

Tumor Necrosis Factor α /Cachectin and Interleukin 1β Initiate Meningeal Inflammation

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Summary

Although previous studies using human cytokines in rabbits and rats have provided evidence of the participation of tumor necrosis factor α (TNF- α) and interleukin 1β (IL- 1β) in the meningeal inflammatory cascade, the results obtained by several groups of investigators have been discordant or, at times, contradictory. In the present study, homologous cytokines were applied to the rabbit meningitis model. Intracisternal administration of 10^2 – 10^5 IU of purified rabbit TNF- α (RaTNF- α) produced significant cerebrospinal fluid (CSF) inflammation. A similar response was observed after intracisternal inoculation of 5–200 ng of rabbit recombinant IL- 1β (rrIL- 1β). Preincubation of these two mediators with their specific antibodies resulted in an almost complete suppression of the CSF inflammatory response. In animals with *Haemophilus influenzae* type b lipooligosaccharide-induced meningitis, intracisternal administration of anti-rrIL- 1β , anti-RaTNF- α , or both resulted in a significant modulation of meningeal inflammation. Simultaneous administration of 10^3 IU of RaTNF- α and 5 ng of rrIL- 1β resulted in a synergistic inflammatory response manifested by a more rapid and significantly increased influx of white blood cells into the CSF compared with results after each cytokine given alone. These data provide evidence for a seminal role of TNF- α and IL- 1β in the initial events of meningeal inflammation.

Despite the availability of advanced medical intensive care and of extraordinarily active antibiotics for treatment of bacterial meningitis, the case fatality and long-term morbidity rates associated with this disease have not appreciably changed (1, 2). As a result, research designed to delineate the molecular pathophysiology of this condition has focused on the mechanisms responsible for the inflammatory response in the subarachnoid space. Initial attention was focused on the specific components of the pneumococcal cell wall (3, 4) and the lipooligosaccharide of *Haemophilus influenzae* type b (Hib)¹ (5, 6), and on their ability to elicit inflammation in the cerebrospinal fluid (CSF) and to alter blood-brain barrier permeability (7, 8).

Two cytokines, TNF- α (cachectin) and IL-1, were shown to play a seminal role in the host's response to these bacterial

elements (9–13). Although the monocyte/macrophage is the principal source of IL-1 and TNF- α , other cells have also been shown to produce these cytokines. Of relevance to meningitis, astrocytes and microglial cells produce IL-1 and TNF- α within the central nervous system (14–17). These mediators have been detected in CSF of patients with bacterial meningitis caused by Gram-positive and Gram-negative organisms (18–23), and in CSF of animals experimentally inoculated with Hib, *N. meningitidis*, Hib lipooligosaccharide (LOS), and pneumococcal cell wall fragments (18, 22, 24–26). Additionally, the availability of rTNF- α and IL- 1β , as well as specific antibodies directed against these molecules, has permitted controlled investigations of their role in the meningeal inflammatory cascade. Experiments in which animals were intracisternally inoculated with these cytokines, and in which anticytokine antibodies were utilized to attenuate the inflammatory response induced by different bacteria or their components, have provided substantial evidence of their participation in this process (24, 27, 28). Results obtained by several groups of investigators, however, do not coincide and, at times,

¹ Abbreviations used in this paper: ANOVA, analysis of variance; CSF, cerebrospinal fluid; Hib, *Haemophilus influenzae* type b; IL-1i, IL-1 inhibitor; LOS, lipooligosaccharide; RaTNF- α , rabbit TNF- α ; rrIL- 1β , rabbit recombinant IL- 1β ; WBC, white blood cell.

appear contradictory, which might result from differences in methodology or in the source of cytokines used in those experiments. Use of human cytokines and their specific antibodies in the rabbit and rat models could explain, at least in part, these discrepancies. Although the amino acid sequence of these cytokines is highly conserved among various species (9, 12), differences in the molecular conformation associated with different epitopes and/or with the repertoire of biological activities (29), and the species-specific nature of the receptor-ligand interaction of TNF- α (30), could account for the dissimilar results observed.

The use of homologous preparations in these animal systems was recently suggested (28) as a means of determining the precise roles of and relationships among these cytokines in the meningeal inflammatory pathway. Rabbit recombinant IL-1 β (rrIL-1 β) (31, 32) and purified rabbit TNF- α (RaTNF- α) (33, 34) were recently made available to us. These molecules and their specific antibodies were applied to the traditional rabbit meningitis model (35) in an attempt to delineate these complex interactions.

Materials and Methods

Preparation of Hib LOS. LOS was purified from cells of Hib strain DL42 by using the hot phenol-water method of Westphal and Jann (36), as modified by Johnson and Perry (37). The purity of this LOS preparation was confirmed by SDS-PAGE, followed by silver staining (38) and Western blot analysis using hyperimmune rat serum to Hib DL42 (39).

It was previously shown that Hib LOS in either its purified form or as an integral part of the outer membrane of Hib, when injected intracisternally, induced meningeal inflammation and alteration of blood-brain barrier permeability (5–8). The minimal amount of LOS necessary to induce CSF pleocytosis was 0.02 ng. Intracisternal injection of 20 ng of Hib LOS, an amount equivalent to 2×10^6 cells, consistently induced substantial changes in the indices of meningeal inflammation (5). Because CSF samples from patients with Hib meningitis contain from 10^4 to 10^7 CFU/ml, 20 ng of Hib LOS was chosen as an appropriate inoculum to induce meningitis in our studies.

Bacterial Strain. Hib strain DL42 is a non- β -lactamase-producing strain isolated from the CSF of a child with meningitis. This strain has been shown to be virulent in the infant rat model for Hib invasive disease and to belong to LOS antigenic group 2, which is the predominate LOS antigenic type among invasive Hib strains (40).

rrIL-1 β . rrIL-1 β was purified from the extract of *Escherichia coli* (HB 101) that was transformed with a rabbit IL-1 β -containing plasmid. The purification was performed by gel filtration, C9 treatment for endotoxin removal, and HPLC on IEX 530 KS (silica-CM type; 7.8 mm \times 30 cm; Toso Co. Ltd., Tokyo). The resulting material was confirmed to show a single band on SDS-PAGE and to have a single amino acid terminus, Ala, and was thought to be composed of 152 amino acid residues. The activity of this material is 1 U/20 pg, as estimated by thymocyte mitogenic assay. Because the endotoxin content of this compound tested in a chromogenic Limulus assay was 10 pg/ μ g, the maximum dose of rrIL-1 β (200 ng), injected intracisternally, contained only 2 pg of endotoxin.

Anti-rrIL-1 β Serum. The anti-rrIL-1 β serum was obtained by immunizing a goat for 8 mo with rrIL-1 β with CFA. This serum neutralized 2 U (40 pg) of rrIL-1 β at a dilution of 2^{-14} . The serum was diluted 1:100 for intracisternal injections in rabbits. The endo-

toxin content of this dilution measured by the chromogenic Limulus assay was 7 pg/ml. Intracisternal injections of 0.1–0.2 ml of the diluted antiserum did not induce significant CSF pleocytosis (maximum 30 white blood cells (WBC)/mm³).

IL-1 Inhibitor (IL-1i). This IL-1 inhibitor is a 22-kD molecule present in the supernatant of human monocytes cultured on adherent immune complexes. It blocks IL-1 augmentation of PHA-induced murine thymocyte proliferation, but not IL-1-induced stimulation of CTLL or HT-2 cell lines. It specifically blocks binding of IL-1 to its receptor on the murine thymoma cell line ELA-6.1. Western blot analysis showed no immunological crossreactivity between IL-1 and this IL-1i (41). The compound was kindly provided by Synergen (Boulder, CO).

Rabbit TNF- α (RaTNF- α). Natural rabbit TNF- α purified from sera of rabbits treated with *Propionibacterium acnes* and endotoxin (33) was kindly supplied by Dainippon Pharmaceutical Co. (Osaka, Japan). The activity of the preparation is 8×10^6 IU/ml. It contained 0.80 ng/ml of endotoxin, as measured by the chromogenic Limulus assay. In our experiments, we inoculated up to 10^5 IU of RaTNF- α that contained 10 pg of endotoxin.

Anti-RaTNF- α . Ascitic fluid from BALB/c mice with hybridoma-secreting monoclonal anti-RaTNF- α (IgG1) (33) was used as a source of anti-RaTNF- α . A 1:100 dilution of ascitic fluid neutralized an equal volume of RaTNF- α with an activity of 11,200 IU/ml in the L929 cytotoxicity assay (34). The endotoxin content of this ascitic fluid preparation was 80 pg/ml. Intracisternal inoculation of rabbits with 50 μ l of the original product diluted in 200 μ l of pyrogen-free saline did not induce CSF inflammation.

Cytolytic Assay for TNF- α . TNF- α activity was determined by modification of a previously described cytotoxic assay (42). Briefly, L929 cells (CCL 1; America Type Culture Collection, Rockville, MD) were maintained in DME supplemented with 10% FCS and 4% penicillin and streptomycin. Confluent cultures were rinsed with sterile PBS, then briefly with 0.05% trypsin, resuspended in fresh medium, and added to 96-well plates (7×10^4 cells/well). After 2–3 h in culture, 50 μ l of cycloheximide (0.3 mg/ml) and 5 μ l of CSF samples were added to each well, and the plates were incubated overnight (5% CO₂, 37°C). After microscopic evaluation, the medium was decanted, and the wells were filled with 100 μ l of 0.5% crystal violet in 25% methanol for 5 min, washed thoroughly with normal saline, and dried. The dye was solubilized with 100 μ l of 50% acetic acid. The degree of cytotoxicity was quantified spectrophotometrically (490 nm) using a computerized automated ELISA plate reader (3550; Bio-Rad Laboratories, Richmond, CA). Equivalent concentrations of rabbit TNF- α were determined for experimental samples by interpolation of the RaTNF- α standard curve run simultaneously (10^{-1} to 10^5 IU/ml). Using this assay, we could reliably detect as little as 5 IU/ml of rabbit TNF- α . The samples were assayed in quadruplicate, and a SD within 10% of the mean was observed.

Detection of IL-1 β . Rabbit IL-1 β was measured by an ELISA developed in Kumamoto University (Japan). In brief, microtiter plates (Immunoplate MaxiSorp F96; Nunc, Kamstrup, Denmark) were coated overnight at 4°C with 100 μ l of goat IgG antibodies directed against rabbit IL-1 β diluted in a 10- μ g/ml concentration in 0.1 M sodium carbonate buffer (pH 9.6). The plates were washed five times with PBS-Tween 20 (0.05%) and then blocked overnight at 4°C with carbonate buffer containing 1% BSA. After five washes, 100 μ l of rabbit CSF was incubated at 4°C overnight, the plates were again washed, and then coated with biotinylated goat anti-rabbit IL-1 β IgG (5 μ g/ml) in PBS-Tween 20 containing 0.1% BSA. After a 3-h incubation at room temperature, the plates were washed, a 100- μ l volume of avidin-peroxidase (Vector Laboratories, Burlin-

game, CA) was added, as recommended by the manufacturer, and the plates were incubated for 90 min at room temperature and then washed. A 100- μ l volume of *O*-phenylen diamine (0.8 mg/ml) in 0.1 M citrate buffer (pH 5.0) containing 0.03% H₂O₂ was added. After 40 min in room temperature, the OD (492 nm) was measured by an ELISA reader (3550; Bio-Rad Laboratories). The concentrations of rabbit IL-1 β in test samples were obtained by comparison with the known concentrations (30–300 ng/ml) of rrIL-1 β . This standard was used on each microtiter plate. The lower limit of detection of this assay was 30 pg/ml.

Measurement of CSF Lactate and Protein. CSF samples were analyzed for lactate with a kinetic enzymatic method that used the reaction of lactate with Nicotinamide-adenine dinucleotide (β -NAD⁺) in the presence of lactate dehydrogenase to produce Nicotinamide-adenine dinucleotide, reduced (NADH) and pyruvate. The production of NADH was quantitatively determined at a 340-nm absorbance on a spectrophotometer (3600; Gilford, Oberlin, OH) with a deuterium source. The precision of the method was tested by running a known standard of 40 mg/dl (UV test 826-UV; Sigma Chemical Co., St. Louis, MO) 10 times in 1 d. The coefficient of variation was 2.2%.

Protein concentrations were measured by a modified biuret assay on the Ektachem 700 (Kodak, Rochester, NY). In this method, protein in a moderately alkaline solution chelates Cu²⁺ ions from an azo dye, resulting in a spectral shift. The decrease in absorbance of the copper-azo dye complex is related to the concentration of protein in the sample. This method detects albumin and globulins.

In Hib LOS-induced meningitis, intracisternal inoculation of anti-RaTNF- α antibody caused a significant early elevation of CSF protein concentration that interfered with measurement of this index of meningeal inflammation. As a result, this variable could not be analyzed in that set of experiments.

Experimental Meningitis Model. The rabbit model of experimental meningitis, originally described by Dacey and Sande (35), was used in a modified form. Approximately 400 New Zealand white male

rabbits (2–3 kg) were anesthetized with intramuscular injections of ketamine (40 mg/kg) and acepromazine (3 mg/kg) and placed in a stereotactic frame. A spinal needle was introduced into the cisterna magna and 0.2–0.3 ml of CSF withdrawn. Animals were then inoculated intracisternally with the different test materials in a total volume of 0.2–0.3 ml. Subsequent CSF samples (0.2 ml) were obtained at 1 (in certain experiments this sample was not obtained to avoid potential removal of the cytokine), 2, 4, 8, 12, 24 and sometimes at 48 h. Immediately after collection, the CSF aliquots were analyzed for cell count using a Neuber hematocytometer (American Optical, Buffalo, NY). The remaining CSF was centrifuged at 5,000 rpm for 5 min, and the supernatants were stored at –70°C until assayed for lactate, protein, and cytokines concentrations.

Statistical Analysis. Two-way repeated measures analysis of variance (ANOVA) was used to assess the effect of independent treatments with time for the following variables: WBC, protein, and lactate concentrations in the CSF. Values of each variable obtained at four or five different time points were included in the analysis.

In Hib LOS-induced meningitis experiments, peak CSF TNF activity among different treatment groups was analyzed by the Mann-Whitney U Test.

All data are expressed as mean \pm SEM, unless otherwise indicated. *p* values <0.05 were considered significant.

Results

Inoculation of rrIL-1 β . Intracisternal injections of 5–200 ng of rrIL-1 β was associated with significant CSF pleocytosis that increased in a dose-dependent fashion up to a 100-ng dose (Fig. 1). When 200 ng of rrIL-1 β was administered intracisternally, the CSF WBC counts observed were comparable with those seen after 100-ng injections. 500 pg of

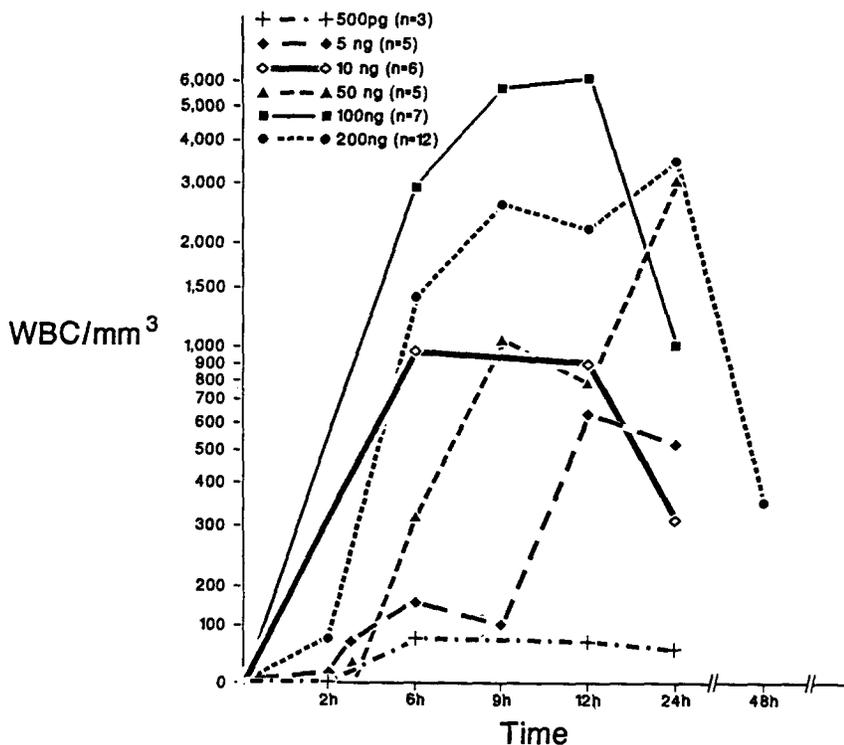


Figure 1. CSF WBC response after varying intracisternal doses of rrIL-1 β . Time represents hours after inoculation.

rrIL-1 β did not produce significant pleocytosis. The specificity of this inflammatory response was evaluated in two sets of experiments.

Preincubation of 200 ng of rrIL-1 β with 100 μ l of 1:100 dilution of anti-rrIL-1 β serum resulted in an almost complete suppression of meningeal inflammation, as evidenced by WBC, protein, and lactate concentrations in CSF (Fig. 2, A and B).

Intracisternal administration of 200 ng of rrIL-1 β with a specific IL-1i (1 mg/kg) yielded a significant reduction of CSF pleocytosis (Fig. 3).

The time pattern of these inflammatory changes induced by rrIL-1 β was substantially delayed compared with those induced by intracisternal inoculation of purified Hib LOS. CSF WBC counts peaked between 6 and 12 h after intracisternal

injection of Hib LOS, compared with 12–24 h in animals injected with rrIL-1 β (Fig. 4). In contrast to the response after Hib LOS, no TNF- α activity was detected in CSF of animals with rrIL-1 β -induced meningeal inflammation (Fig. 4).

Measurement of Rabbit IL-1 β in CSF. CSF rIL-1 β concentrations, when measured by ELISA, were consistently low (range, <30–216 pg/ml) during the 24 h after LOS injections. The largest values were usually observed 6–12 h after intracisternal inoculation with Hib LOS, however, no consistent peaks were detected. Because the rIL-1 β concentrations were unexpectedly low, 36 split CSF samples were independently tested by RIA (43) by Dr. Charles Dinarello, (New England Medical Center, Tufts University School of Medicine, Boston, MA). Those results were similar to the values obtained by ELISA, with no consistent peak concentrations and a range between

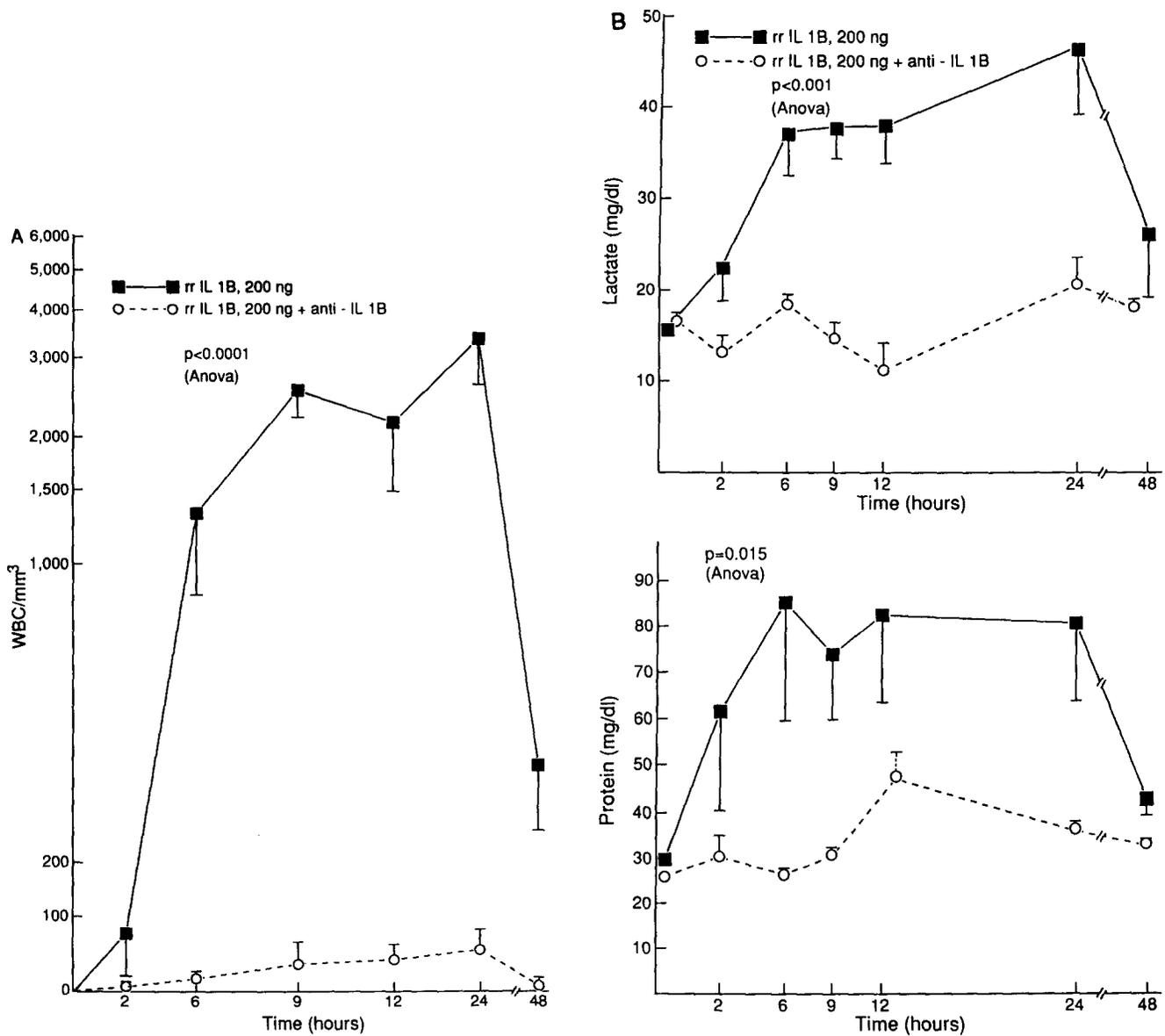


Figure 2. Effect of anti-rrIL-1 β on rrIL-1 β -induced meningitis in rabbits. (A) Effect on CSF WBC counts. (B) Effect on lactate and protein concentrations in CSF.

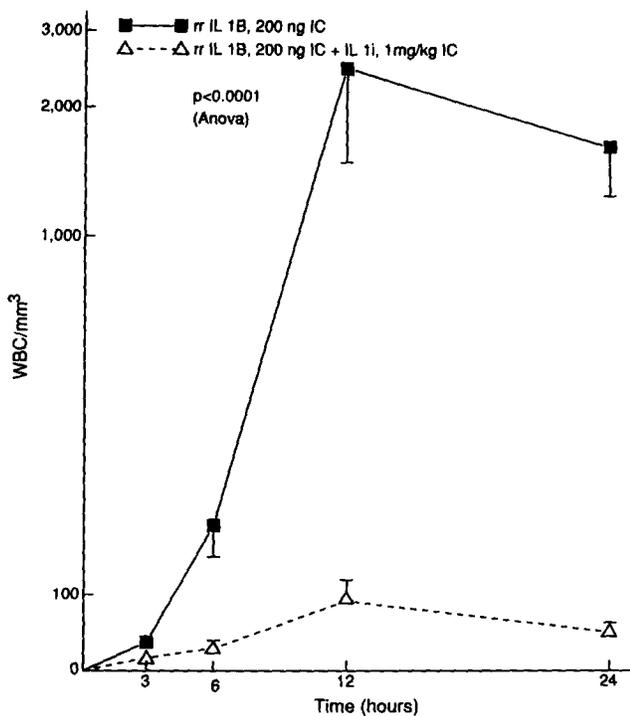


Figure 3. Effect of IL-1i on rrIL-1 β -induced pleocytosis.

56 and 230 pg/ml. Similarly, in rabbits with bacterial meningitis induced by 2×10^4 to 2×10^6 Hib organisms, CSF rIL-1 β concentrations were from <math>< 30</math> pg/ml before, to 352 pg/ml at 6 h after inoculation.

Inoculation of RaTNF. Intracisternal inoculation of 10^2 to 10^5 IU of RaTNF- α was associated with significant CSF pleocytosis. As was observed with rrIL-1 β , CSF WBC counts increased in parallel with increments in RaTNF- α dose, but no significant differences in CSF PMN concentrations were documented after 10^4 or 10^5 IU of RaTNF- α injections (Fig. 5). The timing of the WBC response in CSF after inoculation of RaTNF- α was comparable with that seen after rrIL-1 β , with peak values observed between 12 and 24 h, and persisting pleocytosis for up to 48 h.

Preincubation of 10^4 IU of RaTNF- α with 50 μ l of ascitic fluid containing specific anti-RaTNF- α mAb produced modulation of the inflammatory response induced by RaTNF- α . This was manifested by significantly lower WBC, protein, and lactate concentrations in CSF after inoculation of RaTNF- α and anti-RaTNF- α , compared with RaTNF- α alone (Fig. 6, A and B).

CSF rIL-1 β concentrations were from <math>< 30</math> pg/ml before, to 292 pg/ml after inoculation of RaTNF- α ; although low values were found, all animals had detectable amounts of rIL-1 β in CSF after intracisternal administration of RaTNF- α .

Effect of Anti-rrIL-1 β and Anti-RaTNF- α on Hib LOS-induced Meningitis. The effect of anti-rrIL-1 β and anti-RaTNF- α on Hib LOS-induced meningeal inflammation was assessed in groups of rabbits that received the following intracisternally: (a) 20 ng of Hib LOS + 100 μ l (1:100 dilution) of anti-rrIL-1 β ; (b) 20 ng of Hib LOS + 50 μ l of anti-RaTNF- α ; (c) 20

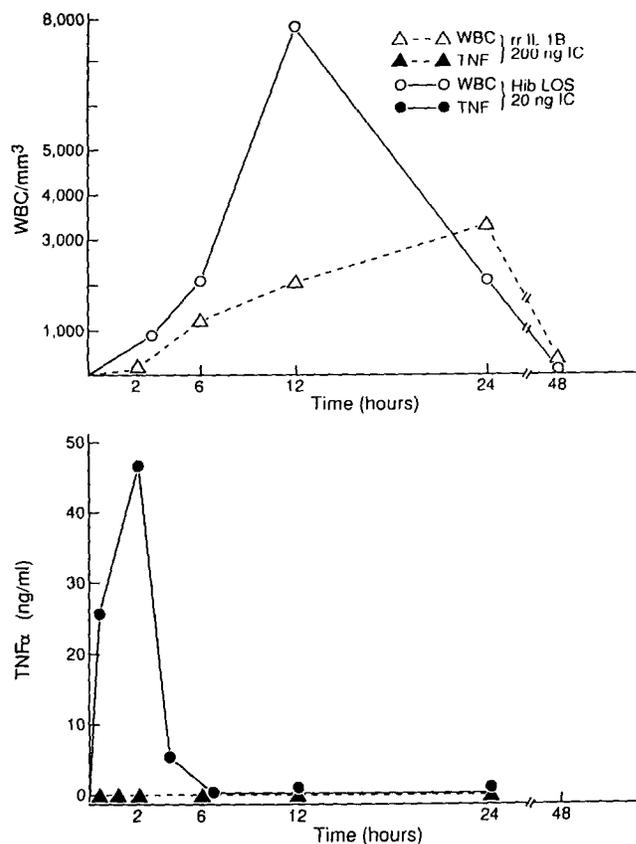


Figure 4. CSF WBC and CSF TNF- α activity after intracisternal inoculation of Hib LOS or rrIL-1 β .

ng of Hib LOS + 100 μ l of anti-rrIL-1 β + 50 μ l of anti-RaTNF- α ; or (d) 20 ng of Hib LOS alone (controls).

In all experiments, the volume of these combinations injected intracisternally was adjusted with pyrogen-free saline to 0.3 ml.

All three treatment regimens (anti-rrIL-1 β , anti-RaTNF- α , and anti-rrIL-1 β + anti-RaTNF- α) had a significant modulatory effect on CSF WBC and lactate concentrations (Fig. 7, Table 1). The most marked attenuation of CSF inflammation was observed in the rabbits treated with both anti-rrIL-1 β and anti-RaTNF- α .

CSF TNF- α activity in the two groups of animals that received anti-RaTNF- α (either alone or in combination with anti-rrIL-1 β) was almost completely suppressed (Fig. 7). Although the meningeal inflammatory response in rabbits treated with anti-rrIL-1 β alone was also significantly reduced at 2 and 6 h, their TNF- α activity in CSF was similar to that of animals given Hib LOS alone.

Combined effect of rrIL-1 β and RaTNF- α . In this set of experiments four groups of rabbits were inoculated intracisternally with one of the following regimens: (a) rrIL-1 β (5 ng); (b) RaTNF- α (10^3 IU); or (c) rrIL-1 β (5 ng) + RaTNF- α (10^3 IU).

Simultaneous administration of 10^3 IU of RaTNF- α and 5 ng of rrIL-1 β resulted in a synergistic inflammatory response manifested by a more rapid and significantly increased influx

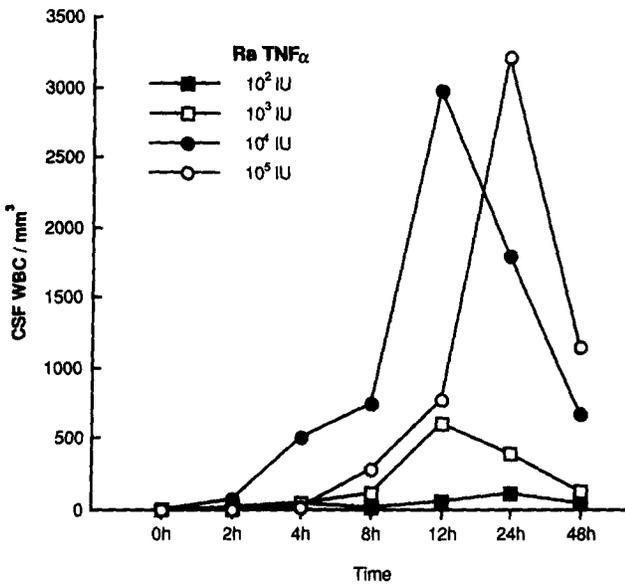


Figure 5. CSF WBC response after varying intracisternal doses of RaTNF- α . Time represents hours after inoculation.

of PMNs into the CSF, compared with each cytokine given alone (Fig. 8). The response to both cytokines was similar to that observed after inoculation of 20 ng Hib LOS. The CSF protein concentrations were significantly larger in rabbits treated with rrIL-1 β + RaTNF- α than in those given either RaTNF- α or rrIL-1 β alone (Table 2). By contrast, CSF lactate concentrations were similar in animals injected with RaTNF- α + rrIL-1 β , and in those inoculated with rrIL-1 β alone, and both groups had values significantly larger than animals that received RaTNF- α alone (Table 2).

Effect of Anti-RaTNF- α on rrIL1 β -induced Meningitis and of Anti-rrIL1 β on RaTNF- α -induced Meningitis. The aim of these experiments was to assess the effect of each antibody on the

CSF inflammatory response generated by the other cytokine. Groups of rabbits were injected intracisternally with the following regimens: (a) rrIL-1 β (200 ng); (b) rrIL-1 β (200 ng) + anti-RaTNF- α (50 μ l); (c) RaTNF- α (10⁴ IU); or (d) RaTNF- α (10⁴ IU) + anti-rrIL-1 β (100 μ l; 1:100 dilution).

Neither antibody exerted a significant modulatory effect on the CSF WBC response induced by the other cytokine (Table 3).

Discussion

Both homologous cytokines, RaTNF- α and rrIL-1 β , when inoculated directly into the subarachnoid space, induced meningeal inflammation in the rabbit meningitis model. The almost complete suppression of the CSF inflammatory changes observed when these cytokines were administered with their respective antibodies demonstrated the specificity of this inflammatory response.

The dose-ranging experiments with rrIL-1 β and RaTNF- α indicated that CSF pleocytosis increased in parallel to increments in cytokine dose, but only to a limited extent. CSF WBC counts generated after intracisternal inoculation of 10⁴ or 10⁵ IU of RaTNF- α were comparable, as were the counts measured after animals were injected with 100 or 200 ng of rrIL-1 β . An analogous dose-limited response was previously observed when purified Hib LOS (5, 7) or Hib OMV (6, 8) were given intracisternally to rabbits or rats. The latter dose-limited inflammatory reactions are likely a reflection of the close interaction of the bacterial components and these two cytokines in the initial steps of meningeal inflammation.

10 ng (5 ng/kg) of rrIL-1 β was required to induce significant and consistent CSF pleocytosis (CSF WBC >1,000/mm³) in our model, the same quantity of rrIL-1 β that was shown to be necessary to elicit a febrile response when injected intravenously into rabbits (43). Likewise, Kawasaki et al. (34) described recently that 3 \times 10² to 3 \times 10³ IU/kg of

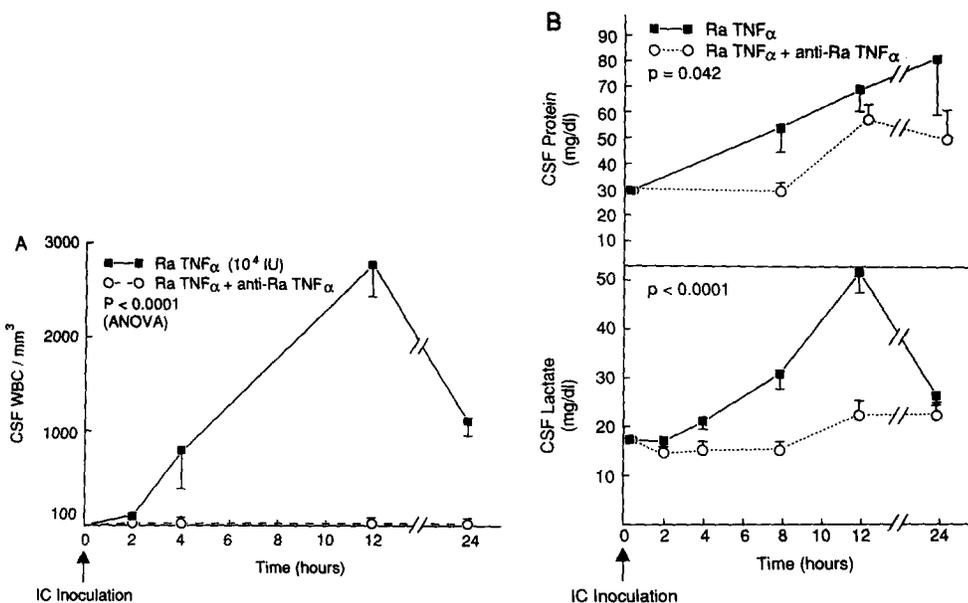


Figure 6. Effect of anti-RaTNF- α on RaTNF- α -induced meningitis in rabbits. (A) Effect on CSF WBC counts. (B) Effect on lactate and protein concentrations in CSF.

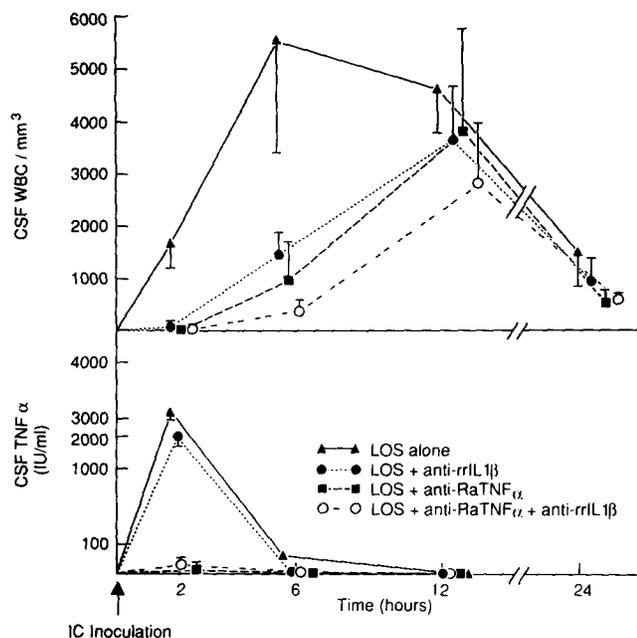


Figure 7. Modulatory effect of anticytokine antibodies on CSF WBC counts (*top*) and on CSF TNF activity (*bottom*) during Hib LOS-induced meningitis. In CSF WBC counts, $p = 0.018$ (ANOVA, over time) in LOS alone (\blacktriangle) vs. LOS + anti-rrIL-1 β (\bullet); $p = 0.026$ in LOS alone (\blacktriangle) vs. LOS + anti-RaTNF- α (\blacksquare); and $p = 0.0023$ in LOS alone (\blacktriangle) vs. LOS + anti-rrIL-1 β + anti-RaTNF- α (\circ). In CSF peak TNF activity (at 2 h), $p = 0.027$ (Mann-Whitney U) in LOS alone (\blacktriangle) vs. LOS + anti-RaTNF- α (\blacksquare); and $p = 0.029$ in LOS alone (\blacktriangle) vs. LOS + anti-RaTNF- α + anti-rrIL-1 β (\circ); $p = 0.114$ in LOS alone (\blacktriangle) vs. LOS + anti-rrIL-1 β (\bullet); and $p = 0.869$ in LOS + anti-RaTNF- α (\blacksquare) vs. LOS + anti-RaTNF- α + anti-rrIL-1 β (\circ).

RaTNF- α evoked a monophasic febrile response, and that 3×10^4 IU/kg was required to produce a biphasic febrile reaction. In our experiments, significant and consistent CSF pleocytosis was attained after intracisternal inoculation of $\sim 10^4$ IU of the same preparation. It is remarkable that these

cytokines possess a similar threshold for fever induction and initiation of meningeal inflammation in the rabbit.

The uniformly delayed appearance of PMNs in the CSF of rabbits with rrIL-1 β - or RaTNF- α -induced meningitis compared with results in Hib LOS-induced meningitis was unexpected. These findings contrast with those using cytokines of human origin in the rabbit and rat models (27, 28), which demonstrated a faster response. However, when low doses of rrIL-1 β and RaTNF- α were given concomitantly, a significant synergistic effect in provoking a more rapid and increased migration of leukocytes into the CSF was observed. The time pattern of this response was similar to that demonstrated in rabbits after intracisternal inoculation of Hib LOS (Fig. 8). We believe these findings suggest that each of these two cytokines, rrIL-1 β and RaTNF- α , alone is capable of initiating the sequence of events that leads to meningeal inflammation. The participation of both cytokines appears to occur in vivo after bacteria reach the subarachnoid space in naturally occurring bacterial meningitis, or after Hib LOS is introduced intracisternally.

Additional evidence for the required involvement of both cytokines is provided by the experiments in which anti-rrIL-1 β and anti-RaTNF- α were utilized to modulate Hib LOS-induced meningeal inflammation. Each antibody inoculated separately or together with Hib LOS exerted a significant modulatory action at 2 and 6 h. Elimination of CSF TNF- α activity was not a prerequisite to attenuate inflammation, as shown by the experiments in which LOS and anti-rrIL-1 β were given. The modulatory effect of these antibodies lasted for at least 6 h, and was not observed at 12–14 h after inoculation. This is likely explained by binding of the antibodies to their respective cytokines and their subsequent elimination from the subarachnoid space in the presence of LOS that can be measured in CSF for at least 12 h after inoculation (unpublished observations). We did not give repeated doses of antibody in these experiments.

Despite the use of amounts of antibody known to be effective in reducing Hib LOS-associated inflammation and in sup-

Table 1. Effect of Anticytokine Antibodies on CSF Lactate Concentrations in Animals with Hib LOS-induced Meningitis

Inoculated material(s)	CSF Lactate concentrations at:				
	0 h	2 h	6 h	12 h	24 h
	<i>mg/dl</i>				
LOS alone	19.0 \pm 1.0*	27.5 \pm 1.7	37.5 \pm 2.6	38.0 \pm 2.6	25.6 \pm 2.1
LOS + anti-rrIL-1 β	15.8 \pm 0.6	19.3 \pm 1.7	38.0 \pm 5.6	34.2 \pm 2.6	23.2 \pm 0.7 [†]
LOS + anti-RaTNF- α	13.7 \pm 0.8	17.0 \pm 1.9	28.4 \pm 7.3	36.6 \pm 3.1	20.9 \pm 2.2 [‡]
LOS + anti-rrIL-1 β + anti-RaTNF- α	14.5 \pm 1.6	16.3 \pm 1.7	21.7 \pm 3.0	33.2 \pm 4.5	20.0 \pm 1.2 [§]

Doses: Hib LOS: 20 ng; anti-rrIL-1 β , 100 μ l of 1:100 dilution; anti-RaTNF- α , 50 μ l.

* Lactate expressed as mean \pm SEM.

[†] $p = 0.04$ (ANOVA, over time) when compared with LOS alone.

[‡] $p = 0.0034$, when compared with LOS alone.

[§] $p = 0.0001$, when compared with LOS alone.

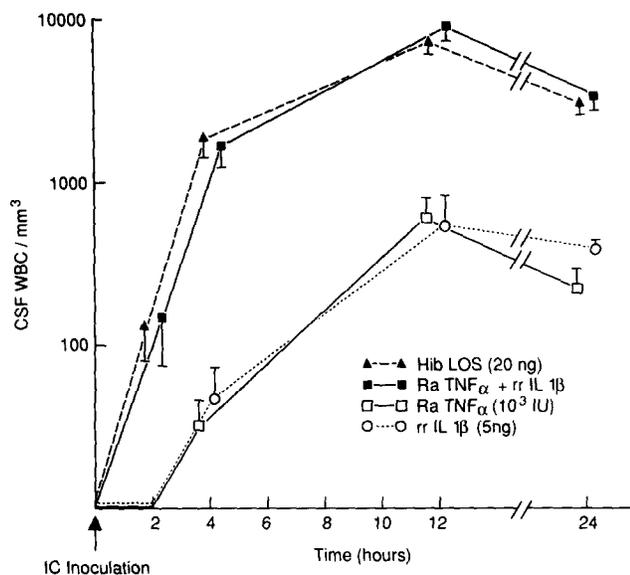


Figure 8. CSF WBC counts after intracisternal inoculation with RaTNF- α , rrIL-1 β , or RaTNF- α and rrIL-1 β . CSF WBC counts for the Hib LOS group represent the mean values of eight rabbits evaluated throughout the study. $p < 0.0001$ (ANOVA, over time) in RaTNF- α + rrIL-1 β (■) vs. RaTNF- α alone (□), and vs. rrIL-1 β alone (○).

pressing the effect of its respective cytokine, we were surprised that there was no inhibitory action of anti-rrIL-1 β on RaTNF- α -induced meningitis and of anti-RaTNF- α on rrIL-1 β -induced meningitis. This observation emphasizes again that both cytokines are capable of independently initiating

Table 3. CSF WBC Counts after Intracisternal Inoculations with Various Cytokines and Anticytokine Antibodies

Inoculated material(s)	CSF WBC at:		
	6 h	12 h	24 h
rrIL-1 β	195 \pm 63*	3,312 \pm 926	2,875 \pm 1,081†
rrIL-1 β + anti-RaTNF- α	135 \pm 52	1,800 \pm 531	3,100 \pm 601‡
RaTNF- α	92 \pm 33	1,480 \pm 713	1,725 \pm 325§
RaTNF- α + anti-rrIL-1 β	77 \pm 42	1,525 \pm 280	1,525 \pm 189§

* CSF WBC per mm³ as mean \pm SEM.

† ANOVA over time, $p = 0.419$.

‡ ANOVA over time, $p = 0.839$.

events that lead to meningeal inflammation. Although neither cytokine is dependent on the presence of the other for inflammatory activity, when combined, they act synergistically.

Our results confirm, in part, and extend observations based primarily on the use of recombinant human molecules in the rat and rabbit models. The actual role of TNF- α in meningeal inflammation has been debated. We showed previously that TNF- α is detected in the CSF of rabbits injected intracisternally with Hib LOS, and that the peak concentration of TNF- α at 2 h after inoculation precedes the appearance of PMNs in CSF (24). In addition, a significant correlation

Table 2. Combined Effect of rrIL-1 β and RaTNF- α on Protein and Lactate Concentrations in CSF

Inoculated material(s)	CSF protein concentrations					
	0 h	2 h	4 h	8 h	12 h	24 h
	<i>mg/dl</i>					
rrIL-1 β (5 ng)	26.2 \pm 3.7*	33.7 \pm 9.4	30.5 \pm 12.0	22.2 \pm 4.9	41.2 \pm 7	40.0 \pm 2.1
RaTNF- α (10 ³ IU)	26.2 \pm 1.1	26.7 \pm 4.9	23.5 \pm 2.3	36.0 \pm 9.9	49.3 \pm 6.7	34.2 \pm 5.2
rrIL-1 β + RaTNF- α	26.7 \pm 0.7	38.2 \pm 12.7	45.2 \pm 14.9	32.0 \pm 7.3	76.7 \pm 11	46.2 \pm 15.5‡
	CSF lactate concentrations					
	0 h	2 h	4 h	8 h	12 h	24 h
	<i>mg/dl</i>					
rrIL-1 β (5 ng)	21.3 \pm 1.9*	15.6 \pm 0.7	21.3 \pm 1.5	29.3 \pm 1.9	49.4 \pm 2.3	32.2 \pm 3.8
RaTNF- α (10 ³ IU)	18.2 \pm 0.8	14.9 \pm 0.7	17.4 \pm 0.9	18.2 \pm 0.6	28.2 \pm 1.9	32.2 \pm 5.2
rrIL-1 β + RaTNF- α	18.8 \pm 0.9	17.1 \pm 1	24.1 \pm 1.6	28.4 \pm 1.1	47.4 \pm 2.3	33.2 \pm 3.7§

* Mean \pm SEM.

† $p = 0.0085$ (ANOVA, over time), when compared with rrIL-1 β alone; and $p = 0.0016$ when compared with RaTNF- α alone.

‡ $p < 0.001$ (ANOVA, over time), when compared with RaTNF- α alone; and $p = 0.992$ when compared with rrIL-1 β alone.

between CSF TNF- α concentrations and the CSF inflammatory indices was demonstrated, and more importantly, intracisternal administration of a polyclonal antibody to recombinant human TNF- α (rhTNF- α) in LOS-induced meningitis caused a significant attenuation of the CSF inflammatory response (24). However, rhTNF- α failed to induce CSF pleocytosis and increased blood-brain barrier permeability when tested in the rat model (44), and we were unable to induce meningeal inflammation after intracisternal inoculation of up to 150 μ g of rhTNF- α (unpublished observations). By contrast, Saukkonen et al. (28), using rhTNF- α in rabbits, observed a marked inflammatory response with a dose of 10^4 IU. The discrepancies between their results and ours might be explained by differences in preparation and storage of the rhTNF- α . We believe that the present data, using the natural rabbit molecule of RaTNF- α , establish clearly a prominent role of TNF- α in the inflammatory cascade.

Two biochemically distinct but structurally related IL-1 molecules (IL-1 α and IL-1 β) have been cloned in humans (12) and also in rabbits (32, 43). There is only 26% amino acid homology between human IL-1 α (hIL-1 α) and human IL-1 β (hIL-1 β), whereas there is 74% homology between hIL-1 β and rIL-1 β . Receptors recognize α and β forms, and both are thought to have similar biological effects. mRNA coding for IL-1 β predominates over that coding for IL-1 α (12, 43). This prevalence is also observed when molecules are measured in body fluids. It is believed that IL-1 β acts as a soluble mediator, whereas IL-1 α remains mostly cell associated (45).

The low rIL-1 β concentrations detected by the ELISA system in CSF of rabbits with LOS-induced meningitis or after intracisternal inoculation of Hib bacteria contrast with the values previously reported by Wispelwey et al. (25), using a bioassay on D10.G.41 cells. They found 10–20 times larger values with a clear peak of activity at 30 min post-inoculation, a kinetic response similar to that demonstrated previously with TNF- α (24). Likewise, Waage et al. (22), by means of a two-step assay on T cell lines NOB and HT2, reported peak CSF IL-1 β concentrations of 600 pg/ml (two to three times our levels), 2 h after the TNF- α peak and 4 h after intracisternal injection with 500 ng of meningococcal LPS. The discrepancy between our data and those of Wispelwey et al. (25) and Waage et al. (22) prompted us to verify our results. Split CSF samples were sent to Charles Dinarello's laboratory, who, using a

specific RIA for rIL-1 β and a different polyclonal antibody to rrIL-1 β (43), confirmed our findings that the concentrations of rIL-1 β in CSF of our animals were low and that there were no clear peak values. The bioassays used by Wispelwey et al. (25) and Waage et al. (22) measured total IL-1 activity, which included IL-1 α and IL-1 β , whereas the ELISA and RIA systems detected only the immunoreactive rIL-1 β , which based on the available information in the rabbit, was expected to be the predominant form (43). Additional studies are required to resolve this discrepancy.

The results with the rabbit recombinant preparation substantiated the important participation of IL-1 β in meningitis. Earlier studies by Quagliariello et al. (27), in the rat model, demonstrated that hrIL-1 β induced increased blood-brain barrier permeability and CSF pleocytosis. The same molecule (hrIL-1 β), however, did not provoke a significant response in rabbits (28), but a very marked inflammatory reaction was seen after injections with hrIL-1 α . The homologous cytokine, rrIL-1 β , elicited a clear inflammatory response in this rabbit model, and its specific antibody, anti-rrIL-1 β , attenuated Hib LOS-induced meningeal inflammation.

Another facet of the complex interactions of IL-1 β and TNF- α is the capacity of each cytokine to stimulate production of the other. Using a very sensitive cytolytic assay, we could not detect any CSF TNF- α activity in rabbits with rrIL-1 β -induced meningitis. By contrast, rIL-1 β was present in low concentrations in CSF of animals with RaTNF- α -induced meningitis. This confirms previous *in vitro* studies by Dinarello et al. (46), in which TNF- α elicited IL-1 production from human mononuclear cells. Recently, Fong et al. (47) demonstrated that passive immunization against TNF- α reduced the appearance of IL-1 β and IL-6 in experimental lethal bacteremia.

Further studies will be required to elucidate the intricate interactions of TNF- α and IL-1 β and other cytokines, such as IL-6 (22), MIP-1, and MIP-2 (28) in the meningeal inflammatory cascade. The activities of these mediators on cerebral capillary endothelial cells (i.e., blood-brain barrier) (48) and the role of specific cytokine and receptor inhibitors will provide a more precise delineation of the molecular events that occur in bacterial meningitis. We believe our data provide evidence for a seminal role of TNF- α and IL-1 β in the initial events of meningeal inflammation.

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