INVESTIGATIONS ON THE IMMUNOPATHOGENESIS OF ATOPIC DERMATITIS IN CATS

PETRA ROOSJE

“The simple things in life…”

INVESTIGATIONS ON THE IMMUNOPATHOGENESIS OF ATOPIC DERMATITIS IN CATS

Onderzoek naar de immunopathogenese van atopische dermatitis bij de kat.

(met een samenvatting in het Nederlands)

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AD	Atopic dermatitis
APC	Antigen presenting cell
APT	Atopy patch test
BG	Birbeck granules
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
C-MC	Mast cell that contains chymase
DDC	Dermal dendritic cell
Dfar	Dermatophagoides farinae
Dpt	Dermatophagoides pteronyssinus
DTM	Dermatophyte test medium
ECP	Eosinophilic cationic protein
ELISA	Enzyme-linked immunosorbent assay
EP	Eosinophilic plaque
FcεR1	High affinity receptor for IgE
FIV	Feline immunodeficiency virus
GM-CSF	Granulocyte macrophage colony stimulating factor
GP	Grass pollen
HDA	House dust allergen
HDM	House dust-mite
H&E	Haematoxylin and eosin staining
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecules
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Langerhans cell
LS	Lesional skin
mAb	Monoclonal antibody
MBP	Major basic protein
MC	Mast cell
MD	Miliary dermatitis
MHC	Major histocompatibility complex
NLS	Non-lesional skin
NU	Noon unit
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCA	Passive cutaneous anaphylaxis
PFD	Pruritic facial dermatitis
P-K	Prausnitz-Küstner
RCA	Reverse cutaneous anaphylaxis
rHuFcεR1a	Recombinant human high affinity IgE receptor alpha chain
SD	Standard deviation
SP	Substance P
TC-MC	Mast cell that contains tryptase and chymase
TCR	T cell receptor
Th	T helper cell
T-MC	Mast cell that contains tryptase
TNF	Tumor necrosis factor
Tyro	Tyrophagus putrescentiae

CONTENTS

Chapter 1

Introduction and aims

Introduction and aims

Atopy in humans is defined as the tendency to develop IgE antibodies to commonly encountered environmental allergens and the subsequent development of disease.[1]

Atopic dermatitis in humans is considered to be a disease with a multifactorial aetiology involving genetic factors, environmental factors and psycho-social influences.[2]

Atopic dermatitis in cats

Atopic dermatitis (AD) in cats was first described in 1982 by Reedy.[3] Until now the diagnosis of feline atopic dermatitis has been dependent primarily on the clinical signs, compatible dermatopathology, and exclusion of other diseases such as parasitic diseases, dermatophytosis, flea bite hypersensitivity and food hypersensitivity reactions.

Apart from allergic dermatitis, cats also develop allergic bronchitis reactions. This condition has been subject of comparative studies in support of the elucidation of respiratory allergy in humans.[4,5]

No breed or sex predilection has been demonstrated in cats with AD. There are however, reports on a familial involvement in feline atopy suggesting a genetic component.[6–8]

The influence of environmental factors on the development of allergic disease in cats is unknown. It is a fact that the life style of cats has changed as many cats live more or strictly indoors, are frequently vaccinated, are regularly treated for endo- and ectoparasites, and are fed commercial pet foods.

The most consistent clinical feature of feline AD is pruritus.[9,10] A distinct common clinical atopic dermatitis phenotype has not been described. The most commonly reported cutaneous reaction patterns are miliary dermatitis, self-induced alopecia, eosinophilic granuloma complex lesions, and an initially non-lesional pruritus of the face, neck and pinnae.[6] Combinations of these patterns are seen. The cutaneous reaction pattern of miliary dermatitis consists of papules and small crusts which can be present virtually all over the body with emphasis on the head, neck, and trunk. Lesions belonging to the eosinophilic granuloma complex (i. e. eosinophilic granulomas and eosinophilic ulcers) however, can have different aetiologies as well. These lesions are usually non pruritic and can be responsive to bacterial therapy.[6] In addition, Power et al.[11] demonstrated a genetic influence in a closed, inbred colony of cats. These cats had eosinophilic granulomas and eosinophilic ulcers and typically outgrew their lesions at 2–3 years of age.

Eosinophilic plaques can vary in size and may have an eroded surface or secondary crusts. These plaques are often observed on the neck, medial thigh, and the abdominal skin.

The histopathological reaction pattern of miliary dermatitis, eosinophilic plaques and pruritic facial and neck dermatoses share the dermal infiltration with mast cells, eosinophils, lymphocytes and macrophages. This archetypal reaction pattern can not distinguish between flea bite hypersensitivity, food hypersensitivity and feline atopic dermatitis.[12–14] Therefore an atopic background can not be excluded in those cases of miliary dermatitis, eosinophilic plaque, and pruritic facial and neck dermatitis, where other underlying causes (such as parasitoses, flea bite hypersensitivity, dietary hypersensitivity, and dermatophytosis) have been ruled out and a histopathology compatible with an allergic reaction has been found. Further evidence for atopic dermatitis could theoretically be obtained from *in vivo* and *in vitro* allergy

tests. Positive reactions in the intradermal allergy test however, are often difficult to interpret because of softer, non-erythematous wheals and wheal formation that occurs often more quickly and disappears faster compared to the reactions seen in dogs. Moreover, only a limited number of publications exist on the validity of the intradermal allergy test as it is used in cats.[15–17] Results of hyposensitization following intradermal allergy testing in cats have only been described of open studies.[3,9,18] The reported success rates ranged from 60–75 % in these studies. *In vitro* testing (ELISA) for feline atopic disease is considered to be unreliable and is at this moment not a useful diagnostic method.[6,19] Limited results of a recently developed test, a FcεRIa-based ELISA, for detection of allergen-specific IgE, have been published.[20]

Atopic dermatitis in humans

Atopic dermatitis (AD) in humans is a common chronic, eczematous skin disease, characterized by remissions and exacerbations. The diagnosis of AD is based on the patient's history, the family history and the distinct clinical morphology and distribution of skin lesions.[21]

The incidence of atopic diseases has increased in many industrialized countries during the past decades. The reasons for this increase in the prevalence of atopy are not known.[2] Nevertheless, various hypotheses have been assessed including genetic susceptibility, changes in the environment including allergen exposure, and psycho-social factors.[2]

Genetic aspects

A multitude of evidence indicates the importance of genetic factors. Twin studies showed an 85 % concordance for AD among monozygotic twins.[22] When both parents have the same type of atopic disease their child has a risk of approximately 70 % of developing a similar phenotype. If parents have different atopic diseases, the incidence of atopic disease in a child is 30 %.[23] Results of a study by Diepgen et al.[24] supported these findings: in some families AD is the clinical disorder in others it is allergic rhinitis or asthma.

Genetic factors relating to a high IgE reactivity include genetic linkages to chromosomes 11q, 5q, and 14q on which likely candidate genes are present. They comprise genes for the high affinity receptor for IgE, (FcεRIβ)[25–28] for IL-4,[29] and the gene for T-cell receptor alpha,[30] respectively. Blumenthal et al.[31] however, could not confirm a linkage between 5q markers and serum IgE levels in a study in atopic families and Coleman et al.[32] could not verify earlier data of IgE reactivity being linked with chromosome 11q13.[26] A genetic variant of mast cell chymase located on chromosome 14q11.2 was found to be associated with AD but not with allergic asthma or allergic rhinitis, suggesting that variants of mast cell chymase may be one source of genetic risk for AD.[33,34] This association however, could not be confirmed by Kawashima et al.[35]

In addition, reports were published that linked abnormal IL-4-gene expression,[36,37] or mutation of the IL-4-receptor,[38] to development of AD. Noguchi et al.[39] could not confirm this linkage in the Japanese population.

Thestrup-Pedersen et al.[40] suggested that AD is a genetically determined change of ectodermal tissue due to a faulty selection of T cells in the thymus. The observation that several chromosomes may be play a role indicates that more than one gene is involved. The increasing prevalence of atopic dermatitis however, is difficult to explain on the basis of genes alone.[41]

Environmental factors

An investigation into the relative importance of genetic factors and environment on the occurrence of atopic diseases demonstrated that for all atopic diseases a stronger correlation was present between sibling parameters than between siblings and parents, indicating that environmental factors, especially during childhood are important determinants.[42]

In recent years the "hygiene hypothesis" has been developed to explain the increased incidence of allergic diseases. This hypothesis states that Th1-skewing infections are necessary to push the immune system of the neonate away from the Th2-skewed pattern that exists after birth. This hypothesis has been based on a large number of observations. Briefly, children who grew up in a "dirty" environment like a farm have less allergies.[43] In addition, children who were more exposed to infections like the younger children in a family have less allergies than their older siblings. However, there are conflicting reports on the possible causes that have been postulated for allergies:(1) more infections and /or less vaccinations, (2) early colonisation of the intestinal microflora with lactobacilli, and (3) low or no usage of antibiotics.[44] Ongoing intervention studies will have to prove the effect of intervention by any of the above methods in stopping the development of allergic disease.

Aeroallergens

Clinical evidence for the role of aeroallergens in the pathogenesis has been presented by several authors.[45–47] Their role was convincingly demonstrated by Tan et al.[48] in a double-blinded-placebo-controlled study that showed a significant improvement of eczematous lesions after house dust-mite (HDM) avoidance in HDM-sensitized patients.

The clinical reaction pattern and the histological features of AD vary according to the patient's age and disease chronicity. Skin lesions observed in humans with AD include erythema, papules, vesicles, excoriations, oozing, crusts, lichenification and dryness.

Histologically, acute lesions contain intercellular edema with spongiotic vesicle formation and a dermal infiltrate consisting of lymphocytes, macrophages and eosinophils. Chronic lichenified lesions are characterized by epidermal hyperplasia with elongation of rete ridges with minimal spongiosis and a lymphohistiocytic dermal infiltrate with increased numbers of mast cells.[49] Macroscopically uninvolved skin of patients with AD is also histologically abnormal and has a mild epidermal hyperplasia, and a sparse dermal lymphohistiocytic infiltrate.[50]

Brief outline of the immunopathogenesis

Although the exact pathogenesis of atopic dermatitis in humans has not been elucidated, there is increasing evidence for the role of IgE. In addition to the involvement of allergen-specific IgE antibodies in immediate-type hypersensitivity reactions, there are indications for a role of IgE antibodies in antigen uptake and processing.[51] Furthermore it is known that the synthesis of IgE is stimulated by T cell derived cytokines.[52] Production of IgE is enhanced by IL-4[53] and IL-13.[54] IFN-γ and IL-12 inhibit its production.[53,55] In the cell infiltrate of lesional skin of AD patients an increased number of T cells can be found including a high CD4+/CD8+ ratio (4.8:1).[56] In addition, IL-4 producing CD4+, allergen-specific T cells occur in considerable number in lesional skin of human AD patients.[57] Moreover, allergen-specific T-lymphocyte clones from atopic donors produced IL-4 and allergen-specific T-lymphocyte clones from non-atopic control donors produce IFN-γ but little or no IL-4.[58]

More recently, also IL-4 secreting CD8+ T cells were described in allergic patients.[59] Studies on IL-4 production by cutaneous mast cells have given conflicting results.[60,61] Recent studies indicate that Th2 as well as Th1 and/or Th0 type cytokines are important for development of AD lesions with the relative contribution of each cytokine type dependent on the acuteness or duration of the skin lesion.[61–63]

Until recently it was unclear whether the atopic antigens were exposed to immune competent cells through the respiratory route or transcutaneously. Nowadays, there is strong evidence that antigens are also presented directly through the skin. The fact that lesional skin, with a cellular infiltrate comparable to spontaneous lesional AD skin, can be induced via epicutaneous application of the allergen in the atopy patch test (APT) supports this idea.[61] Antigen-IgE-complexes are bound to epidermal Langerhans cells with surface high affinity receptors for IgE (FcεRI) and offered to T cells in the skin or draining lymph node.[64] Therefore, IgE-mediated allergen presentation may lead to enhanced activation of the immune system in response to very low allergen concentrations. This in turn causes expansion of the Th2 cell population and thus induces increased production of IgE.[51]

Against the background of the paucity in the knowledge on atopic dermatitis in cats, the present studies aimed at:

1. Reviewing the immunopathogenesis of atopic dermatitis in humans and to make a comparison with the existing knowledge of atopic dermatitis in cats (Chapter 2);

2. Investigation of the presence of IgE and / or other allergen – specific reaginic antibodies in cats with clinical signs of allergic dermatitis by means of the Prausnitz-Küstner (PK) test and the passive cutaneous anaphylaxis (PCA) test (Chapter 3);

3. Identification of antigen-presenting cells in lesional skin and in skin of healthy control animals by means of immunohistochemistry and electronmicroscopy (Chapter 4);

4. Investigation of the occurrence of T cells in lesional skin, nonlesional skin, and peripheral blood of cats with allergic dermatitis in comparison with healthy cats (Chapter 5);

5. To investigate the presence of IL-4 producing cells in skin of cats with allergic dermatitis. To substantiate the findings we investigated the cross reactivity of the monoclonal antibody against human IL-4 with a recombinant feline IL-4 (Chapter 6);

6. To compare eosinophil and mast cell numbers, and mast cell chymase and tryptase expression in skin of atopic cats and control animals (Chapter 7);

7. To elucidate the induction of skin reactions with the atopy patch test and identify sequential cellular reactions (Chapter 8);

8. A summarizing discussion of the results is presented in Chapter 9.

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Chapter 2

Immunopathogenesis of atopic dermatitis

Immunopathogenesis of atopic dermatitis

Introduction

Atopic dermatitis in humans and cats is a chronic, relapsing, highly pruritic inflammatory skin disease. Cutaneous hyperreactivity is a major clinical symptom. Understanding of the immunologic mechanism of the disease is necessary in order to compare "atopic dermatitis" in cats to the disease in humans. The immunological mechanisms underlying cutaneous inflammation in atopic dermatitis in humans are discussed and literature on feline atopic dermatitis is reviewed.

Aeroallergens

The role of aeroallergens in the pathogenesis of atopic dermatitis (AD) in humans has been a subject of debate and investigations. The fact that more than 80 % of the AD patients have elevated serum IgE with specificity to a variety of the aeroallergens and immediate type skin reactions, indicates a role for aeroallergens in sensitization.[1,2] The significant beneficial effect of house dust mite avoidance on the clinical signs of extrinsic AD in children supports this theory.[3] Apart from this so called extrinsic type of AD, there is the intrinsic AD. Patients with this type of AD have symptoms of atopic dermatitis but no association with sensitization to environmental allergens and without increased serum IgE levels.[4]

The current concept is that aeroallergens may enter the body via inhalation or skin contact. A limited number of allergen inhalation studies have been performed in AD patients. Studies demonstrated that skin lesions could be induced or exacerbated by bronchial challenge with aeroallergens in nearly 50 % of the AD patients.[5] In support of the hypothesis of local allergen transmission, Tanaka et al. found house dust mite allergen-bearing Langerhans cells (LC) after epicutaneous application of the allergens.[6] In addition, patients with AD often have dry skin (xerosis) and a disturbed epidermal barrier function, facilitating allergen penetration.[7,8]

Antigen presenting cells

After entering the body, allergens are internalized and processed by antigen presenting cells (APC) that are part of the skin immunosurveillance system. The first APC the antigen encounters in the skin, is the epidermal Langerhans cell (LC). LC are bone marrow-derived cells,[9] that are present in increased numbers in the epidermis of patients with acute and chronic AD.[10] LC express the CD1a molecule which is considered to be part of a family (CD1) of antigen presenting molecules next to MHC class I and MHC class II molecules.[11] The most reliable morphologic way to identify LC is by detection of their Birbeck granules (BG), rod-shaped organelles with unknown function. This can be done by means of electron microscopy or with immunohistochemistry employing an antibody that specifically stains for the Lag-antigen which is part of the membranes of BG and related structures.[12]

The discovery of the expression of FcεRI by LC[13-15] supported earlier findings of the presence of IgE+ dendritic cells in the epidermis and dermis of AD patients.[16,17] Moreover, Langerhans cell-induced allergen-specific T cell activation was reported to occur more efficiently when allergen was taken up via these cell-bound IgE molecules.[18] LC with surface-bound IgE could only be found in AD patients with high serum IgE levels.[19] Klubal et al.

demonstrated that the FcεRI is the predominant IgE-binding structure in lesional skin of AD patients.[20] Moreover, the FcεRI is dramatically upregulated on LC in lesional AD skin, which seems to be highly specific for this disease.[21,22] Other cells that can express the FcεRI are mast cells and basophils, peripheral blood monocytes,[23] dermal dendritic cells and blood dendritic cells.[24] It was demonstrated *in vivo* that freshly isolated monocytes from atopic individuals, but not of nonatopic individuals, carried surface-bound IgE bound to FcεRI[23] and that the majority of IgE-binding sites were occupied by IgE.[25]

After leaving the epidermis LC undergo developmental and functional maturation and form part of the dermal dendritic cell population (DDC). They change from antigen capturing and processing cells into antigen presenting cells.[26,27] During the migratory phase the dendritic cells adapt to antigen presentation to T cells in the dermis or in the draining lymph nodes. Upregulation of MHC class II, adhesion molecules, and costimulatory markers entails these phenotypic changes.[28] Migration of LCs from the epidermis is influenced by cytokines such as TNF-α.[29] TNF-α is produced by activated keratinocytes.[30,31] Activation of keratinocytes can occur via scratching or secondary bacterial infections that commonly occur in AD patients.[32] In addition, Ioffreda et al.[33] demonstrated that TNF-α, released by mast cells, induced the expression of adhesion molecules (α6 integrins) on human LC. These data not only demonstrate the influence of TNF-α on LC but also provide evidence that cells other than keratinocytes provide a source for TNF-α. Treatment of cultured DC with TNF-α resulted in increased expression of another adhesion molecule, CD44.[34] CD44 expression has been shown to markedly influence the mobility and migratory activity of various cells such as leukocytes, parenchymal cells, and carcinoma cells.[35,36]

In the dermis, a number of DC populations exist which express different markers. One group consists of former LC that have lost their BG after migration out of the epidermis. The CD1a+ DC proved to be a more potent APC compared to CD1a+ macrophage-like cells in a stimulation essay of peripheral blood mononuclear cells (PBMC).[37] Osterhoff et al. demonstrated that FcεRI+ cells in the dermis belonged to the LC (CD1a+) / DC (RFD1+) cell lineage or to mast cells, and not to dermal macrophages (FXIIIa+).[15]

T cells

The first step in the process of T cell activation is binding of the antigen presented by the APC in association with MHC class II molecules to the T cell receptor (TCR / CD3). This binding is enhanced by the coupling of the CD4 molecule with the MHC complex.[38] CD4+ T cells recognize exogenous or environmental antigens. For the second signal, costimulatory signals from the APC are important. These signals are instituted by binding of B7-1 (CD80) and B7-2 (CD86) on the APC to CTLA-4 and CD28 on the resting T cell.[39-41] B7 molecules can be found on several professional APC like B cells, dendritic cells, and macrophages. The expression of B7-2 on B cells of patients with AD was significantly higher than that in normal subjects and patients with psoriasis.[42] Total serum IgE in AD patients and normal subjects correlated significantly with B7-2 expression on B cells and purified B7-2+ B cells produced more IgE than B7-2$^-$ B cells *in vitro.*[42]

There is an imbalance in the generation of Th1 and Th2 cytokines in AD in humans which seems to be related to disease symptoms.[43,44] In lesional skin of AD patients a high CD4 / CD8 T cell ratio was found.[45] Van der Heijden et al.[44] and Van Reijsen et al.[46] demonstrated that a considerable part of this T cell population consists of allergen-specific Th2 cells implying an

important role for these cells in the cutaneous inflammatory reaction of AD. By means of *in situ* hybridisation it was demonstrated that acute AD lesions are associated with an increased expression of IL-4 and IL-13 mRNA as compared to chronic lesional skin. In chronic lesions IL-4 producing cells are still found but an increased number of cells expressing IL-5 and IL-12 is present.[47,48] In addition, uninvolved skin of AD patients contains a significant greater number of cells that are positive for IL-4- and IL-13- but not IFN-γ mRNA.[47,48] At least initially Th2 cells are predominant over Th1 and Th0 cells. The increase of IL-12 in chronic lesional skin underlines the biphasic expression pattern as these cytokines plays a role in Th1 cell development. In addition, this increase is thought to initiate the switch from a Th2 into a Th1 cell pattern.

In contrast to the skin, in the peripheral blood is not always an increased number of CD4+ T cells compared to CD8+ T cells.[49,50] However cytokine production by peripheral blood mononuclear cells (PBMC) indicates a tendency towards a Th2 cytokine predominance. PBMC of AD patients with high serum IgE titers produce, after stimulation, significantly lower amounts of TNF-α and IFN-γ compared to healthy individuals and patients with inhalant allergy.[51] Comparable results were published by Ferry et al. who found an increased percentage of CD3+ / $CD8^-$ peripheral blood T cells expressing IL-4 and a decreased percentage of CD3+ / CD8+ T cells expressing IFN-γ.[52] Nakazawi et al. showed a predominance of type 2 cytokine-producing CD4+T cells and CD8+T cells among the PBMC of AD patients.[53] Moreover, elevated serum IL-4 levels were observed.[54] Furthermore, increased numbers of activated T cells with homing receptors to the skin (up-regulated HLA-DR, CD30+ / CLA+) were found in the PBMC of AD patients.[55] In AD patients sensitized to dust mites, the mite-specific- T cell proliferation response in circulating T cells has been attributed to the CLA+ T cells.[56] Moreover it was demonstrated that CLA+ T cells of AD patients are activated and release an IL-13-dominated Th2 cytokine profile and are capable of inducing IgE in autologous B cells without further activation.[57]

Expression of CLA on mitogen-activated T cells can be upregulated by transforming growth factor (TGF-β), IL-6 and by IL-12 in a superantigen dependant manner.[58,59]

IgE

In atopy the immune response is skewed towards IgE production through the T helper 2 cytokines IL-4 and IL-13.[60,61]

Although increased allergen-specific IgE serum levels are present in approximately 80% of patients with AD, its contribution to the development of lesional skin is still not clear. The concept of involvement of allergen specific IgE is based upon the findings that: 1) APC in lesional skin and to a lesser extent in nonlesional skin of AD patients frequently exhibit surface bound IgE molecules,[16,62] 2) LC-induced allergen specific T cell activation and proliferation occur more effectively when the allergen is taken up via these cell surface bound IgE molecules[18] and 3) eczematous reactions to aero-allergens can be most efficiently induced via skin regions harboring IgE-bearing dendritic cells.[18,63]

Mast cells

The exact role of connective tissue mast cells in AD is unknown. In acute lesional AD skin mast cells numbers are not increased but degranulation of mast cells is observed.[64] Mast

cell numbers, however, are increased in more chronic lesional AD skin.[64] Mast cells and basophils were the first recognized cells expressing the high affinity IgE receptor (FcεRI) which triggers the mast cell to release its contents upon bridging of these receptors by the antigen-IgE complex. Klubal et al. demonstrated IgE binding to the FcεRI on mast cells *in situ*.[20] Mast cells can release prestored substances like histamine, proteoglycans, proteases and they secrete synthesized leukotrienes, chemokines and prostaglandines. The collection of cytokines produced includes IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16,[65] granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor α (TNF-α), and IL-12.[66] In accordance to this, it has been demonstrated that leucocyte infiltration at sites of IgE-dependent cutaneous reactions in mice is entirely mast cell dependent and in part dependent of TNF-α.[67] Mast cells can release rapidly pre-stored TNF-α[68] which can influence the inflammatory response by inducing expression of endothelial adhesion molecules ICAM-1 and VCAM-1 and prolonging the survival of inflammatory cells.[69] In addition, mast cells may function as APC and activate T cells as well since mucosal mast cell - like bone marrow -derived mast cells express MHC class II antigens and costimulatory molecules B7-1 (CD80), B7-2 (CD86), ICAM-1 as well as MHC class II antigens.[66]

Furthermore, it has been speculated that the type of APC can determine the pattern of the induced immune response and subsequently regulate the isotype of the resulting antibodies. As mast cells have been demonstrated to produce a pattern of cytokines related to the Th2 phenotype the production of IL-4 is of particular interest with respect to the polarization of the Th phenotype, since the presence of IL-4 is essential for Th0 to differentiate into Th2 cells.[70] In addition to these findings, it is of interest that mast cells in nasal mucosa of allergic individuals contain IL-4 and IL-13 and secrete these cytokines when activated by the specific allergens. These mast cells are able to induce IgE synthesis by B cells in the presence of the specific allergen whereas mast cells of nonallergic tissues do not induce IgE production.[71]

However, the production of IL-4 by mast cells in the skin is still a matter of debate. Thepen et al.[72] described only T cell staining with an antibody against human IL-4 and could not find concurrent staining of mast cells with this antibody. Horsmanheimo et al.[74] and Bradding et al.[73] however described IL-4 immunoreactivity in skin mast cells.

Mast cells are very heterogeneous cells that are strongly influenced by their microenvironment. Therefore, findings in mast cell lines, mucosal mast cells or non-human mast cells cannot be extrapolated to human skin mast cells.

Mast cells are also thought to play a role in the interaction between the immune system and the nervous system. Degranulated mast cells are seen in close proximity of nerve endings in skin lesions of atopic dermatitis.[75] Neuropeptides like substance P (SP) are secreted by nerve fibers and may induce TNF-α mRNA expression and TNF-α secretion in mast cells.[76]

Eosinophils

Although intact eosinophils are not prominent in lesional human AD skin, eosinophil derived proteins such as major basic protein (MBP) and eosinophilic cationic protein (ECP) can be found in abundance, indicating eosinophil degranulation at the site of inflammation.[77,78] In addition, the number of activated circulating eosinophils and serum ECP are increased during disease exacerbation.[79] Eosinophils influence the inflammatory reaction not only by the release of their toxic granule contents but also by the cytokines and chemokines they produce. Eosinophils can produce many cytokines such as IL-1a, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8,

GM-CSF, TGF-α, TGF-β, TNF-α, and IL-10.[80] Moreover eosinophils produce and secrete bioactive IL-12 upon stimulation with IL-4.[81] This eosinophil derived IL-12 can modulate the switch from a Th2 to a Th1/Th0-like immune response which is observed in chronic lesional AD skin where increased levels of IFN-γ mRNA and protein are found.[48,82] In lesional AD skin, enhanced local production of eotaxin and its receptor CCR3 have been described. This finding supports a role for eosinophils in the initiation and maintenance of inflammation as eotaxin is a potent chemoattractant and activator for eosinophils and Th2 lymphocytes.[83] Eosinophils can also modulate the inflammatory process through antigen presentation to T cells and by supplying costimulatory signals.

Atopy patch test in pathogenetic studies

The development of the atopy patch test (APT) has proven to be a valuable tool in elucidating the disease process. The APT bypasses the problem of conflicting results due to differences in chronicity of the lesions of AD patients. Additionally the APT is very specific as only AD patients respond with positive reactions and not patients with other (allergic) disorders, implicating that the APT involves AD-specific triggering mechanisms. Positive reactions can only be evoked in clinically noninvolved skin of AD patients in the presence of IgE+ epidermal LC.[63] These findings substantiate the role of IgE in the induction of cutaneous AD responses.

From investigations using the APT it has become clear that the disease process is initially modulated by Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13.[72,82] During the APT reaction, the first 24 hours are characterized by increased numbers of T cells and eosinophils with an increased production of IL-4 by local T cells.[72] After 48 hours a switch from a Th2 to a Th1/Th0 pattern occurs. Increased levels of IFN-γ mRNA and protein are observed, preceded by increased IL-12 skin levels.[72,82]

This situation is comparable to what is seen in spontaneous lesional human AD skin.[86]

Immunopathogenesis of feline atopic dermatitis

There is little information on antigen presenting cells in cats. In haematoxylin and eosin (H&E) stained sections increased numbers of cells including mononuclear cells have been reported in lesional skin of allergic cats.[87] This population comprises probably LC, dermal dendritic cells (DDC), and macrophages. Factor XIIIa+ cells were demonstrated in this cell population.[88] Other markers for LC, DDC, and macrophages were not used and this Factor XIIIa+ cell population remains unspecified.

Studies on feline T cells have been primarily focussed on virus-induced diseases such as feline immunodeficiency virus infections (FIV).[89] The occurrence of lymphocytes in the cellular infiltrate of lesional skin of allergic cats has only been described by routine H&E staining. Further identification of T cells involved in feline allergic dermatitis has recently been reported in cats with eosinophilic granuloma complex lesions.[90]

Although several studies provided evidence for the existence of a putative IgE in cats its role in the pathogenesis of feline allergic dermatitis is unknown. Reaginic (IgE) hypersensitivity has been demonstrated with passive cutaneous anaphylaxis (PCA) testing in cats infested with *Otodectes cynotis*.[91] Studies in cats experimentally infected with *Brugia pahangi* have provided evidence for the existence of a putative feline IgE.[92,93] DeBoer et al.[94] showed

cross reactivity between monoclonal anti-canine IgE and a putative feline IgE in serum of cats experimentally infected with *Toxocara canis*. In a publication by Gilbert and Halliwell[95] isolation of feline IgE was described. IgE has not been identified *in situ* in skin of allergic cats. Results of comparing *in vitro* serum IgE tests revealed that these tests were not reliable.[96] There is only limited information on a test using a recombinant truncated version of the human high affinity IgE receptor alpha chain (rHuFcεRIα).[97,98]

Mast cells are predominant cells in the perivascular infiltrate in lesional skin of allergic cats. They are present in increased numbers compared to skin of nonallergic cats.[99] Most mast cells of healthy cats contain both chymase and tryptase.[100] As early as 1965 McCusker demonstrated that skin of cats with allergic dermatitis had a 4-fold increase in mast cell numbers and histamine content.[101] Two years later a study was published describing mast cell degranulation after direct skin testing in cats.[102]

Many studies on the identification of feline IgE have used the passive cutaneous anaphylaxis test (PCA), reverse cutaneous anaphylaxis tests (RCA), or the Prausnitz-Küstner test which all are based on the principle of mast cell degranulation upon binding of the antigen to antigen specific IgE.[92,93,95] Other studies on feline mast cells concentrated on electron microscopy. The ultrastructure of mast cell granules of feline mast cells in normal skin and in mast cell tumors has been described by Ward and Hurvitz.[103] Bevier and Dunston reported on the ultrastructural changes of mast cells after intracutaneous injection of allergen in allergic cats.[104]

Eosinophils can be found in increased numbers in lesional skin of allergic cats and often in the peripheral blood. The value, however, of peripheral blood eosinophilia in cats is limited as it can be caused by several conditions including endo-and/or ectoparasite infestations.[105] Although the exact content of feline eosinophil granules is not known similar substances to those of human eosinophils are to be expected as lesions analogous to human flame figures occur in feline eosinophilic dermatoses.[106]

In summary, several immunopathogenic aspects of feline atopic dermatitis are still unresolved.

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Chapter 3

Cytophilic antibodies in cats with miliary dermatitis and eosinophilic plaques: passive transfer of immediate-type hypersensitivity.

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Abstract

Passive cutaneous anaphylaxis tests and Prausnitz-Küstner tests were performed in healthy recipient cats with heated and unheated sera of 17 cats suspected of having an allergic dermatitis and 12 healthy control cats. As positive reactions occurred most often with heated sera we conclude that a heat-stabile cytophilic antibody is involved in the pathogenesis of some cats with eosinophilic plaques and miliary dermatitis.

Introduction

Miliary dermatitis, eosinophilic plaque, and pruritic facial dermatitis are commonly reported clinical manifestations of atopic dermatitis in cats.[1] The diagnosis is usually based on the results of skin tests, the response to treatment with corticosteroids, and the exclusion of other cutaneous diseases such as flea bite allergy, food hypersensitivity, dermatophytosis, and parasitic diseases.[1,2] Histological features of a perivascular dermatitis and dermal infiltration with predominantly mast cells, eosinophils, neutrophils, and macrophages are common in miliary dermatitis, eosinophilic plaque, and pruritic facial dermatitis in cats and mimic those of atopic dermatitis in dogs.[1,3] As in dogs with atopic dermatitis in cats with miliary dermatitis the histamine concentration in the skin is significantly increased above that in normal skin.[4,5]

Only immediate-type reactions have been observed, although skin test results in cats are frequently reported to be inconsistent and difficult to interpret.[6,7] One of the reasons for inconsistent wheal formation may be the release of cortisol subsequent to skin testing in cats.[8]

Whereas canine atopic dermatitis has been associated with IgE and IgGd-mediated hypersensitivity,[9,10] allergen-specific antibodies of similar isotypes have never been isolated in allergic cats. Parasite-associated IgE has been reported by Powell et al.[11] in cats with *Otodectes cynotis* infections and by Baldwin in cats with experimental *Brugia pahangi* infections.[12] Evidence of the existence of feline IgE and crossreactivity with antibodies specific for canine IgE has been described by DeBoer et al.[13] Evidence for mast cell cytophilic antibody mediated reactions has been found by means of immediate-type reactivity in skin tests.[6,7,12-14] Similarity between the clinical manifestations, histological findings, skin histamine concentration, and immediate-type skin test reactivity suggests a comparable pathogenesis for canine and feline atopic dermatitis.

Therefore the aim of this study was to determine cytophilic antibodies in cats with miliary dermatitis, pruritic facial dermatitis, and eosinophilic plaques. For this purpose the Prausnitz-Küstner (P-K) and the Passive Cutaneous Anaphylaxis (PCA) test were used.

Materials and methods

Animals

Seventeen household cats were referred to the Utrecht University Animal Hospital for pruritic dermatitis responsive to corticosteroid therapy. There were 12 European short-haired cats, 4 Persian cats, and 1 Burmese cat, with ages ranging between 1 and 10 years (median 4.5 years). Six cats were female. A routine physical examination revealed no abnormalities other than of the skin. Parasitic disorders and dermatophytosis were ruled out by coat inspection, microscopic examination of skin scrapings and hairs, Wood's light examination, and fungal culture on Dermatophyte Test Medium (DTM). Food hypersensitivity was tested for 6 weeks with a home-prepared diet of lamb heart or turkey. None of the cats showed clinical improvement on this diet or after an intensive flea control program. The histology of multiple skin biopsies was compatible with an allergic dermatitis with increased numbers of mast cells and eosinophils. On the basis of these exclusion and selection criteria and the specific clinical features observed, the cats were divided into three groups:[1,2] 6 cats with miliary dermatitis [MD](2 Persian cats and 4 European short-haired cats [4 male cats] aged 2 to 8 years with a

median age of 5 years), 5 cats with eosinophilic plaques [EP] (1 Burmese cat and 4 European short-haired cats [4 male cats] aged 2 to 10 years with a median age of 5 years), and 6 cats with a pruritic facial dermatitis [PFD](2 Persian cats and 4 European short-haired cats [3 male cats] aged 1 to 7 years with a median age of 4 years). Serum was collected from all cats and immediately stored at -70 °C. Serum was also collected from 12 healthy European short-haired cats without skin problems (3 male cats, age 1 to 10 years with a median age of 3 years).

Passive Cutaneous Anaphylaxis test

Recipient cats – Five healthy European short-haired cats, kept indoors in individual cages, were used for the PCA test. Five days prior to the experiment a jugular catheter was inserted under anesthesia and the hair was clipped bilaterally on the thorax. Each following day the catheters were flushed several times with 1.0 ml of heparin solution (5,000 IU/100 ml of 0.9 % NaCl). In order to minimize stress due to animal handling and restraint, the cats were accustomed to frequent handling and sampling by the same person who performed the final test procedures.

PCA – Sera of 12 healthy control cats and of the 17 cats with MD, EP and PFD were encoded to prevent identification of the sera during the experiment. To inactivate IgE antibodies part of every sample was heated in a water bath at 56 °C for 24 hours. Each recipient cat was sedated with propofol (Diprivan, ICI-Farma, Rotterdam, The Netherlands) (induction dose 4 mg/kg iv) prior to the intradermal injection of 0.1 ml of each of the heated and unheated sera and phosphate-buffered saline solution as a negative control.[15,16] After 24 hours the recipient cats were challenged by the intravenous administration of one of the following allergens (ARTU Biologicals, Lelystad, The Netherlands) dissolved in a saline solution containing 0.5 % Evans blue dye: a grass pollen mixture of *Anthoxanthum odoratum, Dactylus glomerata, Cyrodon dactylum, Holcus lanatus*, and *Phleum pratense*: 50,000 Noon Units, dog dander: 5,000 µg, cat dander: 5,000 µg, human dander: 500 µg, or house dust: 500 µg. Hence each serum was PCA-tested on each of the 5 recipient cats. The selected allergens are common causative allergens of atopic dermatitis in dogs.[1] Skin reactions were read after 15 and 30 minutes, 1 hour, 2, 4, 8, and 24 hours. Reactions were considered positive if there was blue coloration at the injection site with a diameter which exceeding the saline control reaction by at least 5 mm.[17]

Prausnitz-Küstner test

Recipient cats – Three healthy European short-haired cats were used as recipient cats for the P-K test. The same procedure for handling the animals was followed as described for the PCA test.

P-K test – The sera of 11 healthy control cats and of 12 of the 17 cats with atopic dermatitis were encoded to prevent identification before reading the results of the P-K test. To inactivate IgE antibodies part of every sample was heated in a water bath at 56 °C for 24 hours. Of each heated and unheated serum 5 aliquots of 0.1 ml were injected intradermally. The same protocol was followed as for the PCA test. After 24 hours 2 ml of a 0.5 % Evans blue saline solution was injected intravenously, followed after 15 minutes by intravenous sedation and by the intradermal injection of the 5 different allergens at the same sites where the sera were injected 24 hours before. Hence the heated and unheated sera of cats with atopic dermatitis

or healthy control cats were challenged intradermally with each of the five allergens and all allergens were used in all recipients. These allergens (ARTU Biologicals, Lelystad, The Netherlands) were injected in standard skin test concentrations:[14,17] a grass pollen mixture (1000 NU/ml), dog dander (100 µg/ml), cat dander (100 µg/ml),human dander (10 µg/ml), and house dust (10 µg/ml). An 0.01% histamine solution, a phosphate-buffered saline solution, serum (heated and unheated) without subsequent antigen challenge, and allergen solution were used as negative controls. Skin test reactions were interpreted at the same time intervals as for the PCA test. Blue coloring at the injection site was considered positive if the diameter exceeded that of the saline control by 5 mm or more.[17]

Results

PCA – Sera of two cats with EP had positive PCA reactivity: twice with heated serum to cat dander and once with unheated serum to grass pollens. In addition, the heated serum of one cat with MD induced a positive reaction upon challenge with human dander (Table 1).

None of the sera of cats with PFD showed any reactivity. Positive reactions were only observed at 15 minutes after allergen challenge. Of the 12 control sera only serum of one cat gave a positive reaction to cat dander (heated serum) and to grass pollen (unheated serum). The response to phosphate-buffered saline solution was consistently negative.

Table 1. Skin reactivity in the Passive Cutaneous Anaphylaxis (PCA) test with serum of cats with allergic dermatitis (n=17)

Allergen	*Number of positive reactions with* Unheated serum	Heated serum	Both heated and unheated serum
Cat dander	0	2	0
Grass pollen	1	0	0
Human dander	0	1	0
Dog dander	0	0	0
House dust	0	0	0

P-K test – The sera of two cats with EP and one cat with MD, which showed PCA reactivity to cat dander and human dander respectively, also elicited P-K reactivity to these allergens (with heated and unheated serum). In addition, P-K reactivity was seen with heated serum of two cats with MD: once to cat dander, human dander, dog dander, and house dust, and once to dog dander and grass pollen (Table 2).

Tabel 2. Skin reactivity in the Prausnitz-Küstner (PK) test with serum of cats with allergic dermatitis (n=12)

Allergen	*Number of positive reactions with* Unheated serum	Heated serum	Both heated and unheated serum
Cat dander	2	3	2
Grass pollen	0	1	0
Human dander	1	2	1
Dog dander	0	2	0
House dust	0	1	0

No positive reactions were seen with sera of cats with PFD. The serum of the control cat that exhibited PCA reactivity to cat dander and grass pollen showed P-K reactivity to the same allergens with unheated and heated serum. Positive reactions were seen within 15 minutes after antigen challenge and at the injection site of the histamine control solution. The histamine reaction was 10 mm in diameter and had a blue coloration.

No reactions were observed at the injection sites of the negative control solutions: phosphate-buffered saline solution, antigen solution, and heated or unheated serum without subsequent antigen challenge.

Discussion

Reaginic antibodies commonly associated with immediate type hypersensitivity in dogs, horses, man, rats, and mice are of the IgE isotype.[9,18-21] Although IgE antibodies have not yet been isolated in cats with allergic dermatitis, there is evidence for its role in allergic dermatitis in cats.[6,7] In general, IgE antibodies are reported to be heat labile at 56 °C and their activity can be transferred to recipient animals by means of the PCA and the P-K test.[9,22] In addition to IgE, skin sensitizing antibodies of IgG subclasses have been described in the rat, mouse, and guinea pig.[22,23] A heat-stabile allergen specific antibody of the IgGd subclass has also been reported in atopic dogs.[10,24]

The results of the present study indicate that in the P-K test positive reactions are only observed with both heated and unheated sera or with heated sera alone. If both heated and unheated sera cause positive reactions in the PCA or P-K test, the presence of heat-stabile antibodies alone or the presence of both heat-stabile and heat-labile antibodies is indicated. If reactions are only seen with heated sera, there may be no heat-labile antibodies but only low affinity heat-stabile antibodies. Although heating of sera may result in aggregation of allergen- specific antibodies,[25] this is unlikely in this study as heating of the sera did not consistently result in positive reactions. The P-K and PCA reactivity to serum of one of the control cats only indicates that this cat has recently been exposed to the antigens, and does not necessarily means that clinical manifestations will develop.

Therefore our preliminary conclusion is that heat-stabile cytophilic antibodies may play a role in the pathogenesis of miliary dermatitis and eosinophilic plaques of cats. Whether these cytophilic antibodies are of the IgG isotype needs to be investigated.[22,24]

The heated sera of two cats with miliary dermatitis caused P-K reactivity to various danders, grass pollens, and house dust, whereas such activity was not seen in the PCA test. A difference in sensitivity between the PCA and P-K tests or an inadequate antigen challenge dose may be an explanation for this finding. A difference in sensitivity is more likely, as such a difference has also been reported in dogs immunized with *Toxocara canis* eggs or o-dinitrocarboxyphenol-BSA.[24] Because of the intravenous injection route it is more difficult to determine the appropriate antigen dose for challenge in the PCA test than in the P-K test. Whereas for the P-K test the standard skin test concentration is injected directly at the site of the reaction, for the PCA test the antigen is administered intravenously. This can lead to the absence of positive reactions caused by an inadequate amount of allergen. The low total number of positive reactions in both the P-K test and the PCA test, despite histologic features compatible with allergic dermatosis in the affected animals, may be explained by a short half-life of feline reaginic antibodies in serum. A variation in half-life between species from 5-8 hours in the mouse to 2.7 days in man was reported.[26,27] In addition, allergens other than those used for challenge may be involved in the investigated cats, thereby leading to negative P-K and PCA tests, as with these tests only allergen-specific antibodies are detected. The optimal sensitization period for reaginic antibodies of the IgG isotype is 3-4 hours.[22,23] Hence, if some of the reaginic antibodies in the cat belong to this isotype, as was concluded earlier, the 24-hour sensitization period (as used in this study for the P-K and PCA tests) may have contributed to false negative reactions.

It is concluded that the P-K and PCA test results give more evidence of the involvement of heat-stabile than of heat-labile cytophilic immunoglobulins in miliary dermatitis or eosinophilic plaques in cats. Further investigations are needed to elucidate the nature of these antibodies and their role in the pathogenesis of these skin disorders.

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Chapter 4

Feline Atopic Dermatitis: a model for Langerhans cell participation in disease pathogenesis

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Abstract

Atopic dermatitis is a disorder characterized by cutaneous exanthemata as a consequence of exaggerated eczematous reactions to topical and systemic allergens. Langerhans cells, expressing CD1a and HLA-DR, and dermal dendritic cells, expressing HLA-DR, are known to be potent antigen presenting cells and are thought to play an important role in the pathogenesis of atopic dermatitis. The immunophenotype of lesional skin in atopic dermatitis in humans involves increased numbers of CD1a+/MHC class II+ dendritic cells in addition to activated T cells, mast cells, and macrophages.

To establish feline skin as a model for the study of human atopic dermatitis, and to elucidate the role of dendritic cells in feline atopic dermatitis, we investigated the presence of CD1a+ cells and MHC class II+ cells in the epidermis and dermis of lesional feline skin and in skin of healthy control animals. Immunohistochemistry revealed that MHC class II+ epidermal dendritic cells were CD1a+ in normal feline skin and significantly increased numbers of CD1a+ cells and MHC class II+ cells were present in the epidermis and dermis of lesional skin. These data provide the first correlative documentation of CD1a expression by feline dendritic cells containing Birbeck granules, and indicate the utility of feline skin in the study of human cutaneous atopy.

Introduction

Langerhans cells (LC) and dermal dendritic cells (DDC) are known to be potent antigen capturing and presenting cells, and are thought to play an important role in the pathogenesis of atopic dermatitis (AD) in humans.[1,2] Langerhans cells at the site of patch tests to aero-allergens in patients with AD show increased activity.[3] In lesional skin of AD patients, increased numbers of CD1+ and HLA-DR+ cells have been found in the dermis and to a lesser extent in the epidermis.[2] In addition, LC from AD patients depend on surface IgE to mount specific T cell responses to house dust allergen (HDA), both *in vivo* and *in vitro*.[4] After capture, the internalized antigen is processed and presented to T cells in a MHC class II restricted way.[5] T cells and their cytokines are the next step in the complex network which contributes to allergic inflammation.[6,7]

In contrast to humans, little is known about the pathogenesis of atopic dermatitis in feline species. Various observations indicate similarity to the human disease. In cats the histopathology of atopic dermatitis is characterized by perivascular and diffuse dermal infiltrates of lymphocytes, mast cells, eosinophils, and macrophages,[8] findings comparable to the infiltrates seen in humans with AD.[9] Recently, we described an increased number of CD4+ T cells and IL-4 expression by these cells in lesional skin of AD cats, supporting a role for Th2 dependent pathways in disease pathogenesis.[10] Additionally, a putative feline IgE has been described in cats,[10-12] and reports exist which show familial involvement in feline atopic dermatitis.[13] Although cats have been used with success as an experimental model of airway hyperresponsiveness,[14,15] to our knowledge no studies exist on the use of cats as an experimental model for atopic dermatitis in man.

The aim of this study was to explore the suitability of feline skin to serve as a model for participation of Langerhans cells in AD. Specifically, first we determined the presence of CD1a+ cells in lesional skin of allergic cats, and second assessed the relationship of MHC class II expression to CD1a. The data indicate that feline skin harbors CD1a+ Langerhans cells analogous to those in human skin, and suggest active participation of these cells in feline AD.

Materials and Methods.

Animals

Skin biopsies were obtained from 9 domestic short hair cats with a history of recurrent allergic dermatitis. The age of these cats was 3-11 years (median 7 years, 5 were spayed female cats, 4 were castrated male cats). These cats were selected according to the clinical criteria for feline AD,[8] the presence of immediate skin test reactivity, and compatible histopathology of skin biopsies. Food hypersensitivity, dermatophytosis, and ectoparasites were excluded.[8]

Three 6 mm punch biopsies were taken from lesional skin of AD cats (n=9). Two biopsies were used for routine H&E staining to confirm the histopathological features of allergic dermatitis and one biopsy was immediately snap-frozen in liquid nitrogen.

Skin biopsies from the healthy control animals (n=9) were obtained from the same body location and processed in the same manner. The age of these cats was 3-12 years (median 6.6 years, 4 were spayed female cats, 5 were castrated male cats). Before taking skin biopsies all cats were anaesthetized with ketamine, xylazine, and atropine.

Immunohistochemistry

Single staining was performed with a mouse monoclonal antibody (mAb) to feline CD1a (FE1.5F4) and a mouse mAb against feline MHC class II (42.3).[16] From each biopsy 6 μm sections were cut using a cryostat (Reichert Histostat). Sections were air-dried and stored at -80 °C until used. Prior to staining serial sections were fixed with acetone and 0.3 % hydrogen-peroxide to quench endogenous peroxidase. Non-specific binding was blocked by preincubation for 20 minutes with 100 % horse serum. The antibodies to CD1a and MHC class II were applied in different dilutions and sections were incubated overnight at 4 °C. Biotin-conjugated horse anti-mouse antibody was used as a second step followed by incubation with an avidin-biotin-horseradish peroxidase complex (Vector ABC Elite kit, Vector Laboratories, Burlingame, CA).[17] Reactive sites were revealed using 3,3'-diaminobenzidine (Sigma, St. Louis, MO) as the chromogen. Control procedures included substitution of primary mAb with irrelevant isotype-specific mouse monoclonal antibodies. Sections were lightly counterstained with diluted Gill's hematoxylin solution.

Double-immunofluorescence staining with CD1a, MHC class II, and mAb anti-feline CD4 (vpg 34),[18] at different dilutions was also performed. Sections were fixed with acetone, and the mouse anti-feline CD1a was applied for 1 hour at room temperature. A polyclonal rabbit anti-mouse (Dako, Carpenteria, CA) was used as a secondary antibody, followed by a fluorescein-conjugated donkey anti-rabbit antibody (Accurate Scientific, Westbury, NY). After washing the mouse anti-feline MHC class II antibody was administered and left for 1 hour at room temperature. A Texas Red-conjugated sheep anti-mouse antibody (Accurate Scientific, Westbury, NY) was used as a second step. Negative controls consisted of irrelevant isotype-matched antibodies. Sections were evaluated with an Olympus BX 60 fluorescence microscope equipped with the appropriate excitation and emission filters for fluorescein and Texas Red illumination.

Ultrastructural analysis

Skin biopsies of 3 allergic and 3 healthy control animals were processed for electron microscopy. Briefly, tissue was placed in Karnovsky's fixative at 4 °C and stored until further processing.[19] Samples were then post-fixed with 2 % osmium tetroxide for 1.5 hours, dehydrated through graded ethanols followed by propylene oxide, and embedded in Taab Epon 812 (Marivac Ltd., Nova Scotia, Canada). Ultrathin sections were cut on a LKB Ultratome III ultramicrotome, post-stained with uranyl acetate and bismuth subnitrate and viewed on a Hitachi H-7000 electron microscope.

Statistical analysis and cell quantification

Positive staining cells in the epidermis and underlying dermis were counted separately with a square reticule in 5 adjacent high power fields (X400). Results were expressed in cells/linear mm in the epidermis or in cells/mm^2 in the dermis. Only highly dendritic cells with a clearly visible cell body were counted. Positive staining cells in hair follicle walls were not included.

To compare feline AD skin with normal skin for CD1a and MHC class II expression, a Mann-Whitney test was performed for comparison of cell number within the epidermis and within the dermis of both animal groups. The Spearman Rank correlation test was used to test for correlation between the epidermal and dermal parameters.

Results

H&E staining of the skin of the healthy control cats revealed no abnormalities. By immunohistochemistry, the normal feline skin was found to contain CD1a+ dendritic cells primarily within the epidermis: median 10 cells/linear mm [range 4-25] (Figure 1A); 75 % [range 0 %–100 %] of these cells showed immunohistochemical co-expression of MHC class II (Figure 1B), further defining them as Langerhans cells. The median of epidermal CD1a+ dendritic cells in AD skin was 30 /linear mm [range 17-41] (Figure 1C), and the median percentage showing co-expression of MHC class II increased to 100 % [range 17 %–100 %] (Figure 1D).

In the epidermis of the AD cats the number of CD1a+ cells and MHC class II + cells was significantly higher ($P< 0.05$) compared with the normal cats (Table 1). In the dermis, significantly ($P< 0.05$) more CD1a+ cells were present compared to the control group and the number of dermal MHC class II+ cells showed an even larger increase ($P< 0.05$) (Table 1). No correlation was found between the epidermal and dermal parameters.

Table 1. Number of CD1a+ cells and MHC class II+ cells in the skin of cats with atopic dermatitis (n=9) and healthy control cats (n=9). Cells in the epidermis are expressed in median and range per linear mm, whereas cells in the dermis are expressed in median and range per mm^2.

	Cats with atopic dermatitis (n=9)	Control cats (n=9)
Epidermal CD1a+ cells	30 (17-41)	10 (4-25)
Epidermal MHC class II+ cells	30 (10-41)	8 (0-26)
Dermal CD1a+ cells	121 (72-200)	61 (8-136)
Dermal MHC class II+ cells	707 (231-1081)	216 (106-295)

Double immunofluorescence staining of feline AD skin (Figure 1E and F) revealed two populations of dermal MHC class II+ dendritic cells; a larger CD1a-negative cell population (± 80 %) and a smaller CD1a-positive cell population (± 20 %). Whereas most of the CD1a+ cells were concentrated in the superficial dermis, MHC class II+ cells were present throughout the entire dermis. No positive double staining with CD4 and CD1a could be found. Cells staining positive for CD4 lacked dendrites, had a round cell body, and only a few were present in the epidermis. However, in the dermis a significant number of CD4+ round cells was observed.

Electron microscopy confirmed the presence of epidermal dendritic cells containing Birbeck granules (BG) in both normal (not shown) and feline AD skin (Figure 2A). Within the epidermis of control skin only a few epidermal dendritic cells containing a small number of BG were demonstrated, whereas a larger number of tennis racket-shaped BG and zipper-like BG were present in the epidermal dendritic cells of feline AD skin. Additionally, it appeared qualitatively that more BG-containing cells were present in the suprabasal and basal layers of feline AD epidermis whereas in the normal epidermis dendritic cells where primarily present in the basal layer with an occasional dendritic cell in the suprabasal layer. BG-containing cells had a more activated phenotype consisting of a prominent Golgi and many lysosomes (Figure 2B) as compared to normal skin. BG-containing cells could not be found in the normal dermis and neither in the feline AD dermis.

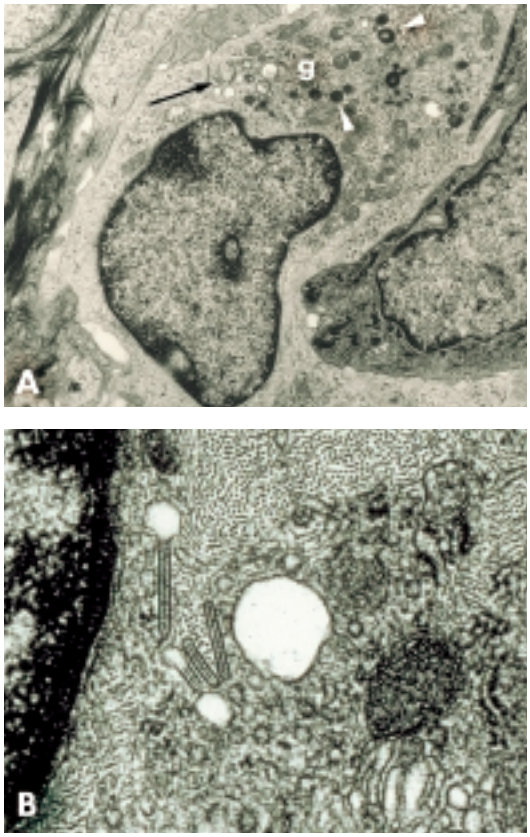

Figure 2. Transmission electron microscopy of intraepidermal Langerhans cell in atopic feline skin. Arrow in **A** indicates Birbeck granules (enlarged in **B**). Also note the prominent Golgi zone (g) and associated lysosomes (arrowheads) consistent with immunologic activation. Magnification, X8,400 (**A**); and X43,500 (**B**)

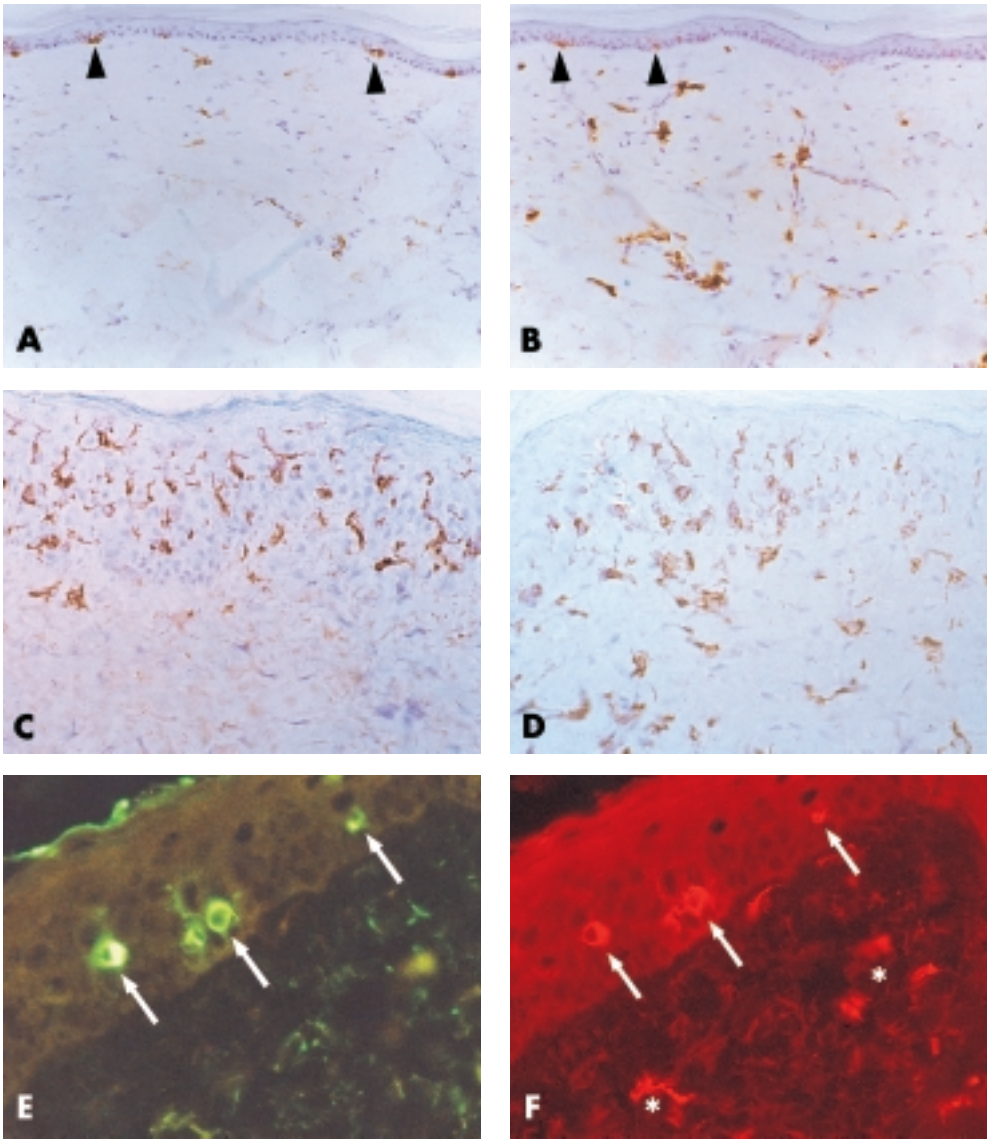

Figure 1. Immunohistochemical and immunofluorescence evaluation of normal and atopic feline skin. **A, C, E**: CD1a+ cells. **B, D**, and **F**: MHC class II + cells are illustrated. In normal skin (**A** and **B**), occasional CD1a+ (**A**) and MHC class II+ cells (**B**) are observed within the thin epidermal layer (arrowheads) and in the underlying dermis (endothelial cells also show variable MHC class II reactivity). In atopic skin (**C** and **D**), there is a marked increase in number of highly dendritic CD1a+(**C**) and MHC class II+ cells (**D**) within the thickened epidermal layer. By double labeling of sections of atopic dermatitis skin (**E** and **F**), the majority of CD1a+ cells (**E**, arrows) are also MHC class II+ (**F**). Note the presence of MHC class II+, CD1a- cells (*, **F**) within the dermis. Magnification, X 400.

Discussion

In the present study we document for the first time correlative evidence for the presence of CD1a glycoprotein on feline epidermal dendritic cells, and utilize this technique to study alterations in CD1a-positive and MHC class II-positive dendritic cells in cats with atopic dermatitis.

CD1a glycoprotein was first described on human Langerhans cells in 1981,[20-22] and on simian Langerhans cells in 1987.[23] In addition to Langerhans cells, it is present on cortical thymocytes, dermal dendritic cells ("indeterminate cells")[24] and on histiocytosis X cells.[25] CD1a expression is closely related to antigen presentation of peptides, lipids and glycolipids and it has served as a relatively specific marker for the immunohistochemical detection of cells with this functional profile. Langerhans cells have been shown to express the high affinity receptor for IgE (FcεRI),[26,27] and serum-IgE-facilitated allergen presentation in atopic disease was demonstrated.[28] LC also play a potentially important role in harboring a certain retrovirus implicated in immunodeficiency.[29] Recognition of CD1a expression by Langerhans cells of an animal species such as the cat which is prone to development of IgE-mediated skin allergy as well as retrovirus-associated immunodeficiency should enhance utilization of this model for investigation of these and related disorders.

Other indications for the suitability of feline atopic dermatitis as a model for human AD were described by the presence of an increased number of CD4+, IL-4 producing T cells in lesional skin of AD cats,[10] and a similar cellular infiltrate containing eosinophils, mast cells, lymphocytes, and macrophages. The presence of eosinophils in lesional skin may be explained by their recruitment elicited by cytokines like IL-4, IL-5, and GM-CSF produced by Th2-cells.[30]

In the atopy patch test in humans, eosinophils were shown to infiltrate into the dermis 2-6 hours after allergen application and were in an activated state after 24-48 hours, however later primarily eosinophil granular products were present.[31] Although in humans with chronic AD the number of mast cells is increased,[9] in acute lesions the mast cells are normal in number but in different stages of degranulation.[9] Their precise role in human AD is still obscure. In cats with AD the number of mast cells is increased and with electron microscopy we noticed different stages of degranulation (data not shown).

In this study, the presence of CD1a+ cells and MHC class II+ cells in the epidermis and dermis of normal cats and of lesional skin of cats with feline atopic dermatitis were investigated to elucidate the role of antigen presenting cells in this disease. In the AD cats, an increased number of CD1a+ cells in the epidermis was detected compared to the control animals, a finding comparable to skin of humans with AD.[2]

The decreased expression by Langerhans cells of MHC class II compared to CD1a in the normal epidermis was similar to that described in humans where antibody to CD1a was found to label more Langerhans cells than HLA-DR.[32] A marked increase in MHC class II+ dendritic cells was also seen in the epidermis of feline AD skin compared to control skin, which is consistent with the upregulation of MHC class II after cytokine-mediated Langerhans cell activation.[33,34] We found only small numbers of intra-epidermal T cells in lesional feline skin in an earlier study[10] and only dendritic cells were counted in this study; therefore we excluded intra-epidermal T cells as MHC class II + cells.

The upregulation of MHC class II is primarily induced by IFN-γ, which is predominantly produced by the Th1 subset, not the Th2 subset implicated in feline AD.[10] However, recently the presence of IFN-γ was demonstrated in lesional skin after atopy patch tests in patients with atopic dermatitis, confirming a shift from a Th2 response to a Th1 and Th0 response.[35,36]

Although all the AD cats had a history of chronic recurrent atopic dermatitis, there were differences in duration of the disease. The wide range in CD1a+ and/ or MHC class II+ cells may reflect a more or less active stage of the disease. Although one animal did not have MHC class II+ cells in the epidermis this could not be attributed to a staining error because MHC class II+ cells were present in the underlying dermis. The marked increase of dermal MHC class II+ cells was comparable to what is seen in humans.[2] Although Langerhans cells in humans express the CD4 marker,[37] we were not able to demonstrate its presence on the feline dendritic cells and we could not demonstrate upregulation of CD4 upon activation. Possible explanations are that the mAb used lacked sensitivity or that the CD4 levels on the Langerhans cells were too low for detection with the technique used.

Based on morphology, the main population expressing the MHC class II consisted of dermal dendritic cells (DDC). Different subsets of this MHC class II+ DDC population in humans have been described each expressing a different combination of markers. [38] In cats only Factor XIIIa+ DDC were described but no double staining for MHC class II or other markers was performed.[39] Therefore, further studies are needed to investigate the existence of similar subsets in cats.

With electron microscopy we were able to identify Birbeck granules which had a similar configuration to BG in humans. Although zipper-like structures were reported in a small proportion of CD18+ feline dendritic cells in an epidermal cell suspension,[40] no tennis racket-shaped BG in feline Langerhans cells were described *in situ*. Small numbers of BG containing cells have been described in DDC in humans defining them as LC,[41] however we could not find BG containing cells in the dermis of normal or feline AD skin. The location of Langerhans cells in normal feline skin was different from the situation in humans where Langerhans cells are normally suprabasilar in location,[42] whereas in the cats they were primarily basilar in location.

In summary it appears that 1) MHC class II+ epidermal dendritic cells are CD1a+ in normal feline skin, 2) increased numbers of MHC class II+ and CD1a+ cells are present in the epidermis and dermis of lesional skin. These data indicate that LC, related DDC, and other MHC class II-positive cells may actively participate in feline AD, and aid in establishing feline atopic dermatitis as a relevant model for the immunopathogenesis of this common and poorly understood human disease.

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Chapter 5

Increased numbers of CD4+ and CD8+ T cells in lesional skin of cats with allergic dermatitis.

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Abstract

The aim of this study was to characterize T cells in the skin of cats with an allergic dermatitis histologically compatible with atopic dermatitis as T cells are important role players in the pathogenesis of atopic dermatitis in humans. We observed a significantly greater number of T cells in lesional skin of domestic short hair cats with allergic dermatitis (n=10; median age 5.8 years.) than in the skin of healthy control animals (n=10; median age 5.0 years). In the skin of the healthy control animals one or two CD4+ cells were found and no CD8+ cells.

A predominant increase of CD4+ T cells and a CD4+/CD8+ ratio (mean ± SD: 3.9 ± 2.0) was found in lesional skin of 10 cats with allergic dermatitis. The CD4+/CD8+ cell ratio in skin of healthy control animals could not be determined because of the absence of CD8+ cells.

The CD4+/CD8+ cell ratio in peripheral blood of 10 cats with allergic dermatitis (mean ± SD: 1.9 ± 0.4) did not differ significantly from that in 10 healthy control animals (2.2 ± 0.4).

The CD4+/CD8+ cell ratio and predominance of CD4+ T cells in lesional skin of cats with allergic dermatitis is comparable to what is found in atopic dermatitis in man.

In addition, the observed increase of CD4+ T cells in non-lesional skin of cats with allergic dermatitis compared with skin of healthy cats is similar to what is seen in humans. Cytokines produced by T cells and antigen-specific T cells are important mediators in the inflammatory cascade resulting in atopic dermatitis in humans. This study is a first step to investigate their role in feline allergic dermatitis.

Introduction

Pruritic miliary dermatitis is one of the clinical manifestations of what is considered to be atopic dermatitis in cats.[1] The diagnosis of feline atopic dermatitis is usually based on a history of pruritus, the presence of miliary papulocrusts, results of skin tests, response to glucocorticoids, histopathology, and the exclusion of other cutaneous diseases such as food hypersensitivity, dermatophytosis, parasitic diseases and flea bite allergy.[1,2] Similar to the cellular components in atopic dermatitis of man[3] on dermatohistopathologic examination perivascular and interstitial dermal infiltrates are found consisting of mast cells, eosinophils, lymphocytes, and macrophages.[4]

Atopic dermatitis in humans is considered to be the result of a dysfunction of the immune system and is associated with elevated serum levels of total and allergen-specific IgE.[5,6] Although the exact pathogenesis of atopic dermatitis has not yet been elucidated, there is increasing evidence for a prominent role of IgE. In addition to the involvement of allergen-specific IgE antibodies in immediate-type hypersensitivity reactions, there are indications for a role for IgE antibodies in antigen uptake and processing.[7] Furthermore it is known that the synthesis of IgE is stimulated by T cell derived cytokines.[5] Production of IgE is enhanced by IL-4 and IL-13[8] and a high frequency of IL-4 producing CD4+ T cells (Th2) can be found in atopic dermatitis lesional skin and peripheral blood.[9] IgE-mediated allergen presentation may lead to enhanced activation of the immune system in response to very low allergen concentrations leading to expansion of the Th2 cell population and thus inducing increased production of IgE.[7] An increased number of T cells, including a high CD4+/CD8+ratio (4.8:1 - 8:1), can be found in the cell infiltrate of atopic dermatitis patients.[10,11]

Reaginic (IgE) hypersensitivity in cats has been demonstrated with passive cutaneous anaphylaxis testing in cats infested with *Otodectes cynotis*.[12] More recent publications also present evidence for the existence of a putative feline IgE in cats experimentally infected with *Brugia pahangi*.[13,14] DeBoer et al.[15] showed cross reactivity between monoclonal anti-canine IgE and a putative feline IgE in serum of cats experimentally parasitized with *Toxocara canis*.[15] In addition, evidence for the existence of a heat stabile cytophilic antibody was found in cats with miliary dermatitis or eosinophilic plaques.[16]

The dermal cell infiltrate in cats with allergic dermatitis (AD) has only been characterized by routine histopathology without further characterization of the cell populations. This despite the fact that T cell markers (pan-T, CD4, CD8) are available and widely used in studies on the pathogenesis of feline immunodeficiency virus infections.[17,18] It was the aim of this study to further characterize T cells present in the skin of cats with AD.

Materials and methods

Animals

Three castrated male and 7 spayed female domestic short hair cats with ages between 3 and 10 years (median 5.8 years), and a history of recurrent miliary dermatitis and pruritus responsive to glucocorticoids, and a histopathology compatible with allergic dermatitis[7] were included in this study. Parasitic skin diseases, dermatophytosis and food hypersensitivity were ruled out by standard diagnostic procedures.[18] Intensive measures to prevent flea infestation were carried out with all animals before skin testing. Except for the described skin problems

the animals were healthy. All cats were withdrawn from injectable glucocorticoids for at least 6 weeks and received no antihistamines or oral glucocorticoids for a minimum of 4 weeks prior to skin testing.

Five castrated male cats and 5 spayed female healthy domestic short hair cats were used as normal control animals. Their ages ranged between 1 and 12 years (median 5 years). The histopathology of skin biopsies revealed no distinct lesions.

Skin testing

All cats were examined with a commercially available test for dogs, containing 22 standardized allergens, the phosphate buffer as negative control solution, and a 0.01% histamine solution as positive control (ARTU Biologicals, Lelystad, the Netherlands).

The cats were skin tested while sedated with 10 $mg.kg^{-1}$ ketamine, 1 $mg.kg^{-1}$ xylazine, and 50 $mcg.kg^{-1}$ atropine sulphate administered intramuscularly. Intradermal skin tests were performed after clipping the hair from non-lesional skin of the lateral thorax. The skin was monitored for 30 minutes after injection of the allergens and reactions were interpreted at 20 minutes after injection with transillumination of the injection site. Reactions were considered positive when larger than half of the sum of the diameter of the two control solutions.

Sample collection

Subsequent to the skin test three 6-mm punch biopsies of lesional skin were obtained under sedation, immediately snap-frozen in liquid nitrogen, and stored at -80 °C until used. Two biopsies of comparable lesions were used for H&E staining to establish the histopathological features of the dermatitis. One biopsy of non-lesional skin was obtained from 4 cats with AD. Blood for flow cytometry was collected by puncture of the jugular vein. The skin biopsies and blood from the control animals were also obtained under sedation. Six skin biopsies were obtained from locations on the body similar to those of biopsies from the AD cats and processed in the same way.

Immunohistochemistry

Biopsy sections (6 μm) were air-dried overnight and fixed with acetone for 7 minutes. Non-specific binding was blocked by preincubation for 25 minutes with phosphate-buffered saline (PBS) containing 10% horse serum and 10% cat serum. Primary antibodies were murine monoclonal antibodies against feline CD4, CD8, and pan-T (vpg34 and vpg9,[17] and 1.572[19] respectively). Biotin-conjugated horse anti-mouse antibody (Vector, Burlington, Ca), was used as a second step, followed by alkaline phosphate-conjugated streptavidin (Dakopatts a/s Denmark). Staining was developed using naphthol As-Biphosphate (N-2250, Sigma, St. Louis, MO) as substrate and New Fuchsin as a chromogen, resulting in pink-red staining. Control staining was performed with isotype-matched antibody (Becton Dickinson, San Jóse, CA) and resulted in no staining. Sections were lightly counterstained with haematoxylin. Two serial sections from 3 different skin biopsies were examined. Positive staining cells in the superficial dermis were counted with a square reticule in 5 adjacent high power fields (X400). Cells in 2 sequential sections of each skin biopsy were counted and the mean of these two values was used. Cells in hair follicle walls and deep dermis were excluded.

Flow cytometry

Fluorescence analysis was performed on whole blood samples. Aliquots of 100 ml heparinized blood were pipetted into round-bottom microtiter plates. The cells were washed with FACS buffer solution (PBS, 0.5% BSA, 0.1% NaN0). Each monoclonal antibody (pan-T, CD4, CD8) was added directly to the well in a dilution of 1:100 (pan-T) or 1:50 (CD4, CD8). Irrelevant antibodies were used as isotype controls. Primary antibodies were incubated in the dark at 4 °C for 30 minutes. The cells were washed twice with FACS buffer solution. As a second antibody, 100 ml of goat antimouse/FITC in a 1:40 dilution (Becton Dickinson, San José, CA) was added to each well and the mixture was incubated in the dark at 4 °C for 30 minutes. The cells were then fixed and lysed with FACS lysis solution (Becton Dickinson, San José, CA). Flow cytometric analysis was performed on 10,000 cells using a FACScan flow cytometer (Becton Dickinson, San José, CA).

Statistical analysis

For the statistical analysis of the flow cytometry data the Wilcoxon two sample rank sum test ($P<0.05$) was used. Descriptive statistics was used for the immunohistochemistry data.

Results

Intradermal skin test

Eight of 10 cats with AD had immediate skin test reactivity to one or more allergens. Five cats reacted only to flea antigen. Two cats had positive reactions to flea antigen and to 5 and 8 other allergens (various tree pollen, grass pollen, weed pollen, *Tyrophagus putrescentiae, Glycyphagus destructor, D. farinae, D. pteronyssinus, Acarus siro*, and crude house dust). One cat had immediate skin test reactivity to *Tyrophagus putrescentiae*, weed mixture and *Glycyphagus destructor*. The group of cats with positive intradermal skin test reactions is further defined as cats with allergic dermatitis which includes cats that may be atopic and others that have flea allergy or both. Two of 10 cats with AD had a positive skin test reaction to histamine but not to the allergens.

Histopathology

The histologic features on HE-stained sections included the following. Epidermal hyperplasia was present in all the sections of lesional skin of cats with AD (n=10). The dermis contained a mixed cellular infiltrate consisting of a variable number of mast cells, eosinophils, neutrophils, and lymphocytes. In all the sections the inflammatory reaction was most prominent in the upper dermis and had a perivascular pattern, combined with a variable diffuse extension (Fig. 1). Eosinophils were present in all sections however in some sections they were more prominent than in others.

The epidermis of the non-lesional skin (n=4) was either normal or showed minor hyperplasia. The cellular infiltrate in the dermis of non-lesional skin contained a smaller number of cells; only few mast cells and eosinophils, but no neutrophils were present.

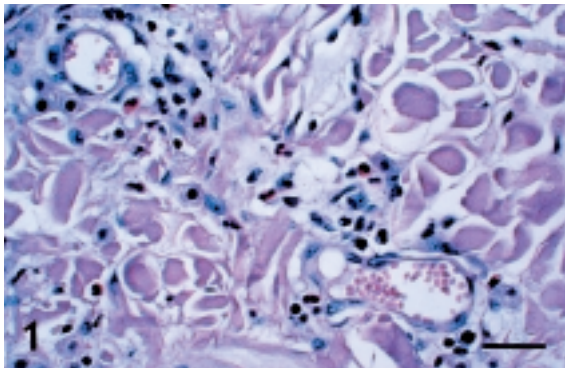

Figure 1. Perivascular infiltrate with eosinophils, mast cells and lymphocytes (X40 objective). HE. Bar=50 μm.

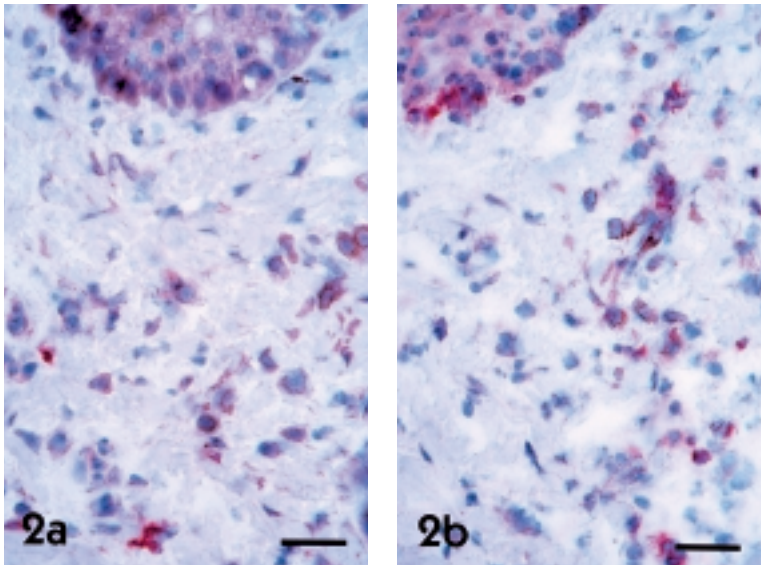

Figure 2. Cryostat section of lesional skin of a cat with allergic dermatitis stained for CD4 and CD8 with a biotin streptavidin alkaline-phosphate complex method, counterstained with haematoxylin. Figure 2a. Section showing a small number of CD8+ pink cells (X100 objective). Bar= 30 μm. Figure 2b. Same section containing a considerable larger number of CD4+ cells when compared to the CD8 staining (X100 objective). Bar= 30 μm.

Immunohistochemistry

The T cell infiltrate in the sections of lesional skin of the AD cats was most prominent in the superficial dermis (Fig. 2a and Fig. 2b). Cells staining positively for pan-T, CD4, and CD8 were also seen in primarily the deeper part of the epidermis.

The dermal cell infiltrate with positively staining cells was sometimes very focal, such that a large number of positively staining cells could be seen within one high-power field (X400), although no positive cells could be found in the adjacent field. The CD4+/CD8+ ratio in the dermis of 3 biopsies of lesional skin of 10 AD cats was 3.9 ± 2.0 (mean ± SD) (Table 1). The CD4+/CD8+ ratio of the 2 cats with negative skin test results were 2.3 and 2.5. Inspite of similar histologic inclusion criteria we found considerable variation in the absolute number of positively staining cells (SD 16.8) and in CD4+/CD8+ ratios (SD 2.0) between cats with AD. The mean value of the CD4/CD8 ratio was 3.9 with a SEM of 0.6 which is significantly higher ($P < 0.05$) than the corresponding value (0) of the control animals since this value does not belong to the 95 % confidence interval of the former value.

There was a predominant increase of CD4+ T cells in non-lesional skin of 4 cats with AD (one biopsy of each cat) with respect to the findings in the skin of healthy cats, where the (mean ± SD) number of CD4+ cells was 0.4 ± 0.7. CD8+ cells were not present.

Flow cytometry

The ratio of CD4+/CD8+ cells in peripheral blood of the 10 cats with AD was 1.9 ± 0.4 (mean ± SD). The CD4+/CD8+ ratio in peripheral blood of 10 healthy control cats was 2.2 ± 0.4 (mean ± SD). This difference is not significant $P > 0.05$.

Discussion

Cats with an allergic dermatitis (AD) based on histopathological features were investigated in this study. Lesions consistent with miliary dermatitis (such as papules and crusts) were biopsied in all cats. There was variation not only in the type of lesions but also in the extent of the skin changes. This implies a difference in chronicity of the lesions, which can influence the intensity of the cell infiltrate and the extent of epidermal hyperplasia.

We included cats with or without concomitant immediate skin test reactivity to aeroallergens in this study. Cats without positive skin test reactions were included because negative skin test results do not exclude allergic dermatitis as intradermal skin tests in cats are considered to be less reliable in cats than in dogs.[20,21] In addition, negative skin test results can also be explained by absence of the causative allergen in the skin test. In the dermal infiltrate of all AD cats a significantly higher number of CD4+ cells than CD8+ cells was found. Also in humans with atopic dermatitis a predominance of CD4+ cells is found with CD4+/CD8+ ratios of 8:1 and 4.8:1.[10,11]

In non-lesional skin of humans with atopic dermatitis an increased number of CD4+ cells is seen if compared to skin of healthy individuals.[22] The normal human skin contains small numbers of T cells in the dermis, primarily perivascularly located. The CD4+/CD8+ ratio varies between 0.00 and 0.32.[23] The biopsies of non-lesional skin of 4 AD cats also showed a significant increase in CD4+ cells when compared to the healthy control animals.

Table 1. Number per 5 high power fields (X400) of pan-T+, CD4+ and CD8+ T cells (mean ± SD) and the CD4+/CD8+ ratio in the dermis of lesional (LS, n=10) and non-lesional (NLS, n=4) skin of cats with allergic dermatitis (AD). For the healthy control cats (n=10) and the CD8 + cells in the NLS, the number of positive cells is depicted as the median and range. From the cats marked with an asterix (*) also non-lesional skin was examined.

	Pan-T	CD4	CD8	CD4/CD8
LS AD				
1*	74.3 ± 3.6	33.3 ± 6.3	14.0 ± 1.0	2.38
2	73.7 ± 5.7	32.3 ± 7.3	15.3 ± 2.3	2.10
3	85.0 ± 5.6	29.3 ± 10.3	4.0 ± 9.0	7.33
4*	97.0 ± 17.6	64.5 ± 24.9	9 ± 4.0.0	7.16
5*	83.3 ± 4.0	51.3 ± 11.3	22.7 ± 9.7	2.26
6	68.7 ± 10.7	46.0 ± 6.4	19.0 ± 6.0	2.42
7*	29.7 ± 49.7	26.7 ± 13.0	10.7 ± 2.3	2.50
8	89.3 ± 10.0	46.0 ± 6.4	12.3 ± 0.7	4.31
9	134.7 ± 60.3	49.0 ± 9.4±	9.7 ± 3.3	5.07
10	66.3 ± 13.0	29.0 ± 10.6	13.3 ± 0.3	3.00
1-10	79.4 ± 27.6	39 ± 16.8.7	13.0 ± 5.5	3.9 ± 2.0
NLS AD				
1	72	11	0	–
4	15	10	0	–
5	14	16	5	3.20
7	54	40	14	2.86
	38.8 ± 29.0	19.3 ± 12.2	2.5 (0–14)	
Control cats				
1	0	0	0	–
2	2	0	0	–
3	0	0	0	–
4	0	0	0	–
5	4	0	0	–
6	4	2	0	–
7	1	0	0	–
8	10	0	0	–
9	2	0	0	–
10	2	0	0	–
	2 (0–10)	0 (0–2)	0	

We found no significant difference in CD4+/CD8+ ratio in peripheral blood of AD cats compared to the control animals. Like in human patients, this may be due to the activity and extent of the disease which influence the results.[24] In humans with atopic dermatitis conflicting results have been published on peripheral blood CD4+/CD8+ ratios. Sowden et al. observed increased percentages of CD2+ cells, increased levels of CD4+ lymphocytes and a decreased percentage of CD8+ cells.[24] These changes became more significant with deterioration of the disease. This is in contrast to the finding of Lever et al.[25] that neither total numbers of circulating T cells nor the CD4+/CD8+ ratio differed significantly between patients with active AD lesions and healthy controls. In the latter study however, the CD4+/CD8+ ratio in the lesional skin of these patients was 7:1. Blok et al.[26] even reported a lower CD4+/CD8+ ratio in peripheral blood of patients with AD than in normal control individuals. Thus it appears that neither in humans nor cats with atopic dermatitis, there is a correlation between CD4+/CD8+ ratios in peripheral blood and skin.

In conclusion, our observations of an increased number of T cells, including a predominant increase of CD4+ T cells in lesional and non-lesional skin, and a substantial CD4+/CD8+ ratio in lesional skin of cats with allergic dermatitis, are analogous to what is seen in atopic dermatitis in man.

As T cells and more specifically T helper 2 cells are thought to play an important role in the pathogenesis of AD in humans by release of their cytokines which initiate, maintain, and mediate the inflammatory cascade resulting in lesional skin. The presence of CD4+ T cells in lesional skin of cats with AD is therefore an important finding requiring further investigation into the role of T cell subsets in the pathogenesis of allergic dermatitis in cats.

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Chapter 6

Interleukin-4 producing CD4+T cells in skin of cats with allergic dermatitis

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Abstract

Lesional skin of cats with allergic dermatitis has a cellular infiltrate and a CD4/CD8 ratio comparable to that in humans with atopic dermatitis. CD4+ helper T cells and in particular cells belonging to the Th2 subset play an important role in disease pathogenesis in humans. Therefore it was the aim of this study to elucidate the cytokine pattern of CD4+ T cells *in situ*, with special emphasis on the putative presence of IL-4 producing cells in cats with allergic dermatitis. Using immunohistochemical procedures we proved that feline CD4+ T cells in lesional and nonlesional skin of cats with allergic dermatitis can produce IL-4, as occurs in humans. In lesional and nonlesional skin of cats with allergic dermatitis significantly more IL-4+ T cells (P=0.001) were found compared to skin of healthy control cats. Double staining indicated that all IL-4+ cells were positive for pan-T or CD4 markers. Double labeling for mast cell chymase and IL-4 stained primarily different cells. Western blotting demonstrated cross reactivity of the used antibody against human IL-4 with a feline recombinant IL-4. These results indicate that IL-4 is primarily produced by CD4+ T cells and is also present in clinically uninvolved skin, indicating a role in the pathogenesis of allergic dermatitis in cats.

Introduction

Atopic dermatitis in humans is mediated by antigen-specific IgE and an important role has been ascribed to CD4+ helper T cells (Th), and in particular to cells of the Th2 subset.[1] IL-4 produced by primarily Th2 cells in humans has many biologic activities and stimulates the production of IgE together with IL-13.[2,3] In lesional skin of human patients with atopic dermatitis IL-4-producing CD4+, allergen-specific T cells occur in high density.[4] Mast cells are considered to be another potential source of IL-4, however studies on IL-4 production by cutaneous mast cells have reported conflicting results.[5,6]

In cats, the diagnosis of atopic dermatitis is generally based on a history of pruritus, recurrent or chronic crusted papules defined as miliary dermatitis, results of intradermal skin tests, response to glucocorticoids, histopathology, and the exclusion of other pruritic skin diseases such as food hypersensitivity, dermatophytosis, flea bite hypersensitivity, and parasitic diseases.[7,8] Histology of lesional skin of cats with allergic dermatitis (AD) shows a perivascular to diffuse infiltrate of mast cells, eosinophils, lymphocytes, and macrophages,[9] comparable to the infiltrate that occurs in humans with atopic dermatitis.[10] Recently we reported that there is a predominant increase of CD4+ T cells and a CD4/CD8 ratio of 3.9 ± 2.0 (mean ± SD) in lesional skin of cats with allergic dermatitis,[11] which is also similar to what is found in humans.[12] In addition, a putative feline IgE,[1,13-15] and the involvement of heat-stabile cytophilic immunoglobulins,[16] have been reported in cats with allergic dermatitis. These observations indicate a comparable disease pathogenesis of atopic dermatitis in cats and atopic dermatitis in humans. Because interleukins play an important role in disease pathogenesis, the next step in the characterization of allergic dermatitis in cats was aimed at the elucidation of the cytokine pattern of CD4+ T cells *in situ*.

Materials and methods

Animals

Skin biopsies were obtained from 5 client-owned domestic short-haired cats with a history of recurrent miliary dermatitis and pruritus responsive to glucocorticoids. These cats were 3-10 years of age (mean 6.5 years; one was a spayed female, 4 were castrated males). Food hypersensitivity, dermatophytosis, and ectoparasites had been excluded by standard diagnostic procedures.[7] Intensive measures to prevent flea infestation were carried out for all animals before skin testing. Except for the described skin problems the animals were healthy. All cats were withdrawn from injectable glucocorticoids for at least 6 weeks and received no antihistamines or oral glucocorticoids for a minimum of 4 weeks prior to skin testing. Only cats with the histopathological features of allergic dermatitis were included in this study.[9]

Five healthy domestic short-haired cats, 2-5 years of age (mean 4.0 years; two were spayed females, three were castrated males), were used as normal control animals. Histopathology of skin biopsies of these cats was compatible with normal skin.

Skin testing

All cats were examined with a commercially available intradermal skin test for dogs, containing 22 standardized allergens, the phosphate buffer as negative control solution, and a

0.01% histamine solution as positive control (ARTU Biologicals, Lelystad, the Netherlands).

The cats were skin tested while sedated with 10 $mg.kg^{-1}$ ketamine, 1 $mg.kg^{-1}$ xylazine, and 50 $mcg.kg^{-1}$ atropine sulphate administered intramuscularly. Intradermal skin tests were performed after clipping the hair from non-lesional skin of the lateral thorax. The skin was monitored for 30 minutes after injection of the allergens and reactions were interpreted at 20 minutes after injection with transillumination of the injection site. Reactions were considered positive when larger than half of the sum of the diameter of the two control solutions.

Sample collection

Subsequent to the skin test one 6-mm punch biopsy of lesional skin and one biopsy of nonlesional skin were obtained under sedation, immediately snap-frozen in liquid nitrogen, and stored at –80 °C until used. Two biopsies of comparable lesions were used for HE staining to establish the histopathological features of the allergic dermatitis. The skin biopsies from the control animals were also obtained under sedation and processed in the same way.

Immunohistochemistry

Single staining was performed with a mouse mAb to human IL-4 (1-41-1, Novartis, Vienna, Austria). Biopsy sections were cut at 6 µm, air-dried overnight, and fixed in acetone for 7 minutes. Nonspecific binding was blocked by preincubation for 25 minutes with phosphate-buffered saline (PBS) containing 10 % horse serum and 10 % cat serum. The mAb to human IL-4 was applied for 1 hour in different dilutions. Horse anti-mouse biotin conjugated antibody (Vector, Burlingame, CA, USA) was used as a second step followed by alkaline phosphate conjugated-streptavidin (Dakopatts a/s, Denmark). Staining was developed using Naphthol AsBi (N-2250, Sigma, St. Louis, MO, USA) as substrate and New Fuchsin as a chromogen, resulting in pink-red staining. The sections were counterstained with diluted hematoxylin. Control staining was performed with an isotype-matched antibody (mouse IgG1 standard 102-01, Southern Biotechnology Assoc. Inc., Birmingham, AL, USA). Two mouse mAbs to human IFN-γ (MD1 and MD2) and a polyclonal rabbit anti-human IFN-γ (these three antibodies were a generous gift from Dr. P.H. van der Meide, TNO, Rijswijk, the Netherlands), tested in different dilutions on cryostat sections did not result in any positive staining.

For the double staining procedures, sections were air-dried overnight and fixed in acetone. Nonspecific binding was blocked by preincubation for 25 minutes with PBS containing 10% horse serum and 10% cat serum. As first primary antibody a dilution of the mouse mAb to human IL-4 was applied for 1 hour. A horse anti-mouse biotin-conjugated antibody (Vector, Burlingame, CA, USA) was used as a second step, followed by blocking with rabbit anti-mouse serum (Dakopatts a/s, Denmark). Murine mAbs to feline pan-T (1.57)[17] or feline CD4 (vpg 34)[18] were administered and left for 1 hour as the second primary antibody, followed by streptavidin-ß-galactosidase (Crowley Down, Sussex, UK) and rabbit anti-mouse alkaline phosphate conjugated serum (Dakopatts a/s, Denmark). After staining of the slides with X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; 651 745, Boehringer Mannheim, Germany) the slides were washed and stained with New Fuchsin as the second chromogen. As a control either of the primary antibodies was omitted, resulting in one color only.

In a combined staining for IL-4 and an enzyme-histochemical staining method for chymase sections were stained first as described with the mouse mAb to human IL-4 (1-41-1,

Novartis, Vienna, Austria). Instead of counter staining the slides were stained for chymase. A freshly prepared mixture containing 0.53 mM Naphthol AS-D-chloroacetate (Sigma, St. Louis, MO, USA) as substrate, 0.3 mM fast blue BB salt (4-benzoylamino-2,5- diethoxybenzenediazonium chloride hemi [zinc chloride] salt (Sigma, St. Louis, MO, USA) as the chromogen, 40mM NaF (sodium fluoride) and 1% Triton X-100 in 50 mM Tris-HCl buffer (pH 6.8). The substrate was first dissolved in dimethylformamide before adding it to the mixture.[19,20] The sections were incubated for 10 minutes, washed in deionized water for 5 minutes and mounted.

Positive staining cells in the superficial dermis were counted with a square graticule in 4 adjacent high power fields (400X). Cells in hair follicle walls were not counted because of differences in hair follicle density and plane of cutting per slide. The deep dermis was excluded because of variation in depth of dermis.

Cloning and sequencing of feline IL-4

Peripheral blood lymphocytes from a specific pathogen free cat were stimulated *in vitro* with 6 µg/ml Con A and 100 U/ml IL-2 for 48 hours. Cells were then pelleted (800xg) for ten minutes and then resuspended in 1 ml Trisolv (Biotecx Laboratories, Inc., Houston, TX, USA) for total RNA extraction according to the procedure recommended by the manufacturer. RNA was resuspended in 40-60 µl DEPC water with 0.5 µl RNase inhibitor and 1 µl DNase. The extracted RNA was incubated for one hour at 37°C to remove DNA, then heated for five minutes at 95°C to inactivate DNase. RNA concentration was determined spectrophotometrically by measuring the A_{260} in a micro-cuvette (Genequant II, Pharmacia Biotech, Inc., Piscataway, NJ, USA). Total RNA (1 µg) was reverse-transcribed (42°C for 1 hour, 95°C for 10 minutes) using an RT-Kit (Promega Corp., Madison, WI, USA) and an oligo-dT primer. The feline IL-4 cDNA was amplified with a single round PCR, performed in a Biotherm Bio-oven III (Biotherm Corp., Fairfax, VA, USA).

The amplification consisted of 35 cycles (30 seconds template denaturation at 94°C, 30 second primer binding at 55°C and 45 seconds elongation at 72°C), with an initial 5 minute denaturation at 94°C and an extra 10 minute elongation at 72°C for completion.

The primers used were derived from the published feline sequence (Genbank accession # X87408) with an added BamHI or EcoR1 restriction enzyme site on the forward

(5' GCGGGATCCATGGGTCTCACCTACCAAC 3') and reverse

(5' GCGGAATCCCAGCTTCAGTGCTTTGAG 3') primer, respectively. The PCR product was ligated into a pCR3.1 plasmid and transformed into E. coli strain One Shot (TOP10F') by TA cloning (Invitrogen Corp., Carlsbad, CA, USA). The feline IL-4 sequence was cut out of pCR3.1 at the BamHI and EcoR1 sites, then ligated into pGEX2T (Pharmacia Biotech, Inc., Piscataway, NJ, USA) and transformed into One Shot cells. Sequences inserted into pGEX2T are expressed as a fusion protein with glutathione-S-transferase (GST). The sequence and coding frame of the fIL-4-pGEX2T was verified using the chain-termination reaction (Sequenase, United States Biochemical, Cleveland, OH, USA) and standard techniques with 5' and 3' pGEX sequencing primers (Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Expression and western blotting of recombinant feline IL-4

The fIL-4-pGEX2T plasmid was transformed into E. coli strain BL-21 (Novagen Inc., Madison, WI, USA). Transformed bacteria were grown at 30°C until the A_{600} reached 0.6. Protein expression was then induced by adding IPTG to a final concentration of 0.1 mM. After 2 hours, shaking at 30°C, bacteria were pelleted and lysed in STE buffer with 420 units/ml lysozyme, 1 unit/ml DNase, 5 mM DTT, and 1.5% sarkosyl. The lysate was clarified by centrifugation and Triton X-100 was added to the lysate to a final concentration of 3% to enhance solubilization of the fusion protein.

Bacterial lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were probed with goat anti-glutathione-S-transferase (GST) polyclonal antibody (Pharmacia Biotech, Inc., Piscataway, NJ, USA) or mouse anti-human IL-4 monoclonal antibody (1-41-1, Novartis, Vienna, Austria). The membranes were then incubated with the appropriate anti-goat or anti-mouse antibody conjugated to horseradish peroxidase. The membranes were developed using Sigma FAST DAB with metal enhancer (Sigma, St. Louis, MO, USA) in 0.1M imidazole (pH 7).

Recombinant feline IL-2, used as a positive control was generated as described for IL-4. The recombinant IL-2 is biologically active and was used to generate monoclonal antibodies against feline IL-2 (G. Dean, unpublished data).

Statistical analysis

For the statistical analysis of the immunohistochemistry data the Student t test ($P<0.05$) was used.

Results

Histopathology

In the biopsies of lesional skin of the 5 cats with AD the epidermis was hyperplastic with occasional exocytosis of lymphocytes. A mixed perivascular infiltrate with a variable number of mast cells, eosinophils, neutrophils, macrophages, and lymphocytes was present in the dermis. The epidermis of the nonlesional skin was either normal or showed minimal hyperplasia. The cellular infiltrate in the dermis of nonlesional skin contained only few mast cells, lymphocytes and sometimes a few eosinophils. Neutrophils however, were not present. In the control cats the epidermis was thin with minimal cellularity of the dermis.

Intradermal skin test

Three out of five cats with AD had immediate skin test reactivity to one or more allergens. These cats had one to three positive reactions to the following allergens: grass pollen, weed pollen, *Tyrophagus putrescentiae, Dermatophagoides farinae,* and *D. pteronyssinus.* One cat had a positive reaction to flea allergen beside the positive reactions to the aero-allergens. Two cats had negative skin test results but showed positive reactions to the histamine control. The group of cats with miliary dermatitis (n=5) is further defined as cats with allergic dermatitis which includes cats that may be atopic and others that may have additional flea bite hypersensitivity. Positive reactions to the allergens did not occur in the control cats.

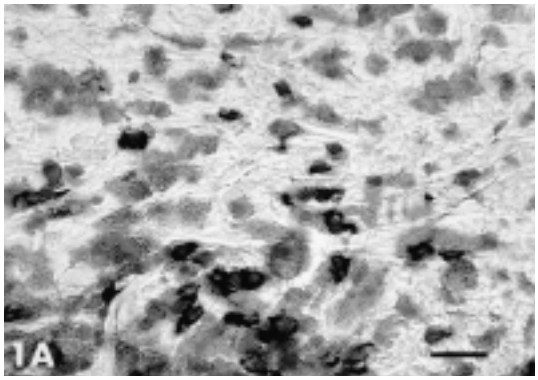
Immunohistochemistry

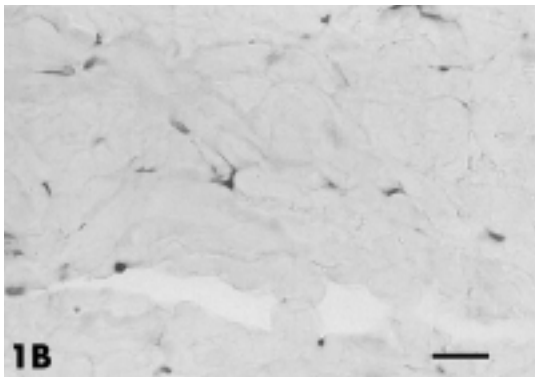
Exocytosis of IL-4 + cells was found in the lower part of the epidermis in lesional skin. In lesional and non lesional skin, predominantly in the superficial dermis a perivascular infiltration with IL-4+ cells was found (Fig. 1A and 1C). The isotype-matched control antibody resulted in no staining. In lesional skin of the allergic cats 58.6 (45.7-77.6) (median and range) IL-4+ cells/mm^2 were found whereas in the nonlesional skin of the same animals 17.6 (12.9-27.1) IL-4+ cells/mm^2 were present (Table 1). Only a very limited number of cells stained positive for IL-4 in the superficial dermis of the control animals (1.4 (0-1.4)) (Table 1 and Fig. 1B). In addition, no intra-epidermal positive staining cells were found. These differences are significant (P=0.001).

To determine whether the IL-4+ cells were T cells, sections were double stained for either pan-T or CD4 in combination with anti-IL-4. All IL-4+ cells also stained positive for pan-T and CD4 within the epidermis and dermis. Single IL-4+ cells were not observed, indicating that in cat skin all IL-4+ cells are indeed CD4+ T cells. In the double labeling experiment for IL-4 and mast cell chymase there was only occasional minimal double staining for IL-4 and mast cell chymase.

Table 1. Number per 4 high power fields (X400) of IL-4+ cells (median and range) seen in one section of skin biopsies of lesional and nonlesional skin of cats with allergic dermatitis (AD, n=5) and in one skin biopsy of healthy control cats (n=5) expressed in cells / mm^2 .

	lesional skin	nonlesional skin
AD cats		
1	58.6	17.6
2	77.6	27.1
3	50	20
4	45.7	12.9
5	77.1	17.1
	58.6 (45.7–77.6)	17.6 (12.9–27.1)
control cats		
1		1.4
2		0
3		1.4
4		1.4
5		0
		1.4 (0–1.4)





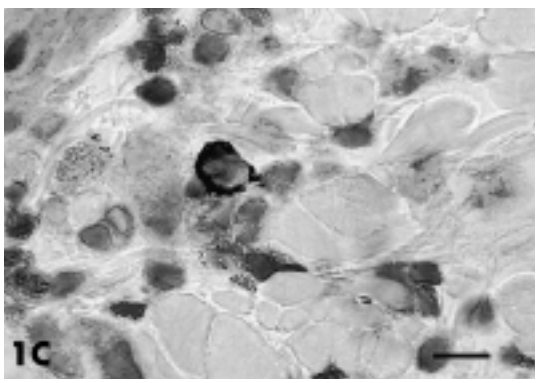
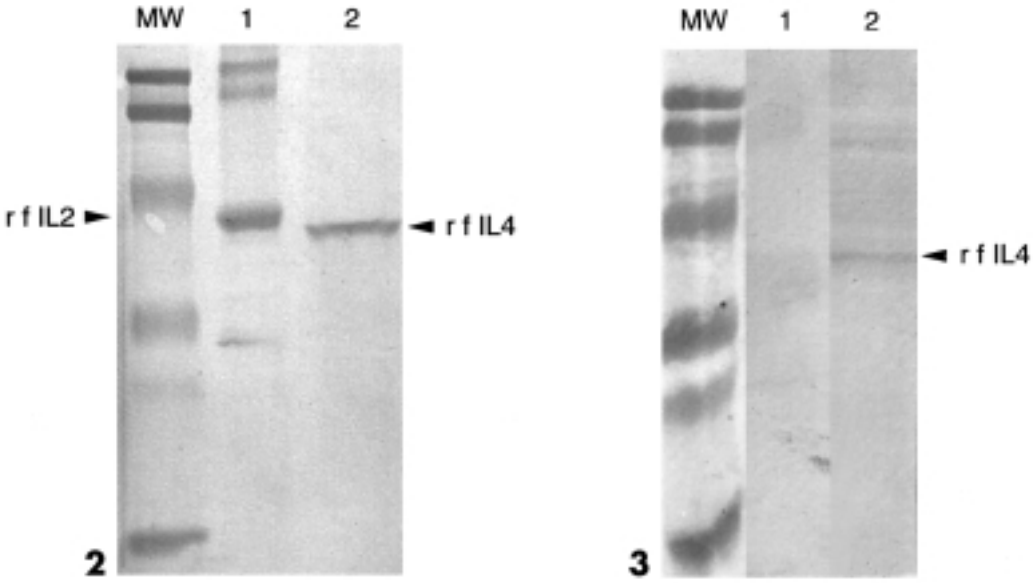


Figure 1A. Cryostat section of lesional skin of a cat with allergic dermatitis stained for IL-4 with a biotin streptavidin alkaline-phosphate complex method showing a number of positive staining cells (40 × objective). Bar = 30 µm.
Figure 1B. Section of skin of a healthy control animal lacking positive staining cells (40 × objective). Bar = 30 µm.
Figure 1C. Detailed photo of a IL-4+ cell demonstrating that the mast cells to the left of the IL-4+ cell did not stain with this antibody (100 × objective). Bar = 80 µm.



Figures 2 and 3. Expression and immunoreactivity of recombinant feline IL-4. Recombinant feline (rf) IL-2 (lane 1) and IL-4 (lane 2) were expressed as fusion proteins with glutathione-S-transferase (GST), run on a 15 % polyacrylamide gel, and transferred to a nitrocellulose membrane. Membranes were then probed with anti-GST polyclonal antibody (Figure 2) or antihuman IL-4 monoclonal antibody (Figure 3). Arrows point to rf-IL-2 in lane 1 at approximately 44 kD and rf-IL-4 in lane 2 at approximately 43 kD. Prestained molecular weight markers (lane MW) are shown for size reference.

Western blotting of recombinant feline IL-4

On Western blot (Fig. 2) recombinant feline IL-4 (rf-IL-4) and recombinant feline IL-2 (rf-IL-2) react with a polyclonal antibody against the fusion protein (glutathione-S-transferase (GST)), and in Fig. 3, the rf-IL-4 but not rf-IL-2 shows cross reactivity with the anti-human IL-4 monoclonal antibody (1-41-1).

Discussion

Atopic dermatitis in humans is considered to be a T cell mediated disorder and an important role has been ascribed to CD4+ T cells and especially the Th2 subset.[21]

In this study T cells of cats with a history and clinical signs of pruritic, miliary dermatitis compatible with an allergic dermatitis (AD) were investigated. AD in cats is associated with a predominant increase of CD4+ T cells in lesional skin.[11] As the next step in the characterization of regulatory mechanisms, the cytokine production by these cells was investigated.

Cats with negative intradermal skin test results (n=2) were included in this study because negative skin test results do not exclude atopic dermatitis and intradermal skin tests are considered to be less reliable in cats than in dogs.[22,23] In addition, in 20% of humans with atopic dermatitis, who have the so called "intrinsic type" skin tests are negative and these patients have no measurable allergen-specific IgE in the serum.[24]

Our findings that AD cats had a significant increase in IL-4+ cells in lesional and clinically non involved skin compared to skin of healthy control cats is compatible to what has been described in humans with atopic dermatitis.[25] Double staining for IL-4 and CD4 or IL-4 and pan-T revealed that all cells staining positively for IL-4 also stained positively for CD4 or pan-T as well. This is in agreement with findings in humans with atopic dermatitis using the same antibody against human IL-4.[6] In that study staining for IL-4 was only present in the CD4+ population; no mast cells or eosinophils were positive.[6]

We did not find a correlation between positive skin test results and number of IL-4+ cells.

In humans with atopic dermatitis, a relationship between skin infiltrating Th2-like cells and allergen-specific IgE response could not be demonstrated.[26] In addition, the expression of IL-4 mRNA changes with chronicity of the lesion in patients with atopic dermatitis.[25] Although clinically similar lesions were biopsied the chronicity of the skin lesions in the cats may have varied.

To our knowledge it is not known if cats display distinct polarized Th cell subsets like mice or if feline Th subsets are more comparable to humans where many of the Th1 and Th2 cells produce a mixed pattern of cytokines.[3] It remains unclear if these feline IL-4+ T cells are Th2-like cells or Th0 cells because staining with anti-human IFN-γ antibodies was unsuccessful and staining for other cytokines was not performed.

In humans, mast cells and eosinophils at mucosal sites and mast cells in skin are able to produce IL-4.[5,27,28] In the present study, the putative feline IL-4 was produced primarily by T cells; no single cells staining positively for IL-4 were found in a double staining for CD4 or pan-T and IL-4. These findings were confirmed by the double labeling experiment for IL-4 and mast cell chymase in which different cells stained positive. This is similar to the results of a study in humans with atopic dermatitis using the same antibody reactive with IL-4. In that study only CD4+ cells and no mast cells or eosinophils stained positive for IL-4.[6]

In conclusion we proved with immunohistochemistry and western blots that feline CD4+ T cells in lesional and nonlesional skin of cats with allergic dermatitis do produce IL-4 and that the anti- human IL-4 antibody cross reacts with the feline IL-4 molecule. This observation is a next step in the characterization of the immunopathogenesis of feline atopic dermatitis.

Acknowledgements

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Chapter 7

Mast cells and eosinophils in feline allergic dermatitis: a qualitative and quantitative analysis

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Submitted

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Abstract

Mast cells and eosinophils are prominent cells in the perivascular infiltrate of cats with allergic dermatitis. It was the aim of our study to investigate quantitative and qualitative aspects in mast cells and eosinophils in these cats. Biopsies of lesional and nonlesional skin of cats with allergic dermatitis were stained with haematoxyline and eosin, Astra blue, Luna stain and with an enzymehistochemical method for the mast cell specific proteases chymase and tryptase. In the skin of allergic cats mast cells were predominantly localized diffusely in the superficial dermis, while the eosinophils were predominantly present in the deep dermis in a perivascular pattern. Mast cell counts were significantly higher in cats with allergic dermatitis ($P< 0.05$) compared to the control cats, however there was a wide range in the number of mast cells. Moreover, the number of eosinophils was significantly higher in the skin of allergic cats ($P < 0.05$) than in the skin of control cats, in which no eosinophils were found. There was no significant correlation between numbers of mast cells and eosinophils in the same biopsy.

With the enzymehistochemical staining for tryptase a significant lower number of mast cells was observed than with the staining for chymase and Astra blue staining in the allergic cats. These changes were observed in lesional and nonlesional skin of cats with allergic dermatitis. These observations indicate a generalized effect on mast cells in allergic dermatitis. In addition, subsequent to clinical signs, eosinophils are an important indicator for allergic dermatitis.

Introduction

Allergic dermatitis in cats is a frequent occurring skin disease with different etiologies. The most common causes of allergic dermatitis in cats are flea bite hypersensitivity, food hypersensitivity, and atopic dermatitis or combinations of these diseases.[1] Miliary dermatitis, is one of the most observed clinical manifestation of allergic dermatitis in cats. The histopathological findings comprise a perivascular to diffuse dermal infiltrate with emphasis on the presence of mast cells and eosinophils among macrophages, and lymphocytes.[2] In general, histopathological features do not allow differentiation between the various etiologies of allergic dermatitis. Hence, disease-specific tests such as intensive flea control programs, dietary tests, and intradermal allergy testing are required for diagnosis.

In addition to the classical histopathological findings, immunohistochemical studies have demonstrated the presence of CD4+ / IL-4+ T cells and an increased number of Langerhans cells and CD1a+ / MHC class II+ dendritic cells in lesional skin of cats with atopic dermatitis.[3-5] In conjunction with the evidence for the existence of feline IgE,[6,7] familial involvement has been described indicating a genetic background.[8-10]

These findings support that the group of cats with miliary dermatitis in which flea bite hypersensitivity and food allergy have been excluded and that preferably has positive skin test reactions towards aeroallergens, may have a pathogenesis comparable to atopic dermatitis (AD) in humans.

Mast cells (MCs) have important regulatory functions in inflammation,[11,12] but their role in the pathogenesis of human AD is still obscure. Contradictory findings were published concerning the production of IL-4 by MCs in skin of humans with AD.[13,14] Increased numbers of tryptase-positive MCs (T-MC) have been found in the superficial dermis of lesional and non-lesional human AD skin compared to skin of healthy individuals.[15] In humans, two different subtypes can be distinguished in the skin: MCs that contain both tryptase and chymase (TC-MC) predominate and few MCs contain only tryptase (T-MC).[16,17]

In human lesional AD skin there is an abundance of depositions of eosinophil derived proteins such as major basic protein (MBP) and eosinophilic cationic protein (ECP), but only few intact eosinophils.[18] The occurrence of eosinophil granule contents in feline lesional skin is unknown.

Healthy feline skin contains substantial numbers of MCs and only few eosinophils.[19] All MCs in healthy feline skin stained positive with an enzyme-histochemical method for tryptase and around 90 % of the MCs stained positive for chymase.[20] However, the existence of different MC subtypes in cats is not known.

The aims of this study were: 1) quantification of MCs and eosinophils in the skin of cats with allergic dermatitis and healthy control cats and 2) search for different subtypes of MCs (TC-MC, T-MC, and C-MC).

Materials and methods

Animals

Ten neutered (3 male and 7 female) domestic short hair cats with ages between 3 and 10 years (median 5.8 years), and a history of recurrent or persistent miliary dermatitis and pruritus were included in this study.

Parasitic skin diseases, dermatophytosis and food hypersensitivity were ruled out by standard diagnostic procedures.[1] An extensive flea control program was performed on all animals. This program included the use of topical parasiticidals, environmental adulticides and insect growth-regulators, and the oral administration of insect growth-inhibitors for at least 3 months prior to the study. Except for the described skin problems the animals were healthy. All cats were withdrawn from injectable glucocorticoids for at least 6 weeks and received no antihistamines or oral glucocorticoids for a minimum of 4 weeks prior to skin testing.

Cats were skin tested with a commercially available test containing 22 standardized allergens, a phosphate-buffered (negative) control solution, and a 0.01% histamine solution as a positive control (ARTU Biologicals BV, Lelystad, the Netherlands), while sedated with 10 mg/kg ketamine, 1 mg/kg xylazine, and 50 μg/kg atropine sulphate, administered intramuscularly. Immediately after skin testing, 1–3 punch biopsies of 6 mm diameter were taken of lesional skin from the dorsal flank area and in 5 cats also from the neck or the abdomen. Biopsies were stored in 10% phosphate-buffered formalin.

For the enzymehistochemistry additional 6 mm punch biopsies from lesional skin from the same body sites (n=8) and nonlesional skin (n=4) of cats with allergic dermatitis (n=8) were taken and immediately snap-frozen in liquid nitrogen and stored at –70 °C until used.

Nine neutered (5 male and 5 female) cats healthy domestic short hair cats owned by the Utrecht University, were used for control studies. Their ages ranged between 1 and 12 years (median 5 years). Skin biopsies were taken from locations similar to those in the cats with miliary dermatitis and tissues were processed as described above.

The study design was approved by the ethical committee of Utrecht University, Faculty of Veterinary Medicine. Control animals were kept according to the Utrecht University animal welfare policy.

Tissues

Paraffin embedded tissue sections were cut at 4 μm and stained with haematoxyline and eosin for routine histology, with Astra blue for detection of MCs and according to Luna's method for eosinophil counting.[21]

Enzymehistochemistry

Frozen tissue sections were air dried overnight and fixed in acetone for 6 minutes immediately prior to staining. For the tryptase staining (red-brown) a modification of the technique described by Caughey was used.[22] The sections were incubated for 30 minutes at 30 °C in a freshly prepared reaction mixture containing 0.54 mM Z-Gly-Gly-Arg-Na (Sigma Chemicals, Zwijndrecht, the Netherlands), 0.22 mM Fast Red salt (Sigma Chemicals, Zwijndrecht, the Netherlands) and 3.8% dimethylformamide in 50 mM Tris-HCl (pH 6.8). Slides were immersed in 1% cupric sulphate for 10 minutes at 20 °C, washed in deionized water for 5 minutes and mounted.

To stain for chymase (blue), a freshly prepared mixture containing 0.53 naphtol AS-D-chloroacetate (Sigma Chemicals, Zwijndrecht, the Netherlands) as substrate, 0.3 mM fast blue BB salt, 40 mM NaF and 1% Triton X-100 in 50 mM Tris-HCl (pH 6.8) was used. Before addition to the mixture, substrate was dissolved in 1ml dimethylformamide. Frozen sections were incubated for 10 minutes, washed in deionized water for 5 minutes and mounted.

A double-staining technique was developed for staining of the frozen sections for chymase as described above, immediately followed by the staining for tryptase omitting the washing step with cupric sulphate.

Immunohistochemistry

For detection of eosinophils, a rabbit polyclonal antibody reactive with human eosinophilic cationic protein (ECP) (a generous gift from Dr. E. Knol, Sanquin Blood Supply Foundation, Division CLB, Amsterdam, the Netherlands) was used for detection of free granule contents. Frozen sections were air-dried overnight and fixed with acetone for 7 minutes. Staining was performed as described previously.[3]

Quantification of cells

In each biopsy the number of MCs and eosinophils was counted twice at a magnification of 250 X over a length of 5 mm as determined using an ocular micrometer. The depth of the test area was limited by an imaginary line, drawn along the basis of the hair follicles.

Statistical analysis

For comparison of groups the paired t-test and variance analysis were used. The correlation analysis was used to examine the relation between eosinophils and MCs. The level of significance was set at 5 %.

Results

Intradermal allergy test

Skin test reactivity is summarized in Table 1. The group of cats with allergic miliary dermatitis is further categorized in three groups: 1. Cats with allergic dermatitis that may be atopic (n = 5), 2. Cats with flea bite hypersensitivity (n =1) and 3. Cats that have atopic dermatitis and flea bite hypersensitivity (n = 4).

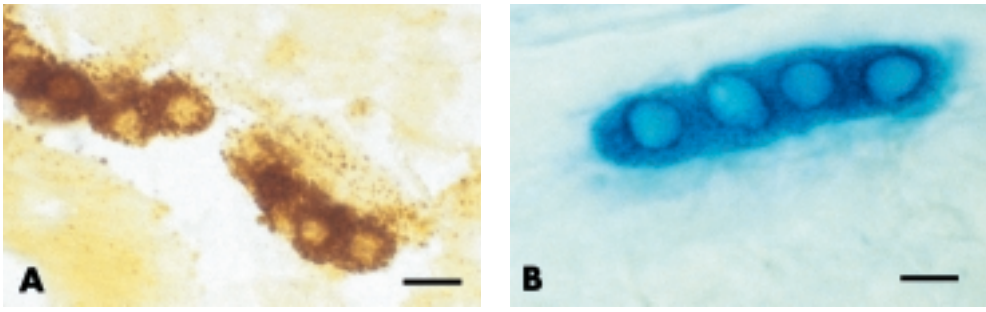

Figure 1. Mast cells stained positive for tryptase (A) and chymase (B). Bar = 25 μm.

Table 1. Results of intradermal skin test reactivity. x= Positive reactions to allergens or no visible reaction to any of the allergens.

Clinical cases	Allergens			
	Aero-allergens	Flea antigen and aero-allergens	Flea antigen	No positive reactions
1			x	
2		x		
3				x
4		x		
5		x		
6	x			
7	x			
8				x
9		x		
10				x

Histology

In the control cats the epidermis was thin (1–2 cell layers). In two cats a slight increase of the cellularity of the superficial dermis was noticed due to MCs.

In the biopsies of all cats with miliary dermatitis a slight to distinct acanthosis was found. In some cases a slight (Nos. 1, 4 and 9) to moderate (Nos. 7 and 8) focal spongiosis was present. Serocellular crusts frequently covered the epidermis. Ulceration was present in five cases. Only in two cases (Nos. 2 and 8) some lymphocytic exocytosis was seen.

In all cases there was a perivascular to diffuse inflammation present, predominantly in the superficial part of the dermis. The inflammatory infiltrate consisted of MCs and eosinophils, mixed with a smaller number of lymphocytes and macrophages, and occasionally neutrophils. Additional findings were folliculitis in case No.7 and focal single cell necrosis in the epidermis of case No. 8.

Mast cells

In the control cats, Astra blue stained MCs, were predominantly present in the superficial dermis in a perivascular to diffuse pattern. In the deep dermis MCs were often found around the adnexa as single cells and as small nests or rows of up to five cells.

In cats with miliary dermatitis MCs were primarily present in the superficial dermis, usually in a diffuse pattern, and in some cases in a more perivascular arrangement (Table 2). In three cats a few MCs were localized within the epidermis. In three other cases some MCs were found within arrector pili muscles.

In the flank skin the number of MCs (Table 3) was significantly higher in clinical cases (mean ± SD: 740 ± 336) than in control cats (mean ± SD: 233 ± 79). In clinical cases as well as in control cats the number of MCs in the flank was not significantly different from other body regions. The results of the first and second cell counts were strongly correlated ($r = 0.9$).

Enzymehistochemistry

In sections stained for tryptase and chymase the positive cells were situated in the superficial and mid-dermis. The chymase positive-MCs consisted of two different populations; i. e. one relatively large group of MCs with fine granules (Fig. 1b) and a small group of MCs containing coarse granules. In lesional skin significantly more coarse-granulated MCs were present compared to nonlesional skin (Table 4). In lesional skin of cases 4, 5, and 8, many extracellular chymase positive granules were scattered throughout the dermis. There was no significant difference between lesional and nonlesional skin with regard to the number of tryptase positive staining cells (Table 4, Fig. 1a).

Results of the double labeling for chymase and tryptase are not included as it was impossible to differentiate between double-labeled cells (brown and blue) with coarse granules and single staining cells for chymase (blue). The coarse blue staining granules masked the finer brown (tryptase) granules.

Table 2. Localisation of eosinophils (Luna stain) and mast cells (Astra blue stain) in the skin of 10 cats with allergic dermatitis.

Cat	Eosinophils						Mast cells				
	Epidermis	Dermis					Epidermis	Dermis			
		superficial			deep			superficial		deep	
	intra-epidermal	focal accumulation	pv	diff	pv	diff	intra-epidermal	pv	diff	pv	diff
1			+		++	±	+	+	++		+
2	+	+		+++	+	+			++		+
3		+		+	+	+			++		+
4		+		+	+	+			+		±
5			+	+	+	±			+	±	±
6	±	+	+	+	+	+			++		+
7	±	+		+	++	+	+		+++	+	+
8				+	+	±	+	+	++		+
9	+	+	+	+	+	+			+		±
10		+	+	++	+	+		±	+		±

+ to +++: depicts the increasing number of cells present; ±: occasional cell; pv: perivascular and diff: diffuse

Table 3. Number of mast cells and eosinophils in the dermis of cats with allergic dermatitis and healthy control cats.

	Clinical cases				Control cats		
	Mast cells		Eosinophils		Mast cells		Eosinophils
Cat	flank	elsewhere	flank	elsewhere	flank	elsewhere	all locations
1	570–620[1]	nd[2]	142–154	nd	180–190	170–190	0
2	443–498	nd	1963–2223	nd	155–190	205–215	0
3	780–830	520–570	730–740	1675–1850	210–225	150–155	0
4	530–560	280–300	140–150	1630–1780	120–130	185–190	0
5	590–620	nd	120–140	nd	215–230	195–200	0
6	710–760	650–690	1250–1355	720–810	190–215	200–218	0
7	963–1070	nd	1013–1092	nd	305–335	378–403	0
8	1560–1820	1840–2070	49–58	515–575	375–390	240–265	0
9	550–580	410–440	610–620	1890–2135	275–290	270–300	0
10	328–490	nd	170–184	nd	nd	nd	0
mean±SD	740±336 (n=10)	777±638 (n=5)	735±725 (n=10)	1358±658 (n=5)	233±79 (n=9)	221±58 (n=9)	0

1) Repeated counts
2) nd = not done

Table 4. Number of cells staining positive for tryptase or chymase in nonlesional skin and lesional of cats with allergic dermatitis.

Clinical cases	Nonlesional skin			Lesional skin					
				site a			site b		
	tryptase	chymase		tryptase	chymase		tryptase	chymase	
		total	coarse		total	coarse		total	coarse
1	103–1111	372–380	0	14–15	160–172	0	180–173	257–245	7–4
2	15–16	242–262	0	146–138	512–477	0	21–22	219–208	14–15
3	63–67	427–396	5–5	56–60	405–396	42–46	356–372	831–879	154–159
4	59–61	515–506	0	295–290	638–666	103–96	135–141	1036–1019	372–394
5				12–14	324–316	75–81	510–535	704–689	9–11
6				37–41	546–550	76–70	22–25	485–503	95–93
7				88–84	895–852	40–47	nd	nd	nd
8				104–110	361–376	71–70	94–90	322–306	28–29
Mean±SD	61.98±37.4	387.3±106.7	1.4±2.8*	94.0±91.9	477.9±217.8	51.1±36.4*	191.1±186.8	550.1±317.11	98.9±137.1*

1) Repeated Counts
* is $P < 0.05$
Site a and site b are biopsies of two different locations

Eosinophils

Eosinophils were not observed in the biopsies of the control cats.

The skin of the patients contained many eosinophils in both superficial and deep dermis (Table 2). In the deep dermis a predominantly perivascular pattern was found, while in the superficial dermis this pattern was less distinct. In seven cases large foci of eosinophils were present in the superficial dermis. In the epidermis of two cats exocytosis of eosinophils was found.

The eosinophil counts in the flank (mean ± SD: 735 ± 725) and other locations (1358 ± 658) were significantly higher than in control cats. The number of eosinophils in the flank did not differ significantly ($P > 0.05$) from other locations (Table 3). No significant correlation was found between numbers of MCs and eosinophils in the same biopsy. The number and distribution of MCs and eosinophils in cats with a positive or negative intradermal test did not differ significantly. The results of the first and second cell counts showed a strong correlation ($r = 0.9$).

Immunohistochemistry

The rabbit polyclonal antibody reactive with human ECP did not stain feline eosinophils or free granule content in frozen sections.

Discussion

Most of the histological features of lesional skin in cats with allergic dermatitis lesions were similar to those described previously.[2] This with exception of epidermal spongiosis that was not prominent in our cats and vesicopustular lesions that were not seen in our cats. In a number of cases there were distinct focal accumulations of eosinophils in the epidermis and superficial dermis. Additionally, a deep perivascular infiltrate with many eosinophils was often present. According to Gross et al.[2] this type of deep dermatitis may be suggestive of food allergy. However food allergy had been excluded in our cases by means of a restricted home-cooked diet for 8 weeks. Therefore our data indicate that this type of inflammation can also occur in cats with allergic dermatitis of different origin. We did not observe any eosinophils in the skin of control cats, although the presence of some eosinophils has been described in healthy cats.[1] No difference was seen in the number of MCs or eosinophils comparing cats with positive intradermal skin test reactions to flea allergen, cats with reactions to other allergens or cats that only had a reaction to the positive control solution (histamine). The value however of positive skin reactions to fleas is a matter of debate as in one study 36 % of the normal cats showed immediate skin test reactions to flea allergen.[23] Moreover, a recent study did not detect a statistical association between presence of clinical signs of flea bite hypersensitivity and positive results on intradermal skin test in cats experimentally exposed to fleas.[24]

Mast cell numbers defined by the Astra blue stain were significantly higher in lesional skin of cats with allergic dermatitis than in control cats. Also in humans with AD, MC numbers are higher in chronic lesional skin.[25] In acute lesional skin however, MC numbers are not different from those in healthy individuals.[26] Although similar lesions were biopsied in the cats with allergic dermatitis, the chronicity of the lesions may have varied.

The role of mast cell proteases in the pathogenesis of atopic dermatitis is still obscure but the various actions of both tryptase and chymase can modulate inflammation (reviewed in Welle, 1997).[27]

Feline MCs were studied for their protease content because changes in MC proteases have been described in skin of humans with AD.[15,28] With tryptase staining significantly less cells were seen in lesional and nonlesional feline skin than with chymase staining (ratio tryptase: chymase =1: 2,54 – 6). In contrast, in healthy cats, slightly more tryptase than chymase positive cells were described.[20]

As we were unable to study MCs by double-labeling we could not determine the changes in the percentages of possible MC subtypes (MC-T, MC-C, and MC-TC). It is however likely that in cats the majority of MC is of the MC-TC subtype, considering information available on human[17] and canine skin,[29] and the findings in healthy cats.[20] In the clinical cases there were changes in both lesional and nonlesional skin. This indicates that allergic dermatitis is associated with a generalized effect on MCs. This is in agreement with earlier findings of increased numbers of CD4+ T cells and increased numbers of IL4-positive cells in lesional and nonlesional skin of cats with allergic dermatitis.[3,4]

A substantial number of the chymase positive MCs in the lesional skin contained coarse granules. These coarse granules have not been described in healthy feline skin[20] and were not present in nonlesional skin except for one cat. Interestingly, to our knowledge, this feature has not been described in dogs with AD[30] or humans with AD. MCs, however, are known to be very heterogeneous cells with differences in histochemical, biochemical, and functional characteristics (reviewed in Welle, 1997).[27] Fusion of mast cell granules was seen with electronmicroscopy in cats after antigen-induced degranulation and this could be an explanation for our observations.[31]

Scattered extracellular chymase but not tryptase deposits were observed in lesional skin. Immuno-electronmicroscopy, has revealed that chymase and tryptase are localized within the same granule in human MC-TC.[16] Nevertheless, within these granules, they are packaged separately.[32] Extracellular chymase has been described in human skin explants after induction of mast cell degranulation.[33] Our enzymehistochemical method may have detected only tryptase bound to heparin in its active tetrameric form as it resides in the granules[34] and not externalized tryptase. Additionally, changes in the mast cell micro-environment in allergic skin may induce production of protease inhibitors which may selectively target extracellular tryptase as was hypothesized by Schechter et al.[35]

With the tryptase staining significantly less MCs were observed in lesional and nonlesional skin of cats with allergic dermatitis than with the Astra blue stain. The reverse is true for healthy cats.[20] Degranulation of MCs is likely to be the cause although the existence of a tryptase -negative MC subtype in allergic dermatitis skin theoretically can not be excluded.

MCs are often prominent cells in the cellular infiltrate of feline allergic dermatitis and are known to produce many mediators and cytokines. Amongst them are substances that can attract eosinophils (e. g. tryptase, IL-4, IL-5).[36,37] Hence, the presence of activated MCs is often associated with eosinophils. In our clinical cases eosinophils were usually present together with an increased number of MCs. However, no correlation was found between numbers of MCs and eosinophils in the same biopsy. The biopsy site appeared not to be crucial, because no significant difference was found between numbers of MCs and eosinophils in different body locations. Although the mean number of MCs in lesional skin was significantly higher in clinical cases than in control cats, the range was wide indicating that in some cats

with allergic dermatitis, mast cell numbers may not differ from those in healthy animals. Thus it seems that eosinophils have the higher specificity. They are usually present in cases of allergic dermatitis and not in skin of healthy control cats.

It was demonstrated that IL-4 induces chemotaxis of blood eosinophils from humans with AD but not from normal individuals.[38] In addition, it was described that eosinophils from human AD patients stimulated with IL-4 and granulocyte-macrophage CSF (GM-CSF) produce biologically active IL-12.[39] Consequently, eosinophils may play a role in the switch from the Th2 to Th1-like immune response in atopic disease.

In summary, the results of our study indicate that: 1) Eosinophils are an important indicator of feline allergic dermatitis and can be present in the deep dermis and without a relationship with food allergy, 2) The number of mast cells is high, predominantly in the superficial dermis, 3) There is no correlation between numbers of mast cells and eosinophils in the same biopsy, 4) The location of the biopsy site does not influence the outcome, 5) The cellular reaction is not different for cats with positive and negative intradermal allergy test results, 6) There is a distinct change in mast cell protease content in cats with allergic dermatitis compared to healthy cats.

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Chapter 8

Atopy patch testing in cats with atopic dermatitis: a role as experimental model for chronic atopic dermatitis in humans?

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Abstract

Background: Cats with spontaneously occurring atopic dermatitis have clinical and immunocytochemical characteristics compatible with these in humans with atopic dermatitis (AD). To explore the suitability of feline atopic dermatitis as an animal model for humans with chronic AD we adapted the atopy patch test (APT) methodology for use in cats.

Objective: To induce macroscopically positive patch test reactions in cats and to investigate the composition of the cellular infiltrate.

Methods: APT were performed in cats (n=6) with atopic dermatitis and healthy cats (n=10). All cats were patch tested with two allergens in three different dilutions according to intradermal test and prick tests results. APT were read after 10, 24 and 48 hours and punch biopsies for immunohistochemical evaluation were collected at these time points.

Results: Macroscopically positive APT reactions were observed in 3 out of 6 cats at 24 and / or 48 hours with allergen concentrations of 25.0000 and 100.000 NU/ml. Reactions were not observed at negative control sites and neither in control animals.

A significantly increased number of IL-4+, CD4+, CD3+, MHC class II+ and CD1a+ cells was found in one AD cat with positive APT reactions. Five out of 6 AD cats had significantly increased IL-4+ T cell numbers at 24 and / or 48 hours.

Conclusion: These observations indicate that in cats macroscopically positive patch test reactions can be induced which have a cellular infiltrate similar to that in lesional skin. We found a high specificity and a macroscopically positive APT reaction in half of the cats which is similar to what is seen in humans. Hence, cats with AD may be a new model for studying chronic AD in humans.

Introduction

Atopic dermatitis (AD) in humans is an IgE-mediated skin disorder with a multifactorial pathogenesis including genetic, environmental, immunologic and pharmacologic factors.[1] In addition, a relationship with allergen exposure and state of disease was demonstrated.[2,3] A double-blind controlled trial, testing the effect of house dust mite allergen avoidance in atopic dermatitis, confirmed this relationship.[4]

The epicutaneous application of aeroallergens, the atopy patch test (APT), has been widely used and has proven to be a good model for studying the onset of allergic inflammation in the skin of humans.[5-8] Analysis of the cytokine pattern expressed in situ revealed a biphasic pattern with an increased expression of IL-4 mRNA and IL-2 mRNA at 24 hours and an increased expression of IFN-γ mRNA and IL-2 mRNA at 48 hours compared to control skin.[9] These findings were confirmed at the protein level by Thepen et al,[10] who described a predominance of IL-4 positive T cells over IFN-γ positive T cells in 24 h APT reactions and an increased number of IFN-γ producing cells compared to IL-4 producing cells in 48- and 72 h APT reactions, and in chronic lesional skin. A comparable switch in cytokine production has been observed in acute versus chronic AD skin. In acute lesional AD skin more IL-4 mRNA expressing cells were found than in chronic lesional skin.[11] For studying chronic AD in humans, there are no appropriate animal models available.

Various observations in cats with AD indicate similarity to the human disease. At present, however limited information is available on the pathogenesis in cats. The histopathology of feline AD is characterised by a perivascular to diffuse dermal infiltrate with lymphocytes, mast cells, eosinophils, and macrophages.[12] Several studies indicate the existence of a putative feline IgE.[13-17] In addition, familial occurrence suggests a role for genetic factors.[18-20] We described an increased number of CD4+ T cells compared to CD8+ T cells in lesional skin of allergic cats[21] and demonstrated an increased IL-4 expression by CD4+T cells in lesional and nonlesional skin of AD cats.[22] Significantly increased numbers of CD1a+ cells and MHC class II+ cells were found in the epidermis and dermis of AD cats suggesting a pathogenic role of Langerhans cells.[23]

Although cats have been used successfully as an experimental model for airway hyperresponsiveness, to our knowledge there are no reports on the use of cats as an experimental model for AD in humans.[24,25]

By investigating if epicutaneously applied allergens can induce macroscopically positive APT reactions in AD cats and characterising the cellular infiltrate at the APT skin sites of cats with spontaneous AD and healthy cats by immunophenotyping, the suitability of feline AD as an experimental model for studying chronic AD in humans, will be explored.

Materials and Methods

Animals

Two castrated male and 3 spayed female domestic short hair cats, and one castrated male Norwegian Forest cat with ages between 1.5 and 11 years (median 5 years), with chronic and recurrent glucocorticoid responsive pruritus and dermatitis, a histopathology of lesional skin indicating allergic inflammation,[12] and positive reactions to intradermal testing were included in this study. Parasitic skin diseases, dermatophytosis and food hypersensitivity were ruled out

by standard diagnostic procedures.[26] Except for the skin problems the animals were healthy. All AD cats were withdrawn from glucocorticoids for at least 6 weeks and received no antihistamines or oral glucocorticoids for a minimum of 4 weeks prior to skin testing.

Five castrated male cats and 5 spayed female healthy domestic short hair cats were used as control animals. Their ages ranged between 1 and 7 years (median 4 years). APT and prick test were applied to the control animals in the same way as in the AD cats.

The healthy control cats were kept under conditions as demanded by the University of Utrecht. The study was approved by the committee for use of experimental animals, University of Utrecht.

Allergy skin tests

In addition to the intradermal test (ARTU Biologicals Europe BV, Lelystad, the Netherlands), which is the standard method for skin testing in cats and dogs,[26] a prick test was performed at the same time. In the prick test a selection of 7 allergens was used, that was also included in the intradermal skin test *(Dermatophagoides farinae, D. pteronyssinus, Tyrophagus putrescentiae),* grass pollen mixture *(Dactylus glomerata, Phleum pratense, Syndactylon, Anthaxatum odoratum,* and *Holcus lanatus), Poa pratensis, Lolium perenne,* and *Ambrosia eliator*. Of each allergen / allergen mixture (ARTU Biologicals Europe BV, Lelystad, the Netherlands) 3 concentrations were used (5.000 NU[Noon Units]/ml, 25.000 NU/ml, and 100.000 NU/ml) together with a positive control (1% histamine solution) and a negative control solution (allergen diluent solution). The allergen diluent solution consisted of a buffered sterile solution of 0.37% sodium chloride, 0.40% phenol, sodiumdihydrogenphosphordihydrate, and disodium hydrogenphosphordihydrate (pH 7.30-7.50) (buffer solution, ARTU Biologicals Europe BV, Lelystad, the Netherlands).

The prick test was performed while the cats were sedated with 10 $mg.kg^{-1}$ ketamine, 1 $mg.kg^{-1}$ xylazine, and 50 $mcg.kg^{-1}$ atropine sulphate, administered intramuscularly. Drops of the allergen dilutions and control solution were applied on the clipped, non-lesional, lateral side of the thorax with the cat in lateral recumbency. The skin was pierced through the drops with a lancet (Unilet GP superlite, Owen Mumford Ltd., Oxford, England). The skin was observed for 30 minutes after pricking and reactions were interpreted at 15 minutes interval after pricking with transillumination and palpation of the prick site. Reactions were considered positive when a distinct erythematous papule was visible and palpable in absence of a reaction of the negative control dilution. The prick test was performed in control cats as in the AD cats.

Atopy patch test (APT)

The patch test in AD cats and healthy control animals was performed according to the procedure described for humans by Langeveld-Wildschut et al.[27] On each animal 3 patch strips containing 7 discs each (Finn chambers, Epitest Ltd. Oy, Tuusula, Finland), one for each time point of evaluation: 10, 24, and 48 hours, were applied on non-lesional skin of the thorax at 24 hours after clipping and chemical epilation of the hairs (Opilca cream, Schwarzkopf & Henkel, Düsseldorf, Germany), and after 10× skin stripping with adhesive tape (Leukofix, Beiersdorf AG, Hamburg, Germany). The strips were protected with an elastic bandage. The allergens (ARTU Biologicals Europe BV, Lelystad, the Netherlands) were applied on the discs

in the three standard dilutions (5.000, 25.000, and 100.000 NU/ml). Because of the limited feline body size only 2 allergens in 3 dilutions and a diluent control could be tested. The allergens for the APT were selected from positive intradermal test and /or prick test results and consisted of: *D. farinae, D. pteronyssinus, Tyrophagus putrescentiae,* and a grass pollen mixture. In the control group, 5 cats were tested with *D. farinae and D. pteronyssinus* and 5 other cats were tested with *T. putrescentiae* and grass pollen mixture dilutions and a diluent control for each time point.

Biopsies

Four mm punch biopsies were taken under sedation with medetomidine HCl (100 μg/kg i.m.) from the macroscopically positive patch test sites and the diluent control sites at 10, 24, and 48 hours after application. When there were no positive reactions visible, the site of the highest concentration was biopsied. After biopsy collection cats received atipamezole HCl (250 μg/kg i.m.) to antagonize the effect of medetomidine. Skin biopsies were immediately snap-frozen in liquid nitrogen, and stored at -70 °C until used.

Immunohistochemistry

Sequential biopsy sections (6 μm) were air-dried overnight and fixed with acetone for 7 minutes. Non-specific binding was blocked by preincubation for 25 minutes with phosphate-buffered saline (PBS) containing either 10 % horse serum and 10 % cat serum or 10 % goat serum and 10 % cat serum depending on the secondary antibodies used. Primary antibodies were murine monoclonal antibodies against feline CD1a (FE 1.5F4),[28] MHC class II (42.3),[29] CD4, CD8 (vpg34 and vpg9),[30] a murine monoclonal antibody against human IL-4 (1-41-1, Novartis, Vienna, Austria) and a polyclonal rabbit antibody against CD3 (AO452, DAKO, Dakopatts A/S, Glostrup, Denmark). Biotin-conjugated horse anti-mouse antibody (Vector, Burlingame, CA) or biotin-conjugated goat anti-rabbit antibody (Vector, Burlingame, CA) was used as a second step, followed by alkaline phosphate-conjugated streptavidin (Dakopatts A/S, Glostrup, Denmark). Staining was developed using naphthol AsBi phosphate (N-2250, Sigma, St. Louis, MO) as substrate and New Fuchsin as a chromogen, resulting in pink-red staining. Control staining was performed with an isotype-matched antibody (Becton Dickinson, San Jóse, CA) and resulted in no staining. Sections were lightly counterstained with hematoxylin.

Positive staining cells in the superficial dermis were counted in 4 adjacent high power fields (400 ×) and expressed as cells/mm^2. Cells in hair follicle walls and deep dermis were not included.

Statistical analysis

ANOVA for repeated measurements was used to compare the number of stained cells at different time points for both AD cats and control cats.

The Students-t-distribution test was used for comparison of pooled data of the three time points. A one sample-t-test was used to compare the pooled data of each marker staining for the group of AD cats with the control animals. A P value of less than 0.05 was considered significant.

Results

Prick test

In the prick test there was a concordance with the intradermal test results in 3 out of 6 tests for *D. farinae,* 4 out of 6 for *D. pteronyssinus,* 3 out of 6 for *T. putrescentiae,* and 4 out of 6 for the grass pollen mixture (Table 1). Despite the negative intradermal test in one AD cat, a reaction to grass pollen mixture was present in the prick test. This was considered relevant as clinical signs of AD were seasonal in this cat. The prick test and the intradermal test were negative in all control animals.

Atopy patch test

Three out of 6 AD cats had erythematous reactions to allergens. These were considered to be positive reactions. None of the control cats (n=10) had positive reactions and no reactions were seen on the negative control site in the AD cats except for one reaction that was related to a clipper burn. Macroscopically positive reactions appeared predominantly after 48 hours. However, in one cat positive reactions were already observed after 24 hours (Table 2). Reactions occurred mainly with allergen concentrations of 100.000 and 25.000 NU/ml. One of these 3 AD cats reacted to the grass pollen mixture in a dilution of 5.000 NU/ml at 48 hours.

Immunohistochemistry

The results obtained with the immunohistochemistry staining are shown in Table 3 for the healthy control animals and Table 4 for the AD cats.

Neither in the AD cats nor the healthy control cats there was a significant difference in the negative control sites (i.e. $NC_{10\ hours}$, $NC_{24\ hours}$, $NC_{48\ hours}$) ($P > 0.05$) (ANOVA for repeated measurements).

There was also no significant difference in time (10, 24, and 48 hours) in the number of stained cells at the allergen site corrected for the negative control value at that time point (e.g. $Dp_{10\ hours} - NC_{10\ hours}$), neither in the group of the AD cats nor in the group of control animals ($P > 0.05$). For that reason we pooled the results of the three time points, for each marker and allergen and for each cat. We then determined, for each marker and allergen, in the group of normal cats the mean and standard deviations and calculated (using Students-t-distribution) the 95 % upper limit values in the group of normal cats. Values in animals exceeding these 95 % upper limits were considered to be abnormal.

We found that one AD cat (no. 1) had a significant higher number of stained cells for IL-4, CD4, CD3, MHC class II, and CD1a at 24 hours compared to the control animals (Table 3; one-sample t-test; values exceeding the 95 % upper limit). This AD cat had macroscopically positive APT reactions for *D. farinae* and *D. pteronyssinus.* At 48 hours APT this cat still showed a significant increase for these markers except for CD1a and MHC class II for the Dfar-APT. For the 48 hours Dpt-APT a significantly increased number of cells staining positive for IL-4, CD3, and MHC class II were found. This cat also had positive APT reactions with Dpt at 25.000 NU/ml and Dfar at 25.000 NU/ml, both at 48 hours APT. The cell numbers in the Dfar reaction were significantly increased for all markers except CD8 when compared to the control cats.

Table 1. Results of the intradermal test (idt) and prick test (pt) in cats with atopic dermatitis (n=6). Only allergens used in both tests are depicted (*Dermatophagoides pteronyssinus* (Dpt), *D. farinae* (Dfar), *Tyrophagus putrescentiae* (Tyro) and grass pollen mixture (GP)). – : negative test result. + : positive test result.

Allergen	Dfar		Dpt		Tyro		GP	
Test	idt	pt	idt	pt	idt	pt	idt	pt
Cat no. 1	+	–	+	+	–	–	–	–
Cat no. 2	–	+	–	+	+	+	–	+
Cat no. 3	–	–	–	–	–	+	–	+
Cat no. 4	+	+	+	+	–	+	–	–
Cat no. 5	+	–	–	–	+	–	–	–
Cat no. 6	+	+	+	–	+	+	–	–

Table 2. Results of the APT in AD cats with one or more positive reactions to aeroallergens. Cat no. 1 was tested with *Dermatophagoides pteronyssinus* (Dpt) and *D. farinae* (Dfar), cat no. 2 was tested with *Tyrophagus putrescentiae* (Tyro) and *D. farinae,* and cat no. 3 with *D. farinae* and grass pollen mixture (GP). * Cat number 3 also had a positive APT reaction to a concentration of 5000 NU/ml of grass pollen mixture at 48 hours.
nd = not done. + = macroscopically positive.

Time after APT	24 h								48 h							
Allergen	Dpt		Tyro		Dfar		Gp		Dpt		Tyro		Dfar		GP	
Allergen concentration ($x10^3$ NU/ml)	25	100	25	100	25	100	25	100	25	100	25	100	25	100	25	100
Cat no. 1	–	–	nd	nd	–	–	nd	nd	+	+	nd	nd	+	+	nd	nd
Cat no. 2	nd	nd	–	–	–	–	nd	nd	nd	nd	+	+	+	+	nd	nd
Cat no. 3*	nd	nd	nd	nd	–	+	–	+	nd	nd	nd	nd	+	+	+	+

Table 3. Pooled number of cells /mm² per allergen (100.000 NU/ml) of five healthy control cats tested with Dpt and Dfar (APT-1) or Tyro and GP (APT-2) at 10, 24, and 48 hours each corrected for the corresponding negative control values. The first numbers in each column depict the mean values. In the second column 95% upper limit values are depicted. DC-values are the means of the diluent control sites of the 2 groups of control cats (DC-1 (APT-1) and DC-2 (APT-2)), respectively.

	APT-1				APT-2				DC-1	DC-2
	Dfar		Dpt		Tyro		GP			
	mean	95 %	mean	95 %	mean	95 %	mean	95 %	mean	mean
IL-4	0.1	2	0.5	3	–0.2	0.5	–0.2	0.5	2	4
CD4	–1	8	1	11	–1	11	1	14	5	5
CD3	5	18	5	19	4	33	–1	21	11	13
CD8	4	21	2	17	0	6	0	5	7	25
MHC II	14	59	10	42	0	20	0	31	34	37
CD1a	–2	10	–1	10	–2	6	1	11	8	9

Table 4. Number of positive staining cells corrected for the negative control values after 24 hours (first number per column) and 48 hours patch testing with allergen concentrations of 100.000 NU/ml *(Dermatophagoides pteronyssinus* (Dpt), *D. farinae* (Dfar), *Tyrophagus putrescentiae* (Tyro) or grass pollen mixture (GP). The patient numbers with an asterix were the cats that had positive APT reactions. Significant values are written in italics.

Allergen	Patient	IL-4		CD4		CD3		CD8		MHC II		CD1a	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Dfar	1*	*8*	*18*	*21*	*12*	*27*	*44*	0	-8	*59*	46	*60*	9
Dfar	2*	*11*	-1	3	*16*	12	*59*	0	-1	36	44	1	*28*
Dfar	3*	1	*3*	6	-4	0	-11	2	-1	-14	*91*	5	8
Dfar	4	0	0	-12	-5	*25*	0	-1	0	7	-4	-10	-12
Dfar	5	*6*	1	6	-5	11	4	0	-1	3	49	-10	*24*
Dfar	6	*5*	*9*	2	1	-16	-2	2	-5	6	34	2	6
Dpt	1*	*10*	*8*	*17*	*7*	*39*	*32*	0	-5	*69*	*47*	*60*	-12
Dpt	4	0	0	-7	4	*23*	15	-1	0	-19	21	-2	*16*
Tyro	2*	*1*	-6	-1	*22*	5	32	0	-1	0	*29*	-2	*13*
Tyro	5	*5*	*4*	9	*19*	14	25	4	2	*31*	*99*	-3	*56*
Tyro	6	*2*	*14*	0	*12*	-7	9	3	0	-10	*25*	*9*	4
GP	3*	*2*	*1*	7	2	8	-3	0	-2	*44*	*59*	*12*	-5

Because only AD cat no 1. had significantly increased cell numbers with the allergen concentration of 25.000 NU/ml as well, cell numbers of positive APT reactions with the lower concentration are not shown in a table. At 24 hours Tyro-APT cells staining positive for IL-4 (cat no. 2) and IL-4 and MHC class II (cat no. 5) were significantly increased. At 48 hours Tyro-APT, AD cats no. 2, and 5 had significantly increased numbers of IL-4+ cells (only cat no. 5), CD4, MHC class II, and CD1a. In 24 hours Tyro-APT skin of AD cat no. 6 significantly increased numbers of IL-4+ and CD1a+ cells were counted. At 48 hours, the numbers of IL-4+, CD4+ and MHC class II+ cells were significantly increased. Skin of GP-APT AD cat no. 3 contained significantly more cells staining positive for IL-4, MHC class II and CD1a at 24 hours and 48 hours (only IL-4 and MHC class II).

In none of the APT reactions in the AD cats we found a significant increase in the number of CD8+ cells.

Discussion

This report is the first to describe atopy patch testing in cats as an approach to study spontaneous AD in cats and to explore its suitability as an experimental model. In humans the APT has been documented to be a very useful test in mimicking the natural disease process.[8,10] The reported number of positive reactions induced with this test in humans varies greatly (15% to 100 %) and seems influenced by the method used (i.e. allergen concentration, tape stripping, allergens dissolved in liquid or petrolatum, and reading time).[27]

Tape stripping was used to remove superficial layers of the stratum corneum, thereby increasing the chance of allergen penetration which may also happen spontaneously when cats lick themselves as part of their natural grooming behaviour or more likely when the licking has increased due to pruritus. Clipping and additional chemical epilation was necessary to ensure adhesion of the tape with Finn chambers.

We observed macroscopically positive APT reactions in 3 AD cats and in none of the healthy control animals. In addition, we did not find positive reactions towards the negative controls (diluent solution) in the AD cats. Hence, we conclude that the APT in cats can elicit positive reactions macroscopically. A dilution of 100.000 NU/ml elicited more macroscopically positive reactions than lower allergen concentrations. In addition, the cellular infiltrate in the positive APT reactions evoked by low allergen concentrations was less prominent. We therefore conclude that a minimum concentration of 100.000 NU/ml is preferable. We did not observe however, a consistent relationship between macroscopically positive reactions and cell numbers at the patch site. Like in cats of the present study, increased T cell numbers, however, have been described in macroscopically negative patch test sites in humans as well.[31]

An important point is the question of whether positive reactions are evoked by a response to the allergen or by an irritant reaction. It has been reported in humans that purified house dust mite major allergens, Der p1 and Der p2, not only can be allergenic, but also can have an additional proteolytic irritant effect, and induce irritant reactions in the APT.[32] The fact that only few of the AD cats and not all of them reacted to the allergen application without reactions at the negative control site speaks in favour of a specific reaction. In addition, all of our healthy controls did not respond to the allergens which favours an allergic response.

We cannot exclude that the allergen solutions prepared from whole mite cultures may have contained such substances that could induce irritant reactions although we have not found any documentation on irritant properties of *D. pteronyssinus* or *D. farinae* related enzymes in cat skin. Recently, it was reported that 68 % of sera of AD cats containing antigen-specific IgE against *D. farinae* reacted with a *D. farinae* chitinase.[33] We cannot exclude that this chitinase has a proteolytic irritant effect as well, causing a positive APT reaction. The positive APT reaction towards *D. farinae* in AD cat no. 3 that had only a positive prick test reaction towards grass pollen could have been caused by this proteolytic irritant effect. However, the significant increase in IL-4+ T cells in the APT in all AD cats except cat no. 4, is in support of a true allergic reaction. This is supported by the fact that in humans with AD it has been reported that in irritant patch test reactions there is no increase of IL-4 mRNA.[9] Moreover, sensitivity to irritants is equal in AD patients having a positive or a negative APT response towards house dust mite.[34]

The cellular infiltrate in the positive APT reactions was very similar to that in lesional skin of AD cats, i.e., an increase in IL-4+, CD4+, CD3+, MHC class II+, and CD1a+ cells.[21-23]

In contrast to the situation for the other markers, in none of the AD cats the number of CD8+ cells was significantly increased. The resulting increased CD4/CD8 cell ratio mimics the natural disease in cats and in humans.[21,35,36] In the APT reaction in humans a rapid increase in T cell numbers is seen until 48 hours, reaching a maximum for the CD8+ cells at 24 hours APT and for the CD4+ cells at 48 hours APT. However, we did not observe a consistent increase in T cells comparing results at 24 hours with 48 hours.

In mice percutaneous sensitisation with allergens through barrier-disrupted skin elicits a Th2-dominant cytokine response.[37] As there was no antibody available that is reactive with feline IFN-γ, we could not differentiate between a Th1, Th2 or Th0 response. Nevertheless, the finding of increased numbers of IL-4+ T cells may indicate a Th2 / Th0 response.

In humans, an increased number of CD1a+ cells, T cells and macrophages has been reported to occur in the dermis of positive and negative patch tests reactions.[38] Similar to these findings we found significantly increased numbers of CD1a+cells and MHC class II+ cells in both macroscopically positive APT sites and in negative APT reactions. We have no explanation for the high concentrations of MHC class II+ cells and the CD1a+ cells at the negative control sites of the control cats, although theoretically an occlusion artefact or a response to depilation may be responsible.

Summarising, we conclude that we were able to induce positive atopy patch test reactions in AD cats with a cellular infiltrate similar to cellular infiltrate in lesional skin. Consequently, the APT is an appropriate tool to study the regulation of the inflammatory response in cats. Additionally, cats with AD may be an interesting new model for studying chronic AD in humans.

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Chapter 9

Summarizing discussion

Summarizing discussion

In cats, the term atopic dermatitis (AD) was introduced in 1982 by Reedy[1] who described immediate skin test reactivity to common aeroallergens in a group of cats that had pruritic, recurrent dermatitis and in which other causes of pruritus had been excluded. Since then the diagnosis of feline AD was based on signs and symptoms, compatible histopathology characterized by a perivascular to diffuse dermal infiltrate with emphasis on the presence of mast cells and eosinophils among macrophages and lymphocytes, and exclusion of other diseases.[2] A distinct clinical atopic dermatitis phenotype however, has not been described. Miliary dermatitis, self-induced alopecia, eosinophilic granuloma complex lesions, and pruritus of the head and neck have been associated with allergy.[2]

Results of intradermal testing in cats are more difficult to interpret than the response in humans and dogs.[2]

The aim of this thesis was to investigate various aspects of the immunopathogenesis in a defined group of cats with signs and symptoms of atopic dermatitis (Table 1) and compare our findings with the immunoregulation of atopic dermatitis in humans.

The criterion for distribution of the lesions has developed during this study. In retrospect, many of the animals with miliary dermatitis displayed involvement of the abdomen and or legs.

Table 1. Initial criteria for the clinical diagnosis of atopic dermatitis in cats

• Pruritus • Lesional features: miliary dermatitis or eosinophilic plaques or self-induced alopecia • A favorable response to glucocorticoids • No response to dietary manipulation and ectoparasite control • Histopathology: superficial to mid dermal perivascular to diffuse cellular infiltrate with eosinophils, lymphocytes, mast cells and macrophages.

Cats with lesions compatible with AD and negative skin intradermal test were often not included, although it is still a matter of debate whether all cases of AD need the association with positive immediate-type skin reactivity. For example in humans with atopic dermatitis, an “intrinsic” type is recognized that is characterized by an absence of allergen specific IgE.[3]

The etiology of AD in humans is multifactorial. Genetic factors, bacterial infections, aeroallergens, irritants, food allergens and psychological factors may play a role.[4] The significance of genetic factors, psychological factors and environmental factors in cats with atopic dermatitis is discussed in **Chapter 1.**

The cellular components of the inflammatory infiltrate in cats are similar to those described in humans. However, the histopathological reaction pattern of feline atopic dermatitis, flea bite hypersensitivity and adverse food reactions is indistinguishable. The value of *in vitro* allergy tests is debatable in cats[5,6] and *in vivo* allergy tests are in general difficult to interpret.

Chapter 2 is a literature review of the immunopathogenesis of AD in humans and in cats. In cats there is only one report describing a variable increase in dermal dendritic cells (Factor XIIIa+ cells) in allergic miliary dermatitis. Further definition of this cell population was not performed.[7] In humans, the Factor XIIIa+ cell population includes CD14+ cells, CD1a+ cells and CD14-/ CD1a- cells.[8] Other cells possibly playing a role in the immunopathogenesis, are discussed in the following chapters.

High serum levels of antigen-specific IgE found in 70 % of humans with atopic dermatitis are considered one of the hallmarks of atopy.[9] In a study described in **Chapter 3** we therefore investigated the presence of antigen-specific IgE in serum of cats with signs and symptoms of atopic dermatitis. For this purpose the Prausnitz-Küstner (PK) test and the passive cutaneous anaphylaxis test (PCA) were used. Positive test results indicate transferability of IgE-mediated reactions via serum. Both tests make use of the heat labile property of the Fc portion of IgE in the serum of the donor. After heating of serum, IgE looses its ability to bind to the high affinity receptor (FcεRI) with the consequent loss of immediate skin test reactivity. PCA and PK- tests were performed in cats with allergic dermatitis and in healthy control cats using five common allergens. With sera of allergic cats positive reactions in the PCA-test were observed with one unheated serum and three heated sera. Moreover, serum of one healthy control cat gave a positive reaction with heated and unheated serum to two different allergens. In both tests, sera of cats with a pruritic facial dermatitis did not elicit any positive reaction. Because in the PK-test positive reactions were observed with both heated and unheated sera, these results indicate that heat-stabile cytophilic antibodies, as well as heat-labile antibodies may be involved. The low number of positive reactions in the PCA versus the PK-test may be due to the fact that the PCA test is a less sensitive test than the PK test or to an inadequate dose of allergen. In addition, differences between the recipient cats in skin reactive capacity, i.e. the number of mast cells and their IgE binding capacity, may have influenced the outcome of both tests. Therefore, it is concluded that IgE, apart form other isotypes, is involved in the pathogenesis of atopic dermatitis in cats.

Langerhans cells (LC) expressing CD1a and MHC class II are potent antigen presenting cells, playing an important role in the pathogenesis of AD in humans.[10] In the study described in **Chapter 4,** we investigated the presence of LC in the epidermis and dermis of cats with allergic dermatitis and healthy control cats. We observed significantly higher numbers of CD1a+ cells and MHC class II+ cells in the epidermis and dermis of atopic cats as compared to healthy cats. A large population of MHC class II+ dendritic shaped cells compared to the number of CD1a+ cells was found in the dermis of AD cats, indicating that other dendritic-shaped cells like dermal dendritic cells and/or macrophages may be involved in the disease process.

Immunofluorescence-double labeling revealed staining of CD1a+ cells with MHC class II antibody. By electronmicroscopic analysis, LC were visualized in the epidermis of both healthy cats and AD cats. Some of the LC contained the typical tennis-racket-shaped Birbeck granules. Moreover, in the epidermis of feline AD skin there was an activated LC phenotype with a prominent Golgi system and many lysosomes, indicating that LC may actively participate in the pathogenesis.

Chapter 5 reports about the occurrence of T cells in the dermis of lesional and nonlesional skin of cats with allergic dermatitis. The findings were compared with those in healthy cats. A significant higher number of CD3+ T cells was observed in lesional and nonlesional skin with predominance of CD4+ T cells. The observed CD4+/CD8+ cell ratio in lesional skin of cats was similar to that in humans with atopic dermatitis.[11,12] In skin of healthy control animals only few T cells, primarily CD4+ T cells, were observed. The CD4+/CD8+ cell ratio in peripheral blood did not differ significantly between cats with atopic dermatitis and control cats. In humans with AD conflicting results have been reported on peripheral blood CD4+/CD8+ cell ratios.[13-15] It has been hypothesized that differences in activity and severity of disease may contribute to these differences in results.[15]

Neither in humans with AD nor in cats with AD, there appears to be a correlation between skin and peripheral blood CD4+/CD8+ cell ratios.

In order to define the T cell isotype involved in feline atopic dermatitis, we investigated the presence of IL-4 **(Chapter 6).** Cross-reactivity of the antibody against human IL-4 with feline IL-4 was confirmed by Western blotting with recombinant fIL-4.

IL-4+ cells were present in the lower part of the epidermis of lesional skin only. Predominantly in the superficial dermis, in both lesional and nonlesional skin, we found cellular infiltrates with IL-4+ cells. Only a very small number of IL-4+ cells was seen in the dermis of the control animals. The IL-4+ cells co-stained with antibodies against CD4 and a pan-T cell whilst double-labeling of IL-4 and mast cell chymase was observed in only a few cells. As a cross-reactive antibody specific for IFN-γ is not available, the T cell population could not be further defined with regard to its Th1-Th0-Th2 cytokine pattern. Hence, the conclusion at this stage is that IL-4+ T cells seem to play a role in the pathogenesis of feline atopic dermatitis.

This is comparable to findings in humans with AD where in acute skin lesions the IL-4 mRNA and protein expression are increased.[16,17] In chronic skin lesions IFN-γ mRNA and protein expression predominates.[16,17] With the atopy patch test (APT) this switch from a Th2–type to a Th1-Th0-type cytokine profile was confirmed at the protein level.[18] Although there is strong evidence in favor of this switch from a Th2 to a Th0/Th1 type response, the extent of IL-4 production by mast cells in the skin remains unclear.[18] Thepen et al.[18] could only demonstrate IL-4+ T cells and no IL-4+ mast cells in a double labeling experiment. In contrast, other reports demonstrated IL-4 production by mast cells with an other antibody and an enzymehistochemical staining method.[19,20] Recently, it was shown in mice that mast cells secreted IL-4 even when a polarized type I immunity was induced.[21] Thus the mere presence of mast cells could contribute to local IL-4 production. As mast cells are very heterogeneous cells and considering interspecies differences, it is at present not clear if this mechanism exists in humans and cats as well.

Chapter 7 focuses on the quantification of mast cells (MC) and eosinophils in the cellular infiltrate in atopic dermatitis. Additionally, MC were characterized according to their protease content, i.e. chymase and tryptase.

In both healthy cats and cats with AD, the MC counts varied considerably, albeit in the diseased cats at a significantly higher level than in the healthy cats.

Eosinophils were exclusively found in cats with atopic dermatitis. The presence of eosinophils is therefore more specific than the presence of MC. No significant correlation was found between numbers of MC and eosinophils in the same biopsy.

In humans with AD a high number of intact eosinophils is found only in early lesions. The commonly observed deposition of extracellular eosinophil granule proteins indicates a pathogenetic role.[22] Extracellular eosinophil granules have been described in cats with eosinophilic dermatoses.[23] Although deposition of extracellular eosinophil granule proteins has not been proven in cats, their existence is supported by the presence and properties of flame figures and "collagen degeneration" in feline eosinophilic dermatoses.[23]

In humans with AD, but not in healthy individuals, IL-4 induces chemotaxis of eosinophils.[24] Although such relation has not been investigated in cats, the significant number of eosinophils and the presence of IL-4+ T cells in atopic dermatitis, make it tempting to believe a similar mechanism is operating in cats.

The enzymehistochemical staining for tryptase showed a significant lower number of MC in comparison with the staining for chymase in lesional and nonlesional skin of cats with AD. In healthy cats however, slightly more MC were stained with tryptase than chymase (chymase: tryptase ratio is 9:10).[25]

Only with the chymase stain coarse granulated MC were found in lesional skin. These coarse granulated MC have not been described in healthy cats. Only very few of this type of MC were found in nonlesional skin of one cat. We did not observe a significant difference in the number of tryptase-positive MC in lesional and nonlesional skin of atopic cats.

In addition, significantly less MC were observed in lesional skin with the staining for tryptase compared with the Astra blue staining. In contrast, in skin of healthy cats MC are less numerous when stained with toluidine blue compared to staining for tryptase or chymase.[25] Astra blue and toluidine are comparable stainings as they both demonstrate sulphated acid glycosaminoglycans.

We conclude that coarse granulated MC are associated with local disease activity and may reflect degranulation as they were primarily present in lesional skin.

Chapter 8 describes immunophenotyping of the cellular infiltrate after atopy patch testing (APT) with 4 allergens (*Dermatophagoides farinae, D. pteronyssinus, Tyrophagus putrescentiae,* and a grass pollen mixture) in 6 cats with AD. Erythematous skin reactions were observed after 24-hours in 1 cat and after 48-hours in 3 cats with AD. No reactions were seen at the negative control sites of atopic cats nor did any of the healthy control cats display positive APT reactions. Immunophenotyping of the cellular infiltrate in the skin of biopsy sites at 24 hours after APT revealed significantly increased numbers of IL-4+, CD4+, CD3+, MHC class II+ and CD1a+ cells in one AD cat with visually positive patch test reactions. All AD cats (except one) had significantly increased IL-4+ T cell numbers at 24 and /or 48 hours patch test sites. These observations indicate that the atopy patch test induces relevant positive reactions and that the atopy patch test is a specific but not sensitive test for cats with atopic dermatitis, which is analogous to human data.[26] Additionally, our findings suggest that a putative Th0-Th2 type of responsiveness is involved in the pathogenesis of atopic dermatitis.

Cats and atopic dermatitis

Our findings indicate that in a defined group of cats with atopic dermatitis similarities can be found with humans with atopic dermatitis (Table 2).

The role of antigen-specific IgE in the immunopathogenesis of AD in cats remains open. We found only few positive reactions in the PCA-test with unheated serum of cats with atopic dermatitis. In contrast, we also observed positive reactions with heated serum possibly indicating a role for heat-stabile immunoglobulins as well.[27] These heat-stabile antibodies are probably IgG antibodies. In atopic cats elevated concentrations of antigen-specific IgG have been described.[28] In humans, IgG anaphylactic antibodies have been described.[29] Although other investigators have reported on the existence of a putative IgE, their findings have been primarily based on data acquired from PCA or PK test results obtained from cats who had been artificially sensitized, and not in cats with spontaneous atopic dermatitis.[30-32]

It was not possible to investigate the presence of IgE on for example epidermal Langerhans cells because a reliable antibody against feline IgE was not available.

Electronmicroscopy in cats with AD revealed activated epidermal LC together with increased numbers of CD1a+ cells and MHC class II+ cells in the epidermis and dermis of lesional skin.[33] Based on morphology the main population expressing MHC class II in the dermis consisted of dermal dendrocytes. This population was much larger than the dermal CD1a+ cell population which can be explained by activation and influx of other dendritic cells, probably also including macrophages.[7]

Although only supported by indirect evidence such as the presence of LC and CD4+/IL-4+ T cells in cats with spontaneous AD, as well as positive APT in some of these cats, it seems likely that – based on what is known in humans with AD – IgE-mediated antigen presentation may play a pivotal role in some cats with atopic dermatitis.

The data from Chapters 5 and 6 support a role for CD4+ T cells in particular, which is comparable to human AD.[11,12] The presence of IL-4 producing CD4+ T cells indicates a role for T cells with a Th0-Th2 type of responsiveness, assuming that feline T cells are similar in their cytokine pattern expression to human T cells. Cytokines, such as IFN-γ that are typically produced by Th1 cells could not be investigated due to unavailability of appropriate antibodies. Therefore the exact role of Th1- and Th2-type cells in the disease process and the occurrence of a Th2-Th1/Th0 type of responsiveness switch remains obscure.

In cats mast cells are more prominent in atopic lesional skin than in humans with AD.[34] Although, it is still not clear which role mast cells play in the pathogenesis of AD in humans, it is known that their protease content is subject to changes.[35] This may modulate the inflammatory response as proteases are known to play a role in recruitment of inflammatory cells and tissue remodelling.[36]

Similar to humans with AD we found changes in mast cell protease content in lesional and non-lesional skin. However, in atopic cats there were high numbers of mast cells staining positive for chymase in lesional and non lesional skin, whereas in humans atopic dermatitis is associated with elevated numbers of tryptase-positive mast cells in the superficial dermis.[35] Additionally, coarse granulated chymase positive mast cells were observed which, to our knowledge, have not been described in humans.

In cats, production of IL-4 was confined to T cells. A similar finding has been described in humans with AD.[18] Production of IL-4 by mast cells in human skin is controversial.[20] A recent study in mice however demonstrated that even in a polarized type I reaction there is IL-4 production by mast cells, albeit little.[21]

In contrast to humans with AD eosinophils are prominent in the cellular infiltrate in lesional skin of cats with atopic dermatitis.[37] However, in humans there is an abundance of eosinophil-derived proteins. These proteins could not be detected in cats due to lack of appropriate antibodies. Nevertheless, free granules can be observed in cats with AD and/or eosinophilic dermatoses.[23]

A species difference could play a role as cats tend to display more intact eosinophils in skin reactions than dogs[38] and most likely also humans.[37] Eosinophils may play an important role in disease pathogenesis as in humans they can influence the switch from a Th2 cytokine-mediated disease to primarily Th1-type cytokines in chronic lesions. IL-12 produced by eosinophils and /or macrophages may be responsible for this cytokine switch.[39]

Positive APT could only be evoked in cats with atopic dermatitis and the induced cellular infiltrate was similar to the infiltrate in lesional skin of cats with atopic dermatitis. The APT is considered to be a highly specific albeit not very sensitive test in humans with AD.[40] The findings of the present study indicate a comparable specificity in cats with atopic dermatitis. With the restrictions mentioned before, the APT results described in this thesis are the first to provide evidence for a role of a percutaneous allergen route in cats with atopic dermatitis.

In conclusion, our observations indicate a variety of immunological similarities between cats with atopic dermatitis and humans with atopic dermatitis. Due to a lack of investigative tools for detection of relevant cytokines and feline IgE, still limited functional insight has gained into the immunopathogenesis of atopic dermatitis in cats. Until then, it might be more prudent to call this type of allergic dermatitis “atopy-like dermatitis”. However, based on what is known from the APT results, it may be that in the future cats with AD become a suitable model for studying chronic AD in humans.

Table 2: Comparison of different aspects of atopic dermatitis in humans and cats.

	Humans	Cats
Distribution preference	Facial and extensor involvement (infants). Flexural side extensors, perioral and periocular, neck (adults).	Medio-caudal side of legs, abdomen, flank, head.
Lesional aspect	Variable with chronicity: xerosis, erythema, vesicles, papules, excoriation, oozing, crusts, lichenification.	Self-induced alopecia, erythema, papulo-crusts, papules, excoriations, eosinophilic plaques.
Involvement of Ag-specific IgE	Around 80 % of patients has increased Ag-specific IgE in serum.	Putative Ag-spec IgE demonstrated by positive PK tests, rHuFcεRIa-ELISA-capture and positive intradermal test; Also healthy animals have occasionally positive reactions.
Total IgE	Relation between total IgE levels and disease severity.	Unknown
Involvement of IgG	Increased total and Ag-specific IgG4 levels.	Increased Ag-spec-IgG levels; IgG class was not determined.
Langerhans cells (LC)	Increased numbers and increased activity of LC in epidermis and dermis.	Increased numbers and increased activity of LC in epidermis and dermis.
Th2 cell involvement	Initial Th2 cell skewed reaction pattern in lesional and nonlesional skin.	Th cells (CD4+) predominate; IL-4 production by T cells; Th1 or Th2 cell type is unknown.
Th2 / Th1 switch	In more chronic lesional skin Th0-Th1 type cytokines predominate.	Unknown.
Mast cells	Most skin MC contain both tryptase (T) and chymase (C); Increased number of T-containing MC in upper dermis of lesional and nonlesional skin; No report of coarse-granulated MC.	Most MC contain both chymase (C) and tryptase (T); Less T-containing MC in lesional and nonlesional skin compared to C-containing MC; Coarse-granulated MC with chymase staining only.
Eosinophils	Only intact eosinophils present in the initial stage; Increased levels of eosinophil granule proteins in lesional skin.	Significant increase of eosinophils in lesional and nonlesional skin; More predominant in AD cats compared to humans.
APT test	20–50 % of macroscopically positive tests (variable with way of testing); APT-induced cellular infiltrate mimics development of lesional skin.	Macroscopically positive test results can be induced; Only one APT method was tested; APT-induced cellular infiltrate is similar to that in lesional skin.

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Chapter 10

Samenvatting

Samenvatting

De term atopische dermatitis (AD) is voor de kat in 1982 geïntroduceerd door Reedy, die bij een groep katten met recidiverende jeuk en huidproblemen een positieve allergietest voor omgevingsallergenen, zoals huisstofmijten en pollen, vaststelde. Sinds die tijd is de diagnose van AD bij de kat gebaseerd op bepaalde verschijnselen, een kenmerkend ontstekingsinfiltraat in de huid (histopathologie) en op uitsluiting van andere aandoeningen zoals ectoparasitaire infecties en voedselintolerantie. Karakteristiek voor AD is verder de aanwezigheid van jeuk en de gunstige reactie op glucocorticoïden. Er is geen kenmerkend distributiepatroon van de huidveranderingen.

De histopathologie van lesionale huid wordt gekenmerkt door een reactiepatroon met lymfocyten, mestcellen, eosinofiele granulocyten en macrofagen. Met histopathologisch onderzoek kan geen onderscheid gemaakt worden tussen voedselintolerantie, vlo-overgevoeligheid en AD.

Ook bij mensen is atopische dermatitis gedefinieerd als een chronisch recidiverende ziekte die gekenmerkt wordt door jeuk. De ziekte wordt primair gediagnosticeerd aan de hand van verschijnselen die echter wel een meer karakteristieke morfologie en distributiepatroon hebben dan bij de kat. Ongeveer 80 % van de patiënten heeft een verhoogd gehalte aan allergeen-specifiek IgE in het serum.

Het onderzoek beschreven in dit proefschrift was gericht op diverse immunopathogenetische aspekten van “atopische dermatitis” bij de kat.

In **hoofdstuk 1** wordt ingegaan op een aantal etiologische faktoren van AD bij mensen en katten. Net zoals bij mensen is de levenswijze van katten sterk veranderd in de laatste 30 jaren. In hoeverre dit ook bij katten heeft geleid tot een toename van AD, is niet bekend.

Hoofstuk 2 is een literatuuroverzicht van de immunopathogenese van AD bij mensen. De rol van allergeen-specifiek IgE, nog steeds een van de hoofdkenmerken van AD, wordt besproken naast die van antigeen/allergeen-presenterende cellen, T cellen, mestcellen en eosinofiele granulocyten bij de ontwikkeling van de ontstekingsreactie. De literatuur met betrekking tot feline AD is beperkt tot klinische studies en de isolatie van IgE.

In **hoofdstuk 3** wordt de aanwezigheid van allergeen-specifiek IgE in serum van een geselecteerde groep katten met symptomen van AD beschreven. De passieve cutane anafylaxietest (PCA) en de Prausnitz-Küstner (PK) test zijn klassieke testen voor het aantonen van dit antilichaam en maken gebruik van de eigenschap dat IgE een hitte-labiel immunoglobuline is.

In de beschreven groep katten zijn positieve PK-test resultaten gevonden met zowel onverhit als verhit serum. Dit betekent dat zowel temperatuur-labiele (IgE) als -stabiele (IgG) immunoglobulinen betrokken zijn bij het proces. De PCA-test induceerde minder positieve reacties dan de PK-test.

Langerhans-cellen (LC) zijn belangrijk voor transport van antigenen en de presentatie ervan aan T cellen **(hoofdstuk 4).** Ook bij katten met AD is een significant groter aantal geactiveerde Langerhans cellen (CD1a+) aanwezig in de epidermis en dermis van lesionale huid dan bij gezonde katten. Verder zijn meer MHC klasse II+ cellen dan CD1a+ cellen aanwezig in de dermis, hetgeen een toename betekent van andersoortige dendritische cellen.

Deze LC lijken geactiveerd doordat meer MHC klasse II tot expressie wordt gebracht in de epidermis. Met electronenmicroscopie zijn Birbeck granulae in een deel der epidermale LC te zien.

Na binding van het antigeen aan LC wordt het door deze cellen opgenomen, verwerkt en aangeboden aan T cellen. Deze T cellen worden op hun beurt geactiveerd en produceren vervolgens cytokinen. Met name de CD4+ T (helper) cellen spelen een belangrijke rol bij het immunologische proces van AD **(hoofdstuk 5).** Uit dit onderzoek is gebleken dat bij katten met AD een verhoogd aantal CD4+ T cellen aanwezig is in zowel lesionale als in niet-lesionale huid. Dit is vergelijkbaar met de ontstekingsreactie bij mensen. Het grote aantal CD4+ T cellen in niet-lesionale huid duidt mogelijk op een systemisch proces en betekent dat het verhoogde aantal T cellen in lesionale huid niet alleen het gevolg is van een locale reactie.

Interleukine-4 (IL-4) en interleukine-13 (IL-13) zijn belangrijk voor de produktie van IgE. Kenmerkend voor de lesionale huid van mensen met AD is, dat in de initiële fase vooral IL-4 geproduceerd wordt door T cellen. Dit past bij een zogenaamde Th2 respons **(hoofdstuk 6).** Bij katten met AD blijkt een verhoogd aantal IL-4 producerende CD4+ T cellen aanwezig te zijn in lesionale en niet-lesionale huid. Hoewel in theorie ook mestcellen IL-4 kunnen produceren, lijkt dit bij de kat niet het geval te zijn.

Mestcellen en eosinofiele granulocyten worden prominent aangetroffen bij routine histologisch onderzoek van lesionale huid. In **hoofdstuk 7** wordt beschreven dat eosinofiele cellen uitsluitend worden gevonden in de huid van katten met AD en niet in die van controle dieren. De lokalisatie van de eosinofiele granulocyten blijkt echter niet specifiek te zijn voor een bepaald type allergische reactie. Met andere woorden: ook bij katten waar AD is uitgesloten kunnen eosinofiele cellen in de diepe dermis voorkomen. Verder is er ook geen verband tussen de aanwezigheid of afwezigheid van een positieve huidtest en het aantal mestcellen of het aantal eosinofiele granulocyten.

Mestcellen daarentegen worden zowel in de huid van gezonde katten als in de huid van katten met AD aangetroffen; bij de laatste groep echter wel in een significant hoger aantal.

Met behulp van enzymhistochemisch onderzoek zijn mestcellen aan de hand van hun protease-inhoud (tryptase en chymase) gekarakteriseerd. Alleen bij katten met AD heeft een aantal mestcellen zeer grote chymase-positive granulae. In niet-lesionale huid is het aantal van deze mestcellen kleiner dan in lesionale huid. Dit betekent waarschijnlijk dat deze grote granulae een rol vervullen bij de degranulatie en het locale ontstekingsproces.

In z'n algemeenheid worden de resultaten van onderzoek aan klinisch materiaal beïnvloed door de chroniciteit van de lesies en mogelijk ook door secundaire onstekingsreacties als gevolg van likken of krabben. Om deze redenen is de methodiek van de zogenaamde atopie patch test (APT) zoals gebruikt bij mensen, aangepast voor de kat **(hoofdstuk 8).** Dit is bij mensen een zeer specifieke maar niet erg gevoelige test voor AD en geeft de mogelijkheid op verschillende tijdstippen de ontstekingsreactie in de huid te onderzoeken.

Bij de helft van de onderzochte katten zijn via de APT macroscopisch zichtbare reacties geïnduceerd na 24 en vooral na 48 uur. Positieve reacties werden gekenmerkt door erytheem. Bij de controle katten zijn geen positieve reacties waargenomen. Bij bijna alle geteste katten werd na 24 uur en of 48 uur een verhoogd aantal IL-4+ T cellen gevonden. Bij 1 kat met een positieve APT werd een verhoogd aantal IL-4+, CD4+, CD3+,CD1a+, en MHC klasse II+ cellen aangetroffen. Dit is vergelijkbaar met de reactie in spontane lesies en duidt op een mogelijk Th2-Th0 type respons. De ontstekingsreactie bij deze katten met AD vertoonde overeenkomsten met die van mensen met atopische dermatitis.

De verschijningsvorm van "atopische dermatitis" bij de kat is heterogeen en slecht gedefinieerd. Wel is door retrospectief onderzoek van katten met de waarschijnlijkheidsdiagnose atopische dermatitis gebleken, dat ze vaak huidveranderingen hebben aan de buik, de kop, de flanken en de poten. Een karakteristiek distributiepatroon, zoals bekend bij de mens, wordt vooralsnog bij de kat niet beschreven.

Het onderzoek voor dit proefschrift laat zien aan dat ook immunophenotypisch AD bij de kat overeenkomsten vertoont met AD bij mensen. Omdat echter een nader onderzoek van deze inflammatoire cellen ook verschillen kan aantonen in cytokinenpatroon en het reactietype, is het vooralsnog beter om in plaats van AD de naam "atopy-like dermatitis" te gebruiken totdat de aandoening beter gekarakteriseerd is **(hoofdstuk 9).** Op grond van de huidige bevindingen en de toepasbaarheid van de APT is mogelijk een rol weggelegd voor katten met AD als model voor het bestuderen van chronische atopische dermatitis bij de mens.

Dankwoord

Dit proefschrift is mede tot stand gekomen met inzet, inspiratie, ondersteuning en advies van vele mensen. Op deze plaats wil ik al die mensen hartelijk danken voor hun inspanningen. Een paar mensen wil ik apart bedanken.

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Luctor et Emergo

Curriculum vitae

De schrijfster van dit proefschrift werd geboren in Wilhelminadorp (gemeente Kattendijke). Na het behalen van het Gymnasium-β diploma aan het Wagenings Lyceum te Wageningen (1971–1977) werd de schrijfster ingeloot voor Diergeneeskunde in 1978.
Deze studie werd in 1985 met goed gevolg afgesloten. Na enkele jaren werkzaam te zijn geweest in diverse kleine huisdierenpraktijken werd de opleiding tot specialist Dermatologie (1989–1993) bij de Vakgroep Geneeskunde van Gezelschapsdieren doorlopen. Daarna bleef de schrijfster als parttime-onderzoeker verbonden aan de vakgroep.
Van 1995–1996 was de schrijfster als lecturer en research fellow in dermatology verbonden aan "the Veterinary Hospital", University of Pennsylvania, Philadelphia, Verenigde Staten.
In 1996 werd het examen van het European College of Veterinary Dermatology met goed gevolg afgelegd (Diplomate of the European College of Veterinary Dermatology).
In 1997 keerde de schrijfster weer terug naar Utrecht en verrichtte verder onderzoek voor dit proefschrift.
Sinds 1998 is zij werkzaam als Oberassistentin und Leiterin der Abteilung Dermatologie, Departement für klinische Veterinärmedizin, Universität Bern, Bern, Zwitzerland.