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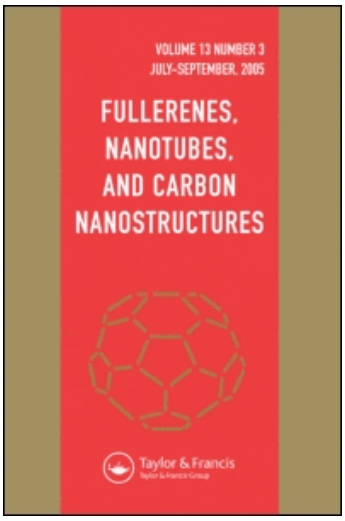

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INHIBITION OF THE INCREASED PERMEABILITY OF BLOOD-BRAIN BARRIER IN ESCHERICHIA COLI-INDUCED MENINGITIS BY CARBOXYFULLERENE

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INHIBITION OF THE INCREASED PERMEABILITY OF BLOOD-BRAIN BARRIER IN *ESCHERICHIA COLI*-INDUCED MENINGITIS BY CARBOXYFULLERENE

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ABSTRACT

The effect of a water-soluble trimalonic acid derivative of fullerene, carboxyfullerene ($C_{63}(COOH)_6$), on the opening of blood-brain barrier (BBB) in *Escherichia coli* (*E. coli*)-induced meningitis was tested. In *E. coli*-induced meningitis model, the increase of BBB permeability was manifested in two distinct phases

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based on cytokine expression pattern and neutrophilic infiltration in B6 mice. An early increase in BBB permeability was occurred at 1–5 h post injection that could be blocked by either anti-TNF-α or anti-IL-1β antibodies. A late one (neutrophil-associated BBB opening) was manifested at 6–12 h that was inhibited by vinblastine pretreatment. Inhibition of the early *E. coli*-induced BBB opening blocked the development of the late one. Furthermore, the neutrophil-associated BBB opening (the late phase), but not cytokine-induced one (the early phase), was inhibited by a water-soluble carboxyfullerene.

Key Words: Blood-brain barrier; Cytokine; Neutrophil; Carboxyfullerene; Meningitis.

INTRODUCTION

Despite the availability of effective antibiotic treatments, bacterial meningitis remains as an infection with a high mortality rate particularly in very young and elderly patients. Recovery is often associated with neurologic sequelae, such as hearing loss and cranial nerve damage (18, 28). The pathophysiology of bacterial meningitis involves the invasion and multiplication of bacteria in the subarachnoidal space of the central nervous system (CNS). The bacterium itself or its degraded products would stimulate the production and release of proinflammatory mediators such as cytokines and prostaglandins by leukocytes, endothelial cells, astrocytes, microglial cells, and other cells in the CNS, which could lead to an increase in the permeability of the blood-brain barrier (BBB). Subsequently the transendothelial migration of neutrophils and leakage of plasma proteins would further damage the brain (2, 17–18, 28). In this study, a model of experimental meningitis induced by direct injection of *Escherichia coli* (*E. coli*) into the brains of B6 mice was set up. As expected, TNF-α and IL-1β production was induced, followed by inflammatory neutrophil infiltration. The vasopermeability of BBB that was monitored by a peripheral tracer was also increased during this process.

Buckminsterfullerene [fullerene (C_{60})] is characterized as a "radical sponge" due to its avid reactivity with free radical (13). A water-soluble trimalonic acid derivative of fullerene, carboxyfullerene [$C_{63}(COOH)_6$], has been synthesized and has been found to be an effective neuroprotective antioxidant both in vivo and in vitro (5, 8–9, 15). Inhibition of *E. coli*-induced meningitis by carboxyfullerene was also reported previously (26). How the carboxyfullerene inhibited *E. coli*-induced meningitis was tested in this study. The results revealed

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that the carboxyfullerene inhibited *E. coli*-induced meningitis through decreasing the damage of infiltrating neutrophils on the BBB.

MATERIALS AND METHODS

Mice

C57Bl/6 (B6) were purchased from The Jackson Laboratory, Bar Harbor, Maine or Charles River Japan, Inc. (Atsugi, Japan). They were maintained on standard laboratory chow and water ad libitum in our animal center. The animals were raised and cared for following the guidelines set up by the National Science Council of the Republic of China. Eight- to 12-week-old female mice were used in all experiments.

$C_{63}(COOH)_6$

Two regioisomers of water-soluble carboxylic acid C60 derivatives with C3 or D3 symmetry were synthesized as described previously (5). Both $C_{63}(COOH)_6$ (C3) and $C_{63}(COOH)_6$ (D3) are effective free-radical scavengers. In this study, we used $C_{63}(COOH)_6$ (C3) dissolved in phosphate-buffered saline (PBS; 2 mg/ml).

Induction of Bacterial Meningitis

E. coli ATCC10536 was cultured in Luria-Bertani (LB) broth (1 % NaCl, 1 % tryptone, 0.5 % yeast extract) for 12 h and was subcultured in fresh medium for another 3 h. The concentration of *E. coli* was determined with a spectrophotometer (Beckman Instrument, Somerset, N.J.), with an optical density at 600 nm of 1 equal to 10^8 CFU/ml (29). Groups of three to four mice were given intracerebral (i.c.) injection directly into the temporal area of a 20-μl volume of 1×10^5 *E. coli* cells diluted in saline (25–26). The 100% lethal dose (LD_{100}) by intracerebral injection in B6 mice is 5×10^5 *E. coli* cells. For inhibition studies, 30 μg of anti-TNF-α or anti-IL-1β mAb was co-injected with *E. coli* into brain. To deplete the circulating neutrophils, the mouse was pretreated with vinblastine (0.5 mg/kg of body weight) intravenously for four consecutive days (14, 24). To each mouse into which vinblastine was treated, 500 *E. coli* cells in 20-μl were given intracerebrally into the brain. In the carboxyfullerene inhibition experiments, the mice were given an intraperitoneal (i.p.) injection of carboxyfullerene (40 mg/kg of body weight) at the same time with intracerebral injection of *E. coli*.

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Detection of Increased Vasopermeability of BBB by M4 Tracer with β-Galactosidase Activity

An *E. coli* mutant (mutant M4) that constitutively expresses β-galactosidase was used as the tracer to detect alterations in the vasopermeability of BBB. M4 was selected from *E. coli* K-12 that grew in an M63 culture plate [0.3% KH_2PO_4, 0.7% K_2HPO_4, 0.2% $(NH_4)_2$ SO_4, 0.1 mM $FeSO_4$] containing 0.2 % lactose, 0.002 % vitamine B_1, 1 mM $MgSO_4$, 0.001 % isoleucine-leucine-valine and 0.002 % 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The M4 mutant constitutively expresses β-galactosidase and has a characteristic blue colony on medium containing X-Gal without induction (25–26). To each mouse into which *E. coli* was injected, 2×10^8 cells of the M4 tracer in 0.1 ml were given intravenously 2 min before the mice were killed. The brains were removed, cyrosectioned, and fixed in 0.2% glutaldehyde (Merck GmbH, Parmstadt, Germany). The M4 in the tissues was detected by X-Gal staining (1 mg of X-Gal per ml in 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, and 2 mM magnesium chloride) at 37°C for 2 h. In the antibody and carboxyfullerene inhibition experiments, the brains were aseptically removed and were homogenized with 3% gelatin (Difco Laboratories, Detroit, Mich.) in PBS. The samples were serial diluted, poured in agar plates, and incubated at 37°C overnight. The colony-forming units (CFU) of M4 mutant were quantitated with X-Gal staining. The number of CFU of M4 mutant was expressed as the mean ± standard deviation per mouse. Representative of three experiments was represented. In some experiment, the mice were perfused with PBS to remove the circulating blood before sacrifice. There is no significant difference from nonperfused one. The significance of differences between treatment groups was determined using Student's *t* test.

Immunohistochemistry

Groups of three mice were killed by perfusion via cardiac puncture with PBS. The brains were removed and embedded in OCT compound (Miles Inc., Elkhart, Ind.) and were then frozen in liquid nitrogen. Four-μm cryosections were made and were fixed with ice-cold acetone for 3 min, then stained with primary rat anti-TNF-α mAb (MP6-XT3, PharMingen, San Diego, Calif.), or hamster anti-IL-1β mAb (Genzyme, Cambridge, Mass.). Secondary antibodies used were peroxidase-conjugated sheep anti-rat IgG or goat anti-hamster IgG (Boehringer Manheim GmbH, Mannheim, Germany). A peroxidase stain with a reddish brown color was developed with an aminoethyl carbazole substrate kit (ZYMED Laboratories, San Francisco, Calif.) (7).

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Transmission Electron Microscopy

Mice under anesthesia were perfused through the heart with saline following by 100–130 ml of fixative containing 4% paraformaldehyde and 0.5% glutaldehyde in PBS. The brains were removed and the cortex areas were cut into small 2 × 4 mm rectangular blocks. The specimen was embedded in warm agar and was chopped at 40 μm. After several washes, tissues were osmicated in 1–1.5% osmium tetroxide in 0.1 M sodium cacodylate solution at 4° C for 1–2 h, and were dehydrated in graded series of ethanol, cleared in propylene oxide, and flat-embedded in plastic. Thin sections were examined in an electron microscope (JEOL JEM-1200EX) at 75kV (16). To determine the microscopic change of BBB, each mouse was given 5 mg horseradish peroxidase (type IV, Sigma Chemical Co., St. Louis, MO) intravenously 10 min before mouse was killed. The chopped tissue sections from the cortex were incubated with diaminobenzidine solution 15–30 min to localize the exogenous peroxidase activity according to the standard protocol (22).

RESULTS

Detection of *E. coli*-Induced Alteration of BBB Vasopermeability by β-Galactosidase Positive M4

Intracerebral injection of sublethal dose (1×10^5 CFU in each mouse) of *E. coli* into B6 mice induced brain inflammation with neutrophil infiltration in meninges beginning at 6–12 h post injection (25). The increase of BBB permeability during the course was manifested using the M4 tracer that constitutively expresses β-galactosidase activity. The X-Gal positive M4 stain was detected along the brain capillaries beginning at 1 h (Fig. 1B) and more M4 tracers were detected in the ventricles and parenchyma together with infiltrating neutrophils at 9 h post injection (Fig. 1D), indicating that peripheral M4 tracer can be permeable to the brain. Furthermore, the increase of BBB permeability was also validated with traditional horseradish peroxidase leakage assay. As shown in Fig. 2A, the transmission electron microscopic examination of brain tissues from PBS-treated mice revealed the presence of tight junctions between endothelial cells. However, at 3 h post *E. coli*-stimulation, exogenous horseradish peroxidase was found to deposit in the basolateral site of blood vessels indicating vascular leakage (Fig. 2C). In addition, the astrocytes surrounded by blood vessels also contained cytoplasmic vacuoles at 3 h post injection (Fig. 2B). The alteration of BBB continued to progress, at 9 h post injection, there were vacuolation of astrocytes, detachment of basement membrane and shriveling of blood vessels (Fig. 2D).

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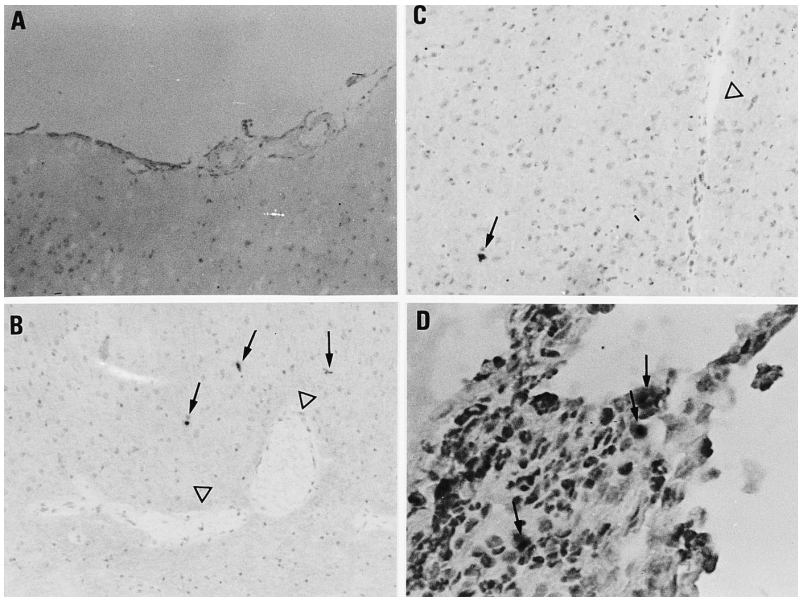

Figure 1. Detection of the peripheral M4 tracer in brain by X-Gal stain after *E. coli*-stimulation. Groups of three B6 mice were inoculated intracerebrally with 1×10^5 *E. coli* per mouse and sacrificed at various times post injection. The M4 tracer (2×10^8) was administrated intravenously 2 min before sacrifice. Four-μm cryosections of frozen brain tissues were stained with X-Gal as described in Materials and Methods. A, mock control, 3 h; B, 1 h; C, 3 h; D, 9 h (400X). " → ", M4 staining; "△", venule.

The Role of Proinflammatory Cytokines and Neutrophils on the Alteration of BBB Permeability

The expression of TNF-α and IL-1β in brain was determined with immunohistochemcial staining. Neither TNF-α nor IL-1β was detected on mock treated mice (Fig. 3A & F). After *E. coli* stimulation, TNF-α staining was found on venules and arterioles (endothelial cells and astrocytes) as early as 1 h, and reached maximum at 3 h after injection (Fig. 3B). The staining of TNF-α on infiltrating neutrophils began at 6 h, and reached maximum at 12 h after injection (Fig. 3C). IL-1β was detected on arterioles, but not on the venules (Fig. 3G). Some of the infiltrating neutrophils were also stained IL-1β (Fig. 3H). Based on the presence of neutrophils, the *E. coli*-induced BBB opening could be separated into two phases. The early phase extended from 1 to 5 h after injection without neutrophil infiltrates. During 6–12 h post injection (the late phase), neutrophil infiltrates became evident. Furthermore, the degree of the increase in BBB perme-

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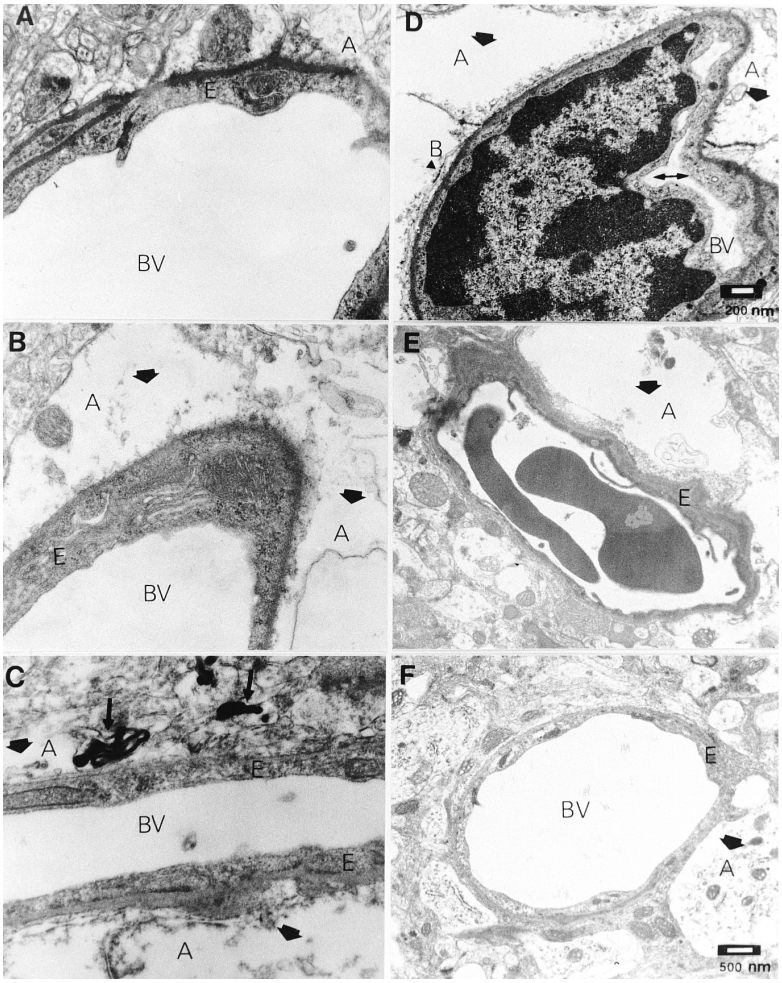

Figure 2. The ultra-structural alteration of BBB after *E. coli*-stimulation by transmission electron microscopic observation. Groups of three B6 mice were inoculated intracerebrally with 1×10^5 *E. coli* per mouse and sacrificed at 3 h or 9 h post injection. The brain tissues were prepared for electron microscopic examination as described in Materials and Methods. In (C), 5 mg of horseradish peroxidase (as a tracer) was administrated intravenously 10 min before sacrifice. In (E) and (F), intraperitoneal injection of C60 (40 mg/kg of body weight) at the same time with intracerebral injection of *E. coli*. A, mock control, 3 h; B–C, *E. coli*, 3 h; D, *E. coli*, 12 h (20,000 X); E, *E. coli* + C60, 3 h; F, *E. coli* + C60, 12 h (12,000 X). " → ", HRP; "→", impaired astrocyte; " A ", astrocyte; " BV ", blood vessel; " E ", endothelial cell; "▲, B ", detached basement membrane.

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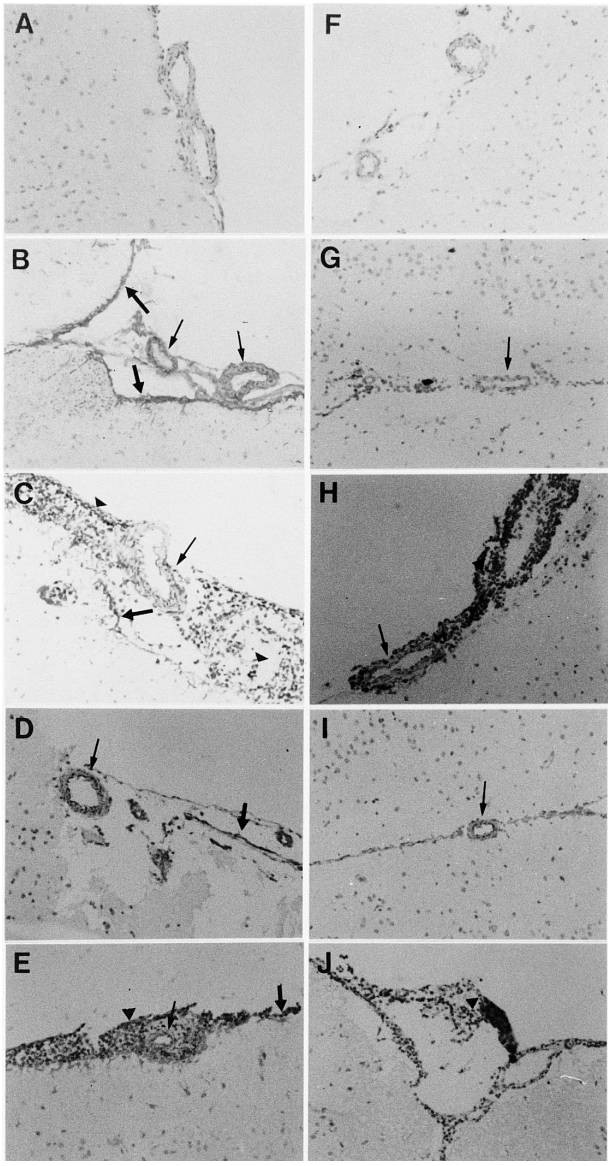

Figure 3. Expression of TNF-α and IL-1β in brain after *E. coli*-stimulation. Groups of three B6 mice were inoculated intracerebrally with 1×10^5 *E. coli* per mouse and sacrificed at various times post injection. Four-μm cryosections of frozen brain tissues were stained with rat anti-TNF-α antibody (A–E), or hamster anti-IL-1β antibody (F–J) as described in Materials and Methods. A, F, mock control, 3 h; B, G, *E. coli*, 3 h; C, H, *E. coli*, 12 h; D, I, *E. coli* + carboxyfullerene, 3 h; E, J, *E. coli* + carboxyfullerene, 12 h (100X). " → ", arteriolar vessel; " → ", meninges; "△", venule; "▲", infiltrating neutrophil.

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ability was determined by quantifying the M4 deposited in brain. There was dramatic increase of M4 in brain at either 3 h or 12 h post injection (Fig. 4).

The factors involved in two phases of BBB opening after *E. coli* stimulation were examined next. Both phases of increases in BBB permeability could be inhibited by co-injection of anti-TNF-α or anti-IL-1β antibodies, and the neutralization was cytokine-specific because heat inactivation of the anti-cytokine antibody abrogated their inhibiting activity (Fig. 4). Inhibition of the early BBB opening blocked the development of the late BBB opening. The strength of TNF-α and IL-1β staining on brain microvascular vessels, as well as the TNF-α on infiltrating neutrophils, were also significantly decreased in the antibody-pretreated mice (data not shown). In order to demonstrate the role of neutrophils in the BBB opening, we used vinblastine to deplete the circulating neutrophils. Pretreatment

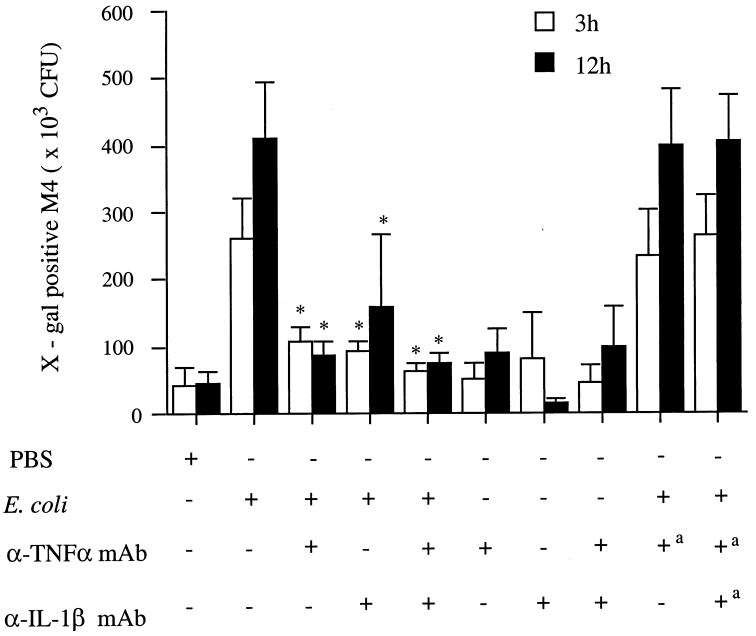

Figure 4. Inhibition of *E. coli*-induced increase of BBB permeability by anti-TNF-α or anti-IL-1β antibodies. Groups of four B6 mice were inoculated intracerebrally with 1 × 10^5 *E. coli* per mouse and sacrificed at 3 h (□) or 12 h (■) post injection. Anti-TNF-α or anti-IL-1β mAbs (30 μg) was co-injected with *E. coli* into brain. The M4 tracer (2 × 10^8) was administrated intravenously 2 min before sacrifice. The colony-forming units of X-Gal positive M4 in brain homogenate were quantitated as described in the Materials and Methods. [a] denotes 95°C treatment for 3 min. * $p < 0.05$ as compared with the *E. coli*-treated mice.

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with vinblastine for four successive days depleted the circulating neutrophils from 70% to 10%, and the vinblastine-treated mice were more susceptible to *E. coli* infection, so we just used low dose of *E. coli* (500 CFU/mouse) to stimulate the mice. There was slight increase of BBB opening after stimulation with low dose of *E. coli*, and the degree of BBB opening was not significantly inhibited by vinblastine at the early phase. While, at the late one, the increase in BBB permeability induced by low dose of *E. coli* was notedly inhibited in the neutrophil-deficient mice (Fig. 5). Moreover, no neutrophil infiltration was found in the vinblastine-treated mice (data not shown). This suggested that neutrophil infiltration played an important role in the late phase of the increase in BBB permeability.

The Neutrophil-Associated BBB Opening, But Not the Cytokine-Mediated One, Was Inhibited by Carboxyfullerene

We previously reported that carboxyfullerene could inhibit *E. coli*-induced meningitis, and its inhibition mechanism was not due to its direct antimicrobial activity (26). To further study the inhibitory effect of carboxyfullerene on *E.*

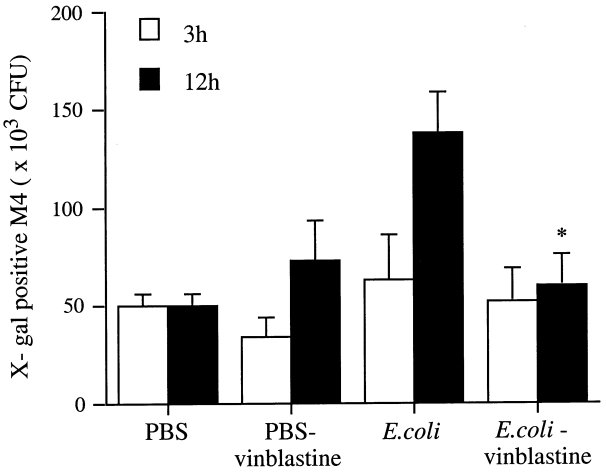

Figure 5. Inhibition of the late opening of BBB after *E. coli*-stimulation by vinblastine. Groups of four B6 mice were pretreated with vinblastine (0.5 mg/kg of body weight) intravenously for four consecutive days before intracerebral inoculation with 500 *E. coli* per mouse. Mice were sacrificed at 3 h (□) or 12 h (■) post injection. The M4 tracer (2×10^8) was administrated intravenously 2 min before sacrifice. The colony-forming units of X-Gal positive M4 in brain homogenate were quantitated as described in the Materials and Methods. * $p < 0.05$ by compared with the *E. coli*-treated mice.

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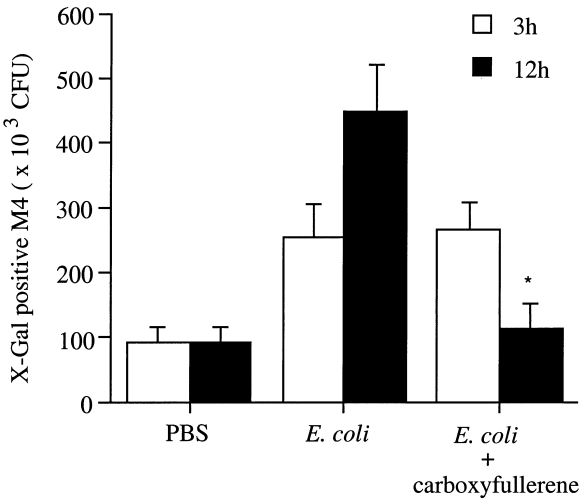

Figure 6. Inhibition of the late opening of BBB after *E. coli*-stimulation by carboxyfullerene. Groups of four B6 mice were inoculated intracerebrally with 1×10^5 *E. coli* per mouse and sacrificed at 3 h (□) or 12 h (■) post injection. Intraperitoneal injected carboxyfullerene (40 mg/kg of body weight) at the same time with *E. coli*. The M4 tracer (2×10^8) was administrated intravenously 2 min before sacrifice. The colony-forming units of X-Gal positive M4 in brain homogenate were quantitated as described in the Materials and Methods. * $p < 0.05$ as compared with the *E. coli*-treated mice.

coli-induced meningitis, carboxyfullerene was given intraperitoneally at the same time with intracerebral injection of *E. coli*. The results revealed that the late phase of BBB opening, but not the early one, was inhibited by carboxyfullerene after stimulation with sublethal dose of *E. coli* (1×10^5 CFU in each mouse) (Fig. 6). This was confirmed by the transmission electron microscopic examination of brain tissues from carboxyfullerene -pretreated mice. The alteration of the BBB at late phase was inhibited in carboxyfullerene -treated mice (Fig. 2F vs. 2D). But, there was no significant difference of the early BBB structural changes between carboxyfullerene -treated and non-treated mice (Fig.2E vs. 2B). The expression of TNF-α and IL-1β in brain after carboxyfullerene treatment was also studied. The pattern of cytokine expression at either early or late phase was not different between carboxyfullerene -treated and non-treated mice (Fig. 3). The degree of neutrophil infiltration in the brain was not inhibited by carboxyfullerene at 12 h post injection (Fig. 3E vs. 3C). Based on the above data, we conclude that the neutrophil-associated BBB opening can be inhibited by carboxyfullerene, and this inhibition is independent of the cytokine expression in the brain.

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DISCUSSION

The CNSs of mammals are considered to be immunologically privileged sites because of a lack of lymphatic drainage and separation from the blood compartment by the BBB. The blood-brain barrier, by virtue of its selective permeability, plays an important role in controlling the migration of inflammatory cells into the brain. The endothelial cells of the brain vessels are unique as they have tight junction, do not form pinocytotic vesicles, and are wrapped by astrocytes that constitute part of the BBB (1, 11, 17). Proinflammatory cytokines such as TNF-α and IL-1β are produced in the CSF during bacterial meningitis. These cytokines are known released from a variety of cells residing in the CNS, such as endothelial cells, microglial cells, and astrocytes (2, 28). Direct injection of recombinant TNF-α or IL-1 into the brain could induce the BBB opening (19, 21). In our study, we found the BBB opening could be divided into two stages in sequence. In the early stage (1–5 h post injection), the BBB opening was mediated by TNF-α and IL-1β, which expressed on blood vessels. In the late one (6–12 h post injection), its opening was mediated by infiltrating neutrophils and could be inhibited by either vinblastine or carboxyfullerene.

Fullerene has a unique cage structure that allows them to interact with biomolecules and to have avid reactivity with free radicals. These properties of fullerene have attracted much attention and generated great interest in use in biomedical research (12–13). It is necessary to convert hyhrophobic C60 into water-soluble derivatives before using it as free-radical scavenger or an antioxidant in medical or therapeutic application. Several strategies have been used to enhance its water solubility and were reported to have protective effects in various systems (3–4, 6, 20, 23, 27). A newly synthesized trimalonic acid derivative of C60, $C_{63}(COOH)_6$, is one of the compounds that not only protected cultured cortical neurons from excitotoxic injury in vitro but that also delayed the neuronal deterioration in a transgenic model of familial amyotrophic lateral sclerosis (5, 8–9, 15). In our previous study, we have demonstrated that carboxyfullerene has a protective effect against the lethal dose of *E. coli*-induced meningitis, and its inhibition mechanism was not due to its direct antimicrobial activity (26). In this study, the sublethal dose of *E. coli* was used to induce the meningitis, and the effect of carboxyfullerene was further examined. Both the BBB opening (Fig. 6) and the expression of cytokines on blood vessels (Fig. 3D & I) at the early stage were not affected by carboxyfullerene treatment. But, the late stage of BBB opening was inhibited by carboxyfullerene. The transmission electron microscopic examination also revealed that the damage of blood vessels was significantly decreased after treatment with carboxyfullerene. Since the late phase of the BBB opening was mediated by infiltrating neutrophils, our result suggests that the inflammatory neutrophils were inhibited by carboxyfullerene.

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Leukocytes recruited from the peripheral would be activated by inflammatory cytokines such as TNF-α and IL-1β, and intense leukocyte infiltration accumulated at perivascular area could damage the endothelial cells and impaired the BBB permeability by releasing reactive free radicals or hydrolytic enzymes (10). We previously also found that the neutrophilic infiltration and cytokine expression on the brain were significantly inhibited at 24 h after carboxyfullerene treatment (26). However, in this study, the TNF-α and IL-1β expression at 3 h, as well as the neutrophil infiltration at 12 h were not inhibited by carboxyfullerene treatment. Apparently, the early induction of TNFα and IL-1β in brain induced by *E. coli* and the recruitment of neutrophils were not affected by carboxyfullerene. Therefore, the inhibition of BBB opening at late phase by carboxyfullerene must be the interruption of the activation of neutrophil and its release of reactive free radicals or hydrolytic enzymes. This indicates that the neutrophil was the major target of carboxyfullerene. Fullerene has avid reactivity for free radicals, carboxyfullerene could inhibit the late stage of the BBB opening in *E. coli*-induced meningitis through neutralization of reactive free radicals released by activated neutrophils.

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