Protective Effect of a 21-Aminosteroid during Experimental Pneumococcal Meningitis

Stefan Lorenzl, Uwe Koedel, Karl Frei,
Andrea Bernatowicz, Adriano Fontana,
and Hans-Walter Pfister

Department of Neurology, Klinikum Großhadern, Ludwig-Maximilians-University of Munich, Germany; Section of Clinical Immunology, University Hospital, Zurich, Switzerland

This study investigated whether the 21-aminosteroid U74389F, an inhibitor of lipid peroxidation, attenuates pathophysiologic changes in experimental pneumococcal meningitis. Infected rats injected intravenously with vehicle and U74389F developed increases in regional cerebral blood flow (rCBF), intracranial pressure (ICP), brain water content, and white blood cells (WBC) in cerebrospinal fluid (CSF) within 8 h after intracisternal challenge. Pretreatment with or administration of U74389F 4 h after infection significantly reduced the increase in ICP but had no effect on rCBF increase. Moreover, U74389F pretreatment significantly reduced brain water content and CSF WBC count. In vitro, U74389F inhibited iron-dependent lipid peroxidation of astrocyte cultures and the production of tumor necrosis factor-α, interleukin-6, and nitric oxide by stimulated macrophages. These data suggest that U74389F modulates early pathophysiologic alterations in experimental pneumococcal meningitis.

Animal models of bacterial meningitis have increased our knowledge of the complex pathophysiologic mechanisms of the disease [1–5]. In a rat model of meningitis, we showed that intracisternal (ic) inoculation of live pneumococci or pneumococcal cell wall components induces an early increase in regional cerebral blood flow (rCBF), intracranial pressure (ICP), and brain water content [6]. Pretreatment with free superoxide dismutase (SOD), polyethylene glycol (PEG)–conjugated SOD, deferoxamine, and catalase greatly attenuates these pathophysiologic changes; the strongest effects are shown with SOD and PEG-SOD [6–8]. Findings by other investigators support a role for reactive oxygen species in the pathophysiology of bacterial meningitis [9, 10]. Cellular injury caused by reactive oxygen species may involve direct damage to proteins and DNA as well as lipid peroxidation [11]. Here we tested the effect of the novel 21-aminosteroid U74389F [12, 13], an inhibitor of lipid peroxidation, for its capacity to alter rCBF, ICP, and brain edema formation and to reduce meningeal inflammation in experimental pneumococcal meningitis.

Materials and Methods

Animal preparation. We used a well-characterized rat meningitis model that has been described in detail [6]. Adult male Wistar rats (250–330 g) were intraperitoneally anesthetized with 100 mg/kg thiobutabarbiturate (Inactin; Byk Gulden, Konstanz, Germany), tracheotomized, and artificially ventilated (small animal ventilator, model 683; Harvard, South Natick, MA). End-expiratory CO_2 was continuously monitored by infrared CO_2 analyzer (model 2200; Heyer, Bad Ems, Germany). Mean arterial blood pressure (MABP) was measured by pressure transducer (Statham P23; Viggo-Spectramed, Oxnard, CA) connected to the femoral artery cannula. Arterial blood gases and hematocrit were determined before ic inoculation and every 2 h thereafter (gas check model 1304; Instrumentation Laboratory, Kirchheim, Germany). Body temperature was maintained at 38°C by a rectal thermometer–controlled heating pad. Rats were placed in a stereotaxic frame, and a burr hole was made in the occipital bone for placement of the cisterna magna catheter. A 5-mm-diameter craniotomy was made in the right parietal bone for the placement of a laser-Doppler probe. rCBF was measured continuously by laser-Doppler flowmetry (model BPM 403a; Vasamedics, St. Paul, MN). Changes in rCBF were expressed as percent changes from baseline. The dura was left intact in all preparations.

Cerebrospinal fluid (CSF; 100 μL) was withdrawn through the cisterna magna catheter, and meningitis was induced by ic injection of 100 μL of pneumococci ($\approx 10^6$ cfu). We used *Streptococcus pneumoniae* type 3 (no. 17260), an isolate from an endotracheal aspiration of a patient with septic infection that was maintained at $-20°C$ in trypticase soy broth (Oxoid, Wesel, Germany) supplemented with 10% glycerol and 1% IsoVitale (Becton Dickinson Microbiology Systems, Heidelberg, Germany). Before use, the bacteria were subcultured on blood-agar plates, checked for purity, inoculated into brain-heart infusion broth (Oxoid), supplemented with 3% horse serum and 1% bovine albumin (Serva, Heidelberg, Germany), and incubated overnight at 35°C. The broth was centrifuged for 20 min at 2500 g, and the sediment was washed once with 0.85% saline and resuspended in saline. The final suspension was turbidimetrically adjusted to an optical density of 0.5 at 546 nm (photometer with 13-mm filter; Eppendorf, Hamburg, Germany), thus achieving a concentration of $\approx 10^7$ cfu/mL.

The following parameters were continuously monitored for 8 h after ic injection by a personal computer system after analog-

digital conversion for signal processing: ICP (measured by Statham P23 pressure transducer connected to the cisterna magna catheter), rCBF, and MABP. Blood and CSF white blood cell (WBC) counts were determined at baseline and 4 and 8 h after ic injection. At the end of the experiment, the reactivity of the cerebral circulation to CO_2 was tested. Hypercapnia was produced with 10% CO_2, 21% O_2, and the balance of N_2. Before and 10 min after hypercapnia, arterial blood samples were drawn for blood gas and pH analysis. To determine brain water content, brains were weighed in glass dishes then dried for 16 h at 130°C to stable weight. Brain water content was calculated by the formula [(wet weight − dry weight)/wet weight] × 100 [6].

We studied 6 groups of rats: There were 6 infected rats in groups 1–3, 6 uninfected animals in group 4, and 3 and 4 infected rats in groups 5 and 6, respectively. Animals in group 1 were injected intravenously (iv) with vehicle of U74389F: 21-[4-(2,6-di-1-pyr-rolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9 (11)-triene-3,20-dione, monomethansulfonate (provided by Upjohn, Kalamazoo, MI). The vehicle was citric acid and citrate (U74389F$_{veh}$). Group 2 animals were treated iv with U74389F 15 min before (3 mg/kg) and 2 h after (1.5 mg/kg) ic pneumococcal challenge. Rats in group 3 were treated iv with U74389F 4 h after (3 mg/kg) and 6 h after (1.5 mg/kg) ic infection. Group 4 rats were injected iv with U74389F 15 min before (3 mg/kg) and 2 h after (1.5 mg/kg) ic injection of PBS. Rats in group 5 were treated iv with U74389F 15 min before (30 mg/kg) and 2 h after (15 mg/kg) ic infection (5-h measurement period). Group 6 rats were pretreated iv with U74389F 15 min before (3 mg/kg) and 2 h after (1.5 mg/kg) ic infection, the NO synthase inhibitor N-nitro-L-arginine (L-NA; 10 mg/kg) was administered iv 4 h after ic infection (5-h measurement period).

In vitro experiments. We investigated whether U74389F is a potent inhibitor of lipid peroxidation when central nervous system (CNS) cells are used. For this purpose, primary rat astrocytes were stimulated with heat-killed pneumococci; lipid peroxidation was assessed by the formation of thiobarbituric acid−reactive products. The data were compared with those of methylprednisolone, a known potent inhibitor of lipid peroxidation.

For initiation of lipid peroxidation, primary rat astrocytes were incubated with 200 μM Fe^{3+} and 50 μM Fe^{2+} in 0.9% saline for 0.5 h at 37°C [14]. Fe^{3+} and Fe^{2+} solutions (Aldrich Chemie, Steinheim, Germany) were prepared fresh in argon-purged H_2O and used immediately. Astrocytes and the supernatant were removed, homogenized, and sonicated for 30 s. After centrifugation (800 g, 5 min, 4°C), 150 μL of the cell suspension was incubated with 1 mL of 0.5% thiobarbituric acid (Aldrich Chemie, Steinheim, Germany) in 12.5% trichloracetic acid (Aldrich Chemie) for 10 min at 90°C. After samples were centrifuged (800 g, 5 min, room temperature), the formation of thiobarbituric acid−reactive oxidation products was determined at A_{532} (Ultrospec III; Pharmacia LKB, Freiburg, Germany). Quantification was based upon a molar extinction coefficient of 1.56 × 10^5 [14]. The following groups were investigated: addition of U74389F$_{veh}$ (n = 16); U74389F, 100 μM (n = 6) and 1 mM (n = 6), 10 mM (n = 4); methylprednisolone vehicle (n = 6); methylprednisolone, 1 mM (n = 6) and 10 mM (n = 4); controls (n = 6; addition of the diluent of Fe^{2+}/ Fe^{3+} plus U74389F$_{veh}$).

Primary astrocyte cultures were prepared from the cerebral cortex of 1-day-old neonatal Wistar rats and grown on 6-well plates

(Falcon; Becton Dickinson, Plymouth, UK) at 37°C in a 5% CO_2 incubator with 95% oxygen. During the first 7 days, the astrocytes were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% heat-inactivated fetal calf serum (FCS). On day 8, the medium was replaced and the astrocytes were maintained in DMEM plus 5% FCS. Astrocytes were cultivated in 6-well plates in culture medium until confluence. When confluence was documented (days 12–14), the astrocytes were shaken at 400 rpm for 3 h to remove microglia and oligodendrocytes. Astrocytes were then maintained 1 day in G5 medium [15] to eliminate microglia and oligodendrocytes. Astrocyte cultures were characterized on the basis of morphologic criteria and by the expression of glial fibrillary acidic protein (GFAP) as detected by immunostaining. These primary astrocyte cultures consisted of >95% GFAP-positive cells. We used only astrocytes without any passage. Before stimulation, astrocytes were washed with PBS without Ca^{2+} and Mg^{2+} and maintained in DMEM with 5% FCS without phenol red and antibiotics.

Peritoneal macrophage cultures. We also questioned whether the activity of U74389F was restricted to inhibition of lipid peroxidation. Thus, we tested to see if it interfered with the production of known mediators of bacterial meningitis, such as cytokines and NO. Peritoneal macrophages were stimulated with heat-killed pneumococci, lipopolysaccharide (LPS), and cytokines. Peritoneal exudate cells were obtained from 8-week-old female Wistar rats that were injected intraperitoneally with 6 mL of Brewer's thioglycollate medium 3 days before isolation. The cells were cultured in DMEM (Biochrom, Berlin) supplemented with 1% FCS (Biochrom), 10 $\mu g/mL$ gentamicin, and 1 mM N-acetyl-L-alanyl-L-glutamine. Cells were stimulated with heat-killed (60°C, 4 h) unencapsulated pneumococci (HKP; isogenic mutant of *S. pneumoniae* type 3, no. 17260) in three different concentrations (10^5, 10^6, and 10^7 cfu/mL), LPS (*Escherichia coli* 0127:B8; 1 $\mu g/mL$) or murine recombinant interferon-γ (rIFN-γ; 100 U/mL) plus murine recombinant tumor necrosis factor-α (rTNF-α; 10 ng/mL). Murine rTNF-α and rIFN-γ were both purchased from Boehringer (Mannheim, Germany). *E. coli* LPS was obtained from Difco (Detroit). Cell cultures were untreated or treated with U74389F (1, 10, or 100 μM) or U74389F$_{veh}$. Interleukin (IL)-6 and TNF-α were measured using 7TD1 cells [16] and L-M cells [17], respectively. NO production in the cell culture supernatant was assessed by measuring nitrite, a stable metabolic product of NO, by the Griess reaction [18].

Statistical methods. Data on rCBF, ICP, and CSF WBC count obtained 4 and 8 h after ic pneumococcal challenge in rats injected iv with U74389F$_{veh}$ or U74389F (groups 1 and 2) were compared by unpaired Student's t test; P values were corrected for repeated measurements using the Bonferroni-Holm procedure. Data on brain water content and CO_2 reactivity at 8 h after ic challenge from groups 1 and 2 were compared by the unpaired Student's t test. Data on rCBF, ICP, and CSF WBC count at 4 h after ic challenge in infected rats injected iv with U74389F in two different dosages (groups 2 and 5) were compared by unpaired Student's t test. Data on rCBF, ICP, and CSF WBC count at 4 and 5 h after ic challenge in infected rats injected iv with U74389F or with U74389F plus L-NA (groups 2 and 6) were compared by unpaired Student's t test; P values were corrected for repeated measurements using the Bonferroni-Holm procedure. One-way analysis of variance and Student-Newman-Keuls multiple comparisons were used

Table 1. Pathophysiologic parameters in different experimental groups of rats.

Group	Regional cerebral blood flow (%) 4 h	Regional cerebral blood flow (%) 8 h	Intracranial pressure (mm Hg) 4 h	Intracranial pressure (mm Hg) 8 h	CSF white blood cell count (cells/μL) 4 h	CSF white blood cell count (cells/μL) 8 h	Brain water content (%) at 8 h	CO_2 reactivity* at 8 h
Infected								
U74389F$_{veh}$ injected	175.3 ± 7.0	211.6 ± 5.2	13.3 ± 1.7	15.4 ± 1.2	2611 ± 551	6710 ± 729	79.09 ± 0.03	1.38 ± 0.56
U74389F pretreated	151.8 ± 7.0	204.4 ± 3.1	9.0 ± 1.4	11.0 ± 1.0[†]	1095 ± 163[†]	4297 ± 289[†]	78.80 ± 0.06[†]	0.86 ± 0.26
Uninfected, U74389F injected	107.9 ± 5.6	115.6 ± 6.5	2.1 ± 0.3	3.8 ± 0.9	6 ± 2	23 ± 10	77.91 ± 0.10	1.34 ± 0.45

NOTE. Each group, $n = 6$.
* Change in regional cerebral blood flow (%)/change in P_{CO_2} (mm Hg).
[†] $P < .05$, vs. infected, U74389F$_{veh}$-injected rats.

to compare data on thiobarbituric acid reactive products. Differences were considered significant when $P < .05$. Data are expressed as mean ± SE.

Results

Physiologic variables. MABP, P_{O_2}, P_{CO_2}, pH, hematocrit, and body temperature were normal throughout the experiment in all groups (data not shown). Cerebrovascular CO_2 reactivity did not differ significantly between groups (table 1).

rCBF, ICP, brain water content, and CSF WBC count. There was an increase in rCBF in U74389F$_{veh}$-injected infected rats (group 1) from a baseline of 100% to 211.6% ± 5.2% within 8 h after pneumococcal challenge (table 1; figure 1). In infected rats pretreated with U74389F (group 2), the mean values of rCBF at 4 and 8 h after ic injection did not differ significantly from that of infected rats pretreated with U74389F$_{veh}$ (table 1).

ICP markedly increased in infected U74389F$_{veh}$-injected rats (group 1) within 8 h after infection from a baseline of 3.6 ± 0.4 mm Hg to 15.4 ± 1.2 mm Hg (figure 1, table 1). Pretreatment with U74389F (group 2) significantly attenuated the increase in ICP at 8 h (table 1). There was no change in rCBF and ICP in uninfected rats pretreated with U74389F and injected ic with PBS (group 4; table 1). Pretreatment with U74389F (group 2) significantly reduced brain water content and CSF WBC counts ($P < .05$; table 1).

The inhibitory effect of U74389F (group 2) on ICP and CSF pleocytosis in infected rats was not enhanced when infected rats were pretreated with the higher dosage of U74389F (group 5). For example, at 4 h after infection, ICP was 10.7 ± 4.2 mm Hg in group 5 versus 11.0 ± 1.0 mm Hg in group 2, and CSF WBC count was 1021 ± 305 versus 1095 ± 163 cells/μL. There was also no effect on rCBF (171.1% ± 26.0% vs. 151.8% ± 7.0% at 4 h after infection).

Compared with effects observed in U74389F$_{veh}$-injected rats (group 1), the administration of U74389F 4 h after ic infection (group 3) significantly attenuated the increase in ICP (7.6 ± 1.5 vs. 15.4 ± 1.2 mm Hg, groups 3 and 1, respectively) but
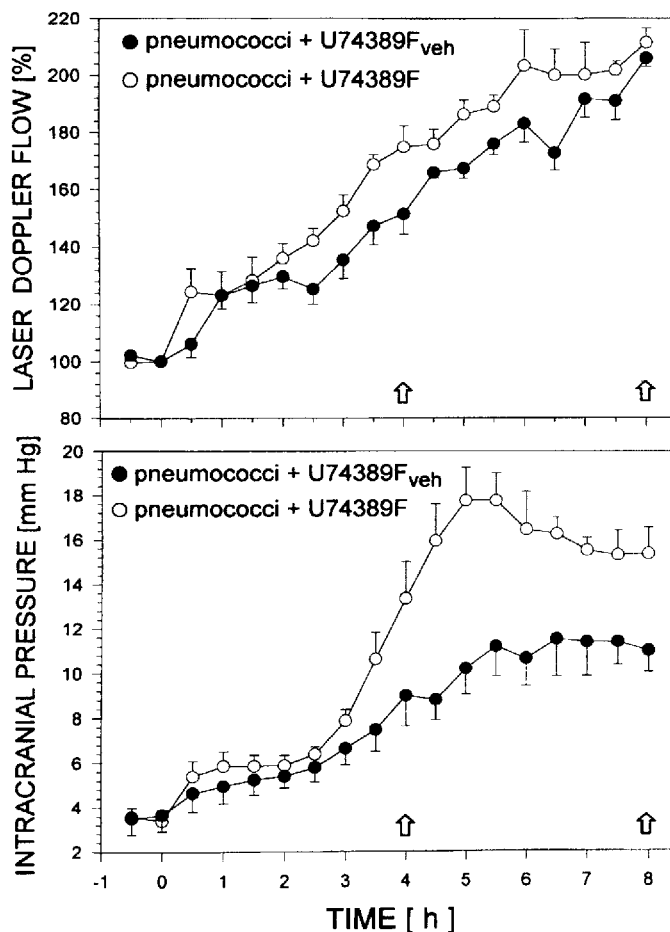
Figure 1. Time course of regional cerebral blood flow (rCBF) and intracranial pressure (ICP) in infected rats injected with U74389F or its vehicle (U74389F$_{veh}$) before infection. rCBF and ICP increased in both groups. Statistical analysis was done 4 and 8 h after intracisternal injection (arrows) by unpaired Student's t test. P values were corrected for repeated measurements by Bonferroni-Holm procedure. Pretreatment with U74389F significantly reduced increase in ICP 8 h after infection (see table 1). Data are mean ± SE.

Lorenzl et al.

Table 2. Effect of different doses of U74389F on the production of nitric oxide, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) by rat peritoneal macrophages.

Group	NO$_2^-$ (μM)	IL-6 (U/mL)	TNF-α* (U/mL)
U74389F$_{veh}$	78.2	1500	<5
+ U74389F, 10 μM	68.3	800	<5
HKP	92.5	72,960	85
+ U74389F, 1 μM	92.9	101,120	120
+ U74389F, 10 μM	85.4	70,400	68
+ U74389F, 100 μM	79.3	24,320	9
LPS	95.6	8640	20
+ U74389F, 10 μM	90.0	4960	<5
+ U74389F, 100 μM	84.5	2050	<5
Interferon-γ + TNF-α	94.6	1650	ND
+ U74389F, 100 μM	78.2	260	ND

NOTE. NO$_2^-$ = nitrite; veh = vehicle of U74389F; HKP = heat-killed pneumococci (10^7 cfu/mL); LPS = *Escherichia coli* lipopolysaccharide; ND = not done.
* Detection limit of TNF-α assay was 5 U/mL.

had no effect on CSF pleocytosis (7240 ± 700 vs. 6710 ± 729 cells/μL), rCBF (189.3% ± 13.3% vs. 211.6% ± 5.2%), or brain water content (79.04% ± 0.03% vs. 79.09% ± 0.03%).

Administration of L-NA 4 h after infection in U74389F-pretreated rats (group 6) produced an increase in MABP from 98 ± 4 to 124 ± 5 mm Hg. The rCBF increase at 4 h after infection (149.1% ± 14.4% for group 6 vs. 151.8% ± 7.0% for group 2) was completely reversed by L-NA (97.4% ± 12.8% for group 6 vs. 167.7% ± 3.4% for group 2 5 h after infection).

Inhibition of release of NO, IL-6, and TNF-α by peritoneal macrophages stimulated with HKP, E. coli LPS, and cytokines. HKP (in a dose-dependent fashion, data shown only for 10^7 cfu/mL), *E. coli* LPS, and murine rIFN-γ plus murine rTNF-α stimulated production of NO, IL-6, and TNF-α in the macrophage cultures (table 2). U74389F inhibited dose dependently NO, IL-6, and TNF-α production by macrophages stimulated with HKP, *E. coli* LPS, or murine rIFN-γ plus murine rTNF-α. HKP (10^7 cfu/mL)-induced production of NO, IL-6, and TNF-α by macrophages was inhibited by U74389F (100 μM) by 92%, 89%, and 67%, respectively.

Inhibition of lipid peroxidation by U74389F in primary astrocytes. Iron-dependent lipid peroxidation in primary rat astrocyte cultures was inhibited by U74389F and methylprednisolone (figure 2).

Discussion

The major finding of this study was that pretreatment with the novel 21-aminosteroid U74389F significantly attenuated increases in brain water content and ICP and CSF leukocytosis during the early phase of experimental pneumococcal meningitis in the rat. U74389F had no effect on the increase in rCBF. Administration of U74389F to animals with established meningitis significantly attenuated the increase in ICP but had no effect on CSF pleocytosis or increases in rCBF and brain water

content. The antiedematous effect of U74389F when given prior to infection corresponds with findings in other pathophysiologic CNS models that documented a protective effect of 21-aminosteroids on brain edema formation [19]. The increase in ICP in the early phase of experimental pneumococcal meningitis is thought to be mainly due to an increase in brain water content and an increase in cerebral blood volume [20]. Our finding that the increase in ICP was reduced but not completely
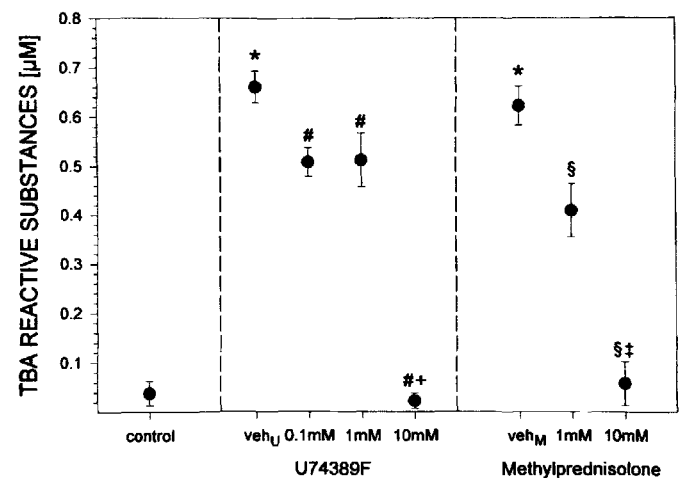
Figure 2. Inhibition of iron-dependent lipid peroxidation in primary rat astrocytes. Lipid peroxidation was assessed by formation of thiobarbituric acid (TBA)—reactive substances (mean ± 99% confidence interval). Addition of Fe^{2+}/Fe^{3+} (+ vehicle of U74389F [veh$_U$] or methylprednisolone [veh$_M$]) to astrocytes induced significant increase of TBA-reactive substances vs. action of diluent of Fe^{2+}/Fe^{3+} (control; * P < .05). Increase was significantly attenuated by methylprednisolone, known inhibitor of lipid peroxidation (§ ‡, P < .05 vs. veh$_M$ and 1 mM, respectively). U74389F also blocked increase of TBA-reactive substances (# +, P < .05 vs. veh$_U$ and vs. 0.1 and 10 mM, respectively). All P values determined by analysis of variance and Student-Newman-Keuls multiple comparisons.

inhibited might be explained by the lack of influence of U74389F on an increase in rCBF, which, in turn, may be caused at least in part by an increase in cerebral blood volume.

The 21-aminosteroids exhibit protective effects in a variety of pathologic conditions, including cerebral ischemia [21], subarachnoid hemorrhage [22], traumatic brain injury [23], vasogenic edema [19], and endotoxemia [24]. Their protective effects have been primarily attributed to their capacity to inhibit membrane lipid peroxidation [12, 25]. We investigated whether U74389F is capable of inhibiting iron-dependent lipid peroxidation in a CNS cell culture system. Our in vitro experiments used primary rat astrocytes and showed that the ability of U74389F to suppress lipid peroxidation is comparable to that of methylprednisolone, a potent inhibitor of lipid peroxidation. By inhibiting lipid peroxidation, the 21-aminosteroids may indirectly exert further inhibitory actions. For example, inhibition of 5-lipoxygenase may prevent the formation of leukotrienes [19], inhibition of NADPH oxidase may prevent the generation of superoxide radical and hydrogen peroxide [26], and inhibition of the destruction of cell membranes may prevent the release of arachidonic acid [27]. Recently, others have suggested that some 21-aminosteroids (e.g., U74389F, U78518F) may have an oxygen radical scavenging effect [28, 29].

We tested whether U74389F, apart from inhibiting lipid peroxidation, also interferes with other mediators (e.g., cytokines and NO) known to be involved in the pathophysiology of bacterial meningitis [4, 30, 31]. Peritoneal macrophages, which we used in our in vitro experiments, are an established cell culture system for the induction of cytokines and NO. There are similarities between rat peritoneal macrophages and brain macrophages and microglia: Both cell types produce cytokines [32, 33] and NO [34, 35] upon stimulation with LPS and *Staphylococcus aureus*. Our data provide evidence that the activity of U74389F is not restricted to inhibition of lipid peroxidation. U74389F inhibited the production of NO, IL-6, and TNF-α by rat peritoneal macrophages stimulated with heat-killed pneumococci, *E. coli* LPS, and cytokines. A previous study showed that another 21-aminosteroid compound (U74500A) interferes with the production of cytokines [36]. Our in vitro studies showed that U74389F inhibited NO production by rat peritoneal macrophages; however, in vivo, U74389F (in two different dosages) surprisingly had no effect on blood flow changes, which are known to be NO-mediated [30, 31]. Thus, in our meningitis model, U74389F did not affect NO production, although the addition of the NO synthase inhibitor L-NA reversed the blood flow increase.

We found that U74389F reduced meningeal inflammation. This observation is consistent with a recent study in which the 21-aminosteroid tirilazad mesylate attenuated the accumulation of neutrophils in ischemic gerbil brain [37]. In contrast, others have reported that U74389F does not inhibit the hyperoxia-induced accumulation of neutrophils in bronchoalveolar lavage fluid [38]. One explanation for the effect observed in our study may be that 21-aminosteroids inhibit the generation of superox-

ide radical and thereby reduce the expression of adhesive glycoproteins [39, 40] and the adherence of neutrophils. Another explanation might be that damage of biomembranes by lipid peroxidation could lead to the release of chemoattractant substances, such as arachidonic acid metabolites or platelet-activating factor, which could be prevented by inhibitors of lipid peroxidation.

Reactive oxygen species are known to play a role in early pathophysiologic changes during experimental bacterial meningitis [6–10]. Along with the data from our previous experiments, the results of the current study suggest that lipid peroxidation induced by oxygen free radicals is involved in the pathophysiologic mechanisms during the early phase of pneumococcal meningitis. However, other activities of the 21-aminosteroid U74389F, such as interference with cytokine production, might contribute to the modulation of the pathophysiologic changes in early experimental pneumococcal meningitis.

Acknowledgments

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