

Characterization of IgE-mediated cutaneous immediate and late-phase reactions in nonallergic horses

Michelle C. Woodward, DVM; Frank M. Andrews, DVM, MS; Michael T. Kearney, MS; Fabio Del Piero, DVM, PhD; Bruce Hammerberg, DVM, PhD; Cherie M. Pucheu-Haston, DVM, PhD

Objective—To characterize the response of skin of nonallergic horses following ID injection of polyclonal rabbit anti-canine IgE (anti-IgE) and rabbit IgG.

Animals—6 healthy horses.

Procedures—Skin in the cervical area was injected ID with anti-IgE and IgG. Wheal measurements and skin biopsy specimens were obtained before and 20 minutes and 6, 24, and 48 hours after injection. Tissue sections were evaluated for inflammatory cells at 4 dermal depths. Immunohistochemical analysis for CD3, CD4, and CD8 was performed, and cell counts were evaluated.

Results—Anti-IgE wheals were significantly larger than IgG wheals at 20 minutes and 6 and 24 hours after injection. There were significantly more degranulated mast cells after anti-IgE injection than after IgG injection. There were significantly more eosinophils at 6, 24, and 48 hours and neutrophils at 6 hours after anti-IgE injection, compared with cell numbers at those same times after IgG injection. There were significantly more eosinophils in the deeper dermis of anti-IgE samples, compared with results for IgG samples. No significant differences between treatments were detected for CD3⁺, CD4⁺, or CD8⁺ cells.

Conclusions and Clinical Relevance—Injection of anti-IgE antibodies was associated with the development of gross and microscopic inflammation characterized by mast cell degranulation and accumulation of inflammatory cells, particularly eosinophils and neutrophils. This pattern appeared to be similar to that of horses with naturally developing allergic skin disease, although lymphocytes were not increased; thus, ID injection of anti-IgE in horses may be of use for evaluating allergic skin diseases of horses. (*Am J Vet Res* 2014;75:633–641)

Hypersensitivity disorders in horses are commonly associated with the development of skin disease. These allergic diseases, such as IBH, atopic dermatitis, and urticaria, cause variable signs in horses, including pruritus, signs of pain, and changes to the coat.¹ Horses with severe disease can lose body condition and may have substantial decreases in athletic performance.

Received January 9, 2014.

Accepted February 18, 2014.

From the Department of Veterinary Clinical Sciences (Woodward, Pucheu-Haston), Equine Health Studies Program and Department of Veterinary Clinical Sciences (Andrews), and Department of Pathobiological Sciences (Kearney, Del Piero), School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803; and the Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607 (Hammerberg).

This manuscript represents a portion of a thesis submitted by Dr. Woodward to the Louisiana State University Department of Veterinary Clinical Sciences as partial fulfillment of the requirements for a Master of Science degree.

Supported in part by a research grant from the Charles V. Cusimano Equine Health Studies Program, Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University. Presented in abstract form at the 2013 North American Veterinary Dermatology Forum, Louisville, April 2013.

Address correspondence to Dr. Woodward (woodwamc@gmail.com).

ABBREVIATIONS

Anti-IgE	Polyclonal rabbit anti-canine IgE
IBH	Insect bite hypersensitivity
T _H 2	T-helper type 2

The prevalence of most allergic skin diseases of horses is unknown, except for IBH, which has a reported prevalence of 11.6% to 71.4%.^{2–6} Research in the field of allergic skin disease in horses has been hampered, at least in part, by the need for a practical model of the disease. In the ideal situation, horses with spontaneous allergic skin disease would be used for study. However, it is difficult to develop a herd of sensitized horses with allergic skin disease, and long-term maintenance of such a herd is expensive.

In addition, generation of allergen-specific responses in sensitized horses typically requires the administration of commercial allergen extracts. Many of these extracts may contain proteolytic compounds or induce nonspecific inflammatory responses, even in nonsensitized horses.⁷ Thus, it would be desirable to develop a model of the disease in nonallergic horses that consistently and easily mimics many or all of the changes associated with spontaneous disease, without

the additional variables associated with administration of allergen extracts. Researchers have effectively developed models of cutaneous allergic inflammation in other species, including dogs^{8,9} and humans.^{10–16}

Briefly, allergic skin disease is often mediated by type I hypersensitivity reactions. These reactions (immediate and late-phase hypersensitivity) are initiated by antigen exposure. On exposure, naïve antigen-specific T cells are induced to differentiate into T_H2 phenotype cells, which then activate B cells. The B cells undergo class switching and produce IgE, which binds to the high-affinity receptor for IgE (ie, FcεRI) on the surface of mast cells. During subsequent exposure to the same antigen, the surface-bound IgE is cross-linked and the mast cells become activated. Mast cells release mediators (histamine, cytokines, and leukotrienes) that lead to the development of immediate and late-phase hypersensitivity reactions. Late-phase reactions are characterized by an influx of inflammatory cells, including neutrophils, eosinophils, basophils and T cells, 6 to 48 hours after challenge exposure to the allergen.^{8,17,18}

Atopic dermatitis¹ and IBH^{1,19} in horses involve type I hypersensitivity reactions. The involvement of IgE, specifically, has been suggested^{7,20} and established^{21,22} in allergic skin disease of horses. Horses affected by IBH have increased expression of both IgE protein and mRNA in dermal cells of affected skin, compared with results for unaffected skin of clinically normal horses, especially in the acute phase.²⁰ Examination of skin biopsy specimens obtained from horses with urticaria also revealed that there were more IgE-bearing cells in the superficial dermis, compared with the number of those cells in skin biopsy specimens obtained from clinically normal horses.²²

Type I hypersensitivity reactions can be simulated by ID injection of anti-IgE antibodies,²³ which functions to cross-link surface IgE and mimic the effects of an allergen. This reaction has been used in humans to evaluate the effects of drugs on IgE-mediated reactions.^{10–16} In dogs, a model of allergic dermatitis has been successfully developed by the use of anti-IgE.^{8,9} To our knowledge, such a model of allergic dermatitis in horses has not been developed.

The objective of the study reported here was to develop a method that would mimic allergic dermatitis in horses. The hypothesis was that ID injection of anti-IgE in clinically normal horses would result in macroscopic and microscopic reactions similar to those of horses with allergic skin disease. Additionally, these reactions would be comparable to those observed in dogs after injection of anti-IgE.

Materials and Methods

Animals—Six horses (3 geldings and 3 mares; 4 to 8 years old) were used for the study. Horses were chosen on the basis of a lack of evidence of current or prior cutaneous or systemic disease (including allergic skin disease) as determined during physical examinations. All horses were vaccinated against eastern equine encephalomyelitis, western equine encephalomyelitis, West Nile virus encephalomyelitis, tetanus, and influenza and dewormed with a product containing ivermectin or moxidectin (200 µg/kg, PO, q 6 mo) as part

of routine herd care. No medications were administered for at least 14 days prior to the study. All experimental protocols were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Generation of IgE-mediated reactions—Hair was shaved from the lateral cervical area of each horse on day 0 in preparation for injections in this location. Horses were sedated by IV administration of xylazine hydrochloride^a (0.4 mg/kg). All horses were injected ID (0.1 mL/injection) at 1 site with PBS solution, at 4 sites with protein G affinity-purified anti-IgE²⁴ (prepared by one of the authors [BH]) diluted to 0.1 mg/mL in PBS solution, and at 4 sites with rabbit IgG^b diluted to 0.1 mg/mL in PBS solution (time of injections was designated as time 0). Anti-canine IgE was used because anti-equine IgE was not available. Furthermore, preliminary experiments conducted by the authors (data not shown) indicated the ability of anti-canine IgE to induce development of gross and microscopic immediate and late-phase reactions in the skin of clinically normal horses. In those preliminary experiments, ID injection of the same anti-IgE product (at a lower dose) into 4 horses resulted in wheals and histopathologic changes consistent with those observed in horses with allergic skin disease.

Rabbit antisera specific for canine IgE was generated by injection of canine monoclonal IgE^c produced by a canine × mouse heterohybridoma.²⁵ Antisera were adsorbed with canine IgG linked to beads to remove antibodies cross-reactive with IgG.

Grading of macroscopic reactions—Sites of anti-IgE and IgG injections were examined 20 minutes and 6, 24, and 48 hours after injection for the development of cutaneous swelling, erythema, or induration. Sites of injection with PBS solution were measured 20 minutes after injection. Diameter of the cutaneous reactions (ie, wheals) was measured in 2 perpendicular directions, and surface area of each reaction was calculated. Dermal thickness of the injected skin was measured with calipers.

Specimen collection and processing—Horses were sedated with xylazine, and areas for acquisition of biopsy specimens were injected SC with 0.5 mL of 2% lidocaine hydrochloride.^d One 8-mm punch biopsy specimen of clinically normal, noninjected (ie, no ID injections) skin on the lateral aspect of the neck was collected at the time of ID injections. Samples were collected at anti-IgE and IgG injection sites 20 minutes and 6, 24, and 48 hours after injection. Each sample was bisected immediately after collection. One half was placed in neutral-buffered 10% formalin for routine processing in paraffin. Fixation time differed but was at least 72 hours. The other half was placed in cryopreservation embedding medium,^e frozen in isopentane cooled in liquid nitrogen, and stored at –80°C until cryosectioning.

Histologic examination—Tissue sections (thickness of 5 µm) were cut from paraffin-embedded blocks, stained with H&E, and used for histologic examination, cell enumeration, and pattern analysis. Luna stain was used to facilitate evaluation of eosinophils.²⁶ A low-

pH (pH, 2.5) toluidine blue stain was used to facilitate evaluation of mast cells.

Immunophenotypical analysis of cutaneous mononuclear cells—A modification of a 3-step streptavidin-labeled method²⁷ was used to characterize the mononuclear cell infiltrate. Briefly, 6- μ m-thick cryosections were air-dried and then immersed in acetone for fixation. Samples were rinsed with PBS solution containing 0.5% Tween 20^f and then blocked with 1% fetal calf serum. Blocking sera were drained, and mouse-origin monoclonal antibodies specific for equine CD4 or CD8 β chain^g were diluted 1:70 in PBS solution and applied to the slides. After the slides were rinsed with PBS solution containing 0.5% Tween 20,^f endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in PBS solution. Biotinylated horse-origin anti-mouse IgG^h diluted 1:400 in PBS solution was applied, which was followed by application of horseradish peroxidase-conjugated streptavidinⁱ diluted 1:400 in PBS solution. Samples were rinsed between steps with PBS solution containing 0.5% Tween 20. Amino-9-ethyl carbazole^j was applied as a chromogen, which was followed by application of hematoxylin counterstain.^k

For CD3 immunohistochemical analysis, slides were deparaffinized by immersion in xylene (3 times, 5 min/immersion, with fresh xylene used for each immersion). Samples were then rehydrated in 100% alcohol (3 times, 5 min/immersion, with fresh alcohol used for each immersion) and then immersion in 95% alcohol for 5 minutes; rehydrated samples were rinsed 3 times with deionized water. Antigen retrieval was performed by immersion in 10mM citrate buffer (pH, 6.0) for 10 minutes at 125° to 127°C, and slides then were allowed to cool for 20 minutes before being rinsed 3 times (each rinse consisted of deionized water followed by Tris-buffer with 1% Tween 20 [pH, 7.6]). The remainder of the procedure was performed in an

autostainer.^l Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 3 minutes, which was followed by rinsing with Tris-buffer with 1% Tween 20. Binding of nonspecific antibodies was blocked by incubation with normal goat serum^m for 30 minutes followed by incubation with primary antibody (rabbit anti-human CD3ⁿ diluted 1:400) for 30 minutes, which was followed by rinsing with Tris-buffer with 1% Tween 20. Samples then were incubated with secondary antibody (biotinylated anti-rabbit secondary antibody^o) for 30 minutes and rinsed with Tris-buffer with 1% Tween 20. Tissue samples were exposed to the detection reagent avidin-biotinylated enzyme complex^p for 30 minutes, which was followed by rinsing with Tris-buffer with 1% Tween 20 and application of membrane stain^q and by additional rinsing with Tris-buffer with 1% Tween 20 followed by deionized water. Samples were counterstained by incubation with Mayer's hematoxylin (Lillie's modification)^k for 5 minutes, rinsed again with Tris-buffer with 1% Tween 20 followed by deionized water, and removed from the stainer. Slides were dried in an oven at 60°C for 30 min-

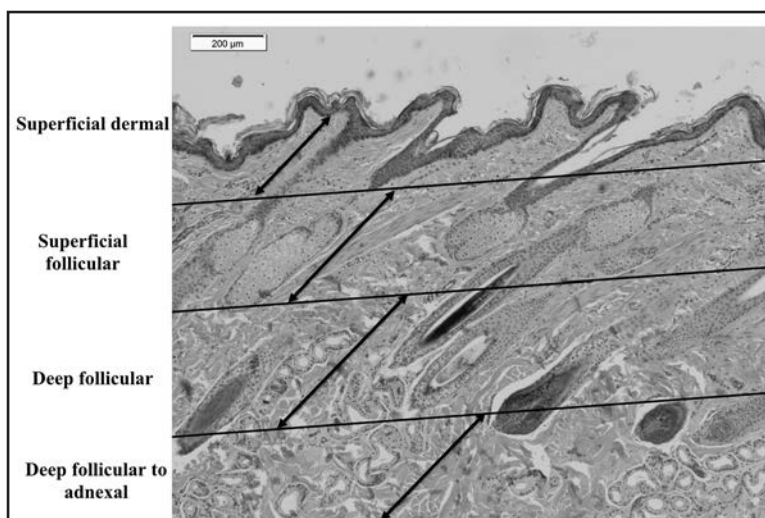


Figure 1—Photomicrograph of a section of skin indicating dermal depths used to evaluate inflammatory cell location in horses. Bar = 200 μ m.

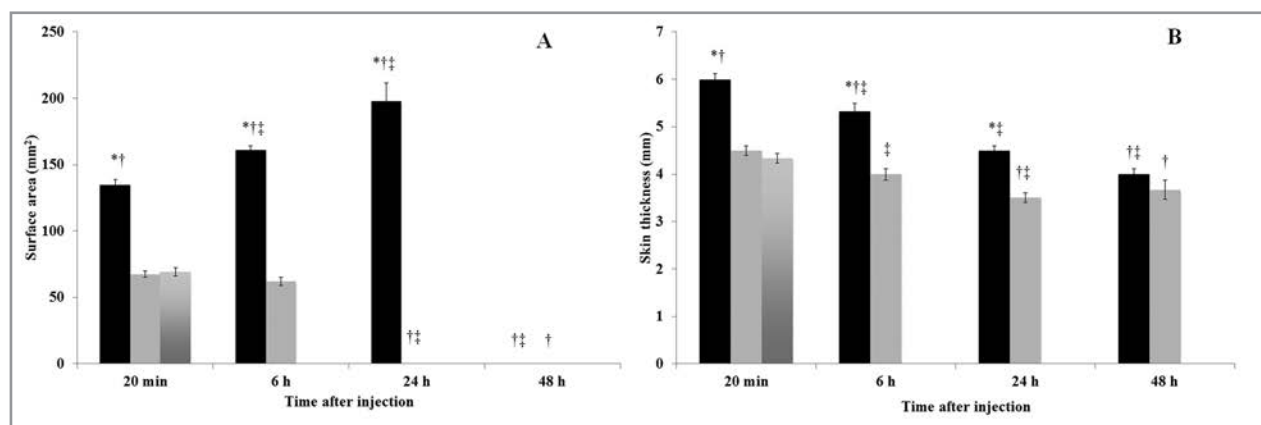


Figure 2—Mean $\pm$ SE surface area (A) and skin thickness (B) of local urticarial reactions after ID injection of anti-IgE at 4 locations (black bars), rabbit IgG at 4 locations (light gray bars), and PBS solution at 1 location (dark gray bars) in 6 clinically normal horses. Skin samples after injection of PBS solution were measured only at 20 minutes. *Within a time period, value differs significantly ($P < 0.001$) from the value for the IgG injection. †Value differs significantly ($P < 0.05$) from the value for the PBS solution injection at 20 minutes. ‡Within a treatment, value differs significantly ($P < 0.05$) from the value at the preceding time point.

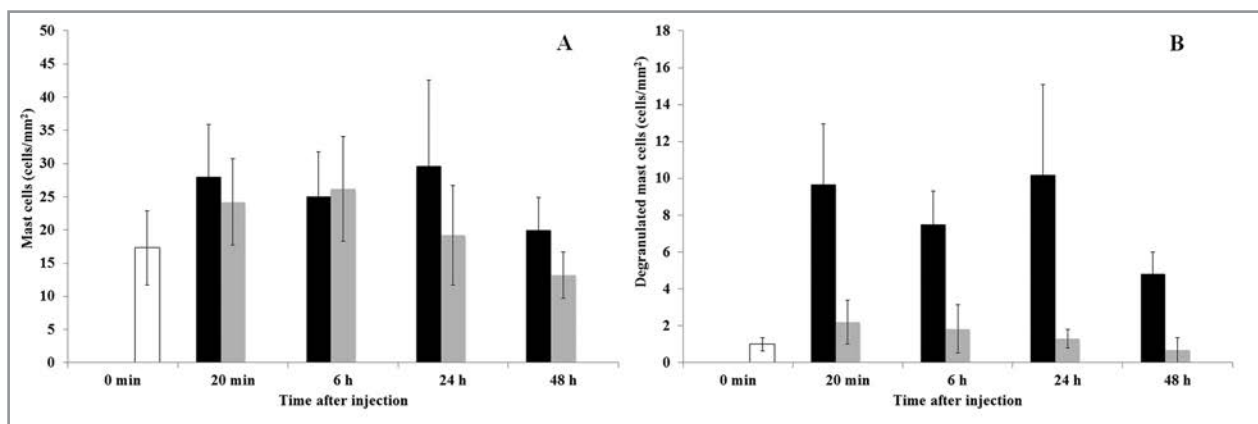


Figure 3—Mean $\pm$ SE number of mast cells (A) and degranulated mast cells (B) in skin samples obtained after ID injection of anti-IgE (black bars) and rabbit IgG (gray bars) or in noninjected skin (white bars) of 6 clinically normal horses. Noninjected skin samples were collected only before injection (time 0). The scale for the y-axis differs between panels. Notice that ID injection of anti-IgE caused degranulation of mast cells.

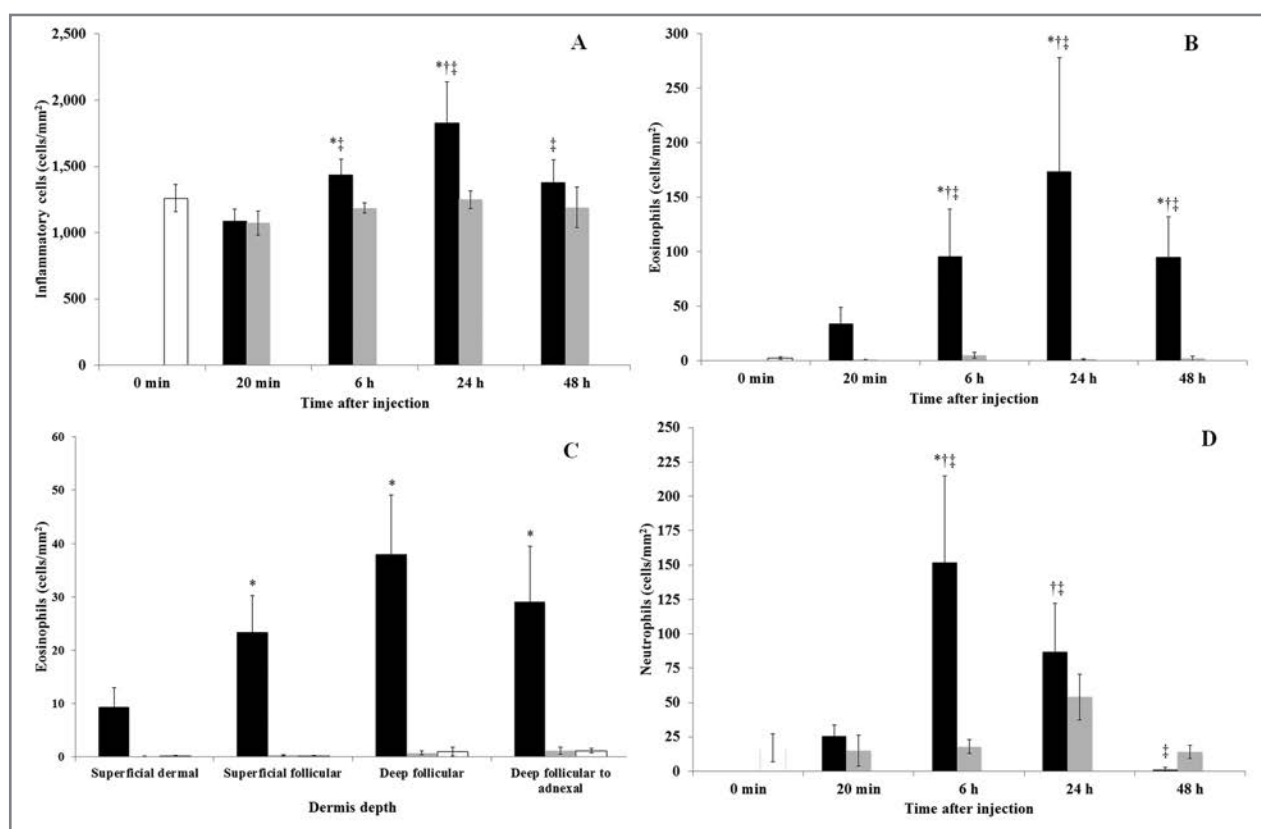


Figure 4—Mean $\pm$ SE number of inflammatory cells (A), number of eosinophils (B), dermal depth of eosinophils (C), and number of neutrophils (D) in skin samples obtained after ID injection of anti-IgE and rabbit IgG or in noninjected skin of 6 clinically normal horses. The scale on the y-axis differs among panels. Notice that ID injection of anti-IgE caused a late-phase cellular response. *Value differs significantly ($P < 0.05$) from the value for the IgG injection within a time point (panels A, B, and D) or within a dermis depth (panel C). †Value differs significantly ($P < 0.05$) from the value for noninjected skin. ‡Within a treatment, value differs significantly ($P < 0.05$) from the value at the preceding time point. See Figure 3 for remainder of key.

utes and then were allowed to cool for 5 to 10 minutes. Slides were rinsed in xylene (3 times, 3 min/rinse, with fresh xylene used for each rinse), and a coverslip was then applied to each slide.

Enumeration of dermal cells—The total number of cells/mm² in the dermis was obtained for all cell types by counting the number of cells in 16 fields (0.25 $\times$ 0.25 mm) of the dermis (excluding epithelial

cells and the adnexa). For slides stained with H&E, Luna stain, and toluidine blue, the 16 fields were allocated evenly among 4 depths of dermis: superficial dermal, superficial follicular, deep follicular, and deep follicular to adnexal (Figure 1). Numbers of nucleated cells, granulocytes, and mononuclear cells were counted on H&E-stained slides. Eosinophils were counted on slides stained with Luna stain. Total neutrophil count was obtained by determining the

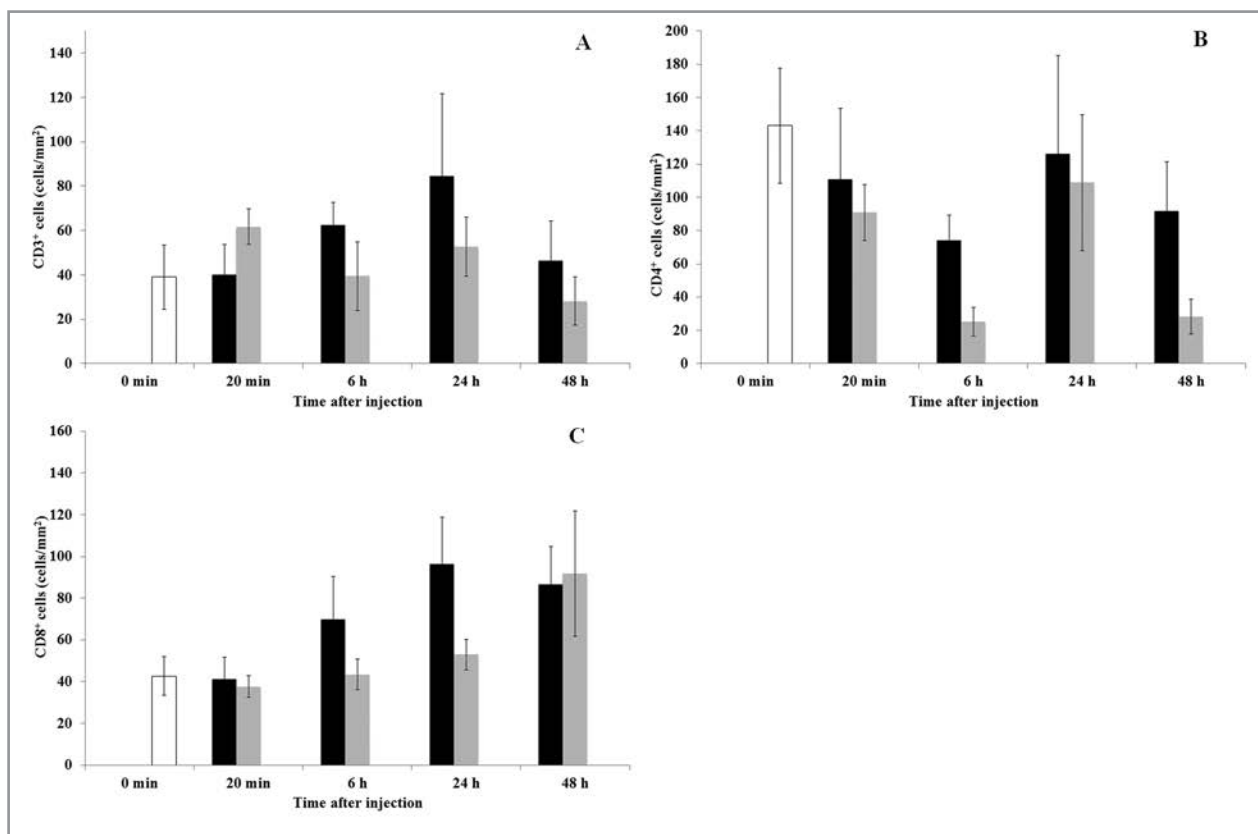


Figure 5—Mean $\pm$ SE number of CD3⁺ (A), CD4⁺ (B), and CD8⁺ (C) cells in skin samples obtained after ID injection of anti-IgE and rabbit IgG or in noninjected skin of 6 clinically normal horses. The scale on the y-axis differs among panels. Notice that ID injection of anti-IgE caused a late-phase cellular response characterized by the recruitment of CD3⁺ cells and CD8⁺ cells. See Figure 3 for remainder of key.

difference between the number of eosinophils/mm² and number of granulocytes/mm². Slides stained with toluidine blue were evaluated to determine the number of intact mast cells as well as the number of degranulated mast cells. The CD3⁺, CD4⁺, and CD8⁺ cells were counted in 16 fields (0.25 $\times$ 0.25 mm) beginning in the superficial dermis (excluding endothelial cells and adnexa) and extending into deeper dermal layers.

Statistical analysis—Data analysis was performed with statistical software.^r A repeated-measures ANOVA with a factorial arrangement of treatments was conducted for dermal cell counts and injection site measurements. Fixed effects included dermal depth (when applicable), time, treatment, and the interactions. Animal was included as a random effect. When significant ($P \leq 0.05$) differences were detected, post hoc comparisons were performed with pairwise *t* tests of least squares means.

Results

Macroscopic reactions—Intradermal injection of anti-IgE induced erythematous and indurated local urticarial reactions within 20 minutes after injection in all horses. The surface areas of those reactions were significantly larger than the surface areas of reactions at 20 minutes and 6 and 24 hours after injection of IgG (Figure 2). The surface area of dermal induration after anti-IgE injection significantly increased from 20

minutes to 6 hours and from 6 to 24 hours. Surface area at sites of anti-IgE injection was significantly larger than the surface area at sites of PBS solution injection at ≥ 20 minutes, except at 48 hours after injection. Surface area at sites of IgG injection, when measurable, never differed significantly from the surface area at sites of PBS solution injection at 20 minutes. There were no measurable wheals or induration at sites of IgG injection at 24 or 48 hours or at sites of anti-IgE injection at 48 hours.

The anti-IgE injection sites were significantly thicker than the IgG injection sites at 20 minutes and 6 and 24 hours after injection (Figure 2). Dermal thickness at sites of anti-IgE injection was greatest 20 minutes after injection and decreased significantly at all time points, compared with the previous values (ie, from 20 minutes to 6 hours, from 6 to 24 hours, and from 24 to 48 hours). Thickness at sites of anti-IgE injection was significantly greater than thickness at sites of PBS solution injection at 20 minutes and 6 hours but was significantly less than thickness at sites of PBS solution injection at 48 hours. Thickness at sites of IgG injection was less than the thickness at sites of PBS solution injection at 24 and 48 hours.

Immediate reactions—No significant difference in the total number of mast cells was found between treatments (Figure 3). The mean number of degranulated mast cells was significantly ($P < 0.001$) higher for anti-IgE injections than for IgG injections, but a treatment-by-time interaction was not detected.

Late-phase reactions—The total number of inflammatory cells increased up to 24 hours after anti-IgE injection, compared with the number of cells at time 0, and there were significantly more inflammatory cells at sites of anti-IgE injection than at sites of IgG injection at 6 and 24 hours (Figure 4). By 48 hours, the total number of inflammatory cells had decreased at sites of anti-IgE injection and did not differ significantly, compared with inflammatory cell counts in noninjected skin at time 0.

Examination of slides stained with Luna stain revealed a significant increase in the number of eosinophils at sites of anti-IgE injection from 20 minutes to 6 hours and from 6 to 24 hours (Figure 4); eosinophil counts significantly decreased from 24 to 48 hours. There were significantly more eosinophils at sites of anti-IgE injection than at sites of IgG injection at 6 hours ($P = 0.002$), 24 hours ($P < 0.001$), and 48 hours ($P = 0.001$). Sites of anti-IgE injection had significantly more eosinophils at 6 hours ($P = 0.001$), 24 hours ($P < 0.001$), and 48 hours ($P = 0.001$), compared with the number of eosinophils in noninjected skin at time 0. Eosinophil counts did not differ significantly between sites of IgG injection at any time point and noninjected skin at time 0.

A significant treatment-by-depth effect was detected for eosinophils. Eosinophil counts were significantly higher in the superficial follicular ($P = 0.001$), deep follicular ($P < 0.001$), and deep follicular to adnexal ($P < 0.001$) dermis for sites of anti-IgE injection, compared with counts at those dermal depths for sites of IgG injection (Figure 4). Eosinophil counts in the superficial dermis did not differ between treatments.

Neutrophils were significantly increased at sites of anti-IgE injections from 20 minutes to 6 hours. Neutrophil counts decreased significantly from 6 to 24 hours and 24 to 48 hours (Figure 4). There were significantly more neutrophils at 6 hours at sites of anti-IgE injection than at sites of IgG injection. Significantly more neutrophils were detected for anti-IgE injection at 6 and 24 hours than for noninjected skin at time 0. Neutrophil counts for IgG injection at any time point did not differ significantly from those of noninjected skin at time 0.

Immunophenotypical analysis of mononuclear cells—No significant difference was detected for CD3⁺, CD4⁺, or CD8⁺ cell counts in the dermis at any time point between sites of anti-IgE injection and IgG injection (Figure 5).

Discussion

Analysis of results of the present study confirmed that ID injection of protein G affinity-purified rabbit-origin polyclonal IgG specific for canine IgE induced many macroscopic and microscopic changes in clinically normal horses comparable to those seen in humans and dogs in similar studies. Anti-equine IgE was not available for use in the present study but would have been expected to yield similar results. The reported protein sequence of the epsilon chain of canine IgE is approximately 65% to 67% identical with that of equine IgE,^{28,29} which would suggest sufficient similarity to permit cross-species use. This assumption is supported

by the fact that some commercially available monoclonal anti-canine IgE antibodies⁵ can cross-reactively bind to equine IgE. In the experience of one of the authors (BH), ID injections of both rabbit-origin polyclonal anti-canine IgE and mouse monoclonal anti-IgE were able to induce immediate wheal-and-flare responses in the skin of clinically normal horses. In addition, a preliminary unpublished study conducted by one of the authors (MCW) indicated that ID injection of the same rabbit-origin polyclonal anti-IgE product, but at a lower dose, in 4 horses resulted in grossly measurable wheals consistent with those seen following allergen injection in allergic horses. Furthermore, histologic examination of those injection sites at various time points revealed mast cell degranulation and eosinophil recruitment. However, the authors acknowledge that the use of anti-canine IgE in horses is less than ideal and may be a contentious issue.

Intradermal injection of cross-linking anti-IgE induced an immediate wheal response at 20 minutes, which continued to increase in surface area through 24 hours (Figure 2). In a similar study⁹ in dogs, a significantly larger wheal was evident for anti-IgE injection than for IgG injection at 20 minutes and 6 hours but not at 24 or 48 hours. In another study⁸ in dogs, urticaria was observed through 24 hours, though lesions decreased in size from the initial examination until 24 hours. Thus, it appears that horses may have a more prolonged macroscopic late-phase reaction than do dogs.

Intradermal injection of PBS solution and IgG also induced measurable distention of the skin, but these areas were significantly smaller and less indurated than were anti-IgE wheals. Additionally, there were no wheals associated with IgG injection by 24 hours. Intradermal injection of any substance will cause transient distention of the superficial dermis, but this distention will dissipate with time as the substance is absorbed. This likely accounted for the initial injection site measurements obtained for injections of IgG and PBS solution.

The use of stereological methods for counting of cells was considered during the design of the present study. However, these programs were not used for several reasons. The first reason was the complex structure of the superficial and middle dermis. In these areas, there are numerous nondermal structures, including the papillary epidermis, hair follicles, sebaceous glands, muscles, blood vessels, and nerves. To consistently count cells in an area of dermis, these structures need to be excluded from that area, which is easiest for a human during manual counting. Additionally, these other structures and their associated cell types (keratinocytes, sebocytes, and endothelial cells) can make it difficult to identify specific cell types of interest (mononuclear cells and granulocytes). For these reasons, we chose to use a manual method for cell counting in this study.

Although ID injection of anti-IgE yielded tissue with significantly more degranulated mast cells than in noninjected tissue at time 0 and IgG-injected tissues at any time point, a treatment-by-time interaction was not apparent. One possible reason for a lack of significant differences with regard to degranulated mast cells was the difficulty in visually identifying them once they had

degranulated and lost their staining capabilities, which leads to variability in cell counts. This degranulation phenomenon has been reported previously in humans³⁰ and has been postulated to occur in horses.²² Investigators in 1 study²⁰ examined mast cells in the skin of horses with IBH by use of toluidine blue stain and immunohistochemical analysis for chymase and tryptase. The effect of fixation time was also evaluated. Analysis of the results of that study²⁰ revealed that toluidine blue staining was significantly decreased in samples that were fixed for > 24 hours. Additionally, more tryptase-positive mast cells were seen in samples fixed for < 24 hours, compared with the number in samples fixed for longer periods. Thus, it might be beneficial in future studies that involve use of the methods described in the present study to decrease fixation times for samples stained with toluidine blue or to include tryptase staining when evaluating mast cells in horses with allergic skin disease.

In the present study, the total number of inflammatory cells was increased at 6 and 24 hours after injection with anti-IgE, compared with the number of cells after IgG injection (Figure 4). This temporal pattern was consistent with a late-phase reaction with influx of inflammatory cells. Eosinophils, in particular, were increased at 6, 24, and 48 hours after anti-IgE injection, compared with results for IgG injection at those same time points and for noninjected skin at time 0. Various chemokines (eg, CCL11, CCL24, and CCL26) recruit eosinophils and are produced by various cell types (eg, epithelial cells).²³ In the present study, eosinophil recruitment was likely attributable to chemotactic factors released from degranulating mast cells because the horses in the study were unlikely to have had a preexisting hypersensitivity to the anti-IgE antibody and thus would not have been expected to have a local population of sensitized T_H2 lymphocytes. Eosinophils are also recruited and activated by T_H2 cell cytokines in late-phase reactions.²³ Future studies could be conducted to evaluate tissue sections for increased concentrations of these chemokines or of interleukin-5, a potent eosinophil-activating cytokine that is produced by mast cells and T_H2 cells.

Historically, eosinophilic dermatitis has been consistently seen with acute allergic skin disease in horses; however, the depth of eosinophilic infiltration was not described or the relative numbers of eosinophils at various depths were not compared.^{1,20,22,31–33} In the present study, more eosinophils were found in the deep layer of dermis in anti-IgE injected skin. Investigators of a previous study⁹ conducted to evaluate effects of ID injection of anti-IgE in dogs described cellular counts only in the most superficial dermis. Results of the study reported here indicated that there were no significant differences in eosinophil numbers in the superficial dermis between anti-IgE and IgG injections (Figure 4). However, eosinophil numbers were increased at the depth of the follicle and deeper dermis after anti-IgE injection, compared with eosinophil numbers after IgG injection. Had this study not evaluated depth as a factor, an important part of the late-phase reaction (eosinophil recruitment) might have been missed. The reason for the increased eosinophil recruitment to the deeper

dermis is not known. Further studies will be needed to evaluate the depth of dermis at which eosinophils are found in horses with naturally occurring allergic skin disease.

Quantitation of CD3⁺, CD4⁺, and CD8⁺ cells over time in the present study did not reveal significant differences between anti-IgE and IgG injections. The CD3-stained slides were assessed for formalin-fixed paraffin-embedded tissue and were evaluated separately from the CD4⁺ and CD8⁺ cells counts, which were obtained from frozen tissue. This may have accounted for the decreased number of CD3⁺ cells, compared with the sum of CD4⁺ and CD8⁺ cell counts (because all of these cells should also have been CD3⁺ cells). The anti-IgE injection resulted in increased numbers of CD3⁺ cells, compared with the number of CD3⁺ cells after IgG injection, from 20 minutes to 24 hours with a subsequent decrease in cell counts at 48 hours, but these values did not differ significantly (Figure 5). This pattern was similar to that for a previous study⁹ in dogs in which CD3⁺ cells increased by 6 hours and peaked 24 hours after anti-IgE injection. Another study⁸ conducted to evaluate late-phase reactions after ID injection of anti-IgE in dogs found that dermal counts of CD3⁺ cells increased from 6 to 48 hours after injection. Investigators evaluated the skin lymphocyte populations in horses with IBH³² and found that there were an increased number of CD3⁺ lymphocytes (many of which were also CD4⁺) in the skin of horses with IBH following injection with *Culicoides* antigen. These late-phase changes were not seen in the present study. However, the investigators in that study³² only evaluated horses with signs of IBH, and none of the horses in the present study had signs of IBH. It is possible that if the present study were repeated with allergic horses injected ID with a specific antigen, the results would be more similar to those seen in that other study.³² Additionally, it must be mentioned that the investigators in that previous study³² evaluated the response to whole-body *Culicoides* extract, which may have included proteases and other irritants. That extract failed to induce a gross wheal response in non-allergic ponies,³⁴ and the *Culicoides* injection sites were not histologically evaluated in those animals. Thus, it is difficult to ascertain whether the lymphocyte responses truly represent an antigen-specific phenomenon.

The CD4⁺ cell counts in the present study were not consistent with findings in other studies.^{32,35} Investigators in 1 study³⁵ on Icelandic horses with IBH found an increase in CD4⁺ cells in skin samples of affected horses. Although the cause of the lack of increase of CD4⁺ cells in the present study is not known, it may have been partially attributable to the difficulty in performing immunohistochemical analysis on frozen tissues. In general, the architecture of the skin in these samples was not maintained well, and the samples were more difficult to stain than were paraffin-embedded tissues. In addition, the use of anti-canine IgE (rather than anti-equine IgE) may have led to the difference in CD4⁺ populations, possibly because of a lower affinity and thus a less robust inflammatory response. However, this possibility appeared to be somewhat unlikely because of the similar reactions for other cell populations.

The CD8⁺ cells have been evaluated less commonly than have CD3⁺ and CD4⁺ cells in horse skin. In the present study, CD8⁺ cells increased in anti-IgE injected skin from 20 minutes to 24 hours and then decreased at 48 hours (similar to the CD3⁺ cell counts). However, the numbers did not differ significantly from those after IgG injection. In 1 study,³⁵ investigators found significantly more dermal CD4⁺ cells, but not more dermal CD8⁺ cells, in lesional skin from horses with IBH, compared with skin from healthy horses.

Horses in the study reported here developed gross and histologic reactions following ID injection of anti-IgE. Therefore, IgE must have been present to some degree at the sites of injection prior to the study. Studies^{7,36,37} have revealed that horses that do not have clinical allergic skin disease can still be sensitized to allergens and have positive reactions to ID injections. In contrast to specific allergens, anti-IgE cross-links all IgE, regardless of allergen specificity. Studies^{21,38,39} in horses have found that IgE on mast cells can be nonspecifically cross-linked by anti-IgE, even in nonallergic horses. Therefore, the ability of the horses in the present study to develop a response to anti-IgE does not necessarily reflect an allergy or parasitism.

As previously mentioned, ID injection of anti-IgE has been used in humans and dogs to mimic allergic skin disease and allow evaluation of the effects of various pharmaceutical agents on immediate and late-phase inflammatory reactions.^{9,10,12–16,40} The study reported here confirmed that injection of anti-IgE induces a dermal inflammatory response with many similarities to those seen in horses with spontaneous allergic skin disease. These similarities were particularly marked for eosinophils and neutrophils, although the lymphocyte response generated in this study was of lower magnitude than would be expected. In the future, controlled studies could be performed by use of these methods to enable evaluation of the efficacy of various treatment modalities and their effects on macroscopic and microscopic reactions for allergic skin disease in horses without the need to maintain a herd of allergic horses.

- a. Lloyd Laboratories, Decatur, Ill.
- b. Jackson ImmunoResearch Laboratories, Westgrove, Pa.
- c. Bethyl Labs, Montgomery, Tex.
- d. Sparhawk Laboratories, Lenexa, Kan.
- e. Tissue-Tek OCT Compound, Sakura Finetek USA Inc, Torrance, Calif.
- f. Amresco LLC, Solon, Ohio.
- g. VMRD, Pullman, Wash.
- h. Vector Laboratories, Burlingame, Calif.
- i. Invitrogen, Life Technologies Inc, Grand Island, NY.
- j. AEC, BioGenex, Fremont, Calif.
- k. Dako North America Inc, Carpinteria, Calif.
- l. DAKO AutoStainer LINK 48, Dako North America Inc, Carpinteria, Calif.
- m. Vector S-1000, Vector Laboratories, Burlingame, Calif.
- n. DAKO A0452, Dako North America Inc, Carpinteria, Calif.
- o. Vector BA 1000, Vector Laboratories, Burlingame, Calif.
- p. Vectastain ABC rabbit IgG kit, Vector PK 6102, Vector Laboratories, Burlingame, Calif.
- q. Vector Sk-4800, Vector Laboratories, Burlingame, Calif.
- r. SAS, version 9.3, SAS Institute Inc, Cary, NC.
- s. MCA1896, AbD Serotec, Raleigh, NC.

References

1. Scott DW, Miller WH Jr. Skin immune system and allergic skin diseases. In: *Equine dermatology*. 2nd ed. St Louis: Saunders, 2010;263–313.
2. Björnsdóttir S, Sigvaldadóttir J, Broström H, et al. Summer eczema in exported Icelandic horses: influence of environmental and genetic factors. *Acta Vet Scand* 2006;48:3–6.
3. Braverman Y, Ungar-Waron H, Frith K, et al. Epidemiological and immunological studies of sweet itch in horses in Israel. *Vet Rec* 1983;112:521–524.
4. van Grevenhof EM, Ducro B, Heuven HC, et al. Identification of environmental factors affecting the prevalence of insect bite hypersensitivity in Shetland ponies and Friesian horses in The Netherlands. *Equine Vet J* 2007;39:69–73.
5. Littlewood JD. Incidence of recurrent seasonal pruritus ('sweet itch') in British and German Shire horses. *Vet Rec* 1998;142:66–67.
6. Halldórsdóttir S, Larsen HJ. An epidemiological study of summer eczema in Icelandic horses in Norway. *Equine Vet J* 1991;23:296–299.
7. Lorch G, Hillier A, Kwochka KW, et al. Results of intradermal tests in horses without atopy and horses with chronic obstructive pulmonary disease. *Am J Vet Res* 2001;62:389–397.
8. Olivry T, Dunston SM, Murphy KM, et al. Characterization of the inflammatory infiltrate during IgE-mediated late phase reactions in the skin of normal and atopic dogs. *Vet Dermatol* 2001;12:49–58.
9. Pucheu-Haston CM, Shuster D, Olivry T, et al. A canine model of cutaneous late-phase reactions: prednisolone inhibition of cellular and cytokine responses. *Immunology* 2006;117:177–187.
10. Grönneberg R, Zetterström O. Inhibition of anti-IgE induced skin response in normals by formoterol, a new beta 2-adrenoceptor agonist, and terbutaline. 2. Effect on the late phase reaction. *Allergy* 1990;45:340–346.
11. Grönneberg R, Zetterström O. Inhibition of anti-IgE induced skin response in normals by formoterol, a new beta 2-adrenoceptor agonist, and terbutaline. 1. Dose response relation and duration of effect on the early wheal and flare response. *Allergy* 1990;45:334–339.
12. Grönneberg R, Strandberg K. Effect of betamethasone on the dual reaction to anti-human IgE in man: influence of time interval between administration of drug and anti-IgE. *Allergy* 1985;40:223–228.
13. Grönneberg R, Zetterström O. Effect of disodium cromoglycate on anti-IgE induced early and late skin response in humans. *Clin Allergy* 1985;15:167–171.
14. Grönneberg R, Zetterström O. Inhibition of the late phase response to anti-IgE in humans by indomethacin. *Allergy* 1985;40:36–41.
15. Grönneberg R. Inhibition of the late phase reaction to anti-IgE by previous mast cell activation with compound 48/80. *Allergy* 1984;39:119–123.
16. Grönneberg R, Strandberg K, Stålenheim G, et al. Effect in man of anti-allergic drugs on the immediate and late phase cutaneous allergic reactions induced by anti-IgE. *Allergy* 1981;36:201–208.
17. Charlesworth EN. The skin as a model to study the pathogenesis of IgE-mediated acute and late-phase responses. *J Allergy Clin Immunol* 1994;94:1240–1250.
18. Frew AJ, Kay AB. The relationship between infiltrating CD4⁺ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988;141:4158–4164.
19. Petersen A. Insect hypersensitivities. In: Robinson NE, Sprayberry KA, eds. *Current therapy in equine medicine*. 6th ed. St Louis: Saunders, 2009;678–680.
20. van der Haegen A, Griot-Wenk M, Welle M, et al. Immunoglobulin-E-bearing cells in skin biopsies of horses with insect bite hypersensitivity. *Equine Vet J* 2001;33:699–706.
21. Wagner B, Miller WH, Morgan EE, et al. IgE and IgG antibodies in skin allergy of the horse. *Vet Res* 2006;37:813–825.
22. Rüfenacht S, Marti E, von Tschanner C, et al. Immunoglobulin E-bearing cells and mast cells in skin biopsies of horses with urticaria. *Vet Dermatol* 2005;16:94–101.

23. Abbas AK, Lichtman AH, Pillai S. IgE-dependent immune responses and allergic disease. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and molecular immunology*. 7th ed. Philadelphia: Elsevier-Saunders, 2012;425–444.
24. Hammerberg B, Olivry T, Orton SM. Skin mast cell histamine release following stem cell factor and high-affinity immunoglobulin E receptor cross-linking in dogs with atopic dermatitis. *Vet Dermatol* 2001;12:339–346.
25. Gebhard D, Orton S, Edmiston D, et al. Canine IgE monoclonal antibody specific for a filarial antigen: production by a canine X murine heterohybridoma using B cells from a clinically affected lymph node. *Immunology* 1995;85:429–434.
26. Luna LG, Armed Forces Institute of Pathology. *Manual of histologic staining methods of the Armed Forces Institute of Pathology*. 3rd ed. New York: McGraw-Hill Blakiston Division, 1968.
27. Affolter VK, Moore PF. Localized and disseminated histiocytic sarcoma of dendritic cell origin in dogs. *Vet Pathol* 2002;39:74–83.
28. Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402.
29. Altschul SF, Wootton JC, Gertz EM, et al. Protein database searches using compositionally adjusted substitution matrices. *FEBS J* 2005;272:5101–5109.
30. Claman HN, Choi KL, Sujansky W, et al. Mast cell “disappearance” in chronic murine graft-vs-host disease (GVHD)-ultrastructural demonstration of “phantom mast cells”. *J Immunol* 1986;137:2009–2013.
31. Foster AP, Lees P, Cunningham FM. Platelet activating factor mimics antigen-induced cutaneous inflammatory responses in sweet itch horses. *Vet Immunol Immunopathol* 1995;44:115–128.
32. McKelvie J, Foster AP, Cunningham FM, et al. Characterisation of lymphocyte subpopulations in the skin and circulation of horses with sweet itch (*Culicoides* hypersensitivity). *Equine Vet J* 1999;31:466–472.
33. Fadok VA, Greiner EC. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. *Equine Vet J* 1990;22:236–240.
34. Foster AP, McKelvie J, Cunningham FM. Inhibition of antigen-induced cutaneous responses of ponies with insect hypersensitivity by the histamine-1 receptor antagonist chlorpheniramine. *Vet Rec* 1998;143:189–193.
35. Heimann M, Janda J, Sigurdardottir OG, et al. Skin-infiltrating T cells and cytokine expression in Icelandic horses affected with insect bite hypersensitivity: a possible role for regulatory T cells. *Vet Immunol Immunopathol* 2011;140:63–74.
36. Wagner B, Miller WH Jr, Erb HN, et al. Sensitization of skin mast cells with IgE antibodies to *Culicoides* allergens occurs frequently in clinically healthy horses. *Vet Immunol Immunopathol* 2009;132:53–61.
37. Lorch G, Hillier A, Kwochka KW, et al. Results of intradermal tests in horses without atopy and horses with atopic dermatitis or recurrent urticaria. *Am J Vet Res* 2001;62:1051–1059.
38. Wilson AD, Harwood L, Torsteinsdottir S, et al. Production of monoclonal antibodies specific for native equine IgE and their application to monitor total serum IgE responses in Icelandic and non-Icelandic horses with insect bite dermal hypersensitivity. *Vet Immunol Immunopathol* 2006;112:156–170.
39. Wagner B, Radbruch A, Rohwer J, et al. Monoclonal anti-equine IgE antibodies with specificity for different epitopes on the immunoglobulin heavy chain of native IgE. *Vet Immunol Immunopathol* 2003;92:45–60.
40. Grönneberg R, Raud J. Effects of local treatment with salmeterol and terbutaline on anti-IgE-induced wheal, flare, and late induction in human skin. *Allergy* 1996;51:685–692.