Immunohistochemical and immunopathologic characterization of superficial stromal immune-mediated keratitis in horses

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Objective—To describe the immunopathologic characteristics of superficial stromal immune-mediated keratitis (IMMK) immunopathologically by characterizing cellular infiltrate in affected corneas of horses.

Animals—10 client-owned horses with IMMK.

Procedures—Immunohistochemical staining was performed on keratectomy samples with equine antibodies against the T-cell marker CD3 and B-cell marker CD79a (10 eyes) and the T-helper cytotoxic marker CD4 and T-cell cytotoxic marker CD8 (6 eyes). Percentage of positively stained cells was scored on a scale from 0 (no cells stained) to 4 (> 75% of cells stained). Equine IgG, IgM, and IgA antibodies were used to detect corneal immunoglobulin via direct immunofluorescence (10 eyes). Serum and aqueous humor (AH) samples from 3 horses with IMMK were used to detect circulating and intraocular IgG against corneal antigens via indirect immunofluorescence on unaffected equine cornea.

Results—Percentage scores (scale, 0 to 4) of cells expressing CD3 (median, 2.35 [range, 0.2 to 3.7]; mean ± SD, 2.36 ± 1.08) were significantly greater than scores of cells expressing CD79a (median, 0.55 [range, 0 to 1.5]; mean, 0.69 ± 0.72). All samples stained positively for CD4- and CD8-expressing cells, with no significant difference in scoring. All samples stained positively for IgG, IgM, and IgA. No serum or AH samples collected from horses with IMMK reacted with unaffected equine cornea.

Conclusions and Clinical Relevance—Pathogenesis of superficial stromal IMMK included cell-mediated inflammation governed by both cytotoxic and helper T cells. Local immunoglobulins were present in affected corneas; however, corneal-binding immunoglobulins were not detected in the serum or AH from horses with IMMK. (*Am J Vet Res* 2012;73:1067–1073)

Immune-mediated keratitis is a recurrent nonulcerative keratitis increasingly recognized as an ophthalmologic disease in horses. Common clinical signs include variable degrees of corneal neovascularization, cellular infiltrate, and edema, occurring more frequently unilaterally than bilaterally. Intraocular inflammation is consistently absent, and secondary corneal changes such as degeneration and fibrosis occur in chronic stages of the disease.[1,2] Histologic findings are supportive of the clinical signs, indicating predominantly lymphocytic-plasmacytic cellular infiltrate with stromal vascularization, edema, hyalinization, and necrosis.[a] Clinical evidence for a possible immune-mediated pathogenesis is provided by the failure to identify infectious agents on corneal cytologic evaluation and microbial cultures and by a positive response to immunosuppressive medications; however, the inciting antigens, mechanisms of activation, and specific nature of the resultant inflammatory response are unknown.

Abbreviations

AH	Aqueous humor
IF	Immunofluorescence
IHC	Immunohistochemistry
IMMK	Immune-mediated keratitis

Geographic differences in the clinical manifestation and response to treatment exist within the spectrum of diseases classified as IMMK. In the southeastern United States, a report[1] of a case series of 19 horses (22 eyes) with IMMK defined 3 clinical manifestations on the basis of the location of

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pathological changes within the cornea: superficial stromal, midstromal, and endothelial. Regardless of the specific location of pathological changes, none of the horses had a history of ocular trauma or previous ocular inflammation and all had a protracted disease course (mean duration, 11.8 ± 8.3 months) with minimal to no ocular pain.[1] Treatments for all horses consisted of combinations of topical administration of corticosteroids, NSAIDs, or cyclosporin as well as systemic administration of corticosteroidal anti-inflammatories or NSAIDs, and a subset of eyes received adjunctive surgical treatment.[1] In that case series,[1] eyes with superficial stromal or midstromal IMMK required either chronic topically applied medications or superficial keratectomy with conjunctival grafting, with progression more likely in eyes with midstromal disease. Endothelial disease was the most difficult to treat, necessitating either chronic medical treatment or penetrating keratoplasty, with a greater chance of progression relative to eyes with more superficial disease. The positive response to surgical treatment was confirmed in another case series,[b] in which no recurrence was reported in 12 of 12 surgically managed eyes.

In the United Kingdom, IMMK has slightly different clinical appearances than that seen in the United States; the appearances are divided into epithelial keratopathy, chronic superficial keratitis, chronic recurrent keratitis, and endothelial variants, with all forms consistently responding more favorably to topically administered immunomodulators without surgical treatment.[2] Epithelial keratopathy is a central epithelial opacity with islands of thickened and elevated epithelium, no vascularization, and only minor ocular pain, requiring only brief topical immunosuppressive treatment for resolution. Chronic superficial keratitis, similar to superficial stromal IMMK in the United States,[1] is initially restricted to the area under the upper eyelid or (less commonly) the third eyelid and responds favorably to chronic topical immunosuppressive treatment. Chronic recurrent keratitis consists of deep stromal edema as well as fibrovascularization, and although similar in appearance to midstromal IMMK in the United States,[1] it is commonly associated with ocular trauma. Additionally, recurrence is at regular intervals and active episodes may subside without treatment. The endothelial classification in the United Kingdom is clinically similar to that in the United States,[1] with central edema and deep stromal vascularization; however, chronic recurrent keratitis responds rapidly to topical administration of corticosteroids and does not recur.[2]

Classically, both effector T cells and antibodies are involved in immune-mediated disease processes. Tissue damage is predominantly caused by either effector T cells or antibodies; however, both are needed to stimulate and perpetuate inflammation.[3] Effector T cells, once presented with an autoantigen by an antigen-presenting cell, cause tissue damage through either a CD4+ T-helper or CD8+ T-cytotoxic response and an apoptosis-inducing response. Typically, T-cell involvement in ocular immune-mediated diseases is immunologically distinguished by a high CD4+:CD8+ ratio (T-helper:T-cytotoxic ratio),[4–7] whereas nondiseased pericorneal tissue (conjunctiva and lacrimal gland) is populated predominantly by T-cytotoxic cells.[8] In addition to T cells, antibodies (IgG, IgM, or IgA) cause local tissue damage through binding cellular or extracellular antigens.[3] This binding either leads to cell lysis or, if the cell is resistant to lysis, stimulates cytokine release; generates a respiratory burst; or activates the arachidonic acid cascade.[3] Additionally, antibodies bind extracellular matrix molecules, which also stimulate complement to activate local inflammation.[3] Increased regional and circulating antibodies have been identified in human ocular autoimmune diseases[9] as well as nonocular autoimmune diseases in veterinary medicine.[10]

Identification of the specific immunopathologic mechanisms involved in IMMK in horses will provide insight into the etiologies and development of the disease in its various forms and will also guide effective therapeutic options. The purposes of the study reported here were to investigate the immunopathologic characteristics of superficial stromal IMMK by use of keratectomy samples obtained from horses with clinical disease to classify the corneal cellular infiltrate as either B cell, T helper, or T cytotoxic; to identify IgG, IgM, or IgA within affected corneas; and to identify the presence or absence of circulating systemic or intraocular antibodies against normally present corneal antigens, thus indicating involvement of a systemic immunologic component in the disease process.

Materials and Methods

Case selection—The use of client-owned horses in the present study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the study protocol was reviewed and monitored by the North Carolina State University Institutional Animal Care and Use Committee. Written consent was obtained from each owner, following thorough explanation of the procedures, for cases in which anything other than routine treatment was performed. Samples used for the present study were collected from horses with nonulcerative superficial stromal IMMK and variable degrees of ≥ 1 characteristic clinical sign, including cellular infiltrate, neovascularization, edema, or secondary degenerative changes, all limited to the superficial third of the corneal stroma. In all horses, clinical signs had improved with topical or systemic administration of anti-inflammatory or immunosuppressive medications prior to referral, and most were receiving medications at the time of surgery but required a superficial keratectomy for definitive treatment. Because all horses were client-owned animals, medical treatment was not discontinued prior to surgery, so as to maintain whatever degree of disease control was present.

Sample collection—To collect keratectomy samples, horses were sedated with detomidine[c] (0.01 to 0.015 mg/kg, IV). The eyelids were regionally anesthetized and immobilized with auriculopalpebral and frontal nerve blocks by use of 2% lidocaine solution[d] (1 and 0.5 mL, respectively, SC), and a retrobulbar block was performed with 10 mL of 2% lidocaine solution[d] to provide extraocular muscle paralysis, as previously

described.[11] Following sterile surgical preparation and draping of the affected eye and topical instillation of proparacaine solution[e] (0.5 mL) for corneal and conjunctival anesthesia, a slightly curved 2- to 3-mm corneal incision was created with a No. 64 microsurgical blade to a depth just deeper than the affected cornea (20% to 40% of the total corneal thickness in all eyes), approximately 1 mm peripheral to the lesion. A Martinez lamellar corneal dissector was inserted through the incision parallel to the surface of the cornea and was used to separate the superficial affected cornea from the deeper normal (unaffected) cornea within 1 corneal lamellar plane, extending 1 mm peripheral to the lesion for 360°. Right and left curved corneal section scissors were used to extend the initial incision to the boundaries of the dissection until the keratectomy site was complete. Keratectomy samples were divided into 2 portions; one section was formalin fixed, and the other was placed in an optical cutting temperature compound, immersed in liquid nitrogen, snap frozen, and stored at –80°C for later sectioning. After surgery, the horses' eyes were treated with neomycin-polymixin-bacitracin ointment[f] (6.35-mm-long [0.25-inch-long] strip, topically, q 8 h) or neomycin-polymixin-gramicidin solution[g] (0.1 mL, topically, q 8 h) until epithelialization of the keratectomy site. Additionally, horses received flunixin meglumine[h] (1.1 mg/kg, PO, q 12 h), and 1% atropine ophthalmic ointment[i] (6.35-mm-long strip, topically, q 12 to 24 h) or ophthalmic solution[j] (0.1 mL, topically, q 12 to 24 h) was used as needed to maintain ocular comfort and minimize reflex uveitis.

To collect AH, paracentesis was performed at the limbus immediately prior to or following the keratectomy. Approximately 0.4 mL of AH was gently removed with a 27-gauge needle attached to a 1-mL syringe. Aqueous humor was stored at –80°C in Eppendorf tubes until analyzed.

Blood samples were collected immediately prior to or following the keratectomy via jugular venipuncture into serum separator tubes. Following centrifugation, the serum was collected and stored at –80°C in Eppendorf tubes until analyzed.

Light microscopy and immunostaining—Formalin-fixed corneas were dehydrated in graded alcohols, followed by xylene and were subsequently paraffin embedded. Paraffin-embedded and frozen samples were cut into 6-µm-thick sections. Paraffin-embedded sections were stained with H&E for histologic evaluation. Separate paraffin-embedded sections were immunostained via the streptavidin-biotin-peroxidase[k] method with antibodies against equine CD3[12] (T-cell) and CD79a[l] (B-cell) surface markers and viewed with 3,3′-diaminobenzidine as the chromogen, whereas frozen sections were immunostained via the streptavidin-biotin-peroxidase[k] method with antibodies against equine CD4 (T-helper) and CD8[m] (T-cytotoxic) cell surface markers and viewed with 3-amino-9-ethylcarbozole as the chromogen. To process paraffin-embedded samples for IHC, sections were placed on charged slides, deparaffinized, and rehydrated according to standard histologic techniques. Following enzyme retrieval with proteinase K, sections were incubated with a 1:150 (CD3) or 1:50 (CD79a) dilution of primary antibody for 30 minutes with EDTA-Tris antigen retrieval (pH, 9.0). To process frozen sections for IHC, tissues were fixed in acetone for 10 minutes and then blocked in 10% goat serum to reduce background staining. Sections were then incubated with a 1:50 dilution of primary antibody (CD4 or CD8) for 30 minutes at room temperature (approx 22°C), followed by incubation for 30 minutes with a biotinylated secondary antibody. Streptavidin-peroxidase conjugate was then added to paraffin-embedded and frozen sections and incubated for 30 minutes. After rinsing with PBS solution, the appropriate substrate-chromogen was added and the color developed for 9 minutes. Normal (unaffected) equine lymph node served as a positive control, whereas affected tissue incubated with PBS solution instead of the primary antibody and normal (unaffected) cornea incubated with the primary antibody served as negative controls.

Antibody (IgG, IgM, and IgA) presence within affected corneal samples was assessed by means of direct IF on paraffin-embedded sections.[10] Briefly, sections were deparaffinized, rehydrated, and digested with 0.1% trypsin[n] for 30 minutes at 37°C for antigen retrieval. Sections were then incubated in 10% fetal calf serum for 20 minutes to reduce background staining, followed by incubation in 1:100 dilutions of anti-horse IgG,[o] IgM, or IgA[p] polyclonal antibodies labeled with fluorescein isothiocyanate for 60 minutes at room temperature. Tissues were then mounted with a mounting medium[q] containing 4′,6-diamidino-2-phenylindole and examined with an epifluorescence microscope. Normal (unaffected) equine lymph node served as a positive control for direct IF studies, whereas affected tissue incubated with PBS solution instead of the primary antibody and normal (unaffected) cornea incubated with the primary antibody served as negative controls.

Antibody (IgG) directed against normal (unaffected) corneal tissue circulating in the serum and AH of horses with IMMK was assessed by indirect IF.[10] Briefly, frozen corneal sections obtained from a horse free of ophthalmic disease and euthanized for reasons unrelated to the present study were fixed in acetone and then blocked in 1% normal goat serum for 20 minutes. The sections were then incubated in 1:10, 1:100, 1:500, and 1:1,000 dilutions of the primary antibody (serum and AH samples collected from horses with IMMK) for 60 minutes and overnight at room temperature for each dilution, followed by a 1:200 dilution of secondary antibody (anti-horse IgG[o]) for 20 minutes at room temperature. Tissue sections were then mounted with the mounting medium[q] containing 4′,6-diamidino-2-phenylindole and examined with an epifluorescence microscope. Normal (unaffected) equine cornea incubated with serum and AH from a clinically normal horse and PBS solution served as negative controls.

Sample evaluation—All tissue sections were assessed by 1 observer (DOP), who was masked to the information about cases at the time of evaluation, following the establishment of grading criteria developed by the authors. The H&E-stained sections were graded for histologic signs of keratitis, including edema, cellular infiltrate, neovascularization, and corneal mineralization, on the basis of previously identified criteria[a] according to the following histologic grading scheme: 0

= normal (no abnormalities observed), 1 = mild, and 2 = marked. The IHC sections were assessed semiquantitatively by assigning a score on the basis of the percentage of mononuclear cells positively stained with each marker, according to the following scheme: 0 = no cells stained, 1 = > 0% to 25% cells stained, 2 = > 25% to 50% cells stained, 3 = > 50% to 75% cells stained, and 4 = > 75% cells stained. A semiquantitative score was used as opposed to cell counts because many of the samples had many positively stained cells oriented in thick mats, making a manual cell count inaccurate. For each corneal sample, the mean score for each lymphocyte marker in each of 10 hpfs (40× magnification) was calculated, and these scores were recorded as a mean ± SD for each lymphocyte marker in all cases combined. The location of positively stained cells within the cornea was noted for each sample (epithelial, basement membrane, or stromal). Direct IF– and indirect IF–stained tissue sections were assessed for location of staining (epithelial, basement membrane, or stromal) and for intracellular versus extracellular staining.

Statistical analysis—Analysis of IHC staining score data, specifically comparison between CD3 and CD79a as well as CD4 and CD8 positive cell-staining scores, was performed via a nonparametric Wilcoxon rank sum test by use of computerized statistical software.[r] Values of $P < 0.05$ were considered significant.

Results

Ten horses with IMMK met the inclusion criteria, including 7 geldings and 3 mares (10 eyes total). Breeds represented consisted of Warmblood (n = 5), Thoroughbred (2), Paint Horse (2), and Quarter Horse (1). The mean duration of clinical signs prior to superficial keratectomy was 17.7 ± 13.4 months (median, 12 months; range, 0.5 to 36 months). Nine of the 10 horses received topical treatment with at least 1 ophthalmic anti-inflammatory or immunosuppressive medication at the time of surgery. Clinical signs included nonulcerative keratitis with variable degrees of corneal cellular infiltrate, neovascularization, edema, and secondary corneal degenerative changes, without signs of pain or uveitis (**Table 1**).

Findings from histologic evaluation of H&E-stained corneal sections from each of 10 eyes were consistent with IMMK. Edema and cellular infiltrate were present in corneal samples from all 10 eyes, neovascularization was present in corneal samples from 5 of 10 eyes, and corneal degeneration was present in corneal samples from 3 of 10 eyes. The mean scores (scale, 0 [normal] to 2 [marked]) for severity of edema and cellular infiltrate were 1.73, and the mean scores for severity of neovascularization and corneal degeneration were 0.73 (data not shown). Cellular infiltrate was composed mainly of lymphocytes with fewer plasma cells and macrophages and infrequent neutrophils.

Immunohistochemical evaluation of paraffin-embedded sections identified cells expressing CD3 in corneal samples from all 10 eyes (**Figure 1**), but only corneal samples from 6 of 10 eyes contained cells staining positively for CD79a (Table 1). For all samples combined, the mean ± SD and median scores (scale, 0 to 4) for the percentage of cells staining positively for CD3 was 2.36 ± 1.08 and 2.35 (range, 0.2 to 3.7), respectively, which was significantly ($P = 0.01$) higher than the mean ± SD and median scores of 0.69 ± 0.72 and 0.55 (range, 0 to 1.5), respectively, for CD79a. Cells expressing CD3 were identified in all regions of the cornea, with more corneal samples having stromal (10/10) rather than epithelial (6/10) or basement membrane (3/10) staining. Cells expressing CD79a were identified in all regions of the cornea, with more samples having basement membrane (6/10) rather than epithelial (1/10) or stromal (5/10) staining. Positive immunostaining was not identified in any of the control samples.

Immunohistochemical evaluation of frozen sections identified cells staining positively for CD4 (**Figure 2**) or CD8 in 6 of 6 corneal sections (Table 1). For all samples combined, the mean ± SD and median scores (scale, 0 to 4) for the percentage of cells staining positively for CD4 (mean ± SD, 1.78 ± 0.78; median, 1.95 [range, 0.8 to 2.7]) were not significantly different than those for CD8 staining (mean ± SD, 2.3 ± 1.22; me-

Table 1—Signalment, clinical manifestation, treatment regimen, and IHC staining scores of 10 horses with superficial stromal IMMK.

	Horse											
Variable	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	**Median (range)**
Sex	MC	MC	MC	MC	F	MC	MC	F	MC	F	NA	NA
Breed	TB	WB	QH	WB	TB	WB	P	WB	WB	P	NA	NA
Duration (mo)	18	UK	24	12	12	0.5	6	2	36	30	15.6 ± 12.4	12 (0.5–36)
Clinical signs	V, I	V, I	I, E	V, I, E	V, I	I, E	V, I	V, I, E	V, I, E	V, I, E	NA	NA
Treatment*	D	None	D	D, B, C, FM	C, FM	C, FM	D, C	B, C	C	PA	NA	NA
CD3†	3.5	1.6	1.8	2.4	0.2	3.7	3.3	2.3	1.7	3.1	2.36 ± 1.08‡	2.35 (0.2–3.7)‡
CD79a†	0	0.1	1.5	1.3	1.5	0	0	1.5	0	1	0.69 ± 0.72‡	0.55 (0–1.5)‡
CD4†	ND	ND	ND	ND	2.1	2.4	0.8	2.7	1.8	0.9	1.78 ± 0.78	1.95 (0.8–2.7)
CD8†	ND	ND	ND	ND	2.8	3	0.6	4	2	1.4	2.3 ± 1.22	2.4 (0.6–4)

*At the time of surgery. †Staining scores on a scale of 0 to 4. ‡Significant ($P < 0.05$) difference between scoring values between CD3 and CD79a.

B = Topically administered bromfenac (0.09%). C = Topically administered cyclosporin (0.02%). D = Topically administrated neomycin-polymyxin-dexamethasone. E = Corneal edema. F = Female. FM = Systemic administration of flunixin meglumine. I = Corneal cellular infiltrate. MC = Male castrated. NA = Not applicable. ND = Not determined. P = Paint Horse. PA = Topically administered prednisolone acetate (1%). QH = Quarter Horse. TB = Thoroughbred. UK = Unknown. V = Corneal vascularization. WB = Warmblood.

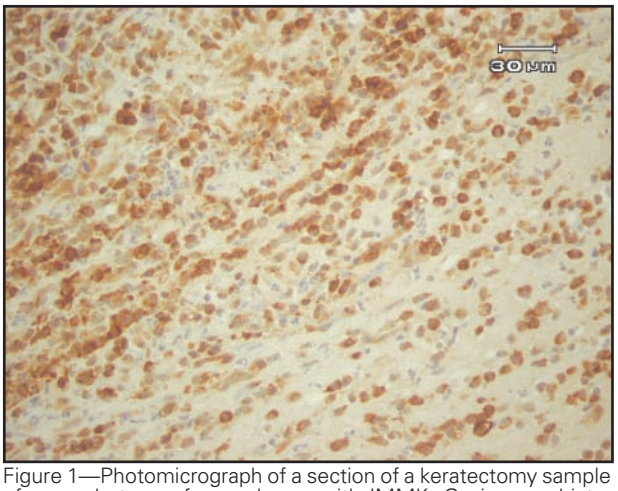

Figure 1—Photomicrograph of a section of a keratectomy sample of corneal stroma from a horse with IMMK. On immunohistochemical evaluation, brown staining of mononuclear cells is indicative of CD3 antigen and therefore T-cell infiltrate. Bar = 30 µm.

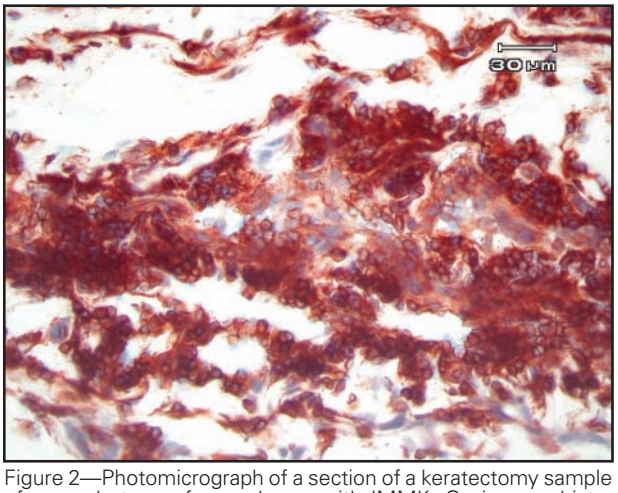

Figure 2—Photomicrograph of a section of a keratectomy sample of corneal stroma from a horse with IMMK. On immunohistochemical evaluation, red staining of mononuclear cells is indicative of CD4 antigen and therefore T-helper infiltrate. Bar = 30 µm.

dian, 2.4 [range, 0.6 to 4]). Cells expressing CD4 were identified in all regions of the cornea, with more samples having stromal (6/6) rather than epithelial (3/6) or basement membrane (2/6) staining. Cells expressing CD8 were identified in all regions of the cornea, with more samples also having stromal (6/6) rather than epithelial (4/6) or basement membrane (1/6) staining. Positive immunostaining was not identified in any of the control samples.

Direct IF performed with equine-specific antibodies against IgG, IgM, and IgA identified positive staining for each antibody in all corneal sections. Immunoglobulin G and IgM staining was identified in the epithelium, basement membrane, and stroma, whereas IgA staining was identified only in the epithelium and stroma. Several staining patterns were present, as determined on the basis of localization within the cornea. All epithelial staining was an extracellular mesh-like (fishnet) pattern (**Figure 3**), whereas basement membrane staining appeared as strong linear staining (paintbrush stroke). Stromal staining occurred in 2 patterns: in sections with substantial cellular infiltrate, strong extracellular staining surrounded

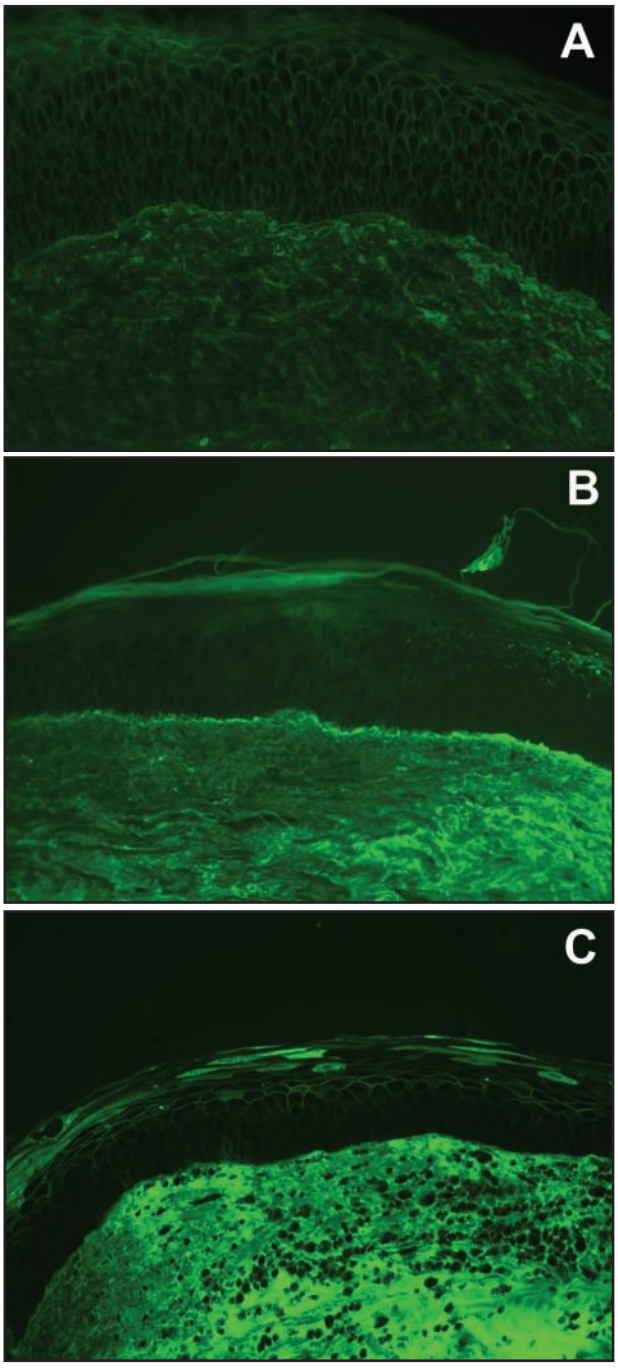

Figure 3—Photomicrographs of sections of keratectomy samples of corneal stroma and epithelium from horses with IMMK. An epifluorescent camera is used to view fluorescent staining indicative of IgG antigen. Notice the extracellular epithelial and diffuse stromal staining (A), the strong linear basement membrane staining (paintbrush stroke; B), and extracellular staining surrounding stromal cellular infiltrate (C).

inflammatory cells, whereas in sections without cellular infiltrate, extracellular staining appeared fibrillar, similar to the histologic appearance of stromal edema. Positive immunoglobulin staining was not identified in any of the normal (unaffected) control samples. Results of indirect IF for identification of antibody directed at corneal antigens performed on 3 AH samples and 3 serum samples were all negative.

Discussion

Results of the study reported here indicated that, in conjunction with nonspecific histopathologic abnormalities of corneal cellular infiltrate, edema, neovascularization, and secondary degenerative changes, keratectomy samples from horses with IMMK have immunopathologic findings suggestive of a predominantly T-cell–driven process. Immunohistochemical evaluation identified a significantly higher score for cells staining positively for CD3, compared with those staining positively for CD79a, with no significant difference in the degree of positive staining for CD4 versus CD8 lymphocyte markers, indicating involvement of both T-helper and T-cytotoxic cells. Additionally, immunoglobulins were detected within diseased cornea, suggestive of local humoral involvement; however, neither circulating serum nor AH antibodies against these antigens were detected, suggesting that systemic humoral immunity may not be a component of IMMK.

T-cell driven immune reactions are involved in other veterinary ophthalmic autoimmune diseases, most notably recurrent uveitis in horses and chronic superficial keratitis in dogs. Gilger et al[13] found a substantial amount of positive staining for CD5 lymphocyte surface markers, indicative of T-cell infiltration, in the uvea of horses with recurrent uveitis, with CD4-positive T-helper cells 2.5 times as prevalent as CD8-positive T-cytotoxic cells. Immunohistochemical studies[14] in dogs of chronic superficial keratitis have shown similar results, with major histocompatibility class II expression further supporting the role of CD4 cells. Although the present study showed a significantly greater degree of T-cell (vs B-cell) staining, we did not find a significant difference between the degree of CD4+ and CD8+ stained cells. This may suggest that immunopathologic changes in IMMK are routinely mediated by both T-helper and T-cytotoxic cells. Another possibility for the variation may be the current medical treatment being administered to the horses of the present study, given that 9 of the 10 eyes were being treated topically with at least 1 ophthalmic anti-inflammatory or immunosuppressive medication at the time of surgery. However, these medications may have affected the local immune response and impacted the results obtained; because of the small number of horses in the present study, comparison of eyes on the basis of previous treatment was not a statistically viable option. The single horse not receiving medical treatment at the time of surgery had a staining pattern consistent with the overall results, with greater CD3 staining and negligible CD79a staining (Table 1). Although it would be ideal to have large numbers of corneal samples from patients affected with IMMK currently not receiving medical treatment, such a study design was not possible with client-owned animals in the stages of disease seen at our referral practice. An additional possibility for the variation in T-cell population may be due to the differing duration of clinical signs among horses. The mean history of clinical signs in horses with IMMK was 17.7 months; however, the range was 0.5 to 36 months; thus, it is possible that disease progression over time influenced the type and degree of cellular infiltrate. Again, the number of horses was too small to evaluate the effect of the duration of clinical signs on the staining properties.

Findings from direct IF revealed immunoglobulins in keratectomy samples from horses with IMMK, suggesting involvement of local humoral immunity in the present study; however, the correlation between the staining observed from IF and the clinical, histopathologic, and immunohistochemical findings was poor. Extracellular staining for immunoglobulins was present throughout the epithelium and stroma of keratectomy samples from all horses, although clinically, all horses with IMMK had stromal keratitis without epithelial involvement. Also unexpected was the diffuse nature of immunoglobulin staining, given that histologic evaluation and IHC revealed focal mononuclear cell staining. Although it is unknown what, if any, importance the patterns of staining observed in the present study indicated, a possible explanation for the findings is nonspecific staining for irrelevant immunoglobulins not involved in the pathogenesis of IMMK but present in the stromal edema, as was suspected for a similar finding in a previous study[15] investigating corneal IgG in chronic superficial keratitis in dogs. Results of that study[15] indicate that staining is attributed to irrelevant IgG, given the lack of correlation between clinical lesion severity and histopathologic location or degree of staining. Although it remains possible that local humoral immunity may be involved in IMMK, indirect IF revealed no evidence of anticorneal IgG in either the serum or AH of horses with IMMK, suggesting systemic humoral immunity may be of limited involvement. At this time, the role of immunoglobulins and humoral immunity is of unknown importance in IMMK.

Resolution of IMMK may be achieved by surgical removal of the affected tissue, with or without placement of a conjunctival graft.[a] In another study,[1] medical treatment was discontinued without disease recurrence during the mean follow-up time of 13.3 ± 11.5 months in 4 eyes with superficial stromal IMMK treated with a keratectomy and conjunctival graft. One explanation for this positive response is that the offending antigen or the sensitized immune cells stimulating inflammation are localized within the cornea and are removed with surgery. An additional explanation is that the blood vessels, growth factors, and fibroblasts provided by the conjunctival graft favorably impacted the healing process.[16] It is important to note that although vessels are beneficial in healing deep or infected corneal wounds, they contribute substantially to the breakdown of ocular immune privilege.[17] Thus, it would be expected that increased vascularization associated with conjunctival grafts might in fact perpetuate an immune-mediated inflammatory episode. Because horses with IMMK included in the present study received superficial keratectomy without conjunctival or other grafting procedures and long-term follow-up was beyond the scope of the present study, definitive statements about the impact of grafting procedures on the overall immune response cannot be made.

A noteworthy limitation of the present study is that all horses were clinically affected client-owned animals and all but 1 horse were receiving various regimens of anti-inflammatory or immunosuppressive medications.

An additional limitation of the present study was the small number of horses with IMMK, which restricted our ability to further categorize the horses with IMMK to determine patterns or subsets of the disease and to draw conclusions regarding the effect of previous medical treatment or duration of clinical signs on the histologic and staining properties of keratectomy samples.

In conclusion, the pathogenesis of IMMK in horses examined in the present study was predominantly T-cell driven, with both cytotoxic and helper T-cell involvement. Additionally, immunoglobulins were present in keratectomy samples; however, corneal-binding immunoglobulins were not detected in the serum or AH of horses with IMMK, leading to uncertainty regarding the involvement of humoral immunity in the disease process.

a. Pate DO, Gilger BC, Clode AB. Histologic evaluation of equine immune-mediated keratitis (abstr), in *Proceedings*. 1st Annu Meet Int Equine Ophthalmol Consort 2009;18.
b. Pate DO, Clode AB, McMullen RM, et al. Superficial keratectomy for the treatment of equine immune mediated keratitis (abstr), in *Proceedings*. 40th Annu Meet Am Coll Vet Ophthal 2009;395.
c. Detomidine hydrochloride (DORMOSEDAN), Pfizer Animal Health, Exton, Pa.
d. Lidocaine hydrochloride 2%, Phoenix Scientific Inc, St Joseph, Mo.
e. Proparacaine hydrochloride ophthalmic solution, Akorn Pharmaceuticals, Lake Forest, Ill.
f. Neomycin-polymixin-bacitracin ophthalmic ointment, Bausch & Lomb Inc, Rochester, NY.
g. Neomycin-polymixin-gramicidin ophthalmic solution, Bausch & Lomb Inc, Rochester, NY.
h. Flunixin meglumine injectable (Banamine), Schering Plough Animal Health, Boxmeer, The Netherlands.
i. Atropine 1% ophthalmic ointment, Falcon Pharmaceuticals, Fort Worth, Tex.
j. Atropine 1% ophthalmic solution, Falcon Pharmaceuticals, Fort Worth, Tex.
k. ABC kit, Vector Laboratories Inc, Burlingame, Calif.
l. Anti-horse CD3, CD79a antibodies, Dako, Carpinteria, Calif.
m. Anti-horse CD4, CD8 antibodies, VMRD Inc, Pullman, Wash.
n. 1% Trypsin, Sigma Chemicals, St Louis, Mo.
o. Anti-horse IgG, Jackson ImmunoResearch Laboratories Inc, West Grove, Pa.
p. Anti-horse IgM, IgA, Bethyl Laboratories Inc, Montgomery, Tex.
q. Vectashield DAPI, Vector Laboratories, Burlingame, Calif.
r. JMP, version 8.0, SAS Institute Inc, Cary, NC.

References

1. Gilger BC, Michau TM, Salmon JH. Immune-mediated keratitis in horses: 19 cases (1998–2004). *Vet Ophthalmol* 2005;8:233–239.
2. Matthews A, Gilger BC. Equine immune-mediated keratopathies. *Vet Ophthalmol* 2009;12(suppl 1):10–16.
3. Murphy KM, Travers P, Walport M, et al. *Janeway's immunobiology*. 7th ed. New York: Garland Science, 2008.
4. Williams DL. Histological and immunohistochemical evaluation of canine chronic superficial keratitis. *Res Vet Sci* 1999;67:191–195.
5. Deeg CA, Kaspers B, Gerhards H, et al. Immune responses to retinal autoantigens and peptides in equine recurrent uveitis. *Invest Ophthalmol Vis Sci* 2001;42:393–398.
6. Baum H, Staines NA. MHC-derived peptides and the CD4+ T-cell repertoire: implications for autoimmune disease. *Cytokines Cell Mol Ther* 1997;3:115–125.
7. Chen YT, Li S, Nikulina K, et al. Immune profile of squamous metaplasia development in autoimmune regulator-deficient dry eye. *Mol Vis* 2009;15:563–576.
8. Knop E, Knop N. Anatomy and immunology of the ocular surface. *Chem Immunol Allergy* 2007;92:36–49.
9. Przyklang U, Nolle B, Reinhold-Keller E, et al. Indirect immunofluorescence study of the cornea and the conjunctiva in a xenogeneic system: analysis of humoral reactivity in patients with Wegener's granulomatosis. *Graefes Arch Clin Exp Ophthalmol* 1996;234:79–86.
10. Olivry T, Joubeh S, Dunston SM, et al. Desmoglein-3 is a target autoantigen in spontaneous canine pemphigus vulgaris. *Exp Dermatol* 2003;12:198–203.
11. Gilger BC, Stoppini R. Equine ocular examination. In: Gilger BC, ed. *Equine ophthalmology*. 2nd ed. Maryland Heights, Mo: Elsevier, 2011;1–51.
12. Jones M, Cordell JL, Beyers AD, et al. Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies. *J Immunol* 1993;150:5429–5435.
13. Gilger BC, Malok E, Cutter KV, et al. Characterization of T-lymphocytes in the anterior uvea of eyes with chronic equine recurrent uveitis. *Vet Immunol Immunopathol* 1999;71:17–28.
14. Williams DL. Major histocompatibility class II expression in the normal canine cornea and in canine chronic superficial keratitis. *Vet Ophthalmol* 2005;8:395–400.
15. Eichenbaum JD, Lavach JD, Gould DH, et al. Immunohistochemical staining patterns of canine eyes affected with chronic superficial keratitis. *Am J Vet Res* 1986;47:1952–1955.
16. Gelatt KN, Gelatt JP. *Handbook of small animal ophthalmic surgery*. New York: Pergamon, 1994.
17. Dana MR, Streilein JW. Loss and restoration of immune privilege in eyes with corneal neovascularization. *Invest Ophthalmol Vis Sci* 1996;37:2485–2494.