Inflammatory neurodegeneration induced by lipoteichoic acid from *Staphylococcus aureus* is mediated by glia activation, nitrosative and oxidative stress, and caspase activation

Agnieszka Kinsner,* Valentina Pilotto,* Susanne Deininger,† Guy C. Brown,‡ Sandra Coecke,* Thomas Hartung*† and Anna Bal-Price*

*European Centre for the Validation of Alternative Methods (ECVAM), European Commission Joint Research Centre, Ispra, Italy
†Department of Biochemical Pharmacology, University of Konstanz, Konstanz, Germany
‡Department of Biochemistry, University of Cambridge, Cambridge, UK

**Abstract**

In this study we investigated the mechanisms of neuronal cell death induced by lipoteichoic acid (LTA) and muramyl dipeptide (MDP) from Gram-positive bacterial cell walls using primary cultures of rat cerebellum granule cells (CGCs) and rat cortical glial cells (astrocytes and microglia). LTA (± MDP) from *Staphylococcus aureus* induced a strong inflammatory response of both types of glial cells (release of interleukin-1β, tumour necrosis factor-α and nitric oxide). The death of CGCs was caused by activated glia because in the absence of glia (treatment with 7.5 μM cytosine-D-arabinoside to inhibit non-neuronal cell proliferation) LTA + MDP did not cause significant cell death (less than 20%). In addition, staining with rhodamine-labelled LTA confirmed that LTA was bound only to microglia and astrocytes (not neurones). Neuronal cell death induced by LTA (± MDP)-activated glia was partially blocked by an inducible nitric oxide synthase inhibitor (1400 W; 100 μM), and completely blocked by a superoxide dismutase mimetic [manganese (III) tetrakis (4-benzoic acid)porphyrin chloride; 50 μM] and a peroxynitrite scavenger [5,10,15,20-tetraakis (4-sulfonatophenyl) porphyrinato iron (III); 100 μM] suggesting that nitric oxide and peroxynitrite contributed to LTA-induced cell death. Moreover, neuronal cell death was inhibited by selective inhibitors of caspase-3 (z-DEVD-fmk; 50 μM) and caspase-8 (z-Ile-Glu(O-Me)-Thr-Asp(O-Me)) fluoromethyl ketone; 50 μM) indicating that they were involved in LTA-induced neuronal cell death.

**Keywords:** Gram-positive bacteria, inflammation, neurotoxicity, reactive oxygen and nitrogen species.


Since the successful elimination of *Haemophilus influenzae* type b disease as a result of vaccination, *Streptococcus pneumoniae*, a Gram-positive bacterium, has been the main pathogen responsible for bacterial meningitis in adults and children (Schuchat et al. 1997). Inflammation-induced brain injury frequently complicates bacterial meningitis despite rapid identification of disease and administration of antibiotics. The mortality and morbidity rate in bacterial meningitis is still high and up to 50% of survivors have permanent neurological damage including seizures, mental retardation, impairment of hearing and loss of cognitive functions (Davis and Greenlee 2003; Kastenbauer and Pfister 2003).

In meningitis, the presence of live bacteria in the CSF does not necessarily cause injury or even symptoms of inflammation. The induction of symptoms requires initiation of the inflammatory response, which is triggered by bacterial components such as lipopolysaccharide (LPS) in Gram-positive bacteria, inflammation, neurodegeneration induced by lipoteichoic acid from *Staphylococcus aureus* is mediated by glia activation, nitrosative and oxidative stress, and caspase activation
negative bacteria, and lipoteichoic acid (LTA) and peptidoglycan (PGN) in Gram-positive bacteria. They are released into the CSF during bacteriolyis resulting from the host defence mechanism or administration of antibiotics (Braun and Tuomanen 1999; Nau and Bruck 2002). In animal models of meningitis, bacterial components, when injected into the cerebrospinal space, induce a rapid inflammatory response (Tuomanen et al. 1985). Although meningitis is an inflammatory disease of the meninges, it is not usually limited only to these membranes, but brain glial cells (astrocytes and microglia) and neurones are also implicated. One of the first hallmarks of neuroinflammation in CNS is glia activation by different inflammatory stimuli (Eddleston and Mucke 1993; Lukii and Bazan 2000; Swanson et al. 2004). On one hand, glia activation is thought to be protective via destruction of pathogens, removal of debris and promotion of tissue repair. However, if it is excessive and long-lasting it might lead to neurotoxicity. Microglial- and astrocyte-derived pro-inflammatory cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-β, IL-6] and related products [nitric oxide (NO), reactive oxygen species (ROS), arachidonic acid metabolites or quinolinic acid] can kill neurones in co-culture, and this may also occur in vivo during neurodegenerative diseases, brain trauma, inflammation and infection (Tuomanen et al. 1985; Eddleston and Mucke 1993; Aloisi 2001).

Most studies have characterized the mechanism of glia activation and subsequent neuronal damage induced by LPS, the main immunostimulatory component of Gram-negative bacterial cell walls (Bal-Price and Brown 2001; Kim et al. 2000; Cai et al. 2003; Qin et al. 2004). However, the pro-inflammatory potential in the CNS of LTA and PGN, the main components of Gram-positive bacterial cell walls, are still poorly understood. Relatively little is known about whether in bacterial meningitis microglia and/or astrocytes become activated in the presence of Gram-positive bacterial products and, if so, whether this might induce neuroinflammation and subsequent neurodegeneration.

In this study we investigated whether LTA and muramyldipeptide (MDP), the smallest bioactive fragment of PGN, could cause neuronal cell death of cerebellar granule cells (CGCs) in culture by direct actions, or indirectly through glia activation. The production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and NO was measured after exposure of pure glial cultures (microglial or astrocytic) or mixed glial–neuronal cultures of CGCs to LTA alone or in the presence of MDP. We demonstrated that LTA and MDP from Staphylococcus aureus induced neuronal cell death indirectly through glia activation, because significant cell death was not observed in the absence of glia. Moreover, we showed that LTA-induced CGC toxicity was mainly mediated by production of ROS and reactive nitrogen species (NO, superoxide and peroxynitrite), and caspase-3 and -8 activation.

Materials and methods

Chemicals and reagents
Reagents for cell culture were purchased from Gibco Invitrogen (Milan, Italy); poly-L-lysine, trypsin, Dnase I, bovine serum albumin (BSA), cytosine-α-arabinoside (ARA-C), isoleucin B4, Triton X-100, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), 3,3′,5,5′-tetramethylbenzidine, sulfanilamide, hydrochloric acid and N-(1-naphthyl)ethylenediamine were from Sigma-Aldrich (Milan, Italy); 1400 W dihydrochloride, Z-Val-Ala-Asp fluoromethyl ketone (z-VAD-fmk), Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-fluoromethyl ketone (z-DEVD-fmk), Z-Ile-Glu(O-Me)-Thr-Asp(O-Me) fluoromethyl ketone (z-IETD-fmk) and Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone (z-LEHD-fmk) were from Alexis Biochemicals (Lausanne, Switzerland); manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) and 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrin iron (III) (FeTPPS) were from Calbiochem (Nottingham, UK); anti-gial fibrillary acidic protein (GFAP) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-OX-42 antibody was from Serotec (Cergy Saint-Christophe, France); secondary antibodies conjugated with FITC or Cy3 were from Chemicon (Chandlers Ford, UK); TNF-α, IL-1β and IL-6 antibodies were from Biotrend (Cologne, Germany); Hoechst 33342 and propidium iodide (PI) were from Molecular Probes Europe (Leiden, The Netherlands); MDP was from Bachem (Bubendorf, Switzerland); LTA was kindly provided by S. Morath (University of Konstanz, Konstanz, Germany).

Neuronal cell culture
The primary culture of CGCs was prepared from 7-day postnatal rat pups (OFA/SPF rat strain) as described previously (Cambray-Deakin 1995) with some modifications. The cerebella were dissociated in Versene solution (1 : 5000) and plated at 0.25 × 10⁶ cells/cm² in 24-well plates coated with poly-L-lysine. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with heat-inactivated horse serum (5%), fetal calf serum (5%), 13 mM glucose, 0.5 mM HEPES buffer, 2 mM l-glutamine, 25 mM KCl and 10 µg/mL gentamicin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air.

In some experiments Ara-C (7.5 µM) was added to the medium 48 h after plating to inhibit proliferation of non-neuronal cells (astrocytes, microglia). In all the experiments the cells were used at 8 days in vitro (DIV) to ensure morphological and physiological maturity.

Astrocyte and microglial cultures
Primary, mixed glial cultures were prepared from the cerebral cortex of 7-day-old rat pups (OFA/SPF rat strain) as described previously (Bal et al. 1994). Briefly, the cells isolated from cerebral hemispheres were dissociated in Hank’s balanced salt solution containing 0.25% trypsin, 0.02 mg/mL Dnase I and 1% BSA. Cells were plated at a density of 1.0 × 10⁶ cells/cm² in 75-cm² culture flasks (Costar, Bethesda, MD, USA), six- or 12-well plates (Costar) coated with poly-L-lysine. The DMEM contained 10% fetal bovine serum and antibiotics (1% penicillin/streptomycin). The medium was changed every 3 days. At confluence (14–16 DIV) the primary mixed glial cultures were used to isolate microglial cells and astrocytes. Briefly, mixed glial cultures were shaken to dislodge microglia that were...
loosely attached to the astrocytes. Microglia were seeded into six- or 12-well plates at a density of 2.0 \times 10^5 or 1.0 \times 10^5 cells/well respectively (2.0 \times 10^5 cells/cm^2) and maintained in astrocyte-conditioned medium (medium collected from astrocyte cultures after 2 days and spun down) mixed 1 : 2 with fresh DMEM (containing 10% fetal bovine serum). The cultures were characterized using immunocytochemical staining with anti-GFAP antibody (astrocytic marker) and anti-OX-42 antibody (microglial marker; an anti-CR3 complement receptor antibody). Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized with 3% Triton X-100 in phosphate-buffered saline (PBS), followed by incubation with anti-GFAP or anti-OX-42 overnight at 4°C. After washing with 1% Triton X-100 in PBS, cells were incubated with secondary antibodies conjugated with FITC or Cy3 for 2 h at 23°C ± 1°C. Cells were washed extensively followed by staining for 10 min with 5 μg/mL Hoechst 33342. The staining was analysed under a confocal laser scanning microscope (Radiance MP2000; Bio-Rad, Hercules, CA, USA).

**Treatment of the mixed neuronal–glial cultures**

Cultures of CGCs (8 DIV) were exposed to LTA (10 or 30 μg/mL) with or without MDP (100 ng/mL) for 24, 48 and 72 h in the presence or absence of a selective inhibitor of inducible nitric oxide synthase (iNOS) (1400 W; 25 μM), a superoxide dismutase (SOD) mimetic (MnTBAP; 50 μM), a peroxynitrite decomposition catalyst (FeTPPS; 25 μM), anti-TNF-α (10 ng/mL), IL-1 receptor antagonist (IL-1Ra; 10 ng/mL), pan-caspase inhibitor (z-DEVD-fmk; 50 μM), and inhibitors of caspase-3 (z-DEVD-fmk; 50 μM), caspase-8 (z-IETD-fmk; 50 μM) and caspase-9 (z-LEHD-fmk; 50 μM).

**Assessment of neuronal cell morphology and viability**

The viability of CGCs and glial cells was estimated by a fluorescent staining with two dyes – PI (20 μg/mL) and Hoechst 33342 (10 μg/mL) using a fluorescent microscope (IX70; Olympus, Hamburg, Germany). PI-positive cells were considered to be necrotic as PI stains only cells with a disrupted cell membrane. The nuclear morphology of the cell (chromatin condensation and fragmentation) was studied using the cell-permeable dye Hoechst 33342 to assess whether apoptotic cells were present. Cells with homogenously stained blue nuclei were considered viable, whereas those with condensed, fragmented blue nuclei were counted as apoptotic. The characteristic shape and size of the nuclei of three types of cells present in CGC cultures (neurons, microglia and astrocytes) together with typical morphology (contrast phase microscopy image) and cell-specific staining (GFAP and OX-42) allowed us to distinguish and quantify the different cell types. Live and dead (necrotic and apoptotic) neurons were counted in five microscopic fields in each well (two wells per treatment) and expressed as a percentage of the total number of neurons. Each experiment was repeated at least three times. To estimate the number of cells that disappeared (owing to phagocytosis by activated microglia), the total number of dead and live cells was quantified and compared with the total number of cells in non-treated control cultures.

**Staining of CGC cultures with rhodamine-labelled LTA**

LTA labelling was performed as follows: 3 mg LTA from *S. aureus*, 4.5 mg sulforhodamine Q 5-acidfluoride (Fluka, Buchs, Switzerland), 2.5 mL dimethylsulfoxide (Wak-Chemie-Medical GmbH, Steinbach, Germany) and 25 μL trimethylamine (Acros Organics, Loughborough, UK) were sonicated for 10 min. The mixture was shaken overnight at 37°C, then centrifuged four times for 90 min at 7000 g in pyrogen-free centrifugal ultrafiltration tubes (Microsep 3K; Centricon, Pall, MI, USA). To remove remaining unbound dye, the labeled LTA was filtered through a pyrogen-free sepharose column (PD-10 desalting column; Amersham Biosciences, Freiburg, Germany). Labelling efficiency, calculated as fluorescence (560 nm/620 nm) per phosphate content, was 1–2 rhodamine molecules per LTA.

**Activation of astrocytes and microglia in culture**

Primary cultures of astrocytes (17 DIV) and microglia (24 h after isolation from mixed glial cultures) were activated by exposure to 10 or 30 μg/mL LTA from *S. aureus*. Before exposure to LTA, some cultures were pretreated with MDP (100 ng/mL) for 30 min. LTA was obtained according to a novel butanol extraction procedure to ensure biological activity, high purity and lack of LPS contamination (determined using LAL assay) (Morath et al. 2001).

The medium was collected at 1, 6, 12 and 24 h after treatment with LTA (± MDP) for assessment of cytokine release, and at 24, 48 and 72 h for measurement NO production. At the end of the experiments astrocytes were gently trypsinized (0.1% trypsin) for 2–3 min and the number of cells was counted using a haemocytometer.

**Assessment of microglia and astrocyte proliferation**

Proliferation of astrocytes and microglia was assessed after exposure to LTA (± MDP) in mixed neuronal–glial cultures by counting (as described in neuronal culture) or in pure glial cultures. Mixed glial cultures (14 DIV) prepared in 75-cm² flasks were shaken to dislodge microglia. Pure microglial cells were isolated and seeded into 12-well plates or 96-well plates at a density of 1.5 \times 10^5 cells/cm². The remaining purified astrocytes were trypsinized and seeded on 12- or 96-well plates at a density of 1.0 \times 10^4 cells/cm². Astrocytes or microglia in the proliferation phase were treated for 72 h with LTA 30 μg/mL in the presence or absence of MDP (100 ng/mL). The cell proliferation of both types of glial cells [control and after LTA (± MDP) treatment] was assessed using a Cell Proliferation (BrdU) ELISA kit (Roche Applied Science, Monza, Italy) or by cell counting after Hoechst 33342 and PI staining as described above.

**Measurement of cytokine release**

Quantification of cytokines in medium collected from LTA ± MDP-activated microglia or astrocytes in culture was performed using a
sandwich ELISA. Maxisorp ELISA plates (Nunc, Wiesbaden, Germany) were coated with TNF-α, IL-1β or IL-6 antibodies diluted in 100 mM NaHCO₃, pH 8.3, at 4°C overnight. After blocking with 3% BSA/PBS for 2 h at room temperature, samples and standard solutions were added. Recombinant TNF-α, IL-1β or IL-6 standard solutions were prepared in 3% BSA/PBS. After incubation for 3 h, biotinylated TNF-α, IL-1β or IL-6 antibodies were added and incubated for 45 min at room temperature, followed by incubation with streptavidin-peroxidase (Biosource, Nivelles, France) for 30 min. Detection of bound cytokines was carried out using 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped using 1 M H₂SO₄ and the absorption was measured at 450 nm in a multiwell spectrophotometer (Spectra Max; Molecular Devices, Sunnyvale, CA, USA). Cytokine concentrations were calculated per 10⁶ cells using standard solutions of recombinant TNF-α, IL-1β or IL-6 and expressed in picograms per millilitre (pg/ml).

Measurement of NO production
The accumulation of nitrite in the medium was assessed by means of the Griess reaction (Green et al. 1982). Sulfanilamide (1 mM), hydrochloric acid (6 mM) and N-(1-naphthyl)ethylenediamine (1 mM) were added to the medium. After 30 min incubation at room temperature the absorbance was measured at 548 nm in a multiwell spectrophotometer (Spectra Max). Nitrite concentrations (micromolar) were calculated using standard solutions of sodium nitrite prepared in culture medium.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA, USA). All data given are mean ± SD (n = number of repeats). Significance of differences was assessed by one-way ANOVA followed by Bonferroni’s post-test.

Results
LTA-induced neuronal cell death in CGC primary cultures is mediated through glia activation
To determine whether LTA could induce neuronal cell death, we used mixed glial-neuronal primary cultures of CGCs (8 DIV) and exposed them for 24, 48 or 72 h to LTA (10 or 30 µg/mL). The chosen concentrations of LTA were based on previous studies performed in vitro in systemic models (human monocytes or human whole blood) (Lehner et al. 2001), as well as on concentrations of LTA found in the CSF of patients suffering from meningitis (Schneider et al. 1999). CGCs were exposed to LTA alone or in the presence of MDP (100 ng/mL). MDP is the minimal active part of PGN and in our previous studies we showed that it significantly potentiates LTA-induced glia activation (Kinsner et al., unpublished observation). Control cultures of CGCs (83 ± 3% neurones, 11 ± 2% astrocytes and 4 ± 1% microglia) contained well differentiated neurones with an extensive neuritic network (Fig. 1a) and little necrotic cell death (Figs 1a and b). After exposure of CGCs to LTA (10 or 30 µg/mL) concentration-dependent neuronal cell death was observed that was only slightly potentiated by MDP as assessed by PI (necrosis) and Hoechst 33342 (apoptosis) staining (Fig. 1b). After 24 h of treatment with LTA (10 or 30 µg/mL) only 11.4 ± 4% of neurones were PI positive (necrosis) or had disappeared (11.2 ± 5%) compared with the number of neurones in control, sister cultures (Fig. 1c), presumably because they were phagocytosed by activated microglia. After 48 h exposure to LTA (10 or 30 µg/mL) in the presence or absence of MDP, the extent of neuronal death did not significantly change as assessed by PI and Hoechst staining. However, prolonged treatment (72 h) with LTA (± MDP) significantly increased cell death (50.0 ± 3.1% phagocytosed, 6.9 ± 3.2% necrotic, 1.3 ± 1.1% apoptotic cells) (Fig. 1c). During 72 h of treatment with LTA (± MDP), the number of apoptotic cells (bright nuclei with condensed or fragmented chromatin) was always low suggesting that neurones died mainly by necrosis. However, it is possible that apoptotic cells were rapidly phagocytosed by activated microglia, so they were not seen.

To determine whether neuronal cell death was induced directly by LTA or mediated indirectly by LTA-activated glial cells, the proliferation of glia in mixed neuronal–glial cultures of CGCs was inhibited by the addition of 7.5 µM Ara-C at 24–48 h after the isolation of CGCs. This concentration of Ara-C almost completely blocked proliferation of glia (less than 2% non-neuronal cells were present) and caused only a slight increase in neuronal cell death as determined by PI and Hoechst 33342 staining (Fig. 2b).

CGCs pretreated at 1–2 DIV with Ara-C (7.5 µM) were exposed to LTA (10 or 30 µg/mL) in the presence or absence of MDP (100 ng/mL) at 8 DIV for 72 h. In the absence of glial cells (after Ara-C pretreatment), LTA-induced neuronal cell death was significantly prevented (43.3 ± 6.2%) compared with cell death observed in CGCs cultured in the presence of glial cells (not treated with Ara-C) (82.1 ± 8.0%) (Figs 2a and b). These results clearly indicate that neuronal cell death in our model was mainly induced by LTA-activated glial cells as neuronal cell death was largely prevented in the absence of glia.

In further studies using LTA labelled with rhodamine we confirmed that LTA did not have a direct action on neurones as CGCs did not show any binding of rhodamine-labelled LTA. In contrast, microglia and astrocytes were stained intensively with rhodamine-labelled LTA in both pure and mixed glial-neuronal cultures (Fig. 3).

LTA induces proliferation of astrocytes and microglia
To assess whether LTA induced changes in the number of astrocytes and/or microglia, the number of both types of glial cell was quantified in CGC cultures after 24, 48 and 72 h of exposure to LTA (30 µg/mL) and MDP (100 ng/mL), and in pure cultures of astrocytes or microglia exposed to LTA (± MDP) for 72 h. In CGC cultures not only was no glial cell death observed, but LTA induced a significant increase in cell
proliferation of both astrocytes and microglia (79 ± 21 and 134 ± 45% respectively) as assessed by GFAP (astrocyte marker) and isolectin B4 (microglial marker) staining (Figs 4a and b). As a high rate of neuronal cell death and phagocytosis was observed after exposure to LTA for more than 72 h, in many microscopic fields astrocytes and microglia were the only cell type that survived (Fig. 1a). The increase in glia proliferation was partly due to a direct effect of LTA on glial cells because in the absence of neurones (pure culture of microglia or astrocytes) an augmentation in the number of glia was still observed (astrocytes: 18.4 ± 7.1 and 22.4 ± 7.2% after LTA and LTA + MDP treatment respectively in comparison with control; microglia: 37.2 ± 17.9 and 39.1 ± 25.7%). However, the effect observed in pure glial cultures was less pronounced than that in mixed neuronal–glial cultures possibly owing to the absence of communication between glia and neurones. The increase in quantity of astrocytes and microglia observed after 72 h might explain why significant cell death was observed only after prolonged (72 h) exposure to LTA. The increased quantity of microglia and astrocytes might potentiate neuronal cell death as observed cell death was mainly mediated by LTA-activated glia.

LTA-activated astrocytes and microglia release pro-inflammatory cytokines TNF-α, IL-1β and IL-6
As neuronal cell death in CGC cultures was mainly mediated by LTA-activated glia, we characterized the response of both cultured astrocytes and microglia to LTA (10 μg/mL and 30 μg/mL) ± MDP (100 ng/mL) stimulation by measuring the time and dose dependence of the release of key pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). Cultures of microglia were pure (> 99% OX-42-positive cells), whereas astrocytic cultures were contaminated with microglia (8% ± 2%). Both types of glial cells (astrocytes and microglia) responded to LTA (30 μg/mL) activation by production of...
comparable levels of IL-1β (maximum levels at 24 h: 3458 ± 342 and 4750 ± 196 pg/mL/10^6 cells in microglia and astrocyte cultures respectively), IL-6 (maximum levels at 24 h: 3639 ± 120 and 4389 ± 145 pg/mL/10^6 cells in microglia and astrocyte cultures respectively) and TNF-α (maximum levels at 6 h: 1930 ± 96 and 2403 ± 50 pg/mL/10^6 in microglia and astrocyte cultures respectively) (Fig. 5). MDP alone did not induce the production of cytokines (IL-1β, IL-6 and TNF-α) and did not have any significant effect on LTA-induced cytokine release (data not shown). Because TNF-α and IL-1β produced by LTA-activated glia might contribute to the observed neuronal cell death, we tested whether pretreatment with an antibody against TNF-α or with IL-1ra would prevent neuronal cell death induced by LTA-activated glial cells. However, neither pretreatment caused any significant protection against neurotoxicity (Fig. 6). These results suggest that IL-1β and TNF-α alone are not involved in the mechanisms of LTA-induced neuronal cell death.

**NO partially contributes to LTA-induced neuronal cell death in CGC cultures**

Microglia and astrocytes in culture exposed to LTA produced NO in a concentration-dependent manner (astrocytes: 10.2 ± 6.7 and 25.1 ± 4.3 μM nitrite/10^6 cells; microglia: 5.3 ± 0.7 and 12.2 ± 3.2 μM nitrite/10^6 cells; levels after 72 h exposure to 10 and 30 μg/mL LTA respectively). As expected, NO production was delayed in relation to cytokine release as it requires iNOS induction that is induced in the presence of pro-inflammatory cytokines. Interestingly, MDP (100 ng/mL) synergized with LTA as comparable levels of NO were produced after exposure of astrocytes to a higher concentration of LTA alone (30 μg/mL) and to a 3-fold lower
dose of LTA (10 μg/mL) but in the presence of MDP (100 ng/mL) (25.1 ± 4.6 and 26.0 ± 2.6 μM nitrite respectively). MDP alone did not induce NO production. As mentioned before, this potentiation did not occur in the case of LTA-induced cytokine release.

To test whether NO contributed to neuronal cell death, CGC cultures were exposed for 72 h to LTA (30 μg/mL) + MDP (100 ng/mL) in the presence of 1400 W (25 μM), a selective inhibitor of iNOS. The production of NO in neuronal–glial CGC cultures pretreated with 1400 W was almost completely blocked (Fig. 7a). In the presence of the iNOS inhibitor neuronal cell death was also partially prevented (decreased by 25% ± 8%) as assessed by PI and Hoechst 33342 staining (Fig. 7b). These results suggest that NO contributed to neuronal cell death of CGCs induced by LTA and MDP-activated glial cells.

LTA-induced neuronal cell death is prevented by MnTBAP and FeTPPS

To determine whether possible ROS production from both glial and neuronal cells upon LTA treatment might be involved in the mechanism of neuronal cell death, we studied whether MnTBAP (a cell-permeable SOD mimetic) and FeTPPS (a peroxynitrite decomposition catalyst) protected CGCs against LTA-induced cell death. The cells were pretreated with MnTBAP (100 μM) or FeTPPS (25 μM) 30 min before exposure to LTA (30 μg/mL) + MDP (100 ng/mL) for 72 h.
The concentrations of both inhibitors were not cytotoxic in CGC cultures. As evaluated using PI/Hoechst 33342 staining, neuronal cell death induced by LTA was entirely prevented in the presence MnTBAP (Fig. 8a) and almost completely blocked by FeTPPS (Fig. 8b), suggesting that both peroxynitrite (ONOO–) and superoxide (O2–) play a major role in the mechanism of LTA-induced cell death of CGCs in culture.

Caspase-3 and -8 activation is involved in LTA-induced neuronal cell death

LTA-induced neuronal cell death of CGCs in culture assessed after 24, 48 or 72 h was mainly necrotic as revealed by PI staining and only a very few Hoechst 33342-positive cells with condensed or fragmented chromatin (apoptotic) were observed. However, it cannot be excluded that secondary necrosis was being observed and that the initial mode of cell death was apoptotic. To test this we used a broad-spectrum non-specific caspase inhibitor, z-VAD-fmk, as apoptosis is mainly mediated by caspase activation. The cells were pretreated with 50 μM z-VAD-fmk for 30 min before LTA (30 μg/mL) + MDP (100 ng/mL) addition. Inhibition of caspase activation by z-VAD-fmk decreased the LTA + MDP-induced neuronal cell death by 35 ± 3% in comparison with CGC cultures that had been exposed to LTA + MDP but not pretreated with z-VAD-fmk (Fig. 9a). This suggests that caspase activation contributed to LTA-induced neuronal cell death.

To determine which caspases were involved in this process, we pretreated CGC cultures with specific inhibitors of caspase-3 (z-DEVD-fmk; 50 μM), caspase-8 (z-IETD-fmk; 50 μM) and caspase-9 (z-LEHD-fmk; 50 μM) and then exposed the cells to LTA (30 μg/mL) + MDP (100 ng/mL) for 72 h. Cell death (apoptotic and necrotic) was assessed after 24, 48 and 72 h using PI and Hoechst 33342 staining. The results suggest that the activation of caspase-3 and caspase-8 (but not caspase-9) was involved in the mechanism of LTA-induced neuronal cell death, as in the presence of specific inhibitors (z-DEVAd-fmk and z-IETD-fmk respectively) we observed a significant decrease in cell death (Fig. 9b). The concentrations of the inhibitors used were not cytotoxic in CGC cultures.

Discussion

In the present study, using in vitro mixed glial–neuronal cultures, we demonstrated for the first time that LTA derived from S. aureus induces neurotoxicity indirectly, through glia activation, as exposure of neuronal cultures with only a low
have shown that Gram-positive bacterial cell wall components are implicated in the pathogenesis of many infectious diseases in the CNS, such as encephalomyelitis and meningitis (Schmidt et al. 2001; Nau and Bruck 2002). However, it is still not clear which component of the Gram-positive bacterial cell wall is mainly responsible and how neuronal cell death is induced. In our experiments we used highly purified LTA from S. aureus prepared according to our recently described butanol extraction procedure (Morath et al. 2001). LTA was tested by Limulus assay to rule out the presence of LPS and ensure that the effects we observed were not due to contamination with LPS, especially with regard to synergy with MDP (Traub et al. 2004).

Exposure of mixed neuronal–glial cultures of CGCs to LTA (10 or 30 µg/mL) caused concentration- and time-dependent neuronal cell death that was most prominent after a prolonged incubation (72 h). The observed cell death was mainly necrotic as the number of apoptotic cells with bright nuclei and condensed or fragmented chromatin (Hoechst positive and PI negative) was always low. However, the possibility that apoptotic cells, once present, were rapidly phagocytosed by activated microglia cannot be excluded, as a high proportion of neurones disappeared after LTA treatment. Indeed, from the changes in microglial phenotype (spherical cells and vacuoles packed with particles) it was obvious that in the presence of LTA microglia had phagocytosed dead cells (Fig. 1a).

A similar phenomenon, that is a high level of phagocytosis of dead neurones by activated microglia, was observed in cultures of CGCs exposed to LPS (Bal-Price and Brown 2001). During the course of Gram-positive bacterial infection, neurones and glial cells are likely to be exposed not only to LTA but also to MDP (the smallest bioactive fragment of PGN). However, the addition of MDP only slightly potentiated LTA-induced neurotoxicity, suggesting that LTA was the main component of the Gram-positive bacterial cell wall responsible for neuronal cell death.

Increasing evidence suggests that in many neurological disorders, such as trauma, infection or inflammation, neuronal injury is mediated by inflammatory molecules released by activated astrocytes and microglia (Eddleston and Mucke 1993; Ruffolo et al. 1999; Lukiw and Bazan 2000; Nau and Bruck 2002). To determine whether in our in vitro model LTA-induced neuronal cell death was mediated through glia activation (indirectly), CGC cultures were pretreated with Ara-C to inhibit glial proliferation. However Ara-C itself slightly increased neuronal cell death (by 13.1% ± 1.3% in comparison to without Ara-C), probably owing to the neuroprotective role of glia (Kirchhoff et al. 2001; Srebro and Dziobek 2001). In these cultures (containing less than 2% glial cells) neuronal cell death was significantly decreased in comparison with that in mixed neuronal–glial cultures (47.6 ± 10.4 vs. 84.5 ± 9.9% respectively) suggesting that neurotoxicity was indeed mediated by LTA-activated...
glial cells. However, in the presence of Ara-C the effect of LTA (+ MDP) was not completely blocked because even 2% activated glia could be neurotoxic. In addition, the involvement of glia in LTA-induced neurotoxicity was confirmed by staining with rhodamine-labelled LTA, which bound only to microglia and astrocytes but not to neurones.

In mixed neuronal–glial cultures treated with LTA there was a marked increase in the number of both microglia (134 ± 45% of control) and astrocytes (79 ± 21% of control) especially after prolonged exposure (72 h) to LTA. It is known from in vivo studies that astrocytes proliferate in the area of a neurodegenerative lesion (gliosis), often stimulated by cytokines released from activated microglia. Gliosis is a prominent feature of many neurodegenerative diseases, including multiple sclerosis, trauma and ischaemia (Dietrich et al. 2003; Liberto et al. 2004). In pure glial cultures the proliferation of astrocytes and microglia was only slightly increased (but still significantly), suggesting that the communication between neurones and glia is important to fully trigger glia proliferation.

The increased proliferation of astrocytes and microglia might have contributed to LTA-induced neuronal death. Indeed microglia and astrocytes recognize and respond to LTA, becoming fully activated, releasing NO and several pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) (see Fig. 5). These factors might subsequently initiate a cascade of events leading to neuronal cell death. TNF-α has been suggested to be an important mediator of LPS-induced inflammation (Waage et al. 1989) and the levels of TNF-α in the CSF of patients with bacterial meningitis correlated well with concentrations of bacterial endotoxin in the brain (Arditi et al. 1990). However, in our model, pretreatment of CGC cultures with anti-TNF-α antibodies or with IL-1ra (an antagonist of IL-1β receptors) did not protect against neurotoxicity, suggesting that other mediators were involved and that it was not enough to block only the effects of IL-1β or TNF-α to prevent LTA-induced neuronal cell death.

The mechanisms by which LTA-activated glia induce neurotoxicity might also involve the release of NO and ROS, both of which are key mediators of the pathophysiological changes that occur during bacterial meningitis (Koedel et al. 1995; Koedel and Pfister 1999; Christen et al. 2001). Under physiological conditions, NO plays an important role in intracellular and intercellular signalling (Garthwaite 1991); however, when produced in excessive levels it can induce neuronal cell death (Boje and Arora 1992; Bal-Price and Brown 2001). Interestingly, in our model MDP significantly potentiated (~3-fold) LTA-induced glial NO production (Kinsner et al, unpublished observation) but did not have a significant effect on LTA-induced neuronal cell death. These results suggest that NO was not the major direct mediator of LTA-induced neurotoxicity. Indeed, in the presence of 1400 W, a selective iNOS inhibitor that completely blocked NO release (measured by nitrite levels in the medium), only partial prevention (25.0 ± 8%) of neuronal cell death was observed. These results are in contrast to in vitro studies on neuronal cell death induced by LPS-activated glia. In this case neuronal cell death was predominantly mediated by NO as pretreatment with iNOS inhibitors almost entirely prevented neuronal cell death (Bronstein et al. 1995; Bal-Price and Brown 2001).

NO produced in large amounts by LTA-activated glial cells might react rapidly with the superoxide anion (O2–) and form a toxic oxidant, peroxynitrite (ONOO–) (Cuzzocrea et al. 2001). Recent evidence has shown that peroxynitrite contributes to neuronal damage, once attributed entirely to NO (Xie et al. 2002; Brown and Bal-Price 2003; Stewart and Heales 2003). Under normal conditions superoxide formation is tightly controlled by SOD (cytoplasmic and mitochondrial). However, in acute and chronic inflammation the production of superoxide is increased at a rate that might exceed the capacity of SOD to remove it. Indeed, neuronal cell death was almost completely blocked in the presence of MnTBA a (cell-permeable SOD mimicetic) (Szabo et al. 1996), suggesting that superoxide contributes to LTA-induced neurodegeneration directly, or indirectly through peroxynitrite formation. The contribution of peroxynitrite to the mechanism of LTA-induced neuronal cell death was confirmed in our model, as strong protection was observed in the presence of FeTPPS, a peroxynitrite decomposition catalyst (Crow 2000). A similar neuroprotective role of FeTPPS was also shown in LPS- (Misko et al. 1998) and amyloid-β peptide-induced cell death (Xie et al. 2002). Taken together, these results suggest that NO plays an important role in LTA-induced neurotoxicity, but indirectly through peroxynitrite production. One of the mechanisms by which NO and peroxynitrite kill neurones is by inactivation of key metabolic enzymes such as succinate dehydrogenase and cytochrome oxidase of the mitochondrial electron transport chain, causing inhibition of mitochondrial respiration and ATP depletion (Brown 1999; Bal-Price and Brown 2000). We were unable to detect any significant changes in ATP and mitochondrial membrane potential levels after exposure of CGCs to LTA (± MDP) (Kinsner et al, unpublished observation). It is possible that cell death assessed by non-specific neuronal endpoints (ATP level or mitochondrial membrane potential) was masked by LTA-induced glia proliferation.

Although in the present study LTA-induced neurotoxicity was mainly necrotic (PI-positive cells) and few cells had typical apoptotic features (condensed or fragmented chromatin; PI-negative/Hoechst 33342-positive staining), it cannot be excluded that the initial mode of cell death was apoptotic and that secondary necrosis was being observed. Indeed, in the presence of z-VAD-fmk, neuronal cell death was significantly decreased (by 35% ± 3%) suggesting that at some stage caspase activation was involved. Caspase-3 and caspase-8 (but not caspase-9) had a major role, as
selective inhibitors of these two caspases significantly blocked LTA-induced neuronal cell death. It is well documented that caspase-8 induces activation of executioner caspase-3, causing cell death (Stennicke et al. 1998). However, it is conceivable that these caspase inhibitors were acting on the glia rather than the neurones, as IL-1β production by microglia requires caspases (Kim et al. 2003).

In conclusion, we found that LTA-induced neuronal cell death is mediated indirectly, through glia activation, as LTA is recognized only by astrocytes and microglia (not neurones) (Fig. 10). LTA-activated glial cells release pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and other mediators (NO, superoxide, peroxynitrite) that could cause oxidative stress, mitochondrial dysfunction, caspase activation and finally induced neuronal cell death.

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References


