Cyanobacterial Microcystis aeruginosa Lipopolysaccharide Elicits Release of Superoxide Anion, Thromboxane B₂, Cytokines, Chemokines, and Matrix Metalloproteinase-9 by Rat Microglia

Alejandro M. S. Mayer,*† Jonathan A. Clifford,*† Monica Aldulescu,*† Jeffrey A. Frenkel,*† Michael A. Holland,*† Mary L. Hall,* Keith B. Glaser,*‡ and John Berry§

*Department of Pharmacology, Chicago College of Osteopathic Medicine; †Biomedical Sciences Program, College of Health Sciences, Midwestern University, Downers Grove, Illinois 60515; ‡Discovery Strategic Portfolio Management, R4SP, Abbott Laboratories, Abbott Park, Illinois 60064; and §Department of Chemistry and Biochemistry, Florida International University, North Miami, Florida 33181

1To whom correspondence should be addressed at Department of Pharmacology, Chicago College of Osteopathic Medicine, Midwestern University, 555 31st Street, Downers Grove, IL 60515. Fax: (630) 515-6295. E-mail: amayer@midwestern.edu.

Received December 17, 2010; accepted February 17, 2011

Microcystis aeruginosa (M. aeruginosa) is a cosmopolitan Gram-negative cyanobacterium that may contaminate freshwater by releasing toxins, such as lipopolysaccharide (LPS) during aquatic blooms, affecting environmental and human health. The putative toxic effects of cyanobacterial LPS on brain microglia, a glial cell type that constitutes the main leukocyte-dependent source of reactive oxygen species in the central nervous system, are presently unknown. We tested the hypothesis that in vitro concentration- and time-dependent exposure to M. aeruginosa LPS strain UTCC 299 would activate rat microglia and the concomitant generation of superoxide anion (O₂⁻). After a 17-h exposure of microglia to M. aeruginosa LPS, the following concentration-dependent responses were observed: 0.1–100 ng/ml M. aeruginosa LPS enhanced O₂⁻ generation, with limited inflammatory mediator generation; 1000–10,000 ng/ml M. aeruginosa LPS caused thromboxane B₂ (TXB₂), matrix metalloproteinase-9 (MMP-9), and macrophage inflammatory protein-2 (MIP-2/CXCL2) release, concurrent with maximal O₂⁻ generation; 1000–10,000 ng/ml M. aeruginosa LPS deactivated O₂⁻ production but maintained elevated levels of TXB₂, MMP-9, tumor necrosis factor-α (TNF-α), interleukin 1-α (IL-1α), and interleukin 6 (IL-6), macrophage inflammatory protein 1x (MIP-1x/CCL3), and MIP-2/CXCL2, with concomitant lactic dehydrogenase release. Although M. aeruginosa LPS was consistently less potent than Escherichia coli LPS, with the exception of O₂⁻, TXB₂, and MCP-1/CCL2 generation, it was more efficacious because higher levels of MMP-9, TNF-α, IL-1x, IL-6, MIP-1x/CCL3, and MIP-2/CXCL2 were produced. Our in vitro studies suggest that one or more of the inflammatory mediators released during M. aeruginosa LPS stimulation of microglia may play a critical role in the subsequent ability of microglia to generate O₂⁻. To our knowledge, this is the first experimental evidence that LPS isolated from a M. aeruginosa strain, can activate brain microglia in vitro, as well as the release of O₂⁻, and other inflammatory mediators hypothesized to be involved in neuroinflammation and neurodegeneration.

Key Words: microglia; cyanobacteria; Microcystis aeruginosa; lipopolysaccharide; superoxide; thromboxane; cytokine; metalloproteinase.
attentive; (Stewart et al., 2006). Interestingly, cyanobacterial
LPS may demonstrate differential toxicity: Thus while
Synechococcus sp. LPS appears to be nontoxic to rodents
(Schmidt et al., 1980), in contrast, Anabaena flos-aquae, A.
cylindrica, Oscillatoria tenuis, O. brevisalve, and Microcystis
aeruginosa LPS were reported to be lethal to mice (Keleti
and Sykora, 1982; Raziuddin et al., 1983).

Systemic Gram-negative LPS may affect the blood-brain
barrier (BBB) directly (Banks and Erickson, 2010) or enter the
brain through regions with defective BBB function and then
activate brain microglia, the macrophage of the brain immune
system (Rock et al., 2004), which participate in neuro-
inflammation (Cunningham et al., 2005). Extensive research
over the past 2 decades has shown that when microglia are
activated by either in vivo or in vitro exposure to Gram-
negative LPS (Rock et al., 2004; Ransohoff and Perry, 2009),
inflammatory mediators may be released (for review, see
Mayer, 1998) including reactive oxygen species, e.g., O2−
(Colon and Gilbert, 1987) which may cause neuronal injury
(Banati et al., 1993; Mayer et al., 1999) and progressive
neurodegeneration (Perry et al., 2010; Qin et al., 2007). To our
knowledge, no studies have been reported on the effects of
cyanobacterial LPS on brain microglia generation of O2−.

Although the chemistry of LPS isolated from several strains
of the cosmopolitan water-bloom forming cyanobacterium
M. aeruginosa has been investigated (Jurgens et al., 1989;
Martin et al., 1989; Raziuddin et al., 1983), there has
been limited research on the toxicology of M. aeruginosa LPS.
Some studies have implicated the immune system in the path-
obiology of M. aeruginosa LPS. Thus, crude M. aeruginosa
extracts, perhaps containing LPS as well as the cyclic he-
petapeptide microcystin, were shown to induce secretion of
the cytokines IL-1 and tumor necrosis factor-α (TNF-α) from
macrophages in vitro and mice in vivo (Nakano et al., 1989,
1991) as well as affecting several parameters of the immune
response in mice (Shen et al., 2003).

The purpose of this investigation was to establish whether
M. aeruginosa LPS might activate neonatal rat microglia and
stimulate release of O2−, a reactive oxygen species that has been
hypothesized to cause injury to the brain (Halliwell, 1992). Our
preliminary studies (Aldulescu et al., 2009; Mayer et al., 2008;
Patel et al., 2010) as well as this study provide experimental
support for our working hypothesis, namely that M. aeruginosa
LPS not only potentiates but also attenuates O2− generation by
rat brain microglia in vitro in a concentration-dependent
manner. Furthermore, the present findings show that inhibition
of O2− generation appears to be concomitant with a progressive
increase in the release of lactic dehydrogenase (LDH), as well as
that of several proinflammatory mediators: thromboxane B2
(TXB2), matrix metalloproteinases (MMP), cytokines, and
chemokines. Our findings are in accordance with our previous
observation that Escherichia coli LPS potentiates as well
as inhibits microglia O2− generation (Mayer et al., 1999),
and further extend this phenomenon to another LPS, namely
M. aeruginosa LPS, which although less potent than E. coli
LPS, was observed for the first time to our knowledge to prime
rat microglia O2− generation in a concentration-dependent
manner in vitro.

MATERIALS AND METHODS

Reagents. Escherichia coli LPS (E. coli) (026:B6) from Difco Laborato-
dies, Detroit, MI.; M. aeruginosa LPS (1.075 × 10^6 endotoxin units/mg) was
prepared from M. aeruginosa strain UTCC 299 by hot phenol/water extraction
from Dr John Berry, Florida International University, Miami, FL as described
(Notch et al., 2010). Dulbecco’s Modified Eagle Medium (DMEM) with
high glucose (4500 mg/l), Hanks’ balanced salt solution (HBSS), penicillin
(P), streptomycin (S), trypsin (0.25%)-EDTA (1mM) were from Gibco
Laboratories, Life Technologies Inc., Grand Island, NY; heat-inactivated fetal
bovine serum (FBS) certified was from HyClone, Logan, UT; ferricytochrome c
(FCC) type III (from horse heart), superoxide dismutase (SOD) (from bovine
liver), phospholipase 12-mytisate 13-acetate (PMA) were from Sigma Chemical Co.,
St. Louis, MO. PMA was maintained at −20°C as a 10mM stock solution
in DMSO.

LPS contamination. All glassware and metal spatulas were baked for 4 h
at 180°C to inactivate LPS (Sharma, 1986). Sterile and LPS-free 225-cm2
vented cell culture flasks were from BD Biosciences, Bedford, MA; 24-well
flat-bottom culture clusters and disposable serological pipettes were from
Costar Corporation, Cambridge, MA. Sterile and pyrogen-free Eppendorf
Biopur pipette tips were from Brinkmann Instruments, Inc., Westbury, NY.

Isolation of rat neonatal microglia. Experiments were performed in
adherence to National Institutes of Health guidelines on the use of experimental
animals, with protocols approved by Midwestern University’s Research and
Animal Care Committee. Rat brain neonatal microglia were isolated and
characterized as previously described (Mayer et al., 1999). Briefly, cerebral
cortices of 1- to 2-day-old Sprague-Dawley rats (Charles Rivers, Hartford, CT)
were surgically removed, placed in cold DMEM containing 120 U/ml P and 12
mg/ml S into each well of nonpyrogenic polystyrene 24-well
culture clusters, with DMEM supplemented with 10% FBS
containing 120 U/ml P and 12
mg/ml S. The meninges removed, and brain tissue minced and
mixed with trypsin-EDTA at 35.9°C for 3–5 min. The mixed glial cell suspension
was plated in 225-cm2 vented cell culture flasks with DMEM medium
supplemented with 10% FBS containing 120 U/ml P and 12 mg/ml S and
grown in a humidified 5% CO2 incubator at 35.9°C for 12–14 days. Upon
confluence (day 14) and every week thereafter, microglia were detached using
an orbital shaker (150 rpm, 0.5 h, 35.9°C, 5% CO2), centrifuged (400 × g, 25
min, 4°C), and cell number and viability assessed by trypan blue exclusion. Rat
neonatal microglia yields averaged 1.1 × 10^6 microglia per tissue culture flask
(225 cm2) per week in our laboratory. Depending on the particular experimental
protocol (see below), microglia averaging > 95% viability were plated in
24-well cell culture clusters, with DMEM supplemented with 10% FBS
containing 120 U/ml P and 12 mg/ml S and placed in a humidified 5% CO2
incubator at 35.9°C 24 h prior to the experiments.

Activation of microglia with LPS (experimental protocol). To determine
the effect of M. aeruginosa LPS on rat neonatal microglia activation and
inflammatory mediator release (O2−, eicosanoids, cytokines, chemokines,
and MMP), 2 × 10^6 rat neonatal microglia were seeded in DMEM + 10% FBS +
120 U/ml P + 12 mg/ml S into each well of nonpyrogenic polystyrene 24-well
flat-bottom culture clusters (Costar, Coming Inc., Coming, NY) and stimulated
with 0.1–100,000 ng/ml M. aeruginosa LPS for 17 h in a humidified 5% CO2
incubator at 35.9°C. E. coli LPS (0.1–100 ng/ml) was used as control in all the
experiments described herein (Mayer et al., 1999). After the 17-h incubation,
conditioned media (1 ml) from each tissue culture well was aspirated and split
into two aliquots. One aliquot (0.1 ml) was used to measure LDH levels, as
a measure of cell viability (Morgenstern et al., 1966). The remaining aliquot
(0.9 ml) was frozen (−84°C) until determination of eicosanoids, cytokines,
chemokines, and MMPs, as described below. Once the conditioned media had been removed, both *M. aeruginosa* and *E. coli* LPS-treated microglia cells were washed with warm (37°C) HBSS, and O₂ was determined as described below.

**Assay for superoxide anion (O₂⁻) generation.** O₂⁻ generation was determined by the SOD-inhibitable reduction of FCC (Mayer et al., 1999). Briefly, PMA (1 μM)-triggered O₂⁻ release from either *E. coli* or *M. aeruginosa* LPS-activated microglia was measured in the presence of FCC (50μM) and HBSS, with or without SOD (700 units), which inhibited > 95% of FCC reduction, during a 70-min incubation. All experimental treatments were run in duplicate and in a final volume of 1 ml. Changes in FCC absorbance were measured at 550 nm using a Beckman DU-800 spectrophotometer. Differences in the amount of reduced FCC in the presence and absence of SOD were used to determine microglia O₂⁻ generation by employing the molecular extinction coefficient of 2.10 × 10⁵ M⁻¹ cm⁻¹ and expressed in nmol.

**Assay for LDH.** To assess cell viability following preincubation of microglia with either *M. aeruginosa* LPS or *E. coli* LPS as described in our experimental protocol, the conditioned media was harvested and LDH release was determined spectrophotometrically as previously described (Mayer et al., 1999; Morgenstern et al., 1966). Microglia LDH release was expressed as a percent of total LDH released into the conditioned media. Total LDH release resulted from 0.1% Triton X-100-lysed microglia (intracellular LDH) plus LDH present in the extracellular media. Because the FBS contains LDH (data not shown), unless LDH release from LPS-treated microglia was greater than 15% of that observed from Triton X-100 (0.1%-treated microglia (total LDH), LPS treatment was considered to have no effect on microglia viability.

**Assay for TXB₂ generation.** Following incubation of microglia with either *M. aeruginosa* LPS or *E. coli* LPS for 17 h, TXB₂ generation in cell-free conditioned media was measured using TXB₂ immunnoassays (Cayman Chemical, Ann Arbor, MI), as indicated by the manufacturer’s protocol. Results were expressed as picogram per ml (pg/ml). The minimum detectable concentration was 7.8 pg/ml TXB₂.

**Gelatinase zymography for MMP-2 and MMP-9 analysis.** Gelatin-containing zymograms are typically used to detect MMP-2 (68 kDa) and MMP-9 (92 kDa), and their identification is based on molecular weight. Following incubation of cultured rat neonatal microglia with either *M. aeruginosa* LPS or *E. coli* LPS, MMP-2 and -9 expression were determined in the cell-free conditioned media. As the rat neonatal microglia cultures were normalized for cell number, equal volumes of harvested media obtained from each condition were analyzed. Briefly, 90 μg of each protein sample were electrophoresed under nondenaturing conditions using a 10% polyacrylamide gel containing 0.1% gelatin. The gels were incubated twice for 30 min in 1X Novex Zymogram Renaturing Buffer (Invitrogen, Carlsbad, CA) and then electrophoresed under nondenaturing conditions using a 10% polyacrylamide gel containing 0.1% gelatin. The gels were incubated twice for 30 min in 1X Novex Zymogram Developing Buffer (Invitrogen, Carlsbad, CA) and then incubated overnight in a 5% CO₂ incubator at 37°C in 1X Novex Zymogram Developing Buffer (Invitrogen). Gels were stained in 0.4% (wt/vol) Coomassie Brilliant Blue R-250 Solution (Bio-Rad, Hercules, CA), followed by destaining in 10% methanol, 10% acetic acid. MMP activity was visualized as clear bands containing zymograms are typically used to detect MMP-2 (68 kDa) and MMP-9 (92 kDa), and their identification is based on molecular weight.

**Assay for cytokines: TNF-α, IL-1β, IL-6, TGF-β₁, and TGF-β₂.** The presence of immunoreactive cytokines in the cell-free conditioned media was determined using rat-specific ELISAs: for TNF-α, interleukin 1-α (IL-1α), interleukin-6 (IL-6), TGF-β₁, and TGF-β₂ from Biosource International (Camarillo, CA) and for TGF-β₁ and TGF-β₂ from R & D Systems (Minneapolis, MN). The results were expressed in pg/ml. The minimum detectable cytokine concentrations were: TNF-α, less than 4 pg/ml; IL-1α, less than 3 pg/ml; IL-6, less than 7 pg/ml; TGF-β₁, less than 15.6 pg/ml; and TGF-β₂, less than 7 pg/ml.

**Assays for chemokines: MIP-1α/CCL3, MIP-2/CXCL2, and MCP-1/CCL2.** The presence of immunoreactive chemokines in cell-free conditioned media was determined using rat-specific ELISAs: for MIP-1α/CCL3 from Koma Biotech, Seoul, South Korea and for MIP-2/CXCL2 and MCP-1/CCL2 from Biosource International. The results were expressed in pg/ml. The minimum detectable chemokine concentrations were: MIP-1α/CCL3, less than 16 pg/ml; MIP-2/CXCL2, less than 1 pg/ml; and MCP-1/CCL2, less than 8 pg/ml.

**Statistical analysis of the data.** Data was expressed as mean ± SEM from 2 to 4 independent experiments (n), each experiment with triplicate determinations. Data were analyzed with Prism software package version 5 from GraphPad, San Diego, CA. LPS-treated microglia were compared with the vehicle-treated microglia (control), shown as 0 in the corresponding figures. One way ANOVA followed by Dunnet’s post hoc procedure was performed on all sets of data. Statistical significance between the effect of a single dose of *E. coli* and *M. aeruginosa* LPS on the release of each mediator studied (e.g., O₂⁻) was determined using 2-way ANOVA. Differences were considered statistically significant at p < 0.05 and reported in each figure legend.

**RESULTS**

**Effect of *M. aeruginosa* LPS on Rat Neonatal Brain Microglia O₂⁻ Generation**

Generation of reactive oxygen species by brain microglia have been implicated in oxidative stress reported in several chronic neurodegenerative diseases (Block et al., 2007; Colton and Wilcock, 2010; Mayer, 1998). We and others have reported that *E. coli* LPS-treated rat microglia release O₂⁻ in vitro (Colton and Gilbert, 1987; Mayer et al., 1999). As shown in Figure 1, PMA-stimulated O₂⁻ production was observed when rat microglia were pretreated with either *M. aeruginosa* or *E. coli* LPS for 17 h. A bell-shaped dose-response curve was observed when rat neonatal microglia were treated with *M. aeruginosa* LPS for 17 h, with maximal O₂⁻ release observed at 1000 ng/ml *M. aeruginosa* LPS which thereafter progressively decreased. In contrast and
as previously reported (Mayer et al., 1999), PMA-stimulated O$_2^-$ generation in *E. coli* LPS-treated microglia cells was bell-shaped but shifted to the left with maximal O$_2^-$ at 1 ng/ml LPS. Thus, *M. aeruginosa* LPS appeared to be a 1000-fold less potent than *E. coli* LPS in inducing O$_2^-$ production from rat microglia in vitro.

**Effect of *M. aeruginosa* LPS on Rat Neonatal Brain Microglia LDH Generation**

In order to determine whether the progressive decrease in O$_2^-$ release generation (Fig. 1) resulted from concentration-dependent toxicity of *M. aeruginosa* and *E. coli* LPS to microglia during the 17-h incubation, LDH release was determined in the tissue culture supernates (Mayer et al., 1999). LDH has frequently been used as a marker for cellular toxicity (Mayer and Spitzer, 1994; Morgenstern et al., 1966).

As shown in Figure 2, there was a concentration-dependent increase in LDH release in *vitro* that closely paralleled the progressive decrease in O$_2^-$ generation observed at higher *M. aeruginosa* and *E. coli* LPS concentrations. Thus, in *M. aeruginosa* LPS-stimulated cells, a maximum 55 ± 19.3% of control LDH release was observed at 100,000 ng/ml. In contrast, in *E. coli* LPS-stimulated microglia, a dose-dependent LDH increase was observed at greater than 1 ng/ml LPS, reaching 67.7 ± 8.2% of control at 100 ng/ml LPS.

**Effect of *M. aeruginosa* LPS on Rat Neonatal Brain Microglia TXB$_2$ Generation**

Eicosanoids released by activated microglia have been proposed to play a proinflammatory role in the pathology of neurological and neurodegenerative diseases (Choi et al., 2009). We and others have shown that *E. coli* LPS–treated rat microglia release TXB$_2$ in *vitro* (Mayer et al., 1999; Minghetti and Levi, 1995). As shown in Figure 3, unstimulated microglia released low levels of TXB$_2$. In *M. aeruginosa* LPS–treated microglia cells, TXB$_2$ generation yielded a sigmoid curve and became statistically significant at 1000 ng/ml, when there was maximal O$_2^-$ generation (Fig. 1) but low LDH release (Fig. 2). Confirming our previous study (Mayer et al., 1999, in *E. coli* LPS-stimulated cells, a concentration-dependent TXB$_2$ release was observed at 1 ng/ml. Thereafter, TXB$_2$ release progressively increased, becoming maximal at 100 ng/ml, when O$_2^-$ generation was attenuated (Fig. 1), and LDH release was maximal (Fig. 2). Thus, *M. aeruginosa* LPS appeared to be less potent than *E. coli* LPS in inducing a concentration-dependent TXB$_2$ release from rat microglia in *vitro* as well as less efficacious than *E. coli* LPS.

**Effect of *M. aeruginosa* LPS on Rat Neonatal Brain Microglia MMP-2 and MMP-9 Generation**

MMPs released by activated microglia have been proposed to play a proinflammatory role in the pathology of sepsis and neuroinflammation (Candelario-Jalil et al., 2009; Vanlaere and Libert, 2009). We and others have reported MMP-2 and MMP-9 release from *E. coli* LPS-stimulated rat microglia in *vitro* (Gottschall et al., 1995; Mayer et al., 1999). As shown in Figure 4, in *M. aeruginosa* LPS-stimulated microglia, MMP-9 but not MMP-2 levels were statistically significant at LPS concentrations equal or greater than 1000 ng/ml. In contrast, when microglia were stimulated with *E. coli* LPS for 17 h, a concentration-dependent increase of MMP-9 but not of MMP-2 was observed at LPS concentrations equal or greater than 1 ng/ml. Thus, similar to O$_2^-$ (Fig. 1) and TXB$_2$ (Fig. 3), *M. aeruginosa* LPS appeared to be a 1000-fold less potent than *E. coli* LPS in inducing concentration-dependent release of MMP-9 from rat microglia in *vitro*. *M. aeruginosa* LPS appeared to be more efficacious than *E. coli* LPS because maximal release MMP-9 was 1.23-fold higher.
Effect of M. aeruginosa LPS on Rat Neonatal Brain Microglia TNF-α, IL-1α, and IL-6 Generation

Presence of the cytokine TNF-α has been considered a hallmark of neuroinflammation as well as numerous neurodegenerative conditions (McCoy and Tansey, 2008). Release of TNF-α triggered by E. coli LPS in vitro has been reported in mouse (Esen and Kielian, 2007; Hausler et al., 2002; Hayashi et al., 1995), human (Lee et al., 1993), and rat microglia (Horvath et al., 2008; Mayer et al., 1999). In the present study, unstimulated rat microglia released low levels of TNF-α. As shown in Figure 5 (panel A), in M. aeruginosa LPS-stimulated rat microglia cells, TNF-α levels increased at greater than 100 ng/ml and became statistically significant at 100,000 ng/ml. In contrast, a concentration-dependent TNF-α release in E. coli LPS-stimulated microglia was observed at greater than 0.1 ng/ml LPS which peaked at 10 ng/ml LPS thus confirming our previous observations (Mayer et al., 1999). Thus, similar to O2− (Fig. 1) and TXB2 (Fig. 3), M. aeruginosa LPS appeared to be a 1000-fold less potent than E. coli LPS in inducing TNF-α production from rat microglia in vitro. Notably, M. aeruginosa LPS was clearly more efficacious than E. coli LPS because maximal TNF-α release was 375% higher.

The cytokine IL-1α appears to be a pivotal mediator in neuroimmune responses and chronic neurodegenerative disorders (Allan et al., 2005; Schultzberg et al., 2007; Simi et al., 2007). Release of IL-1α has been reported from rat microglia stimulated with E. coli LPS in vitro (Giulian et al., 1986). In FIG. 4, the effect of Escherichia coli and Microcystis aeruginosa LPS on rat neonatal microglia MMP-2 and -9 release. Neonatal rat microglia (2 × 10^5 cells/well) were treated with E. coli LPS (0.1–100 ng/ml) or M. aeruginosa LPS (0.1–10^5 ng/ml) for 17-h in vitro. SDS-PAGE zymography (panel A) and bar graphs depicting the quantitated results for E. coli LPS (panel B) and for M. aeruginosa LPS (panel C). MMP-2 and MMP-9 were determined as described in Materials and Methods. Data expressed as mean ± SEM of normalized MMP release from three independent experiments (n). *p < 0.05, **p < 0.01 LPS versus untreated control (0).

FIG. 5. The effect of Escherichia coli and Microcystis aeruginosa LPS on rat neonatal microglia TNF-α, IL-1α, and IL-6 release. Neonatal rat microglia (2 × 10^5 cells/well) were treated with E. coli LPS (0.1–100 ng/ml) or M. aeruginosa LPS (0.1–10^5 ng/ml) for 17-h in vitro. TNF-α (panel A), IL-1α (panel B), and IL-6 (panel C) were determined as described in Materials and Methods. Data expressed as pg/ml is the mean ± SEM from 3 to 4 independent experiments (n), each experiment with triplicate determinations. *p < 0.05, **p < 0.01 LPS versus untreated control (0).
our study, unstimulated microglia released low levels of IL-1α. As shown in Figure 5 (panel B), in M. aeruginosa LPS-stimulated microglia, IL-1α levels increased after 100 ng/ml and became statistically significant at 100,000 ng/ml. In contrast, in E. coli LPS-stimulated microglia, IL-1α levels rose at 0.1 ng/ml and peaked at 10 ng/ml. Thus, similar to O2 (Fig. 1), TXB2 (Fig. 3) and TNF-α (Fig. 5) (panel A) M. aeruginosa LPS appeared to be a 1000-fold less potent than E. coli LPS in inducing IL-1α generation from rat microglia in vitro. Interestingly, as observed for TNF-α (Fig. 5) (panel A) generation, M. aeruginosa LPS appeared more efficacious than E. coli LPS because maximal release of IL-1α was 165% higher.

IL-6 is a multifunctional cytokine involved in a variety of inflammatory conditions, including neuroinflammation (Nishimoto, 2010) and chronic neurodegenerative disorders such as Alzheimer’s disease and multiple sclerosis (Harris and Sadiq, 2009; Maccioni et al., 2009). It has previously been shown that human (Lee et al., 1993), murine (Hausler et al., 2002), and rat (Gottschall et al., 1995; Horvath et al., 2008) microglia release the cytokine IL-6 when stimulated with E. coli LPS in vitro. In our studies, unstimulated microglia released low levels of IL-6. As shown in Figure 5 (panel C), in M. aeruginosa LPS-stimulated microglia, IL-6 levels increased after 100 ng/ml and became statistically significant at 100,000 ng/ml. In contrast, IL-6 levels in E. coli LPS-stimulated microglia rose after 0.1 ng/ml LPS and peaked at 10 ng/ml LPS. Thus, similar to O2 (Fig. 1), TXB2 (Fig. 3), TNF-α (Fig. 5) (panel A), and IL-1α (Fig. 5) (panel B) M. aeruginosa LPS appeared to be a 1000-fold less potent than E. coli LPS in inducing concentration-dependent release of IL-6 from rat microglia in vitro, but as observed with TNF-α (Fig. 5) (panel A) and IL-1α (Fig. 5) (panel B) release, M. aeruginosa LPS was more efficacious than E. coli LPS because maximal release of IL-6 was 233% higher.

**Effect of M. aeruginosa LPS on Rat Neonatal Brain Microglia MIP-1α/CCL3, MIP-2/CXCL2, and MCP-1/CCL2 Generation**

MIP-1α/CCL3 is an inflammatory chemokine involved in a variety of inflammatory conditions, including neuroinflammation (Ubogu et al., 2006) and chronic neurodegenerative disorders such as Alzheimer’s disease and multiple sclerosis (Szczucinski and Losy, 2007). MIP-1α/CCL3 has been shown to be released by LPS-treated mouse (Hausler et al., 2002; Hayashi et al., 1995), rat (Sun et al., 1997), and human microglia in vitro (Peterson et al., 1997). As shown in Figure 6 (panel A), M. aeruginosa LPS stimulated release of MIP-1α/CCL3, which increased at 100 ng/ml and became statistically significant at 100,000 ng/ml. In contrast, E. coli LPS-induced MIP-1α/CCL3 release was bell-shaped, occurred at greater than 0.1 ng/ml and peaked at 1 ng/ml. Thus, similar to O2 (Fig. 1), TXB2 (Fig. 3), TNF-α (Fig. 5) (panel A), IL-1α (Fig. 5) (panel B), and IL-6 (Fig. 5) (panel C), M. aeruginosa LPS was a 1000-fold less potent than E. coli LPS in inducing MIP-1α/CCL3 release from rat microglia. Furthermore, M. aeruginosa LPS was slightly more efficacious than E. coli LPS in releasing MIP-1α/CCL3.

MIP-2/CXCL2 is an inflammatory chemokine involved in a variety of inflammatory conditions, including neuroinflammation (Ubogu et al., 2006). MIP-2/CXCL2 has been shown to be released by LPS-treated murine (Esen and Kielian, 2007; Hausler et al., 2002) and rat (Lafrance et al., 2010) microglia in vitro. Unstimulated microglia released low levels of MIP-2 constitutively. As shown in Figure 6 (panel B), in M. aeruginosa LPS-stimulated microglia, MIP-2/CXCL2 release which progressively increased at 10 ng/ml LPS became statistically significant at 10,000 ng/ml. In contrast, MIP-2/CXCL2 levels in E. coli LPS-stimulated microglia rose after 0.1 ng/ml LPS and increased at greater than 0.1 ng/ml and peaked at 1 ng/ml. Thus, similar to O2 (Fig. 1), TXB2 (Fig. 3), TNF-α (Fig. 5) (panel A), IL-1α (Fig. 5) (panel B), and IL-6 (Fig. 5) (panel C), M. aeruginosa LPS was a 1000-fold less potent than E. coli LPS in inducing MIP-1α/CCL3 release from rat microglia. Furthermore, M. aeruginosa LPS was slightly more efficacious than E. coli LPS in releasing MIP-1α/CCL3.

**FIG. 6.** The effect of Escherichia coli and Microcystis aeruginosa LPS on rat neonatal microglia MIP-1α/CCL3, MIP-2/CXCL2, and MCP-1/CCL2 release. Neonatal rat microglia (2 × 10⁵ cells/well) were treated with E. coli (0.1–100 ng/ml) or M. aeruginosa (0.1–10⁵ ng/ml) LPS for 17 h in vitro. MIP-1α/CCL3 (panel A), MIP-2/CXCL2 (panel B), and MCP-1/CCL2 (panel C) release were determined as described in Materials and Methods. Data expressed as pg/ml is the mean ± SEM from 3 to 4 independent experiments (n), each experiment with triplicate determinations. *p < 0.05; **p < 0.01; ***p < 0.001 LPS versus untreated control (0). ▲ p < 0.05, ▲▲ p < 0.01, ▲▲▲ p < 0.001, ▲▲▲▲ p <0.0001 M. aeruginosa LPS versus E. coli LPS.
were maximal at 10 ng/ml LPS. Thus, similar to $O_2^-$ (Fig. 1), TXB$_2$ (Fig. 3), TNF-$\alpha$ (Fig. 5) (panel A), IL-1$\alpha$ (Fig. 5) (panel B), IL-6 (Fig. 5) (panel C), and MIP-1$\alpha$/CCL3 (Fig. 6) (panel A), *M. aeruginosa* LPS was less potent than *E. coli* LPS in inducing concentration-dependent release of MIP-2/CXCL2 from rat microglia *in vitro*.

MCP-1/CCL2 is an inflammatory chemokine involved in neuroinflammation ([Ubogu et al., 2005](#)) and multiple sclerosis ([Szczucinski and Losy, 2007](#)). MCP-1/CCL2 has been shown to be generated by mouse ([Hauser et al., 2002](#); [Hayashi et al., 1995](#)), rat ([Horvath et al., 2008](#); [Sun et al., 1997](#)), and human microglia *in vitro* ([Peterson et al., 1995](#)). As shown in Figure 6 (panel C), *M. aeruginosa* LPS-induced MCP-1/CCL2 release yielded a flat sigmoid curve with maximal nonstatistically significant release at 100,000 ng/ml. In contrast, *E. coli* LPS-induced statistically significant release of MCP-1/CCL2 yielding a bell-shaped curve which peaked at 1 ng/ml. Thus, similar to the other cytokines and chemokines investigated, *M. aeruginosa* LPS appeared to be less potent than *E. coli* LPS in inducing concentration-dependent release of MCP-1/CCL2. However, in contrast to the other inflammatory mediators investigated, release of MCP-1/CCL2 was less efficacious at the highest *M. aeruginosa* LPS concentration used.

**Effect of *M. aeruginosa* LPS on Rat Neonatal Brain Microglia TGF-$\beta_1$ and TGF-$\beta_2$ Generation**

In order to determine whether *M. aeruginosa* LPS–treated rat neonatal brain microglia affected release of anti-inflammatory cytokines, we investigated the presence of TGF-$\beta_1$ and TGF-$\beta_2$, which have been studied for their neuroprotective effects ([Qian et al., 2008](#)). TGF-$\beta_1$ and TGF-$\beta_2$ are constitutively expressed in rat microglia ([Polazzi et al., 2009](#)) *in vitro*, as well as in *E. coli* LPS-activated human ([Walker et al., 1995](#)) and murine microglia ([Chao et al., 1995](#)). As shown in Table 1, unstimulated rat microglia released TGF-$\beta_1$ constitutively but neither *M. aeruginosa* LPS nor *E. coli* LPS enhanced TGF-$\beta_1$ or TGF-$\beta_2$ release during the 17-h *in vitro* incubation. In fact, a small though nonstatistically significant decrease of constitutive TGF-$\beta_1$ was observed in *M. aeruginosa* LPS–treated microglia.

**DISCUSSION**

The increased generation of reactive oxygen species such as $O_2^-$ in the central nervous system ([Halliwell, 2001](#)) has been hypothesized to be involved in neuronal injury and neurodegeneration in several neuropathologies ([Cunningham et al., 2005](#)). Thus, microglia activation ([Rock et al., 2004](#)) and concomitant $O_2^-$ generation as part of the mechanism of neuroinflammation have been extensively investigated over the past 25 years ([Ranshoft and Perry, 2009](#)). One activator of microglia $O_2^-$ generation that has received considerable attention is LPS ([Holst et al., 1996](#)) because it may activate microglia via the lipid A portion of the macromolecule leading to the *in vivo* and *in vitro* generation of inflammatory mediators such as MMPs, arachidonic acid metabolites, and cytokines ([Mayer, 1998](#)).

The first goal of his study was to determine whether *M. aeruginosa* LPS might induce $O_2^-$ release from brain microglia *in vitro*. Our current data supports the following conclusions: First, confirming our previous observations ([Mayer et al., 1999](#)) after microglia were incubated with *E. coli* LPS for 17 h, PMA-stimulated $O_2^-$ generation yielded a bell-shaped dose-response curve, with potentiation of $O_2^-$ release at 0.1 to 1 ng/ml LPS, followed by a progressive inhibition of $O_2^-$ generation at 10 and 100 ng/ml. The progressive decrease in $O_2^-$ generation was concomitant to an increase in LDH release, revealing that increased concentrations of *E. coli* LPS caused self-injury to microglia cells. Second and for the first time to our knowledge, cyanobacterium *M. aeruginosa* LPS–treated microglia $O_2^-$ generation yielded a bell-shaped curve: thus, following an initial potentiation of $O_2^-$ release at *M. aeruginosa* LPS 10 to 1000 ng/ml, a progressive attenuation of $O_2^-$ generation was observed at *M. aeruginosa* LPS 10,000 and 100,000 ng/ml. Also similar to *E. coli* LPS, after a 17-h incubation with *M. aeruginosa* LPS toxicity to microglia was demonstrated by increased LDH in the tissue culture media. Third, *M. aeruginosa* LPS was less potent than *E. coli* LPS in activating rat microglia $O_2^-$ generation after 17 h of *in vitro* stimulation. Additional studies will be required to further characterize the potentiation and attenuation of $O_2^-$ generation by *M. aeruginosa* LPS to determine whether the kinetics are similar to *E. coli* LPS, where potentiation and inhibition of rat microglia $O_2^-$ generation required 18–24 h ([Mayer et al., 1999](#)).
A second goal of this investigation was to assess whether *M. aeruginosa* LPS–treated microglia might release proinflammatory and antiinflammatory mediators concomitantly with O$_2$ generation. The following results were observed:

First, confirming our previous observations (Mayer et al., 1999), after microglia were incubated with *E. coli* LPS for 17 h, a statistically significant release of TXB$_2$, MMP-9, and TNF-α was observed concomitant with maximal O$_2$ potentiation by 1 ng/ml *E. coli* LPS. Moreover, we observed several additional proinflammatory cytokines and chemokines in *E. coli* LPS–treated microglia tissue culture supernates, in the following rank order: MIP-2/CXCL2 > MIP-1α/CCL3 > IL-6 > IL-1α > MCP-1/CCL2. Second, and for the first time to our knowledge, *M. aeruginosa* LPS–treated microglia were shown to release TXB$_2$, MMP-9, as well as several cytokines and chemokines concomitant with maximal O$_2$ potentiation and in quantities which were ‘enhanced’ when compared with *E. coli* LPS–treated microglia in the following rank order: MIP-2/CXCL2 > MIP-1α/CCL3 > TNF-α > IL-6 > IL-1α > MCP-1/CCL2. Third, and similar to O$_2$ generation, *M. aeruginosa* LPS was less potent than *E. coli* LPS in stimulating release of microglia inflammatory mediators after a 17-h *in vitro* stimulation. However, *M. aeruginosa* LPS was more efficacious than *E. coli* LPS because most of the inflammatory mediators investigated were generated in larger quantities *in vitro*. Fourth, there appeared to be a correlation between both the increased release of LDH and that of TXB$_2$, MMP-9, as well as the cytokines and chemokines we measured in the culture media, and the progressive concentration-dependent attenuation of PMA-elicited microglia O$_2$ generation in *M. aeruginosa* LPS–treated cells. Whether one or several of these inflammatory mediators might be responsible for the mechanism leading to the increased cytotoxicity to neonatal microglia cells *in vitro* is presently unknown. The current study suggests, but does not conclusively prove, that these mediators, and perhaps others, may in some manner contribute to the mechanism of *M. aeruginosa* LPS-induced cytotoxicity to the microglia *in vitro*.

It is important to reflect on potential new lines of inquiry that emerge from the observed effects of *M. aeruginosa* LPS on rat neonatal microglia *in vitro*. First, the present study was completed with *M. aeruginosa* LPS strain UTCC 299, one of several *M. aeruginosa* strains currently available (Rapala et al., 2002). Thus, it would be important to determine whether LPS isolated from other *M. aeruginosa* strains shown to be present in drinking water sources will also be bioactive in the rat microglia model *in vitro*. Second, our study was limited to the effect of *M. aeruginosa* LPS on ‘neonatal’ brain microglia. Thus, it would be important to determine whether ‘adult’ rat microglia, which are known to release higher levels of PGE$_2$ than neonatal microglia (Slepko et al., 1997) may perhaps differ in their capacity to generate O$_2$ and other inflammatory mediators. Third, because human, mice, and hamster microglia produce significantly different amounts of O$_2$ in response to the same activating agents, differences which have been hypothesized to be important when modeling human disease processes from rodent studies (Colton et al., 1996), it should be important to determine whether the observed biphasic effects of *M. aeruginosa* LPS on rat neonatal microglia O$_2$ generation *in vitro* will also occur with human microglia. Interestingly, we have observed that *E. coli* LPS will significantly prime human microglia for O$_2$ release in a concentration-dependent manner (Mayer et al., 2004). Fourth, *in vivo* studies will be required to determine whether *M. aeruginosa* LPS may be pathogenic to the brain immune system because *M. aeruginosa* extracts have been shown to affect the immune response in mice (Shen et al., 2003) and caused IL-1 and TNF-α release from macrophages *in vitro* and mice *in vivo* (Nakano et al., 1989, 1991). Fifth, determining whether LPS isolated from other cyanobacteria such as *A. flos-aquae, A. cylindrica, Oscillatoria tenui*, and *O. brevisothae*, which have been shown to cause mortality in mice (Keleti and Sykora, 1982; Raziuddin et al., 1983), are bioactive in microglia *in vitro* becomes a relevant question meriting further investigation.

In our present investigation, inflammatory products elicited by ‘classical’ activation of rat brain microglia have been observed; however, based on the ‘complexity of microglia activation states’ and the limited nature of the present study (17-h incubation with *M. aeruginosa* LPS *in vitro*), it remains unknown whether additional microglia proinflammatory mediators as well as anti-inflammatory cytokines (e.g., IL-4, IL-13, and IL-10) typical of ‘inflamma-resolution states’ may play an important role in our *in vitro* model (Colton and Wilcock, 2010). We suggest that additional mechanistic investigation of the effect of *M. aeruginosa* LPS on microglia at both the functional and molecular level, both *in vitro* and *in vivo*, will help define the ‘continuum’ of activation states of microglia upon *M. aeruginosa* LPS treatment and the factors involved (receptors, enzymes, proteins, etc.) (Colton 2009). Furthermore, we are hopeful that accumulated data on microglia activation states after *M. aeruginosa* LPS exposure will help identify candidate therapeutic targets and novel treatment strategies to protect and treat humans after environmental exposure to cyanobacterial LPS (Anderson et al., 2002; Stewart et al., 2006, 2009).

**FUNDING**

Office of Research and Sponsored Programs at Midwestern University; Biomedical Sciences Program, College of Health Sciences, Midwestern University.

**ACKNOWLEDGMENTS**

Valuable support by Midwestern University’s animal facility and library staff as well as the excellent secretarial assistance of Mrs Victoria Sears and Ms Laura Phelps is gratefully acknowledged.
REFERENCES

Aldulescu, M., Frenkel, J., Hall, M. L., Powell, J. L., Glaser, K. B., and Mayer, A. M. S. (2009). Effect of Microcystis aeruginosa lipopolysaccharide on neonatal brain microglia cytokine and chemokine release. *FASEB J.* 23, 755–1 (Abstract).

Allan, S. M., Tyrrell, P. J., and Rothwell, N. J. (2005). Interleukin-1 and neuronal injury. *Nat. Rev. Neurosci.* 5, 629–640.

Anderson, W. B., Slawson, R. M., and Mayfield, C. I. (2002). A review of drinking-water-associated endotoxin, including potential routes of human exposure. *Can. J. Microbiol.* 48, 567–587.

Banati, R. B., Gehrmann, J., Schubert, P., and Kreutzberg, G. W. (1993). Cytocotoxicity of microglia. *Glia* 7(1), 111–118.

Banks, W. A., and Erickson, M. A. (2010). The blood-brain barrier and immune function and dysfunction. *Neurobiol. Dis.* 37(1), 26–32.

Bernardova, K., Babica, P., Marsalek, B., and Blaha, L. (2008). Isolation and characterization of green alga and bacteria and green alga. *Appl. Environ. Microbiol.* 74, 567–587.

Banati, R. B., Gehrmann, J., Schubert, P., and Kreutzberg, G. W. (1993). Cytotoxicity of microglia. *Glia* 7(1), 111–118.

Banks, W. A., and Erickson, M. A. (2010). The blood-brain barrier and immune function and dysfunction. *Neurobiol. Dis.* 37(1), 26–32.

Hausler, K. G., Prinz, M., Nolte, C., Weber, J. R., Schumann, R. R., Kettenmann, H., and Hanisch, U. K. (2002). Interferon-gamma differentially modulates the release of cytokines and chemokines in lipopolysaccharide- and pneumococcal cell wall-stimulated mouse microglia and macrophages. *Eur. J. Neurosci.* 16, 2113–2122.

Hayashi, M., Luo, Y., Laning, J., Strieter, R. M., and Dorf, M. E. (1995). Production and function of monocytic chemotactant protein-1 and other beta-chemokines in murine glial cells. *J. Neuroimmunol.* 60(1–2), 143–150.

Hitzfeld, B. C., Hoger, S. J., and Dietrich, D. R. (2000). Cytobacterial toxins: removal during drinking water treatment, and human risk assessment. *Environ. Health Perspect.* 108(Suppl. 1), 113–122.

Holst, O., Ulmer, A. J., Brade, H., Flad, H. D., and Rietschel, E. T. (1996). Biochemistry and cell biology of bacterial endotoxins. *FEMS Immunol. Med. Microbiol.* 16(2), 83–104.

Horvath, R. J., Nuttle-McMenemy, N., Alkaitis, M. S., and Deleo, J. A. (2008). Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures. *J. Neurochem.* 107, 557–569.

Jurgens, U., J., Martin, C., and Weckesser, J. (1989). Cell wall constituents of microcystis sp. *PCC 7806.* *FEMS Microbiol. Lett.* 53(1–2), 47–51.

Keleti, G., and Sylkor, J. L. (1982). Production and properties of cytochalasin-dependent endotoxins. *Appl. Environ. Microbiol.* 43(1), 104–109.

Kauper-Goodman, T., Falconer, I., and Fitzgerald, J. (1999). Human health effects. In *Toxic Cyanobacteria in Water* (L. Chorus and J. Bartram, Eds), pp. 113–153. E and FN Spon, London.

Lafrance, V., Inoue, W., Kan, B., and Luheishi, G. N. (2010). Leptin modulates cell morphology and cytokine release in microglia. *Brain Behav. Immun.* 24, 358–365.

Lee, S. C., Liu, W., Dickson, D. W., Brosnan, C. F., and Berman, J. W. (1993). Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. *J. Immunol.* 150, 2659–2667.

Maccioni, R. B., Rojo, L. E., Fernandez, J. A., and Kuljis, R. O. (2009). The role of neuroinflammation in Alzheimer’s disease. *Ann. N. Y. Acad. Sci.* 1153, 240–246.

Martin, C., Codd, G. A., Siegelman, H. W., and Weckesser, J. (1989). Lipopolysaccharides and polysaccharides of the cell envelope of toxic *Microcystis aeruginosa* strains. *Arch. Microbiol.* 152, 90–94.

Mayr, A. M. S. (1998). Therapeutic implications of microglia activation by lipopolysaccharide and reactive oxygen species generation in septic shock and central nervous system pathologies: a review. *Medicina (B Aires)* 35(4), 377–385.

Mayr, A. M. S., Hall, M. L., and Walker, D. L. (2004). Inhibition of LPS-stimulated human brain microglia superoxide and thromboxanes B2 generation by the marine manzanites. *Inflammm Res.* 53, S217 (Abstract).

Mayr, A. M. S., Holland, M. A., Hall, M. L., Jacobson, P. B., and Berry, J. P. (2008). Effect of *Microcystis aeruginosa* lipopolysaccharide (LPS) on neonatal brain microglia release of superoxide anion, thromboxane B2, tumor necrosis factor alpha, and matrix metalloproteases. *Toxicol. Sci.* 102(Suppl.), 254 (Abstract).

Spitzer, J. A. (1994). Modulation of superoxide anion release and inhibition of rat neonatal microglia superoxide anion generation: correlation with prior lactic dehydrogenase, nitric oxide, tumor necrosis factor-alpha, thromboxane B2, and metalloprotease release. *Shock* 11(3), 180–186.
