Intravitreally Injected Human Immunoglobulin Attenuates the Effects of *Staphylococcus aureus* Culture Supernatant in a Rabbit Model of Toxin-Mediated Endophthalmitis

**Objective:** To determine whether human immunoglobulin attenuates the toxic effects of *Staphylococcus aureus* culture supernatant in a rabbit model of endophthalmitis.

**Methods:** Immunoglobulin binding to products of *S. aureus* strain RN4220 was tested by Western blot analysis using known toxins (β-hemolysin and toxic shock syndrome toxin-1) and a concentrated culture supernatant containing *S. aureus* exotoxins (pooled toxin). To induce endophthalmitis, pooled toxin was injected into the rabbit vitreous. For immunoglobulin treatment, immunoglobulin and pooled toxin were either mixed and injected simultaneously or immunoglobulin was injected immediately after or 6 hours after pooled toxin injection. Severity of endophthalmitis was graded according to a 9-day course with clinical examination (slitlamp biomicroscopy or indirect ophthalmoscopy) and evaluation of histologic sections.

**Results:** The toxic effects of pooled toxin were markedly reduced when immunoglobulin was mixed with pooled toxin and injected simultaneously. Delayed injection of immunoglobulin diminished its ability to reduce toxicity. Clinical and histologic signs of toxicity were partially attenuated when immunoglobulin was injected immediately after pooled toxin, but only minimal clinically detectable reductions in toxicity were observed when immunoglobulin injection was delayed for 6 hours.

**Conclusion:** Pooled human immunoglobulin can attenuate the toxic intravitreal effects of a concentrated culture supernatant containing *S. aureus* exotoxins.

**Clinical Relevance:** Immunoglobulin may represent a novel adjuvant in the treatment of bacterial endophthalmitis. To optimize the potential therapeutic benefit, maximizing the mixture of immunoglobulin with bacterial products and early intervention are likely to be important.

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the vitreous and retina for up to 5 days without producing clinically detectable inflammation (Rajeev Buddi, MD, D.P.H., S.L.P., and J.M.B., unpublished data, 2003). We conducted a “proof of principle” investigation to determine whether pooled human immunoglobulin binds proteins in S aureus culture supernatant and 2 purified S aureus exotoxins and whether immunoglobulin injected into the vitreous reduces the tissue destruction and inflammatory effects produced by an intravitreal injection of S aureus culture supernatant.

POOLED BACTERIAL TOXIN PREPARATION

Staphylococcus aureus strain RN4220 is a derivative of strain 8325-4 modified for genetic manipulation, which primarily produces β-hemolysin (molecular weight, approximately 35 kDa).23 We chose RN4220 because of our extensive laboratory experience with this line and the relative ease with which we can manipulate some aspects of toxin production. The particular strain of RN4220 used in this study also produces toxic shock syndrome toxin-1 (TSST-1) (molecular weight, approximately 22 kDa), α-hemolysin (molecular weight, approximately 32 kDa), and δ-hemolysin (molecular weight, approximately 3 kDa) in small amounts (P.M.S., oral communication, October 2000). The technique for collecting culture supernatant has been described previously.24 Bacteria were propagated overnight with aeration at 37°C in 1200 mL of beef heart extract medium.25 Briefly, this medium is prepared from a tryptic digest of beef hearts that is sterilized with isoelectric focusing using successive gradients of pH 3.5 to 10 and pH 6 to 8.26

The immunoglobulin preparation used in this study is sterile, preservative-free solution of γ-globulin prepared from large pools of human plasma (Gamimmune N, 10%; Bayer Corporation, Pittsburgh, Pa). It is approved for intravenous use in humans for the prevention or attenuation of a variety of infectious diseases. Western blot analysis was performed to determine whether immunoglobulin binds to known toxins produced by S aureus (β-hemolysin and TSST-1; source: laboratory of P.M.S.) and to proteins, including toxins, in pooled toxin. Known toxins, pooled toxin, and control beef heart extract medium were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in reducing conditions (5mM β-mercaptoethanol) using 12.5% separating gels. Protein loading was empirically determined and is reported in the “Results” section. Separated proteins were transferred to nitrocellulose membranes and blotted overnight with a 1:10000 dilution of immunoglobulin. Bands were visualized with a detection system (Enhanced Chemiluminescence; Amersham Biosciences, Piscataway, NJ) after exposure to a donkey antihuman secondary antibody.

Approval from the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee) was obtained prior to initiating animal experiments. New Zealand white rabbits (weight, 2-3 kg) were housed and handled in accordance with the Association for Research in Vision and Ophthalmology’s Statement for the use of Animals in Ophthalmic and Visual Research. Animals were anesthetized prior to intravitreal injection with intramuscular ketamine (Phoenix Scientific Inc, St Joseph, Mo) (20 mg/kg of body weight) and xylazine (Bayer Corporation) (1 mg/kg of body weight) and 0.5% topical proparacaine hydrochloride (Allergan, Hingham, Mass) and mydriacyl (Akorn Inc, and 5% povidone iodine (Alcon Laboratories, Inc, Fort Worth, Tex) was used for asepsis. Preliminary studies indicated that an intravitreal injection of pooled toxin containing 330 µg of protein at concentrations of 6.6 g/L or 66 g/L created reproducible intraocular inflammation with a red reflex but no ophthalmoscopically detectable fundus details. We chose this pathologic outcome because it closely mirrors the degree of inflammation reported in a rabbit model of S aureus endophthalmitis involving live bacteria.12 We also wanted to create a model of intraocular inflammation similar to what is typically seen in a clinical case of S aureus endophthalmitis in which the visual acuity is counting fingers to hand motions.

Rabbits were divided into 3 groups, each with 6 experimental and 6 control animals, in which the timing of the intravitreal injections was varied. In group 1 (simultaneous), pooled toxin and immunoglobulin were mixed and delivered simultaneously; in group 2 (sequential), pooled toxin and immunoglobulin were delivered sequentially with immunoglobulin injected immediately after pooled toxin; and in group 3 (delayed), pooled toxin and immunoglobulin were delivered sequentially with immunoglobulin injected 6 hours after pooled toxin. Group 1 data consisted of 2 sets of 3 experimental and 3 control rabbits evaluated at separate times. The entire protocol was identical except that rabbits from the first set were euthanized on postinjection day 7 and those from the second set on postinjection day 9. Groups 2 and 3 consisted of 6 experimental and 6 control animals evaluated concurrently, and all were euthanized on postinjection day 9.

The volume of immunoglobulin used in this study was determined by 2 competing factors regarding the nonvitrectomized eye. We wanted to inject the largest volume of immunoglobulin possible to maximize the probability of immunoglobulin and toxin interaction yet were limited to volumes less than 250 µL because of associated intraocular pressure elevation. We determined that 200 µL of aqueous humor could be safely aspirated from the anterior chamber without a risk of lens or iris damage. We therefore chose an immunoglobulin volume of 145 µL and adjusted our other parameters. Prior to intravitreal injection, 150 µL (group 1) or 200 µL (groups 2 and 3) of aqueous humor was aspirated with a tuber-
The treated eyes were enucleated and fixed in 10% formalin for tobarbital sodium (25 mg/kg) on postinjection day 7 or 9, and Animals were euthanized with an intracardiac injection of pen-

In both the experimental and control groups. The mean score for each parameter was determined for the 6 examina-
tions in examiner availability, examinations for group 3 (delayed) were performed on postinjection days 1, 3, 5, and 8. Because of limita-
tions (repetitive) and group 2 (sequential), examinations were per-
formed on postinjection days 2, 4, 6, and 9. At each time point, we examined 6 experimental and 6 control eyes except for postinjection day 8 in group 1, for which there were only 3 experimental and 3 control eyes. The anterior chamber reaction and fundus reflex were graded for evidence of ocular inflammation using an adaptation of a grading scale of 0 to 4, as reported by others (Table 1). The mean score for each parameter was determined for the 6 eyes in each group, and control and experimental groups were analyzed for statistically significant differences (P<.05) using the Mann-Whitney test at each time point except for the final day in group 1 (simultaneous), for which there were only 3 eyes in both the experimental and control groups.

HISTOPATHOLOGIC FEATURES

Animals were euthanized with an intracardiac injection of pentobarbital sodium (25 mg/kg) on postinjection day 7 or 9, and the treated eyes were enucleated and fixed in 10% formalin for histopathologic analysis. Eyes were embedded in paraffin, sectioned, and stained with hematoxylin-eosin according to standard protocols. Sections were examined and scored by an investigator masked to the identity of the treatment group. Each eye received scores for 4 tissues using grading scales for severity changes adapted from studies by other investigators.10,27 The cornea, anterior chamber, and vitreous each received a single score of 0 to 3 (Table 2). For the retina, a template was used to divide each retinal section into 6 regions, each of which was graded separately using a scale of 0 to 4 (Table 2). A single retin-
al score per eye was obtained by taking the mean of the 6 regional scores. The Mann-Whitney test was used to determine statistically significant differences (P<.05) between the experimental and control groups.

Table 1. Clinical Grading Scale for Severity of Ocular Inflammation*

<table>
<thead>
<tr>
<th>Grade</th>
<th>Anterior Chamber</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5-10 Cells per field</td>
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</tr>
<tr>
<td>2</td>
<td>10-20 Cells per field</td>
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<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>&gt;50 Cells per field</td>
<td></td>
</tr>
<tr>
<td>Fundus Reflex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Slightly diminished</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Diminished</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderately diminished</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>White</td>
<td></td>
</tr>
</tbody>
</table>

*Based on the scale used by Callegan et al.10

Table 2. Histologic Grading Scale for Severity of Ocular Inflammation*

<table>
<thead>
<tr>
<th>Grade</th>
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<tr>
<td>1</td>
<td>Partial-thickness infiltration of inflammatory cells</td>
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<tr>
<td>2</td>
<td>Segmental full-thickness infiltration of inflammatory cells</td>
</tr>
<tr>
<td>3</td>
<td>Total full-thickness infiltration of inflammatory cells</td>
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<table>
<thead>
<tr>
<th>Anterior Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
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<table>
<thead>
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<tbody>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
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</table>

<table>
<thead>
<tr>
<th>Retina</th>
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</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

*Based on the scales used by Callegan et al10 and Beecher et al.27

RESULTS

REACTIVITY OF IMMUNOGLOBULIN WITH S AUREUS PRODUCTS

When immunoglobulin was used as a primary antibody for Western blot analysis, we observed reactivity with numer-
ous proteins in the pooled toxin, including a promi-

In Western blot analysis, we observed reactivity with numerous proteins in the pooled toxin, including a prominent unidentified high-molecular-weight protein (Figure 1). The absence of comigrating bands in the control beef heart extract medium suggests that the immuno-
reactive proteins in the pooled toxin are bacterial products. To determine whether the immunoglobulin could react with any known S aureus toxins, which may con-


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nal, β-hemolysin and TSST-1 were purified from bacterial supernatants and blotted with immunoglobulin. As shown in Figure 1, immunoglobulin demonstrates reactivity with both β-hemolysin and TSST-1.

CLINICAL EXAMINATION

Pooled Toxin Alone

Intravitreal injection of pooled toxin alone produced a grade 3 to 4 anterior chamber reaction on postinjection day 1. This reaction declined until it resolved by days 5 to 7. The fundus reflex was diminished in all eyes by postinjection day 1. Despite the obscuration of most retinal details in all eyes on postinjection day 1, intraretinal hemorrhages could be observed in some. During the first 5 postinjection days, the fundus reflex worsened in most eyes, at which point it plateaued and remained diminished at approximately the same level on postinjection days 5 through 9. The initial decline in the reflex was attributed to vitreal inflammation. To a variable extent, a white membrane would form on the posterior surface of the lens by postinjection day 2, continue to proliferate from postinjection days 2 through 4, and then stabilize. This membrane would continue to mature even though anterior segment inflammation was resolving, and it prevented adequate evaluation of the posterior segment. The fundus reflex appearance on postinjection day 7 is illustrated in Figure 2A, and scores for the fundus reflex during the 8- to 9-day course are shown in Figure 3 (broken lines).

Pooled Toxin and Simultaneous Injection of Immunoglobulin

When pooled toxin was mixed with immunoglobulin prior to injection, an anterior chamber reaction was seen in only 3 of 6 eyes and was limited to grade 1. The fundus reflex (Figure 2B) was essentially normal throughout the course, and retinal details were easily seen. Fundus reflex scores are graphed in Figure 3A. Compared with eyes receiving pooled toxin and balanced salt solution, the mean fundus reflex scores for eyes receiving immunoglobulin were significantly lower (Figure 3A) at all time points.

Pooled Toxin and Sequential Injection of Immunoglobulin

When immunoglobulin was injected immediately following pooled toxin, the expected inflammatory response was attenuated compared with eyes receiving pooled toxin alone (Figure 3B). The difference in the fundus reflex scores between the 2 groups became greater

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**Figure 1.** Western blot of *Staphylococcus aureus* culture supernatant (pooled toxin) and purified exotoxins. Nitrocellulose membranes were probed and then visualized with the Enhanced Chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Primary antibody: 1:10,000 dilution of 10% Gamimune N (Bayer Corporation, Pittsburgh, PA). Secondary antibody: donkey antihuman. Lanes 1 and 2: toxic shock syndrome toxin-1 (TSST-1), 0.2 µg and 0.5 µg. Lanes 3 and 4: β-hemolysin, 0.2 µg and 1.25 µg. Lanes 5 and 6: Beef heart extract medium. Lanes 7 and 8: concentrated culture supernatant (pooled toxin), 0.5 µg and 1.0 µg. Molecular weight markers (Bio-Rad, Hercules, Calif) indicated on the right are serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Solid arrow indicates β-hemolysin; open arrow, TSST-1.

**Figure 2.** Fundus reflex of rabbit eyes 7 days after pooled toxin injection, without (A) or with (B) simultaneous immunoglobulin.
during the 9-day course and could be attributed to a worsening of the fundus reflex in the eyes not receiving immunoglobulin. The differences were statistically significant at all but the second time point. Compared with eyes receiving simultaneous injections of pooled toxin and immunoglobulin, the fundus scores for eyes receiving sequential injections were higher (ie, more diminished), particularly at earlier time points (Figure 3A and B).

**Pooled Toxin and Delayed Injection of Immunoglobulin**

When the injection of immunoglobulin was delayed 6 hours, only a slight attenuation of the expected inflammatory response was observed. The fundus reflex was diminished more than in eyes receiving simultaneous or sequential injections of immunoglobulin (Figure 3A, B, and C). Despite the diminished reflex, fundus scores were significantly lower at all but the first time point when compared with eyes not receiving immunoglobulin (Figure 3C). Retinal details were obscured throughout the course.

**HISTOPATHOLOGIC EXAMINATION**

**Pooled Toxin Alone**

We found essentially no inflammatory response in the cornea and anterior chamber of these eyes (Figure 4). The vitreous cavity was partially filled with inflammatory cells. Some eyes had abscesses, whereas others did not. Full-thickness retinal disruption was evident with ganglion cell loss, increased vacuolation of the inner nuclear layers, complete loss of photoreceptors, and choroidal thickening observed in most sections (Figure 5A). We noted interspersed areas of focal retinal thinning due to the loss of cellular elements.

**Pooled Toxin and Simultaneous Injection of Immunoglobulin**

In these eyes, we noted a mild inflammatory response in the vitreous cavity. Compared with eyes receiving pooled toxin alone, we observed a marked preservation of retinal architecture with the presence of distinct layers composed of intact cells (Figure 5B). In some sections, vacuolation of the inner nuclear layers and choroidal thickening were observed.

**Pooled Toxin and Sequential Injection of Immunoglobulin**

When immunoglobulin was injected immediately following pooled toxin, we noted an attenuation of the expected histologic response in the retina but not in the vitreous (Figure 4B). Compared with eyes receiving pooled toxin simultaneously with immunoglobulin, the retinal architecture was slightly more disrupted but significantly less than in eyes not receiving immunoglobulin. Focal areas of inner nuclear layer vacuolation, disruption of the ganglion cell layer, retinal edema, photoreceptor loss, and choroidal thickening were noted. We observed other sections with preserved retinal architecture.

**Pooled Toxin and Delayed Injection of Immunoglobulin**

When immunoglobulin was injected 6 hours after pooled toxin, the histologic appearance of all tissues was indistinguishable from eyes receiving pooled toxin alone (Figure 3D).
photoreceptors, and choroidal thickening. As in eyes receiving pooled toxin alone, some sections contained focal areas of retinal thinning due to cell loss.

Staphylococcus aureus endophthalmitis often results in poor visual outcomes with only 50% of patients achieving a final visual acuity of 20/100 or better in the affected eye. Animal studies suggest that poor outcomes are related to secreted bacterial products that are toxic to the retina, are highly inflammatory, and can induce damage similar to that seen with a natural infection. Although cell wall components produce significant intraocular inflammation in this same model, retinal function does not appear to be significantly altered. The fact that retinal damage appears to be secondary to direct toxin effects instead of a host inflammatory response likely explains the lack of a beneficial effect from the adjuvant use of corticosteroids in the treatment of experimental S aureus endophthalmitis. In contrast to the rapid tissue destruction that occurs with intraocular Bacillus cereus infections, the natural history of S aureus endophthalmitis may allow the timely introduction of appropriate antitoxin therapy.

The potential role of antibody therapy in the treatment of ocular disease has been evaluated in the case of a monoclonal antibody as adjuvant therapy for cytomegaloviral retinitis as well as for the neovascular form of macular degeneration. Because the pathogenicity of S aureus endophthalmitis is complex, likely involving numerous exotoxins and regulatory genes and gene products, attempting to treat endophthalmitis by targeting specific bacterial products may prove elusive. We hypothesized that a commercially available pooled human immunoglobulin product might prove to be an effective adjuvant therapy in the treatment of S aureus endophthalmitis. A pooled product offers the potential benefits of targeting numerous exoproteins simultaneously, being widely available, and being approved for human use.

Our model of using pooled toxins extracted from S aureus culture supernatant to induce toxin-mediated endophthalmitis eliminated several experimental variables that would likely occur in a model involving the inoculation of live bacteria, such as the rate and quantity of toxin elaboration, bacterial growth, and antibiotic effect. Such variables would potentially make the evaluation of toxin neutralization by immunoglobulin difficult. The validity of our model as it relates to clinical endophthalmitis is supported by substantial evidence that toxins isolated from complex growth media are biologically active and are functionally involved in many clinical disease states.

The mixing of pooled immunoglobulin with pooled toxin appears to confer neutralization of its toxic effects. In this study, we found that if a volume of immunoglobulin that could be used clinically was allowed to preincubate for as little as 5 minutes with a volume of pooled toxin known to induce significant intraocular inflammation and retinal damage, the pooled toxin was rendered essentially free of such effects according to clinical and histologic measures. The mechanism whereby immunoglobulin is able to render pooled toxin ineffective is unknown,
but we suspect that it is related to the direct binding of antibody to toxin. Similar findings are noted in the case of experimental \textit{B. cereus} endophthalmitis, in which other researchers have demonstrated that specific antibodies against hemolysin, a known \textit{B. cereus} exotoxin, can attenuate the toxin’s effects.27

In our study using Western blot technique, we were able to demonstrate that immunoglobulin could bind purified toxins as well as numerous proteins from \textit{S. aureus} strain RN4220 culture supernatant. These findings suggest that specific antibodies present in commercially available pooled immunoglobulin are capable of binding bacterial products, thus providing a biochemical basis for the attenuated clinical and histologic effect observed when immunoglobulin and pooled toxin interact. Although \textit{S. aureus} RN4220 is a modified laboratory strain that may prove to be more or less virulent than other \textit{S. aureus} strains, it has been well studied and can be manipulated to produce different toxins, thus providing a framework for further study of the concept discussed in this article.

The pharmacokinetics of immunoglobulin remain unknown but certainly influence the therapeutic effects of immunoglobulin in this model. When immunoglobulin was injected immediately following pooled toxin, the toxic effects of the latter substance were attenuated clinically and histologically but less so when compared with eyes receiving pooled toxin simultaneously injected with immunoglobulin. This observation would suggest that optimizing the mixing of the two in vivo might facilitate the treatment effect.

The rapidity with which immunoglobulin is capable of inactivating the biological activity of pooled toxin would suggest that its administration during a critical phase of bacterial toxin production could ameliorate the destructive effects of the toxins. An opportunity for intervention in \textit{S. aureus} endophthalmitis stems from the fact that significant toxin production typically does not occur until the postexponential bacterial growth phase.15 In a rabbit model of endophthalmitis produced by the intravitreal injection of \textit{S. aureus}, bacteria grew exponentially during the first 24 hours.12 Intraocular inflammation was observed at 24 to 48 hours postinjection, whereas attenuation of the electroretinographic recording did not begin until 48 hours postinjection.12 Thus, treatment that occurs within or near this time frame may favorably influence the course of endophthalmitis. The importance of timely intervention prior to the bacterial release of increasingly toxic doses of exotoxins is also suggested by our study. We saw little difference clinically and none histologically when immunoglobulin injection was delayed 6 hours following pooled toxin injection, most likely indicative of the rapidity of tissue destruction from abrupt exposure to a suprathereshold dose of toxin. Because clinical endophthalmitis is probably associated with a gradually increasing concentration of a variety of toxins rather than a bolus dose, we believe that the concept of toxin neutralization early in the course of endophthalmitis retains merit.

The role of immunoglobulin in the clinical treatment of endophthalmitis needs further investigation examining factors such as the interaction of ocular tissues with immunoglobulin-bound toxin, efficacy of immunoglobulin in bacterial models of endophthalmitis, ocular immune host response, immune complex formation, interaction of immunoglobulin with antibiotics, effect of vitrectomy, timing of intervention, and role of hyperimmune immunoglobulin (toxin-specific antibody). Our study provides preliminary support of antibody-mediated antitoxin therapy for bacterial endophthalmitis. We believe that pooled human immunoglobulin may represent a novel adjunctive therapy in the treatment of \textit{S. aureus} endophthalmitis.

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Figure 5. Histopathologic features of rabbit eyes 9 days after pooled toxin injection, without (A) or with (B) immunoglobulin (hematoxylin-eosin, original magnification ×125 [A] or ×82.5 [B]).
REFERENCES


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