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Bilateral acute pyogenic conjunctivitis with iritis induced by unilateral topical application of bacterial peptidoglycan muramyl dipeptide in adult rabbits

Marlyn P. Langford*, Bridgett D. Foreman, Lana Srur [1], James P. Ganley [2], Thomas B. Redens

Department of Ophthalmology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

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ABSTRACT

The factors responsible for the conjunctivitis and iritis associated with acute ocular infection and post enteric inflammatory disease are not fully known. The pro-inflammatory activity of unilateral topical application of muramyl dipeptide (MDP; the smallest bio-active Gram-positive and Gram-negative bacterial cell wall component) was investigated in adult rabbits. The resultant bilateral conjunctivitis/iritis and pyogenic responses were characterized. Bilateral symptoms were graded by slit lamp examinations; tear fluid, Schirmer tests (tear production), blood and aqueous humor (AH) samples were obtained from MDP-treated and untreated rabbits. MDP concentration, gamma-glutamyltranspeptidase activity (GGT; key enzyme in glutathione recapture, xenobiotic detoxification, eicosanoid synthesis and neutrophil function), protein concentration, and tear cell density, cytology, and immunofluorescent antibody reactivity to GGT and calreticulin (CRT; MDP-binding protein) were determined. MDP was cleared from ipsilateral tears and serum by 6 h, but was undetected in mock-treated contralateral tears. Bilateral signs of acute transient pyogenic conjunctivitis, characterized by tearing, lid edema, conjunctival hyperemia, chemosis and leukocytic infiltrate with iritis (erythema and aqueous flare) were detected. Milder symptoms occurred in the mock-treated contralateral eyes. Bilateral symptoms, tear production, tear protein, GGT activity, and mucopurulent discharge (containing up to 2.5–5.0 $\times$ 10^6 cells/mL) were elevated 4–8 h post MDP and resolved to near pre-treatment levels by 24 h. Tear GGT activity and protein levels were higher in MDP-treated and mock-treated contralateral eyes than in eyes of untreated adult rabbits (p's < 0.001). Elevated tear GGT activity was associated with histopathology and increased vascular and epithelial permeability to serum protein, GGT-positive epithelia cells, macrophages and heterophils. Repeat MDP applications induced recurrent induction and resolution patterns of bilateral conjunctivitis/iritis and tear GGT activity, but ipsilateral GGT responses were lower. The results suggest unilateral topical MDP application to adult rabbit eyes induces a bilateral acute pyogenic conjunctivitis/iritis (PCI) characterized by increased vascular and epithelial permeability similar to acute bacterial conjunctivitis in man. The detection of CRT/GGT positive heterophils in tears suggests efferocytosis (phagocytosis of dead/dying cells). Tear GGT activity may be a useful means to quantify MDP-induced toxicity and extraocular inflammation.

Abbreviations: AH, aqueous humor; BAB, blood-aqueous barrier; BCB, blood-conjunctival-barrier; BTB, blood-tear-barrier; CD_{50}, 50% cytotoxic dose; CRT, calreticulin; GGT, gamma-glutamyltranspeptidase; IL-1β, interleukin-1β; MDP, muramyl dipeptide; TNF-α, tumor necrosis factor-α.

* Corresponding author. Department of Ophthalmology, 1501 Kings Hwy., P.O. Box 33932, Shreveport, LA 71130-3932, USA. Tel.: +1 (318) 675 5018; fax: +1 (318) 675 6000.
E-mail addresses: mlangf@lsuhsc.edu (M.P. Langford), BFOREM@lsuhsc.edu (B.D. Foreman), lsrur@med.miami.edu (L. Srur), drganley@comcast.net (J.P. Ganley), TReden@lsuhsc.edu (T.B. Redens).
[1] Present address: Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Health System, Miami, FL 33136, USA.
[2] Present address: 644 Ayrlie Water Rd, Gibson Island, MD 21056, USA.

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1. Introduction

Bacterial overgrowth at the external ocular surface usually induces an inflammatory response involving the eyelids (blepharitis), conjunctiva (conjunctivitis), cornea (keratitis) and/or iris (iritis). In addition, bilateral conjunctivitis and/or iritis can be associated with focal infections (Benedict, 1921), Reiter's syndrome, enteric artropathies (Ford, 1979), inflammatory bowel disease (Geerards et al., 1997), and intravesical injection of Bacille de Calmette et Guérin (BCG) vaccine (Murata et al., 2004). Most bacteria-positive acute conjunctivitis presents as an abrupt onset, unilateral or bilateral, cornea-sparing inflammation with or without mucoid or mucopurulent discharge that resolves in 2–5 days without sequelae (Syed and Chandler, 1995). Pyogenic bacterial conjunctivitis is characterized by mucopurulent tear, containing plasma proteins and leukocytes, causing adhesion of the eyelids and/or crusty eyelashes. Topical application of bacteria alone does not produce conjunctivitis (Behrens-Baumann and Begall, 1993). Current bacteria conjunctivitis/iritis models rely on injection (Liang et al., 2006; McCormick et al., 2011; Oka et al., 2004; Sloop et al., 1999) or topical application of bacteria or lipopolysaccharide (LPS) to abraded cornea (Schultz et al., 1997).

Degraded and phagocytic cell digested Gram-positive and Gram-negative bacteria release muramyl dipeptide (MDP; N-acetyl-muramyl-L-alanyl-D-isoglutamine) (Johannsen et al., 1991). MDP is the smallest component of bacterial cell wall peptidoglycan (Chang et al., 1981) with multiple, dose-dependent, stereo-isomer specific biologically activities (Adam et al., 1981; Cottagnoud et al., 2003; Kotani et al., 1986; Langford et al., 2002), including potent pro-inflammatory, edemagenic, somnogenic, pyrogenic, immunostimulatory (Adam et al., 1981; Chang et al., 1981; Fox et al., 1984; Johannsen et al., 1991; Kotani et al., 1976, 1986; Langford et al., 2006; Oppenheim et al., 1980), and calreticulin (CRT)-dependent cytotoxicity (Chen et al., 2005; Langford et al., 2006). Topical MDP application has been shown to increase protein and immunoglobulins in tears of rabbits immunized with poliovirus vaccine in Freund's complete adjuvant (Langford et al., 2003). However, MDP-induced activities have not been assessed in rabbit conjunctival tissue.

The innate immune response to infection represented by inflammation and infiltrating leukocytes plays an essential role in clearing bacteria. Gamma-glutamyltranspeptidase (GGT; for review see Lieberman et al., 1995) is expressed on infiltrating neutrophils (Brom et al., 1984), monocytes (Grisk et al., 1993) and T cells (Carlisle et al., 2003). GGT activity plays a key role in maintaining glutathione (Jensen and Meister, 1983), leukotriene and prostaglandin synthesis (Black et al., 1994; Shimizu and Wolfe, 1990), and neutrophil function (Kobayashi et al., 2003). GGT activity has been used as a parameter of the inflammatory response in cystic fibrosis (Corti et al., 2012), arthritic (Singh et al., 1986), diabetic (Langford et al., 2007), hepatic (Tahan et al., 2008), and cardiac disease (Mason et al., 2010). Interestingly, tear GGT activity is elevated in eyes of individuals with refraction errors and >40 years of age (Calderón de la Barca Gázquez et al., 1989), but reduced in diabetic individuals (Burnham et al., 2013). MDP has been shown to decrease GGT activity *in vitro* and *in vivo* (Langford et al., 1999, 2002). The levels of GGT activity in tears during ocular inflammation are unknown.

This paper presents the induction of bilateral pyogenic conjunctivitis with iritis (PCI) in adult rabbits following topical unilateral MDP application, the rapid removal of MDP from tear and serum, the bilateral tear GGT levels over the course of the inflammation, and the characterization of the leukocytic infiltrate. Single and multiple unilateral topical MDP applications induced bilateral transient, acute conjunctivitis with chemosis, iritis, increased tear GGT activity and mucopurulent discharge predominantly composed of GGT-positive macrophages and heterophils (neutrophils). Some heterophils expressed CRT on their surface indicative of efferocytosis [the removal of dying/dead cells by phagocytic cells] (Thorp et al., 2011). The results suggest the bilateral PCI induced by MDP is due to cytotoxic and immunomodulatory activities produced in the ipsilateral eye that contribute to an innate consensual response in the fellow eye. The results support the resilience of the adult rabbit conjunctiva and iris to repeat topical MDP application.

2. Materials and methods

2.1. Experimental procedure

Adult New Zealand White male and female rabbits (2–5 yrs-old, 4.0–5.5 kg; Myrtle Rabbitry (Thompson Station, TN)) were acclimated and maintained in an environmentally controlled animal care facility (22 ± 2 °C; 12 h/12 h light/dark cycle). Venous blood (1–2 mL) and tear samples were collected prior to and at times indicated through 48 h after the unilateral topical application of the MDP [50 μg/100 μl; ~10,000 rabbit kidney cell cytotoxic doses (Langford et al., 2002) (Sigma Scientific, St. Louis, MO)] in lubricant eye drops (Optive™; Allergan, Inc., Irvine, CA). The MDP and mock eye drop solutions were applied drop-wise onto the superior palpebral and bulbar conjunctiva of unanesthetized eyes of each test rabbit, while only eye drops were applied to the eyes of untreated control rabbits. The lacrimal ducts were gently occluded for 5 min. Tear, blood, post mortem AH and conjunctival tissue samples were collected at times post treatment and processed for analysis (see below). Three normal cohort untreated adult rabbits were evaluated for tear production by Schirmer tests and provided control tear over a 24 h period, blood, post mortem aqueous humor (AH), and tissue samples.

To investigate the effects of repeat MDP challenges, 6 identical MDP doses were applied at 3–5 day intervals over a 16 d period to the same eyes of 3 rabbits. The bilateral symptoms and tear GGT activities on Schirmer test strips were assessed at 0, 2, 4, 6, 8, 24 and 48 h after each application. Rabbits were housed in an accredited LSUHSC animal care facility and cared for by certified animal care personnel. IACUC approved experiments and euthanasia procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

2.2. Slit lamp examination

Bilateral external ocular and slit lamp examinations were performed through the 48 h post-application period on each rabbit using a Carl Zeiss slit lamp biomicroscope (NAG f Oph-Leu 309620; West Germany), representative photos were taken, clinical symptoms scored hourly using a modified "inflammatory score" system similar to that previously reported by others (Fox et al., 1984; McCormick et al., 2011). Briefly, four clinical symptoms (hyperemia, conjunctival chemosis, exudate and iritis) were scored at each time point by one grader (MPL) using a scale of 0–3; i.e., a score of 0 (absence of symptom), 1 (detectable), 2 (intermediate) or 3 (maximal). The sum of average scores for each eye at each time point for 6 rabbits was plotted to generate the clinical course of the bilateral ocular inflammatory response.

2.3. Tear, blood, AH, and conjunctival tissue samples

Bilateral tear samples were collected over a 5–15 min period by 2 lab personnel using disposable 20 μl borosilicate glass capillary

micropipets (Fisher Scientific, Pittsburgh, PA) as previously described (Langford et al., 1986). Five microliters of each tear sample were pipetted onto glass slides and air-dried (for cytology). The remaining tear was centrifuged (3000 × *g* for 5 min). The clarified tear fluid was stored frozen (−20 °C) in a labeled vial until assayed for GGT activity and protein. Blood samples (0.5–2 mL) were obtained via ear venipuncture using a 5 cc syringe fitted with a 25-gauge infusion needle (Smiths Medical ASD, Inc., Keene, NH). The serum was collected from clotted blood post centrifugation (2000 × *g* for 3 min) and stored at −20 °C. Post mortem AH and conjunctival tissue samples were collected as previously described (Langford et al., 1999, 2006). Conjunctival tissue samples from the MDP-treated and mock-treated control eyes of euthanized rabbits were rinsed gently in 1 mL of cold phosphate buffered saline (PBS; pH 7.4), placed in a plastic embedding trays containing Tissue Tek O.C.T. compound (Miles, Inc., Elkhart, IN) and frozen at −100 °C.

2.4. MDP quantification

MDP was quantified in thawed tear fluids and sera collected at times post unilateral topical MDP application as previously described (Langford et al., 2002). The 50% cytotoxic dose (CD_{50})/tear and serum sample was determined in triplicate bioassays and the mean CD_{50}/mL (±standard error of the mean, SEM)/time point/rabbit group was calculated.

2.5. Tear production

Bilateral tear production was determined by Schirmer tests (Clement Clarke International, Harlow, Essex, UK) (Wright and Meger, 1962). Tear production (mm/5 min) was determined at each time point for untreated control (eye-drops only), MDP-treated and mock-treated contralateral rabbit eyes. The mean (±SD; standard deviation) was calculated for each rabbit group. The Schirmer tear test strips were air-dried and assayed for GGT activity and protein (see below).

2.6. GGT assay

Tear fluid, serum and post mortem AH samples were assayed for GGT as described previously (Burnham et al., 2013; Langford et al., 1999, 2007). Air-dried Schirmer tear test strips were placed in 1 mL of assay medium and incubated at room temperature for 3 h. One unit (U) of GGT activity represents conversion of 1.0 nM of γ-glutamyl-para-nitroanalide. The levels of tear, serum, and post mortem AH GGT activity were expressed as U/mL. Schirmer tear test strip GGT activity was expressed as U/strip.

2.7. Tear protein quantification and gel analyses

The concentrations of protein in tear fluid and AH and adsorbed to Schirmer test strips were determined by colorimetric protein assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA) using bovine albumin as standard (1 mg/mL; Sigma) (Langford et al., 2002). Tear and AH protein profiles were demonstrated by SDS-polyacrylamide gel electrophoresis (PAGE) as previously reported (Chen et al., 2005; Langford et al., 1999). Protein bands were stained with GelCode® Blue Stain Reagent (Pierce, Rockford, IL: www.piercenet.com) as per the manufacturer's instructions.

2.8. Tear cells and conjunctival histology

The tear cell densities (number of cells/mL) for bilateral tears collected over 24 h from 4 MDP-treated rabbits were determined using a Bright Line® hemocytometer per the manufacturer's instructions (Reichert Scientific Instruments, Buffalo, NY). Bilateral tears collected in glass capillary tubes were pipetted onto glass slides (5 μl/drop/slide), air-dried at room temperature and stored at 4 °C. The air-dried cells were fixed with cold 70% ethanol or acetone (4 °C for 15 min) and rinsed 4 times with cold PBS. Similarly, serial cryostat conjunctival tissue sections were cut (2–3 sections placed/5 slides), air-dried, fixed with cold 70% ethanol and stored at 4 °C. Air-dried tear samples, ethanol or acetone fixed conjunctival cryostat-sections and paraffin-embedded formalin-fixed tissue sections on glass slides were stained with Hematoxylin & Eosin (H&E; Core Lab Facility, Department of Anatomy, LSUHSC-Shreveport, LA) and a glass cover-slip applied with Permount® (Fisher Scientific). Cytologies and histopathologies were determined microscopically using an Olympus BX43F light microscope (Tokyo, Japan) as described previously (Langford et al., 2002, 2006). Digital images were created using Olympus Soft Imaging Solutions (CellSons Life Science Imaging Software, Münster, Germany).

2.9. Immunofluorescent antibody detection of GGT/CRT-positive cells

Immunofluorescent antibody (IFA) detection of immunoreactive GGT and CRT in the tear cell infiltrate was performed using the previously described methods (Burnham et al., 2013; Chen et al., 2005). Briefly, the nucleic acid stain DAPI (4′,6-diamidino-2-phenylindole) (Sigma, St. Louis, MO) in PBS was reacted with the air-dried, ethanol fixed, tear cells on glass slide for 1 h (per the manufacturer's recommendation). The slides were rinsed in 3 times in fresh PBS. Murine monoclonal antibody to GGT (1:1000; Cat No. 53506; Ana Spec, Inc., Fremont, CA) and chicken IgY antibody to CRT synthetic peptide (PA1-903; Thermo Scientific/Pierce Biotechnology, Rockford, IL) were applied and incubated overnight at 4 °C. The unbound primary antibodies were removed and the slides rinsed 3 times in PBS. The cells were reacted with tetramethylrhodamine isothiocyanate (TRITC)-labeled $F(ab')_2$ fragment of goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-labeled donkey anti-chicken IgY (1:1000 dilution in PBS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All IFA procedures and digital color images were generated as previously described (Burnham et al., 2013; Langford et al., 2010). Multiple color images were merged using Scanalytics IPLab 3.7 software.

2.10. Statistical analysis

Graphs and statistical analyses of differences between rabbit groups and eyes were determined using Microsoft Excel software (Microsoft, Seattle, WA) or Sigma Plot for Windows 11.1 software (Systat Software, Inc., Chicago, IL). Differences between eye pairs and rabbit groups were considered significant for p-value < 0.05 obtained by paired and unpaired student t-tests, respectively.

3. Results

3.1. Experimental pyogenic conjunctivitis/iritis (PCI)

Topical application of MDP to one eye induced bilateral PCI in both eyes (Fig. 1). Symptoms, especially the amount of chemosis and exudate, varied between eyes and rabbits, but generally increased through 6–7 h. The inflammatory changes in the MDP-treated eyes were characterized by rapid onset (≤3 h) of tearing, redness, lid edema, and chemosis (i.e., diapiresis in the superior and inferior palpebral conjunctival tissue), watery converting to mucopurulent discharge, and iritis (iris erythema, aqueous flare, dilation of iris vessels) without cellular infiltrate (Fig. 1A). Similar, but milder symptoms were detected after an hour delay in the

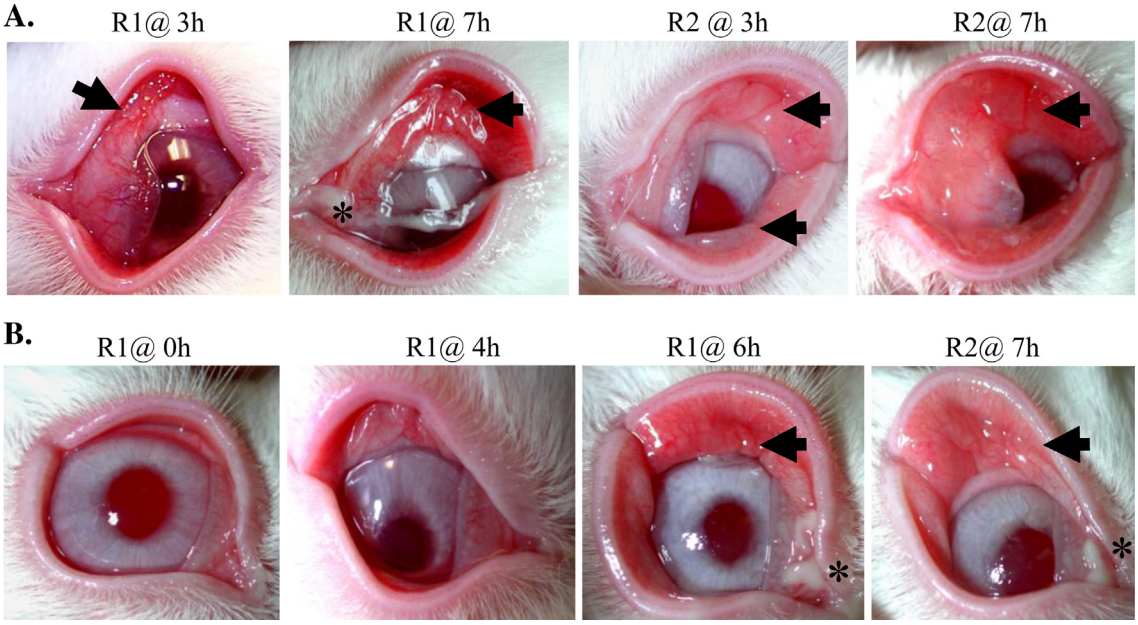

Fig. 1. Clinical symptoms and cytopathology. (A) Appearance of MDP-treated rabbit eyes at 3 and 7 h after topical application. Note the progression over time and the variability in the iritis, conjunctival chemosis (arrows) and mucopurulent exudates (*) between rabbits 1 and 2. (B) Appearance of ipsilateral control eyes at 0, 4, 6 and 7 h.

mock-treated contralateral eyes (Fig. 1B). More severe symptoms in the ipsilateral eyes were generally associated with more severe symptoms in the MDP-treated eyes. The bilateral PCI resolved completely in most rabbits over 48 h.

3.2. Clinical course

The clinical symptoms of hyperemia, chemosis, exudate and iritis were detected by 4 h post topical application in the MDP-treated eyes. Clinical scores were maximal by 5–6 h (scores of 2–3 for each symptom), remained through 8 h, and declined by 12 h (Fig. 2A). Generally, the PCI resolved by 24 h, but signs of conjunctival redness and lid edema were detected up to 48 h in some rabbit eyes. The onset of clinical symptoms in the mock-treated contralateral eye lagged behind the MDP-treated eye by 1–2 h (Fig. 2B). Peak clinical scores were lower in the mock-treated contralateral eyes (5.5 versus 10 in the MDP-treated eyes) than in the ipsilateral eyes. The cumulative clinical scores (sum of average score for all symptoms at a time point) were greatest 5–8 h post application for the ipsilateral and mock-treated contralateral eyes. No membranes, pseudomembranes, follicles, papillae, preauricular lymphadenopathy, corneal involvement, mydriasis or cells in AH were detected.

3.3. MDP levels in tears and serum

High MDP levels were detected in tear ($10^{3.7\pm0.5}$ CD_{50}/mL) between 0.25 and 1.0 h in treated eyes of 6 rabbits. MDP levels declined to undetectable levels (<1.0 CD_{50}/mL) by 6 h in tears of the MDP treated eyes. MDP was not detected in tears of the mock-treated contralateral eyes (Fig. 3). Serum MDP levels were $10^{1.2\pm0.2}$ CD_{50}/mL at 0.25 h and $10^{0.5\pm0.48}$ CD_{50}/mL at 1.0 h post application and declined to undetectable levels by 2 h. Note that MDP declined as bilateral PCI symptoms reached near maximal levels.

3.4. Conjunctival pathology

Histopathological changes consistent with chemosis (fluid accumulation in the interstitial spaces beneath the conjunctival epithelium) and leukocytic infiltration were less prominent in cryosections of ipsilateral (Fig. 4A) than MDP-treated (Fig. 4B) eyes at 5 h. Muco-proteinaceous strands with cells were detectable in of MDP-treated eyes (Fig. 4B). Fewer gross changes (i.e., enlarged interstitial spaces) were detected in the palpebral conjunctiva of the mock-treated contralateral eye at 5 h (Fig. 4A). The conjunctival tissues from the ipsilateral eyes at 5 h were similar to the untreated control rabbit eyes (not shown). H&E staining of paraffin-embedded conjunctival tissue from ipsilateral (Fig. 4C) and MDP-treated (Fig. 4D) eyes identified more heterophils between and beneath the basal epithelial cells and in the lamina propria interstitial spaces of palpebral conjunctival tissue of MDP-treated eyes at 5 h. Notably, heterophils were detected between conjunctival epithelial cells and in the dilated conjunctival venous capillaries in the ipsilateral eyes (Fig. 4E) at 6 h post. Small (Fig. 4F) and broad breaches (Fig. 4G) in the conjunctival epithelium with emerging heterophils were detected within the conjunctival epithelium of MDP-treated eyes at 6 h. Dilation of the iris and ciliary body vasculature, but without cell filtrate in the anterior chamber, was detectable at 6 h (not shown). The conjunctival histopathologies were consistent with bilateral clinical signs and symptoms of vascular dilation, chemosis, and mucopurulent pyogenic exudates.

3.5. Effect of topical MDP on tear production

Bilateral Schirmer tear tests were performed pre and post topical MDP application in 22 rabbits to measure changes in tear production (Fig. 5). Tear production increased above pre-treatment levels in the MDP-treated and mock-treated contralateral eyes (14 ± 7 and 17.3 ± 3.3 mm/5 min, respectively) between 2 and 7 h (all p-values ≤ 0.04), but returned to near pre-treatment levels by 24 h. Tear production was significantly greater in the ipsilateral eyes than in the mock-treated contralateral eyes only at 6 h ($p = 0.02$). The mean (±SEM) tear production was significantly higher in the MDP-treated eyes (24.9 ± 1.1 mm/5 min) than in the mock-treated contralateral eyes (20.7 ± 1.9 mm/5 min; $p < 0.0001$) between 2 and 12 h. The results support acute bilateral increases in tear production with greater tear production in MDP-treated than in mock-treated contralateral eyes.

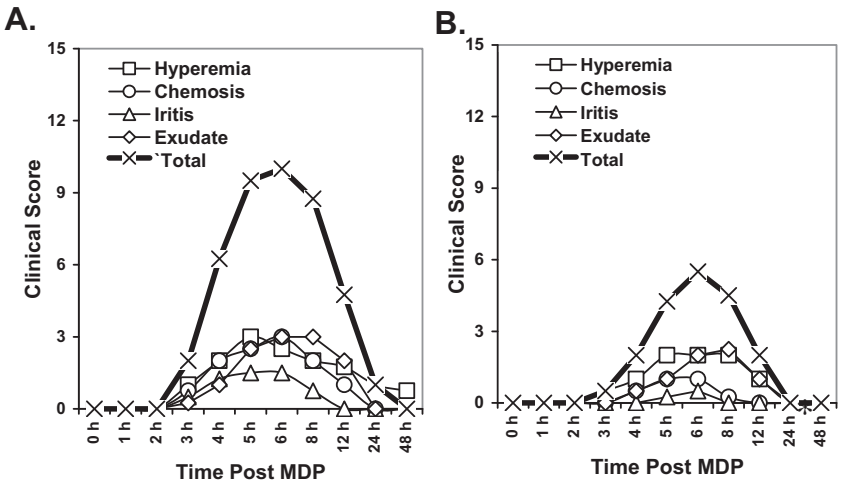

Fig. 2. Clinical course of the MDP-induced bilateral conjunctivitis/iritis. Average scores for clinical symptoms in (A) MDP-treated and (B) contralateral eyes for 4 rabbits. Cumulative clinical scores (bold line) is the sum of average clinical score for all symptoms.

3.6. Effects of topical MDP on tear and AH protein

The tear and AH protein concentrations were determined in the MDP-treated and mock-treated contralateral control eyes to identify MDP-induced blood-conjunctival (BCB) and blood-aqueous barrier (BAB) permeability changes. Tear protein concentrations were increased in both eyes through 6 h and were significantly higher (p-values ≤ 0.01) in MDP-treated than in the mock-treated contralateral control eyes between 3 and 7 h (Fig. 6A). Tear protein concentrations declined to near pre-treatment levels by 12 h. Concomitantly, the PAGE protein profiles for bilateral tear samples were consistent with a 1–2 h lag period between the pronounced BCB disruption in the MDP-treated eyes (Fig. 4) and supported increased BCB permeability in the mock-treated contralateral eyes (Fig. 6B). Accordingly, albumin (68 kDa protein) levels were elevated in the tears of MDP-treated eyes through 6 h and in the mock-treated contralateral eyes through 8 h consistent with the respective bilateral increase in total tear protein. Several protein bands unique to MDP-treated eyes were present in tears collected at 4–8 h; supporting histopathological differences between eyes. The AH protein concentrations were 3.7 times higher at 8 h in MDP-treated eyes (3.45 ± 0.7 mg/mL) than in mock-treated contralateral eyes (0.94 ± 0.37 mg/mL; $n = 4$; $p = 0.0008$), 10 times higher than in AH from untreated adult rabbit eyes (0.33 ± 0.09 mg/mL; $p = 0.0001$), but lower than in serum (7.93 ± 0.85 mg/mL; $p = 0.0002$) (Fig. 6C). PAGE AH protein analysis supported prominent albumin extravasation into the AH and higher protein concentrations in AH of MDP-treated than in mock-treated contralateral eyes at 8 h (Fig. 6D). Taken together, the results support bilateral increases in tear and AH protein levels and suggest the relative increase in BCB and BAB permeability was MDP-treated > mock-treated contralateral > untreated eyes. The higher levels and diversity of ipsilateral tear protein were likely due to a combination of transudate due to vascular leakage, exudate due to permeabilization epithelium, and release of proteins into tear by the cellular infiltrate.

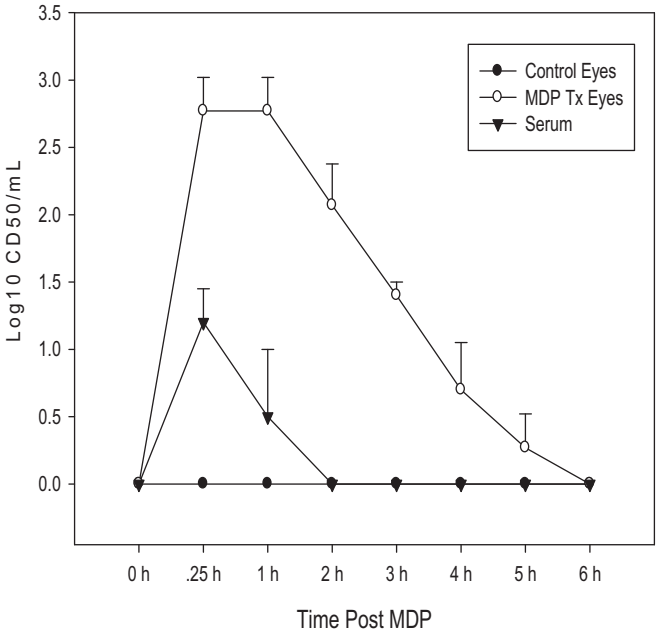

Fig. 3. MDP quantification. Ipsilateral tear and serum MDP levels (mean ± SEM) were elevated after unilateral topical application in 6 rabbits.

3.7. Effect of topical MDP-treatment on tear fluid, Schirmer strip and AH GGT activity

GGT levels were investigated in cell-free tear fluids (infiltrating cells removed), Schirmer test strips (adsorbed tear fluid, proteins and cells), AH and sera to determine the relationship between the tear GGT, edemagenic (chemosis) and pyogenic responses induced by topical MDP application. Over the course of the bilateral conjunctivitis/iritis, elevated tear GGT activities were detected first in the ipsilateral eyes (peaking at 4–5 h) followed closely by elevated levels in the mock-treated contralateral eye (peaking at 6–8 h). Post treatment tear GGT activities (2–8 h) were consistently higher (up to 10-fold) in tear fluids of ipsilateral and mock-treated contralateral eyes than in untreated placebo-control rabbit eyes (p-values ≤ 0.01) (Fig. 7A). The GGT activity levels were significantly higher (p-values ≤ 0.001) in tear fluids from ipsilateral eyes than from mock-treated contralateral eyes at 3–5 h. Bilateral tear fluid GGT activity declined to near pre-treatment levels by 12 h. Similarly, Schirmer test strip GGT activity was detected first in the MDP-treated eyes and was significantly higher in MDP-treated eyes than mock-treated contralateral eyes at 3 and 4 h (Fig. 7B). Post treatment Schirmer test strips GGT activity was significantly higher in the MDP-treated and contralateral eyes than in untreated placebo-

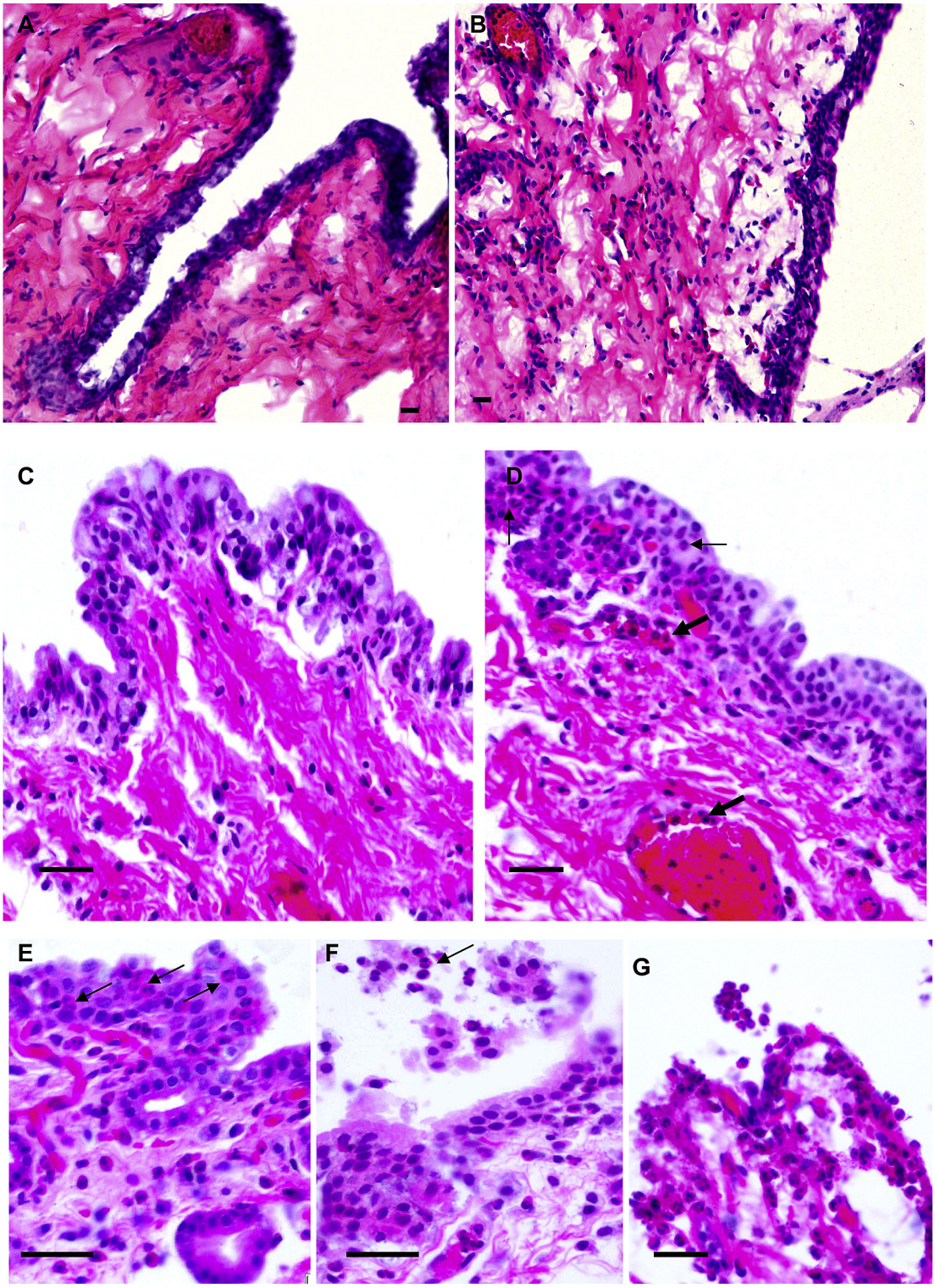

Fig. 4. H&E staining of cryo-sections of rabbit conjunctival tissues from the (A) ipsilateral and (B) MDP-treated eyes of a rabbit at 5 h. Note the proteinaceous exudate and leukocytic infiltration in the MDP-treated eye. H&E staining of paraffin-embedded conjunctival tissue sections from (C) ipsilateral and (D) MDP-treated eyes at 5 h. Note the dilation of the subconjunctival blood vessels and numerous heterophils in the conjunctival vein (large arrow) and epithelium of the MDP-treated eye (small arrow). H&E of paraffin-embedded conjunctival tissue of the (E) ipsilateral and (F) MDP-treated eye at 6 h showing numerous heterophilic leukocytes (small arrows) in the superior palpebral conjunctival epithelium of the ipsilateral eye and the extravasation of heterophils and detached palpebral conjunctival epithelial cells in the MDP-treated eye. (G) MDP-treated rabbit eye at 6 h showing numerous heterophils associated with an area of disrupted superior bulbar conjunctival epithelium. [Bar = 50 μm].

control rabbit eyes at 2–8 h (p-values $\leq$ 0.01). The bilateral Schirmer test tear GGT activities were elevated above untreated placebo-control rabbit tear levels at 24 h. [Note: Tear GGT activity per Schirmer strip did not correlate with tear volume/strip (data not shown).] Notably, the GGT activity levels in pre-treatment cell-free tear (6.2 ± 4.6 U/mL; $p < 0.0001$) were lower than in untreated adult rabbit sera (31.6 ± 6.4 U/mL). GGT activity levels were 2–5-fold higher in bilateral cell-free tears collected 3–8 h post MDP

treatment (70–150 U/mL; p-values $\leq$ 0.01) than in serum (Fig. 7A&C) suggesting a local GGT source. The post mortem AH GGT activity levels were 2-fold higher in ipsilateral eyes (17.4 ± 4.7 U/mL) than in the AH of the mock-treated eyes (8.3 ± 1.9 U/mL; $p = 0.001$) of rabbits euthanized at 8 h. GGT levels were higher in the ipsilateral and mock-treated contralateral eye AH than in untreated control eye AH (3.2 ± 2.5 U/mL; $p = <0.0001$; $p = 0.003$, respectively) (Fig. 7C), but 45% lower than in serum ($p < 0.0001$) consistent with the apparent diapiresis of serum GGT into bilateral AH. The results support a temporal lag between the tear GGT activity responses of the ipsilateral and mock-treated contralateral eyes, and a ≥10-fold increase in bilateral tears and AH GGT activity levels above the levels in tear and AH of untreated control rabbits. The results suggest that tear GGT activity in untreated rabbits was elevated slightly in response to tear collection, that bilateral GGT activity levels in tears of inflamed eyes were due to cell-free and cell-associated GGT, and that elevated AH GGT activity was likely due to increased permeability of the BAB to serum GGT.

3.8. Bilateral cellular responses and tear cell GGT/CRT immunoreactivity

Cell densities, H&E cytology, and immunoreactivity of the cellular infiltrate in tear with antibody to GGT (neutrophil marker) and CRT (MDP-binding protein) were determined in bilateral tears collected over the clinical course to characterize the cellular responses induced by topical MDP. Higher cell numbers were detected in tears from ipsilateral than the contralateral eyes (Fig. 8). Tear cell density increased to maximal levels in the MDP-treated (5.02 ± 0.98 × 10^6 cells/mL) and mock-treated contralateral (2.70 ± 1.04 × 10^6 cells/mL) eyes through 8 h, then declined to near pre-treatment levels by 24 h (Fig. 8A). Higher cell densities were significant only at 2, 3, 7, and 8 h (p-values $\leq$ 0.01). Tear samples collected at 2 and 3 h from ipsilateral eyes contained primarily epithelial cells with few leukocytes (not shown) consistent with early conjunctival cytopathology. Epithelial cells, leukocytes and cell debris were detected in tears from MDP-treated and mock-treated-treated eyes collected between 3 and 8 h, but heterophils were predominant in tears collected between 5 and 12 h (Fig. 8B). The results of the tear immunofluorescent antibody analysis (Fig. 8C–F) suggested that the heterophils, macrophages or conjunctival epithelial cells were positive for extrinsic GGT (Fig. 8D). Also, some heterophils and macrophages were positive for surface CRT (Fig. 8E). Notably, strong surface CRT and GGT immunoreactivity was co-localized between heterophils and between heterophils and macrophages or conjunctival cells in tears (Fig. 8F). Together, the results suggest that the elevated GGT activity in clarified bilateral tear fluids collected post topical MDP application was due to release of GGT from GGT-positive cells in the tear fluid and increased permeability of the BCB to serum GGT. Concomitantly, the increase in Schirmer tear GGT activity was due to free GGT in tear and serum exudate, but was predominantly due to GGT-positive conjunctival/leukocytic cell adsorbed to the test strip.

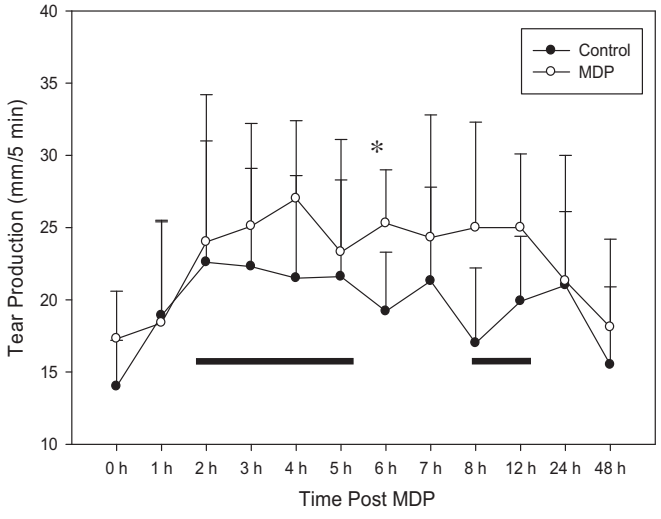

Fig. 5. Tear production. Bilateral tear production by 22 rabbits post-unilateral topical MDP. Each point represents the mean mm (±standard deviation; S.D.) of tear adsorbed in 5 min to Schirmer's test strips placed in unanesthetized eyes. Asterisk (*) indicates a significant difference in tear production between MDP-treated and contralateral eyes at 6 h ($p = 0.02$). Bar indicates significantly higher levels of consensual MDP-treated and contralateral eye tear production above the levels of pre-treatment tear production (p-values $\leq$ 0.04).

3.9. Bilateral tear GGT responses to repeat unilateral topical MDP applications

Since the results suggested that Schirmer test tear GGT activity is temporally and quantitatively associated with the GGT-positive leukocytic infiltration (pyogenic conjunctivitis) induced by topical MDP, tear GGT activity levels on Schirmer test strips were utilized to monitor changes in the inflammatory responses to repeat topical MDP applications in 3 rabbits. The bilateral clinical courses (i.e., exacerbation and resolution of bilateral conjunctivitis/iritis) and GGT responses were recurrent after each unilateral MDP application (Fig. 9). The tear GGT activity was 2–10-fold higher at 2, 4, 6, and 8 h post MDP application in the ipsilateral eyes than in the mock-treated contralateral eyes over the 16 d course. Notably, the average levels (256 ± 13 U/strip) of Schirmer tear GGT activity in the ipsilateral eyes at 4, 6, and 8 h after the first MDP application were significantly greater than the average Schirmer tear GGT activity of the ipsilateral eyes at 4, 6 and 8 h for the 5 subsequent MDP-applications (72 ± 14; 129 ± 37, 185 ± 18, 144 ± 67 and 204 ± 5 U/Strip; p's = 0.0001, 0.004, 0.004, 0.04, and 0.003, respectively). In the mock-treated contralateral eyes, the average levels (84 ± 31 U/strip) of Schirmer tear GGT activity at 4, 6 and 8 h after the initial application were generally lower than in Schirmer test tear GGT activities after 5 repeat MDP applications (33 ± 13, 38 ± 29, 36 ± 29, 25 ± 6, and 40 ± 29 U/strip, respectively), but were not significantly different except for the 4th cycle ($p = 0.03$). The results suggest GGT responses were greater in the MDP-treated eyes, decreased with repeat application in the MDP-treated eyes, but were similar in the mock-treated contralateral eyes after repeat unilateral topical MDP applications.

4. Discussion

Acute onset transient bilateral pyogenic conjunctivitis/iritis (PCI) was induced in adult rabbits by unilateral topical application of the bacterial cell wall peptidoglycan MDP. The PCI occurred first in the MDP-treated eyes and was followed within 1–2 h by a milder PCI in the mock-treated contralateral eye. The MDP-induced PCI exhibited similar clinical signs, symptoms and course as acute bacterial conjunctivitis in man being characterized by acute onset of tearing, lid edema, conjunctival erythema, chemosis, and mucopurulent discharge. In addition, an acute bilateral transient acute iritis (without AH cells) was observed with the edemagenic changes. No corneal involvement, follicles, papillae and membranes were detected. Most symptoms of the bilateral PCI resolved in most rabbits by 48 h.

The acute onset of tearing and inflammatory changes in the conjunctiva and iris by 4 h in the ipsilateral eye suggests topical MDP application caused the PCI. The dissociation and disruption of

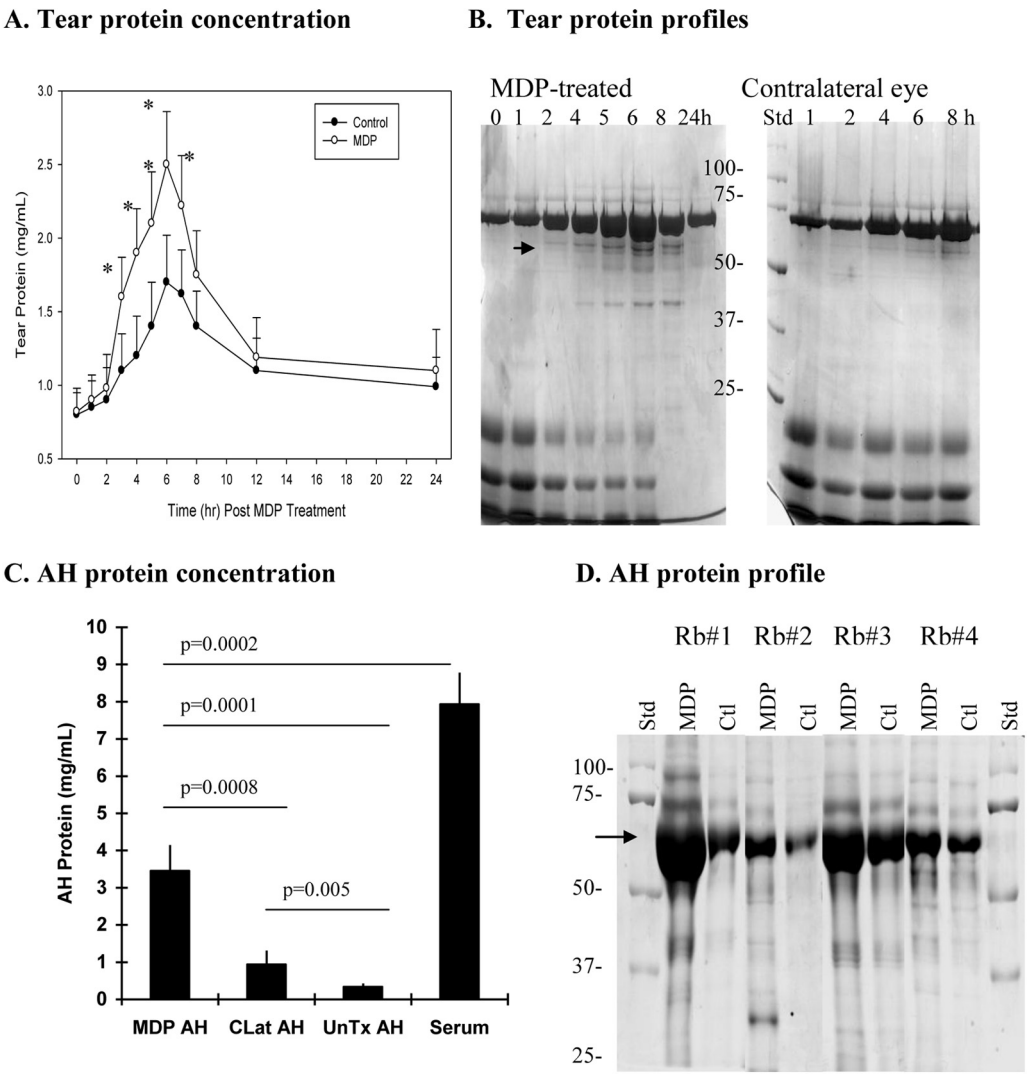

Fig. 6. Tear and aqueous humor (AH) protein. (A) Mean protein concentrations (±SD) in tear samples from MDP-treated and contralateral control eyes of 6 rabbits (*; *p*-values ≤ 0.01). (B) Protein profiles of tear samples (3 μl/lane) collected from MDP-treated and contralateral control eyes of one rabbit. Note the increase in the 68 kDa albumin protein band (arrow) in tears of both eyes and the increase of several 40–70 kDa proteins in the tear fluid from MDP-treated at 4–8 h. (C) Protein concentrations in MDP-treated, mock-treated contralateral (CLat) and untreated (UnTx) AH collected from rabbits at 8 h post treatment were significantly different from each other and from serum (*; *p*-values ≤ 0.005). (D) The protein profile (5 μl/lane) of AH collected at 8 h post mortem from MDP-treated and contralateral control eyes of 4 rabbits.

conjunctival epithelium in the MDP-treated eyes was similar to that observed in the ciliary body epithelium observed in ipsilateral rabbit eyes at 4 h post intracameral MDP injection (Langford et al., 2006). Notably, the conjunctival epithelial cells/debris (consistent with cytopathology), increased levels of cell-free tear GGT activity (consistent with release of GGT), and elevated tear and AH protein levels (consistent with increased permeability of the BCB and BAB) were greater in the ipsilateral eyes than in the mock-treated contralateral and untreated rabbit eyes. Thus, these results suggest that the cytopathologies in the ipsilateral conjunctiva and iris were likely due to the direct cytotoxic effect of MDP and/or pro-inflammatory mediators induced by the interaction of MDP with leukocytes, epithelium, vascular endothelium, and neuronal cells (Adam et al., 1981; Johannsen et al., 1991; Oppenheim et al., 1980; Zídek et al., 1993). Accordingly, the bilateral conjunctival erythema, chemosis, and elevated tear proteins were associated with histopathological changes in the conjunctival vasculature (BCB) and epithelium (BTB) of the MDP-treated eyes, while the iris erythema, aqueous flare and elevated AH proteins were consistent with the BAB disruption observed post intravenous MDP (Kufoy et al., 1990; Lawrence et al., 1992; Waters et al., 1986).

The PCI in the MDP-treated eyes preceded the onset of milder PCI in the mock-treated contralateral eye by 1–2 h. The sequential onset of inflammation and asymmetric bilateral involvement of the conjunctiva and iris following topical MDP application was similar to the bilateral responses previously noted following unilateral MDP administration (Langford et al., 2003, 2006). The possibility of hematogenous spread to the fellow eye is suggested by MDP's small size and detection in serum. The rapid removal of MDP via the lacrimal duct of the ipsilateral eye and rapid decline in serum MDP levels suggests limited tissue exposure and a milder dose-dependent response in the fellow eye. However, the inability to detect MDP (<1 CD_{50}; i.e., <5 ng/mL) in mock-treated contralateral tears argues against hematogenous spread. Also, the absence of conjunctival cells/debris in the tears of the fellow eyes suggests the BCB of the mock-treated contralateral eye may have limited the spread of MDP to the extraocular surface. Thus, the changes observed in conjunctival and iris tissue of the mock-treated

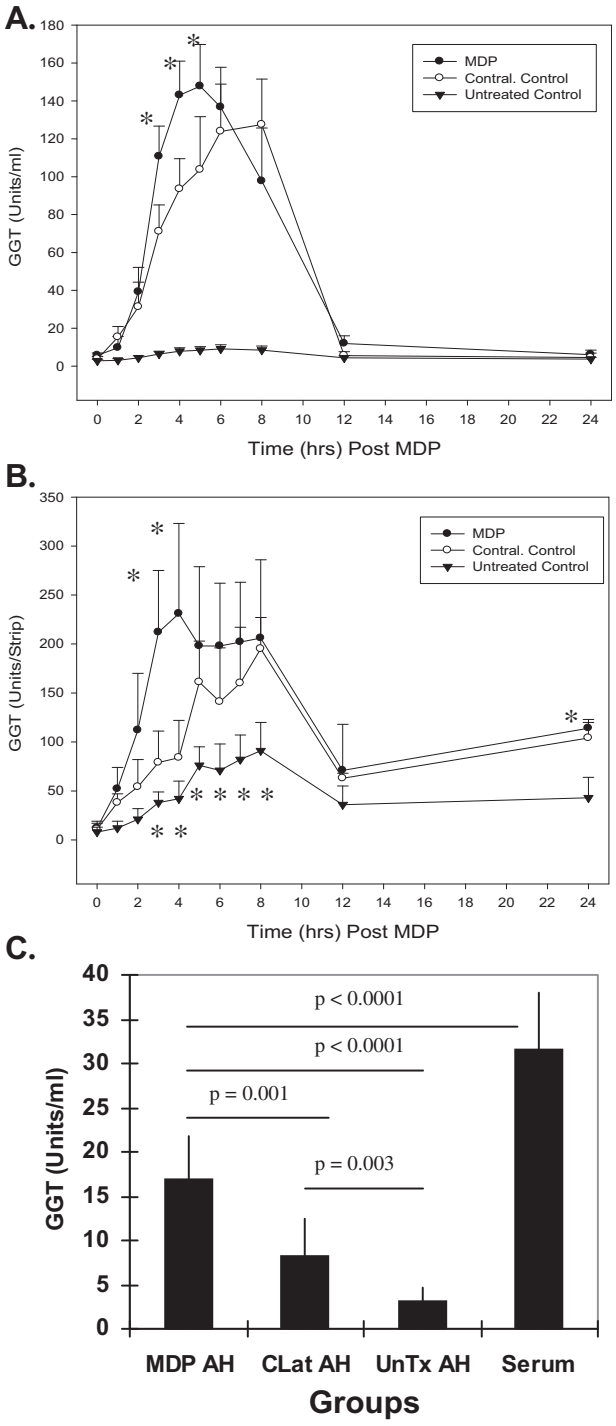

Fig. 7. GGT levels in tear and aqueous humor (AH) post topical MDP. (A) GGT activity was significantly higher in cell-free tear fluid collected from 6 MDP-treated eyes at 3–5 h than in mock-treated contralateral eyes (*; p-values < 0.01; unpaired t-test), but the GGT activity post 2 h was significantly lower in bilateral cell-free tear fluids from untreated control rabbit eyes (p-values < 0.001). (B) Significantly higher GGT activity per Schirmer test strip was detected at 3 and 4 h in 6 MDP-treated eyes than in mock-treated contralateral eyes, but GGT activity on Schirmer test strips from the untreated control rabbit eyes was significantly lower (*; p-values ≤ 0.05). (C) Significantly higher levels of GGT activity were detected in AH collected from MDP-treated and mock-treated contralateral (CLat) eyes of 4 rabbits euthanized at 8 h post topical MDP treatment than in AH and serum of 3 untreated (UnTx) control adult rabbits.

contralateral eye may be a consensual response due to systemic release of chemical mediators from the inflamed eye (Chiang and Thomas, 1972) and/or a neuronal response (Perkins, 1957). Accordingly, the interactions between the MDP applied to conjunctival epithelial cells and/or lymphoid cells associated with the conjunctival and iris tissue could produce pro-inflammatory mediators, such as prostaglandins, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) (Cottagnoud et al., 2003; Langford et al., 2002, 2011; Nagao et al., 1981; Oppenheim et al., 1980; Waters et al., 1986). These chemical mediators could spread to the contralateral eye producing the increased permeability of intraocular blood-barriers in a manner similar to the responses reported in the contralateral eyes post intracameral prostaglandin injection, cataract surgery, and ultraviolet light exposure (Beitch and Eakins, 1969; Podos, 1976; Miyake et al., 1984; Meyer et al., 2013; Shah and Spalton, 1994). The likelihood that topical MDP could induce cytotoxicity and bilateral conjunctivitis with pyogenic discharge, iritis and AH protein mediated by macrophage activation and/or endogenous prostaglandin is supported by its pharmacological properties, endogenous production (Fleisher et al., 1992; Nagao et al., 1990; Podos, 1976; Langford et al., 1999, 2006) and is suggested by indomethacin's inhibition of MDP-induced uveitis, edema, and apoptosis (Langford et al., 2002; Waters et al., 1986; Zídek et al., 1993). However, the possibility that other mediators or mechanisms cannot be ruled out. In this regard, it should be noted that the increased permeability of the human BAB in contralateral eyes post implant surgery was not inhibited by indomethacin (Miyake et al., 1984). Further, MDP application may induce an alteration in the neuronal reflex in the contralateral eye (Leplat, 1924) that could account for the increased tearing and vascular permeability in the conjunctiva and iris of the fellow eye; possibly via binding to serotonin binding sites (Polanski et al., 1995; Root-Bernstein and Westall, 1990; Sevcík and Masek, 1999). Additional studies are needed to detect potential mediators and delineate the mechanism(s) responsible for the putative consensual response.

Elevated GGT activity levels paralleled the respective cellular increases in the tears and occurred earlier and higher in ipsilateral eyes than in contralateral eyes. The elevated bilateral tear GGT activity between 4 and 8 h was associated with GGT-positive heterophils/macrophages and was consistent with GGT as a marker for an innate inflammatory response (Corti et al., 2012; Singh et al., 1986). Notably, the bilateral acute heterophilic tear infiltrate shares some aspects with the neutrophilic sequestration reported with sepsis (Toft et al., 1993), LPS (Erzurum et al., 1992), interferon (Urbaniak et al., 1978), and TNF-α/IL-1β injection (Fleisher et al., 1992). Moreover, acute transient leucopenia has been reported post-intravenous MDP injection (Kotani et al., 1976; Waters et al., 1986). The absence of a detectable leukocytic infiltrate in the post mortem AH of our rabbit eyes with PCI, as well as in rabbits with MDP-induced uveitis was thought to be to immune privilege (Lawrence et al., 1992). Clearly, the bilateral GGT/cellular responses induced by topical MDP were greater than the responses induced by Schirmer test application and/or eye manipulation. Moreover, the inflammatory responses in the contralateral eyes of rabbits treated topically with MDP were more acute, more transient and less severe than the responses reported in the contralateral post monocular trauma (Li et al., 1994), ultra-violet light exposure (Meyer et al., 2013), HSV-1 infection (Atherton et al., 1987), bacterial infection (McCormick et al., 2011), topical LPS (Schultz et al., 1997) and intravitreal peptidoglycan injection (Rosenzweig et al., 2011) supporting an acute innate immune response rather than an adaptive immune response.

The infiltration of heterophils may be to remove MDP, a toileting response to damaged cells and/or contribute to the conjunctival

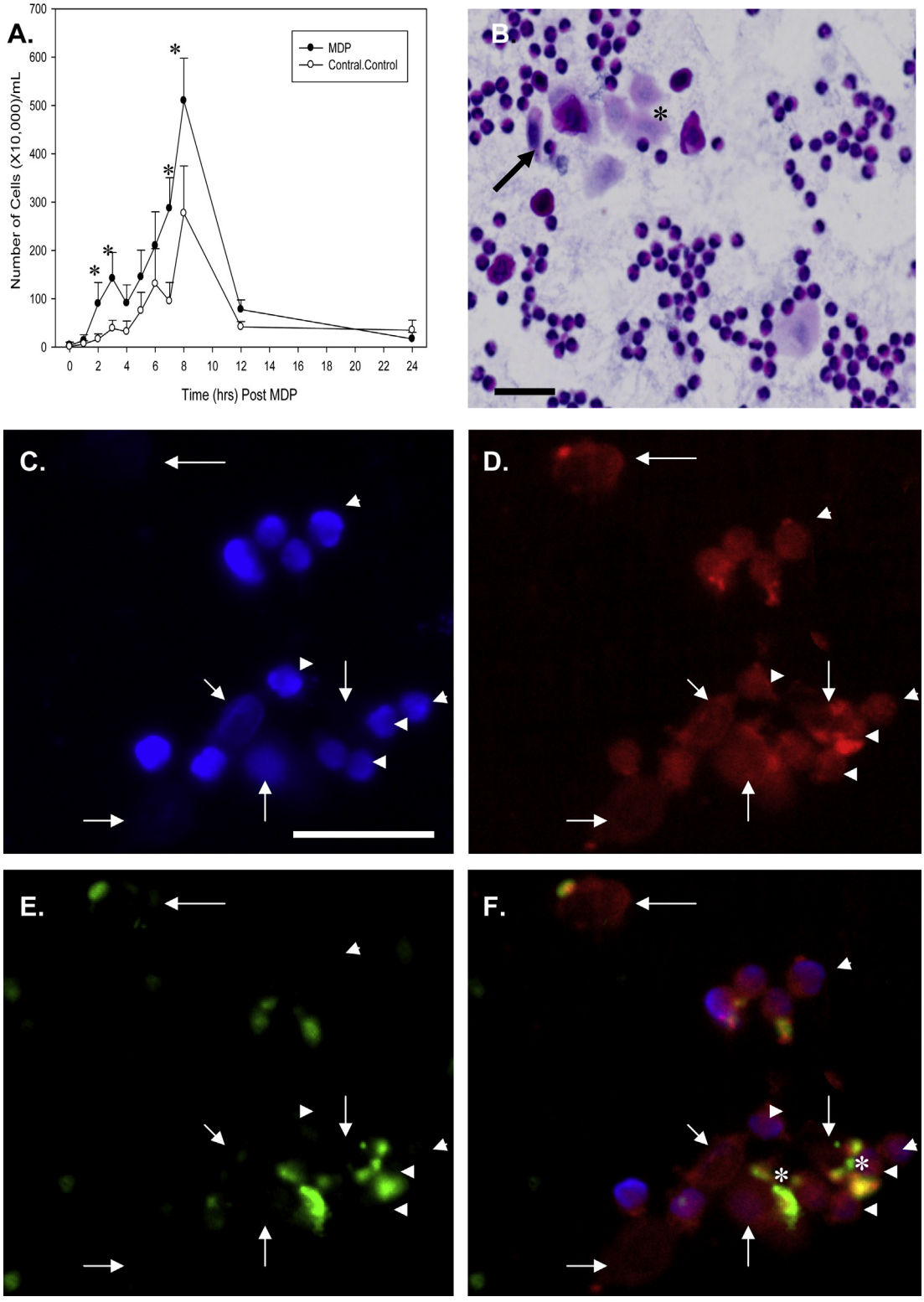

Fig. 8. Tear cytology. (A) Tear cell density (cells/1.0 μL) in MDP-treated and mock-treated contralateral control eyes at times post MDP application. (Each point is the average number of cells in tears of 3 rabbits; *; $p < 0.05$). (B) Tear sample collected at 5 h from a MDP-treated eye showing numerous band and segmented heterophils (PMN), with eosinophils, macrophages (arrow) and epithelial cells (*) (H&E). (C–F) Triple labeling for nuclei (blue), CRT- (green) and GGT- (red) positive cells in tear collected at 6 h from a MDP-treated eye. (C) Strong DAPI-positive (blue) heterophils (arrowheads) and weakly DAPI-positive presumed macrophages or epithelial cells (arrows). (D) Extrinsic GGT expression was detected on and between heterophils and macrophages/epithelial cells. (E) Cell-surface CRT was expressed on heterophils and macrophages. (F) The merged image shows areas of surface CRT co-localized with surface GGT between heterophils and/or macrophages (*). (Bar = 50 μm).

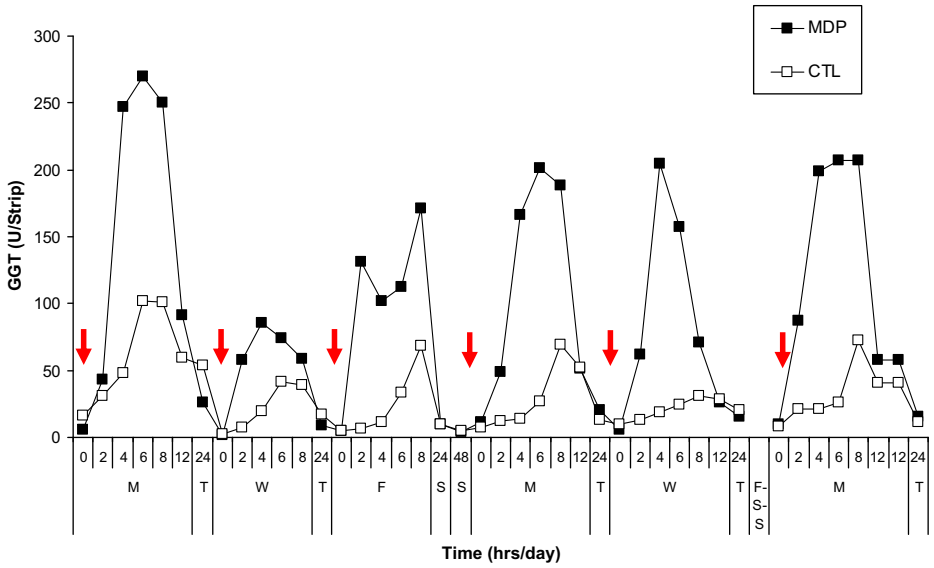

Fig. 9. Repeat unilateral MDP application. Bilateral Schirmer tear GGT activity levels after repeat unilateral topical MDP applications over the course of 16 days. Each point is the average GGT activity on Schirmer test strips from MDP-treated and contralateral untreated eyes of 3 rabbits. Arrows indicate times of topical MDP application.

cytopathology (Gaynor, 1973; Trinkaus-Randall et al., 1991). The decline in tear MDP coincided with the appearance of CRT-positive heterophils suggests MDP clearance by binding to CRT-positive heterophils. MDP binding to CRT on infiltrating leukocytes and conjunctiva-associated cells could affect intracellular calcium homeostasis, cellular stress responses, and innate immunity (Wang et al., 2012; Zeng et al., 2006) and/or induce caspase-mediated apoptosis with activation of IL-1β (Langford et al., 2011). The absence of chronic clinical symptoms after repeat MDP applications may be taken to argue against heterophilic cytopathology. Moreover, surface GGT and CRT were co-localized on the cell surface between tear heterophils and macrophages and conjunctival cells consistent with efferocytosis. That is, CRT expression by human PMN facilitates phagocytosis of live and apoptotic cells (Gardai et al., 2005; Thorp et al., 2011). Concomitantly, the function of the GGT activity in inflamed tear is unknown, but GGT is expressed by neutrophils, macrophages and conjunctival cells (Burnham et al., 2013; Coupland et al., 1993; Gukasyan et al., 2003) and is important to recapture of glutathione, the neutrophil response and resolution of inflammation (Carlisle et al., 2003; Grisk et al., 1993; Kobayashi et al., 2003). Additional studies are needed to ascertain the role of GGT/CRT-positive heterophils in this adult rabbit model of aseptic PCI.

The course of the recurrent bilateral acute conjunctivitis/iritis and Schirmer test tear GGT activity cycles produced following repeat topical MDP applications were temporally similar with resolution within 24 h. The recurrent PCI cycles were similar to the transient anterior uveitis cycles induced by repeat intravenous MDP injections (Li et al., 1993). However, GGT/cellular responses to subsequent topical MDP applications were less intense in the ipsilateral eyes, were similar in the contralateral eyes, and did not cause gross conjunctival pathologies. Cytopathological changes and/or chronic inflammatory disease in uveal, lung, joint, and intestinal tissues have been reported post repeat MDP applications over a more extended period (Gardner et al., 1991; Kuroe et al., 1996; Li et al., 1993; Richerson et al., 1982). The transient PCI course and resolution without sequelae likely depends on rapid MDP elimination.

Taken together, the results show topical application of MDP induces bilateral PCI in adult rabbits and suggest MDP-induced cytotoxicity/immunostimulation should be considered in persons with bilateral PCI of unknown etiology, particularly in the elderly with renal and/or lacrimal dysfunction (i.e., decreased capacity to eliminate MDP) and occult bacterial infection (increased capacity to generate MDP). The detection of GGT/CRT-positive rabbit heterophils in tears supports *in vivo* efferocytosis in eyes with MDP-induced pyogenic conjunctivitis. This reproducible rabbit model with tear GGT monitoring may be useful in investigating anti-inflammatory agents in the treatment of MDP-induced PCI.

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