Listeria Meningitis: Identification of a Cerebrospinal Fluid Inhibitor of Macrophage Listericidal Function as Interleukin 10

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Summary

The killing of bacteria gaining access to the central nervous system is insufficient and requires bactericidal antibiotics for treatment. The inefficient host response in cerebrospinal fluid (CSF) is thought to be due to impaired phagocytosis in CSF, and low local concentration of antibody and complement. In addition, the CSF may contain inhibitors, disabling phagocytes to eliminate bacteria. We have assessed the bactericidal activity of macrophages in the presence of CSF from mice infected intracerebrally with *Listeria monocytogenes* (LM). Pretreatment of J774A.1 macrophages with interferon γ (IFN-γ) resulted in high levels of nitric oxide–dependent intracellular killing of LM. CSF taken from mice 24 h after infection (CSF-LM 24) contained IFN-γ and induced killing of LM by macrophages. However, pulsing J774A.1 cells with IFN-γ in the presence of CSF obtained from mice at later time points (48 h) rendered macrophages partly permissive for intracellular *Listeria* growth. The inhibitor detected in CSF-LM 48 was identified as IL-10 since: (*a*) IL-10 dose dependently impaired the listericidal activity of IFN-γ–activated macrophages; (*b*) anti-IL-10 antibodies abrogated the bacterial growth permissive effect of CSF-LM 48; and (*c*) IL-10 was detected in CSF-LM 48 but not in CSF-LM 24 or CSF of mock-injected animals (CSF-Co). Likewise, IL-10 was found in the CSF of 95% of patients with bacterial meningitis.

Among the factors involved in the high morbidity and mortality rates of bacterial meningitis, the low level of bacterial clearance and the intensity of the inflammatory events in the cerebrospinal fluid (CSF)[1] and meninges may be decisive. The meningeal inflammatory response is elicited by cytokines and arachidonic acid metabolites produced upon stimulation with bacterial compounds intrathecally by invading leukocytes, meningeal macrophages, and microglia as well as astrocytes in brain and spinal cord tissues (for review see reference 1). TNF-α, IFN-γ, IL-1, IL-6, IL-8, M-CSF, G-CSF, and GM-CSF can be detected in the CSF in infectious meningitis (2–9). Intracerebroventricular (i.c.) injection of TNF-α, IL-1β, MIP-1, and MIP-2 causes CSF pleocytosis and brain edema (10, 11). On the other hand, treatment with anti-TNF-α and anti-IL-1β antibodies were found to reduce meningeal inflammation and to be protective in animal models of bacterial meningitis (10, 11). Thus, intrathecally produced cytokines may play a key role in the development of tissue damage in infectious meningitis.

Much less clear, however, are the mechanisms, which despite of the accumulation of large numbers of PMN hinder the numeric reduction of viable bacteria in the CSF compartment (12). Surface phagocytosis, a major host defense mechanism against unopsonized pneumococci within alveoli (13) is poor in the fluid medium of the CSF (14). Furthermore, the relative lack of complement activity in CSF and the low penetration of opsonic antibodies through the blood–brain barrier have been thought to contribute to inefficient host defense mechanisms displayed within the CSF (for review see reference 14). In meningitis caused by intracellular pathogens, e.g., *Listeria monocytogenes* (LM) host factors present in CSF may interfere with macrophage-mediated killing. In the search for macrophage deactivating factors IL-10 has been described to suppress the synthesis of nitric oxide (NO) and reactive oxygen intermediates (ROI), two molecules involved in the antimicrobial defense system (15, 16). In this report we show that IL-10 accumulates in CSF of

[1] *Abbreviations used in this paper:* CSF, cerebrospinal fluid; i.c., intracerebroventricular; LCMV, lymphocytic choriomeningitis virus; LM, *Listeria monocytogenes*; NO, nitric oxide; ROI, reactive oxygen intermediates.

mice with *Listeria* meningitis and impairs the IFN-γ–mediated killing of LM by macrophages.

Materials and Methods

Mice. 6–10-wk-old outbred female ICR mice were obtained from the breeding colony of the Institut für Zuchthygiene (Tierspital Zürich, Switzerland).

Listeria Monocytogenes and Lymphocytic Choriomeningitis Virus. The infectious agents used were kindly provided by Dr. R.M. Zinkernagel (Institute of Experimental Immunology, University Hospital, Zürich, Switzerland). A seed of LM, strain EGD, was kept virulent by passage through mice. Frozen stocks were used to prepare a fresh overnight culture at 37°C giving 1–2 × 10^9 CFU in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) for each experiment. The infectious dose was assessed retrospectively by plating each inoculum. LM and lymphocytic choriomeningitis virus (LCMV) Armstrong were diluted in DMEM containing 1% heat-inactivated FCS.

Reagents. Murine rIL-6 and rIFN-γ were purchased from Boehringer Mannheim (Rotkreuz, Switzerland); rIL-4 and human rM-CSF from Genzyme Corp. (Cambridge, MA). Murine rIL-10 and human rIL-10 were obtained from Pepro Tech., Inc. (Rocky Hill, NJ). Murine rTNF-α generated by Genentech (San Francisco, CA) was kindly provided by Dr. G.R. Adolf (Boehringer-Ingelheim, Vienna, Austria), murine rIL-1β by Dr. A. Shaw (Glaxo, Geneva, Switzerland). Anti–mouse IL-10 mAb (SXC1) and anti–mouse IL-6 mAb (MP5-20F3) were obtained by Pharmingen (San Diego, CA) and a monoclonal hamster anti–mouse IFN-γ Ab by Genzyme. N^G-monomethyl-L-arginine (N^GMA) was purchased from Calbiochem-Behring Corp. (La Jolla, CA), L-arginine and sodium nitrite from Sigma Immunochemicals (St. Louis, MO).

Infection and Harvesting of Blood and CSF. Animals were inoculated intracerebrally with either ~10^3 CFU of LM or 10^2 PFU of LCMV Armstrong as described (2). On days indicated, mice were ether anesthesized and perfused with Ringer solution (Braun Medical AG, Emmenbrücke, Switzerland). The serum was obtained after centrifugation of blood in Microtainers® (Becton Dickinson & Co., Rutherford, NY) and CSF was collected as described by Carp et al. (17); CSF samples of 2 to 10 animals were pooled. After centrifugation, cells in the pellets were counted and supernatants were used in the respective assay.

IL-10 Assays. Murine IL-10 was assayed using a commercially available ELISA (Endogen, Inc., Boston, MA). The limit of detection of IL-10 in CSF and serum samples from mice was 5 U/ml. In human CSF, IL-10 was measured by bioassay using murine MC/9 mast cells as recently described (18). Briefly, MC/9 cells (obtained from the American Type Culture Collection, Rockville, MD) were seeded in 96-well microtiter F plates in RPMI 1640 supplemented with 10% FCS, 5 × 10^{-5} M 2-ME, 1 mM L-glutamine, antibiotics, and murine rIL-4 (3 ng/ml). The cells were cultured at a density of 2 × 10^3 cells per well in the presence of serial dilutions of CSF or human rIL-10 as a standard. For the final 16 h of the 4-d culture, the cells were pulsed with 1 μCi (^{3}H)thymidine (5 Ci/mmol; Amersham Corp., Arlington Heights, IL). Results were expressed in pg/ml in reference to the standard and the detection limit was found to be 100 pg/ml. To characterize the IL-10–like activity in human CSF, a neutralizing anti–mouse IL-10 mAb (SXC1) that crossreacts with human IL-10, was used. After a 3-h incubation of the samples with the mAb (final concentration 1 μg/ml) at 37°C, the residual activity was determined in the bioassay. The following human recombinant cytokines were tested in the MC/9 bioassay in the range of 0.1–10 ng/ml and found to be negative: IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, M-CSF, GM-CSF, G-CSF, IFN-γ, TNF-α, and TGF-β2 (data not shown).

Cytokine Assays. IL-6 was determined using 7TD1 cells as recently described (3). IFN-γ and TNF-α were measured with commercially available ELISAs from Holland Biotechnology (Leiden, The Netherlands) and Genyzme, respectively.

Listeria Monocytogenes Killing In Vitro. The murine macrophage cell line, J774A.1, obtained from American Type Culture Collection, was plated on sterile glass coverslips (9-mm diam) in petri dishes with four inserts (35/10 mm; Greiner GmbH, Nürtlingen, Germany) at a density of 4 × 10^5 cells/coverslip in 150 μl antibiotic-free DMEM containing 5% FCS and 1 mM L-glutamine. After overnight culture, the confluent monolayers were washed once in fresh medium and then cultured in the absence or presence of murine rIFN-γ and/or cytokines, CSF, and antibodies, as noted. The CSF of mice infected with LM (CSF-LM) were sterile filtered (0.22 μm; Acrodisc No. 4192; Gelman Sciences, Ann Arbor, MI) before using. In selected experiments the CSF was incubated with anti-IFN-γ, anti-IL-10, or anti-IL-6 antibodies before testing as described above. 20 h later the macrophages were infected with 4 × 10^5 live bacteria in 25 μl for 45 min. After this phagocytosis period, cover slips were washed five times with warm HBSS and were either reincubated for 8 h in fresh medium containing the respective stimuli (t_8) or the number of intracellular bacteria were assayed microbiologically immediately (t_0) as described recently (19). Sample coverslips, in duplicate, were taken and deposited into plastic tubes containing 5 ml sterile distilled water. Cells were lysed by vigorously vortexing for 20 s and a brief sonication for 10 s, which, in pilot experiments, was found to be innocuous for LM. Serial dilutions were plated in triplicate on brain heart infusion agar (BBL Microbiology Systems, Cockeysville, MD) plates. LM killing was determined by counting the number of CFU per coverslip 24 h later. The number of LM in the supernatants was also determined by plating on agar, the CFU measured never exceeded 10% of the cell-associated bacteria.

Determination of Nitrite Production. Nitrite determinations were made on 50-μl aliquots of sample mixed with 200 μl of the Griess reagent (20). The absorbance was read at 540 nm (Flow ELISA reader) after 10 min of reaction and NO_2^- concentration was determined with reference to a standard curve using concentrations from 1 to 250 μM sodium nitrite in culture medium.

Patients. From October 1988 to January 1993 human CSF samples collected from children with infectious meningitis on admission to the University Children's Hospital (Zürich), were tested for IL-10 in a blind fashion. After collection of CSF the samples were divided in different portions for bacteriological and viral work up, chemistry, and cytokine determination as described (4).

Results

Intracerebral inoculation of mice with LM resulted in first signs of disease after 8–12 h and the death of the animals within 4 d. Pleocytosis in CSF (≥70 cells/mm^3) was noted as early as 4 h after i.c. inoculation and peaked with values of around 10^4/mm^3 after 48 h (Fig. 1 *A*). During the course of LM infection, the expression of cytokines in CSF was monitored. IL-10 was first detected in CSF taken at 24 h, and maximum concentrations were observed 72 h after inoculation (Fig. 1 *C*). Unlike CSF the serum of mice with LM lacked detectable IL-10 at any time point after i.c. LM inoculation. The presence of IL-10 in CSF-LM was not only ascertained by ELISA but also by bioassay on MC/9 cells using a neu-

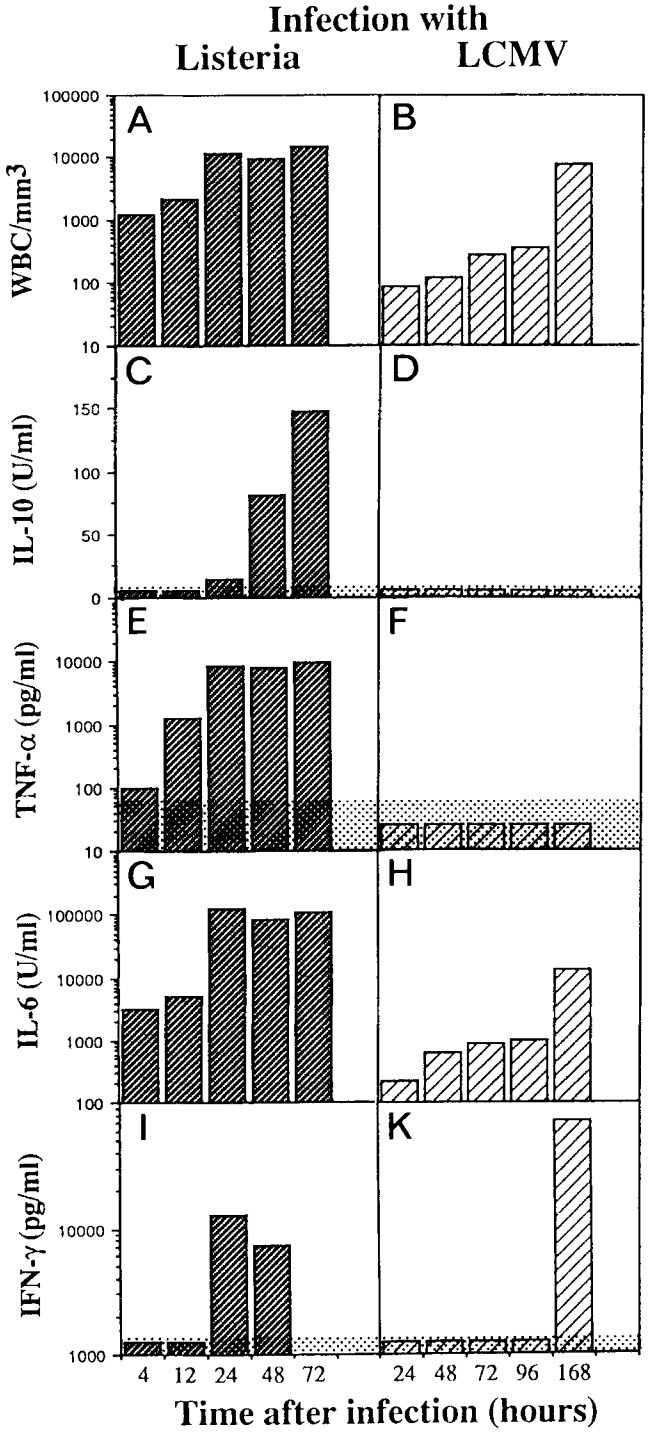

Figure 1. IL-10 is produced intrathecally in bacterial but not viral meningitis. White blood cell counts (*A* and *B*) and pattern of expression of IL-10 (*C* and *D*), TNF-α (*E* and *F*), IL-6 (*G* and *H*), IFN-γ (*I* and *K*) in CSF from mice with LM (▨) and LCMV (◰) meningitis. Shaded area delineate values below the detection limit of the cytokine assay.

tralizing anti-IL-10 antibody (SXC1) (data not shown). In contrast to IL-10, the cytokines TNF-α, IL-6, and IFN-γ appeared in CSF-LM within hours after i.c. inoculation, reaching maximal levels at 24 h (Fig. 1). Thus, compared to the other cytokines investigated, IL-10 appeared only relatively late in the course of infection. The IL-10 concentrations in the CSF differed vastly between mice infected with LM and LCMV. In contrast to what was found in LM infected mice, IL-10 could not be detected in the CSF at any time from i.c. inoculation with LCMV till death on day 7 to 8 (Fig. 1). Besides, IL-10 CSF samples from LCMV-infected mice also lacked TNF-α but were positive for IL-6 and IFN-γ (Fig. 1). The latter data confirm our previous results in these two animal model infections (2, 3).

The listericidal function of macrophages was tested in vitro by using the murine macrophage cell line J774A.1. Macrophages were treated with cytokines and/or CSF 20 h before infection with LM. In untreated J744A.1 cells the survival of LM was followed by bacterial multiplication over a time period of 8 h. Minimal bactericidal effects were observed after pretreatment of macrophages with TNF-α, IL-1β, IL-6, and M-CSF, respectively. However, treatment of macrophages with IFN-γ resulted in high level of intracellular killing of LM (Fig. 2). The effect of IFN-γ on the listericidal activity of J744A.1 cells was enhanced by TNF-α, but not by IL-1β, IL-6, or M-CSF. As shown in Fig. 2 the guanidino-methylated derivative of L-arginine N^GMA, a competitive inhibitor of the L-arginine–dependent macrophage effector pathway, virtually abolished the potent bactericidal effect of IFN-γ–treated J774A.1 cells. The inhibitory effect of N^GMA on activated macrophages was completely reversed by 5 mM of supplemental L-arginine to the culture medium (Fig. 2). Furthermore, IFN-γ was found to stimulate nitrite production by LM-infected J774A.1 cells (Fig. 3). This is in agreement with

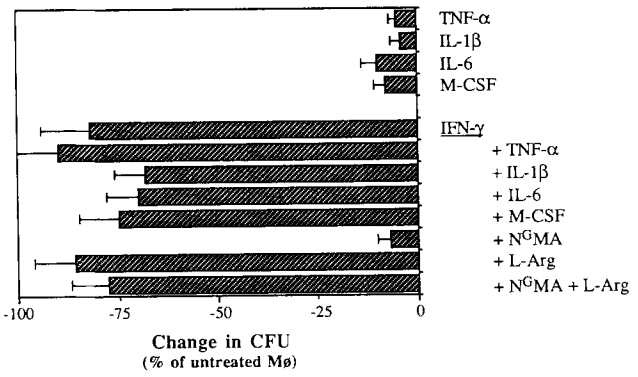

Figure 2. The killing of *Listeria* by macrophages depends on IFN-γ, TNF-α and production of NO. J774A.1 cells were treated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml) or M-CSF (100 U/ml). The latter cytokines were also tested for their listericidal effect in the presence of IFN-γ (200 U/ml). Data are expressed as change in CFU detected intracellularly in J774A.1 cells 8 h (t_8) after infection with LM compared with the CFU values obtained in macrophages not pretreated. In the absence of IFN-γ J774A.1 cells contained 29.6 ± 1.8 × 10^3 CFU of LM at time point zero (t_0) and 2.7 ± 0.4 × 10^6 CFU at t_8. The respective t_8 value in IFN-γ-pretreated macrophages was 0.5 ± 0.1 × 10^6 CFU. Results are expressed as the mean values ± SD from triplicate experiments with duplicate cultures for each group. The influence of NO molecules was tested by the use of N^GMA (1 mM) and L-arginine (5 mM) either alone or in combination.

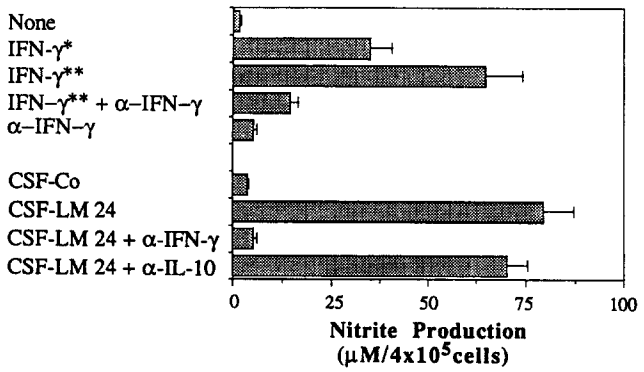

Figure 3. IFN-γ present in CSF of LM-infected mice stimulates NO production by J774A.1 cells. Nitrite production by J774A.1 cells was measured after 20 h of incubation with IFN-γ (*, 20 U/ml; **, 200 U/ml) or CSF as described in Materials and Methods. The CSF were assayed at final concentrations of 20%. CSF-Co refers to CSF samples obtained from mice 24 h after i.c. injection with DMEM containing 1% FCS. CSF-LM 24 was taken from animals at 24 h after i.c. injection with LM. CSF-LM 24 was incubated with anti-IFN-γ mAb (20 μg/ml) or anti-IL-10 mAb (1 μg/ml) for 3 h at 37°C before testing on J774A.1 cells. Results are expressed as the mean values ± SD from one experiment performed in duplicate cultures.

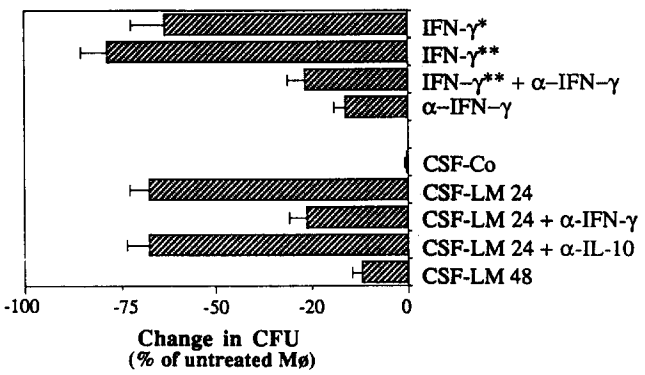

Figure 4. IFN-γ present in CSF obtained from mice early in the course of *Listeria* meningitis augments the listericidal activity of macrophages. IFN-γ (*, 20 U/ml; **, 200 U/ml), CSF-Co, or CSF-LM 24 were added to J774A.1 cells; the killing of LM by the macrophages was assessed 8 h later (see legend to Fig. 2). The CSF samples used corresponded to those described in legend to Fig. 3 and were also tested at 20% in the assay. Results are expressed as the mean values ± SD from one experiment performed in duplicate cultures.

recent data showing production of NO by SCID spleen cells incubated with heat-killed LM (21). The production of NO was dependent on the release of IFN-γ by SCID NK cells. In vivo administration of N^GMA resulted in increased mortality and spleen bacterial loads of LM-infected mice (21). It can be concluded that NO is a critical effector molecule in the killing of LM by IFN-γ–activated macrophages.

We next examined the effect of CSF from mice with LM on NO production. A 20-h incubation of J774A.1 cells with CSF obtained 24 h after intracerebral infection with LM (CSF-LM 24) resulted in high levels of nitrite, a stable oxidative metabolite of NO (Fig. 3). Neutralization experiments indicate that the effect observed depends on IFN-γ present in CSF-LM 24. Unlike anti-IFN-γ antibodies an anti-IL-10 antibody failed to neutralize the capacity of CSF-LM 24 to induce NO production. Control CSF from mice injected intracerebrally with DMEM containing 1% FCS (CSF-Co) was negative for IFN-γ (data not shown) and did not induce nitrite formation (Fig. 3).

Since CSF-LM 24 contains IFN-γ (Fig. 1) in a bioactive form able to trigger NO production (Fig. 3), it was not unexpected to observe killing of LM by J774A.1 cells treated with CSF-LM 24 (Fig. 4). The effect was neutralizable with anti-IFN-γ antibodies but not with anti-IL-10 antibodies and did not occur with CSF-Co. Unexpectedly, however, was the finding that CSF taken from mice 48 h after infection (CSF-LM 48) harbors IFN-γ (Fig. 1) but did not induce a listericidal activity in J774A.1 cells (Fig. 4).

Because of (*a*) recent reports on IL-10 as a macrophage-deactivating factor (15, 16) and (*b*) the presence of IL-10 in CSF at late time points after infection, we examined the effect of IL-10 on the listericidal function of J774A.1 cells. As shown in Fig. 5, IL-10 profoundly inhibited the intracellular killing of LM by IFN-γ–treated macrophages. Half maximal inhibition was obtained with IL-10 at concentrations of ~2 ng/ml. CSF taken 48 h from mice infected with LM mimicked the effect of IL-10: a final dilution of the CSF-LM 48 of 1:10 suppressed the killing of LM by ~70% (Fig. 5). The extent of inhibition is compatible with the amount of IL-10 measured in CSF-LM 48 by the IL-10 ELISA. While pretreatment of CSF-LM 48 with an anti-IL-10 antiserum completely neutralized the inhibitory activity in the CSF, anti-IL-6 antibodies being used as a control had no such effects (Fig. 5). These data show that: (*a*) IL-10 is the CSF mediator responsible for the suppression of *Listeria* killing by CSF taken late in the course of infection; and (*b*) that other molecules in CSF-LM 48 like IFN-γ and TNF-α neither contribute to

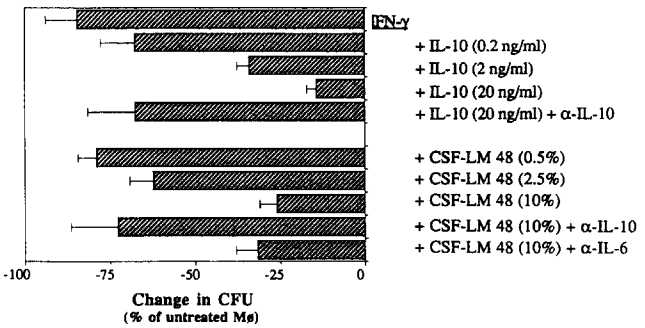

Figure 5. At a later time point of *Listeria* infection IL-10 interferes with the listericidal activity of IFN-γ. Different concentrations of IL-10 and CSF-LM 48 were added together with IFN-γ (200 U/ml) to J774A.1 cells. CSF-LM 48 refers to CSF taken from mice 48 h after i.c. infection with LM. The effect of anti-IL-10 (1 μg/ml) and anti-IL-6 (1 μg/ml) antibodies were evaluated by preincubating CSF-LM 48 with the antibodies prior to testing on J774A.1 cells (see Materials and Methods). The data shown here give mean values ± SD of duplicate cultures in a representative experiment.

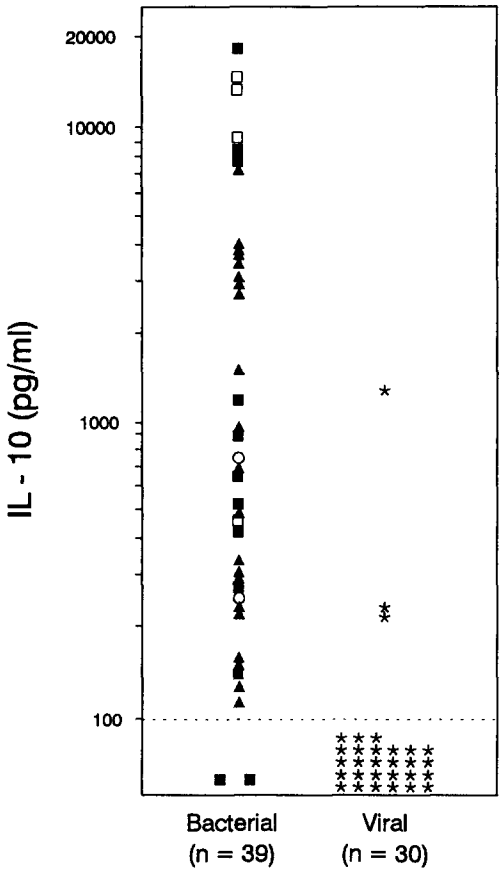

Figure 6. IL-10 is present in CSF of children with bacterial but not viral meningitis. IL-10 concentrations were measured in CSF of 39 children with bacterial and 30 children with viral meningitis. (▲) *H. influenzae* (n = 21); (■) *N. meningitidis* (n = 12); (□) *S. pneumoniae* (n = 4); (○) *E. coli* (n = 1) or *S. mucilagenosus* (n = 1).

nor counteract the macrophage inhibitory effect of IL-10 in CSF-LM.

The results on IL-10 in experimental meningitis in mice are paralleled by detection of IL-10 in children with bacterial meningitis. IL-10 was detected in 37 out of 39 (95%) children with bacterial meningitis caused by *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Stomatococcus mucilagenosus*, and *Escherichia coli* (Fig. 6). The concentrations of IL-10 in CSF ranged from 111 to 18,451 pg/ml. In contrast to bacterial meningitis, IL-10 was found only in CSF of 3 (10%) out of 30 children with viral meningitis. These results are in line with the findings in the two model infections in mice showing IL-10 to be present in LM but not in LCMV-induced meningitis (Fig. 1). Contrasting findings in CSF from bacterial and virus-induced disease has also been noted in studies on TNF-α and IL-1 which are detectable in bacterial but not viral infections (2, 4, 5).

Discussion

This study on *Listeria* meningitis shows a finding relevant to the understanding of the paradox of unrestricted bacterial growth in CSF despite of the presence of large numbers of leukocytes (12). We have identified in the CSF of mice with severe *Listeria* meningitis an inhibitor of the listericidal function of macrophages. In keeping with previous findings by others (21) the killing of the facultative intracellular pathogen LM by IFN-γ–activated macrophages depends on production of NO. CSF of mice with *Listeria* meningitis contains IFN-γ and TNF-α, two cytokines which strongly promote LM killing by macrophages. Indeed, CSF taken 24 h after intracerebral infection with LM induced a potent listericidal state when added to J744A.1 macrophages. Since this effect was almost completely neutralized by anti-IFN-γ antibodies, IFN-γ is the mediator responsible for the effect observed. However, at later time points (48 h) after infection, the effect of IFN-γ is overcome by an inhibitor simultaneously present in CSF-LM. The inhibitor is identified as IL-10 since its inhibitory activity can be neutralized by anti-IL-10 antibodies. IL-10 is secreted by activated monocytes and T lymphocytes and has been shown to inhibit the production of ROI and NO by peritoneal exudate macrophages and macrophage cell lines (15, 16). In the context of the data presented it is interesting that IL-10 also interferes with a recently recognized NO-dependent host defense system in parasitic disease, the killing of *Trypanosoma cruzi* by IFN-γ–activated macrophages (22).

Nitric oxide molecules account for leukocyte-mediated killing of other pathogens causing meningitis such as *Mycobacterium tuberculosis* and *Mycobacterium bovis*, *Staphylococci*, *Toxoplasma gondii*, and *Cryptococcus neoformans* (23–27). Reactive oxygen intermediates have been implicated in the destruction of both grampositive and gramnegative bacteria relevant in meningitis including *Staphylococcus aureus* and *Enterobacteriaceae* (28). Since IL-10 interferes with the generation of both ROI and NO the data presented may be relevant not only for *Listeria* meningitis but also for a number of other microbes which lead to meningitis and are destroyed by ROI- and NO-dependent mechanisms. The presence of IL-10 has not yet been documented in CSF in fungal and parasitic meningitis. However, in children with bacterial meningitis IL-10 is present in CSF in 95% of patients with a variety of causative bacterial strains.

The physiological role of IL-10 produced intrathecally in bacterial meningitis is not known. It is striking that in *Listeria* meningitis IL-10 is produced late in the course of disease compared to other cytokines including TNF-α, IL-6, and IFN-γ. Likewise the production of IL-10 in vitro by LPS-stimulated human monocytes is delayed compared to IL-1, IL-6, and TNF-α (29). Since IL-10 inhibits the synthesis of cytokines by activated monocytes/macrophages (30, 31), the observed expression of IL-10 at later stages of meningeal inflammation may reflect a regulatory circuit which counteracts the inflammatory process maintained by ongoing production of cytokines. This concept is supported by the recent findings that IL-10 is produced in the nervous system of mice having recovered from experimental autoimmune encephalomyelitis rather than during acute disease (32). The latter is characterized by activation of IL-1, IL-2, IL-4, IL-6, and IFN-γ genes.

Furthermore in a murine model of septic shock IL-10 reduces serum levels of TNF-α and protects against the lethality of endotoxin (33, 34). However, in bacterial meningitis production of IL-10 in the presence of those infectious microbes which are eliminated mainly by NO- or ROI-dependent pathways, may deteriorate the course of meningitis by preventing the clearance of pathogens in the CSF compartment.

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