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HIGHLY PURIFIED LIPOTEICHOIC ACID FROM GRAM-POSITIVE BACTERIA INDUCES *IN VITRO* BLOOD–BRAIN BARRIER DISRUPTION THROUGH GLIA ACTIVATION: ROLE OF PRO-INFLAMMATORY CYTOKINES AND NITRIC OXIDE

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Abstract—The co-culture of bovine brain capillary endothelial cells and rat primary glial cells was established as an *in vitro* blood–brain barrier model to investigate the mechanisms by which the Gram-positive bacterial cell wall components lipoteichoic acid and muramyl dipeptide induced injury of blood–brain barrier structure and function. We found that highly purified lipoteichoic acid disrupted blood–brain barrier integrity in a concentration- and time-dependent manner indirectly, through glia activation. Low trans-endothelial electrical resistance and high permeability to fluorescein isothiocyanate-inulin observed in the presence of lipoteichoic acid-activated glial cells were potentiated by muramyl dipeptide and could be reversed only when glial cells were activated by lipoteichoic acid at 10 μg/ml but not with a higher lipoteichoic acid concentration (30 μg/ml). Immunocytochemistry analysis revealed no evident changes in the distribution of the cytoskeleton protein F-actin and tight junction proteins occludin and claudin after lipoteichoic acid treatment. However, the tight junction associated protein AHNAK clearly revealed the morphological alteration of the endothelial cells induced by lipoteichoic acid. Lipoteichoic acid-activated glial cells produced nitric oxide and pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1β) that contributed to lipoteichoic acid-induced blood–brain barrier disruption, since the direct treatment of the endothelial monolayer with tumor necrosis factor-α or interleukin-1β increased blood–brain barrier permeability, whereas the pre-treatment of lipoteichoic acid-activated glial cells with antibodies against these two cytokines blocked lipoteichoic acid effects. Additionally, nitric oxide was also involved in blood–brain barrier damage, since the nitric oxide donor itself (diethylenetriamine-nitric oxide adduct) increased blood–brain barrier permeability and inducible nitric oxide synthase inhibitor (1400W) partially reversed lipoteichoic acid-induced trans-endothelial electrical resistance decrease. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: *Staphylococcus aureus*, astrocytes, endothelial cells, inflammation.

The homeostasis of the CNS environment is maintained by the blood–brain barrier (BBB), which separates the brain from the systemic blood circulation. The highly specialized cerebral endothelial cells (ECs) of the BBB regulate the entry of circulating substances into the brain by the tight junctions and specialized transport functions (de Vries et al., 1997). Our BBB *in vitro* model, consisting of a co-culture of bovine brain capillary endothelial cells (BBCECs) and rat primary glial cells (GCs), results in a well-differentiated cerebral endothelium that displays most of the features observed *in vivo* (Cecchelli et al., 1999; Dehouck et al., 1990), and provides an important tool for studying molecular mechanisms at the BBB level, under both physiological and pathological conditions.

The barrier function of the BBB changes dramatically during various diseases of the CNS, i.e. hypertension (Ueno et al., 2004) or cerebral inflammation (e.g. experimental autoimmune encephalomyelitis, multiple sclerosis) (Lossinsky and Shivers, 2004; Minagar and Alexander, 2003) or cerebral infections (Berger and Avison, 2004; Baldwin and Kielian, 2004).

Certain bacteria, such as Gram-negative *Haemophilus influenzae* and *Neisseria meningitidis* or Gram-positive *Streptococcus pneumoniae*, can cross the BBB without primarily altering its integrity and penetrate into the CNS tissue (Pfister and Scheld, 1997; Swartz, 2004).

During brain swelling (edema) or bacterial meningitis, the BBB permeability increases (Nimmo et al., 2004); however, the mechanisms involved are still poorly understood. A progressive opening of tight junctions resulting in higher BBB permeability was observed in animals after intracerebroventricular administration of the Gram-negative bacterial endotoxin lipopolysaccharide (LPS) as a widely used model for bacterial meningitis. In an early stage of inflammation, the time-dependent effect of LPS-induced BBB

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Abbreviations: BBB, blood–brain barrier; BBCECs, bovine brain capillary endothelial cells; CSF, cerebrospinal fluid; DETA/NO, diethylenetriamine-nitric oxide adduct; DMEM, Dulbecco's modified Eagle's medium; ECs, endothelial cells; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride; FITC, fluorescein isothiocyanate-inulin; GCs, glial cells; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1 beta; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MDP, muramyl dipeptide; NAC, 1 mM *N*-acetyl-L-cysteine; NO, nitric oxide; PG, peptidoglycan; RT, room temperature; RT-PCR, reverse transcriptase–polymerase chain reaction; TEER, trans-endothelial electrical resistance; TNF, tumor necrosis factor.

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disruption (increased permeability) was partially mediated by pro-inflammatory cytokines released from activated glia and ECs (Wispelwey et al., 1988). Tumor necrosis factor (TNF), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) are the main cytokines involved in the development of the inflammatory response after injury or infection (Hashimoto et al., 1991; Kielian et al., 2002) that contribute to BBB damage (de Vries et al., 1996).

During inflammation caused by bacterial infection, BBB disruption could also be mediated by increased production of free radicals, such as nitric oxide (NO). In the presence of pro-inflammatory cytokines, the expression of inducible nitric oxide synthase (iNOS) was observed in ECs of the BBB, astrocytes and microglia followed by high NO production (Feinstein et al., 1994; Morin and Stanboli, 1994). NO or its derivative peroxynitrite ($ONOO^-$, formed in the reaction between NO and superoxide) is a potential cytotoxic agent that contributes to BBB damage by increasing its permeability after LPS administration to rats (Freyer et al., 1999; Mark et al., 2004; Mayhan, 2001; Shukla et al., 1995). The vast majority of studies performed until now characterize the mechanisms of LPS-induced BBB damage. However, the most prevalent and severe infections in the CNS (Ostergaard et al., 2004) and in the peripheral systems (septic shock, organ failure) (Bjerre et al., 2004) are caused by Gram-positive bacteria, such as *Streptococcus pneumoniae*. This bacterium induces meningitis that is still associated with high mortality (28%) and neurological sequelae (50%) (Bohr and Rasmussen, 1988; Durand et al., 1993).

One of the main immune-stimulatory constituents of the Gram-positive bacterial cell wall is lipoteichoic acid (LTA). *In vitro* and *in vivo* studies show that LTA is involved in the pathogenesis of CNS infectious diseases such as encephalomyelitis, meningitis (Nau and Bruck, 2002) and brain abscesses (Baldwin and Kielian, 2004). It is an amphiphilic, negatively charged glycolipid anchored in the bacterial membrane and its chemical structure is closely related to LPS, the endotoxin of Gram-negative bacteria (Weber et al., 2003).

In the present studies, we investigated whether LTA-activated GCs (to mimic CNS inflammation with Gram-positive bacteria) could damage the structure and function of co-cultured BBCECs. We used LTA highly purified according to a special procedure to ensure purity (>99%) and specific bioactivity (Morath et al., 2001) that is not guaranteed by commercially available LTA, often contaminated with LPS (Gao et al., 2001). Trans-endothelial electrical resistance (TEER) and BBB permeability (using fluorescent labeled inulin) were measured after glia treatment with LTA on its own or after pre-treatment with muramyl dipeptide (MDP), minimal active component of peptidoglycan (PG) from the Gram-positive bacterial cell wall. We also studied whether the morphology of the ECs monolayer was affected by LTA-activated glia performing immunocytochemical analysis for the cytoskeleton protein F-actin and the tight junctional-associated proteins occludin, claudin and AHNAK. Moreover, the main aim of these studies was to elucidate the mechanisms of LTA-induced BBB damage. Therefore, we investigated whether NO and the pro-inflammatory cytokines TNF-α and IL-1β produced by LTA-activated GCs contributed to BBB disruption.

EXPERIMENTAL PROCEDURES

Culture of BBCECs

ECs were isolated from the brain of 6-month-old calves purchased from a local slaughterhouse. Capillaries were isolated from the gray matter of brain cortex according to the homogenization technique of Meresse et al. (1989). Brain capillary ECs were cloned as previously described (Meresse et al., 1989). The use of cloned ECs allowed us to obtain a pure EC population without obvious contamination by pericytes. The cells were cultured in the presence of Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA, USA), supplemented with 10% heat-inactivated calf serum (Life Technologies), 10% heat-inactivated horse serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 50 μg/ml gentamycin (Biochrom AG, Berlin, Germany) and 1 ng/ml basic fibroblast growth factor (bFGF, Sigma, St. Louis, MO, USA). The medium was changed every second day.

Primary culture of rat GCs

Primary cultures of rat GCs were prepared from newborn (3-day-old) rat cerebral cortexes. All procedures required to minimize the number of animals used and their suffering were taken. The rats (strain Sprague–Dawley Rjhan) were purchased from Janvier (Le Genest-St. Isle, France). All experiments conformed to French and international guidelines on the ethical use of animals. Briefly, after removing the meninges, the brain tissue was mechanically dissociated by gently forcing it through a nylon sieve, as described by Booher and Sensenbrenner (1972). GCs were then plated on six-well culture plates (Nunc, Roskilde, Denmark) at a density of 1.2×10^5 cells/ml in 2 ml DMEM supplemented with 10% heat-inactivated fetal calf serum (Life Technologies), 2 mM L-glutamine and 50 μg/ml gentamycin. The medium was changed twice a week. Three week-old glial cultures were used for co-culture with ECs. The GC population was characterized by performing immunocytochemical analysis using specific antibodies for three GC types: microglia using the mouse monocytes/macrophages antibody ED-1 (Serotec, Cergy Saint-Christophe, France) followed by the secondary antibody goat anti-mouse Rhodol Green-conjugated (Molecular Probes, Eugene, OR, USA); oligodendrocytes using the rat sulfatide antibody OL-1 (kindly provided by Dr. M. S. Ghandour, Centre de Neurochimie du CNRS, Strasbourg, France) followed by the secondary antibody goat anti-rat-fluoresceine (DTAF) conjugated (Jackson Immunoresearch Laboratories, West Grove, PA, USA); astrocytes using a rabbit glial fibrillary acidic protein (GFAP) antibody (Dakopatts, Älvsjö, Sweden) followed by the secondary antibody anti-rabbit-FITC conjugated (Molecular Probes). Fifty-eight percent of the GC population was GFAP positive, 42% OL-1 or ED-1 positive, as previously described (Descamps et al., 2003).

Co-culture of BBCECs with GCs

Costar Transwell cell culture inserts (0.4 μm pore size, 24 mm diameter, Corning Incorporated, New York, NY, USA) were used for TEER measurements and trans-endothelial transport studies. Millicell-CM cell culture inserts (0.4 μm pore size, 30 mm diameter, Millipore Corporation, Bedford, MA, USA) were used for fluorescent microscopy experiments. Before starting the co-culture, all the inserts were coated on the upper side with a homemade rat tail collagen solution (Cecchelli et al., 1999) and transferred into six-well culture plates containing primary GCs or medium alone. After 24 h, ECs were seeded on the upper side of the inserts at the density of 4×10^5 cells/ml in 1.5 ml medium and cultured in the presence of GCs. Experiments were performed after 12 days of co-culture, to allow the