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# Extracellular Virulence Factors in *Bacillus cereus* Endophthalmitis: Methods and Implication of Involvement of Hemolysin BL

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***Bacillus cereus*** **is a common cause of highly fulminant posttraumatic and metastatic endophthalmitis. Exotoxins or enzymes likely contribute to the severity of the infection, but specific virulence factors have not been identified. We developed two methods for the identification of *B. cereus* ocular virulence factors. In an in vitro assay that allows screening of multiple samples, retinal toxicity was estimated by measuring the release of lactate dehydrogenase from retinal buttons treated with *B. cereus* toxins. The results from this assay were confirmed with a sterile endophthalmitis model in which the histopathologic effect of intravitreally injected toxins was assessed. We tested pure hemolysin BL (HBL), a tripartite dermonecrotic vascular permeability factor of *B. cereus*, and crude exotoxin (CET) preparations, consisting of concentrated, cell-free *B. cereus* culture supernatant. In the in vitro assay, both CET and HBL caused rapid release of lactate dehydrogenase and retinal disintegration. In vivo, the toxins caused endophthalmitis clinically characteristic of *B. cereus* within 4 h. Histological changes included rapid retinal necrosis and detachment, choroidal edema, detachment and disruption of the retinal pigment epithelium, and rapid infiltration of polymorphonuclear leukocytes. Neutralization of HBL in CET preparations inhibited toxicity in vitro by 54%, and pure HBL was less toxic than CET with equal HBL contents in both methods. The results suggest that *B. cereus* ocular virulence is multifactorial and that HBL contributes to virulence.**

*Bacillus cereus* is one of the most common causes of posttraumatic and metastatic bacterial endophthalmitis. *Bacillus* species cause an average of 39% of trauma-associated cases of endophthalmitis (28) and are the most frequent cause of metastatic bacterial endophthalmitis in drug abusers (10).

*B. cereus* is considered one of the most destructive organisms to affect the eye (15, 18, 19, 21). Endophthalmitis caused by this organism is characterized by severe pain, chemosis, periorbital swelling, and extreme proptosis. Late symptoms include corneal edema and corneal ring abscess. Unlike most organisms, intraocular infection with *B. cereus* causes systemic effects, including fever and leukocytosis. This infection typically progresses from injury to enucleation in 24 to 48 h (18). The extreme fulminance of *B. cereus* endophthalmitis highlights the need for simple and rapid diagnostic methods for this and similar infections.

*B. cereus* endophthalmitis is quite refractory, and blindness often occurs even when aggressive and appropriate antimicrobial therapy is instituted before the loss of visual acuity (8, 28). This is probably due to the involvement of toxins. Even if infecting organisms are killed, sufficient intraocular toxin concentrations may continue to damage tissue and promote inflammation. *B. cereus* produces a wide variety of extracellular toxins and potentially toxic enzymes (26, 27). Numerous authors have speculated that the unusual virulence of *B. cereus* is due to these factors (5, 7, 12, 14, 16–18, 23, 24), but there have been no reports confirming this.

Peyman et al. (20) noted an association between the severity of endophthalmitis and the ability of the infecting organism to produce exotoxins and proteases. However, there is little experimental evidence documenting the involvement of specific microbial exotoxins. The best evidence for the participation of a bacterial protein toxin in endophthalmitis is for the pAD1-encoded cytolysin/hemolysin from *Enterococcus faecalis* (13, 25). *E. faecalis* strains expressing the toxin caused more aggressive infections than cytolysin-negative strains.

It may be possible to improve the outcome of endophthalmitis by neutralizing or inactivating specific bacterial virulence factors. First, the relevant factors must be identified. Here, we report two methods for the identification of virulence factors from *B. cereus.*

We developed an in vitro rabbit retinal toxicity assay that permits screening of large numbers of samples. Retinal toxicity was estimated by measuring the cytolytic release of lactate dehydrogenase (LDH) from retinal buttons treated with *B. cereus* toxins. We also developed a sterile endophthalmitis model to confirm in vivo the results of the in vitro method. In vivo toxicity was assessed clinically and histopathologically after intravitreal injection of pure or crude exotoxins.

The results obtained with these methods indicated that cell-free *B. cereus* exotoxins can cause full-blown endophthalmitis. We identified hemolysin BL (HBL) as a potent retinal toxin in vitro and in vivo. HBL is a unique tripartite toxin that is the major dermonecrotic factor of *B. cereus* (1–3). We also determined that HBL accounts for only about 50% of the retinal toxicity of *B. cereus* culture supernatant in vitro, indicating that virulence is multifactorial. The methods described here provide a means of identifying the remaining ocular toxins of *B. cereus.*

## MATERIALS AND METHODS

**Toxin production.** Crude exotoxin (CET) consisted of concentrated culture supernatant from *B. cereus* MGBC 145, an isolate from posttraumatic endophthalmitis (provided by Michael Gilmore). Culture supernatant was prepared in

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TABLE 1. Histological grading scheme for ocular damage in the sterile endophthalmitis model[a]

| Grade | Description |
|---|---|
| 0 | No evidence of retinal disruption. |
| 1 | Mild alteration of retinal architecture, including outer layer invagination and/or mild disruption of inner limiting membrane. |
| 2 | Outer nuclear layer buckling, mild inner layer disruption, and separation from outer nuclear layer. |
| 3 | Full thickness retinal disruption. Photoreceptor layer folds, with retinal detachment. Eosinophilic subretinal fluid not evident. Inner and outer nuclear layers disrupted but still distinguishable; outer nuclear layer contiguous. |
| 4 | Full thickness retinal disruption, with disorganization of all retinal layers, outer nuclear layer disorganized or necrotic, inner layers missing. Subretinal fluid evident. |

[a] Each of eight eye sections was assigned the grade for the most severe damage in that section. The retinal grade assigned to an eye was the average grade for all of the sections graded.

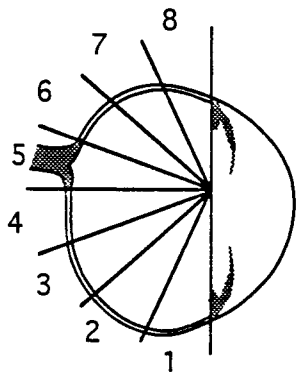


FIG. 1. Method for histopathologic grading of eyes from the sterile endophthalmitis model. A radial grid was printed on clear plastic and laid on top of histopathology slides as illustrated. The base of the grid passed through each ora serrata. The optic nerve was positioned within section 5. Retina within each section was assigned a grade based on the criteria in Table 1. The overall grade was the average for all of the sections graded.

brain heart infusion broth supplemented with 0.1% glucose as described previously (2). The culture was grown for 5 h at 32°C, concentrated by ammonium sulfate precipitation (80% saturation, 4°C), and dialyzed against pH 7.3 Hanks' balanced salt solution (HBSS). Pure HBL was produced and purified to homogeneity as described by Beecher and Wong (3).

**Toxin quantitation.** The concentration of pure HBL components was determined spectrophotometrically ($A_{280}$). The toxic activity of CET involves multiple factors that have not yet been fully characterized. We used HBL as a marker toxin and quantitated CET in terms of its HBL equivalent ($HBL_{eq}$). A dilution series of CET was tested alongside a dilution series of pure HBL of known concentration in the gel diffusion assay for HBL hemolysis that was described previously (4). The CET preparation was assigned an $HBL_{eq}$ concentration based on the HBL activity (as measured by lysis zone diameters) in the crude preparations compared with that of the pure HBL standard.

**Antibody production.** Polyclonal antibodies were produced by injecting electrophoresis gel slices containing separated HBL components into rabbits essentially as described by Harlow and Lane (11). Antisera to all three HBL components each reacted with a single major band from *B. cereus* culture supernatants on Western immunoblots and exhibited reactions of nonidentity with each other in double immunodiffusion assays.

**Tissue culture medium.** UltraCulture (BioWhittaker, Walkersville, Md.) supplemented with 2 mM L-glutamine was used for the in vitro retinal toxicity assay. A serum-free medium was chosen to avoid the potential for serum inactivation of toxins and interference with serum LDH activity in subsequent enzyme assays (9).

**LDH assay.** LDH was measured by the method described in the Worthington Manual (Worthington, Freehold, N.J.). The substrate stock contained 0.2 M Tris-HCl (pH 7.3), 1 mM sodium pyruvate, and enough NADH to bring the $A_{340}$ to 1.4 (ca. 0.22 mM NADH). Fifty microliters of sample was added to 950 μl of substrate in a cuvette. The $\Delta A_{340}$ was measured over 1 to 2 min at room temperature. Activity is reported as $\Delta A_{340}$ per minute.

**Statistical analysis.** Analysis of variance of identically treated retinal buttons was done on the Statistical Analytical System with a split-plot-type model. Heterogeneity of variances evident in residual plots between treated and nontreated buttons was corrected by using a square-root transformation.

**Histopathology.** Formalin-fixed eyes were processed for histopathology at the Eye Pathology Laboratory of the Department of Ophthalmology and Visual Science at the University of Wisconsin–Madison. Eyes were cut in the horizontal plane, and central sections were embedded in paraffin in a Fisher automatic tissue processor. Sections cut 6 μm thick were stained with hematoxylin-eosin and graded in a single masked fashion according to the grading scheme described in Table 1.

**In vitro retinal toxicity assay.** Adult New Zealand White rabbits were used in all experiments. Rabbits were killed, and the eyes were enucleated several minutes postmortem. Eyes were stored in cold HBSS until dissected (30 to 60 min). The eyes were bisected 2 to 3 mm posterior to the ora serrata. The corpus vitreum was teased with forceps from the posterior calotte, which was then placed under tissue culture medium and bisected with scissors 2 to 3 mm inferior to the optic nerve and medullary rays so that the sections would lie flat. Retinal buttons were cut with a 3-mm trephine, taking care not to cut completely through the choroid. The retina surrounding the buttons was teased, usually in one piece, away from each calotte. The buttons were teased from the retinal pigment epithelium-choroid complex with the tips of no. 5 forceps so that they floated freely in the culture medium. Free-floating buttons were handled by drawing them into a wide-terminal-bore micropipette tip. About 12 to 15 buttons were obtained per eye. The buttons were washed with several changes of fresh culture medium.

Individual buttons were transferred in 100 μl of culture medium to separate wells of a 96-well tissue culture plate. Control samples or toxin-containing samples (100 μl each), diluted in culture medium, were added to the wells, and the plate was incubated at 37°C for appropriate times. After the incubation period, all culture medium was drawn from the wells and placed in corresponding wells of a microtiter plate for immediate assay or storage at −20°C. Retinal buttons were left in the wells.

**Sterile endophthalmitis model.** Rabbits were anesthetized by intramuscular injection of ketamine (30 to 50 mg/kg) and xylazine (5 mg/kg) and with topical 0.5% proparacaine. A drop of 0.3% gentamicin was applied topically, as was 1% Cyclogyl (cyclopentolate hydrochloride) and 2.5% Neo-Synephrine Hydrochloride (phenylephrine hydrochloride) to induce mydriasis. Anterior-chamber paracentesis through a 27-gauge needle was performed after placement of a lid speculum. Subsequently, samples of 0.1 to 0.15 ml were injected into the central vitreous cavity. Injections were made 3 mm behind the limbus through a 30-gauge needle. Additional topical gentamicin (0.3%) was applied, and the eyes were examined by indirect ophthalmoscopy for intraoperative complications.

All eyes were injected with samples or negative controls and graded clinically in a single-blind fashion. Samples were chosen on the basis of activity in the in vitro retinal toxicity assay. CET samples (in brain heart infusion broth) were paired with negative controls consisting of brain heart infusion broth or heat-inactivated CET. Pure HBL samples (up to 6 μg per component) in Tris-buffered saline (TBS) were paired with negative controls consisting of 18 μg of bovine serum albumin in TBS. When antisera were added to samples, identical amounts of preimmune serum were added to some paired negative controls. CET doses were quantitated on the basis of the $HBL_{eq}$ as described above for the in vitro assay. Rabbits were monitored continually and anesthetized over the 4-h assay period, after which they were killed. An animal kept for 16 h postinjection was given aspirin (500 mg/kg per os) to prevent pain.

At 4 h postinjection, the eyes were graded clinically by gross appearance and indirect ophthalmoscopy. The rabbits were then killed, and the eyes were enucleated and fixed with 10% buffered Formalin.

**Histological grading.** The extent of histologically evident ocular damage generally varied from one section of an eye slice to another. To get an overall measure of ocular damage, eyes were divided into eight equal sections as depicted in Fig. 1, and each section was given the grade of the most severely damaged tissue in that section. The grades for whole eyes reported below were the averages of the grades for the eight sections (fewer if sections were missing). The extent of retinal disruption was graded with the scheme outlined in Table 1. Choroid thickness was measured with a calibrated ocular micrometer at ×400 magnification.

## RESULTS

**In vitro retinal toxicity assay.** In preliminary studies, we found that CET from *B. cereus* caused rapid and extensive degradation of retinal tissue. All of the toxic activity was likely due to protein components in the *B. cereus* CET because (i) the CET had been dialyzed against buffer through a membrane with a cutoff of 12 to 14 kDa, indicating that large molecules

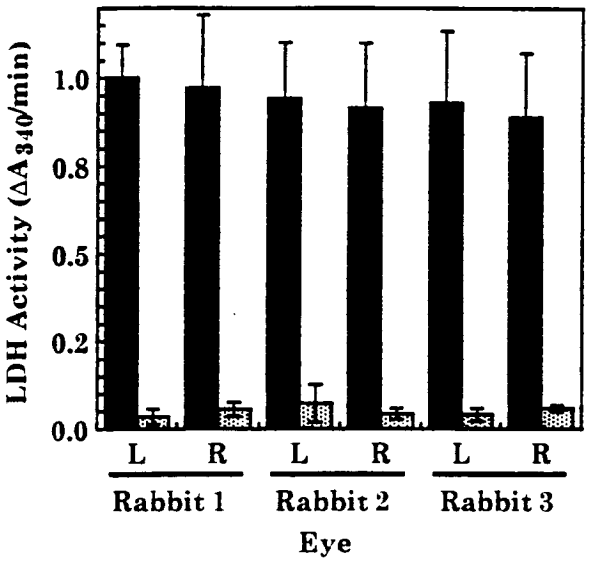


FIG. 2. LDH release from identically treated retinal buttons from different eyes and different rabbits. Solid bars, buttons (six per eye) were treated with 600 ng/ml $HBL_{eq}$ of CET. Shaded bars, buttons (three per eye) were not treated with CET (negative controls). In this and subsequent figures, error bars indicate standard errors. Details are provided in the text. L, left eye; R, right eye.

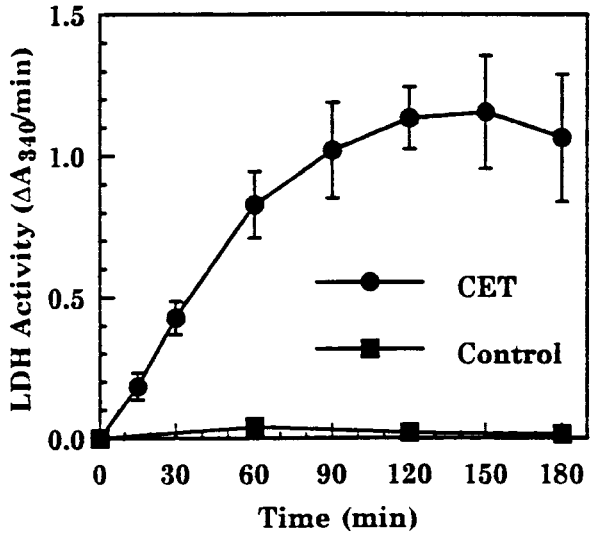


FIG. 3. Time course of LDH release from retinal buttons treated with CET. CET, buttons treated with 100 ng/ml $HBL_{eq}$ of CET in HBSS ($n = 6$). Control, buttons not treated with toxin ($n = 3$).

caused the tissue damage, and (ii) heating of the CET (100°C, 20 min) completely eliminated toxicity.

Preliminary dose-response analysis indicated that total LDH release could be obtained within 2 h with CET concentrations from 100 to 200 ng/ml $HBL_{eq}$. We adopted 100 ng/ml $HBL_{eq}$ as the standard CET dose unless otherwise indicated. For comparison, 1.2 μg of HBL per ml was measured in a late-log-phase brain heart infusion culture (ca. $5 \times 10^8$ cells per ml) of *B. cereus* MGBC 145.

Variation in LDH release from buttons from different eyes or animals was assessed in the experiment shown in Fig. 2. Buttons were prepared from six eyes from three rabbits. Six buttons from each eye were treated for 90 min with 600 ng/ml $HBL_{eq}$ of CET, and three buttons from each eye were not treated with toxin. Buttons were treated with 600 ng/ml $HBL_{eq}$ rather than 100 ng/ml to ensure maximal release of LDH, which would also ensure maximal interbutton variation. Analysis of variance indicated that there were no differences attributable to the source of the buttons. The *P* values for rabbits and eyes were 0.3 and 0.8, respectively. LDH release from toxin-treated buttons was significantly different from that from negative controls ($P < 0.0001$). Standard errors from 76 sets of samples exhibited a mean coefficient of variation of 15.4% ± 9.2%, with a range of 0% to 39.5%.

Figure 3 illustrates the time course of LDH release from retinal buttons treated with 100 ng/ml $HBL_{eq}$ of CET. There was negligible spontaneous release of LDH from negative control buttons after 3 h. From these data, retinal buttons were treated for 1.5 h in subsequent experiments.

Maximal LDH activity ($\Delta A_{340}$ per minute) in the experiments depicted in Fig. 2 and 3 was between 1.0 and 1.2. This level of activity was seen consistently in all experiments in which maximal release occurred and corresponds to release of 0.6 to 0.7 U of LDH per button (1 U causes the oxidation of 1 μmol of NADH per min at 25°C and pH 7.3).

The retinal toxicity of pure HBL was assessed in the experiment depicted in Fig. 4. This dose-response curve confirms that HBL is a potent retinal toxin. Like the other known toxic activities of HBL (3), there was little or no LDH release from retinal buttons treated with the three HBL components individually or in binary combinations.

Retinal buttons treated with either CET or pure HBL became completely disaggregated into cells and cell debris and collapsed upon removal of the supporting supernatant. Buttons treated with 1% Triton X-100 (a nonionic detergent often used for the disruption of cell membranes [9]) remained intact and released only about one-third as much LDH as CET.

Specific antisera were used to estimate the contribution of HBL to the overall retinal toxicity of *B. cereus* MGBC 145. Thirty-six buttons were treated with CET containing 100 ng/ml

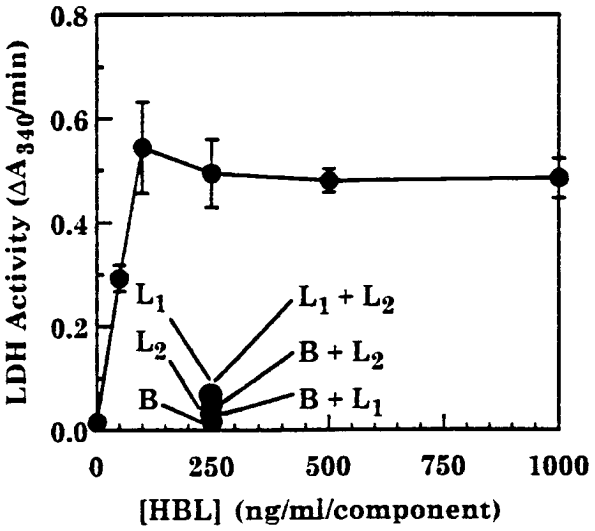


FIG. 4. Retinal toxicity of pure HBL. The dose-response curve was produced by treatment of retinal buttons ($n = 6$) with all three HBL components (B, $L_1$, and $L_2$) at the concentrations indicated on the *x* axis. Retinal buttons treated with 250 ng of each individual HBL component per ml or in binary combinations exhibited little or no toxicity.

$HBL_{eq}$. Groups of six of these buttons received the following additional treatments: (i) none, (ii) antisera to all three HBL components, (iii) antiserum to the B component, (iv) antiserum to the $L_1$ component, (v) antiserum to the $L_2$ component, and (vi) normal rabbit serum. Each antiserum was added to at least a fivefold excess above that required to neutralize HBL, as measured by hemolysis (i.e., 2 μl of antiserum per well).

The combination of all three antisera inhibited the mean release of LDH by 55%, whereas the individual antisera inhibited it by 25, 44, and 54% for anti-B, anti-$L_1$, and anti-$L_2$, respectively. Normal rabbit serum at 2 μl per well did not inhibit LDH release, and 6 μl per well inhibited it by 8%, indicating that serum components do not significantly inhibit toxicity.

**Sterile endophthalmitis model.** Clinically, the response to intravitreal toxins mimicked *B. cereus* endophthalmitis. None of the negative control samples, including heat-inactivated CET, brain heart infusion broth, and bovine serum albumin, produced detectable clinical or histopathological effects. Within 4 h, all eyes receiving ≥0.8 μg of $HBL_{eq}$ of CET exhibited marked exudate, conjunctival edema, and hyperemia. There was no red reflex or view of the retina. In eyes receiving 1 to 4 μg of $HBL_{eq}$ of CET, there was little or no red reflex within 2 h, and by 4 h there was vitreal hemorrhage, hemorrhagic chemosis of the conjunctiva, and corneal haze. Responses to pure HBL were less severe than to CET with comparable HBL contents. Eyes receiving 2.5 to 6 μg of each component of HBL exhibited no fundus details but still had a moderately decreased red reflex. These eyes also exhibited exudate, conjunctival edema, hyperemia, and marked vitreous haze.

Lower amounts of CET (0.125 to 0.5 μg of $HBL_{eq}$) or HBL (0.5 to 1.0 μg per component) produced clinically less severe symptoms. Mild responses to low doses included slight edema, localized retinitis with little or no loss of red reflex, conjunctival edema, mild hyperemia, slight exudate, areas of patchy vitreous haze, and somewhat obscured retinal details.

Eyes that presented relatively minor clinical signs exhibited a broad range of histopathologic changes, ranging in averaged retinal grades from 0.5 to 3.6. Dose-response data for these eyes are presented in Fig. 5.

Representative histopathologic changes are depicted in Fig. 6 and 7. The extent of tissue damage often varied greatly in individual eyes from one section to another. This likely reflects clinical observations of patchy retinitis. The retina exhibited distinctive changes in response to toxin treatments. Most conspicuous was tight "folding" of the photoreceptor layer (Fig. 6B and C) with detachment from the retinal pigment epithelium (RPE). The inner layers did not exhibit folding but became disorganized and detached from the outer nuclear layer. Its limitation to the outer layers suggests that the phenomenon may represent structural disorganization that is not folding per se. Retinal folding may occur as a histological artifact, but it generally involves the entire thickness of the retina. We did not see such a feature in any of our histological preparations.

Necrosis of inner and outer rod segments was common. Folding, detachment, and rod segment necrosis increased with the severity of the response. However, the outer nuclear layer remained identifiable regardless of the extent of retinal necrosis. In severe reactions, the inner layers were not identifiable except for the inner plexiform layer, which often remained contiguous (Fig. 6B). The RPE layer often was detached from Bruch's membrane, and distinct gaps typically appeared between individual RPE cells in toxin-treated eyes (Fig. 6 and 7, panels B and C). In some cases, individual RPE cells were absent between cells still attached to Bruch's membrane. In the most severe reactions, RPE cells were completely disrupted, as evidenced by insoluble melanin globules (Fig. 7D).

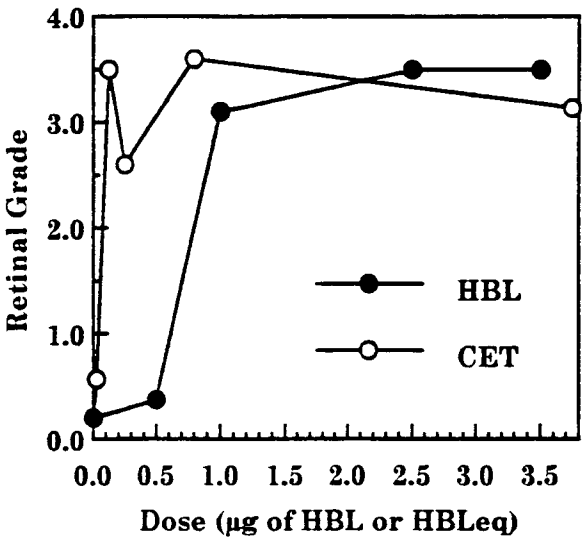


FIG. 5. Dose-response curves of crude *B. cereus* toxin and pure HBL in the in vivo sterile endophthalmitis model. Each rabbit eye received an intravitreal injection of either CET ($HBL_{eq}$) or pure HBL in the quantities indicated on the *x* axis. Toxic activity was recorded as the average retinal histopathology grade for eight sections graded per eye.

Choroidal edema occurred in all instances in which retinal damage was evident. Average choroid thicknesses increased with increasing retinal grade. Choroid thickness ranged from 25 to 40 μm in untreated eyes to ca. 180 μm in toxin-treated eyes with severe retinal damage. Some choroids were too disrupted to measure.

Fibrinous (eosinophilic) fluid was evident under detached RPE cells (Fig. 7B and C) and in the subretinal spaces (Fig. 7C) of eyes with severe reactions. Subretinal fluid was not seen in eyes with less severe reactions.

Ciliary bodies were also damaged by toxin treatment. Typically, there was necrosis of epithelial cells, vessel engorgement, and polymorphonuclear leukocyte (PMN) infiltration. Occasionally, necrosis of pigmented cells caused release of melanin.

The above histopathological descriptions were typical of both CET- and HBL-treated eyes. In addition, erythrocytes were commonly seen in the corpus vitreum of HBL- and CET-treated eyes. There were two notable differences between the responses to CET and HBL. First, pure HBL caused less severe necrotic reactions than CET with equivalent HBL contents. Second, although both treatments promoted vigorous inflammation after only 4 h, there was significantly greater infiltration of PMNs in CET-treated eyes (Fig. 6, compare panel B with C). In HBL-treated eyes, PMNs were generally confined to the choroid after 4 h, whereas CET-treated eyes had sections in which PMNs infiltrated the choroid, retina, and corpus vitreum. Extensive retinal necrosis, RPE detachment, and choroidal edema often occurred in the absence of PMNs or with very few present.

Eyes injected simultaneously with antiserum to the $L_2$ component of HBL and 1 μg of each component of HBL appeared normal clinically and had retinal grades of 0.1 and 0.5. Without antiserum, eyes treated with 1 μg of each component of HBL showed moderate clinical signs and had retinal grades of 3.1 to 3.5.

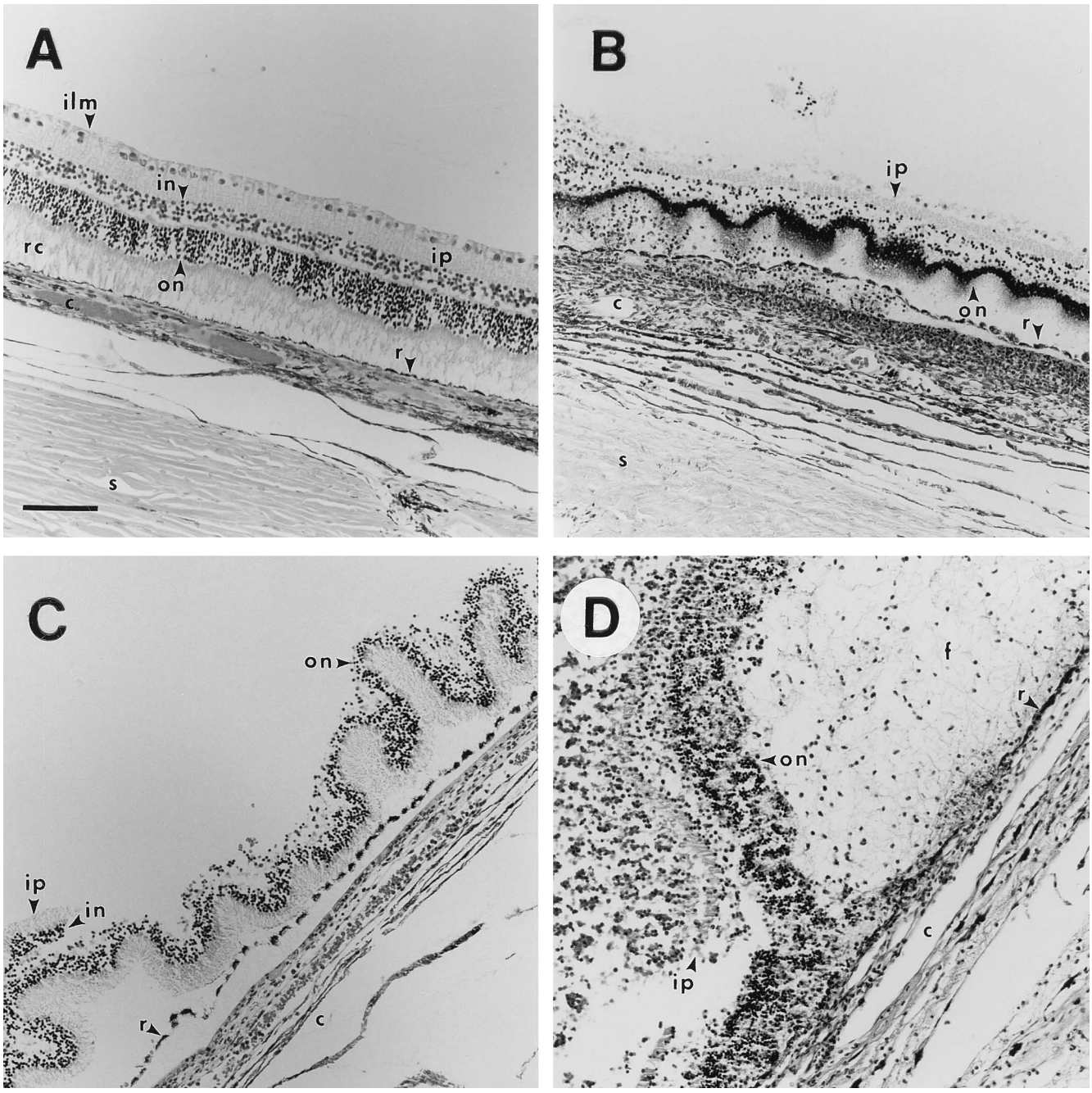


FIG. 6. Histopathologic effects of intravitreal injection of CET and HBL. Eyes were injected (0.1 ml) intravitreally with (A) brain heart infusion broth, (B) 0.8 μg of $HBL_{eq}$ of CET 1 μg each of each component of pure HBL, or (D) 1 μg each of each component of pure HBL. The eyes in panels A, B, and C were fixed at 4 h postinjection; the eye in panel D was fixed after 16 h. Abbreviations: ilm, inner limiting membrane; ip, inner plexiform layer; in, inner nuclear layer; on, outer nuclear layer; rc, layer of rods and cones; r, retinal pigment epithelium; c, choroid; s, sclera; f, subretinal fluid. (A) Bar, 100 μm. Note folding of photoreceptor layer (B and C) and confinement of PMNs to the choroid (C compared with B and D).

One HBL-treated rabbit that had minor retinitis at 4 h was held for 16 h. The entire globe became tightly packed with PMNs, which infiltrated all ocular layers (Fig. 6D). The outer nuclear layer was still discernible and was detached because of subretinal exudate.

## DISCUSSION

We have described two methods for estimation of the ocular toxicity of *B. cereus* exotoxins. Both methods measure gross damage to intact retinal tissue. This is a rational first step to identifying *B. cereus* virulence factors because extreme necrosis is characteristic of *B. cereus* endophthalmitis and retinal damage is the primary cause of visual impairment. The in vitro retinal toxicity assay allowed quantitation of toxic activity without the interference of secondary tissue damage due to inflammation. The sterile endophthalmitis model provided in vivo confirmation of our in vitro observations. In addition, histological observations provide clues to the pathophysiology and

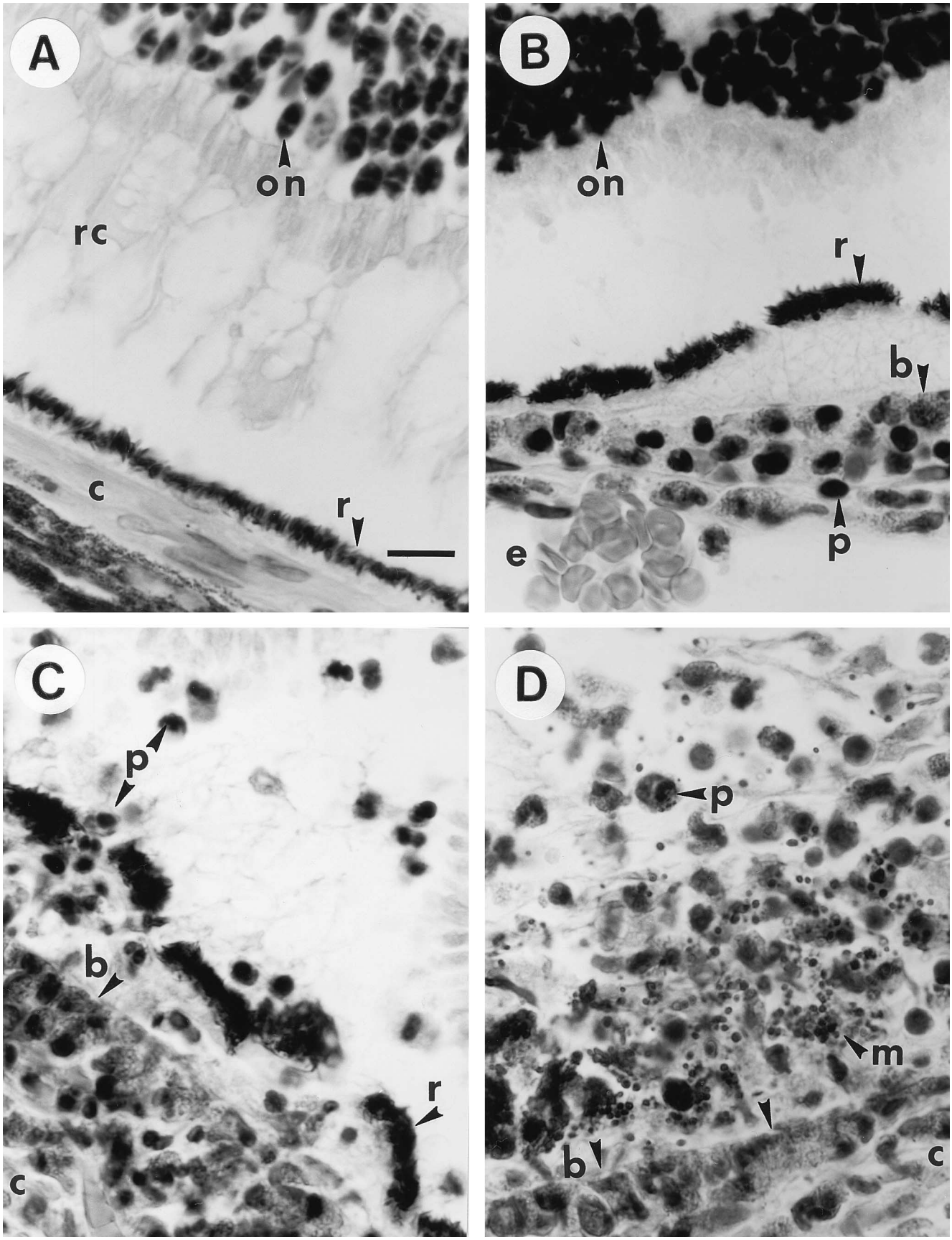


FIG. 7. Representative stages of increasing outer layer necrosis, RPE disruption, and PMN infiltration in toxin-treated eyes. (A) Eye injected (0.1 ml) with brain heart infusion broth. (B, C, and D) Eyes injected with 0.8 μg of $HBL_{eq}$ of CET. All eyes were fixed 4 h postinjection. Abbreviations: on, outer nuclear layer; rc, layer of rods and cones; r, retinal pigment epithelium; c, choroid; b, Bruch's membrane (inner membrane of the choroid); m, melanin; p, PMN; e, erythrocytes. (A) Bar, 10 μm. Note increasing detachment, separation, and disintegration of RPE and progressive infiltration of PMNs from the choroid into the retina.

inflammatory activity of *B. cereus* toxins. By screening samples in vitro for retinal toxicity or inhibition of toxicity, we were able to minimize the number of rabbits needed for in vivo experiments.

It is still unknown if the toxin doses used in these models are germane to actual endophthalmitis, because the kinetics of intraocular toxin production have not been assessed. However, using doses sufficient to cause rapid tissue damage maximizes the likelihood of identifying highly toxic factors and minimizes contributions from host inflammatory factors in vivo and spontaneous LDH release in vitro. Doses of $\leq$100 ng/ml promoted rapid and extensive tissue damage in both methods. It is likely that much lower doses would cause extensive damage in vivo over a longer time, particularly in view of the vigorous inflammatory response that the toxins engender. If toxin production in vivo approaches that seen in vitro (1.2 μg of HBL per ml of a late-log-phase culture), dangerous toxin levels may occur relatively early in the infection.

Both CET and pure HBL reproduced the symptoms of *B. cereus* endophthalmitis in the sterile endophthalmitis model. As predicted by the in vitro assay, pure HBL was less toxic than CET preparations containing equal amounts of HBL activity (Fig. 5).

The production of endophthalmitis symptoms by exotoxins does not prove their participation in the *B. cereus* infection. Other strategies will be more useful for that, e.g., neutralization of specific toxins during active infections. However, these results show clearly that sterile exotoxins are capable of causing endophthalmitis. This supports the notion that appropriate antimicrobial therapy often fails because of continued toxin-mediated ocular damage in the absence of viable bacteria.

*B. cereus* exotoxins may produce two distinct sight-threatening manifestations: direct necrosis and acute inflammation. Direct necrosis was demonstrated in vitro and in vivo, where extensive retinal disruption occurred in the absence of inflammatory cells. Within 4 h, both CET and HBL caused vigorous inflammation, as evidenced by PMN infiltration (Fig. 6B and C). The inflammatory response to HBL was overwhelming after 16 h (Fig. 6D).

The folding of the photoreceptor layer (Fig. 6B and C) may be notable. We are unaware of other instances in which this type of folding characterizes endophthalmitis in animals or humans, even in the presence of extensive retinal disruption and inflammation. The RPE normally controls subretinal fluid volume by pumping it to the choroid (6). The apparent separation of RPE cells (Fig. 6 and 7, panels B and C), along with extensive choroidal edema, suggests that the folding may be the result of fluid flow into the subretinal space. However, subretinal eosinophilic exudate was not usually seen, except in more severe reactions. The inner layers were not typically folded, probably because necrosis broke down the physical connection to the photoreceptors.

The histopathology of the toxin-treated eyes is consistent with known activities of *B. cereus* toxins, particularly HBL. From these observations, we propose some mechanisms for the extraordinary fulminance of *B. cereus* endophthalmitis. The most obvious is direct necrosis of ocular tissue by exotoxins. However, subnecrotic doses of HBL may cause considerable vascular permeability (3), which was likely the cause of the choroidal edema seen in toxin-treated eyes. The action on choroidal capillaries implies access through the RPE, possibly via the apparent physical separations between the RPE cells.

In a normal eye, the vitreal cavity is an immunologically "privileged" site that is sealed by a tight blood-eye barrier that prevents lymphatic drainage (22). The immune system does not have direct access to intravitreal antigens. Tight junctions between normal RPE cells and ciliary body epithelial cells help maintain the external blood-eye barrier (6). *B. cereus* toxins probably rapidly breach the blood-eye barrier by disrupting RPE cells and ciliary body epithelial cells. Retinal vessels may also be compromised, but we did not see this in the relatively avascular rabbit retina. Breach of the blood-eye barrier and increased uveal vascular permeability may provide direct access of the immune system to intraocular antigens, engendering an unusually vigorous inflammatory response.

The dose-response curve in Fig. 4 demonstrates that HBL is a potent retinal toxin. The shape of this curve (i.e., abrupt onset of toxicity, narrow dose-response range, and terminal plateau) is highly unusual, but it closely mimics the dose-response curves for HBL hemolysis (3). As for other known activities of HBL, the combination of all three components promoted maximal retinal toxicity. Individual components or binary combinations had little or no toxic activity.

Pure HBL was less toxic than CET with identical HBL contents, and the retinal toxicity of *B. cereus* culture supernatant was partially inhibited by neutralization of HBL. These results suggest that HBL is a major virulence factor in *B. cereus* endophthalmitis. They also show that another factor(s) contributes to toxicity independently of HBL. We are presently working toward identifying and characterizing the ocular toxicity of all the major *B. cereus* endophthalmitis virulence factors. The in vitro retinal toxicity assay allows the assay of multiple samples from a single animal and will be a useful tool in identifying additional toxins.

If a role for exotoxins in endophthalmitis is firmly established, it will have implications for treatment. The most direct way to avert toxin-mediated tissue damage would be to neutralize specific toxins or otherwise protect ocular tissue. The inflammatory activity of *B. cereus* toxins suggests that corticosteroids may be useful. If it becomes possible to inhibit virulence factors in vivo during an infection, the major threat will be inflammatory necrosis. There is some controversy as to the value of vitrectomy in treating posttraumatic endophthalmitis. If exotoxins modulate the infection, vitrectomy would likely be useful in reducing intraocular toxin concentration as well as inactive factors that may potentiate inflammation.

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