Suppression of Macrophage Activation with CNI-1493 Increases Survival in Infant Rats with Systemic Haemophilus influenzae Infection

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CNI-1493, a potent macrophage deactivator, was used to treat infant rats systemically infected with Haemophilus influenzae type b (Hib). CNI-1493 was injected 1 h prior to bacterial inoculation and 24 h later and resulted in a 75% increased rate of survival compared to that for untreated controls. The effect of CNI-1493 on the inflammatory response was studied by immunohistochemical detection of individual tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and gamma interferon (IFN-γ)-producing cells in the spleen. A significant reduction of the incidence of TNF-α- and IL-1β-expressing cells was found for CNI-1493-treated animals. IFN-γ expression was not suppressed by CNI-1493, indicating that cytokine inhibition was specific in macrophages. CNI-1493 significantly reduced the number of infiltrating granulocytes in the brain from that for controls. This study provides evidence that CNI-1493 protects against lethal Hib infection by deactivating the inflammatory cascade in infant rats.

During the onset of acute invasive bacterial infections such as sepsis and meningitis, activation of cytokine cascades plays a major role in pathogenesis (8, 26). An early event in the host’s inflammatory response is the production of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) (8, 26). These cytokines can be produced by different cells, including monocytes and macrophages and cells within the central nervous system (CNS), such as astrocytes and microglia (13, 20, 27). In sepsis, high levels of these cytokines in serum are associated with mortality (30, 31). During the onset of bacterial meningitis, TNF-α and IL-1β are also present in the cerebrospinal fluid (CSF) (13, 20, 27) and contribute to accumulation of leukocytes in CSF, development of brain edema, and damage of cells within the CNS (13, 20, 27).

Specific therapy targeted against TNF-α has been tried in the search for an effective anti-inflammatory adjunctive treatment. Anti-TNF-α antibodies or soluble TNF-α receptors have been used in experimental sepsis models showing protection against mortality (18). However, clinical studies have not been able to demonstrate such a beneficial effect (18, 26). In recent studies of experimental bacterial meningitis, inhibition of the release of TNF-α with pentoxifylline, thalidomide, glucocorticoids, or specific antibodies to TNF-α receptors have not significantly reduced meningeval inflammation (20, 27). However, treatment of patients with corticosteroids has had a limited efficacy (21). Recent work with a new class of TNF-α synthesis inhibitors, the tetravalent guanylhydrazone CNI-1493, indicates that it acts by inhibition of the phosphorylation of the p38 mitogen-activated protein (MAP) kinase (2, 3, 6). A major effect of CNI-1493 is suppression of TNF-α synthesis, mediated by a dose-dependent inhibition of the translation of TNF mRNA (9, 10). Secondarily, the synthesis of other cytokines and chemokines is also inhibited, including that of IL-1β, macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β (3). CNI-1493 suppresses the production of TNF-α and IL-1 even in the presence of gamma interferon (IFN-γ) (3), which is in contrast to the macrophage-inhibitory action mediated by corticosteroids, the latter appearing to have anti-inflammatory effects only in the absence of IFN-γ (17). Administration of CNI-1493 in a murine model of polymicrobial sepsis significantly reduced levels of TNF-α in serum and increased survival rates (28).

In the present study, we evaluated the protective effects of CNI-1493 on the pathogenic sequelae of cytokine release in an experimental model of Haemophilus influenzae type b (Hib) infection in infant rats. Bacteria were inoculated intraperitoneally (i.p.), resulting in a systemic infection with a hematogenous spread to the CNS (22). The results indicate that CNI-1493 significantly reduces mortality and infiltration of granulocytes in brain tissue and attenuates the systemic proinflammatory cytokine response.

MATERIALS AND METHODS

Hib strain LCR 528 was originally isolated from the CSF of a child with bacterial meningitis. The strain was grown in brain heart infusion broth supplemented with 5% Fildes enrichment medium to late log phase and then frozen at −70°C in Trypticase soy broth with 10% glycerol (pH 7.3) until use. For each experiment, an overnight growth was subcultured on chocolate agar plates and allowed to grow for 8 h in late log phase to facilitate maximal capsule expression and then was centrifuged, washed, and resuspended in phosphate-buffered saline (PBS) to an approximate concentration of 5 × 109 CFU per ml, determined by quantitative subcultures. The final inoculum was 5 × 106 CFU per rat. The weight of each rat was approximately 10 to 15 g.

CNI-1493, the tetravalent guanylhydrazone CAS registration no. 164301-51-3, was synthesized and purified as previously described (2). CNI-1493 is a powerful inhibitor of synthesis of TNF and IL-1. It inhibits macrophage activation and subsequent proinflammatory cytokine production while having no inhibitory activity on T cell proliferation or activation (2, 3). The mechanism by which CNI-1493 inhibits macrophage TNF synthesis is suppression of TNF translation efficiency (9). The purity was ≥98% as estimated by the melting point, nuclear magnetic resonance, elution from high-performance liquid chromatography, and elemental analyses. A stock solution was prepared in sterile, deionized, lipopolysaccharide-free water. Rats were injected with 5 mg/kg of body weight, given as a 0.1-ml injection i.p.

Infant rat model. Five- to seven-day-old outbred Sprague-Dawley rats (Charles River, Uppsala, Sweden) in different litters with their mothers were used as previously described (22). The animals were fed and housed under standard conditions. Animals were inoculated by an i.p. injection of 0.1 ml of the Hib suspension (5 × 109 CFU). The animals were divided into five different groups with 40 to 44 animals per group (three litters per group) and treated as

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follows. Group 1 received two injections of CNI-1493 (5 mg/kg, i.p.), the first 1 h before bacterial inoculation and the second 24 h after. Group 2 received CNI-1493 (5 mg/kg, i.p.) under the same conditions as group 1. This group received additional treatment with a subcutaneous bacterial challenge at 24 h; group 3 was inoculated with Hib only (CNI-1493 was not used). Group 4 was treated with dexamethasone (Deva, Merck & Co., Darmstadt, Germany; 5 mg/kg, i.p.) in 50% BSS-saponin (DS-700; Hoescht Marion Roussel, Stockholm, Sweden) of 50 mg/kg, i.p., started 12 h after bacterial inoculation and given twice daily for 4 days. Group 3 received only cetoxamine, in the same way as group 2. Group 4 was treated with dexamethasone (Deva, Merck & Co., Darmstadt, Germany) and was also treated with a subcutaneous bacterial challenge at 24 h; group 5 was given 5 mg/kg, g.i. 1 h before bacterial injection and 24 h after. The animals received cetoxamine in the same subcutaneous dosage as that for groups 2 and 3. Group 5 served as the control group; i.e., the animals were inoculated and received cetoxamine and saline (Falcon; Becton, Dickinson & Co., Tokyo, Japan) i.p. daily for each group. The animals chosen at random were sacrificed at 3, 24, and 48 h, respectively, after Hib infection. Following decapitation, the spleen and brain were removed and immediately snap-frozen in isopentane and dry ice stored at −70°C until sectioned. For the remaining data, mortality was assessed twice daily for 7 days. The survival data were based on three different experiments. In one set of experiments, animals were monitored for 14 days. The experiment was approved by an animal ethics committee at Huddinge Court House.

Immunohistochemical detection of granulocyte markers in brain sections. Cryostat sections (12 μm) of the brain at 24 h after Hib infection were cut and mounted on glass slides (SuperFrost Plus; Menzel-Gläser). Sections were fixed for 10 min in 2% formaldehyde (Sigma Chemical Co., St. Louis, Mo.) in PBS at room temperature. All slides were subsequently stored at −20°C until stained. The sections were treated with a monoclonal antibody directed against a granulocyte antigen (Mouse anti-granulocyte human/rat; Serotec, Oxford, United Kingdom) (diluted 1:10). The staining method was the same as that described below for TNF-α, but without using saponin. The secondary antibody used was a biotin-labeled Fab2-fragmented donkey anti-mouse antibody (Jackson Immunoresearch Labs) (diluted 1:1,000). The infiltrating granulocytes in the whole section of the brain were then quantified in a computerized image analyzer, as described below.

The specificity of the granulocyte antibody was tested by staining of a blood smear from rat, using the same staining method as for the brain sections.

Immunohistochemical detection of intracellular TNF-α, IL-1β, and IFN-γ in spleen sections. Cryostat sections (10 μm each) of the spleen were cut and mounted on glass slides (SuperFrost Plus; Menzel-Gläser). Sections were fixed for 10 min in 2% formaldehyde (Sigma Chemical Co.) in PBS at room temperature. All slides were subsequently stored at −20°C until stained. TNF-α. The cryopreserved sections were stained for intracellular production of TNF-α as previously described (ref. 25). Briefly, permeabilization of the cell membrane and the Golgi organelle was performed by use of a balanced salt solution (BSS) (GIBCO Ltd., Paisley, United Kingdom) supplemented with 0.1% saponin (Riedel de Haen AG, Seelze, Germany) in all subsequent washes and incubation steps. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide and 0.1% (v/v) sodium chloride dissolved in BSS-saponin for 1 h at room temperature in the dark. Sections were then washed three times in BSS-saponin and were thereafter blocked with either 2% normal goat sera or 2% normal human AB sera in BSS-saponin for 30 min at room temperature to reduce nonspecific binding due to nonspecific binding sites. Slides were then 15 min incubated with 10 μg of cytokine-specific antigen, affinity-purified antibody (polyclonal rabbit anti-rat TNF-α, lot no. 8-14; P. Van de Meide, Biomedical Primate Research Centre, Rijswijk, The Netherlands) (1 μg/ml). Control staining was done by omitting the primary antibody and by a negative control, consisting of an irrelevant mouse immunoglobulin G1 (IgG1) antibody and rabbit IgG (rabbit IgG, mouse IgG1 negative control; Dako, Glostrup, Denmark). The slides were then washed and incubated with appropriate biotin-labeled antibody (Fab2-fragmented donkey anti-rabbit; Jackson Immunoresearch Labs) (diluted 1:1,000) for 30 min at room temperature. After a final wash, the substrate diaminobenzidine (Diaminobenzidine Substrate Kit; Vector) was added. The reaction was stopped after 5 min by washes in BSS, after which sections were counterstained with Mayer’s hematoxylin. Finally, the slides were left to air dry and mounted with buffered glycergel.

IL-1β. The same method as that described above for TNF-α was used. However, the cytokine-specific antibody used was a polyclonal goat anti-IL-1β (lot no. YRO 1/AF-501-NA; R&D Systems, Minneapolis, Minn.) (2 μg/ml) (diluted 1:500). The biotin-labeled antibody used was a Fab2-fragmented donkey anti-goat antibody (Jackson Immunoresearch Labs) (diluted 1:1,000).

IFN-γ. The same method as that described above was used. The cytokine-specific antigen affinity-purified antibody used was a monoclonal mouse anti-rat IFN-γ (DB-1; P. Van der Meide, Biomedical Primate Research Centre, Rijswijk, The Netherlands) (2 μg/ml). The biotin-labeled antibody used was a Fab2-fragmented donkey anti-mouse antibody (Jackson Immunoresearch Labs) (diluted 1:1,000).

Semiquantification of cytokine-producing cells by computerized in-situ imaging. The immunocytochemically stained cells were examined with a Leica DMI microscope (Leica, Wetzlar, Germany) equipped with a 3-chip charge-coupled device (CCD) camera (CXC-7500; Canon, Tokyo, Japan). The photomicrographs were taken with a Nikon (Tokyo, Japan) a 20×/0.50 objective. The images were analyzed in an image analyzer (Quantimet QW 550; Leica, Cambridge, United Kingdom). The image was directed by a PC computer. Special software for cell detection and measurement of intensity (Detect A Cell) was written in QUIPS. The methodology has recently been described (1, 4, 5). For data analysis, cell size was expressed in cell area (square microns), mean total cell intensity was expressed in gray levels (0 to 255), and the frequencies of positive and negative cells were calculated for at least 1.5 × 104 cells per section. The data were imported into a Microsoft Excel dedicated macro setup (IMAGE2XL, developed by T. E. Fehniger, Department of Infectious Diseases, Huddinge University Hospital, Stockholm, Sweden), which provided statistical analysis using the Astute program (University of Leeds, Leeds, United Kingdom) by calculating the positive stained area versus the total stained area.

Culture of Hib in presence of CNI-1493. In order to evaluate if CNI-1493 had any direct antimicrobial effects, the Hib strain used in the animal experiments was cultured in broth with and without addition of CNI-1493 in a high concentration. A Hib inoculum of 5.5 × 107 CFU/ml was cultured in Müller-Hinton broth with CNI-1493 at a concentration of 30 mg/liter. Control cultures were grown without CNI-1493. Quantitative subcultures were done at 5 and 24 h, respectively.

Statistical analysis. Analysis of variance was used for comparison of multiple groups, while the Mann-Whitney U test was used to compare two groups. Differences were considered significant at a P value of <0.05. Data are expressed as means ± standard errors.

RESULTS

Effect of CNI-1493 on survival in an infant rat model. Mortality rates were assessed for all groups twice daily for 7 days (Fig. 1). For group 1, among animals treated with CNI-1493 only (n = 25), 76% survived 1 week after bacterial inoculation (P = 0.0001 compared to results for the control; P = 0.0001 compared to results for animals treated with cetoxamine only). In group 2, treatment with CNI-1493 plus (n = 29), 72% survived after 1 week (P = 0.0001 compared to results for the control). For group 3, treatment with cetoxamine alone (n = 28), a 14% survival rate was seen after 1 week. Group 4, treatment with dexamethasone plus cetoxamine (n = 26), showed a mortality rate of 100% within 2 days. Group 5, the control animals (infected without treatment; n = 25), showed a mortality rate of 100% within 3 days. Animals surviving to day 7 appeared to be in an unaffected and normal condition and were considered cured of Hib infection. Observation of a subgroup for a total of 14 days indicated that all animals were healthy (as judged by observation of movements, fur, suckling, and growth). These data indicate that the treatment with CNI-1493 conferred protection against lethality.

Detection of granulocyte markers in brain sections. Brain sections at 24 h after Hib inoculation from the group of animals treated with CNI-1493 only and from the control group were analyzed to determine the number of infiltrating granulocytes. In both groups, single granulocytes were found scattered in the brain parenchyma and the meninges, and clusters of infiltrating cells were detected, especially in perivascular areas (see Fig. 3). The granulocyte antibody was also incubated with a normal rat blood smear in order to test its specificity, showing that granulocytes stained in the same way as the granulocytes in the brain sections.

Semiquantification by computerized in-situ imaging of positively stained cells was done for both groups of animals (n = 5 per group) and showed that control animals (infected but not treated) had a mean presence of infiltrating granulocytes of 3.52% ± 0.61% of the total cell area. In the group of animals pretreated with CNI-1493, the mean number of infiltrating granulocytes was significantly reduced at 1.76% ± 0.37% (P = 0.019) positively stained cells per total cell area (see Fig. 3C and D).
Effect of CNI-1493 on TNF-α-producing cells in spleen. To
determine the effect of treatment on cellular cytokine synthe-
sis, splenic cells were analyzed at time points of 3, 24, and 48 h
(n = 5 at each time point) after bacterial inoculation. We did
perform two color staining procedures using MAC 387 and
CD68 monoclonal antibodies in order to show that all IL-1β-
and IL-1α-expressing cells were CD68- and MAC 387-positive
cells, while the TNF-α-expressing cells costained with CD68
and MAC 387 at a rate of 80 to 95%. The rest of the TNF-α-
positive cells were CD3-positive T cells in the spleen. Semi-
quantification by computerized in-situ imaging of TNF-α-pro-
ducing cells showed that the control animals had a mean
prevalence of TNF-α-producing cells per total cell area of
8.13% ± 1.88%, 0.57% ± 0.25%, and 1.52% ± 1.19% positive
cells at 3, 24, and 48 h, respectively. Pretreatment with CNI-
1493 significantly reduced the mean number of TNF-α-produc-
cing cells at every time point compared with results for the
control group: 2.03% ± 0.19% (P = 0.007), 0.04% ± 0.02% (P = 0.01),
and 0.07% ± 0.02% (P = 0.02) positive cells, respectively (Fig. 2A and
3A and B).

Effect of CNI-1493 on IL-1β-producing cells in the spleen.
Semi-quantification by computerized in-situ imaging of IL-1β-
producing cells was done at the same time points as those used
for TNF-α, with and without CNI-1493 treatment. This showed
that the control animals had a mean prevalence of IL-1β-
producing cells per total cell area of 0.86% ± 0.23%, 1.87% ±
0.93%, and 0.78% ± 0.29% positive cells at 3, 24, and 48 h,
respectively. In animals pretreated with CNI-1493, the mean
number of IL-1β-producing cells was significantly reduced at
all time points compared with results for the control group:
0.37% ± 0.10% (P = 0.04), 0.12% ± 0.05% (P = 0.007), and
0.18% ± 0.06% (P = 0.04) positive cells at 3, 24, and 48 h,
respectively (Fig. 2B).

Effect of CNI-1493 on IFN-γ-producing cells in the spleen.
To obtain evidence that the cytokine-suppressing effects of
CNI-1493 were specific, semi-quantification by computerized
in-situ imaging of IFN-γ-producing cells was done for both
groups. The control animals had a mean prevalence of IFN-γ-
producing cells per total cell area of 3.33% ± 1.02%, 4.55% ±
0.77%, and 4.86% ± 1.06% positive cells at 3, 24, and 48 h,
respectively. In animals pretreated with CNI-1493, the mean
number of IFN-γ-producing cells was not significantly different
at any time point compared with results for the control group:
3.2% ± 0.93%, 4.63% ± 0.92%, and 4.35% ± 1.42% positive
cells, respectively (Fig. 2C).

Antibacterial effect of CNI-1493 on Hib. No antibacterial
effect of CNI-1493 could be demonstrated. Quantitative cul-
tures at 5 h in media without CNI-1493 resulted in 4.0 × 10⁷
CFU/ml, and in media with CNI-1493, results were 3.9 × 10⁷
CFU/ml. At 24 h, the corresponding values were 4.5 × 10⁷
and 4.3 × 10⁷ CFU/ml, respectively.

DISCUSSION
These results indicate that treatment with CNI-1493 reduced
mortality by 75% with experimental Hib infection of infant
rats. CNI-1493 therapy resulted in a significant decrease in the
number of TNF-α- and IL-1β-producing cells in the spleen and
the number of infiltrating granulocytes in the brain, compared
with results for untreated animals. The meningeal inflam-
mation seen with Hib infection in the infant rat model has previ-
ously been well described (19, 22) and has recently been used
for studies of the CNS inflammatory response in Hib infection
(11, 12). However, this model has some drawbacks, including
the difficulties of obtaining CSF or blood samples due to the
small size of the animal, and if CSF is taken, the amount of
blood contamination has to be determined to avoid false pleo-
cytosis or a positive CSF culture (29). In the present study,
CNS inflammation was measured immunohistochemically by
determining the number of infiltrating granulocytes in the
whole brain section, including the meninges. Despite the 50% 
reduction in the number of infiltrating granulocytes in
CNI-1493-treated animals, our belief is that the significant reduc-
tion in mortality mainly results from suppression of the sys-
temic inflammation rather than of the local CNS inflammation.
The model used here therefore more closely resembles neo-
natal sepsis with CNS engagement than meningitis alone.

Previous in vitro studies revealed that CNI-1493 effectively
down-regulates proinflammatory cytokine synthesis, in partic-
ular, TNF-α synthesis in cultured endotoxin-stimulated murine

FIG. 1. Life table analysis illustrating the percentages of surviving animals at the indicated time points after i.p. injection of Hib at time point 0 h (all drugs were
also given i.p.). Treatment groups are indicated by the following abbreviations. CNI, CNI-1493 (5 mg/kg) injected 1 h before bacterial inoculation and 24 h after
(n = 25). CNI + CTX, CNI-1493 (as previous dosage) with cefotaxime (50 mg/kg), twice daily for 4 days, first dose 12 h after bacterial inoculation (n = 29). CTX, cefotaxime
alone (as previous dosage) (n = 28). Dexamethasone (0.15 mg/kg), first dose 1 h before bacterial inoculation and 24 h later with cefotaxime (as previous
dosage) (n = 26). Control, infected animals without any treatment (n = 25). A significant increase in the survival rate over that for controls was noticed for all
CNI-1493-treated animals both with and without antibiotic treatment.
and human macrophages (3, 6). However, suppression of TNF-α is not complete, since CNI-1493-treated macrophages still can produce about 10% of the amount of TNF-α produced by nontreated macrophages (3, 6). It has recently been deduced that the mechanism by which CNI-1493 exerts its inhibitory effects on macrophages is predominantly suppression of TNF-α mRNA translation, while the expression of TNF-α mRNA is not significantly affected by CNI-1493 (9). The p38 MAP kinase signaling cascade has been demonstrated to be crucial in the posttranscriptional regulation in the synthesis of some proinflammatory cytokines (15). Recent findings indicate that CNI-1493 inhibits the phosphorylation of p38 MAP kinase, thereby providing the molecular background for its action as an inhibitor of cytokine translation in the macrophage (10). It has previously been shown in vitro that CNI-1493 fails to have any suppressive effect on cytokine expression in lymphocytes and that IFN-γ production is not blocked (6). In that study, proinflammatory cytokine synthesis was studied at the single-cell level (using computerized image analysis) following different routes of cell activation, and it was demonstrated that the production of IL-2, IFN, and TNF by activated T cells was not affected by CNI-1493 treatment (6). On the other hand, similar treatment resulted in a profound inhibition of lipopolysaccharide-induced production of TNF, IL-1, IL-6, and IL-8 by macrophages, independently of IFN priming (6). This is in line with the present study, because the number of IFN-γ-producing cells in the spleen was unaffected by the administration of CNI-1493. Additionally, the capacity of CNI-1493 to override IFN-γ-induced steroid-resistant inflammation was also shown in this study, in accordance with previous reports (3, 6, 17).

Our results indicate that inhibition of TNF-α production in the spleen in CNI-1493-treated animals was associated with reduced mortality rates compared with results for nontreated animals. Previously, it has been demonstrated in vitro that secondarily to inhibition of TNF-α by CNI-1493, the production of other proinflammatory cytokines and chemokines, including IL-1, IL-6, IL-8, MIP-1α, and MIP-1β, was also suppressed (3, 6). In the present study, numbers of IL-1β-producing cells in the spleen were significantly reduced in CNI-1493-treated animals, possibly also contributing to the low mortality rate in this group.

The accumulation of leukocytes in CSF has been demonstrated to be one main contributing factor in the CNS injury associated with bacterial meningitis (24). It has previously been shown that blocking of receptors for leukocyte-endothelial adhesion prevents transmigration of leukocytes into the CSF, reduces neuronal cell apoptosis (7), and increases survival in experimental bacterial meningitis (24). Our findings that the numbers of infiltrating granulocytes in brain sections were significantly reduced in CNI-1493-treated animals, possibly also contributing to the low mortality rate in this group.

The accumulation of leukocytes in CSF has been demonstrated to be one main contributing factor in the CNS injury associated with bacterial meningitis (24). It has previously been shown that blocking of receptors for leukocyte-endothelial adhesion prevents transmigration of leukocytes into the CSF, reduces neuronal cell apoptosis (7), and increases survival in experimental bacterial meningitis (24). Our findings that the numbers of infiltrating granulocytes in brain sections were significantly reduced in CNI-1493-treated animals compared to results for controls indicated a protective effect on the CNS inflammatory response by CNI-1493. It is likely that this contributed to increased survival rates in this model, although the mechanism by which CNI-1493 inhibited granulocyte traversal of the blood-brain barrier was not clear. One explanation could be that several mediators in the granulocyte extravasation process were suppressed by CNI-1493. TNF-α and IL-1β are known to activate leukocyte adhesion receptors (selectins and integrins), which is a prerequisite for extravasation of leukocytes to the site of inflammation (14). Chemokines, such as MIP-1α, MIP-1β, and MIP-2, activate and attract leukocytes, leading to extravasation and accumulation of these cells in the inflamed area (23). Moreover, it has recently been shown with the infant rat model with Hib-induced CNS inflammation that neutralization of MIP-1α and MIP-2 with monoclonal antib-
ies significantly reduced the number of neutrophils in brain tissue (11). Other possible protective effects exerted by CNI-1493 in vivo could be the reduction of nitric oxide and cellular apoptosis, as indicated by Villa et al. (28). There is evidence that CNI-1493 has the capacity to interfere in the inflammatory cascade on several different levels, conferring protection against lethal Hib infection in infant rats. However, the compound does not seem to have any direct antimicrobial properties in itself, since bacterial growth was unaffected in the presence of even a high concentration of CNI-1493.

In the present study, three groups of animals received a suboptimal dose of cefotaxime (50 mg/kg twice daily for 4 days; first dose, 12 h after infection). The main reason for this treatment was not to sterilize the CSF compartment but rather to observe possible differences in survival rates of CNI-1493-treated animals with different kinetics of bacterial growth. The survival rate, however, was the same for the group treated with CNI-1493 only and the group treated with CNI-1493 plus cefotaxime. Treatment with cefotaxime alone protected 14% of the animals from death in this model. When animals receiving cefotaxime were pretreated with glucocorticosteroids (dexamethasone, 1 h before infection and 24 h later), mortality was increased to 100% within 48 h. One explanation for this fast death could be that it is due to the immunosuppressive effects exerted by glucocorticosteroids. With this dosage (pretreatment) of corticosteroids the negative effects obviously outweighed the positive, immunomodulatory effects documented for the treatment of bacterial meningitis when an adequate dose of antibiotics is given at the same time. Additionally, IFN-γ expression was already noticed in the spleen 3 h after Hib exposure, leaving a very short time frame for steroid-mediated antiinflammatory action.

In conclusion, our data indicate that by down-modulating the very initial inflammatory response with prophylactic treatment with a tetravalent guanylhydrazone (CNI-1493) (5 mg/kg, given i.p.), survival rates were increased by 75% for infant rats with systemic and CNS inflammation induced with Hib. Treatment with CNI-1493 was demonstrated to strikingly reduce the number of TNF-α- and IL-1β-producing cells in the spleen and the number of infiltrating granulocytes in the brain. Our find-

FIG. 3. (A and B) Microphotographs of cryopreserved spleen sections immunohistochemically stained for TNF-α 3 h after Hib inoculation. Sections from an untreated control animal (A) and a CNI-1493 treated animal, where the number of positively stained cells was reduced (B), are shown. TNF-α-expressing cells stain brown with diaminobenzidine. Please note the extracellular deposition of TNF surrounding producer cells. The nuclei of all cells were counterstained with hematoxylin (blue). (C and D) Microphotographs of cryopreserved brain sections immunohistochemically stained for infiltrating granulocytes 24 h after Hib inoculation. Sections from an untreated control animal (C) and a CNI-1493-treated animal, showing a reduced number of granulocytes (D), are shown. Granulocytes stained brown with diaminobenzidine. The nuclei of all cells were counterstained with hematoxylin (blue).
ings illustrate a potential treatment strategy with a macrophage suppressive compound as a novel therapeutic approach to reduce CNS inflammatory damage in bacterial meningitis.

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ERRATA

Autonomous Expression of the slo Gene of the Bicistronic nga-slo Operon of Streptococcus pyogenes

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Volume 70, no. 5, p. 2730–2733, 2002. Page 2732, column 1, line 7 from bottom: “phoR” should read “pfoR.”
Page 2732, column 1, lines 6, 4, and 3 from bottom: “phoA” should read “pfoA.”
Page 2732, column 1, line 4 from bottom: “PhoR” should read “PfoR.”
Page 2732, column 2, line 4: “phoR” should read “pfoR.”
Page 2733, column 1, line 17: “phoA” should read “pfoA.”

Transcript Heterogeneity of the p44 Multigene Family in a Human Granulocytic Ehrlichiosis Agent Transmitted by Ticks

Ning Zhi, Norio Ohashi, Tomoko Tajima, Jason Mott, Roger W. Stich, Debra Grover, Sam R. Telford III, Quan Lin, and Yasuko Rikihisa

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Volume 70, no. 3, p. 1175–1184, 2002. Page 1177, column 1, line 1: “P44-7, AF412812” should read “P44-7, AF412822.”
Page 1179, column 2, lines 9 and 10: “lacked a 44-kDa band corresponding to P44-18 in the 37°C sample” should read “with a faint 44-kDa band corresponding to P44-18 (44-kDa) in the 24°C sample.”

Suppression of Macrophage Activation with CNI-1493 Increases Survival in Infant Rats with Systemic Haemophilus influenzae Infection

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Volume 68, no. 9, p. 5329–5334, 2000. Page 5329, the byline should appear as shown above.