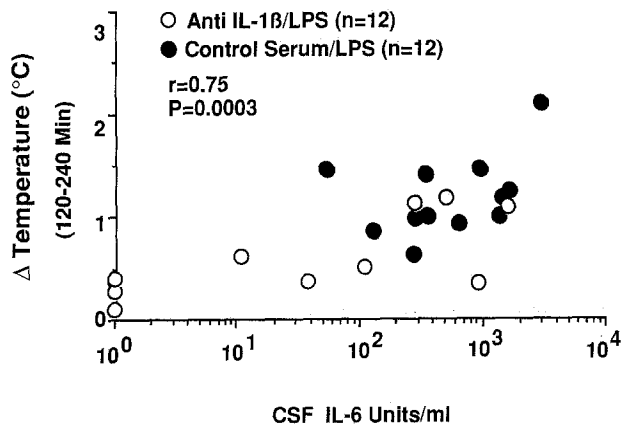


Figure 7. CSF IL 6 activity vs change in body temperature for rats injected with control serum plus 10 $\mu\text{g}/\text{kg}$ LPS or with antiserum to IL 1 β plus 10 $\mu\text{g}/\text{kg}$ LPS.

The data used are the same as those from Figs. 3 and 5 and are for individual rats at the relevant time. r = correlation coefficient, which was calculated based on best-fit logarithmic regression, and P = level of statistical significance.

LPS-treated animals that inhibit or facilitate the B-9 assay. Identical results were obtained from recovery studies done in the plasma of IL 1 β -treated animals (data not shown).

DISCUSSION

Our data qualitatively support the hypothesis that during LPS fever IL 1 β is released locally and may act to stimulate the release of another pyrogen, IL 6, which circulates to the brain to cause fever. Injection of fever producing doses of rm IL 1 β resulted in significant increases in both the plasma and CSF IL 6 concentrations and in body temperature. Approximately two-thirds of the rise of core temperature and rise of IL 6 in

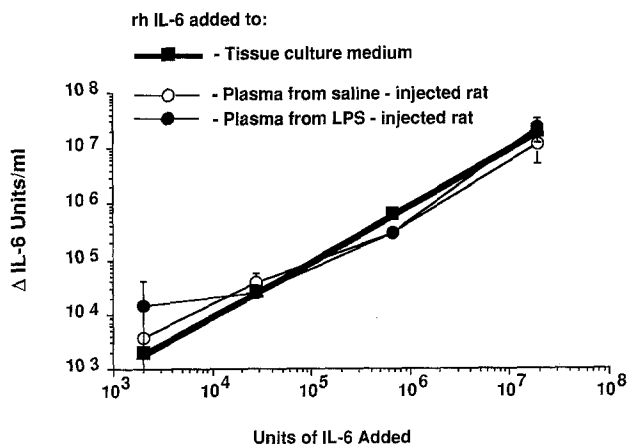


Figure 8. Change in IL 6 activity from base line after addition of known quantities of rh IL 6.

Rat plasma was harvested 4 hr after injection of either saline or 10 $\mu\text{g}/\text{kg}$ LPS. Base values were 80 U/mL for the saline group and 88,000 U/mL for the LPS treated group. The tissue culture medium was free of IL 6, and so the baseline value was 0.

LPS fever was blocked by pretreatment with antiserum to IL 1 β . There was a positive correlation between the changes in plasma and CSF IL 6 and body temperature in these experiments.

However, quantitatively, the data pose a problem for this hypothesis. The plasma IL 6 (1,230 U/mL) in IL 1 β fever was much lower than the plasma IL 6 (50,184 U/mL) in LPS fever, but the magnitude of the IL 1 β fever was similar to the magnitude of the LPS fever. LPS induces the release of IL 1 β and TNF, and there is a known synergy between these two cytokines in inducing the production of IL 6.²³ This synergy may account for our finding higher IL 6 levels with LPS than with IL 1 β alone. Whatever the explanation, the important point, which casts doubt on the hypothesis that IL 6 is the major circulating pyrogen in LPS fever, is that the fevers induced by LPS and IL 1 β were similar, even though the plasma IL 6 levels were very different.

We speculate several possible explanations for this quantitative discrepancy. The first possibility is that the hypothesis is wrong, that plasma IL 6 is not the mediator of LPS fever, and that IL 1 β results in fever directly or through other circulating cytokines (e.g., NAF/IL 8, or MIP-1), without the participation of circulating IL 6. Further evidence, which is consistent with IL 6 not being the major pyrogen in LPS fever, comes from studies demonstrating that anti-tumor necrosis factor (anti-TNF) antibody enhanced rather than reduced fever^{16,29} and that anti-TNF antibody suppressed rather than enhanced IL 6 production.²³ These results together imply that the fever enhancement with anti-TNF antibody occurred in the face of decreased IL 6 levels, and such results call into question a major role for IL 6 as a pyrogen in LPS-induced fever.

A second possibility, one that preserves a major pyrogenic role for IL 6 in LPS fever, is that it is the concentration of IL 6 in the brain rather than in the plasma that is important for the development of fever. This possibility is raised by the fact that, despite the quantitative discrepancy between plasma IL 6 values after LPS and IL 1 β , the value of CSF IL 6 (578 U/mL) in IL 1 β fever was close to the value of CSF IL 6 (877 U/mL) measured during LPS fever. A recent study showed that hypothalamic tissue can release IL 6. This supports the hypothesis that the rise of IL 6 can come directly from the brain.²⁵ Therefore, it is possible that locally released IL 1 β stimulates the release of IL 6 in brain tissue and that it is this IL 6 that causes fever.

A third possibility, one that also preserves a major pyrogenic role for IL 6 in LPS fever, is that when large amounts of IL 1 β are injected systemically, a high concentration of circulating IL 1 β is created that permits sufficient quantities of circulating IL 1 β to reach the brain and cause fever by a direct action on the hypothalamus, without involvement of IL 6. In contrast, this situation probably does not occur during LPS fever, because there are few data demonstrating an elevation

of IL 1 β in the plasma of febrile animals. In other words, it is possible that the fever caused by LPS may be entirely the result of the direct action on the brain of IL 6 alone, while the fever caused by injection of IL 1 β reflects the combined action of circulating IL 1 β and IL 6 on the central nervous system.

The above three possibilities cannot be distinguished by the present data, and a definitive answer to the hypothesis that IL 6 is the mediator of LPS fever awaits experiments using antibodies to IL 6.

Finally, it should be emphasized that, to the best of our knowledge, these data provide the first evidence that the rise of IL 6 in LPS fever *in vivo* can be blocked significantly by antiserum to IL 1 β , and supports the hypothesis that much of the rise in plasma IL 6 during LPS fever is stimulated by IL 1 β .

MATERIALS AND METHODS

Animals

Fifty specific-pathogen-free male Sprague-Dawley rats weighing 180 to 220 g were obtained from Charles River (Portage, MI). Rats were housed at 23 to 25°C with a 12/12 hr light-dark cycle and given *ad libitum* tap water and rodent chow.

Measurement of Body Temperature

Core temperature was measured by biotelemetry, using transmitters implanted intraperitoneally (*i.p.*) (Mini Mitter, Inc., Sunriver, OR).²⁶ The transmitters were implanted at least 4 days before the experiments. Each transmitter was calibrated before implantation. Output (frequency in Hz) was monitored by a mounted antenna placed under each rat's cage and fed into a peripheral processor (Dataquest III system, Mini Mitter, Inc.) connected to an IBM-PC. Temperatures were recorded at 5 min intervals. To avoid any circadian variation in body temperature or plasma IL 6 activity, all injections were made between 8:00 AM and 10:00 AM, except for the injections of control serum or antiserum to IL 1 β , which were at 5 PM.

IL 6 Bioassay

IL 6 activity in plasma and CSF was measured using the IL 6-dependent B-9 hybridoma cell line.²⁷⁻²⁹ The B-9 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technology, Inc.) supplemented with human recombinant IL 6 (8 U/mL obtained from Dr. Gordon Wong, Genetics Institute), 20 μ M 2-mercaptoethanol, 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin and 100 μ g/mL streptomycin. Cells were washed once in the above medium without added IL 6, before the addition of the plasma samples or known amounts of human rIL 6.

To run the IL 6 assay, a 5 μ l sample to be assayed was combined with 5000 B9 cells in 195 μ l IMDM/10% FCS in flat-bottom microtiter plates (Corning) for a final volume of 200 μ l. All samples were run in duplicate. The control medium, which contained no IL 6, was run in quadruplicate. In addition to the undiluted plasma samples, a serial dilution of each

sample was assayed. Serial dilution was performed because our standard curve indicated that high levels of IL 6 result in an inhibition of growth of the B-9 cells. Each sample was serially diluted initially to a maximum of 1:62,500.

Cells were pulsed at 68 to 72 hr with 0.5 μ Ci 3H thymidine, harvested 4 hr later onto glass fiber filter strips (Cambridge Technology, Inc), and the radioactivity incorporated into DNA was counted by a β -Scintillation Counter (Packard Instrument Company). For each assay, a standard curve was run with recombinant human IL 6. One unit of IL 6 is equal to the amount that caused half-maximum proliferation in the standard curve. From this standard curve, a best-fit regression was calculated for the rising portion of the curve in the linear range. The equation for this best fit line was used to calculate IL 6 activity in plasma samples.

All samples were serially diluted until they became undetectable. All counts per minute (cpm) that fell within 2 standard deviations of the baseline cpm were excluded to reduce the potential error resulting when converting IL 6 from cpm to units, by multiplying by the dilution factor. The largest calculated IL 6 activity, which fell outside two standard deviations of the baseline and fell well below the peak, was taken as the IL 6 value. This ensured that all data used fell on the steep linear portion of the standard curve, which increased the accuracy of the value.

We also ran rh IL 6 from National Biological Standards Board (NBSB) in the B-9 assay to compare the units obtained from our B-9 assay and units from NBSB (Dr. Craig Reynolds, National Cancer Institute, Md.). This comparison indicated that 1 U of NBSB IL 6 is equal to approximately 300 U in the B-9 assay. In other words, approximately 0.0034 NBSB U were required to produce half maximal proliferation of the B-9 assay. In this paper, all units refer to the B-9 assay half-maximum units.

Antiserum to IL 1 β and LPS

Recombinant mouse IL 1 β and rabbit antiserum to mouse IL 1 β were obtained from Pfizer Research, Groton, CT. The specific activity of recombinant IL 1 β was 9×10^7 U/mg based on lymphocyte activating factor assay (LAF). The antiserum to mouse IL 1 β was made from rabbits immunized with murine recombinant IL 1 β (Daumy et al., submitted for publication). The resulting antibody was found to be specific for both mouse and rat IL 1 β by Western blotting. Blocking antibody was titered *in vitro* against recombinant murine IL 1 β using the LAF assay, and it was found that 2 μ g of antibody would neutralize 1 ng of IL 1. Approximately 4 mg of antibody were administered per rat in each *in vivo* blocking experiment.

Purified LPS (*E. coli* endotoxin 0111:B4 # L 97F-4089) was obtained from Sigma Company.

CSF and Plasma Samples

To collect fluids, rats were anesthetized by intramuscular injection of a combination of 70 mg/kg ketamine hydrochloride and 10 mg/kg xylazine. Blood was then collected within 1.5 min in a heparinized syringe by cardiac puncture. Plasma was separated by centrifugation of the freshly drawn blood and stored at -20°C. Immediately after the blood sample was collected, about 60 μ l to 100 μ l of CSF was drawn from the cisterna magna into a 0.5 mL syringe through a 28 gauge 1/2 inch needle. The percent of contamination of the CSF

with blood was estimated by visually comparing the color of tubes containing artificial CSF, which were serially diluted following the addition of a known amount of blood. In virtually all of the samples, the level of contamination was 0.001 parts of blood or lower. The actual CSF IL 6 activity was calculated by subtracting the IL 6 activity in the blood multiplied by the contamination factor from the measured IL 6 activity in the CSF.

IL 6 Recovery

We tested whether, after LPS and IL 1 β injection, there were any factors present in the plasma sample that inhibited or enhanced the B-9 assay. We added known quantities of rh IL 6 to the plasma samples obtained from rats at 4 hr following injection of sterile pyrogen free 0.9% sodium chloride (saline), IL 1 β , or 10 μ g/kg LPS and then ran the B-9 assay to determine the amount of IL 6 recovered from these samples.

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