Bacillus cereus–Induced Permeability of the Blood–Ocular Barrier during Experimental Endophthalmitis

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PURPOSE. The purpose of this study was to determine to what extent blood-retinal barrier (BRB) permeability occurred during experimental Bacillus cereus endophthalmitis and whether tight junction alterations were involved in permeability.

METHODS. Mice were intravitreally injected with 100 colony-forming units of B. cereus, and eyes were analyzed at specific times after infection for permeability to fibrin and albumin, quantitation of intraocular plasma constituent leakage, production of inflammatory cytokines, and alterations in tight junction protein localization and expression at the level of the retinal pigment epithelium.

RESULTS. B. cereus induced the leakage of albumin and fibrin into the aqueous and vitreous humor by 8 hours after infection. BRB permeability occurred as early as 4 hours and increased 13.3-fold compared with uninfected controls by 8 hours. Production of proinflammatory cytokines IL-6, MIP-1α, IL-1β, and KC increased over the course of infection. In the retina, ZO-1 disruption began by 4 hours and was followed by decreasing occludin and ZO-1 expression at 4 and 8 hours, respectively. Tubulin condensation and RPE65 degradation occurred by 12 hours. A quorum-sensing mutant B. cereus strain caused BRB permeability comparable to that of wild-type B. cereus. Wild-type and mutant B. cereus sterile supernatants induced blood–ocular barrier permeability similarly to that of wild-type infection.

CONCLUSIONS. These results indicate that BRB permeability occurs during the early stages of experimental B. cereus endophthalmitis, beginning as early as 4 hours after infection. Disruption of tight junctions at the level of the retinal pigment epithelium may contribute to barrier breakdown. Quorum-sensing dependent factors may not significantly contribute to BRB permeability. (Invest Ophthalmol Vis Sci. 2009;50: 3783–3793) DOI:10.1167/iovs.08-3051

The introduction of bacteria into the posterior chamber of the eye can provoke rapid inflammation known as endophthalmitis. Pathogens usually enter the vitreous through a penetrating eye injury (after trauma), contamination during ocular surgery (after surgery), or spread from an extraocular infection into the eye (endogenous). The severity and outcome of endophthalmitis are dependent on the nature of the offending organism.1–4 Mild infection with a fairly avirulent organism may be cleared, and visual function can be retained with prompt treatment. However, infection with a virulent organism, such as Bacillus cereus, can often result in loss of vision or of the affected eye itself in less than 48 hours, regardless of antibiotic treatment or surgical intervention.5–12

B. cereus virulence has been generally attributed to toxin production.2 Most extracellular B. cereus toxins are produced under the control of a global regulator, plcR. However, non-plcR-regulated toxins have been found to be important in inducing retinal pigment epithelial (RPE) toxicity and blood-retinal barrier (BRB) permeability in vitro.13–15 B. cereus and its toxins induce an explosive infection that poses a significant threat to vision, causing loss of retinal function and complete destruction of retinal architecture in a murine model by 12 hours after infection.14 In this mouse model, significant inflammation occurs, with polymorphonuclear leukocytes (PMNs) infiltrating the vitreous as early as 4 hours after infection.14 The presence of these inflammatory cells in the posterior chamber is atypical and may interfere with preserving clarity of the visual axis, which is necessary for proper visual function. The eye is protected from inflammatory cells and blood constituents by the blood–ocular barrier (BOB), which includes the blood aqueous barrier (BAB) and the BRB. The BAB is composed of iridial vessel endothelium, iridial epithelium, and ciliary epithelium. Inner and outer BRBs are composed of retinal endothelium and retinal pigment epithelium, respectively. These barriers manage blood supply to the retina and are responsible for the homeostasis of the neural retina. Intercellular tight junctions contribute to a tightly regulated diffusion barrier, resulting in highly selective permeability. Ocular inflammation can damage this delicate system, resulting in barrier breakdown, which may contribute to vision loss during endophthalmitis.

Inflammation is linked to BRB permeability in many ocular diseases, such as uveitis15–17 and glaucoma.18 Barrier changes can be a result of the dysfunction of tight junction proteins such as ZO-1 and occludin in mouse and rabbit models of experimental autoimmune uveoretinitis (EAU).19 Tight junctions of the BRB normally maintain a barrier between the blood and the retinal tissues. Dysfunction or degradation of these proteins could cause unregulated permeability and contribute to the observed disease. Endophthalmitis is an inflammatory disease, yet to our knowledge the integrity of the BOB and alterations in tight junction structure during active infection have not been analyzed. Barrier changes during endophthalmitis could contribute to loss of retinal structure and function. Outer BRB permeability has been demonstrated in an in vitro BRB system composed of polarized RPE monolayers. B. cereus infection resulted in the permeability of RPE monolayers and
the disruption and degradation of ZO-1 and occludin. Rapid BRB permeability occurred in vitro. However barier integrity has not been analyzed during active infection.

We hypothesized that tight junction disruption and BOB permeability occur during experimental B. cereus endophthalmitis. Analyzing the changes in barrier function during B. cereus infection can lead to a greater understanding of mechanisms of inflammation and vision loss during B. cereus endophthalmitis. This may lead to the development of novel therapeutics aimed toward preventing barrier dysfunction, limiting inflammation, and preserving vision.

**Methods**

**Experimental Bacillus cereus Endophthalmitis**

Male C57BL/6 mice (4–5 weeks of age; Jackson Laboratories, Bar Harbor, ME) were maintained according to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All mice were anesthetized with a mixture of ketamine (85 mg/kg body weight; Ketavet; Phoenix Scientific, Inc., St. Joseph, MO) and xylazine (14 mg/kg body weight; Rompun; Bayer Corp., Shawnee Mission, KS). Ophthalmic anesthesia (0.5% proparacaine HCl; Ophthalmic Allergan, Inc., Irvine, CA) was administered topically before intravitreal injections. Intravitreal injections were performed as previously described. Briefly, 0.5 μL containing PBS (surgical control), brain heart infusion (BHI) medium alone (control), approximately 100 colony-forming units (CFUs) of *B. cereus*, or sterile cell-free *B. cereus* supernatants were injected into the midvitreous. The contralateral eye was not injected (absolute control).

**Bacterial Inoculum and Sterile Supernatant Preparation**

Wild-type *B. cereus* strain 14579 (American Type Culture Collection [ATCC], Manassas, VA) or a *pckR*-deficient quorum-sensing isogenic mutant strain of *B. cereus* (BCpckRkanR) was used to induce experimental endophthalmitis. Wild-type or *pckR*-deficient *B. cereus* were grown to early stationary phase in BHI for 18 hours and subcultured to 100 CFU/0.5 μL for injection into the midvitreous. Sterile cell-free supernatants were prepared from the 18-hour bacterial cultures described. Wild-type or *pckR*-deficient *B. cereus* bacterial cultures were centrifuged at 37°C for 15 minutes at 378,000 g to pellet bacteria. Supernatants were filtered through 0.02-μm filters (Millex-GV; Millipore, Billerica, MA). 0.5-μL supernatants were injected intravitreally into the midvitreous.

**Histology**

Eye globes were harvested at 0, 4, 8, and 12 hours after infection and were incubated for 24 hours at room temperature. Globes were then transferred to 70% ethanol and embedded in paraffin, sectioned, and xylazine (14 mg/kg body weight; Rompun; Bayer Corp., Shawnee Mission, KS). Ophthalmic anesthesia (0.5% proparacaine HCl; Ophthalmic Allergan, Inc., Irvine, CA) was administered topically before intravitreal injections. Intravitreal injections were performed as previously described. Briefly, 0.5 μL containing PBS (surgical control), brain heart infusion (BHI) medium alone (control), approximately 100 colony-forming units (CFUs) of *B. cereus*, or sterile cell-free *B. cereus* supernatants were injected into the midvitreous. The contralateral eye was not injected (absolute control).

**Immunohistochemistry**

Fluorescence Detection. Sections were deparaffinized and incubated in a surfactant (PBS + 0.02% Tween 20) for 10 minutes. Samples were blocked in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 1 hour at room temperature (Gibco, Grand Island, NY). Slides were treated with primary antibody diluted in 0.5% bovine serum albumin (BSA) in PBS with 0.02% NaN₃ overnight at 4°C, followed by secondary antibody for 1.5 hours at room temperature. Coverslips were mounted with fluorescence mounting medium (Dako, Copenhagen, Denmark) or fluorescence microscopy (Eclipse E800; Nikon, Tokyo, Japan). Images of at least three eyes were analyzed (Fluoview, version 4.3; Olympus) at each time point. Secondary-alone controls were used to verify the absence of nonspecific fluorescence. Primary antibodies included rabbit anti–rat albumin (1:200; Accurate Chemical and Scientific Corp., Westbury, NY), rabbit anti–ZO-1 (1:200; Zymed, San Francisco, CA), mouse anti–occludin (1:200; Zymed), rat anti–tubulin (1:200; Chemicon, Temecula, CA), or mouse anti–RP-E65 (1:200; Chemicon). Secondary antibodies included goat anti–rabbit (Alexa Fluor 594) or goat anti–mouse (Alexa Fluor 488) prepared at 1:200 (Molecular Probes-Iovitrogen, Carlsbad, CA). The mouse anti–ZO-1 antibody required epitope unmasking immediately after deparaffinization (1 mg/mL protease, 10 minutes at 25°C).

**Polymer Detection.** Sections were deparaffinized and incubated in a steam bath of 1 mL EDTA (pH 8.0) for 75 minutes to retrieve antigens. This was followed by a 20-minute cool-down and wash in 0.3% potassium permanganate for 10 seconds to remove pigment from the retinal pigment epithelium. This was followed by a 5-second 1% oxalic acid wash and a 10-minute water wash. Samples were blocked with DMEM + 10% FBS. Detection methods were in accordance with the Thermo Fisher Scientific detection system (UltraVision LP; Thermo Fisher Scientific, Fremont, CA). Endogenous peroxidases were blocked by the addition of 3% H₂O₂. Primary antibody (mouse anti–ZO-1 [1:200; Zymed]) was added for 15 minutes at 25°C. Secondary antibody (goat anti–mouse, 1:200; Alexa Fluor 488; Molecular Probes) conjugated to horseradish peroxidase, was incubated with samples for 30 minutes at 25°C. The substrate 3,3-diaminobenzidine (DAB) was added to the sample for 1 minute. Enzyme cleavage of DAB produced an insoluble brown product.

**Quantitation of Vascular Permeability**

Albumin leakage from blood vessels into the retina was measured to quantify vascular permeability using a modified Evans blue protocol. The Evans blue dye (Sigma, St. Louis, MO) was dissolved in normal saline (30 mg/mL) sonicated for 5 minutes, and filtered (0.45 μm; Millipore, Bedford, MA). After intravitreal injection of *B. cereus* and 2 hours before enucleation of the eye, mice were anesthetized, and Evans blue dye (30 mg/kg) was injected into the tail vein. Mice were kept on a warm pad for 2 hours to ensure complete distribution of the dye. Mice were perfused with 1% paraformaldehyde in citrate buffer (pH 4.2, 37°C, 120 mm Hg). Eyes were harvested every 2 hours after injection up to 12 hours. Retinas were dissected out and placed in 150 μL of formamide to extract the dye, incubated at 70°C for 18 hours, and centrifuged for 20 minutes at 378,000 g. Optical densities of 100-μL supernatants were determined by spectrophotometry (OD620). The concentration of Evans blue was calculated from a standard curve and normalized to the total protein per sample (calculated by bicinchoninic acid [BCA] protein assay). Results were expressed in micrograms of Evans blue per milligrams of total protein content. Values represent mean ± SEM for *N* = 12 eyes per time point.

**Western Blot of Tight Junction Proteins**

Eyes were harvested and dissected to remove the retinas. For each sample, two retinas were pooled in 200 μL of 1X lysis buffer (Cell Signaling, Danvers, MA) supplemented with phenylmethylsulfonyl fluoride (1 mM final concentration) and sonicated. Lysates were centrifuged at 16,000 rpm for 5 minutes. Supernatant protein concentrations were determined by BCA. Lysate samples prepared at 50 μg were analyzed by gel electrophoresis. Proteins were transferred to nitrocellulose for immunoblotting. Membranes were blocked with 5% nonfat milk in 0.1% Tween-20 in Tris-buffered saline (TBST) for 1 hour at
25°C. The same membrane was probed for actin and occludin or ZO-1. Membranes were treated with primary antibody (rabbit anti–occludin [1:200] or rabbit anti–ZO-1 [1:100; Zymed], or mouse antiactin [1:500; Affinity Bioreagents, Golden, CO]) overnight at 4°C and secondary antibody (anti–mouse or anti–rabbit immunoglobulin G-horseradish peroxidase [1:2500]; Sigma) for 1 hour at 25°C. All antibodies were prepared in TBST with 0.02% NaN₃. Membranes were developed (ECL Plus Detection System; GE Healthcare, Piscataway, NJ) and imaged (Storm 860 Imager [Amersham Biosciences, Pittsburg, PA] or ImageStation 2000R [Eastman Kodak, Rochester, NY]). Densitometry was performed using Kodak software (IM; Eastman Kodak). Expression was calculated in arbitrary units, normalized to actin, and presented as percentage change compared with 0 hour.

Quantitation of Cytokines and Chemokines

After intravitreal injection of B. cereus, eyes were analyzed for the presence of proinflammatory cytokines, shown to be involved in models of ocular inflammation.15–18 Globes were harvested and homogenized with glass beads in a protease inhibitor cocktail (Triton X-100, 0.5 M EDTA, 10 mM sodium orthovanadate [Sigma] and Protease Inhibitor [Calbiochem, La Jolla, CA] in PBS, pH 7.4). Supernatants were then analyzed for the presence of interleukin (IL)-6, macrophage inflammatory protein 1 alpha (MIP-1α), interleukin-1 beta (IL-1β), or keratinocyte-derived chemokine (KC) with the use of commercial ELISA kits (Quantikine; R&D Systems, Minneapolis, MN) in accordance with the manufacturer’s instructions. Cytokine and chemokine concentrations were interpolated from standard curves. Values are expressed as mean ± SEM for at least six analyses per time point.

Statistical Analysis

Results were the arithmetic mean ± SEM of all the samples in the same experimental group. A two-tailed Student’s t-test was used to determine the statistical significance of the data. Statistical significance was determined at P ≤ 0.05.

RESULTS

Blood–Ocular Barrier Permeability

Our experimental B. cereus endophthalmitis infection course was consistent with that previously published.14 One hundred CFUs of B. cereus injected into the midvitreous resulted in similar intraocular bacterial growth, inflammation, and loss of globe architecture. Leakage of blood constituents within the eye during B. cereus endophthalmitis is depicted in Figure 1. Trichrome staining of infected eyes demonstrated that fibrin had seeped into and filled the anterior and posterior chambers by 8 hours after infection (Fig. 1a). Fibrin was still visible in both the posterior and the anterior chambers at 12 hours as eyes began to lose structural integrity and severe retinal detachment was apparent. Mock-infected and uninjected eyes retained structural integrity, and no fibrin was detected in the vitreous or aqueous humor of these control eyes.

Blood–Retinal Barrier Disruption

Because of the parallel of inflammatory eye disease and outer BRB breakdown19,23,24 and the early leakage of albumin into the vitreous, we analyzed and quantified BRB leakage (Fig. 2). There was minimal leakage of dye in retinas after BHI mock injection when compared with un.injected controls (P = 0.9).
Leakage of albumin into the retina increased above mock and un.injected controls as early as 4 hours after infection ($P = 0.04$). These results correlated with PMN infiltration into the eye at 4 hours after infection. $^{14}$ By 8 hours after infection, BRB permeability had increased 13.30-fold over controls ($P = 0.003$; Fig. 2). Decreasing retinal albumin after 8 hours was attributed to the loss of structural integrity of the eyes and the consequential leakage of ocular fluids in highly inflamed eyes.

**Tight Junction Alterations in the Outer BRB**

To understand the mechanism of BRB permeability during endophthalmitis, we analyzed the integrity of the tight junctions occludin and ZO-1 in the retina, focusing on the retinal pigment epithelium of the outer BRB (Fig. 3). Both ZO-1 and occludin are expressed in the retina, but control eyes had little visible immunofluorescent signal because of the quenching of the fluorescent signal by the RPE pigment. The ZO-1 signal was continuous in the adherens junctions of the outer limiting membrane. By 4 hours, the ZO-1 signal was laterally elongated. Abnormal distribution of ZO-1 in the RPE layer continued 8 hours after infection. Retinal detachment was evident, and ZO-1 signal in the retinal pigment epithelium continued to elongate. By 12 hours after infection, retinal destruction was severe, and no signal was detected. Throughout the course of infection, B. cereus induced abnormal redistribution of ZO-1 away from the cell-cell junctions.

To quantify alterations in tight junction proteins, retinal lysates were analyzed by immunoblot (Fig. 5a). The occludin signal was detectable in mock, 0-hour, and 4-hour samples. The occludin signal significantly decreased ($P = 0.001$) at 8 hours and was almost undetectable by 12 hours. ZO-1 was detected in retinal lysates at 0, 4, and 8 hours but not at 12 hours after infection. Expression was further quantified by densitometry (Fig. 5b), demonstrating that ZO-1 and occludin expression were decreased during the course of infection. This significant loss of ZO-1 ($P < 0.0001$) and occludin ($P < 0.0003$) by 12 hours after infection indicated retinal tight junction disruption, which could contribute to barrier permeability during infection.

**Proinflammatory Cytokines and Chemokines**

Proinflammatory cytokine and chemokine synthesis in whole eyes during experimental B. cereus endophthalmitis is summarized in Figure 6. Production of TNFα was previously described, reaching 49.05 ± 4.9 pg/eye by 10 hours after infection. $^{14}$ Similarly, other proinflammatory cytokines were significantly elevated during B. cereus infection. IL-6 increased 52.57-fold over 0-hour controls, reaching 455.18 ± 15.83 pg/eye by 12 hours after infection ($P < 0.0001$). Similarly, IL-1β and MIP-1α expression increased 31.00-fold ($P = 0.0001$) and 29.04-fold ($P < 0.0001$) over 0-hour controls by 12 hours, reaching 335.50 ± 22.32 and 494.90 ± 9.10 pg/eye, respectively. KC expression was not detectable at 0 hour, but expression drastically increased to 751.23 ± 204.9 by 4 hours ($P = 0.02$). KC concentrations continued to increase, peaking at 1627.09 ± 15.37 pg/eye by 12 hours after infection ($P = 0.03$).

**plcR-Deficient B. cereus–Induced BOB Permeability**

Given that in vitro data suggested that plcR-deficient B. cereus induced the permeability of polarized RPE monolayers in a manner similar to that of wild-type B. cereus, $^{15}$ we analyzed whether plcR-deficient B. cereus induced the leakage of fibrin across the BOB during experimental B. cereus endophthalmitis. Histologic analysis by trichrome staining demonstrated fibrin leakage into the anterior and posterior chambers by 8 hours, with similar dispersal throughout the eye by 12 hours after infection (Fig. 7). plcR-Deficient B. cereus appeared to induce BOB permeability comparable to that induced by wild-type B. cereus (Figs. 1, 7), suggesting that a non-plcR-regulated toxin, or group of toxins, might have contributed to the loss of barrier function during experimental B. cereus endophthalmitis.
FIGURE 3. Tight junction protein redistribution, tubulin condensation, and RPE65 degradation at the level of the retinal pigment epithelium during experimental B. cereus endophthalmitis. Retinas of B. cereus–infected eyes were harvested, and paraffin-embedded sections were used for histologic analysis and immunohistochemistry. Intact retinas had little fluorescent signal in the retinal pigment epithelium because of quenching by the pigment. However, at 12 hours, ZO-1 and occludin expression were intermittent and scattered. Additionally, tubulin condensed, and RPE65 expression was not detected by 12 hours after infection. Control samples were developed with secondary antibody alone. Images represent three or more individual experiments. Images were acquired at 100× magnification.

FIGURE 4. Lateral redistribution of ZO-1 in the retina during B. cereus endophthalmitis. Retinas of B. cereus–infected eyes were harvested, and paraffin-embedded sections were used for histologic analysis and immunohistochemistry. Bleaching RPE pigment before immunohistochemistry resulted in a visible brown punctate ZO-1 signal in the retinal pigment epithelium (white arrows) and a continuous signal along the outer limiting membrane (white arrowheads). ZO-1 lateral redistribution was evident at 4 and 8 hours after infection (white arrows). By 12 hours, severe damage to retinal layers had occurred, and ZO-1 signal was not detectable (top). Isotype controls confirmed the lack of nonspecific antibody (bottom). Images were acquired at 40× magnification. RPE, retinal pigment epithelium; PCL, photoreceptor cell layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
During experimental endophthalmitis, which is a uniquely severe ocular inflammatory disease that often has devastating consequences, such as rapid loss of vision or loss of the affected eye itself despite treatment. During experimental 

\( B. \) \textit{cereus} endophthalmitis, PMNs infiltrate into the posterior segment as early as 4 hours after infection, indicating BOB permeability. As with uveitis and other ocular inflammatory diseases, this barrier breach prevents the proper separation of blood constituents from the neural retina, which can allow an unregulated influx of blood constituents into the eye. Because of parallels between inflammation in uveitis and endophthalmitis and the lack of information about mechanisms of barrier changes during endophthalmitis, we sought to determine to what extent the BOB is compromised during 

\( B. \) \textit{cereus} endophthalmitis and to examine whether the disruption of tight junctions contributing the BRB contributed to this dysfunction.

To examine changes in the BOB, we analyzed whole globes by histology and used trichrome staining and immunohistochemistry to identify infiltrating fibrin or blood albumin, respectively. Fibrin and albumin penetrated the blood-retina and blood-aqueous barriers by 8 hours after infection. Leakage of albumin appeared to occur more rapidly from the BRB than from the BAB. Fibrin and albumin moved from the blood into the ocular tissues and filled the aqueous and vitreous humor, contributing the occlusion of the visual axis. These data are consistent with reports of BRB disruption in conjunction with ocular inflammation and infection. In patients with endophthalmitis, serum albumin was observed in the retina but was not quantified. To further analyze changes in the BRB during experimental 

\( B. \) \textit{cereus} endophthalmitis, we quantified albumin accumulation in the retina. The robust leakage of albumin into the retinal tissues from 4 to 8 hours corroborated the histology showing fibrin and albumin leakage in the vitreous at 8 hours after infection. Decreasing albumin in the retina after 8 hours might have occurred as a result of the leakage of eye constituents during dissection because of the dissolution of retinal tissues late in infection. Taken together, these data suggest that early loss of BRB integrity might have permitted infiltration of PMNs into the vitreous as early as 4 hours after infection. BRB permeability, infiltration of PMNs, and loss of more than 60% of A- and B-wave function occurred in parallel through 8 hours after infection. These events also correlate with the course of inflammation during infection, which was reported to be mild at 6 hours and moderate to severe by 10 to 12 hours after infection. Early BRB permeability may allow the influx of fluids into the posterior segment, contributing in part to the occlusion of the visual axis. These data are consistent with reports of BRB disruption in conjunction with ocular inflammation and infection.
to edema, retinal detachment, and ultimately loss of vision. However, the contribution of BRB breakdown to vision loss has yet to be determined. Breakdown of the BRB during endophthalmitis may be due, in part, to the production of bacterial toxins or other secreted factors during infection. B. cereus produces a plethora of toxins that could contribute to permeability, including phospholipases, metalloproteases, enterotoxins, cytolysins, and proteins of unknown function.

Before the role of secreted factors in barrier permeability was investigated, the integrity of RPE tight junctions was analyzed by immunohistochemistry and Western blot analysis. By Western blot analysis, occludin appeared as a set of 110-kDa, 65-kDa, and 60-kDa bands. The 65-kDa and 60-kDa bands were consistent with reports of occludin at various molecular weights. The 110-kDa band might have represented a dimerization of occludin, similar to that reported by McCaffrey et al. Degradation of RPE tight junctions occurred during experimental B. cereus endophthalmitis. Retinal ZO-1 and occludin expression decreased throughout the course of infection when compared with actin controls, a decrease that paralleled increasing barrier leakage through 8 hours after infection. Western blot analysis did not detect ZO-1 expression and showed occludin expression to be less than 5% by 12 hours after infection. Retinal occludin signal was lost more rapidly than ZO-1 during early infection, corroborating similar findings in RPE monolayers in vitro. ZO-1 and occludin were detected at 12 hours after infection by immunohistochemistry, but signals were intermittent. This discrepancy might have resulted from the superior sensitivity of fluorescence detection compared with that of Western blot analysis. Redistribution of tight junction proteins in the retinal pigment epithelium could have been sufficient to cause the disruption of the tight junction, rendering the junction and the RPE barrier permeable, similar to that observed during EAU. These data correlated with in vitro data demonstrating the degradation of ZO-1 and occludin in polarized RPE monolayers after B. cereus infection. Changes in the RPE tight junctions could have been the result of toxins or other factors secreted by B. cereus. B. cereus is a gastrointestinal pathogen that can opportunistically infect the eye. Some gastrointestinal pathogens synthesize toxins that can alter tight junctions and can compromise epithelial barriers during infection. These tight junction-damaging tox-

**Figure 7.** plcR-deficient B. cereus–induced barrier permeability similar to that in wild-type B. cereus during experimental endophthalmitis. One hundred CFUs of plcR-deficient (BC-plcR-kanR) Bacillus were injected into C57BL/6j mouse eyes. Eyes were analyzed by histology and were trichrome stained to detect fibrin in the aqueous and vitreous of the anterior chamber (AC) and posterior chamber (PC). Fibrin leaked across the BOB, filling the aqueous and vitreous humor by 8 hours after infection. Images represent three or more individual experiments. Images were acquired at 5× magnification.

**Figure 8.** Sterile cell-free wild-type and plcR-deficient B. cereus supernatants were sufficient to induce BOB permeability. C57BL/6j mouse eyes were intravitreally injected with 0.5 μL sterile B. cereus supernatant prepared from either wild-type (BCWT) or plcR-deficient (BC-plcR-kanR) cultures. Eyes were analyzed by histology. Fibrin (A) and albumin (B) leakage into the anterior chamber (AC) and posterior chamber (PC) were analyzed by trichrome staining. Wild-type and plcR-deficient Bacillus sterile supernatants caused BOB permeability to fibrin, with wild-type inducing fibrin leakage more rapidly than the mutant strain. Images represent three or more individual experiments. Images were acquired at 5× magnification.
ins include *Pseudomonas* elastase and alkaline protease, *Vibrio cholera* hemagglutinin/protease, *Bacteroides fragilis* metalloproteases, and *Clostridium difficile* exotoxins. Proteases produced by nonpathogenic organisms have also been shown to cause the breakdown of tight junctions.  

In the present study, ZO-1 was redistributed laterally in the RPE cell layer by 4 hours after infection. ZO-1 and occludin have been reported to be redistributed and dissociated from the cellular junction of primary human gastric epithelial cells as early as 2 hours after infection with *Helicobacter pylori*. However, alterations in ZO-1 structure of retinal pigment epithelium during active infection in vivo have not previously been reported. In the present study, alterations in retinal ZO-1 were visible by 4 hours after infection, correlating with BRB permeability. Disruption of ZO-1 might have contributed to the tight junction dysfunction that renders the BRB permeable during *B. cereus* endophthalmitis.  

Tubulin and RPE65 localization in retinal pigment epithelium during endophthalmitis was also analyzed. The eukaryotic cytoskeleton is composed of actin microfilaments, intermediate filaments, and microtubules consisting of tubulin. Microtubule integrity is vital for epithelial barrier function. During endophthalmitis, the tubulin signal in the retina became intensified, indicating condensation, and was intermittent in the disrupted retinal layers. Several pathogens, including *B. cereus*, can target cytoskeletal elements during infection. Although we did not analyze the effect of *B. cereus* infection on actin in the retina, *B. cereus* has been shown to disrupt actin in intestinal epithelium. *B. anthracis* disrupts actin in neutrophils, and *H. pylori* and enteropathogenic Escherichia coli target host cell actin. *B. cereus*, or its toxins or other secreted factors, may disrupt the cytoskeleton of retinal pigment epithelium by targeting tubulin. However, alterations in tubulin may also be an indirect result of other cellular changes. The direct effects of *B. cereus* and its products on cytoskeletal elements in barrier cells have yet to be defined. RPE65 expression is an indicator of epithelial function. Because RPE65 is essential for the regeneration of 11-cis-retinol, loss of RPE65 has been reported to directly correlate with loss of vision. Patients with mutations in RPE65 lose vision during adolescence, and mice with a knock-in R91W mutation of RPE65 experience loss of retinal function. In the present study, RPE65 expression was not detectable by 12 hours after infection, which is consistent with a loss of retinal function by 12 hours during experimental *B. cereus* endophthalmitis. However, whether the loss of RPE65 contributes to loss of vision during endophthalmitis is an open question.  

BRB permeability during *B. cereus* endophthalmitis may also be a consequence of proinflammatory cytokine production during infection. The host response during endophthalmitis has not been well defined. Examining the proinflammatory mediators produced in other models of ocular inflammation (i.e., uveitis) may provide insight into the contributions of these molecules during endophthalmitis. In the present study, expression of IL-1β, MIP-1α, KC, and IL-6 was analyzed by ELISA. These candidate cytokines were selected because they are detected in the eye during uveitis and are thought to be directly linked to BRB permeability.  

Specifically, IL-1β is associated with BRB permeability and induction of inflammation involving PMN and mononuclear cell infiltration when this cytokine is intravitreally injected into Lewis rats, produced during EAU, or produced in the vitreous of patients with uveitis. This IL-1β could be derived predominantly from vascular endothelium and may contribute to PMN infiltration seen during *B. cereus* endophthalmitis. IL-6, MIP-1α, and IL-8 have been detected in the eyes of patients with uveitis and in models of experimental uveitis. Each mediator has the capacity to increase neutrophil recruitment. IL-6 levels were elevated in mouse eyes during experimental *B. cereus* endophthalmitis. IL-6 expression by polarized ARPE-19 monolayers was increased on exposure to *B. cereus*, but production of IL-6 by other cell types has not been examined. KC (an IL-8 homolog) may significantly modulate the immune response during *B. cereus* endophthalmitis by recruiting PMNs to the site of infection. KC and its homologs have been detected in experimental and human ocular infections, including keratitis caused by fungi, adenosviruses, *Pseudomonas*, and *Staphylococcus*, and in acute bacterial conjunctivitis and uveitis. Several of these studies noted that increases in PMN likely resulted from KC production. The increase in PMN infiltration and MPO activity between 4 and 8 hours during experimental *B. cereus* endophthalmitis might have been the result of the robust production of KC at the respective time points. In experimental *B. cereus* endophthalmitis, KC increased before the onset of EAU, suggesting that this mediator contributes to initial cellular recruitment. MIP-1α has been reported to play a role in the recruitment of circulating monocytes. However, during experimental *B. cereus* endophthalmitis, monocytes and macrophages were detected in small numbers in the eye (less than 5%), whereas more than 70% of the infiltrating cells were identified as PMNs. It is possible that PMNs synthesize MIP-1α or that local cells in the retina, such as astrocytes, produce MIP-1α, but the infection may reach an experimental end point before monocyte recruitment occurs. MIP-1α may also be produced to recruit neutrophils. MIP-1α mediates lung leukocyte recruitment in mice challenged with LPS. Injection of MIP-1 into the mouse footpad results in a local influx of PMNs followed by a monocyte infiltrate. MIP-1α may play a similar role in PMN recruitment during *B. cereus* endophthalmitis.  

TNFα has also been detected in the ocular fluid of patients with uveitis, during EAU, and in mouse eyes infected with *B. cereus* and may induce neutrophil proliferation during inflammation in both models. During experimental *B. cereus* endophthalmitis in TNFα knockout mice, decreased intraocular PMN infiltration was reported. In addition, the increase in intraocular *B. cereus* growth and the decrease in retinal function occurred more rapidly in the eyes of TNFα knockout mice than in control mice. These data suggested that TNFα played an important role in the inflammatory response that attempts to control *B. cereus* intraocular infection.  

Production of proinflammatory cytokines during experimental *B. cereus* endophthalmitis may contribute to permeability of the BRB. The complex synthesis and interaction of proinflammatory mediators during ocular inflammation makes identification of their specific roles difficult. Although proinflammatory cytokines have been shown to contribute to BRB breakdown in uveitis, their activity in directly altering tight junctions is an open question. In a rabbit model of EAU, treatment of ex vivo retinas (with retinal pigment epithelium) with proinflammatory cytokines (including TNFα, IL-1β, and MIP-1α) did not result in the downregulation of tight junction proteins. The role of individual proinflammatory mediators in endophthalmitis could be further analyzed by establishing in infections in mice deficient in individual cytokines of interest. However, compensation for the absence of an individual cytokine and the production of other cytokines by multiple pathways could confound such studies. For example, IL-1β and TNFα act on similar cell types during inflammation, and both signal through the NFκB pathway. The absence of one cytokine...
may induce the synthesis of compensating cytokines, as has been reported during *B. cereus* endophthalmitis in TNFα knockout mice and in experimental EAU in which cytokine receptors were deleted. The ability of one cytokine to compensate for the absence of a different cytokine makes it difficult to analyze the contribution of individual cytokines to barrier permeability and tight junction disruption.

Although the contribution of proinflammatory cytokines must be further investigated, understanding the role of *B. cereus* toxins and other secreted factors is equally important. Previous studies indicate that intraocular injection of the *B. cereus* cell wall alone causes transient inflammation but not loss of vision, whereas the injection of *B. cereus* toxins and other secreted factors results in rapid loss of vision. This highlights the importance of identifying the *B. cereus*-secreted factors that contribute to tight junction disruption and BRB permeability. The role of *plcR*-regulated secreted factors in BOB permeability was analyzed by histology. By 8 hours, *plcR*-deficient *B. cereus* caused RPE toxicity and in vitro BRB permeability to the same extent as did the wild-type strain. To determine whether *plcR*-deficient *B. cereus* induced BOB permeability during experimental endophthalmitis, we analyzed fibrin leakage in the aqueous and vitreous humor. Infection with *plcR*-deficient *B. cereus* resulted in seepage of fibrin in the anterior and posterior chambers by 8 hours after infection, similar to what was seen during infection with wild-type *B. cereus*, suggesting that *plcR*-regulated toxins were not required for BOB permeability to fibrin. We also examined the ability of sterile cell-free supernatants from wild-type or *plcR*-deficient *B. cereus* to induce BOB permeability. Eyes injected with these sterile cell-free supernatants also exhibited permeability to fibrin, but fibrin accumulation was less dense than that induced by viable bacteria and was cleared by 24 hours after infection, possibly because of the absence of replicating bacteria and the continual replenishing of toxic factors. Fibrin accumulated in the posterior and anterior chambers of eyes injected with wild-type sterile supernatant more rapidly than in mice injected with supernatant from the *plcR*-deficient mutant strain. This difference in the rate of leakage was consistent with reports in which viable wild-type *B. cereus* caused inflammation more rapidly than did the *plcR*-deficient mutant in rabbit or mouse eyes. In both studies, infection with either strain eventually caused significant inflammation, retinal detachment, and complete loss of retinal function. These data indicate a role for non-*plcR*-regulated secreted factors in BOB dysfunction during endophthalmitis. *B. cereus* produces a plethora of factors that could contribute to permeability, including phospholipases, metalloproteases, enterotoxins, cytolsins, and proteins of unknown function. Several proteins are not, or are only partially, regulated by *plcR*, including neutral protease, InhA2, enolase, oligopeptidase permease A, penicillin-binding protein B, or other proteins of unknown function. Previous data indicated that barrier permeability of ARPE-19 monolayers in vitro was caused by *B. cereus*-secreted factors. In that study, toxins and other secreted factors not regulated by *plcR* contributed to BRB disruption in vitro. In the present study, *plcR*-deficient *B. cereus*, as well as *plcR*-deficient *B. cereus* sterile cell-free supernatants, induced permeability of the BOB, suggesting that non-*plcR*-regulated secreted factors may contribute to the loss of BRB integrity. Unpublished data indicate the production of a 20- to 30-kDa protease and a 160-kDa protease by wild-type and *plcR*-deficient *B. cereus*. Of the *B. cereus* proteases in this size range, 94% do not encode a *plcR* box upstream of the protease gene, indicating that they are produced independently of *plcR* regulation. Such proteases may include camelysin or a zinc metalloprotease, whose activities include cleavage of extracellular matrix proteins. *B. cereus* proteases could similarly target components of the extracellular matrix, disrupting the RPE barrier and indirectly inducing tight junction dysfunction. The identities and roles of the secreted factors that facilitate barrier permeability in vivo are still under investigation.

This study is the first to characterize permeability of the BRB and to demonstrate alterations in RPE tight junctions during bacterial endophthalmitis. The rapid production of toxic secreted factors by *B. cereus* may cause significant barrier dysfunction through the alteration of tight junctions at the level of the retinal pigment epithelium, but proinflammatory mediators may also be indirectly involved in BRB permeability during infection. These findings are a platform for further studies to understand the mechanisms of BRB dysfunction, the role of bacterial products and inflammatory cytokines in this process, and the contribution of these factors to inflammation and vision loss during endophthalmitis. Understanding the mechanism of barrier dysfunction may facilitate the identification of therapeutic targets designed to improve the stability of the BRB, obstruct cellular influx, and protect the retina from explosive inflammation during *B. cereus* endophthalmitis.

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**References**


