ABSTRACT

PUCHEU-HASTON, CHERIE. Role of epicutaneous exposure to *Dermatophagoides farinae* in the development of IgE-dependent and independent allergic dermatitis in the dog.
(Under the direction of Dr. Bruce Hammerberg.)

Atopic dermatitis (AD) is an inflammatory and pruritic allergic skin disease of humans and several species of domestic animals. It presents as a clinical syndrome with characteristic features, including a young age of onset, genetic predisposition, dermatitis of flexural skin and predisposition to secondary infections. Clinical disease is most frequently observed with IgE antibodies to environmental allergens, especially those associated with several species of housedust mite (HDM), including *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*.

Although the immune response in established AD has been well studied, many questions remain regarding the development and perpetuation of clinical disease. For example, it remains unclear as to whether naturally occurring sensitization and the development of clinical disease can be induced by cutaneous exposure to HDM allergens. Most research on the role of epicutaneous allergen exposure during the sensitization phase of AD has been performed in mouse models. However, extrapolation of the results of murine studies to spontaneous human disease is limited by the existence of subtle but important
differences in the cellular distribution and function of several receptors and mediators critical to the development and maintenance of cutaneous hypersensitivity.

A complementary alternative model is the dog, a species in which spontaneous allergic diseases are common and artificial sensitization is both possible and commonly performed. Historically, research in this species has been limited due to the lack of canine-specific reagents, but recent advances in the development of monoclonal and polyclonal antibodies and primer sequences for use in the dog now allows research to be performed at a high level of sophistication.

The current work describes the results of three experiments designed to further understanding of the role of epicutaneous allergen exposure in the development and perpetuation of AD. First, the gross, microscopic and inflammatory mediator responses of normal dog skin following IgE-mediated challenge were evaluated by intradermal injection of cross-linking anti-IgE antibodies. This study provided important baseline data and established optimized sample processing protocols for the following two studies. Second, the cutaneous and systemic response to cutaneous allergen exposure was evaluated by repeated epicutaneous application of a sonicated slurry of Dermatophagoides farinae house dust mites to the intact skin of allergy-predisposed Maltese-Beagle cross-bred dogs. This study demonstrated that both cutaneous and systemic inflammation similar to naturally-occuring AD can be induced in the dog by cutaneous exposure to a mix of mite allergens. Finally, the role of allergen proteolytic activity in facilitating epicutaneous sensitization was evaluated by repeated epicutaneous application of either proteolytically-active or –inactive Der f 1 house
dust mite allergen. This study demonstrated demonstrate that cutaneous exposure to Der f 1 allergen through intact canine skin may be sufficient to induce sensitization, and suggest that this response may be facilitated by the proteolytic activity of the allergen.
Role of epicutaneous exposure to *Dermatophagoides farinae* in the development of IgE-dependent and independent allergic dermatitis in the dog

By

Cherie Pucheu-Haston

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Approved by

Dr. Thierry Olivry

Dr. Gregg Dean

Dr. Keith Linder

Dr. Bruce Hammerberg
Chair of Advisory Committee
DEDICATION

To absent friends, and to those who yet remain…
BIOGRAPHY

Dr. Pucheu-Haston graduated from Louisiana State University School of Veterinary Medicine in 1992. She completed an internship in small animal medicine and surgery at Louisiana State, and completed a residency in dermatology at North Carolina State University College of Veterinary Medicine. She became a diplomate of the American College of Veterinary Dermatology in 1995. She was in private practice for seven years before returning to North Carolina to pursue a PhD in immunology. Her major research interest is atopic dermatitis, with a special focus on the roles of mast cells and epicutaneous allergen penetration during the sensitization phase.
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**Introduction:**

Atopic dermatitis (AD) is an inflammatory and pruritic allergic skin disease of humans[1] and several species of domestic animals, including dogs[2]. It presents as a clinical syndrome with characteristic features[3], including a young age of onset, genetic predisposition, dermatitis of flexural skin and predisposition to secondary infections with bacteria and yeast[4]. Clinical disease is most frequently observed with IgE antibodies to environmental allergens[3] Although many environmental antigens have been associated with AD (including pollens, danders and molds)[4], the most common allergens appear to be those associated with several species of housedust mite (HDM), especially *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*[5].

AD is associated with significant co-morbidities and health care costs. A recent study estimated an annual treatment expenditure of $464 million in the United States alone[6]. Pruritus and dermatitis associated with AD may be severe, resulting in disfiguring damage to the skin and emotional distress. Furthermore, evidence suggests that the development of AD (and the immunologic changes that accompany it) may be the first step in a progression of allergic diseases such as asthma—the “*atopic march*”[4, 7]. Investigation of the cutaneous immunologic interactions with allergens (especially those associated with *Dermatophagoides*) during sensitization may facilitate further understanding of the pathogenesis of AD and enhance investigation of novel preventative and therapeutic interventions.
Although the immune response in established AD has been well studied, many questions remain regarding the development and perpetuation of clinical disease. It remains unclear as to whether naturally occurring sensitization and the development of clinical disease can be induced by cutaneous exposure to HDM allergens. Epicutaneous sensitization has often been considered unlikely due to the relative impermeability of the stratum corneum[8]. Substances known to cross the stratum corneum are typically small and/or lipophilic, whereas HDM antigens are large, ranging from 14kD to over 100kD[9]. However, recent evidence suggests that the barrier presented by the stratum corneum can be circumvented by particles as large as 1 micron by entering the dermis through the hair follicles[10]. In addition, *Dermatophagoides* allergens include a number of highly active proteases (especially the mite allergen Der f 1)[11, 12], several of which have been shown to increase paracellular permeability in a number of in vitro culture systems[12]. These proteolytic enzymes have the potential to increase allergen absorption through both the follicles and stratum corneum.

Research into the role of epicutaneous absorption during sensitization has been hampered by the lack of a suitable animal model. Most research on the pathogenesis and treatment of AD has been performed in mouse models, such as spontaneously allergic NC/Nga or passively sensitized BALB/c mice[13, 14]. However, it must be considered that information derived from groups of inbred, genetically identical and/or artificially or passively sensitized subjects may not necessarily be suitable for extrapolation to spontaneous disease in genetically predisposed humans, which entails complex interactions between
genetic predisposition and host environment, skin barrier defects, microbial skin infections and other immunologic factors[4]. The risk of over-extrapolation from mouse to man is further highlighted by the existence of subtle but important differences in the cellular distribution and function of several receptors and mediators critical to the development and maintenance of cutaneous hypersensitivity, such as the high- and low-affinity receptors for IgE [15, 16] and IgG [17-19].

Canine models of AD can produce information complementary to that generated by murine studies. The domestic dog has a high incidence of naturally occurring AD[2, 20]. Furthermore, the canine cutaneous immune system and function is very similar to that of humans. In the dog, surface expression of IgE has been detected in situ on epidermal Langerhans cells, dermal dendritic cells and dermal mast cells[21], as well as circulating B cells, CD14+ mononuclear cells and CD1c+ dendritic cells[22]. Aggregation of this IgE by the intradermal injection of cross-linking anti-canine IgE antibodies has been demonstrated to produce immediate and late phase reactions (LPR) in the skin of both normal dogs and dogs with naturally occurring AD[23]. These reactions grossly and microscopically resemble those generated by the intradermal injection of allergen or anti-IgE in humans with atopic disorders, including AD[23-27].

This work describes the results of three experiments that were designed to further our understanding of the role of epicutaneous exposure to allergen in the development and perpetuation of AD.
1. The gross, microscopic and inflammatory mediator responses of normal dog skin following IgE-mediated challenge were evaluated by intradermal injection of cross-linking anti-IgE antibodies. This study provided important baseline data and established optimized sample processing protocols for the following two studies.

2. The cutaneous and systemic response to cutaneous allergen exposure was evaluated by repeated epicutaneous application of a sonicated slurry of *Dermatophagoides farinae* house dust mites to the intact skin of allergy-predisposed Maltese-Beagle cross-bred dogs. This study demonstrated that both cutaneous and systemic inflammation similar to naturally-occurring AD can be induced in the dog by cutaneous exposure to allergen.

3. The role of allergen proteolytic activity in facilitating epicutaneous sensitization was evaluated by repeated topical application of either proteolytically active or inactive *Der f 1* house dust mite allergen. This study demonstrated that cutaneous exposure to *Der f 1* allergen through intact canine skin may be sufficient to induce sensitization, and suggest that this response may be facilitated by the proteolytic activity of the allergen.
Literature Review:

Atopic dermatitis (AD) is a common inflammatory skin disorder of humans and dogs which is frequently associated with hypersensitivity to house dust mites.

AD is a genetically-predisposed, chronic inflammatory and pruritic allergic skin disease characterized by cutaneous hyperreactivity to a number of environmental antigens[4]. This hyperreactivity is most commonly associated with IgE antibodies to environmental allergens (extrinsic AD), although an IgE-independent form (intrinsic AD) has also been described[4, 28-31]. AD usually appears as a syndrome with characteristic clinical features. Facial and extensor dermatitis and pruritus may appear as early as infancy[4, 32]. This often progresses to a chronic pruritic flexural dermatitis and an increased susceptibility to cutaneous infections by adulthood[4, 33-35]. Pruritus and dermatitis associated with AD may be severe, resulting in disfiguring excoriation and lichenification of the skin, as well as significant emotional distress. Furthermore, evidence also suggests that the development of AD (and the immunologic changes that accompany it) may be the first step in a progression of allergic diseases such as asthma—the so-called “atopic march”[4, 7]. Although the prevalence of AD is much higher in children than in adults (10-20% vs 1-3%, respectively)[4, 32], there is ample evidence demonstrating that the incidence in both populations is rapidly increasing.

A spontaneous, pruritic dermatitis has also been described in a number of domestic animal species, and is particularly common in the dog[20]. In canines, the syndrome is strikingly similar to human AD[36-38]. It is characterized by an early age of onset
(frequently <1 year of age) of pruritus and dermatitis, usually involving facial and flexural skin[36, 37]. Secondary infections with *Staphylococcus* and *Malassezia* sp. are common[39, 40]. In addition, affected dogs usually have high levels of IgE specific for one or more environmental antigens[41-43].

**The major route of allergen exposure resulting in the development of AD and perpetuation of clinical disease remains controversial.**

AD has been associated with a variety of “triggering” environmental antigens, including pollens, danders and molds[44-46]. The most common allergens for both humans and dogs are those associated with several species of housedust mite (HDM), especially *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*[9, 46-48]. However, the major route of allergen exposure resulting in the development and perpetuation of clinical disease remains controversial.

Inhalation of allergenic particles has frequently been proposed as the exposure route most likely to result in the induction of AD[49]. At least one study has demonstrated an exacerbation of existing lesions and the development of new ones in some human AD patients challenged by inhalation of HDM extracts[50]. Also, Basenji-Greyhound crossbred dogs experimentally sensitized by repeated inhalation of *Ascaris suum* extracts were reported to develop pruritus and dermatitis in addition to airway hyperreactivity[51]. However, these results are by no means universal, in that other studies have failed to demonstrate the development or exacerbation of AD after aerosol challenge[52].
The epidermal barrier: an insurmountable obstacle?

We propose that transcutaneous absorption of allergen may provide an alternative route of sensitization. Support for this hypothesis is found in a small number of studies in which HDM antigens have been detected by immunohistochemistry in naturally occurring lesions of human AD[53, 54]. Despite these findings, naturally occurring epicutaneous sensitization has often been dismissed as unlikely due to the presence of the relatively impermeable “epidermal barrier”. The major component of this barrier is the stratum corneum[8]. The stratum corneum is comprised of multiple layers of tightly apposed cornified keratinocytes embedded in a matrix of highly ordered lipid sheets[8, 55]. These organized sheets (or lamellae) contain a mixture of cholesterol and unbranched, tightly packed free fatty acids and ceramides[55]. The embedded keratinocytes are terminally differentiated, anuclear cells that have a peripheral envelope comprised of cross linked structural proteins (such as involucrin and loricin)[8, 56, 57]. Keratin filaments inside the cell are linked to this envelope, and in turn are linked to other keratinocytes by intercellular adhesive junctions called desmosomes[8, 55]. This pattern of overlapping, linked keratinocytes and extracellular lipids has been described as having a “bricks in mortar” appearance. Together, these components of the epidermal “wall” form an efficient hydrophobic barrier, preventing excessive movement of most hydrophilic substances into or out of the skin[58]. Those substances that are capable of crossing the stratum corneum are typically small and/or lipophilic[58]. In contrast, the smallest HDM allergens are 14-15kD, while other HDM allergens can be greater than 100kD[9, 48, 59, 60].
It is important to remember, however, that the epidermis and stratum corneum are dynamic, living structures. The supply of keratinocytes is continually being replenished by rapidly dividing cells in the basal layer of the epidermis. At the same time, the top layers of the stratum corneum are constantly being shed in a process called desquamation, during which the desmosomes are degraded by a collection of serine and cysteine proteases[61-63]. This continual cycle of loss and replenishment is vital for the maintenance of epidermal homeostasis, and demands a delicate balance between basal cell proliferation and proteolytic digestion.

Additionally, the “epidermal barrier” is not an uninterrupted wall. Hair follicles are present at varying densities over most areas of mammalian skin. Although hair follicle ostia represent only 0.1% to 10% of the skin surface area (depending upon the body location)[64], the total area occupied by these invaginated structures is likely far greater. Although the epidermis lining the most superficial area of the follicle (infundibulum) contains a mature stratum corneum similar to that on the skin surface, deeper areas of the follicle (the isthmus and inferior segment) do not have this protection. As a result, transfollicular absorption of substances may be much greater than previously estimated.

Dermatophagoides allergens include a number of highly active proteases that may contribute to increased epidermal permeability.
Dermatophagoides farinae and Dermatophagoides pteronyssinus are closely related members of the family *Pyroglyphidae* [5, 9, 48]. Allergens derived from these ubiquitous mites include a number of highly active proteolytic compounds [5, 9, 48, 65-68]. The most well-studied of these are the Group 1 allergens (*Der f 1* and *Der p 1*, respectively). These 24kD proteins are derived from the digestive tract of the mites, and are particularly prevalent in mite fecal pellets [5, 9, 48, 68]. Both *Der f 1* and *Der p 1* have potent cysteine protease activity [68-72], and may also have some degree of serine protease activity [9, 73]. These activities are usually lost during the processing and purification of commercial mite extracts, but can be readily restored in vitro [67, 70, 71, 74, 75]. Proteolytically active *Der p 1* has been demonstrated in vitro to directly and/or indirectly degrade intercellular components of tight junctions, such as occludin, claudin and ZO-1 (and to a lesser extent, E-cadherin) [76-78]. In other studies, proteolytically active *Der p 1* has been shown to induce apoptosis in some cell lines independent of tight junction degradation [79]. The net effect of these changes is to markedly increase paracellular permeability and cellular detachment, allowing migration of *Der p 1* through these normally impermeable cellular barriers [78].

Other *Dermatophagoides* proteases include Group 3 trypsin-like proteases, Group 4 amylase-like proteases, Group 6 chymotrypsin-like proteases and Group 9 collagenases [5, 9, 44, 48, 67]. These allergens have been studied in far lesser detail than the Group 1 allergens. However, *Dermatophagoides pteronyssinus* extracts enriched for serine proteases (including Group 3, 6 and 9 allergens) have been shown to produce epithelial tight junction disruptions similar to those seen with *Der p 1* [77].
Proteolytically active Der p 1 has also been shown to disrupt desmosomal adhesions in a variety of cell lines including MDCK canine kidney cells and canine tracheal epithelial cells[76]. Similar effects have been noted for less purified preparations of Dermatophagoides serine and cysteine proteases[77]. In these studies, the disruptive effects on desmosomes were less pronounced and more transient than those seen on the “classical” tight junction proteins, such as occludin. However, these results do suggest that Dermatophagoides proteases may be able to produce similar disruptions of stratum corneum desmosomes, perhaps by degrading the same proteins that are the targets of endogenous serine and cysteine desquamation proteases. Such an interaction would be by no means unique, as disruption of the stratum corneum by exogenous proteases has been demonstrated in a number of other models. For example, *Staphylococcus aureus* exfoliative toxin (a serine protease) has been demonstrated to cleave desmoglein 1 directly[80, 81], Group A *Streptococcus* pyrogenic exotoxin (a cysteine protease) has been shown to play a critical role in the development of Streptococcal impetigo[82], and epidermal invasion with some *Candida* isolates appears to be dependent upon a secreted aspartic protease[83].

**Dermatophagoides** proteases play an active and critical role in the induction and polarization of immune responses by interacting with a number of surface molecules and receptors.

Proteases derived from *Dermatophagoides* may facilitate the induction and perpetuation of hypersensitivity disorders not only by increasing epithelial permeability and
allergen exposure, but also by interacting with a number of cell surface molecules and receptors. The most well-studied of these are the protease activated receptors (PARs). PARs are 7-transmembrane G protein-coupled receptors[84, 85]. Serine proteases activate these receptors by cleaving part of the extracellular N-terminus of the receptor, producing a “new” N-terminus at the cut end[84, 85]. This new terminus then binds to another extracellular site on the same molecule, activating the receptor. Alternately, a synthesized peptide mimicking the “new” terminus (commonly called an activating peptide, or AP) can also bind to and activate the receptor[84, 86, 87]. In either case, the activated receptor then binds to intracellular G proteins, triggering a cascade of protein kinases and ultimately activating nuclear transcription factors[84].

PARs are expressed on a variety of cell types, including keratinocytes, mast cells, dermal endothelial cells, eosinophils and antigen presenting cell[84-86, 88-91]. Nucleotide sequences similar to all four known human PARs have been identified in the canine genome[92-95]. Also, PAR-activating peptides have been reported to induce cellular activation in canine endothelial cells[96] as well as in a canine-derived pancreatic cell line[97]. Furthermore, antibodies recognizing human and rat PAR-2 also bound to cells from this pancreatic cell line[97].

PARs are not ligand-specific[84], and each receptor can be activated by a variety of endogenous and exogenous serine proteases, including those derived from *Dermatophagoides. Der p 3* and *Der p 9* have been reported to induce cellular activation and
release of GM-CSF and eotaxin from pulmonary epithelial cells in a protease-dependent manner via PAR-2[98]. Also, proteolytically active Der p 1 has been demonstrated to induce the release of IL-6 and IL-8 in the human respiratory cell line A549 via PAR-2, although it appeared to inhibit activation via PAR-1[11]. The ability of Dermatophagoides proteases to directly contribute to an inflammatory response in an IgE-independent manner suggests one mechanism by which they may facilitate sensitization. Furthermore, there is evidence that PAR expression may be upregulated on a number of cells in hypersensitivity disorders (including AD)[91], implying that mite protease/PAR interactions may also play a role in the perpetuation of allergic inflammation.

Dermatophagoides proteases could well set the stage for the induction and perpetuation of allergic diseases by interacting with a number of other receptors and surface molecules as well. Proteolytically-active (but not inactive) Der p 1 has been shown to cleave CD23 from the surface of B cells, negating an important negative feedback mechanism for IgE production[65]. It has also been shown to cleave CD25 from the surface of both CD8 and CD4 positive T cells, resulting in increased production of interleukin 4 (IL4), decreased production of interferon gamma (IFN-gamma) and decreased T cell proliferation after stimulation with anti-CD3 and anti-CD28[99, 100]. Later co-culture of these T cells with naïve autologous B cells resulted in a greatly enhanced production of IgE as compared to co-cultures with T cells stimulated by proteolytically-inactive Der p 1[101]. Active Der p 1 has been reported to induce IgE-independent production and release of IL4,
IL5, IL13 and histamine from human mast cells and basophils[74]. Active Der f 1 has also
been demonstrated to induce degranulation and activation of eosinophils[71].

**A model to investigate the role of Dermatophagoides protease activity during the
development of naturally occurring AD is critically needed to elucidate the pathogenesis of the disease and to investigate novel therapeutic interventions.**

The studies cited above have identified a number of possible mechanisms and pathways by which proteolytically-active Dermatophagoides allergens may act to facilitate the development and perpetuation of allergic inflammation associated with AD. These mechanisms include:

- Disruption of intercellular adhesion molecules resulting in increased epidermal permeability to allergens
- Activation of protease activated receptors on a variety of cutaneous cells, resulting in IgE-independent release of inflammatory mediators and activation of antigen presenting cells
- Induction of IgE-independent release of mediators from mast cells, eosinophils and basophils, providing an inflammatory milieu highly conducive to Th2 polarization and the development of hypersensitivity
- Directly influencing T cell proliferation and cytokine production towards a Th2 phenotype by cleaving membrane-bound CD25
- Direct interference with negative feedback mechanisms regulating IgE production by cleaving membrane-bound CD23 from B cells
Each of these pathways offers a potential opportunity for preventative and therapeutic intervention. However, it must be emphasized that these mechanisms have primarily been investigated in vitro. Although some in vivo studies have demonstrated an important role for Dermatophagoides protease activity in the development and perpetuation of experimental asthma in mouse models[102, 103], very little work has been performed to determine the role of protease activity with regards to the development and perpetuation of naturally occurring allergic diseases, including AD.

**Information derived from traditional mouse models of epicutaneous sensitization may not necessarily be suitable for extrapolation to spontaneous disease in humans.**

Most research on the pathogenesis and treatment of AD (including research on the role of cutaneous exposure to allergens in the development of sensitization) has been performed in inbred mouse strains, such as spontaneously allergic NC/Nga mice and either actively or passively sensitized BALB/c mice[13, 14]. The ready availability of murine subjects meeting exact genetic and phenotypic specifications (and of appropriate research reagents for the study of these mice) has made the mouse an invaluable asset to the study of AD.

However, mouse models also have their disadvantages. A number of significant differences exist between the cutaneous immune system of the mouse and that of humans. Perhaps the most conspicuous of these differences is the lack of the high-affinity receptor for IgE on murine Langerhans cells and dendritic cells[16, 18, 104], as well as on circulating and
tissue-resident monocytes, macrophages and eosinophils[15, 18, 104]. In humans, cross-linking of surface-bound IgE can induce full activation of Langerhans cells and dendritic cells[105, 106]. These activated cells are then able to elaborate a number of inflammatory and chemotactic mediators (such as IL-16) that recruit cells important to hypersensitivity responses, such as CD4+ T cells and eosinophils[107, 108]. Langerhans cells and dendritic cells activated in this manner are able to efficiently prime naïve T cells [107, 109]. This priming is not restricted to T cells specific for the IgE-cross linking allergen, and is believed to play a major role in the sensitization to new allergens (“epitope spreading”) as well as the perpetuation of existing hypersensitivity. The absence of the high-affinity IgE receptors on LC and DC in mouse skin suggests the likelihood of significant differences between mice and humans in the phenomena resulting in sensitization and perpetuation of AD. Indeed, further evidence of significant interspecies differences is provided by the fact that mouse IgG subclasses (especially IgG1) are able to mediate many of the immune phenomena typically associated with IgE responses in humans, such as cutaneous and/or systemic anaphylaxis[18, 19, 110]. Finally, it must be considered that artificial sensitization of groups of inbred, genetically identical murine subjects may not necessarily produce results analogous to those seen in spontaneous disease in outbred populations of genetically predisposed humans. For these reasons, murine models of AD may produce information of limited utility for extrapolation to human disease, which entails complex interactions between genetic predisposition and host environment, skin barrier defects, microbial skin infections and other immunologic factors[4]. Clearly, some form of alternate model is needed to complement the results obtained from murine studies.
The canine cutaneous immune system bears important similarities to that of humans and is an important complement to mouse models of AD.

One candidate for such a complementary model is the dog, a species in which spontaneous allergic diseases are common and artificial sensitization is both possible and commonly performed. Historically, research in this species has been limited due to the lack of canine-specific reagents. However, recent advances in the development of monoclonal and polyclonal antibodies and primer sequences for use in the dog allows research to be performed at a high level of sophistication.

The canine cutaneous immune system shares many features with that of humans, which are not shared by the mouse. For example, surface expression of IgE has been detected in situ on epidermal Langerhans cells, dermal dendritic cells and dermal mast cells[21], as well as circulating B cells, CD14⁺ mononuclear cells and CD1c⁺ dendritic cells[22]. The cutaneous distribution of these IgE-bearing cells is similar to that in human skin, and appears to be upregulated in the face of active atopic disease in a comparable fashion[21]. Aggregation of this IgE by the intradermal injection of allergen has been demonstrated to produce immediate and late phase reactions (LPR) in the skin of dogs with naturally occurring AD[23]. Similar reactions may be produced in the skin of normal dogs or dogs with spontaneous AD by the intradermal injection of cross-linking anti-canine IgE antibodies[23]. These reactions grossly and microscopically resemble those generated by the intradermal injection of allergen or anti-IgE in humans with atopic disorders, including
AD[23-27]. These facts indicate that significant similarities in the pathogenesis and perpetuation of AD between dogs and humans may exist, and suggest the need for further investigation into the utility of this model for the study of the development of AD.

The current work describes the results of three experiments that were designed to further our understanding of the role of epicutaneous exposure to allergen in the development and perpetuation of AD.

1. The gross, microscopic and inflammatory mediator responses of normal dog skin following IgE-mediated challenge were evaluated by intradermal injection of cross-linking anti-IgE antibodies. This study provided important baseline data and established optimized sample processing protocols for the following two studies.

2. The cutaneous and systemic response to cutaneous allergen exposure was evaluated by repeated epicutaneous application of a sonicated slurry of *Dermatophagoides farinae* house dust mites to the intact skin of allergy-predisposed Maltese-Beagle cross-bred dogs. This study demonstrated that both cutaneous and systemic inflammation similar to naturally-occurring AD can be induced in the dog by cutaneous exposure to allergen.

3. The role of allergen proteolytic activity in facilitating epicutaneous sensitization was evaluated by repeated topical application of either proteolytically-active or –inactive *Der f 1* house dust mite allergen. This study demonstrated that cutaneous exposure to *Der f 1* allergen through intact canine skin may be sufficient to induce sensitization, and suggest that this response may be facilitated by the proteolytic activity of the allergen.
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Chapter one:

Title:

A canine model of cutaneous late phase reactions: Prednisolone inhibition of cellular and cytokine responses

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Authors/Affiliations:


* North Carolina State University, College of Veterinary Medicine, Department of Population Health and Pathobiology, 4700 Hillsborough Street, Raleigh, NC 27606

† Drug Discovery, Schering-Plough Animal Health, Union, NJ

‡ North Carolina State University, College of Veterinary Medicine, Department of Clinical Sciences, 4700 Hillsborough Street, Raleigh, NC 27606

§ Schering-Plough Animal Health, Terre Haute, IN

‖ DNAX Research Institute, Palo Alto, CA
Abstract:
IgE-mediated late phase reactions (LPR) can be induced in atopic humans by intradermal injection of relevant allergens or anti-IgE antibodies. The histology of these reactions resembles that of naturally occurring atopic dermatitis. Strikingly similar responses can be induced in dogs, suggesting that a canine model could prove valuable for pre-clinical investigation of drugs targeting LPR. This study was designed to characterize the cellular, cytokine and chemokine responses after intradermal anti-IgE injection in untreated and prednisolone-treated dogs. Normal Beagle dogs were untreated or treated with prednisolone before intradermal injection of polyclonal rabbit anti-canine IgE or normal rabbit IgG. Biopsies were taken before injection and 6, 24 and 48 hours after injection. Samples were evaluated by histological and immunohistochemical staining, as well as by real-time quantitative polymerase chain reaction analysis. Dermal eosinophil and neutrophil numbers increased dramatically within 6 hours after injection of rabbit anti-canine IgE, and remained moderately elevated 48 hours later. The numbers of CD1c⁺ and CD3⁺ mononuclear cells were also increased by 6 hours. Real-time quantitative PCR analysis demonstrated marked increases in mRNA expression for interleukin-13 (IL-13), CCL2, CCL5 and CCL17. Levels of mRNA for IL-2, IL-4, IL-6 and IFN-gamma did not change within the limits of detection. Prednisolone administration suppressed the influx of neutrophils, eosinophils, CD1c⁺ and CD3⁺ cells, as well as expression of IL-13, CCL2, CCL5 and CCL17. These data document the cytokine and chemokine response to anti-IgE injection in canine skin, and they
demonstrate the ability of the model to characterize the anti-inflammatory effects of a known therapeutic agent.
Introduction:

Atopic dermatitis (AD) is a common inflammatory skin disorder of humans that is showing increasing prevalence[1]. Similarly, dogs have been shown to manifest spontaneous atopic dermatitis[2, 3]. In humans and dogs, the disorder is often characterized by genetic predisposition, early age of onset, dermatitis and pruritus (predominantly on flexural surfaces), elevated IgE antibodies to environmental allergens, and a predisposition to secondary infections with bacteria and yeast[3].

Research into the pathogenesis of this disorder and the development of new therapies has been hampered by the lack of a suitable animal model. Most research on the pathogenesis and treatment of AD has been performed in mouse models, such as spontaneously allergic NC/Nga or passively sensitized BALB/c mice[4, 5]. However, it must be considered that information derived from groups of inbred, genetically identical and/or artificially or passively sensitized subjects may not necessarily be suitable for extrapolation to spontaneous disease in genetically predisposed humans, which entails complex interactions between genetic predisposition and host environment, skin barrier defects, microbial skin infections and other immunologic factors[1]. The risk of over-extrapolation from mouse to man is further highlighted by the existence of subtle but important differences in the cellular distribution and function of several receptors and mediators critical to the development and maintenance of cutaneous hypersensitivity, such as the high- and low-affinity receptors for IgE [6, 7] and IgG [8-10].

In the dog, surface expression of IgE has been detected in situ on epidermal Langerhans cells, dermal dendritic cells and dermal mast cells[11], as well as circulating B
cells, CD14+ mononuclear cells and CD1c+ dendritic cells[12]. Aggregation of this IgE by the intradermal injection of cross-linking anti-canine IgE antibodies has recently been demonstrated to produce immediate and late phase reactions (LPR) in the skin of both normal dogs and dogs with naturally occurring AD[13]. These reactions grossly and microscopically resemble those generated by the intradermal injection of allergen or anti-IgE in humans with atopic disorders, including AD[13-17]. These similarities suggest a need for further investigation into the utility of this model for the study of the development of LPR and for use in pre-clinical studies of new treatments targeted to the LPR.

The specific aims of this study were two-fold. Our first objective was to expand upon previous work describing canine anti-IgE induced LPR by extending the study observation period and by determining the cytokine expression profile of these reactions. This objective was achieved by obtaining biopsies of normal dog skin, before and six, twenty-four and forty-eight hours after intradermal injection of anti-IgE. These samples were submitted for routine histology, immunohistochemistry and quantitative mRNA analysis.

Our second objective was to investigate the potential utility of this model for the evaluation of the effects of pharmacologic therapy upon the LPR. This was achieved by administering prednisolone before and during the experimental period.

Normal dogs (rather than dogs with AD) were used in this study to maximize and further evaluate the practicality of this model. Although AD is common in dogs, it would be difficult, expensive and impractical for most research laboratories to maintain a dedicated colony of dogs with AD. In contrast, a model designed for use in normal dogs could be instituted at any research laboratory. Similar studies using intradermal injection of anti-IgE in
healthy, non-allergic humans have been used to evaluate the efficacy of a variety of drugs upon immediate wheal and flare reactions and LPR[18-20].

Methods:

Subjects: Thirteen normal, sexually intact, male and female Beagle dogs (mean age 32 months) were used for this study. These dogs were chosen based upon lack of clinical or historical evidence of allergic skin disease, cutaneous bacterial infections or systemic disease. Dogs that had received medication in the 14 days prior to the study were excluded. Physical examinations were performed on all dogs one day prior to the start of the study. Housing and experimental samplings were in accordance with the National Research Council’s 1996 Guide for Care and Use of Laboratory Animals. All experimental protocols were approved by an Institutional Animal Care and Use Committee.

Generation of IgE-mediated LPR: All dogs were shaved on their lateral thorax on study Day 0. On Day 3, each dog was sedated with medetomidine (Domitor®, Pfizer, Exton, PA, USA) injected intravenously. Five dogs received no medication before the study, and were injected intradermally (0.05 ml each injection) at two sites with phosphate-buffered saline and at eight sites with protein G affinity-purified rabbit polyclonal IgG specific for canine IgE (anti-canine IgE)[21] diluted to 0.08 mg/ml in PBS. Three dogs were injected at eight sites with 0.05 ml of normal rabbit IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) diluted to 0.08 mg/ml in PBS, instead of anti-canine IgE. The optimal injection volume and concentration had been previously determined by serial titration.
Determination of the effect of prednisolone upon IgE-mediated LPR: Five remaining dogs were given a moderate anti-inflammatory dose (0.5 mg/kg) of prednisolone orally twice daily, starting on study Day 0, and continuing through the study. On Day 3, these dogs were sedated, injected at two sites with PBS and at eight sites with anti-IgE and biopsied as described below.

Grading of macroscopic reactions: Injected skin sites were examined at 20 minutes and at 6, 24 and 48 hours after injection. The diameter of cutaneous reactions was measured in two perpendicular directions and used to determine the reaction area.

Specimen collection and processing: Two 8 mm punch biopsies of normal skin on the lateral thorax were collected before injection. Similar paired samples were collected at injection sites 6, 24 and 48 hours after injection with anti-canine IgE or normal rabbit IgG. One sample from each pair was immediately placed into a cryotube, snap-frozen in liquid nitrogen and stored at –70 degrees Celsius for gene expression analysis. The other sample was bisected immediately after collection. One half was placed in 10% neutral buffered formalin for routine processing in paraffin. The other half was placed in Optimal Cutting Temperature medium (OCT Tissue Tek, Baxter Diagnostics Inc., McGaw Park, IL, USA), immersed in isopentane cooled in liquid nitrogen and stored at –70 degrees Celsius until cryosectioning.

Histological staining: Five-micrometer sections were cut from paraffin blocks and stained with hematoxylin-eosin for examination and pattern analysis. Eosinophils were visualized and counted using Luna’s stain for eosinophils[22]. A low-pH (1.5) toluidine blue stain[23, 24] was used to facilitate evaluation of dermal mast cells.
**Immunophenotyping of cutaneous mononuclear cells:** A three-step labeled streptavidin method modified from Affolter and Moore[25] was used to characterize the mononuclear cell infiltrate. Briefly, 6-micrometer cryosections were fixed by immersion in acetone. Endogenous peroxidase activity was quenched by immersion in hydrogen peroxide (diluted to 0.3% in PBS) containing sodium azide (0.01%), then blocked with 1% fetal calf serum in PBS. Monoclonal antibodies specific for canine CD1c, CD3, CD4 and CD8 (courtesy of Dr. Peter F. Moore, University of California, Davis, CA, USA) were used as cell culture supernatant diluted 1:10 in PBS. Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was diluted 1:400 in PBS, followed by horseradish peroxidase-conjugated streptavidin (Zymed, San Francisco, CA), diluted 1:400 in PBS. Amino-9-ethyl-carbazole (AEC, AEC Substrate Kit, Biogenix, San Ramon, CA, USA) was applied as a chromogen, followed by a hematoxylin counterstain (Sigma-Aldrich, St. Louis, MO, USA).

**Enumeration of dermal cells:** The number of total nucleated cells/mm² in the dermis was obtained by counting 51 consecutive 0.14 mm x 0.14 mm fields of superficial dermis (excluding endothelial cells and adnexae). Neutrophils, mast cells and eosinophils, as well as CD1c⁺ and CD3⁺ mononuclear cells/mm² of dermis were determined by counting 13 consecutive 0.24 mm x 0.32 mm fields of superficial dermis.

**RNA Expression analysis:** For real-time quantitative PCR analysis, the Biopulverizer (BioSpec #59013, Biospec Inc., Bartlesville, OK, USA) was used to crush the skin biopsies on dry ice and total RNA was isolated using Qiagen RNeasy Midi kit according to manufacturer's instructions (Qiagen RNeasy Midi handbook, second edition pgs 41-47 and Appendix E pgs 91-92, respectively; Qiagen Inc., Valencia, CA, USA). Five micrograms of
total RNA was subjected to further treatment with DNase (Ambion Inc., Austin, TX, USA) according to manufacturer's instructions to eliminate possible genomic DNA contamination. Real-time quantitative PCR for gene expression: DNase-treated total RNA was reverse-transcribed using Superscript II (Gibco/BRL, Carlsbad, CA) according to manufacturer's instructions. Primers were designed using Primer Express (PE Biosystems, Foster City, CA). Real-time quantitative PCR on 10 ng of cDNA from each sample was performed using two gene-specific unlabelled primers at 400 nM in a Perkin Elmer SYBR green real-time quantitative PCR assay utilizing an ABI 5700 or 7900 instrument. The absence of genomic DNA contamination was confirmed using primers that recognize genomic region of the canine MMP-9 promoter. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the δ-δ Ct method. (Using the mean cycle threshold value for ubiquitin and the gene of interests for each sample, the equation 1.8 e (Ct ubiquitin minus Ct gene of interest) x 10^4 was used to obtain the normalized values.)

Statistical analysis: Unless otherwise specified, all statistical analyses were performed using SAS Software, Version 8.2 of the SAS system for Windows (SAS institute, Cary, NC, USA). Statistical analysis of dermal cell counts or mRNA expression within groups was performed by evaluating differences of least squared means using a PROC MIXED analysis of log-transformed raw data. Comparisons between groups were performed by evaluating differences of least squared means/PROC MIXED analyses of fold changes from baseline. Comparisons of injection site areas were performed using two-tailed paired and unpaired T-Tests, and correlation analyses were performed using a two-tailed Spearman Correlation Test, both calculated with GraphPad Prism Software, version 3.03 for Windows.
Results:

Intradermal injection of anti-IgE produces macroscopic immediate and late phase reactions:

Intradermal injection of anti-IgE produced mildly to moderately erythematous and indurated focal urticarial reactions within twenty minutes (Figure 1). These areas were significantly (P<0.01) larger than paired PBS injection sites (mean 105.3 mm² and 37.5 mm², respectively). By six hours, mild erythema and/or induration remained at anti-IgE injection sites in three dogs. Twenty-four and forty-eight hour macroscopic LPR were not seen.

In contrast, sites injected with normal rabbit IgG did not differ significantly in area or appearance from paired PBS injection sites, and they were significantly smaller than anti-IgE injected sites (mean 67.3 mm² and 105.3 mm², respectively; P<0.05).

Intradermal injection of anti-canine IgE induces degranulation of dermal mast cells: Low pH toluidine blue staining revealed large, granular, oval cells throughout the dermis and clustered around blood vessels, hair follicles and adnexae. Although toluidine blue stains cytoplasmic granules in both canine basophils and mast cells, basophils are rarely found in the skin of either normal dogs or dogs with AD[13]. For this reason, positively staining cells were considered to be mast cells. Injection of anti-IgE resulted in a nearly nine-fold decrease in intact mast cells by six hours (P<0.01) and a ten-fold decrease by twenty-four hours (P<0.001), followed by a return to near pre-injection levels by forty-eight hours (Figure 2A).

Faintly stained cells and cells with dermal granule dispersion were frequently seen. In contrast, sites injected with normal rabbit IgG exhibited a decrease of 2.3-fold compared to baseline, and did not show an increase in visibly degranulated mast cells (data not shown).
The LPR induced by anti-IgE is characterized by a rapid influx of neutrophils and eosinophils, followed by CD1c⁺ dendritic cells and CD3⁺ lymphocytes. Sites injected with anti-IgE had significant (P<0.001) increases in total dermal nucleated cell numbers after injection (Figure 2B, Table 1). These cells were increased greater than two-fold relative to baseline by six hours, with cell counts continuing to increase at twenty-four hours, and remaining elevated forty-eight hours after injection. The six-hour influx was dominated by a significant increase in eosinophils (P<0.01) and neutrophils (P<0.001), with peak accumulations at six and twenty-four hours, respectively (Figure 2, C and D, Figure 3, A and B, Table 1). All six and twenty-four hour samples contained eosinophils exhibiting granule aggregation or dispersion consistent with degranulation. Both CD1c⁺ dendritic cells and CD3⁺ lymphocytes were increased as early as six hours after injection, and peaked at twenty-four hours (Figure 2, E and F, Figure 3, C and D). These increases became significant at six hours for dendritic cells (P<0.01) and at twenty-four hours for lymphocytes (P<0.001).

Compared to sites injected with anti-IgE, intradermal injection with normal rabbit IgG produced considerably lesser infiltrates, with notable exceptions. Dermal neutrophils were significantly increased relative to baseline six and twenty-four hours after injection of IgG (Table 1, P<0.001 and <0.01, respectively). Total nucleated cells were moderately increased at six hours, but had returned to near baseline by twenty-four hours (Table 1).

Injection of normal rabbit IgG produced minimal influx of other inflammatory cells. A small increase in eosinophils was noted six hours after injection (Table 1), but this increase was not statistically significant. CD1c⁺ and CD3⁺ cells increased minimally at twenty-four hours (1.6-fold and 3.3-fold, respectively), and had returned to baseline levels by forty-eight
hours (Table 1). Fold increases in CD1c+ cells after normal rabbit IgG injection were significantly smaller at all time points compared to those seen in dogs injected with anti-IgE (P<0.001-0.05; Table 1). Fold increases in CD3+ cells at twenty-four and forty-eight hours were also significantly lower than those seen in dogs injected with anti-IgE (P<0.05).

The LPR induced by anti-IgE is characterized by increased expression of mRNA for Interleukin-13, Interleukin-5, CCL2, CCL5 and CCL17: Significant increases in expression of mRNA for Interleukin (IL) 13 (P<0.001), IL-5 (P<0.01), CCL2 (Monocyte chemotactic protein-1; MCP-1; P<0.001) CCL5 (Regulated Upon Activation, Normal T cell Expressed and Secreted; RANTES; P<0.01) and CCL17 (Thymus and Activation Regulated Chemokine; TARC; P<0.001) were noted six hours after injection of anti-IgE (Figure 4, A-D). However, mRNA expression of all three mediators rapidly decreased by twenty-four hours.

Minor increases were noted in mRNA for Tumor Necrosis Factor alpha (TNF-alpha) and IL-10, while expression of mRNA for IL-2, IL-4, IL-6 and Interferon (IFN) gamma remained negligible at all times.

At sites injected with normal rabbit IgG, six-hour elevations were also seen in expression of mRNA for IL-13, CCL2, CCL5 and CCL17, as well as TNF-alpha. These increases were not significant for CCL5 and CCL17. However, the increases in mRNA for IL-13, CCL2 and TNF-alpha were more robust, and did reach statistical significance (P<0.01, <0.01 and <0.05, respectively). Expression of mRNA for IL-2, IL-4 and IL-6 remained negligible, and small, non-statistically significant increases in mRNA expression for IL-5, IFN-gamma and IL-10 were seen (Table 2).
Treatment with prednisolone has no significant effect on anti-IgE induced immediate wheal and flare reactions, but abrogates macroscopic late phase reactions: Intradermal injection of anti-IgE produced wheal and flare reactions within twenty minutes in prednisolone-treated dogs, similar in appearance and area to those seen in untreated dogs injected with anti-IgE (Figure 1). However, unlike anti-IgE injected untreated dogs, prednisolone treated dogs did not have macroscopically evident LPR at any time after injection of anti-IgE.

Prednisolone completely inhibits eosinophil influx and greatly decreases recruitment of dermal neutrophils, dendritic cells and lymphocytes in anti-IgE induced LPR: Although treatment with prednisolone completely inhibited macroscopic LPR, treated dogs did not appreciably differ from untreated dogs with regards to post- injection changes in intact and visibly degranulated mast cells (Figure 2A, Table 1). Regardless, prednisolone treatment greatly inhibited the infiltration of most cell types. Total nucleated cell counts were elevated at twenty-four hours after injection, but the relative increase was still markedly lower than that seen in untreated dogs at the same time point (Figure 2B; Table 1).

Prednisolone also inhibited infiltration of neutrophils, CD1c⁺ dendritic cells and CD3⁺ lymphocytes (Figure 3, E and F). Most dramatically, administration of prednisolone was associated with an almost complete abrogation of the eosinophil response (Figure 2, C-F).

Prednisolone inhibits expression of mRNA for IL-13, CCL2, CCL5 and CCL17 in LPR: Although prednisolone-treated dogs did have six-hour increases in mRNA expression for IL-13, CCL2, CCL5 and CCL17 (Figure 4, A-D), only IL-13, CCL2 and CCL17 increases reached significance. Furthermore, the relative increase in CCL17 was significantly (P<
0.05) decreased compared to that seen in untreated dogs. Prednisolone had no appreciable inhibitory effect upon mRNA expression of IL-10 and TNF-alpha, but did significantly inhibit post-injection expression of IL-5 (P<0.05).

Discussion:

The study of the delayed inflammatory response exhibited by some eczema patients after cutaneous challenge with allergens is not a new discipline. Indeed, the suggestion that observation of cutaneous LPR could provide information regarding the nature and etiology of AD was published in the scientific literature almost one hundred years ago[26]. Great progress has been made since that time, both in the understanding of the phenomena that comprise the LPR, and in the development of safer and more efficient ways to study those phenomena. Many excellent studies have been performed to evaluate the inflammatory response in allergic and normal human volunteers. However, most LPR research has been performed in mice, using actively or passively sensitized subjects. The ready availability of murine subjects meeting exact genetic and phenotypic specifications (and of appropriate research reagents for the study of these mice) has made the mouse an invaluable asset to the study of AD.

However, mouse models also have their disadvantages. A number of significant differences exist between the cutaneous immune system of the mouse and that of humans. Perhaps the most conspicuous of these differences is the lack of the high-affinity receptor for IgE on murine Langerhans cells and dendritic cells[7, 9, 27], as well as on circulating and tissue-resident monocytes, macrophages and eosinophils[6, 9, 27]. In humans, cross-linking
of surface-bound IgE can induce full activation of Langerhans cells and dendritic cells[28, 29]. These activated cells are then able to elaborate a number of inflammatory and chemotactic mediators (such as IL-16) that recruit cells important to hypersensitivity responses, such as CD4+ T cells and eosinophils[30, 31]. Langerhans cells and dendritic cells activated in this manner are able to efficiently prime naïve T cells [30, 32]. This priming is not restricted to T cells specific for the IgE-cross linking allergen, and is believed to play a major role in the sensitization to new allergens (“epitope spreading”) as well as the perpetuation of existing hypersensitivity. The absence of the high-affinity IgE receptors on LC and DC in mouse skin suggests the likelihood of significant differences between mice and humans in the phenomena resulting in sensitization and perpetuation of AD. Indeed, further evidence of significant interspecies differences is provided by the fact that mouse IgG subclasses (especially IgG1) are able to mediate many of the immune phenomena typically associated with IgE responses in humans, such as cutaneous and/or systemic anaphylaxis[9, 10, 33]. For this reason, murine models of AD that are designed solely to investigate IgE-related responses (such as IgE passive sensitization models) may be of limited utility for extrapolation to human disease. Clearly, some form of alternate model is needed to complement the results obtained from murine studies.

One candidate for such a complementary model is the dog, a species in which spontaneous allergic diseases are common and artificial sensitization is both possible and commonly performed. Historically, research in this species has been limited due to the lack of canine-specific reagents. However, recent advances in the development of monoclonal
and polyclonal antibodies and primer sequences for use in the dog now allows research to be performed at a high level of sophistication.

The canine cutaneous immune system shares many features with that of humans, which are not shared by the mouse. For example, surface-bound IgE has been detected on canine Langerhans cells and dendritic cells[11], as well as circulating monocytes, dendritic cells and B cells[12]. The distribution of these IgE-bearing cells is similar to that in human skin, and appears to be upregulated in the face of active atopic disease in a comparable fashion[11]. Furthermore, canine skin has been demonstrated to display more “typical” IgE-mediated, mast cell driven inflammatory responses as well, such as the development of immediate and late phase reactions after cutaneous challenge with relevant allergens or cross-linking anti-canine IgE antibodies[13]. These facts, when considered together, suggest the likelihood of significant similarities in the pathogenesis and perpetuation of AD between dogs and humans.

The current study expands upon previous work by our laboratory and others in the characterization of the LPR in canine skin[13, 14, 34]. Specifically, our study provides new information regarding the cellular and cytokine/chemokine response in LPR following intradermal injection of anti-IgE in dogs[13, 14, 34]. We have confirmed that the anti-IgE induced LPR in healthy research dogs are grossly and microscopically similar to allergen-induced LPR in dogs with AD[13], as well as allergen and anti-IgE induced LPR in humans[16, 35-37]. We have shown that anti-IgE induced canine late phase reactions exhibit increases in mRNA transcription of several mediators typical of Th2-dominated responses, notably IL-13, IL-5, CCL5 and CCL17. It was also demonstrated that these cellular and
mediator responses can be inhibited by prednisolone therapy, in a manner similar to that seen in allergen-induced LPR in humans[38-41]. Finally, our isotype-matched controls produced new information that underscores the importance of this control in interpreting the results of anti-IgE induced LPR.

Intradermal injection of anti-IgE in normal dogs induced immediate development of erythematous and indurated urticarial plaques, which were indistinguishable from wheal and flare reactions seen after intradermal injection of allergen extracts in dogs with AD[13], as well as those described after intradermal injection of allergen or anti-IgE in humans[18, 41]. These reactions were followed by macroscopic erythema and induration at six hours but not at later times. These areas were similar to LPR following injection of allergen or anti-IgE in humans or dogs with AD[13, 16, 18, 41].

Biopsies of injected sites exhibited a clear decrease in the number of intact mast cells and an increase in the number of visibly degranulated mast cells. These changes were seen not only in samples from macroscopic LPR, but also in twenty-four and forty-eight hour samples. Similar changes have been reported in biopsies taken after intradermal injection of allergen in humans[35, 42, 43].

Injection of anti-IgE resulted in a rapid influx of inflammatory cells. The observed biphasic increase in granulocytes followed by mononuclear cells (including both CD1c+ dendritic cells and CD3+ lymphocytes) is typical of those described in LPR after injection of allergen or anti-IgE in both dogs[13] and humans[16, 35, 41, 43].

Our results indicated that IgE-mediated canine LPR are characterized by rapid but transient increases in mRNA transcription for IL-13, IL-5, CCL2, CCL5 and CCL17.
Spearman analysis demonstrated that increases in mRNA expression for IL-13, CCL2 and CCL17 correlated significantly with increases in tissue eosinophil numbers (P<0.01, P<0.05 and P<0.05, respectively) in untreated dogs injected with anti-IgE. Expression of mRNA for IL-10 was also detected, but increased minimally post-injection. Remarkably, only negligible levels of mRNA for IFN-gamma, IL-2, IL-4 and IL-6 were found at any time. Our first samples were taken six hours after injection, which corresponded to the acme of the clinical LPR, but may have been too late to detect transient increases.

In this study, administration of prednisolone for three days prior to injection of anti-IgE had no appreciable effect upon the appearance of immediate reactions, nor upon the numbers and appearance of intact and degranulated mast cells. Corticosteroid therapy has been reported to have varying effects upon allergen or anti-IgE induced wheal and flare reactions, such that, although topical application of either a 0.015% triamcinolone solution[34] or a 1% hydrocortisone conditioner[14] before anti-IgE injection decreased wheal diameter in two studies, oral administration of prednisolone at varying doses (40 mg, single dose[38]; 60 mg daily for three days[41] or 20 mg daily for five )before injection of allergen did not. Regardless, glucocorticoids have been almost universally reported to significantly inhibit LPR following injection of allergen in humans[39-41]. In our study, prednisolone-treated dogs did not develop visible LPR at any point after injection.

Prednisolone sharply decreased cellular infiltration at all time points, resulting in marked decreases in neutrophils, CD1c⁺ dendritic cells and CD3⁺ lymphocytes, compared to untreated dogs. However, prednisolone had the most marked effect upon eosinophil response, which was inhibited greater than 97% relative to that seen in untreated dogs. Similar degrees
of inhibition have been seen in human allergen-induced LPR, in which eosinophil influx was inhibited 70% after a single dose of prednisolone[40], and completely inhibited after three days of prednisolone administration[41]. Cytokine and chemokine responses were also inhibited at six-hours by 58%, 59%, 86%, 73% and 90.8% (for IL-13, IL-5, CCL2, CCL5 and CCL17, respectively) in prednisolone-treated dogs as compared to placebo-treated dogs. Glucocorticoid treatment has been noted to decrease transcription of all of these mediators both in vitro[44-48] and/or in vivo[49-51].

Finally, although our results demonstrate many similarities between anti-IgE induced LPR in normal dog skin and allergen-induced LPR in allergic dogs and humans, they also highlight the requirement for appropriate control samples. Intradermal injection of normal rabbit IgG produced neither visible wheal and flare responses nor macroscopic LPR, failed to induce significant increases in dermal infiltration of eosinophils, CD1c+ and CD3+ cells and failed to significantly increase transcription of IL-5, CCL5 and CCL17. However, it did produce a significant increase in neutrophils and in transcription of TNF-alpha, IL-13 and CCL2. Although marked neutrophilia has been reported as a characteristic of both allergen and anti-IgE induced LPR in humans and dogs[13, 15, 16, 41, 52], it is not a typical feature of uninfected AD skin in either species[11, 35, 53, 54]. However, the significance of this response can be difficult to evaluate when “irrelevant” antigen challenges have not been included in experimental protocols. Our results suggest that the contribution of nonspecific inflammatory responses within the LPR may be underestimated if only diluent controls are used.
In summary, our study has expanded upon previous work investigating the use of a canine model of IgE-mediated inflammation for the study of the pathogenesis and treatment of AD. We have confirmed that intradermal injection of anti-IgE produces an inflammatory response essentially identical to the LPR seen after intradermal injection of allergen. We have shown that this response is present even in normal, outbred research dogs, thus eliminating several potential problems associated with the study of artificially sensitized or inbred subjects. Furthermore, we have demonstrated that this response is diminished by prednisolone in a fashion similar to that seen in human allergen-mediated LPR, thus supporting the potential utility of this model in studying therapies targeted to the LPR. We have also provided evidence that some of the inflammatory response seen in experimentally induced LPR may in fact be artefactual, and suggest that the ready availability of isotype-matched control antibodies provides the anti-IgE model of LPR with a clear advantage over traditional allergen-injection models.

In closing, we feel that our results indicate great promise for this model in the evaluation of the pathogenesis of LPR, and in the development of drugs designed to target LPR. It should be pointed out that this model is most reflective of the inflammatory response associated with acute onset or exacerbation of atopic dermatitis. Chronically active AD is associated with the development of a number of cutaneous alterations (such as dermal fibrosis, epidermal hyperplasia and hyperkeratosis, and upregulation of IgE expression on Langerhans cells and dendritic cells), which would not be well represented by this model as it is currently designed. It is likely that the continued use of allergen-sensitized models will be necessary for the evaluation of events and therapies relating to chronic inflammation.
associated with AD. Nonetheless, the current model can be easily modified to accommodate sensitized subjects as well, which will allow evaluation of IgE-mediated exacerbations of chronic disease. In this fashion, the majority of AD-associated inflammatory responses can be efficiently modeled.

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Figure 1: Intradermal injection of anti-canine IgE (but not normal rabbit IgG) induces the formation of an immediate wheal and flare response that is not inhibited by prednisolone. Normal Beagles (treated or not with prednisolone, 0.5mg BID) were injected intradermally with PBS and either normal rabbit IgG or polyclonal rabbit IgG anti-canine IgE. Wheal dimensions were measured twenty minutes after injection.
Figure 2: Intradermal injection of anti-IgE produces mast cell degranulation and a biphasic cellular response. Skin biopsies were taken before and after anti-IgE injection in untreated (□) or prednisolone-treated (■) dogs. A. Intact mast cells  B. Total nucleated cells  C. Neutrophils  D. Eosinophils  E. CD1c+ dendritic cells F. CD3+ lymphocytes. Data represent dermal cell counts/mm² from individual dogs and group means. ***P<0.001, **P<0.01, *P<0.05 compared to baseline.
Figure 3: Intradermal injection of anti-IgE produces a rapid cellular influx that is markedly inhibited by prednisolone. A. Perivascular accumulation of neutrophils and eosinophils six hours after injection in untreated dog skin, hematoxylin-eosin stain, bar = 100μm. B. Higher magnification of area outlined in A, bar = 50μm. C-F. Immunohistochemical staining of biopsies taken forty-eight hours after injection in untreated dogs demonstrates aggregates of CD1c⁺ dendritic cells (C) and CD3⁺ lymphocytes (D). In contrast, biopsies taken forty-eight hours after injection in prednisolone-treated dogs exhibit few CD1c⁺ dendritic cells (E) or CD3⁺ lymphocytes (F). Amino-9-ethyl-carbazole chromogen with hematoxylin counterstain, bar = 100μm.
Figure 4: Intradermal injection of anti-IgE produces rapid increases in mRNA expression for IL-13, CCL2, CCL5 and CCL17. Fold changes (relative to baseline levels) were calculated for mRNA expression of IL-13 (A), CCL5 (B), CCL17 (C) and CCL2 (D) after intradermal injection of anti-IgE. Data represent group means and individual changes in untreated (■) or prednisolone-treated (□) dogs. ***P<0.001, **P<0.01, *P<0.05 compared to baseline.
Table 1: Dermal cellular infiltrate before and after intradermal injection of polyclonal rabbit IgG anti-IgE or normal rabbit IgG

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<tr>
<th></th>
<th>Normal Rabbit IgG (n=3)</th>
<th>Anti-IgE, no treatment (n=5)</th>
<th>Anti-IgE, Prednisolone treated (n=5)</th>
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<td></td>
<td>Baseline</td>
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<td><strong>Total nucleated cells</strong></td>
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<tr>
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<td><strong>Intact Mast Cells</strong></td>
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<td>18.3 (9.4)</td>
<td>15.3 (5.4)</td>
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<td>Mean (SE)</td>
<td>2.0 (1.5)</td>
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<td>Median</td>
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<td>55.3** (20.5)</td>
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<tr>
<td><strong>CD1c+</strong> Mean (SE)</td>
<td>82.7 (9.8)</td>
<td>45 (5.2)</td>
<td>117 (17.3)</td>
<td>73.7 (18.2)</td>
<td>23 (5.1)</td>
<td>53.8** (11.8)</td>
<td>150.4*** (11.7)</td>
<td>107.2*** (19.2)</td>
<td>23 (5.1)</td>
<td>47* (8.7)</td>
<td>81.8* (35.6)</td>
<td>59.6** (18.6)</td>
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<td>45</td>
<td>117</td>
<td>76</td>
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<td>40</td>
<td>158</td>
<td>95</td>
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<td>45</td>
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<td>-0.6††</td>
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<td>2.8††</td>
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<td>88.7 (14.3)</td>
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<td>12.6 (3.3)</td>
<td>19 (3.3)</td>
<td>73.6*** (16.4)</td>
<td>52.2*** (10.8)</td>
<td>14 (2.7)</td>
<td>11.6 (2.6)</td>
<td>32 (13.4)</td>
<td>23.4 (10.4)</td>
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<td>Median</td>
<td>40</td>
<td>20</td>
<td>96</td>
<td>38</td>
<td>11</td>
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<td>13</td>
<td>11</td>
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<td>-0.1</td>
<td>0.4††</td>
<td>0.1†</td>
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**Footnote:** ***P<0.001, **P<0.01, *P<0.05, relative to baseline cell numbers. †††P<0.001, ††P<0.01, †P<0.05, decreased fold change compared to that seen in time-matched, anti-IgE injected skin in untreated dogs.
Table 2: Changes in cytokine and chemokine mRNA expression after intradermal injection of polyclonal rabbit IgG anti-IgE or normal rabbit IgG

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<tr>
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<th>Normal Rabbit IgG (n=3)</th>
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<th>Anti-IgE, Prednisolone treated (n=5)</th>
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<td>CCL2 (MCP-1)</td>
<td>Mean (SE)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3.4 (0.8)</td>
<td>90.7** (10.2)</td>
<td>26.7 (7.2)</td>
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<tr>
<td></td>
<td>Median</td>
<td>3.1</td>
<td>90.4</td>
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<td>Fold change</td>
<td>28.2</td>
<td>7.8</td>
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<td>CCL5 (RANTES)</td>
<td>Mean (SE)</td>
<td>1.1 (0.1)</td>
<td>3.9 (1.2)</td>
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<td>3.1</td>
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<tr>
<td></td>
<td>Fold change</td>
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<td>3.6</td>
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<td>CCL17 (TARC)</td>
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<td>0.6 (0.3)</td>
<td>1.4 (0.6)</td>
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<td></td>
<td>Median</td>
<td>0.4</td>
<td>1.2</td>
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<tr>
<td></td>
<td>Fold change</td>
<td>2.9†††</td>
<td>1.2</td>
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<tr>
<td>IL-13</td>
<td>Mean</td>
<td>0.5 (0.1)</td>
<td>3.6** (1.0)</td>
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Table 2, continued

| Table 2, continued |
|---------------------|-----------------|-----------------|-----------------|-----------------|
|                     | (SE)            | (0.8)           | (0.3)           | (13.0)          | (1.8)           | (8.8)           | (6.1)           | (1.0)           |
| **IL-5**            |                 |                 |                 |                 |                 |                 |                 |                 |
| Mean (SE)           | 0.6 (0.1)       | 0.8 (0.2)       | 0.4 (0.1)       | 0.6 (0.2)       | 2.9 (0.1)       | 8.8** (3.8)     | 2.8 (0.6)       | 2.1 (0.2)       |
| Median              | 0.5             | 0.7             | 0.3             | 0.7             | 3.0             | 5.2             | 2.8             | 1.9             |
| Fold change         | 0.8             | -1.3            | -1.3            | 0.4             | 3.0             | -0.1            | -1.5            | 0.6†            |
| **TNF-alpha**       | 12.2 (4.0)      | 28.0* (9.8)     | 26.0* (5.1)     | 11.6 (1.8)      | 16.8 (4.5)      | 22.4 (4.6)      | 18.0 (1.9)      | 13.0 (1.7)      |
| Mean (SE)           | 8.8             | 27.1            | 21.1            | 10.1            | 13.5            | 20.3            | 15.7            | 13.5            |
| Median              | 2.4             | 2.3             | 0.3             | 1.2             | 0.8             | -0.4            | 2.4             | 0.7             |
| Fold change         | 1.9             | 1.9             | 0.7             | 1.5             | -0.4            | 1.2             | 1.1             | -0.3            |
| **IL-10**           | 0.6 (0.1)       | 1.1 (0.2)       | 1.2 (0.3)       | 0.8 (0.1)       | 11.9 (1.2)      | 17.4 (2.3)      | 11.6 (2.5)      | 18.9 (3.1)      |
| Mean (SE)           | 0.7             | 1.2             | 1.3             | 0.7             | 11.8            | 20.2            | 10.8            | 20.2            |
| Median              | 1.9             | 1.9             | 0.7             | 1.5             | -0.4            | 1.2             | 1.1             | -0.3            |
| Fold change         |                 |                 |                 |                 |                 |                 |                 |                 |

Footnote: ***P<0.001, **P<0.01, *P<0.05, relative to baseline mRNA expression. †††P<0.001, ††P<0.01, †P<0.05, decreased fold change compared to that seen in time-matched, anti-IgE injected skin in untreated dogs.
Chapter two:

Title:
Epicutaneous sensitization with *Dermatophagoides farinae* slurry induces generalized allergic dermatitis and elevated mite-specific IgE levels in a canine model of atopic dermatitis

Authors and affiliations:
Cherie M. Pucheu-Haston, DVM*, Hilary A. Jackson†, Thierry Olivry, DrVet, Ph,D.†, Bruce Hammerberg, DVM, Ph.D.*

*North Carolina State University, College of Veterinary Medicine, Department of Population Health and Pathobiology, 4700 Hillsborough Street, Raleigh, NC, 27606

†North Carolina State University, College of Veterinary Medicine, Department of Clinical Sciences, Raleigh, NC, 27606
Abstract:

Atopic dermatitis (AD) is a cutaneous hypersensitivity disorder associated with elevated levels of antigen-specific IgE[1], commonly to house dust mites (HDM)[2]. It remains controversial as to whether sensitization and clinical disease development can be induced by cutaneous exposure to HDM. We hypothesized that cutaneous application of Dermatophagoides farinae slurry would result in elevations of HDM-specific IgE and the development of gross and microscopic lesions typical of AD in high-allergy risk Maltese-Beagle crossbred puppies. Puppies received weekly HDM slurry applications to the axilla and groin, and were patch tested at 120 days, or were patch tested at days 1, 60 and 120, but did not receive further slurry application. Patch test and/or control sites were biopsied and serum samples obtained on days 1, 60 and 120. Pruritus and dermatitis was seen in 3/4 puppies by day 30 and in all puppies by day 60. D. farinae-specific IgE levels were elevated by day 60, and were similar to or higher than those of a control AD dog by day 120. Histologic examination of HDM application sites revealed increasingly severe inflammation. Early applications were characterized by a mild perivascular accumulation of mononuclear cells and sparing of the epidermis. Later applications were dominated by a dense, heavily eosinophilic infiltrate and edema in both the dermis and epidermis. This study demonstrated that epicutaneous application of HDM slurry to Maltese-Beagle puppies results in elevations of HDM-specific IgE, localized and generalized pruritus and dermatitis similar to clinical AD, and histologic changes typical of IgE-driven inflammation.
**Introduction:**

Atopic dermatitis (AD) is a common inflammatory skin disorder of both humans[1] and several domestic animals, including dogs[3]. It is a genetically-predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features[1, 4], which include a young age of onset, flexural dermatitis and predisposition to secondary infections with bacteria or fungi[4]. It is associated most commonly with IgE antibodies to environmental allergens[1]. Although many environmental antigens have been associated with AD (including pollens, danders and molds)[4], the most common allergens appear to be those associated with several species of housedust mite (HDM), especially *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*[2].

Although the inflammatory response in established AD has been well studied, the major route of allergen exposure resulting in the development of AD and perpetuation of clinical disease remains controversial. Inhalation of aeroallergens has frequently been proposed as the exposure route most likely to induce the development of AD. At least one study has demonstrated an exacerbation of existing lesions and the development of new ones in some human AD patients challenged by inhalation of HDM extracts[5]. Also, Basenji-Greyhound crossbred dogs experimentally sensitized by repeated inhalation of *Ascaris suum* extracts have been reported to develop pruritus and dermatitis in addition to airway hyperreactivity[6]. However, these results are by no means universal, with other studies failing to demonstrate the development or exacerbation of AD after aerosol challenge[7].
An alternative route of sensitization could be by transcutaneous absorption of allergen. This possibility has often been dismissed as unlikely due to the presence of the stratum corneum, which is impenetrable to many materials[8]. Substances known to be capable of crossing the stratum corneum are typically small and/or lipophilic[9]. In contrast, the smallest HDM allergens are 14-15kD, while other HDM allergens can be larger than 100kD[10, 11].

Most studies of epidermal penetration of HDM allergens in humans (as well as those studies evaluating cellular, cytokine and clinical responses to epicutaneously applied HDM) have incorporated some method of facilitating epidermal penetration, such as removing or disrupting the stratum corneum, or adding a detergent or lipophilic carrier to the extract[12, 13]. Such studies have demonstrated a high percentage of “positive” patch test reactions in AD patients[12-16], with house dust mite antigens readily demonstrable in the epidermis and dermis of biopsies taken from application sites[15, 16]. Grossly positive patch test reactions were not seen in non-manipulated patch sites or those in which the test compound was not suspended in a detergent or lipophilic medium[12-16]. However, HDM antigens can be found in non-manipulated lesional skin of AD patients even if these areas have not been patch tested[17, 18]. Furthermore, although no grossly positive patch test reactions were found in normal individuals (or in non-manipulated, alesional AD skin) in these studies, none of the studies attempted to ascertain whether or not allergen had penetrated the epidermis (e.g., only visibly “positive” patch test sites were biopsied). For this reason, the extent (or lack thereof) of epidermal penetration of HDM in intact normal skin or alesional AD skin remains unknown.
One pathway by which allergens could potentially bypass the stratum corneum and gain access to the dermis is by traveling down the hair follicles. Depending upon the body location, follicular ostia represent only about 0.1-10% of the surface area of the skin[19-21]. However, the total surface area represented by the invaginated walls of the follicles is likely far greater. Although it has been long assumed that the absorption of substances through the hair follicles and their associated adnexae was minimal, recent work demonstrates that this is not always the case. Particles as large as one micron (or proteins as large as 47kD) have been visualized by a variety of methods (laser scanning confocal microscopy, conventional fluorescent microscopy) at the level of the sebaceous ducts or within the dermis itself following epicutaneous application of microspheres or microparticles[19, 22, 23]. This process is enhanced if these microparticles are applied to areas of skin undergoing flexion[22]. Additional particles can be seen to cross directly through the stratum corneum in these areas of flexed skin as well[22].

It is possible that some allergens may possess properties that can serve to facilitate their passage into the deeper layers of skin. Allergens derived from both *D. farinae* and *D. pteronyssinus* include a number of highly active enzymatic and proteolytic compounds, including cysteine and serine proteases, amylases and chitinases[24, 25]. Both the serine and cysteine proteases associated with *D. pteronyssinus* have been demonstrated to degrade tight junctions in cultures of respiratory epithelium, resulting in an increase in permeability to mannose or albumin[26-29]. It is known that much of the protease activity of HDM allergens can be inactivated during routine preparation of commercial HDM extracts[29-31].
Most of the respiratory epithelial studies have addressed this problem by reactivating the proteases before use. However, to our knowledge this has not been attempted in any of the patch test studies. This leaves open the possibility that proteolytic activity (which may facilitate passage through an undamaged stratum corneum) may not have been present in the test extracts used in these studies.

Proteolytic allergens derived from *Dermatophagoides* may contribute to the development of cutaneous hypersensitivity in a direct fashion as well as by enhancing epidermal permeability. Initial capture and processing of epidermally absorbed antigens involves both Langerhans cells (LC) and dermal dendritic cells (DC)[32]. Antigen capture in the absence of inflammatory stimuli results in antigen presentation to T cells by LC or DC expressing low levels of costimulatory molecules, resulting in T cell anergy or tolerance to the presented antigen[33, 34]. However, if antigen capture occurs in the presence of inflammatory stimuli (i.e., Toll-like receptor [TLR] ligands such as lipopolysaccharide, peptidoglycan and other microbial products, or “inflammatory” cytokines), the LC or DC will become activated, increasing expression of costimulatory molecules (such as CD80, CD86 and CD40) and elaborating inflammatory cytokines such as interleukin-12 (IL-12)[33-35]. Cognate antigen presentation to T cells under these conditions results in activation of naïve T cells, or loss of anergy or tolerance in memory T cells. *Dermatophagoides* allergens have been demonstrated to induce IgE-independent degranulation of mast cells, basophils[36] and eosinophils[24]. Such non-specific degranulation results in the release of inflammatory (TNFα, histamine) and Th2-type cytokines (IL4 and IL13). These cytokines not only promote the activation of LC / DC (resulting in T cell activation as described...
above), but also provide an environment rich in Th2 cytokines, which may bias recruited T cells towards a Th2 phenotype.

In a study performed by McCall, et al., neonatal high IgE-responder beagle pups that were treated with repeated topical applications of a saline-based slurry containing minimally processed, sonicated *D. farinae* mites later developed dermatitis, bronchial hyperreactivity, increased serum *D. farinae*-specific IgE titers and increased *D. farinae*-stimulated T cell proliferation as compared to untreated dogs[37]. Later patch tests on these dogs using the same slurry produced gross and microscopic inflammatory reactions typical of AD[37]. It is important to note that the preparation of this slurry involved far less processing than that used in the production of commercially available mite extracts, and that some or all of the proteolytic activity may have remained intact.

Although these results strongly suggest that an epicutaneous route of sensitization can exist in the dog, three basic questions remain. First, is this phenomenon restricted to neonatal dogs, or could older patients also be sensitized in this fashion? Second, is this phenomenon restricted to this particular line of hypersensitivity-predisposed dog, or could other breeds of dog (including other breeds in use as canine models of AD) also be sensitized by cutaneous exposure to allergen? Finally, can alternate exposure routes be ruled out--e.g., could the puppies have ingested the slurry?

This study was designed to provide more information regarding the relevant allergen-cellular interactions during the sensitization period. The specific aims of this study were
three-fold. First, we wanted to determine if repeated epicutaneous applications of a sonicated *D. farinae* slurry to intact canine skin results in the development of clinical, gross or microscopic abnormalities similar to spontaneous canine AD. Second, we wanted to determine if repeated applications of this slurry would result in the development of elevated levels of serum mite-specific IgE and/or IgG. Finally, we wanted to determine if epicutaneous applications of this slurry would produce detectable levels of mite antigens in skin biopsies taken from the application sites.

**Methods:**

**Subjects:** Our laboratory has developed a breeding colony of Maltese-Beagle cross-bred atopic dogs (Malteagles; Maltese Beagle atopic dogs). These dogs are unique and distinct from dogs bred for high IgE responses in that they spontaneously develop hypersensitivities following controlled exposure to allergens[38]. These responses do not require that the allergen be administered parenterally or with an adjuvant. Dogs from this colony have been demonstrated to spontaneously develop sensitivities when introduced to food items containing corn, soy[38], peanuts and chicken. Oral challenge with these items induces the rapid development of clinical signs of pruritus, otitis and GI signs. Dogs in this colony are maintained with aggressive dietary management and in a rigorously controlled housing environment, which has been highly effective in preventing spontaneous hypersensitivity to environmental allergens. However, dogs taken out of this colony and adopted into homes as pets have been reported to develop pruritus and/or rhinitis after environmental exposure to household allergens.
Four Malteagle puppies (three males, one female) were selected for this study. These puppies were littermates, and were five months of age at the time sensitization was begun. At this time, the puppies were all healthy and had neither dermatitis nor any other signs of allergic disease. The puppies were housed with the remainder of the colony in a facility determined by ELISA (Indoor Biotechnologies, Charlottesville, VA, USA) to be free of detectable levels of the house dust mite allergens Der f 1 and Der p 1.

Epicutaneous sensitization of Malteagle puppies with sonicated D. farinae slurry:

Sensitization protocol: The puppies were divided into two groups of two puppies each. This division was deemed necessary to allow comparison of the efficacy of two different protocols of cutaneous sensitization, as well as to allow control for any confounding effects induced by occlusion of the test substance against the skin. This preparation was a minimally processed, sonicated slurry of D. farinae mites in PBS, (courtesy Heska), as had been previously used by McCall et.al[37]. This slurry was applied to the skin “as-is”--no detergents or lipophilic carriers were added at any time. In all cases, sensitization was performed by application of the slurry to intact, unmanipulated skin—i.e., the skin was not tape stripped, pricked, scratched or otherwise disrupted.

Occluded group: These puppies were patch tested with the slurry on study days 1, 60 and 120, but did not have the slurry applied at any other times. Patch testing was performed by lightly painting 30 µl of the slurry onto an approximately 1 cm² area of skin on the lateral thorax, then covering the area with a minimally occlusive dressing (Op-Site, Smith and Nephew, Largo, FL). The puppies were then placed in restrictive Elizabethan collars (to
prevent ingestion of the extract), then separated and placed into individual runs to minimize disturbance of the application sites. Biopsies were taken of the slurry application sites (as well as of adjacent, non-treated areas of control skin) at six and twenty-four hours after application, using an 8mm biopsy punch. Biopsies were halved immediately after acquisition and placed in 10% formalin or frozen in OCT tissue cutting medium.

**Non-occluded group:** These puppies received sixteen weekly applications of slurry, alternating between their right axillary and groin areas. Each slurry application was performed by lightly painting 30 µl of the slurry onto an approximately 1 cm² area of skin in either the axilla or groin. After application, the puppies were placed in restrictive Elizabethan collars to prevent ingestion of the extract, then separated and placed into individual runs for twenty-four hours. The next day, the application sites were examined then gently wiped clean. These areas were not biopsied, with the single exception of study day 70 (see “Immunofluorescent detection of *Der f 1*” below).

Non-occluded group puppies were mock-patch tested on study days 1 and 60. This procedure was identical to that used on the occluded group puppies, except no slurry was applied at these times. However, skin covered by the Op-site dressing was biopsied at six and twenty-four hours as in the occluded group puppies. On study day 120, the puppies were patch tested (including slurry application) and biopsied as described above.

**Clinical monitoring:** All puppies were given a physical examination and a complete dermatologic examination twice weekly. In addition, the puppies were observed for a one
hour period two to three times weekly. Any behavior suggestive of pruritus (scratching, chewing, rubbing) was noted.

**Antibody titers:** Serum samples were obtained from all puppies on study days 1, 60 and 120. Season-matched control samples were also taken at these times from two normal dogs and one dog with spontaneous AD. Sera were divided into 100:l aliquots, and frozen at –20°C until analysis. All samples were analyzed by ELISA (in triplicate) to determine total IgE levels, as well as IgE and IgG levels specific for purified Der f 1. Briefly, round-bottomed 96 well ELISA plates were coated overnight in either mouse-origin monoclonal anti-canine IgE (developed in our laboratory) or purified Der f 1. All coating reagents were diluted in a carbonate/bicarbonate coating buffer. Nonspecific binding was inhibited by a blocking step with PBST. Dilutions (1:5 for IgE; 1:100 for IgG) of patient and control sera were added and incubated at room temperature for two hours. Bound IgE was detected by incubating with biotinylated mouse-origin monoclonal anti-canine IgE, followed by an incubation with horseradish peroxidase conjugated streptavidin (Zymed, San Francisco, CA, USA). Bound IgG was detected using a peroxidase-labeled, goat-origin polyclonal anti-canine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reactions were developed by the addition of ABTS horseradish peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and read at 30 and 60 minutes at 405nm.

**Processing and staining of formalin-fixed skin:** Formalin-fixed skin samples were dehydrated and embedded in paraffin-containing tissue blocks. Five micrometer sections were cut, then stained with hematoxylin and eosin, as well as with Luna’s stain and low pH
(1.5) Toluidine blue stains (for eosinophils[39] and mast cell enumeration[40, 41], respectively). All slides were randomly numbered and evaluated by the same investigator on a blinded basis. This evaluation included standard pattern analysis and enumeration of the total nucleated cells, neutrophils, eosinophils and mast cells per square millimeter of dermis.

Immunohistochemical staining: A three-step labeled streptavidin method modified from Affolter and Moore[42] was used to characterize the mononuclear cell infiltrate. Briefly, 6-micrometer cryosections were fixed by immersion in acetone. Endogenous peroxidase activity was quenched by immersion in hydrogen peroxide (diluted to 0.3% in PBS) containing sodium azide (0.01%), then blocked with 1% fetal calf serum in PBS. Monoclonal antibodies specific for canine CD1c and CD3 (courtesy of Dr. Peter F. Moore, University of California, Davis, CA, USA) were used as cell culture supernatant diluted 1:10 in PBS. Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was diluted 1:400 in PBS, followed by horseradish peroxidase-conjugated streptavidin (Zymed, San Francisco, CA, USA), diluted 1:400 in PBS. Amino-9-ethyl-carbazole (AEC, AEC Substrate Kit, Biogenix, San Ramon, CA, USA) was applied as a chromogen, followed by a hematoxylin counterstain (Sigma-Aldrich, St. Louis, MO, USA).

Enumeration of dermal cells: The number of total nucleated cells/mm$^2$ in the dermis was obtained by counting 51 consecutive 0.14 mm x 0.14 mm fields of superficial dermis (excluding endothelial cells and adnexae). Neutrophils, mast cells and eosinophils, as well as CD1c$^+$ and CD3$^+$ mononuclear cells/mm$^2$ of dermis were determined by counting 13 consecutive 0.24 mm x 0.32 mm fields of superficial dermis.
Immunofluorescent detection of Der f 1 in application site biopsies: Immunofluorescent staining was performed on skin biopsy samples from application sites and control skin. Briefly, five micrometer cryosections were fixed in acetone, followed by blocking in 1% fetal calf serum in PBS. Mouse-origin monoclonal anti-Der f 1 antibodies (4C1, Indoor Biotechnologies, Charlottesville, VA, USA) were diluted 1:2000 in PBS containing 10% normal dog serum. FITC-labeled horse-origin affinity purified anti-mouse gamma chain antibodies (Vector Laboratories, Burlingame, CA, USA) was diluted 1: 100 in PBS, then slides were coverslipped using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). For double-stained sections, the blocking solution was changed to 1% fetal calf serum and 10% normal donkey serum. Normal donkey serum (10%) was also added to the secondary antibody solution. Otherwise, slides were stained with anti-Der f 1 antibodies and horse-origin anti-mouse gamma chain antibodies as described above, then rinsed in PBS. These steps were followed by the addition of biotinylated mouse-origin monoclonal antibodies specific for either CD1c or CD3 (courtesy Dr. Peter Moore), diluted 1:50, followed by the addition of phycoerythrin-conjugated streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:200 in PBS.

Assay of slurry for protease activity: Serial dilutions of the slurry were tested for proteolytic activity using a commercially available assay kit (QuantiCleave Protease Assay Kit, Pierce Biotechnology, Rockford, IL, USA), either in the presence or absence of reversible or irreversible protease inhibitors (PMSF, aprotinin, leupeptin, E64, antipain).
Results:

Repeated applications of *D. farinae* slurry induce the development of a pruritic dermatitis similar to spontaneous canine AD: In occluded group puppies, pruritus and dermatitis were not detected at the time of the first patch test. However, both puppies developed severe pruritus within twenty-four hours of slurry application at the Day 60 and Day 120 patch tests. Although the pruritus appeared to be focused on the application sites themselves, generalized pruritus was also noted.

In addition to the pruritus associated with slurry applications, one puppy also developed a gradually worsening, generalized pruritic dermatitis by study day 42 (e.g., before the second application of slurry), which persisted until the end of the study. Clinically, erythema, chewing and self-inflicted hair loss were most prominent in the antecubital and tarsal skin folds. Erythema and alopecia were also noted in the retro-patellar fold areas, on the medial thighs, ventral abdomen and face.

Neither puppy in the non-occluded group developed significant pruritus after the mock patch tests on Day 1 and Day 60. At the Day 120 patch test, one puppy became moderately pruritic by six hours after slurry application, and both puppies were severely pruritic by twenty-four hours. Both puppies became slightly pruritic after the initial weekly application of slurry, and pruritus and/or application site dermatitis were seen intermittently until the sixth application (study Day 45). After this point, both pruritus and application site erythema were consistently seen following slurry applications. After study Day 60, slurry
application was also associated with the development or exacerbation of dermatitis in areas other than the application site. This effect was particularly pronounced in the contralateral axillary and groin skin. For this reason, a single set of biopsies (application site and contralateral control skin) was taken on Day 70.

Within four to six weeks of the beginning of the study, both puppies also developed a gradually worsening, more generalized pruritic dermatitis. Mild erythema and alopecia were initially noted on the ventral abdomen and feet, and eventually progressed to also involve the face, antecubital, tarsal and retropatellar folds. These areas of dermatitis were exacerbated by slurry applications to groin or axillary skin, but were also seen to persist between applications.

Repeated applications of *D. farinae* slurry induces the development of elevated serum mite-specific IgE and IgG levels: Serum levels of IgE and IgG specific for both denatured and non-denatured crude *D. farinae* extract (Greer Laboratories, Lenoir, NC, USA), as well as serum levels of IgE specific for purified *Der f 1* and *Der f 2* (Indoor Biotechnologies, Charlottesville, VA, USA), and total serum IgE levels were measured using ELISA, with each sample analyzed in triplicate. Allergen-specific antibody titers were calculated relative to a reference serum derived from a polyallergic dog. To minimize inter-assay variability, analysis was delayed until the end of the study. This allowed all of the serum samples to be evaluated at the same time for each of the measured parameters.
Mite-specific IgE and IgG levels were virtually undetectable in all puppies at the beginning of the study, although total IgE levels were comparable to those from control dogs (Figure 1). Both groups developed increases in mite-specific IgE and IgG by day 60, and antibody levels remained elevated at day 120. These increases were somewhat higher in occluded group puppies than non-occluded group puppies. However, by day 120 both mite-specific IgE and IgG levels in both groups of puppies were higher than those from a normal dog as well as a dog with spontaneous AD. Similar increases were seen in Der f 1-specific IgE levels in both groups of puppies (Figure 1). Notable increases in Der f 2-specific IgE levels were not seen (data not shown). The increases in mite-specific IgE did not appear to be an artefact of non-specific stimulation of IgE production, as total IgE levels in both groups of puppies actually decreased with time.

Epicutaneous application of D. farinae slurry induces degranulation of dermal mast cells:
Low pH toluidine blue staining revealed large, granular, oval cells throughout the dermis and clustered around blood vessels, hair follicles and adnexae. Although toluidine blue stains cytoplasmic granules in both canine basophils and mast cells, basophils are rarely found in the skin of either normal dogs or dogs with AD[43]. For this reason, positively staining cells were considered to be mast cells. Compared to control biopsies, biopsies taken from the first slurry application site exhibited fewer total toluidine blue-positive mast cells per square millimeter of dermis, presumably secondary to complete degranulation[44](Figure 2). Of the cells that could be seen in slurry application sites, higher percentages of mast cells were visibly degranulated (faintly stained cells and/or cells with dermal granule dispersion)
compared to those seen in control skin. These differences between control and slurry application sites became even more apparent by the third application (Figure 2).

Repeated applications of *D. farinae* slurry induce the progressive development of a heavily eosinophilic dermatitis: Histological examination of *D. farinae* application sites in occluded group puppies revealed the development of progressively more severe inflammatory dermatitis (Figure 3). Biopsies of application sites taken after the first patch test (first exposure to *D. farinae* slurry) had a mild to moderate increase in cellularity compared to the control sections. This infiltrate was primarily located around superficial vessels in the dermis, and was primarily composed of CD1c+ dermal dendritic cells, CD3+ T lymphocytes and neutrophils. Several visibly degranulated dermal mast cells were evident amongst these cells. Epidermal changes were minimal at this time. However, biopsies of *D. farinae* application sites from the second and third patch test were characterized by a severe, progressive, diffuse cellular infiltration of the dermis, which was accompanied by moderate to marked dermal edema and pronounced degranulation of dermal mast cells. The cellular infiltrate was largely eosinophilic, but large numbers of dermal dendritic cells, T lymphocytes and neutrophils were also present (Figure 3). This infiltrate was heavily concentrated in the superficial dermis, although in some specimens notable infiltration of the mid- and deep dermis was also seen. The epidermis was frequently spongiotic and infiltrated by inflammatory cells, most commonly eosinophils. In some areas, the eosinophilic infiltrate completely effaced the epidermis and superficial dermis or formed epidermal microabscesses.
Since non-occluded group puppies were not patch tested with *D. farinae* extract until study Day 120, slurry application sites were not available from the first two patch tests. Microscopically, slurry application sites from the third patch test were characterized by a severe, diffuse infiltration of inflammatory cells, similar to that seen in the occluded group puppies. This infiltrate was seen predominantly in the superficial dermis, but also involved the epidermis and deeper dermis (data not shown). Marked dermal and epidermal edema was noted in these samples, and visibly degranulated mast cells were frequently seen. The cellular infiltrate in both the dermis and epidermis was a mixture of mononuclear cells and granulocytes, but was even more heavily eosinophilic than that seen in occluded group puppies (Figure 4).

Microscopic evaluation was also performed on biopsies obtained from both the right and left axillary and groin areas (e.g., from weekly slurry application sites and contralateral skin) on study Day 70. Samples obtained from slurry application sites were characterized by a perivascular to diffuse cellular infiltrate in the superficial dermis, which consisted primarily of neutrophils, eosinophils and CD1c+ dendritic cells. Samples obtained from contralateral skin also displayed perivascular to diffuse cellular infiltration of the superficial dermis. However, this inflammatory infiltrate was of a lesser magnitude and contained somewhat fewer eosinophils than that seen at the application sites themselves.

The major mite allergen *Der f 1* can be demonstrated by immunofluorescent staining at *D. farinae* slurry application sites: Immunofluorescent staining using mouse-origin monoclonal anti-*Der f 1* antibodies (4C1, Indoor Biotechnologies, Charlottesville, VA, USA) was
performed on frozen biopsy samples obtained from patch test slurry application sites to
determine whether mite allergens could be demonstrated within the skin after epicutaneous
application of slurry.

Biopsies obtained from slurry application sites frequently contained foci of brightly-
staining cells located predominantly within subepidermal cellular infiltrates (Figure 5) and
immediately adjacent to the upper regions of the hair follicles. These brightly staining cells
were not seen in matched sections stained with a mouse-origin IgG1 isotype control (Zymed
Laboratories, San Francisco, CA, USA). Overnight incubation of the antibodies with a ten-
fold molar excess of purified Der f 1 (Indoor Biotechnologies, Charlottesville, VA, USA)
was performed as a further control for staining specificity. This preadsorption suppressed all
but the most intense foci of staining.

Immunofluorescent staining was also performed on biopsies of axillary skin (slurry
application sites and skin from the contralateral axilla) obtained on study Day 70. Brightly
staining cells were seen scattered throughout the superficial dermis in samples obtained from
application sites. This staining does not appear to be an artefact of nonspecific interactions
between the detection antibodies and FcR-bearing cells in the samples, as positive staining
was not seen in samples obtained from the contralateral axilla, despite the presence of
substantial dermal inflammation.

Double-staining was performed on these axillary biopsies to determine the identity of
the dermal cells containing Der f 1 (Figure 6). Biotinylated mouse-origin anti-canine CD1c
or anti-CD3 antibodies were detected using phycoerythrin-labeled streptavidin. Slides double-stained for Der f 1 and CD1c demonstrated the presence of many double-positive (yellow) cells scattered throughout the dermis, indicating localization of Der f 1 on or within dermal dendritic cells. Although double-positive cells could also be seen on slides stained for Der f 1 and CD3, most cells were single positive for either antigen.

D. farinae slurry is proteolytically active: The crude D. farinae slurry exhibited moderate proteolytic activity, as determined by cleavage of a succinylated casein substrate (Figure 7). Treatment of the slurry with protease inhibitors of varying specificity suggest that the slurry contains both serine and cysteine protease activity.

Discussion:

This study expands upon previous work by McCall and others[37] in the characterization of epicutaneous sensitization using a canine model of AD. Specifically, it was determined that repeated epicutaneous exposure to a sonicated slurry of D. farinae mites to the intact skin of Maltese-Beagle cross-bred puppies results in the development of clinical, microscopic and serologic abnormalities similar to those seen in spontaneous canine AD. It is not necessary to begin the process in the neonatal period, nor is successful sensitization dependent upon the use of high-IgE responder Beagles as subjects. This sensitization can be achieved even if reasonable measures are taken to ensure that the slurry is not ingested by the puppies, and is enhanced by (but does not require) that the slurry be applied under occlusion. Finally, immunofluorescent staining was able to demonstrate the presence of large numbers of Der f 1-containing cells in the superficial dermis and near the sebaceous ducts, suggesting
that both transepidermal and transfollicular penetration may play a role in the development of epicutaneous sensitization.

Repeated epicutaneous applications of a minimally processed, sonicated slurry of *D. farinae* mites in saline to the intact skin of Malteagle puppies induced the development of a pruritic dermatitis very similar to naturally-occurring canine AD. Minimal to no clinical response was seen in either group of puppies following the initial application of slurry. However, later applications were associated with the progressive development of pruritus and erythematous dermatitis not only at the application sites themselves, but also at areas distant from those sites. It is noteworthy that the areas affected by the dermatitis (elbow and stifle folds, face) were those typically affected in spontaneous human and canine AD.

None of the puppies had detectable levels of mite-specific IgE at the beginning of the study. Mite specific IgG levels could be detected, but were far lower than those seen in the normal and allergic controls. By day sixty, *D. farinae*-specific antibodies of both isotypes were markedly elevated in both groups of puppies. Serum *D. farinae*-specific IgG levels continued to increase with time in all of the puppies. In contrast, mite specific IgE levels were seen to peak at sixty days in three of the four puppies. This peak was seen in the allergic control dog as well. The reason for this peak is unclear, but it may be related to a heightened sensitivity to seasonal fluctuations in environmental *Dermatophagoides* allergens.

Serum IgE levels directed against *Der f 1* were also determined. In humans, *Der f 1* represents the major allergen from *D. farinae*[2, 45], although spontaneous canine reactivity
to *Der f 1* is uncommonly seen[46]. *Der f 1* specific IgE was seen to increase with time in both occluded and non-occluded group puppies. However, at no point were notable levels of *Der f 1* specific IgE seen in either the nonallergic or allergic control dogs.

Total serum IgE levels were evaluated to ascertain the specificity of the observed IgE responses. Total IgE levels were seen to actually decrease with time, making it unlikely that the observed mite-directed IgE response was simply due to nonspecific induction of IgE antibody production.

Gross and microscopic examination of application sites revealed the development of an eosinophilic dermatitis of progressive severity. Erythema and edema were not observed at any of the initial application sites in any puppy. However, by the third patch test, all of the puppies exhibited varying degrees of erythema and dermal induration at the application sites. These gross findings were echoed on the microscopic level. Histologic examination of biopsies taken from the first slurry application sites demonstrated a mild perivascular cellular infiltrate consisting mostly of neutrophils, CD1c+ dendritic cells and CD3+ lymphocytes. In contrast, biopsies taken from the third application sites were characterized by a dense, heavily eosinophilic perivascular to diffuse cellular infiltrate and edema involving both the dermis and the epidermis. These changes are similar to those described in mite-patch test reactions in humans with AD.
Finally, immunofluorescent staining of biopsies obtained from slurry application sites demonstrated a population of dermal cells that stained positive with monoclonal antibodies directed against the mite allergen Der f 1. This effect was seen regardless of application site occlusion, and it demonstrated the ability of D. farinae allergens to access the dermis even in intact, unmanipulated canine skin. Immunofluorescent double staining demonstrated the frequent co-localization of Der f 1 with CD1c, suggesting the phagocytosis of Der f 1 (among other mite-origin antigens) by dermal dendritic cells. In contrast, co-localization with CD3+ lymphocytes was seen only occasionally. The inflammatory response to epicutaneous application of D. farinae slurry would be expected to induce recruitment of lymphocytes of many different specificities to the application sites. For this reason, the relatively low number of CD3+ lymphocytes demonstrating co-localization with any single antigen (here, Der f 1) is not surprising.

In summary, our study demonstrated that repeated epicutaneous applications of a sonicated D. farinae slurry to the intact, unmanipulated skin of high-allergy risk Malteagle puppies resulted in the development of a pruritic dermatitis essentially indistinguishable from spontaneous canine AD. This dermatitis was associated with elevations in mite-specific IgE and IgG levels, without concomitant increases in total IgE. These effects were not dependent upon, but were greatly enhanced by, occlusion of the application sites. Histologic analysis of slurry application sites revealed the progressive development of a severe, predominantly eosinophilic infiltration of the superficial dermis and epidermis, which was associated with marked dermal and epidermal edema and mast cell degranulation. Finally, immunofluorescent staining demonstrated the presence of Der f 1-containing cells within the
dermis itself, predominantly within CD1c⁺ dermal dendritic cells. We feel that these results suggest that epicutaneous exposure to allergen may play an important role during both the sensitization and perpetuation of AD. Further studies are indicated to confirm these results and to investigate the roles played by individual allergens (both proteolytically-active and – inactive) during this process.
References:


46. Nuttall TJ, Lamb JR, Hill PB. Characterisation of major and minor 

Figure 1: Epicutaneous application of sonicated *D. farinae* mites induces elevations in serum mite-specific antibodies without concomitant increases in total serum IgE. Serum IgE (A) and IgG (B) directed against a crude *D. farinae* extract, as well as IgE specific for the major mite allergen *Der f 1* (C) increase relative to baseline levels in puppies receiving sonicated *D. farinae* topically to intact skin. These increases do not require occlusion of the application sites but are markedly enhanced by it. In contrast, total serum IgE levels (D) do not increase with time. Antibody levels from non-allergic and spontaneously allergic control dogs are also displayed for reference.
Figure 2: Epicutaneous application of *D. farinae* slurry induces mast cell degranulation.

Compared to control sites, skin treated with *D. farinae* slurry (HDM) contained fewer toluidine blue-positive mast cells (open bars) and a greater percentage of degranulated mast cells (shaded bars). This phenomenon was most evident after the third slurry application (Control 3 and HDM 3, right) but could be seen even following the first application (Control 1 and HDM 1, left). Bars indicate averages from Occluded group puppies.
**Figure 3:** Epicutaneous applications of sonicated *D. farinae* mites induce the recruitment of an evolving population of inflammatory cells. Comparison of skin biopsy samples taken twenty four hours after the first (left) and third (right) mite applications demonstrates differences in both the magnitude and character of the cellular influx. Biopsies were obtained from mite application sites (HDM) and adjacent untreated skin (control), both under occlusion. Bars indicate average cell numbers from both Occluded group puppies.
Figure 4: Epicutaneous applications of sonicated *D. farinae* mites induce the development of epidermal and dermal edema and inflammatory cell recruitment. Biopsies taken from Non-occluded group dogs on Day 120 after 24 hours’ exposure to *D. farinae* demonstrate marked dermal edema and epidermal spongiosis, with a perivascular to diffuse infiltrate of eosinophils into the dermis and epidermis. Hematoxylin-eosin, 4X (left) from Dog One; 20X (right) from Dog Two.
Figure 5: The mite allergen *Der f 1* can be detected in the dermis following epicutaneous application of sonicated *D. farinae* slurry. Biopsies taken from an Occluded group dog after six hours’ exposure to *D. farinae* demonstrate the presence of *Der f 1* allergen in cells infiltrating the superficial dermis. (A) and (B) Immunofluorescent detection of *Der f 1* using a mouse-origin monoclonal antibody, followed by FITC-conjugated horse-origin anti-mouse gamma chain. (C) and (D) Mouse origin IgG1 isotype control antibody. Sections are shown with and without DAPI counterstain. 40X magnification; Bar = 100 micrometers.
Figure 6: Immunofluorescent staining demonstrates frequent co-localization of the mite allergen \textit{Der f} 1 with CD1c$^+$ dendritic cells in the dermis, but only occasional co-localization is seen with CD3$^+$ lymphocytes. (A) Immunofluorescent double staining using mouse-origin monoclonal antibodies specific for \textit{Der f} 1 and CD1c demonstrates the presence of the mite allergen within dermal dendritic cells (co-localization indicated in yellow). Mouse anti-\textit{Der f} 1 was detected using FITC-conjugated horse origin anti-mouse gamma chain (green), while biotinylated mouse anti-CD1c was detected using phycoerythrin-labeled streptavidin (red). (B) In contrast, co-localization is less commonly observed when double staining using mouse-origin monoclonal antibodies specific for \textit{Der f} 1 and CD3 is performed.
Figure 7: *D. farinae* slurry is proteolytically active. Serial dilutions of a slurry of sonicated *D. farinae* mites in saline induce dose-dependent cleavage of a casein substrate. (A) Succinylated casein was incubated with increasing concentrations of slurry in the absence of exogenous reducing or activating agents. Incubations were performed for 20 minutes in a borate buffer (50mM, pH 8.5, 37°C), after which casein degradation was analyzed using a commercially available protease assay kit (QuantiCleave Protease Assay Kit, Pierce Biotechnology Inc., Rockford, IL, USA). (B) Inhibition of slurry protease activity was determined by incubating succinylated casein with a fixed concentration of slurry (300 ug/ml) in the presence or absence of increasing concentrations of five protease inhibitors.
**Chapter three:**

**Title:**

Epicutaneous sensitization with purified *Der f 1* allergen induces allergic dermatitis and elevated mite-specific IgE levels in a canine model of atopic dermatitis

**Authors and affiliations:**

Cherie M. Pucheu-Haston, DVM*, Hilary A. Jackson†, Thierry Olivry, DrVet, Ph.D.†, Bruce Hammerberg, DVM, Ph.D.∗

∗North Carolina State University, College of Veterinary Medicine, Department of Population Health and Pathobiology, 4700 Hillsborough Street, Raleigh, NC, 27606

†North Carolina State University, College of Veterinary Medicine, Department of Clinical Sciences, Raleigh, NC, 27606
Abstract:

In humans, atopic dermatitis (AD) is a cutaneous hypersensitivity disorder associated with elevated levels of antigen-specific IgE[1], commonly to house dust mites (HDM)[2]. It remains controversial as to whether spontaneous sensitization and clinical disease development can be induced by cutaneous exposure to HDM. *Dermatophagoides* allergens include a number of highly active proteases (especially the mite allergen *Der f 1*[3, 4]), several of which have been shown to increase paracellular permeability[4] and facilitate the induction and perpetuation of hypersensitivities by interacting with cell surface molecules and receptors[5, 6]. We hypothesized that application of purified *Der f 1* to intact canine skin would induce the development of a cutaneous and systemic immune response resembling that seen in spontaneous AD, and that this effect would be facilitated by allergen cysteine protease activity. Adult allergy-predisposed Maltese-Beagle cross-bred dogs (Malteagles, Maltese Beagle atopic dogs) received weekly applications of proteolytically active or inactive purified *Der f 1* allergen to the axilla and groin, and were patch tested with the allergen at days 1, 30 and 60. Patch test and/or control sites were biopsied on days 1, 30 and 60, and serum samples were obtained before, during and after the study. Topical application of proteolytically active *Der f 1* was associated with the progressive development of pruritic dermatitis in all dogs. Dogs receiving active *Der f 1* allergen developed increases in both mite specific IgE and IgG during the study. In contrast, dogs receiving inactive allergen had lesser increases in mite specific IgE and did not develop appreciable increases in mite specific IgG. Microscopic examination of skin biopsy samples demonstrated a predominantly mononuclear cell response (CD1c+ and CD3+ cells) in dogs receiving proteolytically-active *Der f 1*, with a weaker response seen in dogs receiving proteolytically-
inactive allergen. In summary, our results demonstrate that cutaneous exposure to Der f 1 allergen through intact canine skin may be sufficient to induce sensitization, and suggest that this response may be facilitated by the proteolytic activity of the allergen.
**Introduction:**

Atopic dermatitis is a genetically-predisposed, chronic inflammatory and pruritic allergic skin disease characterized by cutaneous hyperreactivity to a number of environmental antigens[1]. This hyperreactivity is most commonly associated with IgE antibodies to environmental allergens (extrinsic AD), although an IgE-independent form (intrinsic AD) has also been described[1]. Although many environmental antigens have been associated with AD (including pollens, animal danders and molds), the most common allergens appear to be those associated with several species of house-dust mite (HDM), especially *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*.

Although much is known about the immunologic phenomena associated with established disease, the major route of allergen exposure resulting in the development of AD and perpetuation of clinical disease remains controversial. Inhalation of allergenic particles has been proposed as the exposure route most likely to result in the induction of AD. At least one study has demonstrated an exacerbation of existing lesions and the development of new ones in some AD patients challenged by inhalation of HDM extracts[7]. However, other studies have failed to demonstrate the development or exacerbation of AD after aerosol challenge[8].

An alternative route of sensitization could be by transcutaneous absorption of allergen. Tentative support for this hypothesis may be found in a small number of studies in which HDM antigens have been detected in naturally occurring lesions of human AD[9]. Despite these findings, naturally occurring epicutaneous sensitization is often dismissed as
unlikely due to the presence of the relatively impermeable “epidermal barrier”. The major component of this barrier is the stratum corneum[10], which is comprised of multiple layers of tightly apposed cornified keratinocytes embedded in a matrix of highly ordered lipid sheets[10]. Together, the lipid and protein components of the stratum corneum form an efficient hydrophobic barrier, preventing excessive movement of large hydrophilic substances (such as most allergens) into or out of the skin. However, the epidermis and stratum corneum are dynamic structures. The supply of keratinocytes is continually replenished by division of basal layer cells, and the top layers of the stratum corneum are constantly shed during desquamation. This process involves the degradation of desmosomes by a series of endogenous serine and cysteine proteases[11]. The continual cycle of loss and replenishment is vital for the maintenance of epidermal homeostasis, and demands a delicate balance between basal cell proliferation and proteolytic digestion.

It is possible that some allergens can to exploit these normal processes to facilitate their passage into the deeper layers of skin. For example, allergens derived from both *D. farinae* and *D. pteronyssinus* include a number of highly active proteases, especially the Group 1 allergens *Der f 1* and *Der p 1*[4, 12]. Both *Der f 1* and *Der p 1* have potent cysteine protease activity[4], and may also have some degree of serine protease activity[12, 13]. These activities are usually lost during the processing and purification of commercial mite extracts, but may be readily restored in vitro[4]. Group 1 proteases have been demonstrated to degrade intercellular tight junctions and disrupt desmosomes in several in vitro culture systems[4, 14], and to induce apoptosis independent of tight junction degradation[15]. The net effect of these changes is to increase paracellular permeability and cellular detachment,
allowing migration of allergens through these normally impermeable cellular barriers[14]. These results suggest that *Dermatophagoides* proteases may be able to produce similar disruptions of the epidermal barrier, perhaps by degrading the same proteins that are the targets of endogenous desquamation proteases.

However, transcutaneous penetration need not occur through the epidermis alone. The total absorption of external substances through hair follicles and their associated adnexae has typically been considered minimal, largely due to the fact that follicular and adnexal ostia represent only between 1-10% of the body surface area[16-18]. Nonetheless, if the inner surfaces of the follicles and adnexae are included in this calculation, the total surface area represented by these structures is likely far larger. Penetration of external substances into the deeper areas of the hair follicles (and from there into the dermis) can be considerable. Particles as large as one micron (or proteins as large as 47kD) have been visualized by a variety of methods (laser scanning confocal microscopy, conventional fluorescent microscopy) at the level of the sebaceous ducts or within the dermis itself following epicutaneous application of microspheres or microparticles[16, 19, 20]. This process is enhanced if these microparticles are applied to areas of skin undergoing flexion[19]. Additional particles can be seen to cross directly through the stratum corneum in these areas of flexed skin as well[19].

Allergens from *Dermatophagoides* may facilitate the induction and perpetuation of hypersensitivity disorders not only by increasing epithelial permeability and allergen exposure, but also by directly interacting with a number of cell surface molecules. Several
Dermatophagoides allergens have been reported to induce cellular activation and release of GM-CSF, eotaxin, IL-6 and IL-8 from epithelial cell lines in a protease-dependent manner via interactions with protease activated receptors (PARs)[3]. Proteolytically-active Group 1 Dermatophagoides allergens have been demonstrated to cleave CD23 from the surface of B cells, to cleave CD25 from the surface of both CD8⁺ and CD4⁺ T cells, to induce degranulation and activation of eosinophils[21], and to induce IgE-independent cytokine and histamine production and release from human mast cells and basophils[21]. The ability of Dermatophagoides proteases to directly contribute to an inflammatory response in an IgE-independent manner suggests mechanisms by which they may facilitate sensitization as well as perpetuation of allergic diseases.

In a previous study, we performed repeated applications of a slurry of D. farinae mites (courtesy Heska) to the intact skin of four hypersensitivity-predisposed Maltese-Beagle cross-bred (Malteagle; Maltese Beagle atopic dog) puppies. Two puppies received a total of three applications under occlusion (“Occluded group”) on days 1, 60 and 120. The other two puppies did not receive slurry under occlusion until day 120 (“Non-occluded group”), but did receive sixteen weekly applications of slurry to their axillary and groin skin. Serum samples and biopsies of application sites and adjacent untreated skin were obtained from all puppies on days 1, 60 and 120. By the end of the study, all four puppies had developed localized and generalized pruritic dermatitis. This dermatitis was exacerbated by slurry applications, but also persisted between exposures. Serum samples demonstrated the development of D. farinae-specific IgE and IgG antibodies in all puppies. Histological examination of D. farinae application sites revealed the development of an inflammatory dermatitis of
progressive severity. Biopsies taken from early slurry application sites were characterized by a mild perivascular accumulation of mononuclear cells and had no epidermal lesions, while later applications were dominated by a dense, heavily eosinophilic cellular infiltrate and edema in both the dermis and epidermis.

These results suggested that transcutaneous allergen penetration may play a role in the development and perpetuation of the immunologic abnormalities associated with canine AD. However, the slurry mixture that we had used was a crude preparation of sonicated *D. farinae* mites. The nature of this mixture made it impossible to determine the role of any particular allergen (proteolytically-active or not) in the development of sensitization. To clarify our results, we asked whether a similar response could be obtained by application of a single allergen component of the slurry, and whether the development of such a response would be influenced by the proteolytic activity of that allergen.

The specific aims of this study were three-fold. First, we wanted to determine if repeated epicutaneous applications of proteolytically-active (or –inactive), purified *Der f 1* allergen to intact canine skin would result in the development of clinical pruritus and/or dermatitis similar to naturally occurring canine AD. Second, we wanted to determine if applications of proteolytically-active or inactive *Der f 1* would induce the development of microscopic inflammatory changes similar to those seen with spontaneous canine AD and patch test reactions with crude mite preparations. Finally, we evaluated if repeated applications of proteolytically-active or -inactive allergen would result in the development of elevated levels of serum mite-specific IgE and / or IgG.
**Methods:**

**Test substance:** This allergen was a commercially prepared extract of affinity purified natural *Der f 1* in preservative and carrier free phosphate buffered saline, pH 7.4 (Indoor Biotechnologies, Charlottesville, VA). We chose to evaluate the effects of epicutaneously applied *Der f 1* rather than the larger molecular weight *Dermatophagoides* allergens (*Der f 15, Der f 18*) that may be more commonly associated with canine AD[22]. This choice was based upon careful consideration of the biochemical properties of the Group 1 allergens, with regards to their potential penetration-enhancing or adjuvant effects. The proteolytic activities of the Group 1 allergens have been well described, as have the environmental conditions which favor maximal protein digestion[23]. These conditions include a preference for a neutral to acidic pH, and a requirement for the presence or availability of reducing agents for proteolytic activity. All of these requirements may be met on the surface of human and canine skin[10, 24, 25]. Furthermore, these environmental optima are very similar to those described for endogenous cysteine proteases involved in the desquamation of the outermost layers of the stratum corneum[11]. The significance of these findings becomes evident when one considers the well-documented ability of the Group 1 allergens to disrupt or degrade intercellular adhesion molecules, including those known to be present in the epidermis.

Cysteine proteases are readily inactivated, and require the presence of a reducing agent to regain proteolytic activity[23, 26, 27]. For this reason, the stock preparation was diluted before each use in sodium phosphate buffer, pH 7.6, containing cysteine hydrochloride monohydrate (Sigma Aldrich, St. Louis, MO) at a final concentration of 6
mM. The pH of this diluted solution was 6.3, which is similar to the pH optimum for Der f 1 proteolytic activity[23], and approximates the pH of normal stratum corneum[28]. The diluted solution was then placed in a 37°C incubator until use.

Assay of Der f 1 for protease activity: The diluted Der f 1 was tested for proteolytic activity using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC (Sigma Aldrich, St. Louis, MO) in a variation of a previously described technique[23]. Aliquots were prepared with and without the activating agent cysteine hydrochloride, as well as in the presence or absence of the irreversible cysteine protease inhibitor, E64 (Sigma Aldrich, St. Louis, MO).

Subjects: Our laboratory has developed a breeding colony of Maltese-Beagle cross-bred dogs (Malteagles; Maltese Beagle atopic dogs). These dogs are unique and distinct from dogs bred for high IgE responses in that they spontaneously develop hypersensitivities following controlled exposure to allergens[29]. These responses do not require that the allergen be administered parenterally or with an adjuvant. Dogs from this colony have been demonstrated to spontaneously develop sensitivities to food items containing corn, soy[29] and chicken. Oral challenge with these items induces the rapid development of clinical signs of pruritus, otitis and GI signs. Dogs in this colony are maintained with controlled dietary management and in a rigorously controlled housing environment, which has been highly effective in preventing spontaneous hypersensitivity to environmental allergens. However, dogs removed from this colony and adopted into homes as pets have been reported to develop pruritus and/or rhinitis after environmental exposure to household allergens.
Four Malteagle dogs (two sexually intact males, one sexually intact female and one ovariohysterectomized female) were selected for this study. These dogs were between six and eight years of age at the time sensitization was begun. At this time, the dogs were all healthy and had neither dermatitis nor any other signs of allergic disease. The dogs were housed with the remainder of the colony in a facility determined by ELISA (Indoor Biotechnologies, Charlottesville, VA, USA) to be free of detectable levels of the house dust mite allergens Der f 1 and Der p 1.

Sensitization protocol: To minimize degradation or freeze-thaw damage to the extract, individual aliquots containing 60 micrograms of the stock solution were prepared and frozen at -20°C until use. This dosage was chosen to approximate the Der f 1 concentration of the slurry used in the preliminary study (as determined by ELISA analysis), to facilitate data interpretation and comparison between the two studies. Individual aliquots of the test extract were thawed and diluted immediately before use as described above (either with or without a three-fold molar excess of the irreversible cysteine protease inhibitor E64) to a final volume of 80 microliters per application.

For patch tests, each dog received two applications—one for each side of the thorax. For weekly applications, only a single aliquot was used per dog per week. In all cases, sensitization was performed by application of the diluted Der f 1 to intact, unmanipulated skin—i.e., the skin was not tape stripped, pricked, scratched or otherwise disrupted. Dogs were randomly assigned to receive proteolytically-active or -inactive Der f 1 throughout the study (“Active” and “Inactive” groups; two dogs each).
All dogs were patch tested with the preparation on study days 1, 30 and 60. The Der f 1 preparation used in this study lacked the paste-like consistency of the slurry used in our initial work. For this reason, the original patch test protocol required modification to prevent excessive movement or loss of the test extract. Whatman Grade 1 cellulose filter paper (Whatman Inc., Florham Park, NJ) was cut into 1.5cm squares, which were placed on a rectangle of minimally occlusive dressing (Op-Site IV 3000, Smith and Nephew, Largo, FL). Aliquots of Der f 1 and control applications of diluent were prepared (either with or without the addition of E64) and were applied to the filter paper squares immediately before use. The treated filter paper squares were then secured on the skin of the lateral thorax with the Op-Site dressing. The dogs were placed in Elizabethan collars to prevent ingestion of the extract, then placed into individual runs. Biopsies were taken of Der f 1 and diluent application sites at six and twenty-four hours after application, using an 8mm biopsy punch. These biopsies were halved immediately after acquisition and placed in 10% formalin or frozen in OCT tissue cutting medium.

In addition to the three patch tests, these dogs received weekly applications of Der f 1 between patch tests, for a total of six weekly applications. The test extract was prepared as described above to a final volume of 80 microliters per application. This volume was dispensed onto an approximately 1-1.5cm² area of skin. Application sites alternated weekly between the right axillary and groin skin. After application, the dogs were placed in Elizabethan collars to prevent ingestion of the extract, then placed into individual runs for
twenty-four hours. The next day, the application sites were examined then gently wiped clean.

Clinical monitoring: The dogs were given a physical examination and a complete dermatologic examination twice weekly. In addition, the dogs were observed for a one hour period twice weekly. Any behavior suggestive of pruritus (scratching, chewing, rubbing) was noted.

Antibody titers: Serum samples were obtained from the dogs before the first patch test. After the first test, further samples were drawn every seven to fourteen days for the duration of the study. Sera were divided into 100:1 aliquots, and frozen at –20C until analysis. All samples were analyzed by ELISA (in triplicate) to determine total IgE levels, as well as IgE and IgG levels specific for purified Der f 1. Briefly, round-bottomed 96 well ELISA plates were coated overnight in either mouse-origin monoclonal anti-canine IgE (developed in our laboratory) or purified Der f 1. All coating reagents were diluted in a carbonate/bicarbonate coating buffer. Nonspecific binding was inhibited by a blocking step with PBST. Dilutions (1:5 for IgE; 1:100 for IgG) of patient and control sera were added and incubated at room temperature for two hours. Bound IgE was detected by incubating with biotinylated mouse-origin monoclonal anti-canine IgE, followed by an incubation with horseradish peroxidase conjugated streptavidin (Zymed, San Francisco, CA, USA). Bound IgG was detected using a peroxidase-labeled, goat-origin polyclonal anti-canine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reactions were developed by the addition of
ABTS horseradish peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and read at 30 and 60 minutes at 405nm.

**Processing and staining of formalin-fixed skin:** Formalin-fixed skin samples were dehydrated and embedded in paraffin-containing tissue blocks. Five micrometer sections were cut, then stained with hematoxylin and eosin, as well as with Luna’s stain and low pH (1.5) toluidine blue stains (for eosinophil[30] and mast cell[31, 32] enumeration, respectively). All slides were randomly numbered and evaluated by the same investigator on a blinded basis. This evaluation included standard pattern analysis and enumeration of the total nucleated cells, neutrophils, eosinophils and mast cells per square millimeter of dermis.

**Immunohistochemical staining:** A three-step labeled streptavidin method modified from Affolter and Moore[33] was used to characterize the mononuclear cell infiltrate. Briefly, 6-micrometer cryosections were fixed by immersion in acetone. Endogenous peroxidase activity was quenched by immersion in hydrogen peroxide (diluted to 0.3% in PBS) containing sodium azide (0.01%). The sections were then blocked with 1% fetal calf serum in PBS. Monoclonal antibodies specific for canine CD1c and CD3 (courtesy of Dr. Peter F. Moore, University of California, Davis, CA, USA) were used as cell culture supernatant diluted 1:10 in PBS. Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was diluted 1:400 in PBS, followed by horseradish peroxidase-conjugated streptavidin (Zymed, San Francisco, CA, USA), diluted 1:400 in PBS. Amino-9-ethyl-carbazole (AEC, AEC Substrate Kit, Biogenix, San Ramon, CA, USA) was applied as a chromogen, followed by a hematoxylin counterstain (Sigma-Aldrich, St. Louis, MO, USA).
Enumeration of dermal cells: The number of total nucleated cells/mm² in the dermis was obtained by counting 51 consecutive 0.14 mm x 0.14 mm fields of superficial dermis (excluding endothelial cells and adnexae). Neutrophils, mast cells and eosinophils, as well as CD1c⁺ and CD3⁺ mononuclear cells/mm² of dermis were determined by counting 13 consecutive 0.24 mm x 0.32 mm fields of superficial dermis.

Results:

Purified Der f 1 allergen is proteolytically-active in the presence of a reducing agent, and this activity can be suppressed by a class-specific protease inhibitor: The proteolytic activity of purified Der f 1 allergen was evaluated by measuring the fluorescence generated by liberation of 7-amino-4-methylcoumarin (AMC; Sigma Aldrich, St. Louis, MO, USA) from the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC (Sigma Aldrich, St. Louis, MO, USA). Der f 1 prepared in sodium phosphate buffer (0.05M) produced appreciable digestion of the peptide substrate only in the presence of cysteine hydrochloride (final concentration 6mM; Sigma Aldrich, St. Louis MO) (Figure 1). Similar digestion reactions were performed in the presence or absence of the cysteine protease-specific inhibitor E64. E64 induced almost complete suppression of Der f 1 protease activity (Figure 1).

Repeated topical applications of purified Der f 1 allergen to intact canine skin induce the development of pruritic dermatitis in a non-protease dependent manner: Topical application of purified Der f 1 allergen was associated with the progressive development of a distinct, repeatable (albeit subtle) cutaneous response in all dogs. As expected, none of the dogs
displayed evidence of dermatitis or appreciable pruritus following the initial applications. However, later applications were associated with pruritic dermatitis of variable onset, severity and distribution regardless of treatment group. Pruritus was seen after the second application in two dogs (one “active group” and one “inactive group” dog), after the third application in the second inactive group dog and after the fourth application in the remaining active group dog. Subsequent applications were frequently followed by an exacerbation of pruritus. This pruritus was seen to become more severe and/or persistent at later times in the study in three of the four dogs, although the fourth dog (which belonged to the “inactive group”) actually seemed to improve somewhat by the end of the study. A pruritic, erythematous dermatitis was occasionally noted at the application sites themselves, and could also be seen in areas distant from the application sites. The distribution and severity of this pruritic dermatitis was highly variable between individuals, and showed no apparent correlation with treatment group. Affected areas included the feet, elbow and hock folds, periocular skin, and the concave surface of the pinnae. Serous to seromucoid ocular discharge was occasionally seen in all dogs following application of allergen. However, other signs of systemic hypersensitivity (sneezing, coughing, urticaria, collapse, etc.) were not seen in any dog at any point.

Repeated topical applications of proteolytically-active purified *Der f 1* allergen induce increases in serum mite-specific IgG and enhance induction of serum mite-specific IgE without increases in total serum IgE: Serum levels of IgE and IgG specific for purified *Der f 1* were measured by ELISA from samples obtained before, during and after sensitization. To
minimize inter-assay variability, analysis of all samples was delayed until the end of the study, at which time they were analyzed in a single batch.

None of the dogs had appreciable levels of serum *Der f 1*-specific IgE prior to the beginning of sensitization (Figure 2). Both groups of dogs developed some increase in *Der f 1*-specific IgE by the end of the study. However, this response was more pronounced in the dogs receiving proteolytically-active allergen. Serum *Der f 1*-specific IgG levels did not change above background levels in either group of dogs until the fifth week of the study. By the end of the study dogs in the active group had developed considerable increases in serum mite-specific IgG, while this increase was not observed in the inactive group dogs (Figure 2).

Total IgE levels were measured to investigate the possibility that the observed increases in mite-specific IgE were an artefact of non-specific polyclonal antibody stimulation, especially for dogs receiving proteolytically-active allergen. However, total serum IgE levels remained essentially unchanged (both within individual dogs and within treatment groups) over the entire study period. These levels were roughly equivalent for three of the dogs (Figure 2) but the fourth dog (in the inactive group) had considerably lower levels of total serum IgE at all times. The reason for these lower levels (other than simple individual variation) is not immediately apparent. The relevance of this finding is questionable, as this dog did not appear to have an impairment in the development of allergen-specific IgE or IgG, nor in the development of allergen-stimulated gross or microscopic inflammation (individual data not shown).
Epicutaneous application of purified *Der f 1* allergen induces mast cell degranulation: Low pH toluidine blue staining revealed large, granular, oval cells throughout the dermis and clustered around blood vessels, hair follicles and adnexae. Although toluidine blue stains cytoplasmic granules in both canine basophils and mast cells, basophils are rarely found in the skin of either normal dogs or dogs with AD[34]. For this reason, positively staining cells were considered to be mast cells. Application of proteolytically-active purified *Der f 1* allergen was associated with a decrease (as compared to diluent treated skin) in the total number of mast cells visible using toluidine blue, presumably secondary to complete degranulation of these cells (Figure 3A)[35]. In addition, the percentage of faintly stained cells and cells with dermal granule dispersion (degranulated mast cells) was higher in proteolytically-active allergen treated skin (Figure 3A) than in diluent-treated skin.

In contrast, skin treated with proteolytically-inactive allergen exhibited mild to moderate increases in mast cell numbers as compared to diluent-treated skin (Figure 3B). Some increase in the percentage of degranulated mast cells was seen at the initial allergen application, but this increase was not evident in biopsies taken from the final allergen application (Figure 3B).

Epicutaneous application of purified *Der f 1* allergen induces the recruitment of CD1c⁺ and CD3⁺ mononuclear cells into the superficial dermis: Histologic examination of the first allergen patch test sites demonstrated an increase in dermal inflammatory cells and mild superficial dermal edema as compared to diluent-treated skin (Figure 4A). These inflammatory cells were clustered around blood vessels in the superficial dermis, and were
predominantly CD1c⁺ dendritic cells and CD3⁺ lymphocytes. Neutrophils were also seen scattered throughout the dermis, but these cells did not appear to be differentially increased in allergen-treated skin. Only rare eosinophils were seen in either allergen-treated or diluent-treated skin. Occasional foci of inflammation could be seen surrounding the sebaceous ducts and the hair follicles at the isthmus region. This perivascular to periadnexal inflammatory pattern was also seen in allergen-treated skin after the third patch test (Figure 4B) in these dogs. Although allergen-treated skin biopsies from dogs receiving proteolytically-inactive Der f 1 displayed a similar pattern of perivascular to periadnexal inflammation, fewer inflammatory cells were seen than in samples from dogs receiving proteolytically-active allergen at both the first and third patch tests (Figures 4C, 4D).

Discussion:

This study expands upon our previous work investigating the role of cutaneous exposure to protein allergens in the induction of systemic sensitization. Specifically, this work demonstrates that repeated cutaneous applications of a single protein allergen (the mite cysteine protease Der f 1) to the intact skin of adult dogs can induce the development of localized and generalized pruritic dermatitis, elevations in serum mite-specific antibody levels, cutaneous mast cell degranulation and a mild to moderate superficial perivascular to periadnexal dermal inflammatory cellular infiltrate. Systemic sensitization did not appear to be dependent upon the proteolytic activity of Der f 1 in these dogs, in that similar results were obtained for several of the measured parameters. However, several important, albeit subtle, differences between the two groups could be ascertained. First, dogs receiving proteolytically-active Der f 1 developed a broader humoral response to the allergen, in that
both mite-specific IgG and IgE were seen, as were higher serum levels of mite-specific IgE. These dogs also developed decreases in total visible mast cells suggestive of cutaneous mast cell degranulation (as well as higher percentages of visibly degranulated mast cells) and a more intense dermal inflammatory infiltrate. We suggest, therefore, that while cutaneous sensitization with \textit{Der f 1} may not be critically dependent upon allergen proteolytic activity, the presence of that activity does serve to enhance sensitization.

Repeated cutaneous applications of either proteolytically active or inactive \textit{Der f 1} were associated with the development of localized and generalized pruritic dermatitis. This dermatitis could be noted not only at the application sites themselves but was also evident in areas typically affected in spontaneous canine AD, including the flexural skin of the hocks and elbows, the concave surface of the pinnae and the periocular skin. The severity and distribution of these signs was highly variable between individuals and had no apparent correlation with treatment group. This lack of correlation is somewhat surprising, given the differences that were observed between the groups in some of the other parameters measured (such as serum mite-specific antibody levels). Nonetheless, it must be remembered that a variety of interconnected chemical and neurologic pathways are involved in the generation and perpetuation of cutaneous inflammatory responses, and that individual sensitivity to cutaneous inflammatory stimuli can be highly variable. While many known triggers of pruritus might be expected to be preferentially triggered by proteolytic allergens (for example, PAR-mediated release of neurogenic mediators such as substance P), this group of triggers certainly is not all-inclusive.
Epicutaneous application of proteolytically-active *Der f 1* allergen was associated with slowly progressive elevations in serum levels of mite-specific IgG and IgE. Dogs receiving proteolytically-inactive allergen produced somewhat lower levels of mite-specific IgE throughout the study. By the end of the study, serum mite-specific IgE was seen to be slowly increasing in these dogs as well. However, increases in serum mite-specific IgG were still not apparent at this point.

Skin biopsies obtained from proteolytically-active allergen patch test sites demonstrated fewer toluidine-blue dermal mast cells (suggestive of complete degranulation) and increased percentages of visibly degranulating mast cells as compared to matched diluent treated skin samples. These findings were noted not only at the last patch test (when IgE-mediated cell degranulation might be expected to have come into play) but also at the first patch test. In contrast, similar decreases in mast cell number were not seen at either patch test in dogs receiving proteolytically-inactive allergen, and visible evidence of degranulation was seen only at the final patch test.

Although cross-linking of IgE is perhaps the most well known method of mast cell degranulation, other pathways do exist. For example, mast cells express protease-activated receptors and can be degranulated by exposure to both cysteine and serine proteases[36, 37]. Degranulation has also been reported secondary to exposure to microbial products[35] and complement fragments[38]. During degranulation, mast cells release a variety of inflammatory mediators, including tumor necrosis factor alpha (TNF-alpha)[35, 39], interleukin-5 (IL-5)[40], IL-13[40] and IL-4.[38, 41]. These mediators not only increase
recruitment of other cells of the innate immune system (notably eosinophils) but also create an environment highly conducive to the development of Th2 polarization in naïve T lymphocytes. It is tempting to speculate that repeated protease-mediated mast cell degranulation may have contributed to the enhanced sensitization seen in dogs receiving proteolytically-active allergen.

Dogs receiving proteolytically-active allergen developed an influx of inflammatory cells into the superficial dermis. This inflammatory infiltrate was comprised primarily of CD1c⁺ and CD3⁺ mononuclear cells and was concentrated around blood vessels and occasionally around sebaceous ducts and the isthmus regions of hair follicules as well. Neutrophils did not appear to be appreciably increased relative to their numbers in the corresponding diluent treated samples. Eosinophils were rarely seen in any sample.

It must be acknowledged that many of the responses to cutaneously-applied Der f 1 were fairly subtle in both groups of dogs. This is particularly true when the results are compared to the inflammatory response to sonicated D. farinae slurry seen in our previous study, and in other studies using an identical slurry[42]. However, the current study differed from our previous one in several important respects.

First and foremost is the difference in total allergen dose. In the current study, each dog received individual aliquots of sixty micrograms of purified protein. This quantity was based upon Der f 1 ELISA analysis of the slurry, and was calculated to approximate the average dose of Der f 1 contained in individual 30 microliter applications of slurry. We do
not consider this to be either an unreasonably high or low weekly exposure dosage, given that “high” levels of environmental Der f 1 have been defined as 10 micrograms or more per gram of household dust[43]. Although Der f 1 has been estimated to comprise approximately 10% of commercial D. farinae extracts[2], the crude slurry used in our previous study was a very concentrated preparation. As a result, 60 micrograms represents less than one percent of the total protein concentration of the slurry. While not all of this protein could be expected to be allergenic (or biologically available), this still represents a many-fold decrease in weekly challenge dose. As such, the inflammatory response generated in the current study would not and should not be expected to approximate that generated against D. farinae slurry.

Other factors which must be considered when comparing the study results are the route of patch test challenge (prior adsorption onto filter paper versus direct application of slurry), the relative age of the subjects (middle-aged adult dogs versus puppies) and the duration of the studies (60 days versus 120 days).

In summary, the current study demonstrated that repeated epicutaneous applications of a purified preparation of proteolytically-active or –inactive Der f 1 allergen to intact canine skin will induce the development of a generalized pruritic dermatitis similar to that seen in spontaneous canine AD. This dermatitis is accompanied by increases in serum allergen-specific IgE, but increases in allergen-specific IgG are seen only in dogs receiving proteolytically-active allergen. Both groups of dogs develop a mild perivascular inflammatory cell infiltrate at application sites, consisting predominantly of CD1c+ dendritic cells and CD3+ lymphocytes. This infiltrate is accompanied by dermal mast cell
degranulation after even the first application of proteolytically-active allergen. Dogs receiving proteolytically-inactive allergen develop less-dense cellular infiltrates, and notable mast cell degranulation is not seen until later in the sensitization process. These results suggest that epicutaneous exposure to either proteolytically-active or inactive Der f 1 allergen may be sufficient to induce sensitization in adult dogs. However, differences in the magnitude and quality of the serologic and dermal inflammatory response suggest that the presence of proteolytic activity may serve to enhance the development of sensitization while not being strictly required for it. This enhancement may occur by antigen-nonspecific (but protease-dependent) mechanisms including mast cell degranulation, with subsequent cytokine/chemokine release and enhanced inflammatory cell recruitment.
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Figure 1: Purified *Der f 1* allergen becomes proteolytically active upon the addition of cysteine, and this activity is suppressed by E64. (A) Purified natural *Der f 1* allergen (Indoor Biotechnologies, Charlottesville, VA, USA) was prepared in a sodium phosphate buffer (0.05M; pH 6.6, 37°C) in the presence or absence of cysteine hydrochloride (6mM), then added to the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC (Sigma Aldrich, St. Louis, MO, USA). Proteolytic activity was detected by fluorescence of the digested substrate. (B) The proteolytic activity of purified *Der f 1* is suppressed by the addition of the cysteine protease-specific inhibitor E64 (Sigma Aldrich, St. Louis, MO, USA). Results are expressed as micromoles of fluorescent AMC liberated from the digested peptide substrate. The fluorescence profile of undigested Boc peptide is shown for reference.
Figure 2: Epicutaneous application of proteolytically-active Der f 1 induces mite-specific IgG and enhances mite-specific IgE production without increasing total IgE. (A) Serum samples taken before, during and after epicutaneous sensitization from dogs receiving proteolytically-active purified Der f 1 allergen demonstrate greater increases in mite-specific IgE as compared to dogs receiving proteolytically-inactive allergen. (B) Dogs receiving proteolytically-active allergen also developed increases in serum mite-specific IgG, where no such increase was observed in dogs receiving proteolytically-inactive allergen. (C) Changes in mite-specific antibody levels were not associated with appreciable changes in total serum IgE levels in any dog. Although one dog did have constitutively lower total serum IgE levels than the other three, this was not associated with an impaired serologic response (individual data not shown).
Figure 3: Epicutaneous application of purified Der f 1 allergen induces mast cell degranulation. (A) Compared to diluent-treated sites, skin treated with proteolytically-active Der f 1 contained fewer toluidine blue-positive mast cells (open bars) and a greater percentage of degranulated mast cells (shaded bars). This phenomenon was most evident after the first allergen application (Diluent 1 and Der f 1 1, left) but could also be seen following the third application (Diluent 3 and Der f 1 3, right). In contrast, skin treated with proteolytically-inactive Der f 1 (B) had more toluidine-blue positive mast cells than matched diluent-treated skin.
Figure 4: Epicutaneous application of purified *Der f 1* allergen induces the recruitment of mononuclear cells into the dermis. 

(A) Compared to control diluent-treated skin, skin from the initial patch test application of proteolytically-active *Der f 1* exhibited an increased perivascular and periadnexal accumulation of inflammatory cells, predominantly CD1c⁺ and CD3⁺ mononuclear cells. This pattern remained largely unchanged following the third patch test application (B). Although skin from the initial and final patch test applications of proteolytically-inactive *Der f 1* allergen exhibited a similar inflammatory pattern (C, D), the magnitude of the total and mononuclear cell responses was notably decreased compared to proteolytically-active allergen treated skin.