Methotrexate Reduces the Complications of Endophthalmitis Resulting from Intravitreal Injection Compared with Dexamethasone in a Rabbit Model

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Purpose. The incidence of infectious endophthalmitis associated with intravitreal injection (IVI) of steroid has been reported to be as high as 0.87%. This study was designed to investigate whether the antimicrobial activity of intravitreal methotrexate (MTX) alters the incidence or course of bacterial endophthalmitis associated with IVI in a rabbit model.

Methods. A rabbit model of endophthalmitis induced by Staphylococcus epidermidis (S) was established. Six groups of rabbits had IVI of sterile balanced salt solution (BSS), MTX (400 μg), dexamethasone (Dex, 200 μg), S, S and MTX (S-MTX), or S and dexamethasone (S-Dex). On days 0, 1, 3, 6, 10, and 14, total intraocular inflammation was measured in each animal. Vitritis was graded by the degree of vitreal haze. An intravitreal tap was performed on two animals from groups S, S-Dex, and S-MTX. A histopathologic study was performed on day 14.

Results. No endophthalmitis was observed in the control groups BSS, MTX, and Dex. Group S-Dex had the highest and group S-MTX had the least total intraocular inflammation and vitritis scores from days 3 to 14. The difference in total inflammation and vitritis among groups S, S-Dex, and S-MTX is significant (P = 0.046 and P = 0.001, respectively). Live bacteria were isolated only from groups S and S-Dex. Pathology revealed severe ocular destruction in groups S and S-Dex and intact structures in group S-MTX.

Conclusions. MTX appears to reduce the risk of development of bacterial endophthalmitis and ocular destruction associated with IVI compared with Dex. IVI of MTX may be a safer alternative than steroid injection in treating noninfectious uveitis. (Invest Ophthalmol Vis Sci. 2006;47:1516–1521) DOI: 10.1167/iovs.05-0880

Methotrexate (MTX) is a competitive inhibitor of dihydrofolate reductase. Systemic MTX has been included in many standard treatments for a variety of malignancies, including acute lymphocytic leukemia, non-Hodgkin’s lymphoma, osteosarcoma, breast cancer, and choriocarcinoma.1 It is also a commonly used steroid-sparing agent for the treatment of systemic inflammatory disorders, such as systemic lupus erythematosus, rheumatoid arthritis, and psoriasis and a range of non-infectious uveitides.2–5 A recent study documented a 76% control of inflammation and a 56% corticosteroid-sparing effect in patients with various ocular inflammatory conditions treated with MTX.6 Systemic MTX carries well-known serious toxic effects that include cytopenia, hepatotoxicity, and interstitial pneumonitis. In that study, MTX was discontinued in 18% of patients due to such side effects. In addition, systemic administration of the drug may not reach therapeutic levels in the eye because of the blood–ocular barrier.

Local or organ-specific administration of drug is desirable because of the potential to reduce or eliminate systemic toxicities and to improve therapeutic efficacy by ensuring organ-specific drug concentration. Intravitreal injection [IVI] of MTX has been shown to be clinically safe and effective in treating ocular lymphoma that is refractive to systemic chemotherapy and radiation.7 Concentration at 400 μg of MTX in human eyes appears to be clinically well tolerated.8,9 Velez et al.10 have shown that at this concentration, intravitreal MTX is nontoxic by ERG study and remains at therapeutic levels in rabbit eyes for 48 hours. Preliminary data have demonstrated that intravitreal MTX appeared to reduce inflammation effectively in a rabbit model of S-antigen-induced uveitis (Civelek ML et al. IOVS 2004;45:ARVO E-Abstract 557). Intravitreal MTX has not been reported to induce ocular hypertension, which is a frequent complication of intravitreal triamcinolone. Its use for uveitis has not yet been studied in humans, largely due to its unknown long-term safety and toxicity profile.

IVI of steroids has been the only intraocular medication successfully treating refractory cytokid macular edema from noninfectious uveitis, diabetes, central retinal vein occlusion, and pseudophakia.11–16 Its use has increased dramatically in recent years. Side effects of intraocular steroid include elevation of intraocular pressure,17 development or progression of cataract, and sterile and infectious endophthalmitis.18,19 A major safety concern related to intravitreal injection is the development of infectious endophthalmitis. A recent multicenter retrospective study has shown that the rate of bacterial endophthalmitis associated with intravitreal triamcinolone treatment was 0.87%.20 This is higher than that seen with cataract surgery, which was recently reported to be 0.215%.21 The most common pathogens isolated from postcataract endophthalmitis are Staphylococcus epidermidis and Staphylococcus aureus,22 which also have been isolated from endophthalmitis as a complication from intravitreal injections. Of interest is that MTX has been shown to have antimicrobial activity against many common pathogens in vitro, such as S. aureus,23,24 Streptococcus pneumoniae, Streptococcus pyogenes,23 and Pneumocystis carinii.25 In this article, the in vitro study was designed to investigate whether MTX has anti-S. epidermidis activity in vitro and whether this anti-S. epidermidis activity can reduce the risk of endophthalmitis in vivo, and therefore result in a safer complication profile resulting from intravitreal injection of bacteria in a rabbit model. To replicate...
the clinical setting closely, the most common pathogen from postoperative endophthalmitis, *S. epidermidis*, was selected for use in this study.

### Materials and Methods

#### Bacterial Preparation and Antimicrobial Activity Against *S. epidermidis* In Vitro

*S. epidermidis* (ATCC 12228), obtained from the American Type Culture Collection (Manassas, VA), was stored at −80°C and underwent three subcultures on agar plates (Remel, Lenexa, KS) before susceptibility testing and inoculation of the animals. The minimum inhibitory concentration (MIC) of methotrexate (methotrexate LPF sodium; Xanodyne Pharmaceutical Inc., Florence, KY) against this organism was determined by the microbroth dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards. The methotrexate was diluted with Mueller-Hinton broth (Difco, Detroit, MI) supplemented with 22.5 mg/L calcium and 11.25 mg/L magnesium (csMHB) to obtain MIC test concentrations ranging from 0.06 to 64 μg/mL. An inoculum of the *S. epidermidis* strain was prepared by direct suspension and adjusted with sterile saline until the turbidity matched a 0.5 McFarland standard, using a spectrophotometer at 625 nm. The suspension was further diluted in csMHB to obtain a final inoculum of approximately 10^5 CFU/mL. In addition to the standard inoculum, a series of 1:10 dilutions in MHB were performed to obtain suspensions of approximately 10^4, 10^3, and 10^2 CFU/mL. The exact inoculum size of each bacterial suspension was determined by colony counts. MIC microtiter plates were set up in duplicate and were read after 20 hours of incubation at 35°C. The MIC was defined as the lowest concentration at which there was no visible growth.

Preparations of bacteria for intravitreal injection were done as follows: A suspension of *S. epidermidis* was washed once in 10 mL of sterile irrigating physiologic saline (BSS; Alcon Laboratories Inc., Fort Worth, TX) using nonpyrogenic polypropylene tubes. The organism was resuspended in the BSS to an absorbance of 0.15 on a spectrophotometer at 625 nm, which would give a density of bacteria at 10^7 CFU/mL. Further dilution in BSS was made to obtain the desired concentration of bacteria for intravitreal injection. The final dilution was replated on blood agar to confirm the actual CFU.

### Establishing an In Vivo Experimental Model of *S. epidermidis* Endophthalmitis

New Zealand albino rabbits weighing 2 to 3.5 kg were obtained from New Franken (New Franken, WI) and housed at the University of Illinois Biological Research Laboratory. All animal handlings were approved by the Biological Research Laboratory and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was induced by intramuscular injection of a 50/50 mixture of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg). Pupillary dilation was achieved with 1 drop each of tropicamide 1% and phenylephrine 2.5%. Fundus examination was performed in all rabbits before injection, to rule out any baseline retinal disease. All intravitreal injections were performed approximately 2 mm posterior to the limbus with a 27-gauge needle. To maintain normal IOP, we performed paracentesis of the anterior chamber before the intravitreal injection. The left eye was used as an undisturbed control, and two eyes were injected with BSS as a control for the injection. Different amounts of bacteria ranging from 10 to 5000 CFU were injected into the vitreous cavity to determine the optimal CFU that was needed to create endophthalmitis reliably. Animals were evaluated on days 0, 1, 3, 6, 10, and 14. Ocular inflammation was graded based on a modified ophthalmic grading system from Pleyer et al. as described in Table 1. All animals were euthanized by intracardiac injection of 5 mL of 2% lidocaine.

### Effect of Intravitreal MTX on the Course of *S. epidermidis* Endophthalmitis

Anesthesia was achieved as described in the prior section, and baseline fundus examination was performed in all animals before intravitreal injection. Six groups of animals (total, 39), were used. Only the right eye of each animal received intravitreal injection with the appropriate substance(s) as depicted in Table 2. The left eye was used as a control.

In groups *S. epidermidis* (S)-MTX and S-dexamethasone (Dex), 50 μL of BSS containing 18 CFU bacteria, as determined by plating of final dilution on blood agar, was injected into the vitreal cavity, as described in the prior section, followed by injection of 50 μL of BSS containing either 400 μg of MTX or 200 μg of Dex at the same site. Therefore, the total volume of intravitreal injection was kept at 100 μL per eye in all groups. Again, all animals were examined on days 1, 5, 6, 10, and 14. A clinical score was given to each eye as described earlier. Vitreous tap was performed on two animals with significant vitritis from groups S, S-Dex, and S-MTX on day 6. The recovered vitreal samples were plated.
Histology

One animal from groups BSS, MTX, and Dex, and two animals from groups S, S-Dex, S-MTX were euthanatized and then enucleated after eye examination on day 14. The globes were fixed in 10% buffered formaldehyde for 96 hours. All eyes were sectioned through the optic nerve and any gross abnormalities were recorded. Specimens were stained with hematoxylin and eosin (H&E) and examined by microscope.

Data Analysis

The sum of clinical scores at each observation interval from all animals in each group were compiled. Average score ± standard errors at each observation interval were calculated for each group at each observation point. The data were analyzed with the analysis of variance method for repeated measures data (SAS software; SAS, Inc., Cary, NC). The model includes terms for treatment groups, study days, and an interaction term between groups and days, to test for an overall profile difference among the three groups was tested on days 3, 6, 10, and 14 separately. The model also adjusts for possible correlation of the repeated measures in the same animal over time. Pair-wise difference in response among the three groups was tested on days 3, 6, 10, and 14 separately. The Bonferroni procedure was applied to adjust the probabilities for the multiple comparisons made on each day (i.e., the adjusted probability on each day for each comparison was calculated as the original probability multiplied by 3). Then this probability was compared to the multiple comparisons made on each day (i.e., the adjusted probability on each day for each comparison was calculated as the original probability multiplied by 3). Then this probability was compared to the probability on each day for each comparison was calculated as the original probability multiplied by 3). Then this probability was compared to

Effect of Intravitreal MTX on the Course of *S. epidermidis*-Induced Endophthalmitis

Mild conjunctival inflammation was noted at the injection site in all groups on day 1, which was completely resolved by day 6. No vitritis or anterior chamber reaction was observed in any of the three control groups (BSS, MTX, or Dex), indicating that intraocular MTX and Dex, like BSS, alone did not elicit intraocular inflammation. A representative fundus photograph of an eye that was injected with Dex alone is shown in Figure 2 (Dex). Eyes injected with BSS or MTX alone showed a similar picture (data not shown). All animals in group S-Dex (100%) and seven (87.5%) of eight animals in the S group had vitritis develop, whereas it developed in only five (56%) of nine animals in group S-MTX, as shown in Figure 3. The likelihood of the development of endophthalmitis was compared among these three groups. As shown in Table 4, the presence or absence of Dex did not alter the risk of development of infection (S-Dex versus S, *P* = 1.00). However, MTX appeared to provide some protection from infection compared with Dex (*P* = 0.08, Fisher two-tailed test). Two of the five animals in...
group S-MTX, none in group S, and 1 in group S-Dex that developed endophthalmitis recovered from the infection as shown in Figure 3.

The course of endophthalmitis showed a similar trend among all three experimental groups S, S-MTX, and S-Dex. It peaked on days 3 to 6 and then slowly declined (Fig. 1). The total inflammation (i.e., the average score of the sum of conjunctival inflammation, anterior chamber reaction, and vitritis of each animal) was compared among all groups (Fig. 1). Group S-Dex had the most inflammation, followed by group S, and group S-MTX had the least inflammation from days 3 to 14. The differences in total inflammation among these three experimental groups were statistically significant from days 3 to 14 ($P = 0.046$, analysis of variance). The total inflammation in group S-MTX was significantly lower than that in group S-Dex on days 3, 6, and 14 by pair-wise comparison (adjusted $P < 0.05$, Bonferroni procedure). The difference between groups S and S-MTX, was significant on day 6 only (adjusted $P = 0.02$) and between S and S-Dex on day 3 only (adjusted $P = 0.04$), most likely because of the small sample size.

Vitritis is another measure of the infection process. When the degree of vitritis was compared among all three experimental groups, a similar trend was observed. The severity of vitritis reached a maximum on day 6 and then slowly improved. From days 3 to 14, group S-Dex had the highest vitritis score, followed by group S, and group S-MTX had the lowest (Fig. 4). Again, the degree of vitritis among these three groups was significantly different from days 3 to 14 ($P = 0.001$, analysis of variance). The difference between groups S-Dex and S-MTX was statistically significant by pair-wise comparison on days 3, 6, and 14 (adjusted $P < 0.05$, Bonferroni procedure). However, the difference between groups S and S-Dex and between S and S-MTX was only significant on day 3 ($P = 0.002$ and $P = 0.014$, respectively). Two of five animals in which vitritis developed in group S-MTX recovered completely on day 14, by clinical examination. Representative fundus photographs of one animal from each of these groups are shown in Figure 2.

Vitreous taps were performed on two animals with significant vitritis (score, $\geq 2.5$) from each of the three experimental groups on day 6. Cultures of the vitreal samples on blood agar recovered viable bacteria from both animals in groups S and S-Dex, but from neither animal in group S-MTX (Table 5). Vitreous tap was also performed on the animal in group S-Dex, in which extremely severe endophthalmitis and orbital cellulitis developed. The morphology of the colonies was distinct from that of $S. \text{epidermidis}$. They were grayish and mucoid, instead of the typical white, rounded colony of $S. \text{epidermidis}$. This pathogen was identified as non-$S. \text{epidermidis}$. Further isolation of the pathogen was attempted but was not successful.

Histopathologic studies were performed on two animals from each group on day 14. Neither Dex nor MTX alone induced microscopic structural changes (Fig. 5). Vitritis, large vitreal abscesses, and extensive retinal necrosis were seen in groups S and S-Dex (Fig. 5). In comparison, the eye from group S-MTX which had grade 3 vitritis on day 6 improved significantly and showed only mild pars planitis and optic neuritis (not shown) on day 14 (Fig. 5). No retinal necrosis was seen, and the retinal structures were largely intact. The other eye from the S-MTX group show did not show any inflammation in all structures (data not shown).

**DISCUSSION**

This is the first study to demonstrate that MTX has both in vitro and in vivo anti-$S. \text{epidermidis}$ activity. $S. \text{epidermidis}$-induced endophthalmitis associated with intravitreal injection was established in a rabbit model of this study. $S. \text{epidermidis}$ generally is known to induce a milder and less acute form of endophthalmitis in human and animal models, compared with $S. \text{aureus}$. In our study, only 10 to 20 CFU of live bacteria was sufficient to induce significant infection and inflammation. In some of the published animal studies that used the same strain of bacterium, a larger number of bacteria, 7,000 to 10,000 CFU, was used to induce endophthalmitis. $27$ We used the colony counts from the plating of the final dilution on blood agar rather than the calculated CFU from the serial dilution. We found that there could be up to a 10-fold difference between these two counts, the calculated one always being higher.

**TABLE 4.** Comparison of the Likelihood of Development of Endophthalmitis on Day 14

<table>
<thead>
<tr>
<th>Groups</th>
<th>Animals with Endophthalmitis (%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S vs. S-Dex</td>
<td>87.5 vs. 100</td>
<td>1.00</td>
</tr>
<tr>
<td>S vs. S-MTX</td>
<td>87.5 vs. 56</td>
<td>0.29</td>
</tr>
<tr>
<td>S-Dex vs. S-MTX</td>
<td>100 vs. 56</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Fisher exact test, two-tailed.

**TABLE 5.** Presence of Viable Bacteria from the Vitreal Taps on Day 6

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vitritis Scores</th>
<th>Bacteria in Vitreous</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>3</td>
<td>Present</td>
</tr>
<tr>
<td>S</td>
<td>4</td>
<td>Present</td>
</tr>
<tr>
<td>S-Dex</td>
<td>4</td>
<td>Present</td>
</tr>
<tr>
<td>S-Dex</td>
<td>4</td>
<td>Present</td>
</tr>
<tr>
<td>S-MTX</td>
<td>4</td>
<td>Absent</td>
</tr>
<tr>
<td>S-MTX</td>
<td>2.5</td>
<td>Absent</td>
</tr>
</tbody>
</table>

CONTROL GROUPS INCLUDE GROUPS BSS, MTX, AND DEX. THE DIFFERENCE AMONG GROUPS S, S-MTX, AND S-DEX WAS SIGNIFICANT FROM DAYS 3 TO 14 ($P = 0.01$). *SIGNIFICANT DIFFERENCE IN A PAIR-WISE COMPARISON BETWEEN GROUPS S-DEX AND S-MTX (ADJUSTED $P < 0.05$) ON THE SPECIFIC OBSERVATION DAY.

**FIGURE 3.** Number of animals in which vitritis developed in groups S, S-Dex, and S-MTX.

**FIGURE 4.** Comparison of vitritis per eye in each group. Controls include groups BSS, MTX, and Dex. The difference among groups S, S-Dex, and S-MTX is significant from days 3 to 14 ($P = 0.01$). *Significant difference in a pair-wise comparison between groups S-Dex and S-MTX (adjusted $P < 0.05$) on the specific observation day.
Whatever the difference in the amount of bacteria used in our study, the course of endophthalmitis was the same as in other studies. It reached a maximum within 7 days and then slowly improved. However, the possibility that the severity of the infection was slightly milder in our study is not excluded.

The antimicrobial activity of MTX appeared to prevent the development and lessen the severity of infection. Endophthalmitis developed in only 56% of eyes in group S-MTX compared with 100% in group S-Dex. Among the eyes in which endophthalmitis developed, 40% recovered completely from the infection in the presence of MTX, whereas only 11% did so in the presence of Dex and 0% in group S. This was probably achieved by inhibition of bacterial growth by MTX in the vitreous cavity. The observation that no viable bacteria were recovered from the vitreous cavity in the S-MTX group supports this notion. The actual mechanism of antimicrobial activity of MTX is not entirely clear. It is a synthetic analogue of folic acid that binds to dihydrofolate reductase and inhibits DNA synthesis in eukaryocytes. It has been postulated that a similar mechanism may account for its anti-Staphylococcus activity. Further study is needed to confirm this hypothesis.

MTX modulates inflammation by two mechanisms: inhibition of DNA synthesis and therefore cell proliferation, and release of adenosine into extracellular matrix. Binding of adenosine to its receptor inhibits polymorphonuclear leukocyte chemotaxis and secretion of TNF-α, IL-8, and IL-6 by monocytes. The anti-inflammatory activity of MTX can reduce the immune response that is associated with infection. It is believed that ocular damage resulting from endophthalmitis is primarily a result of bystander effects from the intense inflammatory response against pathogens. Hence, less vigorous inflammation would lead to less ocular damage. It is possible that both the antimicrobial and anti-inflammatory effects of MTX work in concert to eliminate the infection, and at the same time, reduce the associated inflammation. Our clinical observations and the results of our histopathologic studies support this hypothesis. Group S-MTX had the lowest inflammation scores, and the ocular structures remained largely intact in this group, whereas extensive retinal necrosis was seen in groups S and S-Dex.

The presence of Dex induced a more severe course of endophthalmitis in our study. A similar observation was made in a previous study in humans. Intraocular steroids appeared to complicate the recovery from postoperative bacterial endophthalmitis. However, the underlying mechanism is unclear. Steroids have a complex mechanism of anti-inflammatory activity that acts on many different intracellular pathways. One of them is inhibition of NF-κB and AP1 signaling pathways, which are crucial to the regulation of cytokines and adhesion molecules that modulate the activation and function of inflammatory cells. It is possible that with the initial immunosuppression of inflammatory cells, the intraocular bacteria continue to grow unchecked. An increased pathogen load in turn elicits a much larger immune response. The effects of a more substantial infection and the associated increase in inflammation would result in more tissue damage. This hypothesis must be confirmed in future studies. Of note, the use of steroid in treating infectious endophthalmitis is still controversial due to the conflicting data from animal and human studies. Our study indicates that introduction of steroid when there are viable pathogens worsens the course of infection. However, introduction of steroid after pathogens are cleared could reduce the immune response and diminish the bystander ocular destruction. Therefore, the timing of steroid treatment in infectious endophthalmitis could be the most crucial factor.

The relatively high incidence of infectious endophthalmitis from intravitreal steroid injections is troubling. This procedure is used mostly in the outpatient office setting. The fact that a more recent retrospective study showed a lower incidence of infectious endophthalmitis from intravitreal injection of triamcinolone from one medical center indicates that sterile, aseptic environment and techniques play a role. It is likely that the incidence of infection is between 0.10% to 0.87%. Nevertheless, our study, along with studies from others, indicate that endophthalmitis in the presence of steroids can result in a more severe infection course and ocular damage. The search for an alternative with a safer complication profile is warranted.

In summary, our study has demonstrated that MTX has both in vitro and in vivo antimicrobial activity. This unique property appears to prevent the development of bacterial endophthalmitis associated with intravitreal injection and to reduce the severity of the associated ocular inflammation and hence the subsequent ocular destruction. Intravitreal MTX may be a safer option than intravitreal steroids for the local treatment of refractory uveitis.

**Acknowledgments**

The authors thank M. Young for help with the statistical analyses, photographer Mark Janowicz for help with the fundus photography, and Rashmi Kapur for assistance in animal handling.
References