Effects of Immunomodulatory and Organism-Associated Molecules on the Permeability of an In Vitro Blood-Brain Barrier Model to Amphotericin B and Fluconazole

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Amphotericin B (AMB) is used to treat fungal infections of the central nervous system (CNS). However, AMB shows poor penetration into the CNS and little is known about the factors affecting its permeation through the blood-brain barrier (BBB). Therefore, we studied immunomodulatory and organism-associated molecules affecting the permeability of an in vitro BBB model to AMB. We examined the effects of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), lipopolysaccharide (LPS), lipoteichoic acid (LTA), zymosan (ZYM), dexamethasone (DEX), cyclosporine, and tacrolimus on transendothelial electrical resistance (TEER); endothelial tight junctions; filamentous actin; and permeability to deoxycholate AMB (DAMB), liposomal AMB (LAMB), and fluconazole. Prolinflammatory cytokines and organism-associated molecules significantly decreased the mean TEER by 40.7 to 100% (P < 0.004). DEX increased the mean TEER by 18.2 to 26.4% (P ≤ 0.04). TNF-α and LPS increased the permeability to AMB by 8.2 to 14.5% compared to that for the controls (1.1 to 2.4%) (P ≤ 0.04). None of the other molecules affected the model’s permeability to AMB. By comparison, the BBB model’s permeability to fluconazole was >78% under all conditions studied, without significant differences between the controls and the experimental groups. LPS and TNF-α decreased tight-junction protein zona occludens 1 (ZO-1) between endothelial cells. In conclusion, IL-1β, ZYM, and LTA increased the permeability of the BBB to small ions but not to AMB, whereas TNF-α and LPS, which disrupted the endothelial layer integrity, increased the permeability to AMB.

Materials and Methods

Cells. Bovine brain microvascular endothelial cells (BBECs; Cell Applications Inc., San Diego, CA) were grown in 75-cm² collagen-coated tissue culture flasks with BBEC medium (Cell Applications Inc.). The BBECs were propagated and harvested at passage 5. C6 rat glioma cells (ATCC, Sterling, VA) were grown in 75-cm² tissue culture flasks with modified Ham’s F-12K medium enriched with 12.5% horse serum and 2.5% fetal calf serum.

Materials. Bovine interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) were purchased from AbDSerotec (Raleigh, NC). Lipoteichoic acid (LTA), lipopolysaccharide (LPS), zymosan (ZYM), cyclosporine (CSA), tacrolimus (FK-506), dexamethasone (DEX), and general chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated anti-zona occludens 1 (anti-ZO-1) antibodies, 4’,6-diamidino-2-phenylindole (DAPI), and Texas Red-conjugated phalhinidin were purchased from Invitrogen Corporation (Carlsbad, CA). Fluconazole, deoxycholate AMB (DAMB), and liposomal AMB (LAMB) were purchased from the Washington Hospital Center pharmacy (Washington, DC). Lab-Tek slide chambers were purchased from Nalge Nunc International (Naperville, IL).

BBB model. The in vitro BBB model consisted of cocultured BBECs and C6 rat glioma cells. BBECs (10⁴) were plated on permeable polyester transwell insert membranes (Corning, NY) with pore sizes of 0.8 µm on 24-well plates, and C6 (10⁴) cells were plated on the bottom of the wells (Fig. 1). The growth medium was exchanged daily, and on day 5, the medium was supplemented with forskolin (an adenylylcyase inducer) and Ro20-1127 (a cyclic AMP [cAMP]-specific phosphodiesterase inhibitor) to a final concentration of 50 µM each to increase the quantity and the complexity of the tight junctions in the endothelial cells (17).

Pharmacology. On day 5, 200 µl of medium containing 5 µg/ml of DAMB, 50 µg of LAMB, or 50 µg/ml of fluconazole was placed on the vascular (upper) compartment of the BBB model and tested under one of the experimental conditions evaluated (IL-1β at 0.01 and 0.1 µg/ml, TNF-α at 0.01 µg/ml and 0.1 µg/ml, ZYM at 1 and 10 µg/ml, LTA at 0.1 and 1 µg/ml, DEX at 0.1 and

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and then multiplying it by the surface area of the transwell inserts (0.33 cm²) [i.e., Precision Instruments, New Haven, CT]). TEER was calculated by subtracting across the endothelial cell layer was calculated by using a volt-ohm meter (World the electrical resistance approximately 10-fold higher peak serum concentrations ultraperformance liquid chromatography (UPLC). LAMB and fluconazole achieve the drug concentration. DAMB was added at a concentration of 5 of AxioCam MRm monochrome digital camera and were analyzed by the use of AxioVision (version 4.6) software.

Amphotericin B assay. Assays for AMB were performed by UPLC. Briefly, the samples were deproteinized with acetonitrile at a ratio of 1:1.5, vortexed, and left at 4°C for 60 min. The samples were then centrifuged and the supernatant was transferred to autosampler vials. The UPLC system consisted of an Acquity photodiode array detector, column manager, sample manager, and binary solvent manager. The chromatograms were analyzed with the Empower (version 2) program. Separation was achieved with an Acquity UPLC BEH C18 column (2.1 by 50 mm; particle size, 1.7 μm; Waters, Ireland) and the acetonitrile-EDTA buffer (2 mM) at a gradient run (20:80 to 80:20) over 4 min. The injection volume was 3 μl. The elution time for AMB under these conditions was 3.3 min. The detector was set at 409 nm. The lower limits of detection and quantitation were 0.01 and 0.025 μg/ml, respectively. The inter- and intraday variations were <8%.

Fluconazole assay. Assays for fluconazole were performed by high-performance liquid chromatography (HPLC), as described previously (1). The HPLC system consisted of two Varian (Palo Alto, CA) ProStar 210 pumps, a ProStar 410 autosampler, and a ProStar 325 detector. Briefly, fluconazole and the internal standard, metoclopramide, were extracted by adding dichloromethane to the samples at a ratio of 5:1 and vortexing well. The samples were then centrifuged, and the top aqueous layer was aspirated and discarded. The bottom layer was evaporated in a 50°C water bath. The evaporated samples were then reconstituted in 100 μl of the mobile phase. The mobile phase consisted of 20% acetonitrile and 80% of a 0.05 M ammonium acetate buffer containing 0.1% triethylamine. The mobile phase was run at 1 ml/min, the injection volume was 75 μl, and the detector was set at 262 nm. Under those conditions, fluconazole and the internal standard eluted at 5 and 11 min, respectively. The lower limits of detection and quantitation were 200 μg/ml. The interday variation was <11%.

Statistics. The data are expressed as the means ± standard errors of the means. Differences in continuous variables were analyzed by the Mann-Whitney U test by use of the Statview (version 5.0.1) program (SAS Institute). A two-sided statistical significance level of 0.05 was considered significant. Each experimental condition was tested in three wells, and the entire experiment was then performed in triplicate.

RESULTS

Alteration of TEER. After a 24-h exposure, the average percent change in TEER compared to that for the controls is

1 μg/ml CSA at 0.1 and 1 μg/ml, FK-506 at 0.01 and 0.1 μg/ml). Following a 24-h incubation, both the vascular and the CNS compartments were sampled for assay of the drug concentration. DAMB was added at a concentration of 5 μg/ml, to allow accurate measurement of at least 1% penetration through the BBB model by ultraperformance liquid chromatography (UPLC). LAMB and fluconazole achieve approximately 10-fold higher peak serum concentrations in vivo than DAMB. We therefore added those agents at a concentration of 50 μg/ml.

TEER. The permeability of the BBB to small ions was assessed by measuring the transendothelial electrical resistance (TEER). The electrical resistance across the endothelial cell layer was calculated by using a volt-ohm meter (World Precision Instruments, New Haven, CT). TEER was calculated by subtracting the electrical resistance of the model from that of a blank transwell membrane and then multiplying it by the surface area of the transwell inserts (0.33 cm²) [i.e., TEER = (resistance_model – resistance_blank) × 0.33], expressed in ohms · cm² (11). TEER was assessed at day 5 and 24 h later.

Cell preparation for cytoarchitectural studies. BBECs were grown to confluency on slide chambers with astrocyte conditioned medium and the cAMP inducers. Astrocyte conditioned medium was produced by allowing 15 ml of BBEC medium to remain in a confluent flask of C6 glial cells for 24 h. This was then aspirated and mixed with unused medium at a ratio of 1:1 before it was applied to the BBECs in the slide chambers. Those cells were then exposed to the same experimental conditions stated above for 24 h. Following the incubation,
shown in Fig. 2. TNF-α, IL-1β, LPS, LTA at 1 μg/ml, and ZYM at 10 μg/ml significantly decreased the TEER. DEX increased the TEER, while LTA 0.1 μg/ml, CSA, and FK-506 had no statistically significant effect on the TEER.

Alteration of permeability to AMB. The mean percent penetrations of DAMB and LAMB across the BBB in the in vitro BBB model are shown in Fig. 3A and B. TNF-α and LPS both significantly increased the permeability of the in vitro BBB.

FIG. 3. Effects of cytokines, immunosuppressants, and organism-associated molecules on the permeability of the BBB to DAMB (A) and LAMB (B), expressed as the percent permeation across the endothelial monolayer. All P values are for comparison to the results for the controls: (A) *, P < 0.01; (B) *, P < 0.05.

FIG. 4. Effects of cytokines, immunosuppressants, and organism-associated molecules on the permeability of the BBB to fluconazole, expressed as the percent permeation across the endothelial monolayer.
Alterations of permeability to AMB. None of the conditions tested affected the permeability of the in vitro BBB model to AMB over 24 h. There was no significant difference in the percent penetration of the BBB between the two AMB formulations.

Alterations of permeability to fluconazole. None of the conditions tested had a significant effect on the 24-h permeability of the in vitro BBB model to fluconazole (Fig. 4). In contrast to AMB, the permeability of the BBB model to fluconazole was >78% under all conditions studied, with no significant differences occurring between the controls and the experimental groups.

Immunofluorescent staining of the BBB. Loss of the tight junctions, which corresponds to a decreased signal from fluorescently stained ZO-1 proteins, was visually determined after endothelial cell exposure to TNF-α and LPS (Fig. 5B). In comparison, tight junctions were present under all of the other experimental conditions tested, including conditions with exposure to IL-1β (Fig. 5C). Further differences in tight-junction structures were not apparent from the immunofluorescent staining.

The structure of filamentous actin changed to a more granular appearance under the influence of TNF-α, IL-1β, and the organism-associated molecules (Fig. 6). The endothelial cell monolayers remained intact under the other conditions tested. DEX, CSA, and FK-506 did not exert an apparent effect on the cytoarchitectural pattern of filamentous actin.

TNF-α and LPS disrupted the integrity of the endothelial cell monolayer and caused a marked loss of cells. By comparison, the other immunomodulatory and organism-associated molecules studied had no effect on the integrity of the endothelial cell monolayer.

DISCUSSION

This report describes the effects of immunomodulatory and organism-associated molecules on the permeability of an in vitro BBB model to AMB and fluconazole. LPS, TNF-α, IL-1β, ZYM, and the higher concentration of LTA increased the permeability of the model to small ions, as measured from the TEER. By comparison, the permeability of the in vitro BBB to AMB increased only for cells exposed to TNF-α and LPS. This may indicate that because of the higher molecular weight, the low ionic strength, and high protein and lipoprotein affinities, permeability to AMB is modulated by factors different from those affecting permeability to small ions (9). Disruption of the continuous endothelial cell layer may be necessary to enhance the permeability of the BBB model to AMB. This observation would also be consistent with the findings of our recent study showing that the permeability of the BBB to AMB is enhanced only in areas of the CNS with microabscesses caused by Candida albicans in a rabbit model of experimental hematogenous candida meningoencephalitis (15).

In contrast to AMB, fluconazole extensively permeated the in vitro model, achieving concentrations in the CNS compartment ≥78% of the concentrations in the vascular compartment for all the variables studied. These findings parallel those of in vivo and clinical studies, which have demonstrated a high level of penetration of fluconazole into the CNS (3, 4, 13, 14, 19). The smaller size, hydrophilic nature, and lower level of protein
binding of fluconazole compared to those of AMB may explain the large differences in the permeability of the BBB to these agents.

The cytoskeletal changes in actin filaments were mediated in this model by the proinflammatory cytokines (TNF-α and IL-1β) and the organism-associated molecules (LPS, LTA, and ZYM). However, the altered actin filaments did not independently induce increased BBB permeability to AMB. Oxidation of the cytoskeleton and disruption of its smooth filamentous structure have previously been documented in vivo and in vitro under inflammatory conditions (6, 12).

 Tight junctions were lost in cells treated with LPS and TNF-α but were preserved under the other conditions tested. The persistence of tight junctions with significant decreases in TEER may reflect changes in the complexity of the tight junctions, which have been shown to impair the permeability of BBB to small ions but not necessarily to larger molecules (17).

 No significant differences between the permeabilities of the in vitro BBB model to LAMB and DAMB were noticed under the conditions tested. Although both compounds are active in treating fungal infections in the CNS, neither achieves high concentrations in the CSF (10). In the same in vivo study (10), the brain parenchyma showed higher concentrations of LAMB than of DAMB. However, as the current in vitro study did not measure the concentrations of LAMB and DAMB within the astrocytes, a comparison of the level of penetration into the brain substance is not possible.

The BBB model demonstrated that DEX did not adversely affect the permeability of the BBB model to AMB. While our study and that of Cucullo et al. demonstrated that DEX increased the TEER, these observations further illustrate the dissociation between permeability to small ions and permeability to AMB (5). Concerns have been raised about decreasing the BBB permeability to antimicrobial agents by administering corticosteroids in patients with CNS infections (8). Reduced permeability to AMB was not observed when the effect of DEX was studied in this in vitro model. To our knowledge this is the first study of the factors influencing the permeability of the BBB to AMB. While IL-β, TNF-α, and all of the organism-associated molecules increased the permeability of the BBB model to small ions and altered the cytoskeletal morphology, only the conditions disrupting the integrity of the BBB endothelial monolayer (TNF-α and LPS) increased the permeability to AMB.

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