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# Characterization of IgE-mediated Cutaneous Immediate and Late-Phase Reactions in Non-Allergic Horses

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CHARACTERIZATION OF IgE-MEDIATED CUTANEOUS IMMEDIATE  
AND LATE-PHASE REACTIONS IN NON-ALLERGIC HORSES

A Thesis

Submitted to the Graduate School of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Sciences

in

The Interdepartmental Program in Veterinary Medical Sciences  
through the Department of Veterinary Clinical Sciences

by

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May 2014

I would like to dedicate this to my husband, Kevin O’Gorman, and my parents, Carl and Tracey Woodward, for their support, understanding, and encouragement.

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Finally, I would like to thank my family. My husband has demonstrated incredible patience and support in my academic career. Without him, and my parents, I would not have had the opportunities that allowed me to be where I am today.

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## ABSTRACT

**Objectives** – To characterize the response of horse skin following intradermal injection of polyclonal rabbit anti-canine IgE (anti-IgE) and rabbit immunoglobulin G (IgG) in an attempt to develop a model of equine allergic skin disease.

**Study design** - *In vivo* study.

**Animals** – 10 Adult Thoroughbred horses.

**Methods** –Horses were injected intradermally with one of two different concentrations of anti-IgE and rabbit immunoglobulin G (IgG). Wheal measurements and injection site biopsies were obtained before and 20 min, 6 hr, 24 hr, and 48 hr after injection. Tissue sections were stained with Hematoxylin and Eosin, Luna, and Toluidine Blue. Immunohistochemistry for CD3+, CD4+, and CD8+ cells was performed. Cells were counted in 1 mm<sup>2</sup> of dermis and divided over four depths for standard stains: superficial dermal, superficial follicular, deep follicular, and deep follicular to adnexal. The superficial dermis was evaluated in immunohistochemistry sections.

**Results** – Lower concentrations of anti-IgE produced suboptimal responses, so the higher concentration injections were evaluated. Anti-IgE wheals were significantly larger than IgG wheals at 20 min, 6 hr, and 24 hr after injection. Anti-IgE injected skin had significantly more degranulated mast cells than IgG injected skin and there were significantly more inflammatory cells (6 hr, 24 hr), eosinophils (6hr, 24hr, 48hr), and neutrophils (6 hr). Eosinophil counts significantly increased in anti-IgE samples in the deeper but not superficial dermis when compared to IgG samples. There were more eosinophils in the deeper dermis of anti-IgE injected skin. There were no significant differences between anti-IgE and IgG injected skin for CD3+, CD4+, or CD8+ cells.

**Conclusion** - Injection of anti-IgE antibodies at a higher concentration was associated with the development of gross and microscopic inflammation that was characterized by mast cell

degranulation and accumulation of inflammatory cells, particularly eosinophils and neutrophils.

This pattern was similar to that seen in horses with spontaneous allergic skin disease, although lymphocytes were not increased.

**Clinical Relevance** - This study documents the response to intradermal anti-IgE injection in horses and demonstrates the potential use of this model for studying equine allergic skin disease.



## CHAPTER 1 GENERAL INTRODUCTION

### 1.1 Type I Hypersensitivity Reactions

Hypersensitivity reactions are allergic reactions that are provoked by exposure (and subsequent re-exposure) to an antigen (allergen) and can involve skin, eyes, the respiratory system, and the gastrointestinal tract. The most common allergens include proteins in pollen, house dust mites, animal dander, and other chemicals (Abbas et al., 2012). Upon initial exposure to an allergen, naïve T cells differentiate into  $T_H2$  T cells.  $T_H2$  cells interface with naïve B cells via CD40-CD40 ligand interactions. They also secrete  $T_H2$  cytokines such as IL-4 and IL-13. Together, these signals induce B cells to produce antibodies and promote the switch of antibody forms (isotypes) from IgM to other classes, especially IgE. In some species, certain isotypes of IgG (such as IgGT in the horse (Wagner et al., 2006)) may also mediate hypersensitivity reactions, but the relevance of these isotypes to clinical disease is unclear.

Perhaps the most common clinical forms of hypersensitivity involve Type I (immediate, IgE mediated) hypersensitivity reactions. These reactions may manifest in a biphasic fashion, consisting of immediate and late-phase reactions (not to be confused with Type IV delayed hypersensitivity reactions). The immediate reaction begins upon re-exposure to an antigen to which an individual has already become sensitized. Allergen-specific IgE is bound to high-affinity receptors (FcεRI) on the surface of mast cells. These receptors may also be found on other cells, such as basophils, eosinophils, epidermal Langerhans cells, dermal macrophages and activated monocytes (Abbas et al., 2012). Exposure to allergen results in allergen binding to this surface bound IgE and “cross-linking” these receptors. This cross-linking activates the mast cells and causes them to release a variety of mediators. One subset of mast cell mediators includes the biogenic amines (ie. histamine, serotonin) that cause the immediate reaction by inducing vasodilation and vascular leakage. Histamine causes vasodilation by stimulating endothelial cells to release smooth muscle relaxants. Histamine binding also causes

endothelial cell contraction, leading to increased vascular permeability (Abbas et al., 2012). It is these effects of histamine that are responsible for the typical wheal-and-flare response that is associated with immediate hypersensitivity reactions. Mast cells also release granule enzymes and proteoglycans that contribute to tissue damage in the immediate phase (Abbas et al., 2012). Mast cell activation also leads to the synthesis and release of lipid-derived mediators, the most important of which are derived from arachidonic acid. Prostaglandin D<sub>2</sub> binds to smooth muscle cell receptors and acts as a vasodilator, a bronchoconstrictor, and promotes neutrophil chemotaxis to sites of inflammation (Abbas et al., 2012). Leukotrienes are also produced, which promote bronchoconstriction and possibly contribute to the wheal-and-flare reaction. Platelet activating factor, also mast-cell derived, can promote bronchoconstriction, vascular smooth muscle relaxation, and endothelial cell retraction (Abbas et al., 2012). In addition to aiding B class switching, T<sub>H</sub>2 cells are involved in immediate hypersensitivity in other ways. T<sub>H</sub>2 secreted IL-5 activates eosinophils and increases their maturation while IL-13 stimulates increased secretion of mucus by epithelial cells (such as those in the airways) (Abbas et al., 2012).

The second aspect of Type I hypersensitivity reactions is the late phase reaction. Late-phase reactions are mediated by cytokines from T<sub>H</sub>2 cells and mast cells, as well as lipid mediators from mast cells. The late-phase reaction is characterized by increased numbers of neutrophils, eosinophils, macrophages, and CD4<sup>+</sup> cells. Mast cell derived cytokines include tumor necrosis factor, which activates endothelial cell expression of adhesion molecules and contributes to neutrophil and monocyte infiltration. Mast cells and T<sub>H</sub>2 cells produce IL-4, which enhances the endothelial expression of adhesion molecules for eosinophils, such as E-selectin and VCAM-1. This contributes directly to the accumulation of eosinophils and other inflammatory cells at the site of the reaction. Epithelial cells at allergic reaction sites produce CCL11 (eosinophil chemotactic protein), which binds to CCR3 on eosinophils. Eosinophils, once recruited to the site of inflammation, may be activated by cross-linking of

surface-bound IgE. They then secrete granule proteins (such as platelet activating factor, prostaglandins and leukotrienes) that further contribute to allergic disease by inducing tissue damage and continuing inflammatory cell recruitment. Neutrophils and other cells can also be recruited to the site of inflammation in late-phase reactions.

## 1.2 Anti Immunoglobulin-E

Anti-IgE is an antibody that is generated in one species (rabbits in Chapters 2 and 3) that is capable of binding and cross-linking mast cell bound IgE molecules in another species (dogs in Chapter 2 and 3). This binding and cross-linking produces activation of mast cells in a manner similar to that seen with naturally occurring Type I hypersensitivity reactions and has been used as a model for spontaneous disease. The benefit of using anti-IgE rather than allergen to produce mast cell degranulation is that (instead of requiring a population of specifically-sensitized individuals), the anti-IgE antibodies can bind any IgE and induce cross-linking and activation. Using anti-IgE also means that a large amount of IgE specific for any particular antigen isn't needed. Additionally, even normal, non-allergic individuals have some IgE bound to their mast cells, making them acceptable subjects for studies using anti-IgE.

Intradermal injection of anti-canine IgE antibodies has been demonstrated in dogs to produce immediate and late-phase reactions in the skin that mimic those of spontaneously occurring allergy (Olivry et al., 2001; Pucheu-Haston et al., 2006). Urticarial reactions were seen in atopic and normal dogs 20 minutes following injection of anti-canine IgE (Olivry et al., 2001). There continued to be grossly visible lesions characterized by erythema and dermal thickening in the same subset of dogs up to 24 hours later. These gross lesions were similar to those seen in mite- allergic atopic dogs following exposure to *Dermatophagoides farinae* (Olivry et al., 2001). Late-phase reactions in normal and atopic dogs following injection of anti-IgE were characterized by early recruitment of neutrophils and

eosinophils and later recruitment  $\alpha\beta$  T-lymphocytes and dermal dendritic cells. This appearance was generally similar to the reaction seen in atopic dogs exposed to *Dermatophagoides farinae* in the same study (Olivry et al., 2001). These results of normal dogs exposed to anti-IgE were later confirmed in another study (Pucheu-Haston et al., 2006).

One use for this model is in the pre-clinical evaluation of treatments for allergic disease. In dogs, studies have characterized the anti-inflammatory effects of drugs such as prednisolone (Pucheu-Haston et al., 2006), hydrocortisone (Rivierre et al., 2000), and triamcinolone (DeBoer, 2000). Similar studies have been performed in humans for different potential allergy treatments (Gronneberg and Raud, 1996; Gronneberg and Strandberg, 1985; Gronneberg et al., 1981; Gronneberg and Zetterstrom, 1985b, 1990b).

### 1.3 IgE-Mediated Allergic Skin Disease in Horses

Allergic skin disease in horses includes, but is not limited to, insect bite hypersensitivity and atopic dermatitis. Insect bite hypersensitivity (IBH) (a.k.a. sweet itch, summer eczema) is the most common allergy in horses, and has subsequently become the most studied. The causative insects are frequently *Culicoides spp.* midges, and the allergens responsible are likely salivary in origin (Hellberg et al., 2006; Langner et al., 2009; Schaffartzik et al., 2011; Wilson et al., 2008). The prevalence of IBH varies based on breed and geographic location, but has been reported to be up to 71.4% (Bjornsdottir et al., 2006; Braverman et al., 1983; Halldorsdottir and Larsen, 1991; Littlewood, 1998; van Grevenhof et al., 2007). The disease severity is variable and clinical signs include pruritus, papules, hyperesthesia, urticaria, edema, and secondary self-trauma (Brostrom et al., 1987; Scott, 2010). Lesion location varies based on the type of insect involved. Research has demonstrated that the disease is IgE-mediated (van der Haegen et al., 2001; Wagner et al., 2006; Wilson et al., 2001). There is also evidence supporting a Th2/Th1 imbalance (Heimann et al., 2011), but this is not yet conclusive.

Less information is available about non-IBH related atopic dermatitis in horses. Clinical signs include pruritus, recurrent urticaria, and lesions secondary to itch (Fadok, 2013; Scott, 2010). Reports of prevalence and breed predisposition are anecdotal and are generally not confirmed by population-controlled analyses. Much of the information that is assumed about atopic dermatitis in horses is extrapolated from humans and dogs (Fadok, 2013). There are some retrospective studies available detailing the success (or lack thereof) of treatments for atopic dermatitis in horses, but the information is lacking when compared what is known about treatment options and efficacy in humans and dogs (Olsen et al., 2011; Rees, 2001; Stepnik et al., 2012). Effective pre-clinical evaluation of the efficacy of novel anti-allergic therapies requires the ability to consistently produce or reproduce allergic inflammation. For this reason, there is a great need for the development of a practical model that induces responses similar to those seen in atopic horses.

While a large amount of information is available about IBH, including prevalence and histopathology findings, there is little peer-reviewed information regarding various treatments and long-term outcomes (O'Neill et al., 2002; Olsen et al., 2011; van den Boom et al., 2010). There is even less information regarding treatment and prognosis associated with non-IBH related atopic dermatitis. 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 studied. However, development and long-term maintenance of a dedicated herd of sensitized horses with allergic skin disease is difficult and expensive. In addition, generation of allergen-specific responses in these sensitized horses typically requires the administration of commercial allergen extracts. Many of these extracts may contain proteolytic compounds and/or induce nonspecific inflammatory responses even in non-sensitized horses (Lorch et al., 2001b). Thus, it would be desirable to develop a model that can consistently and easily mimic many or all of the changes associated with

spontaneous disease using non-allergic horses, without the additional variables associated with allergen extract administration.

## CHAPTER 2 CHARACTERIZATION OF IgE-MEDIATED (LOWER CONCENTRATION) CUTANEOUS IMMEDIATE AND LATE-PHASE REACTIONS IN NON-ALLERGIC HORSES

### 2.1 Introduction

Hypersensitivity disorders in the horse are frequently associated with the development of skin disease. These allergic diseases, such as insect bite hypersensitivity, atopic dermatitis, and urticaria, cause variable signs in horses, including pruritus, pain, and changes to coat and hair (Scott, 2010). In severe cases, horses can lose body condition and exhibit significant decreases in athletic performance. The true prevalence of most equine allergic skin diseases is unknown with the exception of insect bite hypersensitivity, for which the prevalence has been reported to vary from 11.6 to 71.4% (Bjornsdottir et al., 2006; Braverman et al., 1983; Halldorsdottir and Larsen, 1991; Littlewood, 1998; van Grevenhof et al., 2007). 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 studied. However, development and long-term maintenance of a dedicated herd of sensitized horses with allergic skin disease is difficult and expensive. In addition, generation of allergen-specific responses in these sensitized horses typically requires the administration of commercial allergen extracts. Many of these extracts may contain proteolytic compounds and/or induce nonspecific inflammatory responses even in non-sensitized horses (Lorch et al., 2001b). Thus, it would be desirable to develop a model that can consistently and easily mimic many or all of the changes associated with spontaneous disease using non-allergic horses, without the additional variables associated with allergen extract administration. Similar approaches have been used to effectively model cutaneous allergic inflammation in other species, including dogs (Olivry et al., 2001; Pucheu-Haston et al., 2006) and humans (Gronneberg, 1984; Gronneberg and Strandberg, 1985; Gronneberg et al., 1981; Gronneberg and Zetterstrom, 1985a, b, 1990a, b).

Briefly, allergic skin disease is often mediated by Type I hypersensitivity reactions. These reactions (immediate- and late-phase hypersensitivity) are initiated by antigen exposure. Upon exposure, naïve antigen-specific T cells are induced to differentiate into T<sub>H2</sub> phenotype cells which then activate B cells. The B cells undergo class switching and begin to produce IgE, which binds to the high affinity receptor for IgE (FcεRI) on the surface of mast cells. Upon future exposure to the same antigen, the surface-bound IgE is cross-linked and the mast cells become activated. The mast cells release mediators (histamine, cytokines, 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 six to 48 hours after challenge (Charlesworth, 1994; Frew and Kay, 1988; Olivry et al., 2001).

Atopic dermatitis (Scott, 2010) and insect bite hypersensitivity (Petersen, 2009; Scott, 2010) in horses involve type I hypersensitivity reactions. The involvement of IgE, specifically, has been suggested (Lorch et al., 2001b; van der Haegen et al., 2001) and established (Rufenacht et al., 2005; Wagner et al., 2006) in equine allergic skin disease. Horses affected by insect bite hypersensitivity have been shown to have increased expression of both IgE protein and mRNA in dermal cells in affected skin when compared to unaffected skin from normal healthy horses, especially in the acute phase (van der Haegen et al., 2001). Skin biopsies from horses with urticaria also revealed more IgE-bearing cells in the superficial dermis when compared with skin biopsies obtained from normal horses (Rufenacht et al., 2005).

Type I hypersensitivity reactions can be simulated by intradermal injection of anti-IgE antibody (Abbas et al., 2012). The anti-IgE functions to cross-link surface IgE, mimicking the effects of an allergen. This reaction has been used in humans to evaluate the effects of drugs on IgE-mediated reactions (Gronneberg, 1984; Gronneberg and Strandberg, 1985; Gronneberg et al., 1981; Gronneberg and Zetterstrom, 1985a, b, 1990a, b). In dogs, allergic dermatitis has been successfully modeled using



anti-IgE (Olivry et al., 2001; Pucheu-Haston et al., 2006). Such a model has not been developed in the horse.

The hypothesis of this pilot study was that intradermal injection of 0.08 mg/ml protein G affinity-purified anti-canine IgE will cause macroscopic and microscopic reactions similar to those seen in horses with allergic skin disease.

## 2.2 Materials and Methods

Four horses (3 geldings, 1 mare) aged 3 to 8 years were used for this study. These horses were chosen based upon lack of evidence of current or prior cutaneous or systemic disease (including allergic skin disease). All horses were vaccinated for Eastern Equine Encephalitis, Western Equine Encephalitis, West Nile Virus Encephalitis, Tetanus, and Influenza, and dewormed with a product containing ivermectin or moxidectin (200 µg/kg body weight, orally, twice yearly) as part of routine herd care. No medications were given for 14 days prior to the study. Physical examinations were performed on Study Day 0. All experimental protocols were approved by the Louisiana State University Institutional Animal Care and Use Committee (Protocol #11-072)

All horses were shaved on the lateral cervical area on Study Day 0 in preparation for injections in this location. Horses were sedated with intravenous xylazine<sup>a</sup> (0.4 mg/kg). All horses were injected intradermally (0.1 ml each injection) at one site with phosphate-buffered saline (PBS), at four sites with protein G affinity-purified anti-IgE<sup>b</sup> (Hammerberg et al., 2001) diluted to 0.08 mg/ml in PBS, and at four sites with IgG<sup>c</sup> diluted to 0.1 mg/ml in PBS. Anti-canine IgE was used as anti-equine IgE was not available. Furthermore, preliminary work (data not shown) had demonstrated the ability of anti-canine IgE to induce the development of gross and microscopic immediate and late phase reactions in the skin of normal horses.

Rabbit antisera specific for canine IgE was generated by injection of canine monoclonal IgE<sup>d</sup> produced by a canine x mouse heterohybridoma (Gebhard et al., 1995). Antisera were adsorbed with canine IgG linked to beads to remove antibodies cross-reactive with IgG.

Anti-IgE and IgG injected skin sites were examined at 20 minutes, and at 6, 24, and 48 hours after injection for the development of cutaneous swelling, erythema or induration. The PBS injected sites were measured at 20 minutes. The diameter of the cutaneous reactions (wheals) was measured in two perpendicular directions and the reaction surface areas were calculated. Dermal thickness of the injected skin was also measured with calipers.

Horses were sedated with xylazine prior to all skin biopsy acquisitions. All biopsy areas were injected subcutaneously with 0.5 ml of 2% lidocaine hydrochloride<sup>e</sup>. One 8-mm punch biopsy of normal, non-injected skin on the lateral neck was collected at the time of injection. Samples were collected at anti-IgE and IgG injection sites at 20 minutes, and 6, 24, and 48 hours after injection. Each sample was bisected immediately after collection. One half was placed in 10% neutral buffered formalin for routine processing in paraffin. Fixation time was variable, but was a minimum of 72 hours after sample acquisition. The other half was placed in Optimal Cutting Temperature medium<sup>f</sup>, frozen in isopentane cooled in liquid nitrogen and stored at -80° until cryosectioning.

Five-micrometer tissue sections were cut from paraffin blocks and stained with hematoxylin and eosin for examination, cell enumeration, and pattern analysis. Eosinophils were better visualized using Luna's stain(Luna et al., 1968) for eosinophils . A low-pH (2.5) toluidine blue (T-blue) stain was used to facilitate evaluation of dermal mast cells.

A three-step labeled streptavidin method(Affolter and Moore, 2002) was modified and used to characterize the mononuclear cell infiltrate. Briefly, 6-µm cryosections were air dried, then immersed in acetone for fixation. The samples were rinsed with PBS containing 0.5% Tween 20 (0.5% PBST)<sup>g</sup> and

then blocked with 1% fetal calf serum. The blocking sera was drained and mouse-origin monoclonal antibodies specific for equine CD4 or CD8 beta chain<sup>h</sup> were diluted 1:70 in PBS and applied to the slides. After rinsing, endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in PBS. Biotinylated horse-origin anti-mouse IgG<sup>i</sup> was applied diluted 1:400 in PBS, followed by horseradish peroxidase-conjugated streptavidin<sup>j</sup> diluted 1:400 in PBS. Samples were rinsed with 0.5% PBST between steps. Amino-9-ethyl carbazole<sup>k</sup> was applied as a chromogen, followed by hematoxylin counterstain<sup>l</sup>.

For all cell types, the total number of cells/mm<sup>2</sup> in the dermis was obtained by counting sixteen 0.25 mm x 0.25 mm fields of dermis (excluding epithelial cells and adnexa). For the H&E, Luna, and T-blue slides, the sixteen fields were split evenly between four depths of dermis: superficial dermal, superficial follicular, deep follicular, and deep follicular to adnexal (Figure 2.1). Total numbers of nucleated cells, total granulocytes, and total mononuclear cells were counted using H&E stained slides. Eosinophils were counted using the Luna stained slides. A total neutrophil count was obtained by determining the difference between the number of eosinophils/mm<sup>2</sup> and the number of total granulocytes/mm<sup>2</sup>. The T-Blue stained slides were evaluated for total numbers of intact mast cells as well as degranulated mast cells. CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> dermal cells were counted on sixteen 0.25 mm x 0.25 mm fields beginning in the superficial dermis (excluding endothelial cells and adnexa) and extending deeper as needed.

Data analysis was performed with statistical software<sup>s</sup>. A repeated measures analysis of variance with a factorial arrangement of treatments was conducted. Fixed effects in the model included depth (when applicable), time, treatment, and their interactions. Animal was included as a random effect. When overall significance was detected ( $p \leq 0.05$ ), post hoc comparisons were made with

pairwise t tests of least-squares means. This was done for all dermal cell counts as well as injection site measurements.

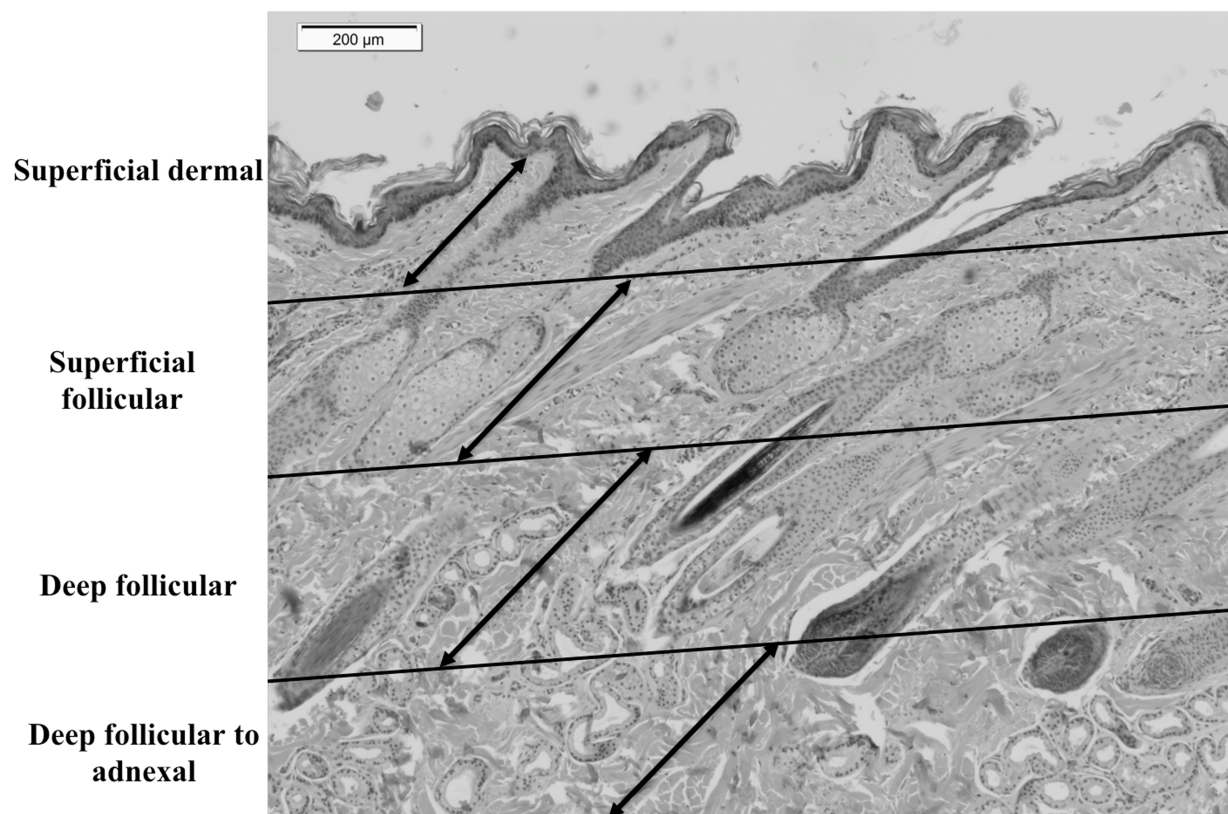


Figure 2.1: Dermal depths used to evaluate inflammatory cell location in horses. Bar = 200 μm.

### 2.3 Results

**Macroscopic Reactions:** Overall, anti-IgE injection resulted in significantly larger wheal surface areas compared to IgG injection, but there was not a significant Treatment x Time interaction (Figure 2.2A). The anti-IgE injection sites were significantly thicker than IgG injected skin at 20 minutes and 6 hours (Figure 2.2B). Dermal thickness in anti-IgE injected skin was greatest 20 minutes after injection and decreased significantly at 6 hours.

Immediate-Phase Reactions: Anti-IgE injected skin had significantly more total mast cells (Figure 2.3A) and degranulated mast cells (Figure 2.3B) noted than IgG injected skin, but a significant Treatment x Time interaction was not observed.

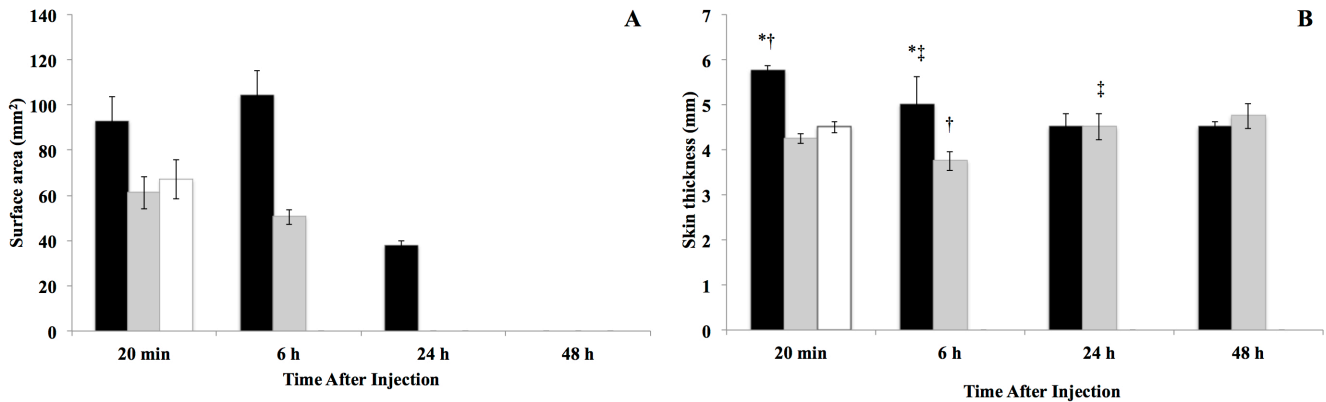


Figure 2.2: Mean ± SE surface area (A) and skin thickness (B) after intradermal injection of anti-IgE at 4 locations (black bars), rabbit IgG at 4 locations (light gray bars), and PBS solution at 1 location (white bars) in 4 clinically normal horses. PBS injected skin samples were measured only at 20 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.

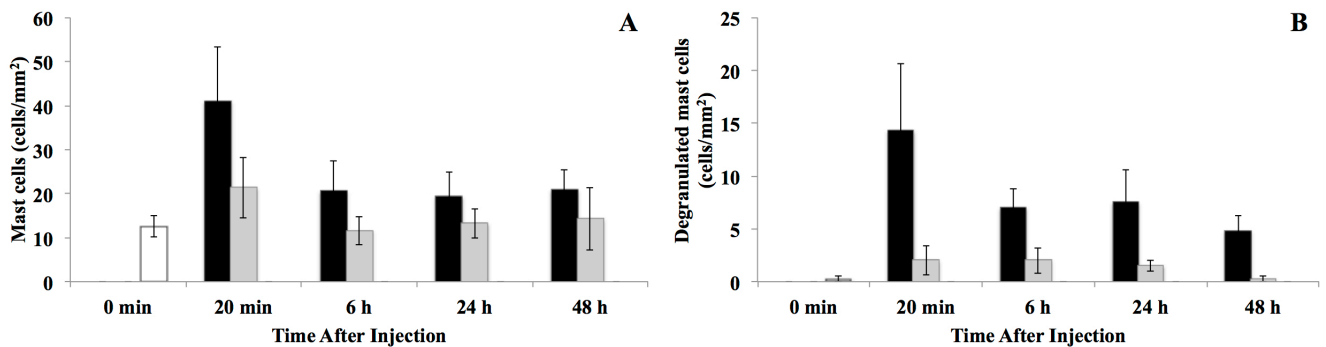


Figure 2.3: Mean ± SE number of mast cells (A) and degranulated mast cells (B) in skin samples obtained after intradermal injection of anti-IgE (black bars) and rabbit IgG (gray bars) or in noninjected skin (white bars) of 4 clinically normal horses. Noninjected skin samples were collected only before injection (time 0). The scale for the y-axis differs between panels. Notice that intradermal injection of anti-IgE causes degranulation of mast cells.

Late-Phase Reactions: The total number of inflammatory cells was not significantly different between treatment groups (Figure 2.4A). There were significantly more eosinophils in anti-IgE injected skin compared to IgG injected skin, but a significant Treatment x Time interaction was not seen (Figure 2.4B). There are significantly more eosinophils in the deep follicular and deep follicular to adnexal depths for anti-IgE injected skin compared to IgG injected skin. The neutrophil count was not significantly different between treatment groups (Figure 2.4C).

Lymphocyte Profile: There were significantly more CD4<sup>+</sup> cells at 24 and 48 hours for IgG injected skin when compared to anti-IgE. There was also a significant increase in CD4<sup>+</sup> cells from 6 to 24 hours for IgG injected samples (Figure 2.5A). There was no significant difference between treatment groups for CD8<sup>+</sup> cell counts (Figure 2.5B).

## 2.4 Discussion

This study found that intradermal injection of anti-canine IgE (0.8 mg/ml) in normal horses failed to produce significant reactions, both macro- and microscopically. This lack of significance may have been due to both the concentration of anti-canine IgE used, as well as the low sample size (n=4).

With a larger number of test subjects, more significant differences may have been seen between treatment groups. There was not a significant Treatment x Time effect for wheal surface area (Figure 2.2A), which would be expected if adequate amounts of anti-IgE were used. Additionally, the only time that there was a significant injection site thickness difference between treatment groups was at 20 minutes. (Figure 2.2B) In a similar study in dogs, wheals were significantly larger for anti-IgE injection sites compared to IgG at 20 minutes and 6 hours (Pucheu-Haston et al., 2006). In another study in dogs, significant wheal persistence was observed through 24 hours following anti-IgE injection (Olivry et al., 2001). At the concentration used in the present study and with the current sample size, anti-IgE did not create the expected macroscopic reactions.

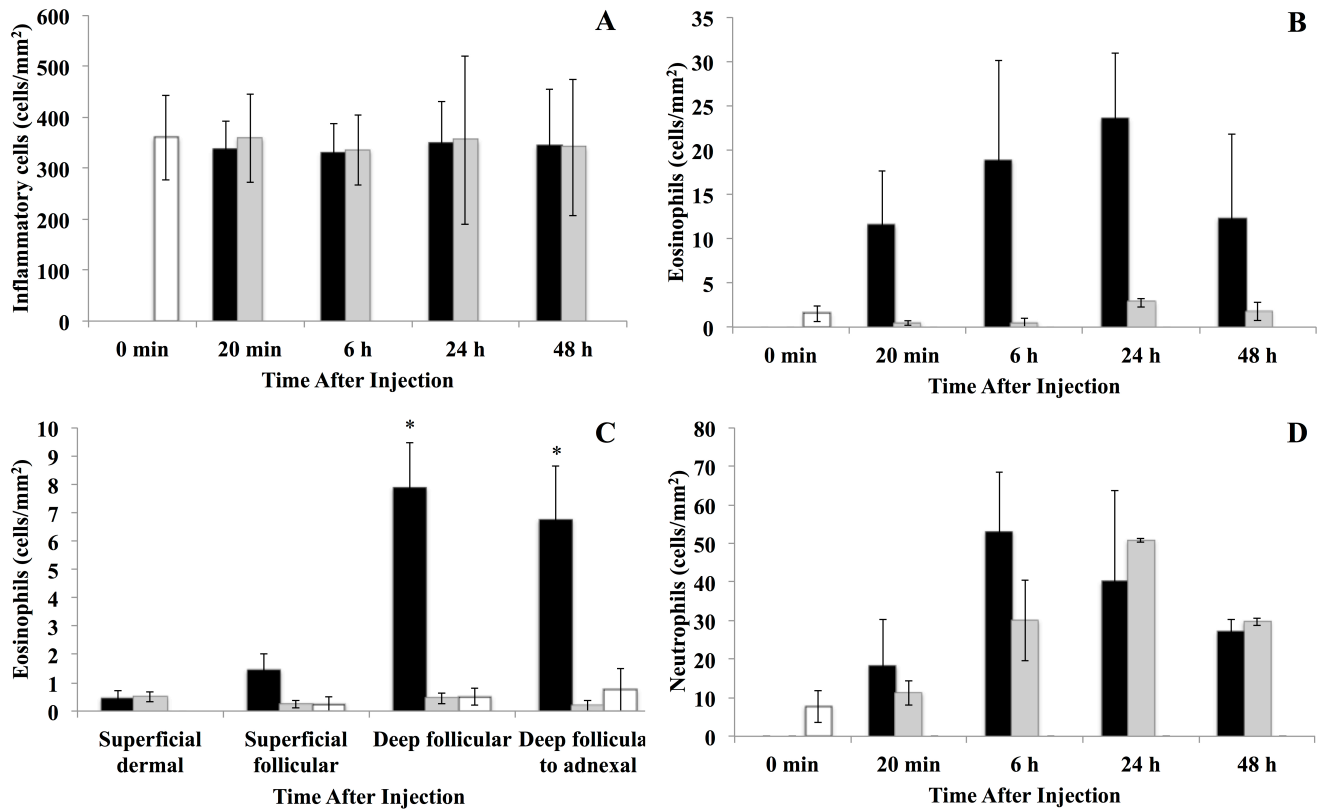


Figure 2.4: Mean  $\pm$  SE number of inflammatory cells (A), number of eosinophils (B), eosinophil depth (C), and number of neutrophils (D) in skin samples obtained after intradermal injection of anti-IgE and rabbit IgG or in noninjected skin of 4 clinically normal horses. The scale on the y-axis differs among panels. Notice that intradermal injection of anti-IgE causes a late-phase cellular response. \*Value differs significantly ( $P < 0.05$ ) from the value for the IgG injection within a dermis depth (panel C).

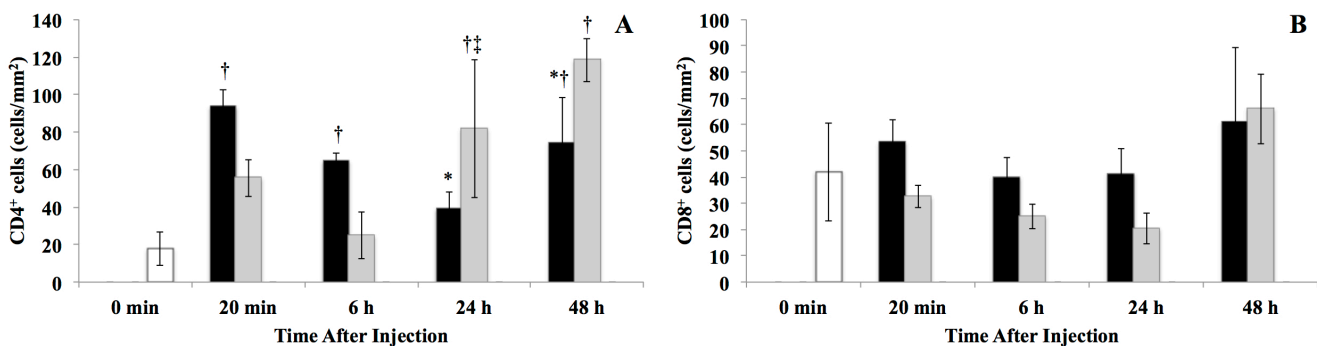


Figure 2.5: Mean  $\pm$  SE number of CD3<sup>+</sup> (A), CD4<sup>+</sup> (B), and CD8<sup>+</sup> (C) cells in skin samples obtained after intradermal injection of anti-IgE and rabbit IgG or in noninjected skin of 4 clinically normal horses. The scale on the y-axis differs among panels. Notice that intradermal injection of anti-IgE causes a late-phase cellular response characterized by the recruitment of CD3<sup>+</sup> cells and CD8<sup>+</sup> cells. \*Value differs significantly ( $P < 0.05$ ) from the value for the IgG injection within a time point. <sup>†</sup>Value differs significantly ( $P < 0.05$ ) from the value for noninjected skin. <sup>‡</sup>Within a treatment, value differs significantly ( $P < 0.05$ ) from the value at the preceding time point.

Anti-IgE intradermal injection resulted in significantly more mast cells and degranulated mast cells than IgG injection skin, but a treatment x time interaction was not noted (Figure 2.3). It is possible that an increased concentration of anti-IgE would make the results significant, but this did not prove true for the concentration of anti-IgE used in the study presented in Chapter 3. Further possible causes for the lack of mast cell significance are addressed in that chapter.

In the present study, the total number of inflammatory cells was not significantly different between treatment groups (Figure 2.4A). This lack of response may be due to the decreased concentration of anti-IgE used in the study, as significant differences between the two groups were noted when a higher concentration of anti-IgE was used. (Chapter 3). While there were overall significantly more eosinophils in anti-IgE injected skin compared to IgG, a significant Type x Time interaction was not noted (Figure 2.4B). This is in contrast to the subsequent high-dose study (Chapter 3), where eosinophils were significantly increased following anti-IgE injection at 6, 24, and 48 hours. Again, the lack of significance shown in this study is assumed to be due to the decreased concentration of anti-IgE, as well as the decreased sample size of the other study (4 horses vs. 6 horses).

There were significantly more CD4<sup>+</sup> cells in IgG injected skin compared to anti-IgE samples at 24 and 48 hours (Figure 2.5A). As discussed in Chapter 3, the results may also have been affected by the difficulty encountered in performing and evaluating IHC on frozen tissues. This unexpected difference in CD4<sup>+</sup> cells was no longer apparent when a higher concentration of anti-IgE was used (Chapter 3). There was not a significant difference between treatment groups for CD8<sup>+</sup> cells (Figure 2.5 B). Similar results were seen with higher concentrations of anti-IgE (Chapter 2). This cell type has not been frequently evaluated in horses with allergic skin disease, so expected results are unknown.

Intradermal injection of anti-IgE has been used in human and dogs to create a model of allergic skin disease and to evaluate the effects of different pharmaceutical agents on immediate and late-phase



inflammatory reactions (Gronneberg, 1984; Gronneberg and Raud, 1996; Gronneberg and Strandberg, 1985; Gronneberg et al., 1981; Gronneberg and Zetterstrom, 1985a, b, 1990b; Pucheu-Haston et al., 2006). At the concentrations used in the present study, anti-IgE did not produce expected results, suggesting that an anti-IgE concentration of 0.08 mg/ml would not be suitable for use in modeling equine allergic skin disease.

## 2.5 Endnotes

<sup>a</sup> Lloyd Laboratories, Decatur, IL

<sup>b</sup> Bruce Hammerberg, North Carolina State University

<sup>c</sup> Jackson ImmunoResearch Laboratories, Westgrove, PA

<sup>d</sup> Bethyl Labs, Montgomery, TX

<sup>e</sup> Sparhawk Laboratories, Lenexa, KS

<sup>f</sup> O.C.T. Compound Tissue Tek®, Sakura Finetek USA Inc., Torrance, CA

<sup>g</sup> Amresco LLC, Solon, OH

<sup>h</sup> VMRD, Pullman, WA

<sup>i</sup> Vector Laboratories, Burlingame, CA

<sup>j</sup> Invitrogen, Life Technologies, Grand Island, NY

<sup>k</sup> AEC, BioGenex, Fremont, CA

<sup>l</sup> Dako, Carpinteria, CA

<sup>m</sup> Vector S1000, Vector Laboratories, Burlingame, CA

<sup>n</sup> DAKO A 0452, Dako North America, Inc, Carpinteria, CA

<sup>o</sup> Vector BA 1000, Vector Laboratories, Burlingame, CA

<sup>p</sup> Vectastain ABC Rabbit IgG Kit, Vector PK 6102, Vector Laboratories, Burlingame, CA

<sup>q</sup> Vector Sk-4800, Vector Laboratories, Burlingame, CA

<sup>r</sup> Dako, North America, Inc, Carpinteria, CA

<sup>s</sup> SAS, version 9.3, SAS Institute Inc, Cary, NC.

## CHAPTER 3 CHARACTERIZATION OF IgE-MEDIATED (HIGHER CONCENTRATION) CUTANEOUS IMMEDIATE AND LATE-PHASE REACTIONS IN NON-ALLERGIC HORSES

### 3.1 Introduction

Anti-IgE antibodies have been used in the past to model allergic skin disease in dogs and humans (Gronneberg and Raud, 1996; Gronneberg and Strandberg, 1985; Gronneberg et al., 1981; Gronneberg and Zetterstrom, 1985a, b, 1990a). However, no work is available regarding the use of anti-canine IgE in horses and the ideal concentration of anti-IgE needed to induce significant immediate- and late-phase reactions is unknown.

The purpose of this study was to expand upon our prior work in which we found that intradermal injection of 0.08 mg/ml of anti-IgE produced a weak gross and microscopic inflammatory response with some similarities to the inflammation seen in spontaneous allergic disease. The hypothesis of the present study was that normal horses would have macroscopic and microscopic reactions similar to horses with allergic skin disease following intradermal injection of anti-IgE at a concentration 0.1 mg/ml. Additionally, these reactions would be comparable to those seen in dogs following similar injection.

### 3.2 Materials and Methods

Six horses (3 geldings, 3 mares) aged 4 to 8 years were used for this study. These horses were chosen based upon lack of evidence of current or prior cutaneous or systemic disease (including allergic skin disease). All horses were vaccinated for Eastern Equine Encephalitis, Western Equine Encephalitis, West Nile Virus Encephalitis, Tetanus, and Influenza, and dewormed with a product containing ivermectin or moxidectin (200 µg/kg body weight, orally, twice yearly) as part of routine herd care. No medications were given for 14 days prior to the study. Physical examinations were performed on Study

Day 0. All experimental protocols were approved by the Louisiana State University Institutional Animal Care and Use Committee (Protocol #11-072)

All horses were shaved on the lateral cervical area on Study Day 0 in preparation for injections in this location. Horses were sedated with intravenous xylazine<sup>a</sup> (0.4 mg/kg). All horses were injected intradermally (0.1 ml each injection) at one site with phosphate-buffered saline (PBS), at four sites with protein G affinity-purified anti-IgE<sup>b</sup> (Hammerberg et al., 2001) diluted to 0.1 mg/ml in PBS, and at four sites with IgG<sup>c</sup> diluted to 0.1 mg/ml in PBS. Anti-canine IgE was used as anti-equine IgE was not available. Furthermore, preliminary work (data not shown) had demonstrated the ability of anti-canine IgE to induce the development of gross and microscopic immediate and late phase reactions in the skin of normal horses.

Rabbit antisera specific for canine IgE was generated by injection of canine monoclonal IgE<sup>d</sup> produced by a canine x mouse heterohybridoma (Gebhard et al., 1995). Antisera were adsorbed with canine IgG linked to beads to remove antibodies cross-reactive with IgG.

Anti-IgE and IgG injected skin sites were examined at 20 minutes, and at 6, 24, and 48 hours after injection for the development of cutaneous swelling, erythema or induration. The PBS injected sites were measured at 20 minutes. The diameter of the cutaneous reactions (wheals) was measured in two perpendicular directions and the reaction surface areas were calculated. Dermal thickness of the injected skin was also measured with calipers.

Horses were sedated with xylazine prior to all skin biopsy acquisitions. All biopsy areas were injected subcutaneously with 0.5 ml of 2% lidocaine hydrochloride<sup>e</sup>. One 8-mm punch biopsy of normal, non-injected skin on the lateral neck was collected at the time of injection. Samples were collected at anti-IgE and IgG injection sites at 20 minutes, and 6, 24, and 48 hours after injection. Each sample was bisected immediately after collection. One half was placed in 10% neutral buffered formalin for routine

processing in paraffin. Fixation time was variable, but was a minimum of 72 hours after sample acquisition. The other half was placed in Optimal Cutting Temperature medium<sup>f</sup>, frozen in isopentane cooled in liquid nitrogen and stored at -80° until cryosectioning.

Five-micrometer tissue sections were cut from paraffin blocks and stained with hematoxylin and eosin for examination, cell enumeration, and pattern analysis. Eosinophils were better visualized using Luna's stain (Luna et al., 1968) for eosinophils. A low-pH (2.5) toluidine blue (T-blue) stain was used to facilitate evaluation of dermal mast cells.

A three-step labeled streptavidin method (Affolter and Moore, 2002) was modified and used to characterize the mononuclear cell infiltrate. Briefly, 6-µm cryosections were air dried, then immersed in acetone for fixation. The samples were rinsed with PBS containing 0.5% Tween 20 (0.5% PBST)<sup>g</sup> and then blocked with 1% fetal calf serum. The blocking sera was drained and mouse-origin monoclonal antibodies specific for equine CD4 or CD8 beta chain<sup>h</sup> were diluted 1:70 in PBS and applied to the slides. After rinsing, endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in PBS. Biotinylated horse-origin anti-mouse IgG<sup>i</sup> was applied diluted 1:400 in PBS, followed by horseradish peroxidase-conjugated streptavidin<sup>j</sup> diluted 1:400 in PBS. Samples were rinsed with 0.5% PBST between steps. Amino-9-ethyl carbazole<sup>k</sup> was applied as a chromogen, followed by hematoxylin counterstain<sup>l</sup>.

For CD3 immunohistochemistry, slides were deparaffinized in three changes of xylene for five minutes each. The samples were then rehydrated in three changes of 100% alcohol and one change of 95% alcohol for five minutes each before being rinsed three times in deionized water. Antigen retrieval was performed with 10mM citrate buffer (pH 6.0) for 10 minutes at 125-127° C (Biocare Decloaker) and then slides were cooled for twenty minutes before being rinsed three times each with deionized water first and Tris-buffer with 1% tween (pH 7.6). The rest of the procedure was performed in a DAKO

AutoStainer LINK 48. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for three minutes before a buffer rinse. Nonspecific antibody binding was blocked by incubation with normal goat serum for 30 minutes<sup>m</sup> followed by incubation with the primary antibody of 1:400 rabbit anti-human CD3<sup>n</sup> for 30 minutes followed by a buffer rinse. The sample was then incubated with secondary antibody of biotinylated anti-rabbit secondary antibody<sup>o</sup> for 30 minutes followed by a buffer rinse. The tissue samples were then exposed to the detection reagent avidin/biotinylated enzyme complex<sup>p</sup> for 30 minutes before a buffer rinse and application of NovaRed<sup>q</sup> followed by a buffer rinse and then deionized water. The samples were counterstained for five minutes with Mayers hematoxylin (Lillie's modification)<sup>r</sup> rinsed again with buffer and then deionized water, and removed from the stainer. Slides were dried in an oven at 60° C for thirty minutes before being removed and cooled for five to ten minutes. Slides were rinsed in three changes of xylene for three minutes each before being coverslipped.

For all cell types, the total number of cells/mm<sup>2</sup> in the dermis was obtained by counting sixteen 0.25 mm x 0.25 mm fields of dermis (excluding epithelial cells and adnexa). For the H&E, Luna, and T-blue slides, the sixteen fields were split evenly between four depths of dermis: superficial dermal (A), superficial follicular (B), deep follicular (C), and deep follicular to adnexal (D) (Figure 2.1). Total numbers of nucleated cells, total granulocytes, and total mononuclear cells were counted using H&E stained slides. Eosinophils were counted using the Luna stained slides. A total neutrophil count was obtained by determining the difference between the number of eosinophils/mm<sup>2</sup> and the number of total granulocytes/mm<sup>2</sup>. The T-Blue stained slides were evaluated for total numbers of intact mast cells as well as degranulated mast cells. CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> dermal cells were counted on sixteen 0.25 mm x 0.25 mm fields beginning in the superficial dermis (excluding endothelial cells and adnexa) and extending deeper as needed.

Data analysis was performed with statistical software<sup>s</sup>. A repeated measures analysis of variance with a factorial arrangement of treatments was conducted. Fixed effects in the model included depth (when applicable), time, treatment, and their interactions. Animal was included as a random effect. When overall significance was detected ( $p \leq 0.05$ ), post hoc comparisons were made with pairwise t tests of least-squares means. This was done for all dermal cell counts as well as injection site measurements.

### 3.3 Results

**Macroscopic Reactions:** Intradermal injection of anti-IgE produced erythematous and indurated local urticarial reactions within 20 minutes in all horses. The surface areas of these reactions were significantly larger than normal rabbit IgG injected sites at 20 minutes, 6 and 24 hours after injection (Figure 3.1A). The areas of dermal induration in anti-IgE injected skin significantly increased from 20 minutes to 6 hours and from 6 to 24 hours. Anti-IgE injection sites were significantly larger than PBS injected skin at 20 minutes, except at 48 hours. IgG injection sites, when measurable, were never significantly different from PBS injected skin at 20 minutes. There were no measurable wheals or induration for IgG injected skin at 24 or 48 hours or for anti-IgE injected skin at 48 hours.

The anti-IgE injection sites were significantly thicker than IgG injected skin at 20 minutes, 6 hours, and 24 hours (Figure 3.1B). Dermal thickness in anti-IgE injected skin was greatest 20 minutes after injection and decreased significantly compared to the previous values at all time points (e.g., from 20 minutes to 6 hours, from 6 to 24 hours and from 24 to 48 hours). Anti-IgE injection site thickness at 20 minutes and 6 hours was significantly greater than PBS thickness, but was significantly less than PBS thickness at 48 hours. IgG injection site thickness at 24 and 48 hours was less than the thickness of PBS injection.

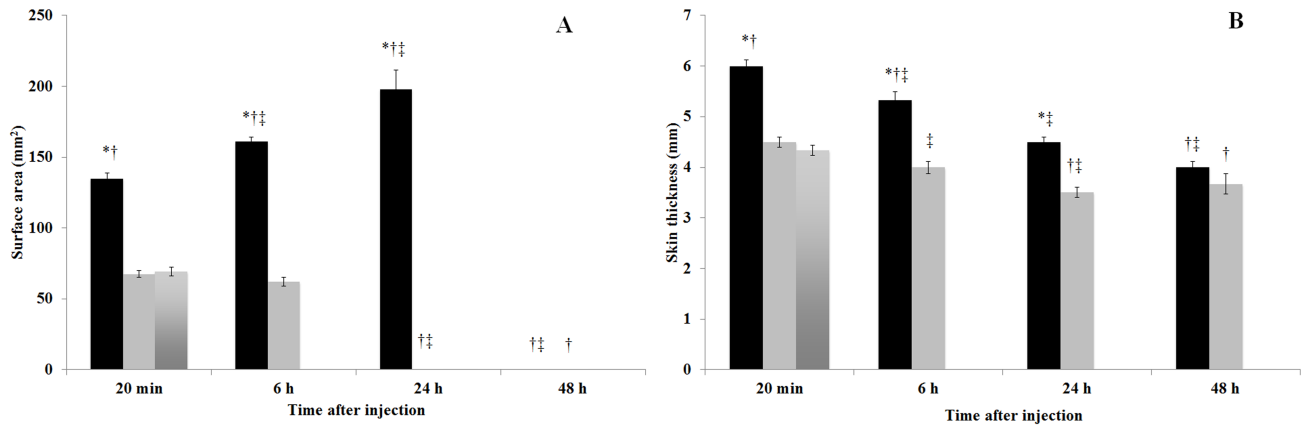


Figure 3.1: Intradermal injection of anti-canine IgE (but not normal rabbit IgG) induces the formation of a measurable wheal. (A) Injection site surface area and (B) injection site thickness. \* Value differs significantly ( $P < 0.001$ ) between treatment groups. † Significant difference ( $P < 0.05$ ) from previous measured value for the same treatment group.

**Immediate-Phase Reactions:** There was no significant difference in the total number of mast cells seen between treatment groups (Figure 3.2A). The mean number of degranulated mast cells were significantly ( $P < 0.0001$ ) higher in anti-IgE treated skin when compared to IgG injected skin, but a treatment x time interaction was not found (Figure 3.2B).

**Late-Phase Reactions:** The total number of inflammatory cells increased up to 24 hours after anti-IgE injection when compared to time 0 (Figure 3.3A) and there were significantly more inflammatory cells in anti-IgE injected skin compared to IgG injected skin at 6 and 24 hours. By 48 hours, the total number of inflammatory cells decreased in anti-IgE injected skin and were no longer significantly different when compared to inflammatory cell counts in non-injected control skin.

Luna staining revealed a significant increase in the number of eosinophils in anti-IgE injected skin from 20 minutes to 6 hours and 6 to 24 hours (Figure 3.3C). Then, eosinophil counts significantly decreased from 24 to 48 hours. There were significantly more eosinophils for anti-IgE injected skin compared to IgG injected skin at 6 hours ( $P = 0.0015$ ), 24 hours ( $P < 0.0001$ ), and 48 hours ( $P = 0.0013$ ). Anti-IgE injected skin had significantly more eosinophils compared to non-injected skin at 6 hours



( $P=0.0011$ ), 24 hours ( $P<0.0001$ ), and 48 hours ( $P=0.0013$ ). Eosinophil counts were not significantly different in IgG injected skin when compared to non-injected skin at any time point.

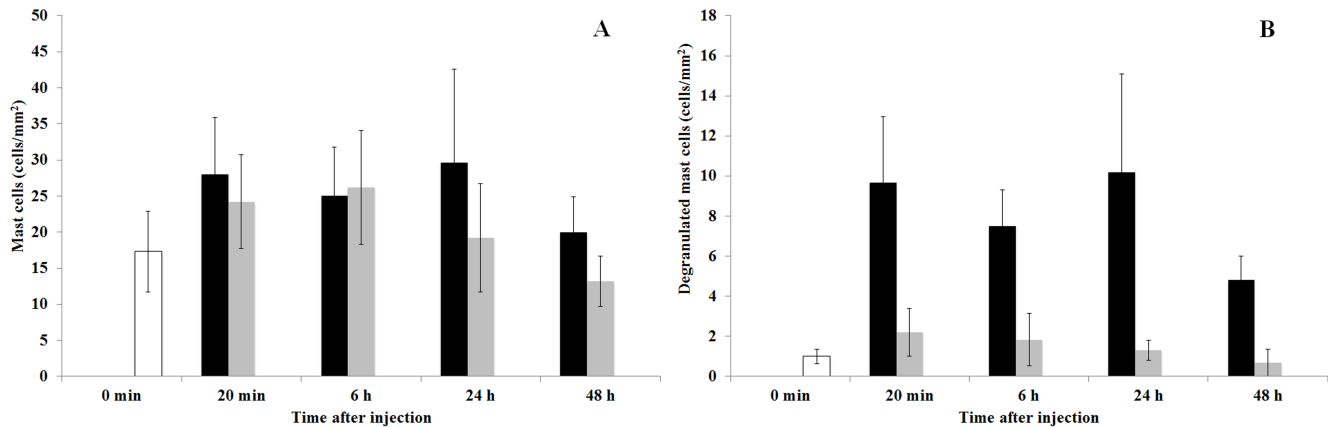


Figure 3.2: Intradermal injection of anti-canine IgE produces mast cell degranulation. (A) Total mast cells and (B) degranulated mast cells.

There was a significant treatment x depth effect for eosinophils. Eosinophil counts were significantly higher in the deeper dermis at the superficial follicular ( $P<0.0014$ ), deep follicular ( $P<0.0001$ ), and deep follicular to adnexal ( $P<0.0001$ ) depths, for anti-IgE samples when compared to IgG tissue (Figure 3.3C). Eosinophil counts did not differ between treatment groups at the depth of the superficial dermis.

Neutrophils were significantly increased in anti-IgE injected skin from 20 minutes to 6 hours. Neutrophil counts decreased significantly from 6 to 24 hours and 24 to 48 hours (Figure 3.3D). For anti-IgE injected skin, there were significantly more neutrophils at 6 hours compared to IgG injected skin. Significantly more neutrophils were seen in anti-IgE injected skin compared to non-injected skin at 6 and 24 hours. Neutrophil counts in IgG injected skin did not differ from non-injected skin at any time point.

Lymphocyte Profile: There was no significant difference between  $CD3^+$ ,  $CD4^+$ , or  $CD8^+$  (Fig 3.4) cell counts in the dermis at any time point for anti-IgE and IgG injected skin.

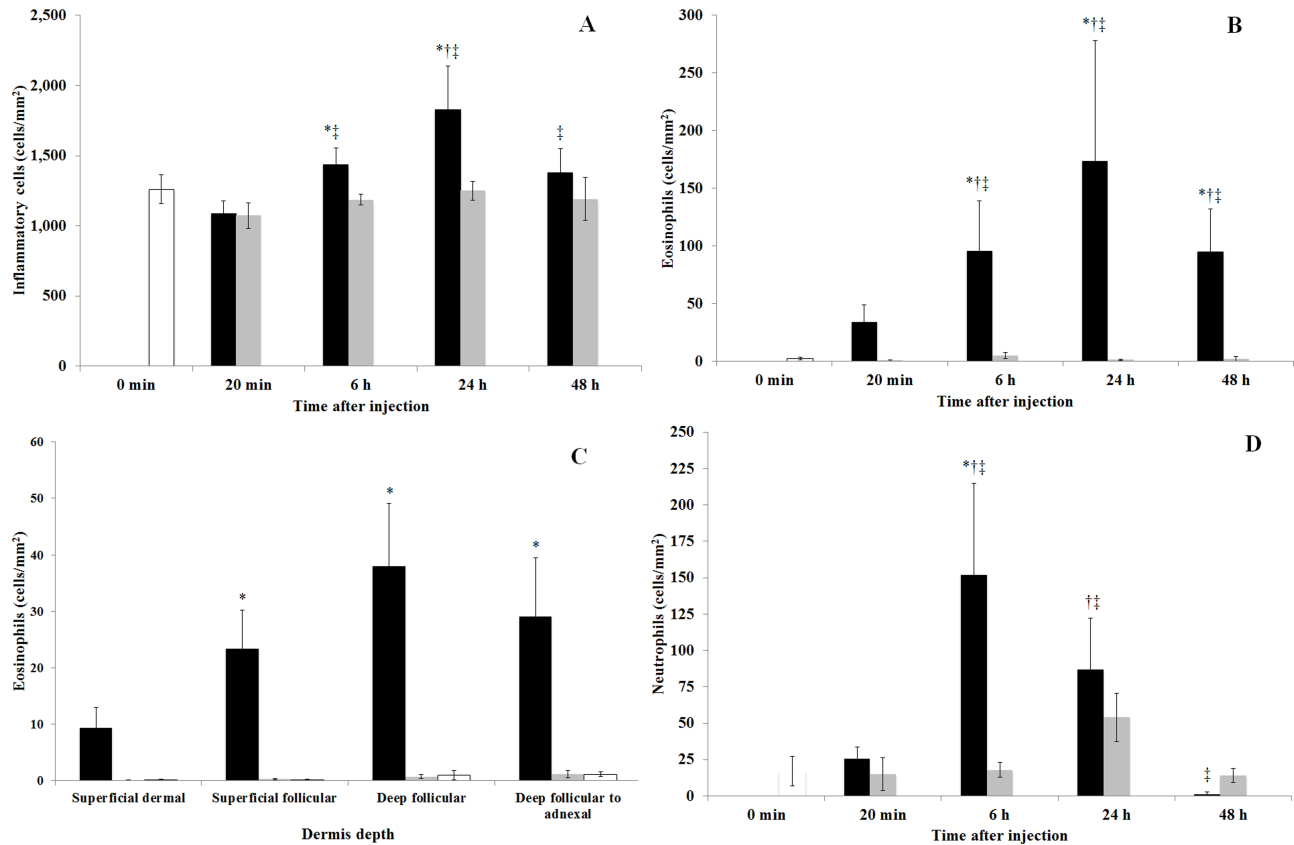


Figure 3.3: Intradermal injection of anti-canine IgE produces a late-phase cellular response. (A) Total inflammatory cells, (B) eosinophils, (C) eosinophil depth, and (D) neutrophils. \* Value differs significantly ( $P<0.05$ ) between treatment groups. † Significant difference ( $P<0.05$ ) from previous measured value for the same treatment group. ‡ Significant difference ( $P<0.05$ ) from noninjected skin.

### 3.4 Discussion and Conclusions

The present study confirmed that intradermal injection of protein G affinity-purified rabbit-origin polyclonal IgG specific for canine IgE produced many macro- and microscopic changes in normal horses similar to those seen in humans and dogs in similar studies. Anti-equine IgE was not available for use in the present study, but would be expected to produce similar results. The reported protein sequence of canine IgE is approximately 65-67% identical with that of equine IgE (as determined by BLAST<sup>t</sup> (Altschul et al., 1997; Altschul et al., 2005)), which would suggest sufficient similarity to permit cross-species use. This assumption is supported by the fact that some commercially available anti-canine IgE antibodies<sup>u</sup> have been demonstrated to bind cross-reactively to equine IgE. Furthermore, previous work

(B. Hammerberg, unpublished study) has demonstrated that both rabbit polyclonal anti-canine IgE and mouse monoclonal anti-IgE were able to induce immediate wheal-and-flare responses in the skin of normal horses following intradermal injection.

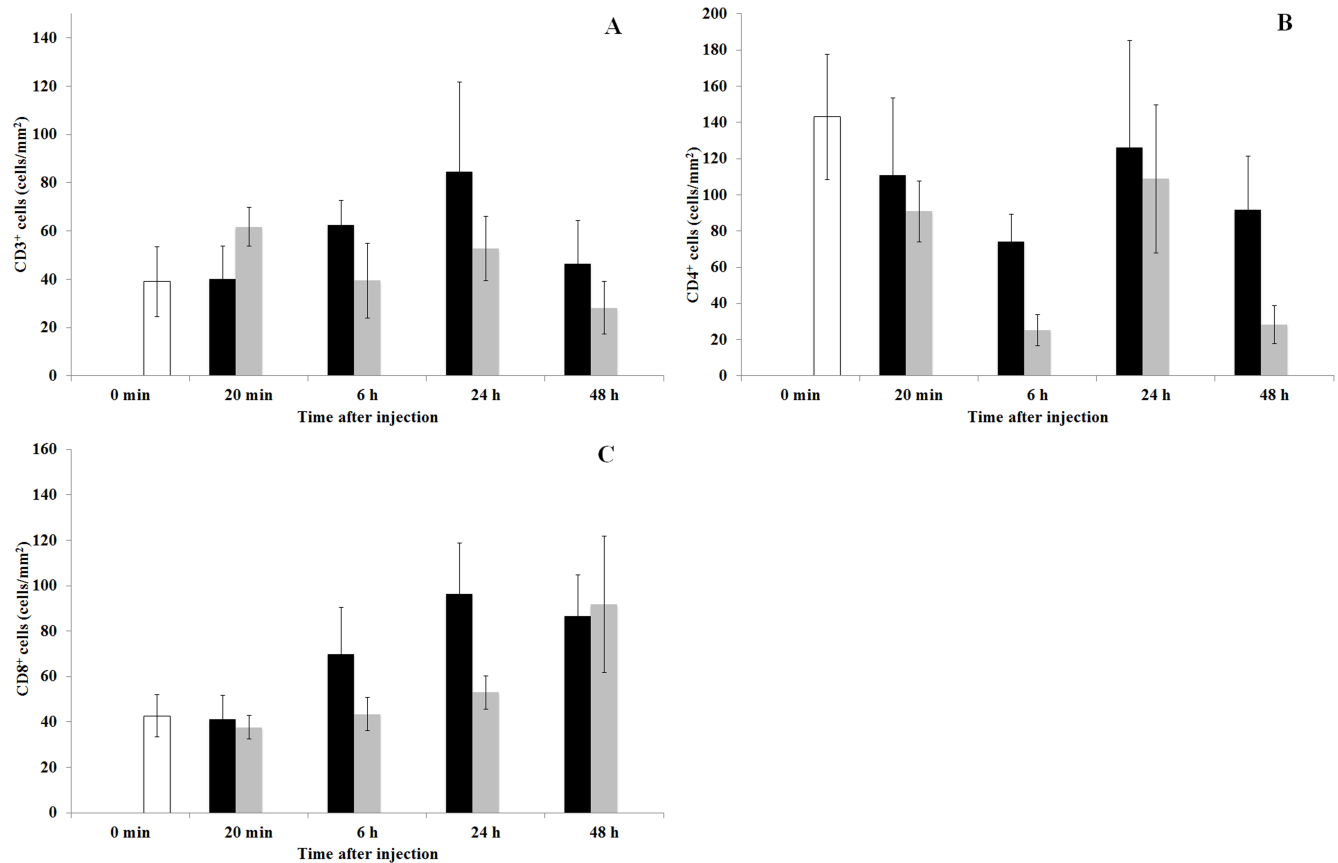


Figure 3.4: Intradermal injection of anti-canine IgE produced a late-phase cellular response characterized by the recruitment of CD3 and CD8 positive cells. (A) CD3, (B) CD4, (C) CD8.

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 3.1A). In a similar study in dogs, a significantly larger wheal was noted for anti-IgE injection when compared to IgG at 20 minutes and 6 hours, but was not seen at 24 or 48 hours (Pucheu-Haston et al., 2006). In another study in dogs, urticaria was observed through 24 hours, though lesions decreased in size up to that point (Olivry et al., 2001). Thus, it appears that horses may have a more prolonged macroscopic late-phase reaction when compared to dogs.

Intradermal injection of PBS and IgG also produced measurable distention of the skin, but these areas were significantly smaller and less indurated than anti-IgE wheals (Figure 3.1B). Additionally, injection site surface area associated with IgG injection was absent by 24 hours. Injection of any substance intradermally will cause a transient distention of the superficial dermis, but this distention will become absent with time as the substance is reabsorbed. This likely accounts for the initial injection site measurements seen with IgG and PBS.

The use of stereological methods of counting cells (rather than manual cell counting, as was performed here) was considered in the design of this study. However, these programs were not used for several reasons. The first reason is the complex structure of the superficial and middle dermis. In these areas, there are numerous non-dermal structures, including the papillary epidermis, hair follicles, sebaceous glands, muscles, blood vessels, and nerves. In order to count a consistent area of dermis, these structures need to be excluded from the area to be evaluated, which is easiest for a human to do. Additionally, these other structures, and their associated cell types (keratinocytes, sebocytes, endothelial cells) can make identifying specific cell types of interest (mononuclear cells, granulocytes) very difficult. For these reasons, we chose to employ a manual cell count method in this study.

Although anti-IgE intradermal injection tissue had significantly more degranulated mast cells than non-injected and IgG injected skin, a treatment x time interaction was not apparent. One possible reason for a lack of statistical significance with regards to degranulated mast cells is the difficulty in visualizing them once they have degranulated and lose their staining capabilities, leading to variability in cell counts. This degranulation phenomenon has been noted previously in humans (Claman et al., 1986) and has been postulated to occur in horses (Rufenacht et al., 2005). Van der Haegen et al (van der Haegen et al., 2001) examined mast cells in skin of horses with IBH and evaluated their numbers with toluidine-blue stain and immunohistochemistry for chymase and tryptase. The effect of fixation time

was also evaluated. The results of that study revealed that toluidine-blue staining was significantly decreased in samples that were fixed for periods longer than 24 hours. Additionally, more tryptase+ mast cells were seen in samples fixed for less than 24 hours when compared to samples fixed for longer periods of time (van der Haegen et al., 2001). Thus, it might be beneficial, in future studies using the model developed in the present study, to either shorten fixation times for toluidine-blue stained samples, or to additionally include tryptase staining when evaluating mast cells in equine 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 to IgG injection (Figure 3.3A). This timing was consistent with a late-phase reaction with influx of inflammatory cells. Eosinophils, in particular, were increased at 6, 24, and 48 hours for anti-IgE injected skin when compared to IgG and non-injected skin. Various chemokines (e.g. CCL11, CCL24, and CCL26) have been shown to recruit eosinophils and are produced by different cell types (e.g. epithelial cells) (Abbas et al., 2012). In the current study, eosinophil recruitment was likely due to chemotactic factors produced by degranulating mast cells, as the horses in this study were unlikely to have a pre-existing hypersensitivity to the anti-IgE antibody and thus would not be 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 (Abbas et al., 2012). Future studies could evaluate tissue sections for increased levels of these chemokines or of IL-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 the horse, however the depth of eosinophilic infiltration was either not described or comparisons of the relative numbers of eosinophils at different depths were not made (Fadok and Greiner, 1990; Foster et al., 1995; McKelvie et al., 1999; Rufenacht et al., 2005; Scott, 2010; van der Haegen et al., 2001). In the present study, more eosinophils were found in the deeper layers of dermis in anti-IgE injected skin.

Previous studies evaluating anti-IgE intradermal injection in dogs have only described cellular counts in the most superficial dermis (Pucheu-Haston et al., 2006). The results of the study reported here demonstrated that there was no significant difference in eosinophil numbers between anti-IgE and IgG injected skin in the superficial dermis (Figure 3.3C). However, eosinophil numbers were increased at the depth of the follicle and deeper dermis in anti-IgE injected skin. Had this study not evaluated depth as a factor, a significant 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 would be useful to evaluate the depth of eosinophils in naturally occurring equine allergic skin disease.

Quantitation of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells over time in this study did not demonstrate significant differences between anti-IgE and IgG injected skin. The CD3 stained slides were assessed on formalin fixed, paraffin tissue and should be evaluated separately from the CD4 and CD8 cells counts, which were obtained from frozen tissue. This may account for the decreased number of CD3<sup>+</sup> cells seen compared to the sum of CD4<sup>+</sup> and CD8<sup>+</sup> counts (as all of these cells should also be CD3<sup>+</sup>). The anti-IgE injected skin showed increased numbers of CD3<sup>+</sup> cells, when compared to IgG injected skin, from 20 minutes to 24 hours with a subsequent decrease in cell counts at 48 hours, but these trends were not significant (Figure 3.4A). This trend was similar to results from a previous study in dogs in which CD3<sup>+</sup> cells increased by 6 hours and peaked 24 hours after anti-IgE injection (Pucheu-Haston et al., 2006). Another study evaluating late-phase reactions after intradermal injection of anti-IgE in dogs found that CD3<sup>+</sup> dermal cell counts increased from 6 to 48 hours after injection (Olivry et al., 2001). McKelvie et al. evaluated the skin lymphocyte populations in horses with IBH (McKelvie et al., 1999) and found that there were an increased number of CD3<sup>+</sup> lymphocytes (many of which were also CD4<sup>+</sup>) in the skin of horses with IBH following injection with *Culicoides* antigen. These late-phase changes were not seen in the present study. However, McKelvie et al. only evaluated horses with signs of IBH, which horses in the present study did not demonstrate. It is possible that if the present study's methods were repeated

with allergic horses that were injected intradermally with specific antigen, the results would be more similar to those seen by McKelvie et al. Additionally, it must be kept in mind that the McKelvie study evaluated the response to whole-body *Culicoides* extract, which may have included proteases and other irritants. This extract failed to produce a gross whealing response in non-allergic ponies in a prior study and the histology of the *Culicoides* injection sites was not evaluated in these animals (Foster et al., 1998). For this reason, it is difficult to ascertain whether these lymphocyte responses truly represent an antigen-specific phenomenon.

The CD4<sup>+</sup> cell counts in this study were not consistent with findings in previous studies (Heimann et al., 2011; McKelvie et al., 1999). One study evaluating Icelandic horses with insect bite hypersensitivity found an increase in CD4<sup>+</sup> cells in skin samples of affected horses (Heimann et al., 2011). Although the cause of the lack of increase of CD4<sup>+</sup> cells in this study is not known, it may be partially attributed to the difficulty in performing and evaluating IHC on frozen tissues. These samples, in general, did not maintain the architecture of the skin well and were more difficult to stain than paraffin tissue. In addition, the use of anti-canine IgE (rather than anti-equine IgE) may have led to the difference in CD4<sup>+</sup> populations, possibly due to a lower affinity and thus a less robust inflammatory response. However, this possibility seems somewhat unlikely due to the similar reaction in other cell populations.

CD8<sup>+</sup> cells have been evaluated less often than CD3<sup>+</sup> and CD4<sup>+</sup> in horse skin. In the present study, CD8<sup>+</sup> cells increased in anti-IgE injected skin from 20 minutes to 24 hours and then decreased at 48 hours (similar to the CD3<sup>+</sup> cell counts) (Figure 3.4C). However, the increase was not significantly different from IgG injected skin. One study evaluating CD8<sup>+</sup> cells found significantly more dermal CD4<sup>+</sup>, but not more CD8<sup>+</sup>, cells in horses with IBH lesional skin compared to healthy horses (Heimann et al., 2011).

The horses in this study developed significant gross and histologic reactions following intradermal injection of anti-IgE. In order to do so, IgE must have been present to some degree at the sites of injection prior to the study. Previous studies have shown that horses that do not have clinical allergic skin disease can still be sensitized to allergens and show positive reactions to intradermal injection (Lorch et al., 2001a; Lorch et al., 2001b; Wagner et al., 2009). Anti-IgE, unlike specific allergens, crosslinks all IgE, regardless of allergen specificity. Previous studies in horses have demonstrated that IgE on mast cells can be non-specifically cross-linked by anti-IgE, even in non-allergic horses (Wagner et al., 2006; Wagner et al., 2003; Wilson et al., 2006). The ability of the horses in this study to develop a response to anti-IgE therefore does not necessarily reflect allergy or parasitism.

As previously discussed, intradermal injection of anti-IgE has been used in human and dogs to create a model of allergic skin disease and to evaluate the effects of different pharmaceutical agents on immediate and late-phase inflammatory reactions (Gronneberg, 1984; Gronneberg and Raud, 1996; Gronneberg and Strandberg, 1985; Gronneberg et al., 1981; Gronneberg and Zetterstrom, 1985a, b, 1990b; Pucheu-Haston et al., 2006). The study reported here confirms that injection of anti-IgE produces a dermal inflammatory response with many similarities to that seen in spontaneous allergic skin disease in horses. These similarities were particularly marked for eosinophils and neutrophils, although the lymphocyte response generated in this study was lesser in magnitude than what would be expected. In the future, controlled studies using this model could be performed to evaluate the effectiveness of different treatment modalities and their effects on macro- and microscopic reactions for allergic skin disease in horses without having to maintain a herd of allergic horses.

### 3.5 Endnotes

<sup>a</sup> Lloyd Laboratories, Decatur, IL



- <sup>b</sup> Bruce Hammerberg, North Carolina State University
- <sup>c</sup> Jackson ImmunoResearch Laboratories, Westgrove, PA
- <sup>d</sup> Bethyl Labs, Montgomery, TX
- <sup>e</sup> Sparhawk Laboratories, Lenexa, KS
- <sup>f</sup> O.C.T. Compound Tissue Tek®, Sakura Finetek USA Inc., Torrance, CA
- <sup>g</sup> Amresco LLC, Solon, OH
- <sup>h</sup> VMRD, Pullman, WA
- <sup>i</sup> Vector Laboratories, Burlingame, CA
- <sup>j</sup> Invitrogen, Life Technologies, Grand Island, NY
- <sup>k</sup> AEC, BioGenex, Fremont, CA
- <sup>l</sup> Dako, Carpinteria, CA
- <sup>m</sup> Vector S1000, Vector Laboratories, Burlingame, CA
- <sup>n</sup> DAKO A 0452, Dako North America, Inc, Carpinteria, CA
- <sup>o</sup> Vector BA 1000, Vector Laboratories, Burlingame, CA
- <sup>p</sup> Vectastain ABC Rabbit IgG Kit, Vector PK 6102, Vector Laboratories, Burlingame, CA
- <sup>q</sup> Vector Sk-4800, Vector Laboratories, Burlingame, CA
- <sup>r</sup> Dako, North America, Inc, Carpinteria, CA
- <sup>s</sup> SAS, version 9.3, SAS Institute Inc, Cary, NC.
- <sup>t</sup> BLASTP 2.2.29+, National Center for Biotechnology Information, Bethesda, MD
- <sup>u</sup> MCA1896, AbD Serotec, Raleigh, NC

## CHAPTER 4 FINAL DISCUSSION AND CONCLUSIONS

Intradermal injection of anti-IgE induced macroscopic and microscopic reactions in horses that were similar to those seen in naturally occurring allergic disease, but only at a higher concentration of 0.1 mg/ml. At a lower concentration (0.08 mg/ml), the difference between anti-IgE injection and IgG injection was less significant, suggesting that this lower dose was suboptimal for modeling dermal IgE-mediated inflammation. Although the results were not precisely what was expected when compared to previous studies in other species, this may be secondary to species-specific differences. In the future, the model developed in this paper could be used to evaluate different medical therapies for equine allergic skin disease secondary to type I hypersensitivity reactions mediated by IgE.

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