

ORIGINAL PAPER

Epicutaneous sensitization with *Dermatophagoides farinae* induces generalized allergic dermatitis and elevated mite-specific immunoglobulin E levels in a canine model of atopic dermatitis

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Clinical and Experimental Allergy

Summary

Background Atopic dermatitis (AD) is a cutaneous hypersensitivity associated with elevated levels of antigen-specific IgE, commonly to house dust mites (HDMs). It remains controversial as to whether sensitization and clinical disease are induced by cutaneous exposure to HDM.

Objectives The objectives of this study were to determine whether repeated applications of *Dermatophagoides farinae* slurry to intact skin of Maltese–Beagle atopic (MAB) dogs would result in the development of clinical signs or lesions resembling spontaneous canine AD, to determine whether repeated slurry applications would induce elevations in mite-specific IgE and/or IgG, and to determine whether mite antigens could be demonstrated within the dermis of application sites.

Methods Dogs received weekly 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 applications. Skin biopsies and serum samples were obtained on days 1, 60 and 120.

Results Pruritic dermatitis was seen in all dogs by day 60. *D. farinae*-specific IgE was elevated by day 60. Histologic examination of early application sites revealed mild, mononuclear perivascular dermatitis. Later application sites were characterized by a dense inflammatory infiltrate and oedema in both the dermis and the epidermis. Immunofluorescent staining confirmed the presence of *D. farinae* antigens in the dermis.

Conclusions This study demonstrated that epicutaneous application of HDM slurry to MAB dogs results in elevations of HDM-specific IgE, localized and generalized pruritic dermatitis resembling spontaneous canine AD, and histologic changes typical of IgE-driven inflammation. We feel that these results suggest that epicutaneous exposure to allergen may play an important role during both the sensitization and the perpetuation of AD, and provide support for the use of a canine model in the investigation of the pathogenesis of AD.

Keywords allergy, animal models, atopic dermatitis, atopy, *Dermatophagoides farinae*, dogs, skin (dermatology) immunology

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Introduction

Atopic dermatitis (AD) is a common inflammatory skin disorder of both humans [1] and several domestic animals, including dogs [2, 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], especially those associated with house dust mites (HDMs), including *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* [5].

Although the inflammatory response in established AD has been well studied, the major route of allergen exposure resulting in the development and perpetuation of AD remains controversial. Inhalation of aeroallergens has 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 [6]. 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 [7].

An alternative route of sensitization could be by transcutaneous/transepidermal absorption of allergen. A potential obstacle to this route is the stratum corneum, which is impenetrable to many substances [8]. Substances known to be capable of crossing the stratum corneum are typically small and/or lipophilic [9]. In contrast, HDM aeroallergens range from 14 kD to > 100 kD [10, 11].

One pathway by which allergens might bypass the stratum corneum and gain access to the dermis is by travelling down the hair follicles. Depending on the body location, follicular ostia represent only about 0.1–10% of the surface area of the skin [12–14]. However, the total surface area represented by the invaginated walls of the follicles is likely far greater. Particles as large as 1 µm (or proteins as large as 47 kD) have been visualized by confocal and conventional fluorescent microscopy at the level of the sebaceous ducts or within the dermis following epicutaneous application of microspheres or microparticles [12, 15, 16]. This process is enhanced if these microparticles are applied to areas of skin undergoing flexion [15]. Additional particles can be seen to cross directly through the stratum corneum in these areas of flexed skin as well [15].

It is also possible that passage into the dermis may be facilitated by some property or properties of the allergens themselves. 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 [17, 18]. Both serine and cysteine proteases from *D. pteronyssinus* have been demonstrated to degrade tight junctions in cultures of respiratory epithelium, resulting in increased permeability to mannose or albumin [19–22]. These proteases may facilitate not only the passage of HDM allergens into the skin but may also create sufficient disruption of the stratum corneum to allow entry of non-proteolytic allergens as well.

Support for a role of epicutaneous sensitization in the development of AD comes from a study performed by McCall *et al.* In this study, neonatal high IgE-responder beagle dogs were treated with repeated topical applications of a saline-based slurry containing minimally processed, sonicated *D. farinae* mites. These dogs later developed dermatitis, bronchial hyperreactivity, increased serum *D. farinae*-specific IgE titres and increased *D. farinae*-stimulated T cell proliferation as compared with untreated dogs [23]. Later patch tests on these dogs

using the same slurry produced gross and microscopic inflammatory reactions typical of AD [23–25].

The specific aims of our study were fourfold. First, we wanted to determine whether repeated epicutaneous applications of sonicated *D. farinae* slurry to intact skin of 5-month-old Maltese-Beagle dogs (a line of dogs prone to food allergy and AD) results in the development of clinical, gross or microscopic abnormalities similar to spontaneous canine AD. Second, we wanted to determine whether repeated applications of this slurry would result in the development of elevated levels of serum mite-specific IgE and/or IgG. Third, we wanted to determine whether mite antigens could be detected in skin biopsies following epicutaneous applications of this slurry. Finally, we wanted to determine whether this slurry was proteolytically active.

Materials and methods

Subjects

Our laboratory has developed a breeding colony of Maltese-Beagle atopic (MAB) dogs. These dogs are distinct from dogs bred for high IgE responses in that they spontaneously (e.g. without requiring parenteral administration or an adjuvant) develop hypersensitivities following controlled exposure to food items containing corn, soy [26], peanuts and chicken. Oral challenge with these items induces the rapid development of pruritus, otitis and gastrointestinal signs. These dogs also display a predisposition for the spontaneous development of hypersensitivities to environmental allergens, as dogs taken out of the colony and adopted into homes as pets have been reported to develop pruritus and/or rhinitis after environmental exposure to household allergens. These signs are similar to those seen in dogs with spontaneous AD, but it must be noted that the concurrent predisposition of these dogs to food hypersensitivity is not typically seen in dogs with naturally occurring AD (recent studies have demonstrated comorbid adverse food reactions in fewer than 10% of canine AD patients) [27].

Dogs in this colony are maintained with restricted dietary management (Hill's Prescription Diet Salmon and Rice) and in a controlled housing environment. These measures are extremely effective in preventing the development of spontaneous allergy to both environmental and dietary allergens (full control has been achieved for 112 dogs from 25 litters).

Four MAB dogs (three males, one female) were selected for this study. These dogs were littermates and were 5 months 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 HDM allergens Der f 1 and Der p 1.

Epicutaneous sensitization of Maltese-Beagle atopic dogs with a sonicated Dermatophagoides farinae slurry

Sensitization protocol. The dogs were divided into two groups of two dogs 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 substance was a minimally processed, sonicated slurry of *D. farinae* mites in phosphate-buffered saline (PBS) (Heska, Loveland, CO, USA), as has been described previously by McCall et al. [23]. This slurry was applied to the skin 'as-is' – no detergents or lipophilic carriers were added. In all cases, the slurry was applied to intact, unmanipulated skin – i.e. the skin was not tape stripped, pricked, scratched or otherwise disrupted, although overlying fur was clipped to approximately 2 mm when necessary to facilitate application.

Occluded group. These dogs 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 clipping an area of fur on the lateral thorax to approximately 2 mm in length (to minimize disruption of the epidermis), and then lightly painting 30 µL of the slurry onto an approximately 1 cm² area of skin. The area was covered with a minimally occlusive dressing (Op-Site, Smith and Nephew, Largo, FL, USA). The dogs were then placed in restrictive collars to prevent ingestion of the extract 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 normal-appearing skin) at 6 and 24 h after application, using an 8 mm biopsy punch. Biopsies were halved immediately after acquisition, with one half placed in 10% formalin and the other half frozen in OCT tissue cutting medium.

Non-occluded group. These dogs received 16 weekly applications of slurry, alternating between the axillary and groin skin (right side only). Slurry (30 µL) was lightly painted onto an approximately 1 cm² area of skin in these areas. After application, the dogs were placed in restrictive collars to prevent ingestion of the extract, and then placed into individual runs. The next day, the application sites were examined and gently wiped clean. These areas were not biopsied, with the exception of study day 70 (see 'Immunofluorescent detection of Der f 1 in application site biopsies').

Non-occluded group dogs were mock-patch tested on study days 1 and 60. This procedure was identical to that used on the occluded group dogs, except that no slurry was applied at these times. However, skin covered by the Op-site dressing was biopsied at 6 and 24 h as in the occluded group dogs. On study day 120, the dogs were patch tested (including slurry application) and biopsied as described above.

Clinical monitoring. All dogs were given a physical examination and a complete dermatologic examination by a board-certified veterinary dermatologist (C. P. H.) twice weekly. At this time, any lesions observed were photographed and described by location, character and severity. In addition, the dogs were observed for a 1-h period two to three times weekly. Any behaviour suggestive of pruritus was noted and graded on a scale of 0–5 (0 = no pruritus; 5 = consistent and urgent pruritus, difficult or impossible to distract).

Antibody titres. Serum samples were obtained on study days 1, 60 and 120. Control samples were also taken at these times from two normal dogs and a housedust mite-sensitive dog with spontaneous AD. Sera were divided into 100 µL aliquots, and frozen at –20 °C until analysis at the end of the study. All samples were analysed by ELISA to determine the total IgE levels, as well as IgE and IgG specific for crude *D. farinae* extract, purified Der f 1 and Der f 2. Briefly, round-bottomed 96-well ELISA plates were coated in mouse-origin monoclonal anti-canine IgE (developed in our laboratory), *D. farinae* extract (Greer Laboratories, Lenoir, NC, USA), purified Der f 1 or Der f 2 (Indoor Biotechnologies), diluted in carbonate/bicarbonate buffer. Non-specific binding was inhibited by blocking with PBS with 0.05% Tween 20. Dilutions (1 : 5 for IgE; 1 : 100 for IgG) of patient and control sera were added and incubated at room temperature for 2 h. Bound IgE was detected with biotinylated mouse-origin monoclonal anti-canine IgE, followed by horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA, USA). Bound IgG was detected using a peroxidase-labelled, goat-origin polyclonal anti-canine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reactions were developed using 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonate) and hydrogen peroxide 0.02% horseradish peroxidase substrate (Kirkegaard & Perry Laboratories) and read at 405 nm.

Processing and staining of formalin-fixed skin. Formalin-fixed skin samples were dehydrated and embedded in paraffin-containing tissue blocks. Five micrometre sections were stained with haematoxylin and eosin, as well as with Luna's stain and low-pH (1.5) toluidine blue stain (for eosinophil [28] and mast cell enumeration [29, 30], respectively). All slides were evaluated by the same investigator

on a blinded basis. This evaluation included pattern analysis and enumeration of total nucleated cells, neutrophils, eosinophils and mast cells/mm² of the dermis.

Immunohistochemical staining. A three-step labelled streptavidin method modified from Affolter and Moore [31] was used to characterize the mononuclear cell infiltrate. Briefly, 6 µm cryosections were fixed by immersion in acetone. Endogenous peroxidase was quenched with hydrogen peroxide (0.3% in PBS) containing sodium azide (0.01%). Samples were blocked with 1% fetal calf serum (FCS) 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 the cell culture supernatant diluted 1 : 10 in PBS. Bound antibodies were detected with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 400 in PBS, followed by horseradish peroxidase-conjugated streptavidin (Zymed Laboratories), diluted 1 : 400 in PBS. Amino-9-ethyl-carbazole (AEC Substrate Kit, Biogenix, San Ramon, CA, USA) was applied as a chromogen, followed by haematoxylin (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 × 0.14 mm fields of superficial dermis at × 40 magnification on an Olympus BH2 microscope (Olympus America, Melville, NY, USA) (excluding endothelial cells and adnexae). Neutrophils, mast cells and eosinophils, as well as CD1c⁺ and CD3⁺ mononuclear cells/mm² of the dermis were determined by counting 13 consecutive 0.24 mm × 0.32 mm fields of superficial dermis at × 20 magnification using a didymium filter on an Olympus VANOX AHS-3 microscope.

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, 5 µm cryosections were fixed in acetone and blocked with 1% FCS in PBS. Mouse-origin monoclonal anti-Der f 1 antibodies (4C1, Indoor Biotechnologies) were diluted 1 : 2000 in PBS containing 10% normal dog serum. Bound antibodies were detected with fluorescein isothiocyanate-labelled horse-origin anti-mouse γ chain antibodies (Vector Laboratories) diluted 1 : 100 in PBS. Slides were coverslipped using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories).

For double-stained sections, the blocking solution was changed to a mixture of 1% FCS and 10% normal donkey serum. Normal donkey serum (10%) was also added to the secondary antibody solution. Slides were stained with anti-Der f 1 antibodies and horse-origin anti-mouse antibodies as described above. These steps were followed by the addition of biotinylated mouse-origin monoclonal antibodies specific for canine CD1c or CD3 (courtesy

Dr Peter Moore), diluted 1 : 50, which were detected with phycoerythrin-conjugated streptavidin (Kirkegaard & Perry Laboratories) 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 in the absence of reversible or irreversible protease inhibitors [phenylmethylsulphonyl fluoride (PMSF), aprotinin, leupeptin, E64, antipain].

Results

Repeated applications of Dermatophagoides farinae slurry induce the development of a pruritic dermatitis similar to spontaneous canine atopic dermatitis

Pruritus and dermatitis were not detected at the time of the first patch test in occluded group dogs. However, both dogs developed severe pruritus (clinical grades 4–5) within 24 h of slurry application at patch tests days 60 and 120. Although the scratching appeared to be focused on the application sites themselves, rubbing, chewing or scratching was also observed on the muzzle, groin and lateral thigh regions after the day 60 patch, and on the muzzle, periocular skin (associated with serous ocular discharge), paws and pinnae after the day 120 patch.

One dog also developed a gradually worsening, generalized pruritic dermatitis by study day 42 (e.g. before the second application of slurry). Clinically, papules, erythema, chewing and self-inflicted hair loss were most prominent in the antecubital and tarsal skin folds. Papular erythema and alopecia were also noted in the knee fold areas, on the medial thighs, ventral abdomen, muzzle, periocular skin and on the paws. This dermatitis persisted until the end of the study, but did improve noticeably between patch exposures. In contrast, the other dog in this group exhibited minimal or no dermatitis, except in conjunction with patch exposures.

Neither dog in the non-occluded group developed significant pruritus after the mock patch tests on days 1 and 60. At the day 120 patch test, one dog became moderately pruritic 6 h after slurry application, and both dogs were severely pruritic 24 h later. Both dogs were 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 and papules were consistently seen following HDM slurry applications (Fig. 1). After study day 60, slurry application was also associated with the development or exacerbation of papular dermatitis in areas other than the application sites. This effect was particularly pronounced in the contralateral axillary and groin skin.

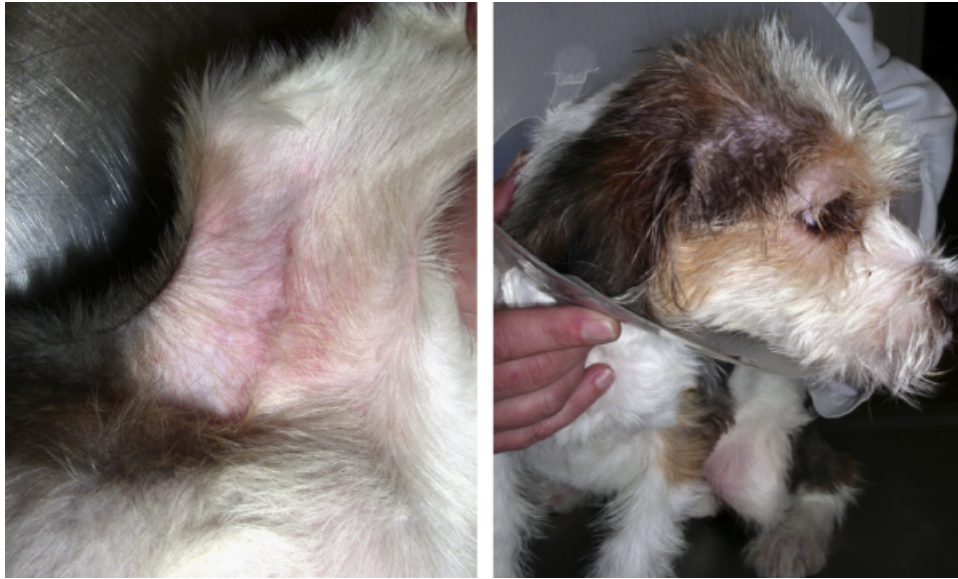


Fig. 1. Epicutaneous application of sonicated *Dermatophagoides farinae* mites induces the development of localized and generalized pruritic dermatitis. A papular, erythematous dermatitis is noted at the slurry application site 24 h after non-occlusive exposure (left). Erythema and self-inflicted alopecia are observed on the muzzle and periocular skin of the same dog (right). Erythema is also visible on the medial stifle region and the dorsal surface of the rear paws.

To evaluate these areas more closely, a single set of biopsies (application site and contralateral control skin) was taken on day 70.

Within 4–6 weeks of the beginning of the study, both dogs also developed a gradually worsening, more generalized pruritic dermatitis with papules. Mild erythema and alopecia were initially noted on the ventral abdomen and paws, and eventually progressed to also involve the face (Fig. 1), pinnae, antecubital, tarsal and retropatellar folds. These areas of dermatitis were exacerbated by slurry applications to groin or axillary skin, but some dermatitis persisted between applications.

Repeated applications of Dermatophagoides farinae slurry induce the development of elevated serum mite-specific immunoglobulin E and G levels

Serum levels of IgE and IgG specific for crude *D. farinae* extract (Greer Laboratories), as well as serum levels of IgE specific for purified Der f 1 and Der f 2 (Indoor Biotechnologies), and total serum IgE levels were measured using ELISA, with each sample analysed in triplicate. Allergen-specific antibody titres were calculated relative to a reference serum derived from a polyallergic dog.

Mite-specific IgE and IgG levels were virtually undetectable in all MAB dogs at the beginning of the study, although total IgE levels were comparable to those from control dogs (Fig. 2). Both groups developed increases in mite-specific IgE (Fig. 2a) and IgG (Fig. 2b) by day 60, and antibody levels remained elevated at day 120. These increases were somewhat higher in occluded group dogs than non-occluded group dogs. By day 120, both mite-

specific IgE and IgG levels in both groups of dogs 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 dogs (Fig. 2c). 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 induction of IgE, as total IgE levels in both groups of dogs decreased with time (Fig. 2d).

Epicutaneous application of Dermatophagoides 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 [32]. For this reason, positively staining cells were considered to be mast cells. Compared with control biopsies, biopsies taken from the first slurry application site exhibited fewer total toluidine blue-positive mast cells per square millimetre of dermis, presumably secondary to complete degranulation (Fig. 3) [33]. 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 with those seen in control skin. These differences between control and slurry application sites became more apparent by the third application (Fig. 3).

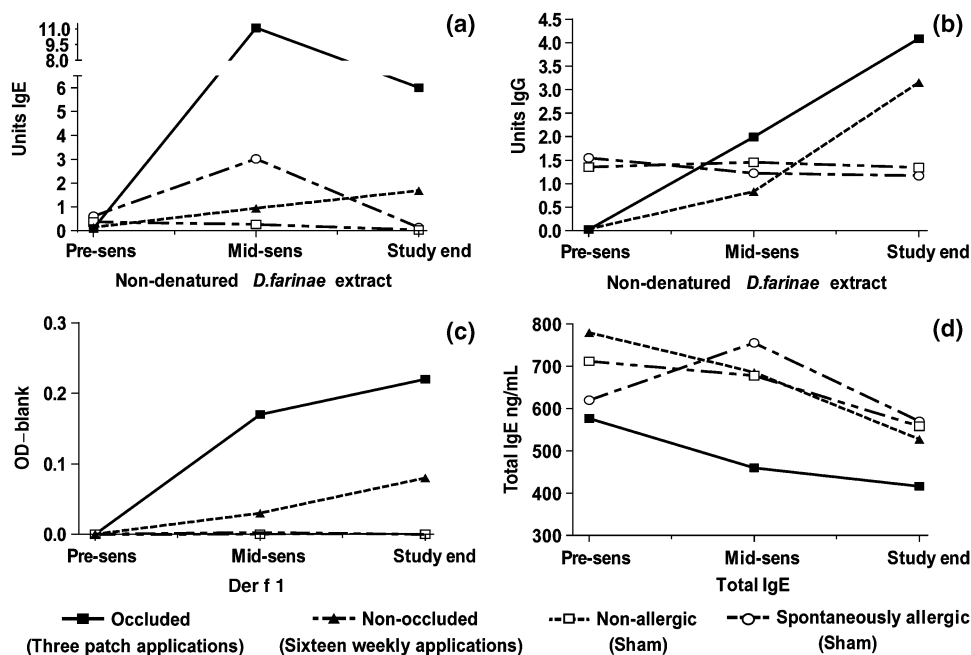


Fig. 2. Epicutaneous application of sonicated *Dermatophagoides farinae* mites induces elevations in serum mite-specific antibodies without concomitant increases in total serum IgE. Serum IgE (a) and IgG (b) directed against a crude *D. farinae* extract, as well as IgE specific for the major mite allergen Der f 1 (c) increase relative to baseline levels in dogs 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.

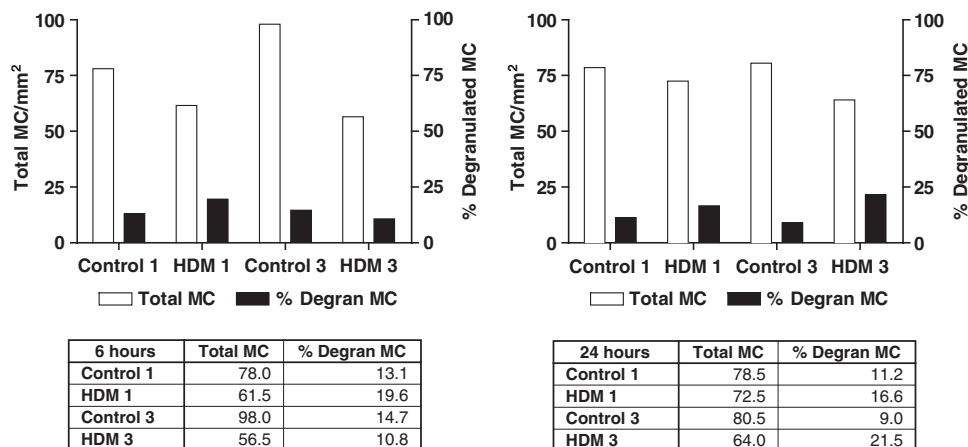


Fig. 3. Epicutaneous application of *Dermatophagoides farinae* slurry induces mast cell degranulation. Compared with control sites, skin treated with *D. farinae* slurry [house dust mite (HDM)] contained fewer toluidine blue-positive mast cells (open bars) and a greater percentage of degranulated mast cells (shaded bars). This phenomenon was most evident after the third slurry application (Control 3 and HDM 3) but could be seen even following the first application (Control 1 and HDM 1). Bars indicate averages from occluded group dogs at 6 h (left) and 24 h (right) after patch test application.

Repeated applications of *Dermatophagoides farinae* slurry induce the progressive development of a mixed inflammatory dermatitis

Histological examination of *D. farinae* application sites in occluded group dogs revealed the development of progressively more severe inflammatory dermatitis (Figs 4 and 5). 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 with the control sections. This infiltrate was primarily located around superficial vessels in the dermis, and was largely composed of CD1c⁺ dermal dendritic cells, CD3⁺ T lymphocytes and neutrophils. Several visibly degranulated mast cells were evident among these cells. Epidermal changes were minimal at this time. However, biopsies of *D. farinae*

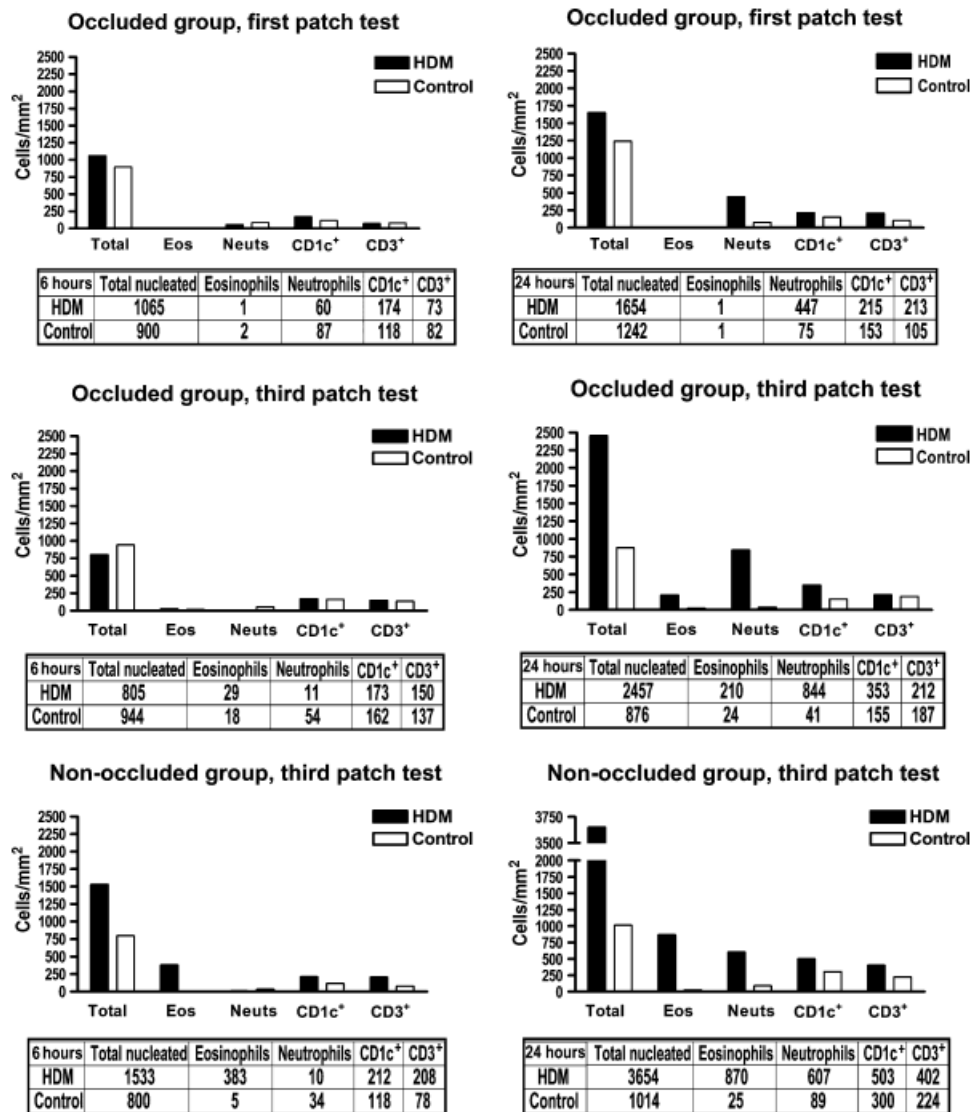


Fig. 4. Epicutaneous applications of sonicated *Dermatophagoides farinae* mites induce the recruitment of an evolving population of inflammatory cells. Comparison of skin biopsy samples taken from occluded group dogs 6 (left) and 24 h (right) after the first and third mite applications demonstrates differences in both the magnitude and the character of the cellular influx. Biopsies taken from non-occluded group dogs 6 (left) and 24 h (right) after the final slurry application demonstrate inflammation of a similar character but more severe in magnitude. Biopsies were obtained from mite application sites (house dust mites) and adjacent untreated skin (control), both under occlusion.

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 oedema and pronounced degranulation of dermal mast cells. The cellular infiltrate contained large numbers of dermal dendritic cells, T lymphocytes and neutrophils, as well as increased numbers of eosinophils (Figs 4 and 5). This infiltrate was concentrated in the superficial dermis, but occasionally extended to the mid- and deep dermis as well. The epidermis was frequently spongiotic and infiltrated by inflammatory cells, most commonly eosinophils. In some areas, the eosinophilic infiltrate effaced the epider-

mis and superficial dermis or formed epidermal microabscesses.

Because non-occluded group dogs 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 heavy, diffuse infiltration of inflammatory cells, similar to that seen in the occluded group dogs. This infiltrate was concentrated in the superficial dermis, but also involved the epidermis and deeper dermis. Marked dermal and epidermal oedema was noted in these samples, and degranulated mast cells were seen frequently. The cellular infiltrate in both the

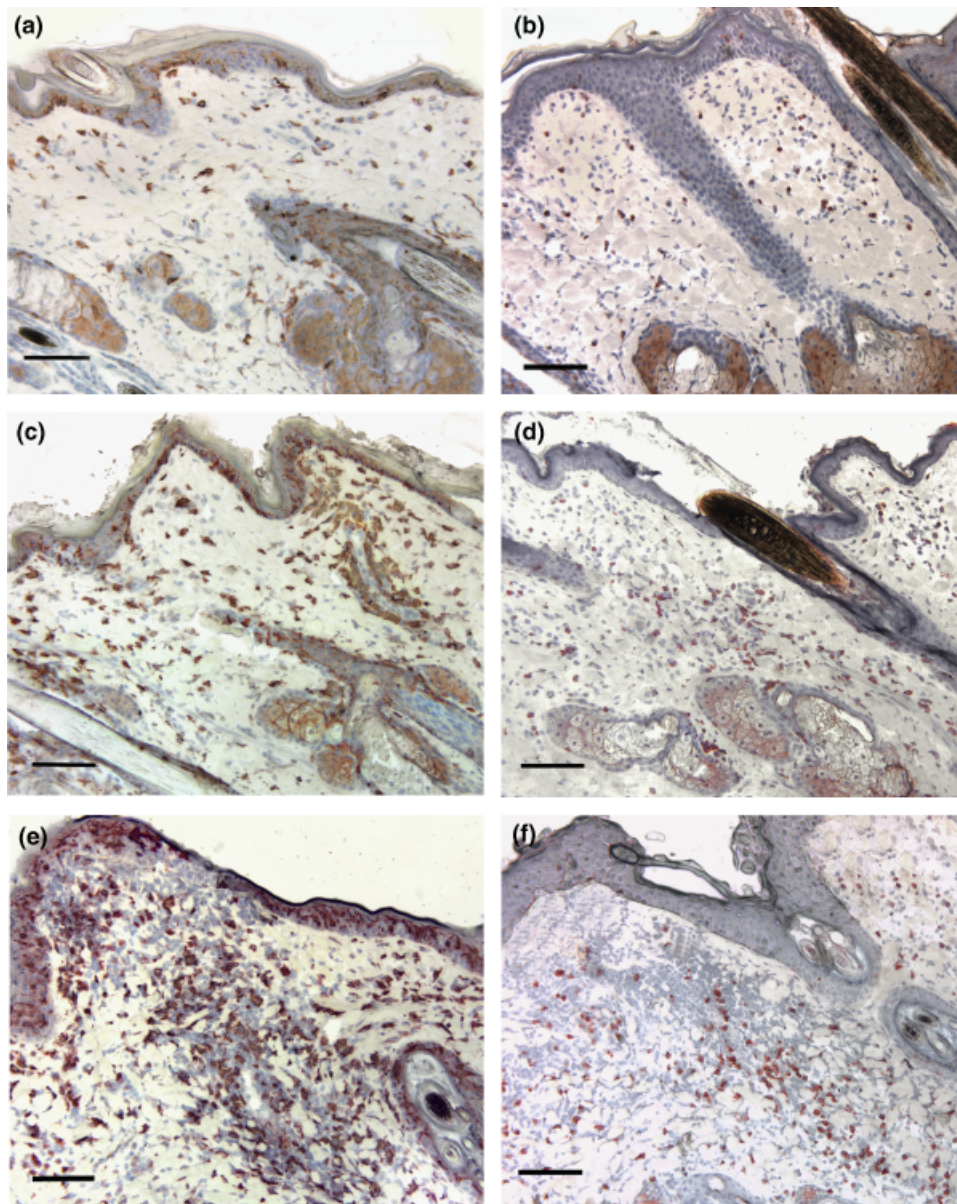


Fig. 5. Biopsies of *Dermatophagoides farinae* mite slurry application sites reveal an inflammatory cell recruitment of progressive severity. Biopsies taken from occluded group dogs after the first mite slurry application (a, b) demonstrate low numbers of CD1c⁺ dendritic cells (a) and CD3⁺ lymphocytes (b) scattered throughout the superficial dermis. In contrast, biopsies taken from occluded group dogs after the third slurry application (c, d) display a more dense, largely perivascular dermal infiltration of CD1c⁺ (c) and CD3⁺ (d) cells. Biopsies taken from non-occluded dogs (e, f) demonstrate a similar but more intense infiltrate. Amino-9-ethyl-carbazole chromogen with haematoxylin counterstain, $\times 40$ magnification; scale bar = 100 μ m.

dermis and the epidermis was a mixture of mononuclear cells and granulocytes (Figs 4 and 5), and was more heavily eosinophilic than that seen in occluded group dogs (Figs 4 and 6).

Microscopic evaluation was also performed on biopsies obtained from both the right and the 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, CD1c⁺ dendritic cells and CD3⁺ lymphocytes. Samples obtained from contralateral skin also displayed perivascular to diffuse cellular infiltration of the superficial dermis. However, this infiltrate was of a lesser magnitude and contained fewer eosinophils than that seen at the application sites themselves.

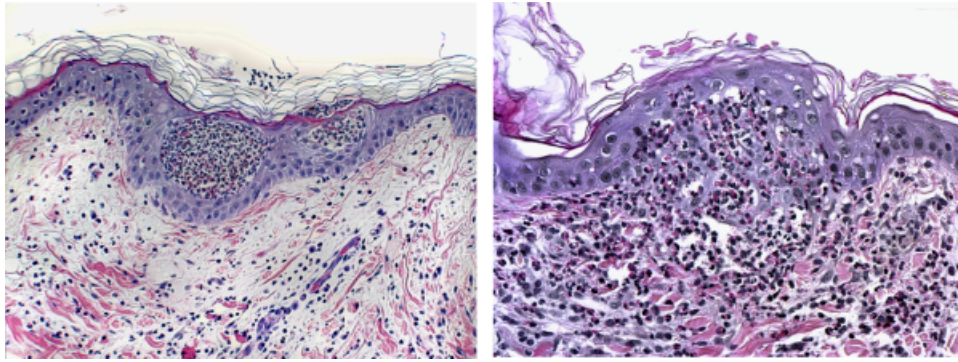


Fig. 6. Epicutaneous applications of sonicated *Dermatophagoides farinae* mites induce the development of epidermal and dermal oedema and inflammatory cell recruitment. Biopsies taken from non-occluded group dogs on day 120 after 24 h exposure to *D. farinae* demonstrate marked dermal oedema and epidermal spongiosis, with a perivascular to diffuse infiltrate of eosinophils into the dermis and epidermis. Haematoxylin–eosin, $\times 4$ (left) from dog 1; $20\times$ (right) from dog 2.

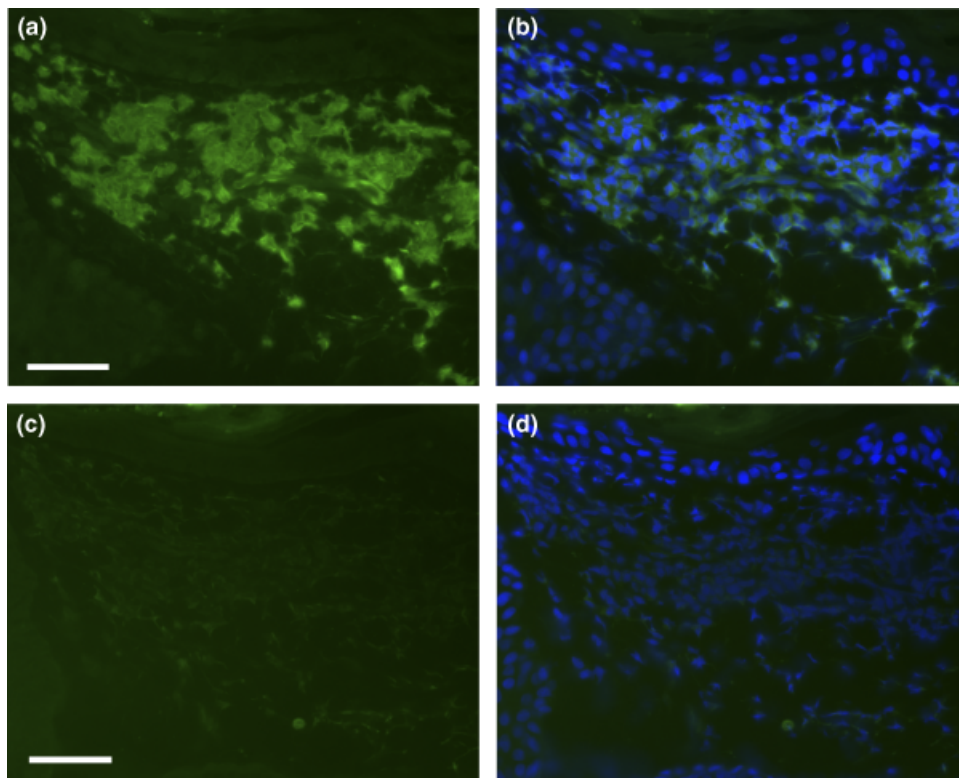


Fig. 7. The mite allergen Der f 1 can be detected in the dermis following epicutaneous application of sonicated *Dermatophagoides farinae* slurry. Biopsies taken from an occluded group dog after a 6-h exposure to *D. farinae* demonstrate the presence of Der f 1 allergen in cells infiltrating the superficial dermis. (a and b) Immunofluorescent detection of Der f 1 using a mouse-origin monoclonal antibody, followed by fluorescein isothiocyanate-conjugated horse-origin anti-mouse γ chain. (c and d) Mouse-origin IgG1 isotype control antibody. Sections are shown with and without DAPI counterstain. $\times 40$ magnification; scale bar = $100\ \mu\text{m}$.

The major mite allergen Der f 1 can be demonstrated by immunofluorescent staining at Dermatophagoides farinae slurry application sites

Immunofluorescent staining using mouse-origin monoclonal anti-Der f 1 antibodies (4C1, Indoor Biotechnologies) 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.

Immunofluorescent staining for Der f 1 demonstrated foci of brightly staining cells located within subepidermal cellular infiltrates (Fig. 7) in biopsies from slurry application sites. Brightly stained cells were also found

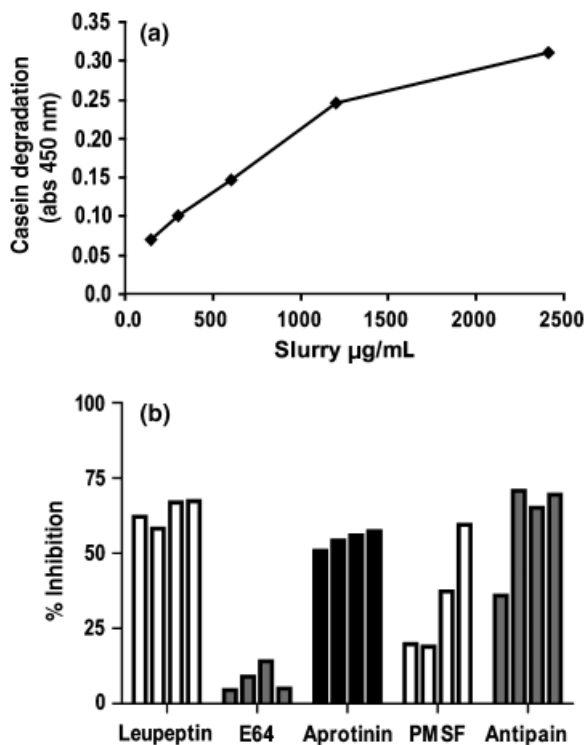


Fig. 8. *Dermatophagoides farinae* slurry is proteolytically active. Serial dilutions of a slurry of sonicated *D. farinae* mites in saline induce dose-dependent cleavage of a casein substrate. (a) Succinylated casein was incubated with increasing concentrations of slurry in the absence of exogenous reducing or activating agents. Incubations were performed for 20 min in a borate buffer (50 mM, pH 8.5, 37 °C), after which casein degradation was analysed using a commercially available protease assay kit (QuantiCleave Protease Assay Kit, Pierce Biotechnology Inc.). (b) Inhibition of slurry protease activity was determined by incubating succinylated casein with a fixed concentration of slurry (300 µg/mL) in the presence or in the absence of increasing concentrations of five protease inhibitors.

frequently immediately adjacent to the upper regions of the hair follicles, and near the sebaceous glands and ducts. These cells were not seen in matched sections stained with a mouse-origin IgG1 isotype control (Zymed Laboratories). Pre-adsorption of the antibodies with a 10-fold molar excess of purified Der f 1 (Indoor Biotechnologies) suppressed all but the most intense foci of staining.

Immunofluorescent staining was also performed on biopsies of axillary skin 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 non-specific 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 staining

positive for Der f 1. Biotinylated mouse-origin anti-canine CD1c or anti-CD3 antibodies were detected using phycoerythrin-labelled streptavidin. Slides double stained for Der f 1 and CD1c demonstrated the presence of many double-positive 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.

Dermatophagoides farinae slurry is proteolytically active

The crude *D. farinae* slurry exhibited moderate proteolytic activity, as determined by cleavage of a succinylated casein substrate (Fig. 8). Treatment of the slurry with protease inhibitors of varying specificity suggested that the slurry contained both serine and cysteine protease activity.

Discussion

This study expands upon previous work by McCall et al. [23] in the characterization of epicutaneous sensitization using a canine model of AD. Specifically, it was determined that repeated epicutaneous exposure to a proteolytically active, sonicated slurry of *D. farinae* mites to the intact skin of MAB dogs results in the development of clinical, microscopic and serologic abnormalities similar to those seen in spontaneous canine AD. Our results suggest that it is not necessary to begin the process in the neonatal period, nor is a successful sensitization dependent on 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 dogs, and is enhanced by (but does not require) application of the slurry under occlusion. Immunofluorescent staining demonstrated the presence of large numbers of Der f 1-containing cells in the superficial dermis of slurry application sites but not of contralateral control skin. This pattern of distribution suggests that a transepidermal pathway of allergen penetration may play a role in the development of epicutaneous sensitization. Der f 1-containing cells were also seen distributed around hair follicles near the sebaceous ducts in these biopsies. We hypothesize that these foci of positively staining cells may be the result of additional antigen entry into the dermis via the poorly cornified follicular epithelium. If this is indeed the case, then it is possible that transfollicular penetration of allergen may also play a role during sensitization.

Repeated epicutaneous applications of a minimally processed, sonicated slurry of *D. farinae* mites in saline to the intact skin of MAB dogs induced the development of a pruritic dermatitis similar to that of naturally occurring canine AD. Minimal or no clinical response was seen

in either group of dogs following the initial application of slurry. However, later applications were associated with the progressive development of an erythematous, pruritic papular dermatitis at the application sites themselves and at areas distant from those sites. It is noteworthy that the areas affected by the dermatitis (including skin folds and the face) were those typically affected in spontaneous human and canine AD.

None of the MAB dogs had detectable levels of mite-specific IgE at the beginning of the study, and mite-specific IgG could be detected only at low levels (lower than the non-allergic and allergic controls). By day 60, *D. farinae*-specific antibodies of both isotypes were markedly elevated in both groups of dogs. Serum *D. farinae*-specific IgG levels continued to increase with time in all the dogs. In contrast, in three of the four dogs, mite-specific IgE levels were actually somewhat higher at 60 days than they were at the end of the study. This 60-day 'peak' was observed in samples from the allergic control dog as well. The reason for this variability is unclear, but may be related to the short serum half-life of IgE (as compared with IgG), in conjunction with seasonal fluctuations in exposure to *Dermatophagoides* allergens. We have been unable to detect *D. farinae* allergens in environmental dust samples taken from the MAB housing facility. However, the dogs could have been exposed to very small amounts of mite allergen carried on the body or clothing of personnel in contact with the colony [34, 35]. We consider it unlikely that this degree of exposure would have a biologically relevant impact on these dogs prior to experimental sensitization. In fact, no MAB tested to date has developed spontaneous hypersensitivity to *Dermatophagoides* while housed in this colony. However, sensitized dogs might be more easily affected by exposures to small amounts of environmental antigen, especially if they are simultaneously exposed to experimentally administered allergen.

Serum IgE levels directed against Der f 1 were also determined. In humans, Der f 1 represents the major allergen from *D. farinae* [5, 36], although spontaneous canine reactivity to Der f 1 is uncommonly seen [37]. Der f 1-specific IgE was seen to increase with time in both occluded and in non-occluded group dogs. However, at no point were notable levels of Der f 1-specific IgE seen in either the non-allergic or the allergic control dogs.

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

Gross and microscopic examination of application sites revealed the development of a mixed dermatitis of progressive severity. Erythema and oedema were not observed at any of the initial application sites in any dog. However, by the third patch test, all the dogs 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, perivascular to diffuse cellular infiltrate and oedema involving both the dermis and the epidermis. These changes are similar to those described in mite-patch test reactions in humans with AD, dogs with natural AD and dogs experimentally sensitized to HDM [24, 25].

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 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.

Finally, the *D. farinae* slurry used in our study was found to have moderate proteolytic activity (as determined by dose-dependent digestion of a succinylated casein substrate). This activity was inhibited to varying degrees by specific inhibitors of serine proteases (PMSF) and cysteine proteases (E64), as well as by protease inhibitors of mixed specificity (leupeptin, aprotinin and antipain). This pattern of inhibition implies that the slurry contains proteases from at least the serine and cysteine protease groups, and that these proteases are active 'as is'; i.e., without the need for additional activating or reducing agents. Several *in vitro* and *in vivo* studies have identified a number of protease-dependent mechanisms by which *Dermatophagoides* allergens may act to facilitate the development and perpetuation of allergic inflammation. These mechanisms include IgE-independent degranulation of mast cells and basophils [38], cleavage of protease-activated receptors [39], cleavage of cell-associated CD23 [40] and CD25 [41] and increased epithelial permeability [19, 21, 22]. Because of the small number of subjects in this study and the mixed composition of the test substance, it was not possible to determine the role of allergen protease activity in the development of sensitization in

these dogs. However, the results of this study suggest that a relationship between the two is possible.

In summary, our study demonstrated that repeated epicutaneous applications of a sonicated *D. farinae* slurry to the intact, unmanipulated skin of high-allergy risk MAB dogs resulted in the development of a pruritic dermatitis similar to 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 on, but were greatly enhanced by, occlusion of the application sites. Histologic analysis of slurry application sites revealed the progressive infiltration of the superficial dermis and epidermis with a mixed population of inflammatory cells, which was associated with marked dermal and epidermal oedema 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 the 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.

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