Département de recherche clinique et santé publique vétérinaire
de la Faculté Vetsuisse Université de Berne
Division de recherche clinique expérimentale
(Prof. Dr. A. Zurbriggen,
PD. Dr. E. Marti)

et

Département de médecine vétérinaire clinique
Division de Dermatologie clinique
(PD. Dr. P. J. Roosje)

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Drs. E. Marti et P. J. Roosje

Influence of allergen-specific immunotherapy on allergen-specific IgG subclasses in dogs with atopic dermatitis

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Julien Dandrieux
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Influence of allergen-specific immunotherapy on allergen-specific IgG subclasses in dogs with atopic dermatitis.

Julien Dandrieux[1*], Eliane Marti[2], Michael J. Day[3], Marcus Doherr[2], Beat Bigler[4], Petra J. Roosje [1, 5]

[1] Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, PO Box 8466, 3001 Bern, Switzerland

[2]Departement of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, PO Box 8466, 3001 Bern, Switzerland

[3] School of Clinical Veterinary Science, University of Bristol, Langford, Bristol BS40 5DU, UK

[4] Labor Laupeneck AG / imovet bg, 3008 Bern, Switzerland

[5] DermFocus, Vetsuisse Faculty, University of Bern, Switzerland

[*]Corresponding author: Tel. +41316312315; Fax. +41316312275

E-mail address: julien.dandrieux@kkh.unibe.ch

Address: Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Länggassstrasse 128, 3012 Bern, Switzerland

Abstract

Canine atopic dermatitis (AD) is one of the most common pruritic skin diseases in dogs and is diagnosed based on compatible history, clinical signs and exclusion of other pruritic skin diseases. Allergen-specific immunotherapy (ASIT) is widely used to treat AD but the precise mechanism of action is unknown. The aims of our study were to investigate the influence of ASIT on levels of *Dermatophagoides farinae* (*D. farinae*) specific IgG (*D. farinae*-IgG) subclasses and to explore whether changes in IgG subclasses are associated with the efficacy of ASIT.

Sera from 98 dogs were collected before and during ASIT (duration of at least 2 years) with *D. farinae*. All dogs had serum IgE specific for *D. farinae* (imovet bg assay). Atopic dogs were divided into two groups: ASIT Group (n=48, ASIT as the sole therapy) and ASIT+ Group (n=50, insufficient control with ASIT requiring additional glucocorticoid treatment). A control group (CTRL Group, n=32) consisted of dogs without dermatological disease.

Allergen-specific IgG subclass antibodies were detected by ELISA using monoclonal antibodies specific for canine IgG1 – IgG4. *D. farinae*-IgG1 and IgG4 were detected in $\geq$ 78% of all sera before ASIT while *D. farinae*-IgG2 and IgG3 were found in $\leq$ 31%. Prior to therapy, dogs from the ASIT Group had significantly higher serum *D. farinae*-IgG1 than dogs in the ASIT+ Group (p<0.05). ASIT led to a significant increase in *D. farinae*-IgG1 in dogs from the ASIT (p<0.05) and ASIT+ (p<0.01) groups. *D. farinae*-IgG2, IgG3 and IgG4 concentrations were comparable for all groups before and during ASIT. Allergen-specific IgE concentration was not influenced by ASIT and the concentrations of IgG1 and IgG4 specific to an irrelevant antigen (*Betula*; birch pollen) were not influenced by ASIT against *D. farinae*. We conclude that long term ASIT increases levels of *D. farinae*-IgG1 and that dogs responding well to ASIT have a higher *D. farinae*-IgG1 concentration before therapy than partial responders.

Keywords

Dog; allergen-specific immunotherapy; atopic dermatitis; IgG subclass; ELISA

Abbreviations

AD: canine atopic dermatitis; ASIT: allergen-specific immunotherapy; *Betula*-Ig: *Betula* specific immunoglobulin levels; *D. farinae*: *Dermatophagoides farinae*; *D. farinae*-Ig: *D. farinae* specific immunoglobulin levels; EU: ELISA unit; Group ASIT: patients suffering from AD under ASIT as sole therapy; Group ASIT+: patients suffering from AD under ASIT and concurrent glucocorticosteroid therapy; Group CTRL: dogs without dermatological problems and without therapy; LOQ: limit of quantification; Treg: regulatory T cells; SAT: serologic allergy test.

Introduction

Canine atopic dermatitis (AD) is a frequent cause of pruritus in dogs that is characterized by the production of IgE against environmental antigens (Ag) (Halliwell, 2006). The genetically predisposed dog overreacts to these antigens, usually before 3 years of age, and shows pruritus at typical anatomical locations (Griffin and DeBoer, 2001). The diagnosis of AD is reached after fulfillment of clinical criteria and exclusion of other differential diagnoses including: adverse food reaction, flea bite hypersensitivity, scabies or other pruritic mite infestation, pruritic bacterial folliculitis, *Malassezia* dermatitis and less commonly contact dermatitis (DeBoer and Hillier, 2001). Additionally, the presence of allergen-specific IgE demonstrated by means of an intradermal (i.d.) test or a serological allergy test (SAT) is required for the diagnosis of AD (Halliwell, 2006).

Many studies on AD in humans and dogs have been performed to elucidate different aspects of the pathomechanism of this multifactorial disease. Studies have shown remarkable similarities between AD in humans and dogs. However, the role of IgG antibody (Ab) in AD in both species remains controversial. Four IgG subclasses (IgG1, G2, G3 and G4) are described in humans and dogs (Mazza et al., 1994). The canine subclasses were named to correlate to those of humans with respect to electrophorectic mobility and relative serum concentration, however it is not known whether there is functional similarity between the human and canine molecules (Day, 2007). A fifth type of IgG, IgGd, a non-IgE anaphylactic Ab, was also described in dogs (Willemse et al., 1985) but antiserum to IgGd myeloma protein was not able to detect purified canine IgG1 – IgG4, making IgGd of questionable significance (Day et al., 1996). Most human patients with AD have greater amounts of total IgG4 and of allergen-specific IgG4 compared with clinically normal individuals (Gondo et al., 1987). Patients who had received hyposensitization had greater amounts of IgG4 than atopic dermatitis patients not so treated (Gondo et al., 1987). Atopic dogs also have serum allergen-specific IgG and different subclasses may predominate for different allergens (Day et al.,

1996). In that study the IgG response to *D. farinae* was primarily of the IgG4 subclass (Day et al., 1996).

A recent study using a western blot analysis system showed that both healthy dogs and dogs with AD mounted similar *D. farinae*-IgG1 and -IgG4 responses (Hou et al., 2006). Another study reported a higher IgG response against *D. farinae* in atopic dogs compared with normal dogs, but this was not statistically significant (Hou et al., 2005). It has also been reported that atopic dogs have a significant increase in total serum IgG1 in comparison with non-atopic dogs (Fraser et al., 2004) but that study was performed with reagents that are unlikely to detect the same protein as the IgG subclass reagents used in the other investigations (Day, 2007).

Allergen-specific immunotherapy (ASIT) or "hyposensitization" is often used as therapy for management of clinical signs of AD in dogs and allergic rhinitis in humans (Ross et al., 2000). It is a safe treatment without side effects in dogs. There is much debate as to the mechanism of ASIT in human and veterinary medicine. Human patients treated with ASIT show an increase in circulating allergen-specific IgG, specifically of the IgG1 and IgG4 subclasses. Both subclasses have been shown to be able to block allergen binding to IgE in a quantitative manner to birch pollen (Ejrnaes et al., 2004) which may account for part of the clinical effect of ASIT.

The aim of the present study was to investigate the effect of long term ASIT against *D. farinae* on the concentration of serum allergen-specific IgG subclasses in dogs with AD. We hypothesized that successful ASIT correlated with changes in allergen-specific IgG subclasses.

Materials and Methods

Selection procedure for atopic dogs

Sera from the serum bank of imovet bg, Bern, Switzerland were selected for use in this study. Sera were retrieved from dogs with a presumptive diagnosis of AD that had been submitted during the previous years by the dermatology service, Vetsuisse Faculty University of Bern and private practioners for detection of allergen-specific IgE by PolyCheck Allergy Strips (BioCheck GmbH, imovet bg Bern).

Veterinarians and owners of these dogs were contacted through a questionnaire to verify and ensure that the dogs were suffering from AD and were currently under ASIT. Questions were based on the exclusion of other diseases and the clinical criteria for AD defined by Willemse (Willemse, 1990) and Prélaud (Prélaud et al., 1998). The first section of the questionnaire included general information about the patients (date of birth, breed, gender, previous history, type of feeding, deworming, and ectoparasitic control). The second section consisted of questions related to the development of the clinical signs (age at onset and seasonality). Owners and practitioners assessed severity of pruritus before and during ASIT on a scale ranging from 0 (no clinical signs) to 5 (highest level), and localization of the symptoms (head, ear, legs, feet, dorsum and abdomen). Dogs with lesions on the dorsum were excluded from the study.

The effect of therapy was assessed in the third section of the questionnaire. Information was collected concerning the duration of ASIT (in years), time until an effect was noticed (3 months, more than 3 to 6 months and more than 6 months) frequency of injections (every 4 weeks, every three weeks, other), need for and type of adjunctive therapies and effect of the ASIT (good effect, partial effect defined as effect in conjunction with other therapy, and no effect).

Dogs and serum samples

Ninety-eight dogs with AD (*n*=98) were included in the study based on history and clinical signs (pruritus, exclusion of ectoparasites and other skin diseases) consistent with atopic dermatitis and the presence of at least one positive reaction for *D. farinae*-specific IgE (*D. farinae*-IgE) on PolyCheck Allergy Strips (BioCheck GmbH, imovet bg, Bern, Switzerland). Dogs with positive reactions to flea allergen were not included in the study. Sera from the dogs were taken before beginning ASIT and frozen at –20°C for later use. All dogs received ASIT for at least two years and were still under ASIT with at least *D. farinae* when a second serum sample was taken. All dogs received ASIT using commercial, aluminiumhydroxid adsorbed allergen extracts assembled by one company (Dr. E. Graeub AG, Bern, Switzerland) and according to the same protocol.

A control group (Group CTRL, *n*=32) consisted of 32 healthy dogs without history of dermatological disease, pruritus or systemic conditions likely to affect immune function. These dogs were older than 2 years and had not received corticosteroids or other medication for at least one month prior to sampling. Breed and age distribution of the control dogs was matched with the breeds of the atopic dogs.

Atopic dogs were divided into two groups: Group ASIT (*n*=48, ASIT as the sole therapy) and Group ASIT+ (*n*=50, insufficient control with ASIT requiring additional glucocorticoid treatment) based on the questionnaires. The additional glucocorticoid treatment was defined as any type of systemic glucocorticoid or the need for topical corticosteroid at any frequency to control clinical signs.

Determination of allergen-specific IgG subclasses by ELISA

Relative concentration of the four IgG subclasses (IgG1-4) specific for *D. farinae* (*D. farinae*-IgG; antigen from Allergomed AG, 4106 Therwil, Switzerland) and *Betula* (*Betula*-IgG; birch pollen) (antigen from Allergomed AG, 4106 Therwil, Switzerland) were determined in the

sera of AD and control dogs before and during ASIT (after a minimum of 2 years of therapy), using the ELISA described by Day et al. (1996). *Betula* was used as a negative control allergen as none of the selected sera had *Betula*-specific IgE (*Betula*-IgE) and no dog received ASIT containing *Betula*.

IgG1 and IgG4 levels were measured in all dogs. IgG2 and IgG3 were measured only in a subset of atopic dogs chosen randomly: for IgG2, Group ASIT (n=28), Group ASIT+ (n=23), and for IgG3, Group ASIT (n=28), Group ASIT+ (n=22) and in all dogs of the control group.

Flat-bottomed 96-well ELISA plates (Immulon 2HB Plates, Catalys AG, 8304 Wallisellen, Switzerland) were coated overnight at 4°C with 75 µl of allergen diluted 1/50 in PBS (VWR International AG, 8953 Dietikon, Switzerland). Plates were washed 3 times in PBS before being blocked with 100 µl 1% polyvinylpyrrolidone (SIGMA, Fluka Chemie GmbH, 9471 Buchs SG, Switzerland) in PBS for one hour at room temperature. Serum samples were then diluted 1/25 in PBS containing 5% milk powder and 0.5% Tween 20, (PBS-milk-Tween; SIGMA, Fluka Chemie GmbH, 9471 Buchs SG, Switzerland). For the determination of IgG1 and IgG4 subclasses, duplicate titration curves were serially made from 1/25 to 1/200 for each serum tested. Serum from a dog immunized with *D. farinae* and *Betula* (courtesy of Novartis, Centre de Recherche Santé Animale SA, 1651 St Aubin, Switzerland) was used as standard. This standard serum was serially diluted on each plate from 1/200 to 1/6400, for *D. farinae*-specific IgG1 and IgG4 and 1/50 to 1/1600 for *Betula*-specific IgG1 and IgG4. To determine the interplate coefficient of variation a serum diluted from 1/25 to 1/800 was included on each plate. As the majority of the sera produced very low optical density (OD) values when measuring IgG2 and IgG3 subclasses, only two serum dilutions (1/25 and 1/50) were used for detection of these two IgG subclasses. The sera were incubated for two hours at 37°C.

After washing as above, 50 µl of monoclonal Ab specific for the different canine IgG subclasses (Day et al., 1996) were added to the plate and incubated for two hours at 37°C. The supernatants of clones B6 (specific for canine IgG1), E5 (IgG2) and A3G4 (IgG3) were used at a dilution of 1/20 and A5 (IgG4) at a dilution of 1/50 in PBS-milk-Tween. After washing, 50 µl of alkaline phosphatase-conjugated affinity purified goat anti-mouse IgG (Milan Analytica AG, 1634 La Roche, Switzerland) was added at a dilution of 1/2000 in PBS-milk-Tween and incubated for one hour at 37°C.

After final washing, 100 µl of p-nitrophenyl phosphate (SIGMA, Fluka Chemie GmbH) was added to each well and the plates were incubated for one hour at room temperature. Absorbance was recorded at 405 and 492 nm. Mean background absorbance of a series of control wells was deducted from each test value (one component of the ELISA omitted in each).

The parallelism of the binding curves between the reference serum and the test sera was confirmed with a covariance analysis in preliminary experiments (data not shown). The interplate coefficient of variation was 10% for IgG4 and 14% for IgG1. All Ig levels of the sera were expressed as ELISA units (EU), the standard being defined as 100 EU. A limit of quantification (LOQ) was defined as follows. The mean value and the standard deviation of the blank of each series of plates were calculated. The LOQ equaled the mean value plus ten times the SD. The relative amount of Ab was calculated for all the sera that had at least three values above the LOQ against the standard. Sera with IgG levels above the range of the standard curve were diluted appropriately and measured again. Sera with less than three measurable dilutions were considered as under the limit of detection of the assay and were given an arbitrary value of 1 EU for further analysis.

Determination of allergen-specific IgE

Sera from a randomly selected subgroup of atopic dogs (Group ASIT (*n*=28) and Group ASIT+ (*n*=24)) before and during ASIT and from all dogs of Group CTRL (*n*=32) were tested for specific IgE by BioCheck GmbH, Germany using PolyCheck Allergy-Test (BioCheck GmbH, imovet bg). In this test, a cellulose membrane (so called 'chip card') coated with allergens is incubated with dog serum and specific IgE is detected using a biotinylated monoclonal Ab (D9, DeBoer et al., 1993) against dog IgE. These membranes are then incubated with streptavidin-alkaline-phosphatase conjugates and the reaction is visualized using 5-bromo-4-chlor-3-indolyl-1- phosphate/nitro blue tetrazolium. Intensity of coloration is scanned and analyzed using Biocheck Image Software® to give the concentration of IgE (kU/l). Sera with undetectable IgE levels were given an arbitrary value of 0.1 kU/l for further analysis.

Statistical analyses

Information from the questionnaires and the laboratory results were analyzed using the program Number Cruncher Statistical Systems (NCSS 2004, Utah, USA). Two-Sided Fisher's Exact Test and Chi Square Test were used to analyze results from the questionnaires. Specific breed distribution was compared to the Swiss breed population using a two-Sided Fisher's Exact Test. Non distributive parameters were analyzed with a Kruskal-Wallis One-Way ANOVA on ranks. A *p* value less than 0.05 was considered as significant.

Results

Characteristics of the dogs with atopic dermatitis

Breed was known for 97 dogs representing a total of 35 breeds and cross breeds. The most represented breeds were Labrador Retriever (*n*=15; 15%), German Shepherd Dog, GSD (*n*=11; 11%), Boxer (*n*=8, 8%), cross breed (*n*=8; 8%), Golden Retriever (*n*=7, 7%), Yorkshire Terrier (*n*=5; 5%), Poodle (*n*=5; 5%), West Highland White Terrier (*n*=5; 5%) and Jack Russell Terrier (*n*=3; 3%). There were two each (2%) of the Bernese Mountain Dog, Border Collie and Hovawart. The remaining 24 breeds were represented only once (1%). Compared to the dog breed distribution in Switzerland (data from the dog databank in Switzerland, ANIS, for 2005), only Boxers were found to be significantly over represented in the AD dog population (*p*=0.03) (Labrador *p*=0.28 and GSD *p*=0.10). There were 52 female dogs (52/97, 54%) and 45 male (45/97, 46%). Nearly all of them were neutered.

The median age at onset was 19 months (*n*=67, range 2 months to 8 years). Sixty seven dogs (69%) were ≤ 3 years old and 30 (31%) were > 3 years old (Table 1). Forty-two (43%) dogs out of 98 were receiving no other specific treatment for AD than ASIT. The drugs used in the other 56 dogs (57%) included corticosteroids (systemic and topical at different frequencies) in 50 dogs (51%), antibiotics in 14 dogs (14%, cases of recurrent pyoderma or otitis), anti-histamines in 6 dogs (6%), only topical corticosteroids in 19 dogs (19%), additional essential fatty acids in 3 dogs (3%) and various shampoos in 42 dogs (43%). Alternative medicine was used only in a small number of dogs: acupuncture in one and homeopathy in nine dogs (8%).

A pruritus grading was given by the veterinarian for 88 dogs out of 98. Seventy-eight percent (*n*=69) of the dogs were graded as having disease severity between 4 and 5 before therapy and 22% (*n*=16) between 1 and 3 (Figure 1). At the time of completing the questionnaire, 44% of 98 dogs did not have any pruritus, 48 dogs (51%) were scored between 1 and 3 (moderate pruritus) and 5 dogs (5%) were scored 4 (marked pruritus).

Factors influencing the outcome of ASIT

The duration of ASIT was not significantly (p = 0.09) associated with the outcome of ASIT. Sixty percent of the 50 dogs that were under ASIT for 2 to 4 years but only 35% of the 29 dogs that received ASIT for 4 to 6 years needed corticosteroids in addition to ASIT. Fifty-three percent of the 19 dogs that were under ASIT for > 6 years received corticosteroids. The other factors related to the ASIT itself (time until improvement and frequency of injections) were also not significant (Table 1).

Influence of the treatment on the severity of pruritus

We analyzed the disease severity score assigned by the veterinary surgeon using a subdivision of the symptoms into two classes: absent (Score 0) to moderate (score 3) or severe (score 4 to 5). The distribution of the severity of the symptoms was not significantly different between Group ASIT and Group ASIT+ before ASIT: pruritus (n=88; p=0.44), alopecia (n=82; p=0.81) and pyoderma (n=84; p=0.63).

The decrease in pruritus was assessed by subtracting the score before therapy from the score during therapy for each dog. In both groups, the average difference was significantly greater than zero, which means that the score during ASIT was significantly lower than the score before any therapy (Group ASIT (n=41), p<0.001 and Group ASIT+ (n=47), p<0.001). The median of the differences in Group ASIT was 4, range [1 to 5] and in Group ASIT+ 3, range [-1 to 5]. The difference between the two medians was statistically significant (p<0.001). Hence, the pruritus of Group ASIT during ASIT was significantly lower than in Group ASIT+.

D. farinae-IgG subclasses

D. farinae-IgG1 and IgG4 were above the detection limit of the assay in 72% to 91% of the dogs in the different groups before and during therapy (Table 2).

D. farinae-IgG1: Before therapy, Group ASIT had a median of 14.5 EU (*n*=42, range [1-144]), Group ASIT+ a median of 9.0 EU (*n*=39 dogs [1-38]), and Group CTRL a median of 11.5 EU (*n*=27, [0-40]). The only significant difference between groups before therapy was that the median value in Group ASIT was significantly higher than in Group ASIT+ (*p*=0.03). During therapy, Group ASIT had a median of 17.0 EU (*n*=43 dogs, [1-386]) and Group ASIT+ a median of 13.0 EU (*n*=45 dogs, [1-69]). Conversely to what was found before therapy, the difference between the two groups was no longer significant during therapy.

D. farinae-IgG4: Before therapy, Group ASIT had a median of 94.5 EU (*n*=42 dogs, [1-2601]), Group ASIT+ a median of 89.5 EU (*n*=40, [1-3245]), and Group CTRL a median of 53.5 EU (*n*=24, [1-2348]). During therapy, Group ASIT had a median of 80.5 EU (*n*= 39, [1-2903]) and Group ASIT+ a median of 71.0 EU (*n*=36, [1-725]). There were no significant differences between the groups before or during therapy.

D. farinae-IgG2 and -IgG3 were detected in less than one third of the dogs at 1/25 serum dilution before or during therapy (Table 3). Because most of the sera were below the detection limit of the assay for these two subclasses, it was not possible to quantify *D. farinae*-IgG2 and IgG3 levels. Thus only the percentage (number) of dogs with IgG2 or IgG3 levels above the detection limit of the assay is indicated.

D. farinae-IgG2: 25 % (*n*=7) of the dogs of Group ASIT were positive before therapy and 18% (*n*=5) during therapy. Seven dogs (30%) of Group ASIT+ were positive before and 5 (22%) during therapy. Thirty-one percent of the dogs of Group CTRL were positive. There were no significant differences in distribution between the different groups before or during therapy.

D. farinae-IgG3: 21% (*n*=6) of the dogs of Group ASIT were positive before and 7% (*n*=2) during therapy. Fourteen percent (*n*=3) of the dogs of Group ASIT+ were positive before and 5% (*n*=1) during therapy. Sixteen percent (*n*=5) of the control dogs were positive. There were no significant differences in the distribution between the different groups before or during therapy.

D. farinae-IgE

Group ASIT had a median value for *D. farinae*-IgE of 12.0 kU/l (range: 0.1-42) before and 9.8 kU/l (range: 0.1-53) during therapy (Table 4). Group ASIT+ had a median of 9.1 kU/l (range: 0.3-33.0) before and 7.5 kU/l (range: 0.1-40.0) during ASIT. In comparison, the Group CTRL had a median of 5.7 kU/l (range: 0.1-33.0) (Table 4).

The median concentration of *D. farinae*-IgE in the control dogs was significantly lower than the median of Group ASIT ($P<0.01$) before therapy, but not compared to Group ASIT+. There was no significant difference in level between Group ASIT and Group ASIT+, or within groups when comparing levels before and during treatment.

Comparison of D. farinae-IgG subclass and -IgE Ab levels before and during ASIT

The ratios of IgG and IgE levels before and during therapy were calculated (Table 2 and Table 4). The analysis of these ratios revealed that the ratio for IgG1 was significantly higher than 1 for group ASIT (median ratios of 1.09; $p=0.02$) and ASIT+ (median ratio of 1.35; $p<0.01$). This means that IgG1 levels increased significantly during therapy in both groups. There was no significant difference between the medians of Group ASIT and ASIT+ ($p=0.23$) (Table 2). The IgG4 ratios were lower then 1 in both groups ASIT and ASIT+ but this decrease was not statistically significant. There was no significant difference between the medians of the IgG4 ratios of Group ASIT and ASIT+ ($p=0.98$). IgE levels were also not influenced significantly by ASIT as for both groups the ratio of IgE during/before therapy was

one. There was no significant difference between the medians of this IgE ratio for Group ASIT and ASIT+ (Table 4).

We also analyzed IgG4/IgG1 ratios before and during therapy (data not shown). Before therapy, Group ASIT had a median of 6.8 and Group ASIT+ of 11.2. There was no significant difference between the medians of Group ASIT and ASIT+ (p=0.70). During therapy, Group ASIT had a median of 3.9 and Group ASIT+ of 4.2. There was no significant difference between the medians of Group ASIT and ASIT+ (p=0.84).

Betula-IgG subclasses

Betula was used as a negative control allergen, as none of the dogs had been subjected to ASIT with this Ag.

Betula-IgG1: Ab were present in 29 to 41% of the tested dogs (highest percentage for dogs of Group ASIT+ before ASIT). The 3 groups presented a median of 1 EU before ASIT (range: Group ASIT: 1-80 EU, Group ASIT+: 1-65 EU and Group CTRL 1-260 EU) and during ASIT (range: Group ASIT: 1-270 EU and Group ASIT+: 1-59 EU). There were no significant differences between the medians of the three groups before and during ASIT.

Betula-IgG4: The percentage of dogs with detectable Ab levels was lower than for IgG1, varying between 13 and 21%. The 3 groups presented a median value of 1 EU before therapy (range: Group ASIT: 1-692 EU, Group ASIT+: 1-204 EU and Group CTRL: 1-121 EU) and during ASIT (range: Group ASIT: 1-1326 EU and Group ASIT+: 1-148 EU). There were no significant differences between the medians of the three groups before and during ASIT.

Hence, Betula-IgG1 and –IgG4 levels were not influenced by *D. farinae* SIT (Table 2).

Because of the high percentage of sera that were below the detection limit of the assay for *D. farinae*-IgG2 and - IgG3 Ab levels, we did not measure the *Betula*-IgG2 and IgG3 subclasses.

Betula-IgE

Group ASIT had a median value of 0 kU/l (range: 0.0-0.4) during ASIT and Group ASIT+ had a median value of 0 kU/l (range: 0.0-9.7) before ASIT. There were no detectable IgE levels for the other categories (Table 4).

Comparison of Betula-IgG subclass antibody levels before and during ASIT

We analyzed the following ratios: IgG1 during/IgG1 before and IgG4 during/IgG4 before ASIT (Table 2). For IgG1 during/IgG1 before ASIT, Group ASIT and Group ASIT+ had a median of 1. There was no significant difference between the medians of both groups (p=0.53). For IgG4 during/IgG4 before ASIT, Group ASIT and Group ASIT+ had a median of 1. There was no significant difference between the medians of both groups (p=0.31).

Discussion

Atopic dermatitis is a multi-factorial disease and a frequent cause of chronic or recurrent pruritus in dogs. In this study we reviewed 98 Swiss dogs with increased serum concentrations of *D. farinae*-specific IgE which were treated with long term ASIT. All dogs included in this study had clinical signs consistent with canine atopic dermatitis and were free of ectoparasites. Specific IgE against *D. farinae* was present in all dogs, whereas none had specific IgE against flea Ag (data not shown) or *Betula*. The aim of this study was to determine whether changes in allergen-specific IgE and IgG-subclasses occur during ASIT and to identify prognostic factors that may indicate the successful outcome of this therapy.

Canine atopic dermatitis is characterized by the presence of a number of clinical signs and exclusion of other diseases. Therefore strict criteria were used in order to provide assessment of the patient population and enable us to possibly link clinical parameters such as outcome of ASIT with immunological parameters. The distribution of breeds of the investigated dogs was comparable with other studies reporting breed predilections for AD (Nuttall et al., 1998; Mueller et al., 1999; Zur et al., 2002; Nodtvedt et al., 2006)

Clinical parameters (sex, age at onset of dermatitis, anatomical localization of lesions, presence of recurrent otitis, presence of adverse food reaction and seasonality) were not significantly different between dogs from Group ASIT and Group ASIT+. These findings are comparable to other studies (Nuttall et al., 1998; Saridomichelakis et al., 1999; Park et al., 2000; Zur et al., 2002; Schnabl et al., 2006). Adverse food reactions (as defined by Hillier and Griffin, 2001) were diagnosed by feeding a restricted protein diet in 7% of the atopic dogs. It was not possible to assess adequately this part of the treatment for each dog, because the information needed (type of food, duration of the trial and food challenge) was seldom thoroughly recorded. The same type of difficulty was encountered in a retrospective study made in Sweden based on records of 335 insured dogs in the year 2002 (Nodtvedt et al., 2006).

As many dogs had been treated additionally with corticosteroid (topically or systemically), we divided the dogs in two groups: Group ASIT (ASIT only) and Group ASIT+ (ASIT and corticosteroid) in order to be able to measure antibody responses and clinical signs only influenced by ASIT.

There was no difference in clinical signs before treatment between the experimental groups. However, the reduction in pruritus was significantly more pronounced in the Group ASIT compared to the Group ASIT+. One reason for this difference might be that proper treatment of atopic dermatitis requires a management strategy that should be tailored to the individual patient when ASIT only is not effective or insufficient. Experience in management strategies, regular visits and good owner compliance are a necessity and may explain in part these results. Another possibility is that dogs from Group ASIT+ had reactions to additional allergens that were not influenced by *D. farinae* ASIT and hence responded less favorably to ASIT alone. This hypothesis could be supported by the fact that the *D. farinae* IgG1 levels were significantly lower than in the Group ASIT before therapy, possibly reflecting a lower exposure to this antigen than in the dogs that responded well to ASIT. This could mean that successful ASIT is related to a certain level of exposure to *D. farinae* represented by higher levels of *D. farinae*-IgG1. A good outcome (Group ASIT) in this study was observed in 49% of the population which is comparable to what is normally cited (between 52 and 68%; (Nuttall et al., 1998; Park et al., 2000; Saevik et al., 2002; Schnabl et al., 2006; Zur et al., 2002).

The first aim of our serological investigation was to compare *D. farinae*-IgG subclasses in the sera of healthy dogs and dogs with AD that responded well to ASIT alone and dogs that only partially responded to ASIT. We found that before ASIT most of the atopic and healthy dogs had *D. farinae*-IgG1 and IgG4, while allergen-specific IgG2 and IgG3 was often undetectable in the sera of these dogs. These findings are concordant with those of Day et al. (1996) and Hou et al. (2006). Thus, *D. farinae*-IgG2 and IgG3 do not seem to play an

important role in the immunopathogenesis of AD. However, dogs can respond with up-regulation of specific Ab of all IgG subclasses to other antigens as was shown in dogs with visceral leishmaniasis (Quinnell et al., 2003).

There were no significant differences for *D. farinae*-IgG1 and IgG4 concentrations between atopic and healthy dogs before ASIT, suggesting that an IgG response to this allergen is a marker of exposure to *D. farinae* and possibly part of a normal immune response after exposure to this allergen. These findings are consistent with the study of Hou et al. (2006) who basically found no difference between healthy and atopic dogs except that atopic dogs showed more often IgG1 binding to a 180 kDa protein band in immunoblots of *D. farinae* extract than healthy dogs. However, for the other IgG binding proteins in *D. farinae* extracts no differences were found between atopic and healthy dogs. In our study, dogs that did not respond well to ASIT alone had significantly lower *D. farinae*-IgG1 levels before ASIT compared with dogs that responded well to ASIT. This finding is difficult to explain; it may be that ASIT+ dogs had more frequently received corticosteroid treatment before ASIT. Alternatively, the atopy symptoms in these dogs may not have been caused only by allergy to *D. farinae*, i.e. that although they had measurable *D. farinae*-IgE and IgG1 they had reactions to other allergens as well. Although there was no statistically significant difference between the median *D. farinae*-IgG1 levels of Group ASIT and the control group, the range of the Group ASIT + and CTRL was comparable and lower than the Group ASIT which could indicate a better response to ASIT in the presence of increased *D. farinae*-IgG1 levels. However, due to the great overlap in the ranges between Group ASIT and Group ASIT+, specific-IgG1 levels cannot be used to discriminate dogs which are going to respond well to ASIT before instigating this therapy. Interestingly, although not statistically significant, the Group ASIT+ had a lower median serum concentration of *D. farinae*-IgE but this was not as low as in the control group.

The second aim of the serological study was to test whether ASIT influenced *D. farinae*-IgG subclasses and IgE levels in dogs with AD. In both groups, ASIT with *D. farinae* led to a significant increase of *D. farinae*-IgG1 while IgG4, IgG2, IgG3 and IgE concentrations were not influenced significantly by ASIT. This is the first study investigating the influence of ASIT on four allergen-specific IgG subclasses in atopic dogs. However, a previous study where total serum IgG1 levels were measured had shown that ASIT induces a significant increase of total IgG1 levels (Fraser et al. 2004) although the reagent used to detect IgG1 in that study is known not to be specific for this IgG subclass alone (Day, 2007). This result, together with our study, suggests that in dogs ASIT leads to increases of IgG1 levels. Furthermore, we demonstrate in our study that the increase in IgG1 is antigen specific, as IgE and IgG subclasses against *Betula*, used as a negative control, were not influenced by ASIT with *D. farinae*.

In human patients, successful ASIT is accompanied by an increase of specific IgG4, and to a lower extent also of IgG1 (Jutel et al. 2005), and by a slow decline in IgE levels (Shida et al., 2004). The role of IgG in ASIT is still not clear but was thought to be due to a 'blocking' effect whereby the IgG captured the allergen before it was able to reach the mast-cell bound IgE, thus preventing degranulation of such effector cells. However, the relationship between efficacy of ASIT and the induction of allergen-specific IgG subclasses remains a controversial issue, with serum levels of allergen-specific IgG correlating with clinical improvement in some studies but not in others. In humans, the role of increased allergen-specific IgG4 Ab in ASIT is still debated. Study results suggest that successful ASIT is associated with an increase in IgG blocking activity that is not solely dependent on the quantity of IgG (Wachholz and Durham, 2004). This finding is in contrast to the results of another study in which the blocking activity of ASIT-induced allergen-specific IgG4 was only dependent on the quantity of IgG4 (Ejrnaes et al., 2004). More recent studies propose that ASIT acts by modulating the balance between the allergic Th2 cell responses and regulatory T

cells (Treg). In humans ASIT induces allergen-specific Treg which produce suppressive cytokines such as IL-10 and TGF-β (Akdis et al. 2005). IL-10 is a potent suppressor of IgE and enhances allergen-specific IgG4 production (Akdis et al. 1998).

In conclusion, we have demonstrated an increase of allergen-specific IgG1 during long term immunotherapy of canine AD, which was not indicative of success of treatment. Because an increase in serum concentration of allergen-specific IgG1 does not correlate with clinical improvement, we extrapolate that ASIT must have an influence on other pathways to promote a disease free status. Further studies are warranted to investigate these mechanisms, in particular, the involvement of regulatory T cells. Future studies on allergen-specific Treg may provide more insight into the mechanism of ASIT or improve its efficacy.

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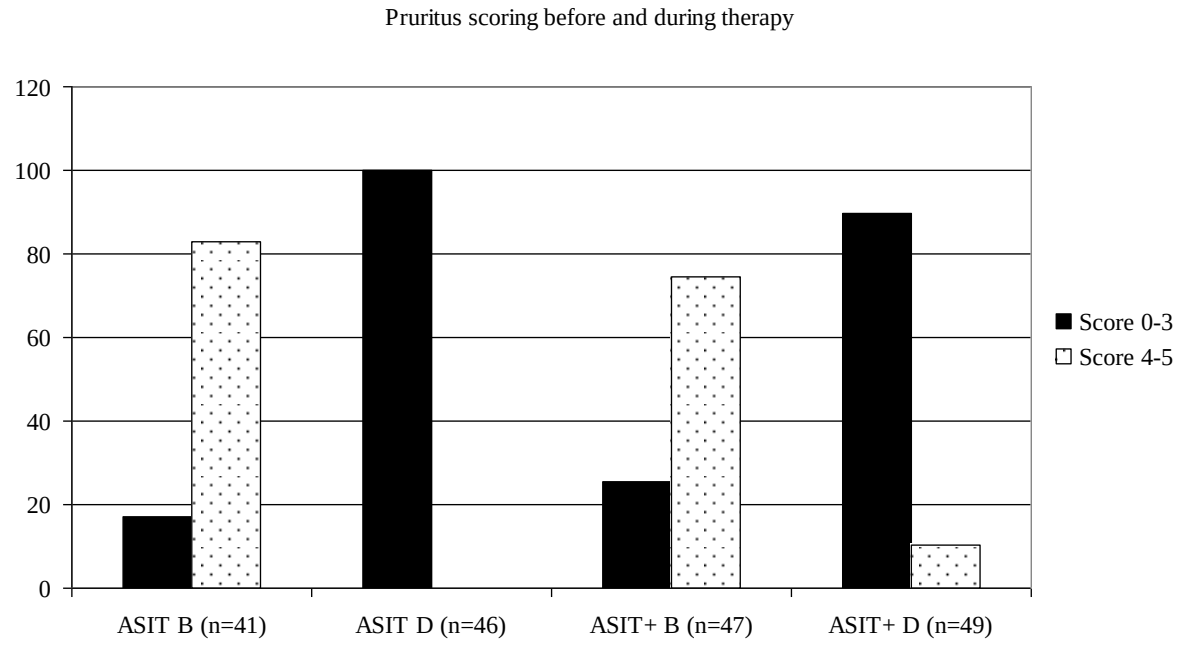

Figure 1. *Pruritus graded by the veterinarian before and during therapy*. Group ASIT: atopic dogs receiving only ASIT, Group ASIT+: atopic dogs receiving ASIT and corticosteroids. B, before therapy, D, during therapy. Symptom score is divided as 0 to 3 (black) and 4 to 5 (dots). The percentage of dogs in each group is given on the Y-axis. The distribution of the pruritus severity was the same in both groups before therapy and both groups show a marked decrease in pruritus during therapy.

Table 1A

Parameter	Total (*n*)	Group ASIT (*n*)	Group ASIT+ (*n*)	*p* Fisher
Number of dogs	98	50	48	
Female / Male	52 / 45	26 / 21	26 / 24	0.84
Onset $\leq$ 3 years	67	34	33	0.52
Localization	92	43	49	0.11
Otitis	32	18	14	0.39
Food allergy	7	2	5	0.44
Seasonality	37	19	18	0.67
Therapy	54	18	36	<0.001

Table 1B

Parameter	Total (*n*)	Group ASIT (*n*=48)		Group ASIT+ (*n*=50)	
		Median	Range	Median	Range
Dermatitis onset [month]	98	18.5	1.0 - 96.0	21.0	41.0 - 84.0
Therapy duration [year]	98	4.2	2.0 - 8.3	3.4	2.1 - 8.3
Time until effect [month]	98	2	0 - 12	1.5	0 - 24

Table 1A. *Patient signalment and clinical symptoms* and 1B. *Onset of symptoms and ASIT parameters*.
Localization: compatible with AD; *n*: number of dogs; *p* with corresponding test; seasonality: seasonality in the symptoms; therapy: need for concurrent therapy other than ASIT. Group ASIT required less concurrent therapy than group ASIT+, no other parameters were significant between the two groups.

D. farinae	IgG1 B [EU]	IgG1 D [EU]	IgG4 B [EU]	IgG4 D [EU]	**IgG1 D/B**"	IgG4 D/B
Group ASIT (*n*=48)	**14.5*** [1-144] 88%, 42	17.0 [1-386] 90%, 43	94.5 [1-2601] 88%, 42	80.5 [1-2903] 81%, 39	**1.1 "** [0-42]	0.8 [0-91]
Group ASIT+ (*n*=50)	**9.0*** [1-38] 78%, 39	13.0 [1-69] 90%, 45	89.5 [1-3245] 80%, 40	71.0 [1-725] 72%, 36	**1.4 "** [0-39]	0.9 [0-48]
Group CTRL (*n*=32)	11.5 [1-40] 84%, 27	ND	53.5 [1-2348] 75%, 24	ND	ND	ND

Betula	IgG1 B [EU]	IgG1 D [EU]	IgG4 B [EU]	IgG4 D [EU]	IgG1 D/B	IgG4 D/B
Group ASIT (*n*=48)	1 [1-80] 29%, 14	1 [1-270] 35%, 17	1 [1-692] 21%, 10	1 [1-1326] 17%, 8	1 [0-270]	1 [0-135]
Group ASIT+ (*n*=49)	1 [1-65] 41%, 20	1 [1-59] 35%, 17	1 [1-204] 14%. 7	1 [1-148] 16%, 8	1 [0-52]	1 [0-124]
Group CTRL (*n*=32)	1 [1-260] 34%, 11	ND	1 [1-121] 13%, 4	ND	ND	ND

Table 2. *Summary of changes in levels of IgG1 and IgG4 subtypes against D. farinae* and *Betula*. Group ASIT, therapy with ASIT no corticosteroid; Group ASIT+, therapy with ASIT and corticosteroids; Group CTRL, dogs without dermatological signs and without therapy. B, before therapy; D, during therapy; D/B, ratio of IgG during and before therapy. EU: ELISA Unit.
First line: results expressed as median with [range]. Second line: percentage and number of dogs with detectable antibody levels. All zero levels were arbitrary defined as 1EU.
*****, significant difference between the median (Kruskal-Wallis One-Way ANOVA on Ranks, not corrected for Ties p =0.03).
", ratio significantly higher than 1 for Group ASIT and Group ASIT+ (respectively p=0.017 and p <0.01).

D. farinae	IgG2 B	IgG2 D	IgG3 B	IgG3 D
Group ASIT	25%	18%	21%	7%
(*n*=28)	7	5	6	2
Group ASIT+	30%	22%	14%	5%
(*n*=23)	7	5	3	1
Group control	31%	ND	16%	ND
(*n*=32)	10		5	

Table 3. *Summary of IgG2 and IgG3 detection against D. farinae*. Group ASIT, therapy with ASIT no corticosteroid; Group ASIT+, therapy with ASIT and corticosteroids; Group CTRL, dogs without dermatological signs and without therapy. B, before therapy; D, during therapy. First line: percentage of dogs with detectable IgG, second line: number of dogs with detectable IgG. There was no significant difference in the distributions between the groups for both subtypes (Chi-Square Test).

	D. farinae			*Betula*		
	IgE B [kU/l]	IgE D [kU/l]	IgE D/B	IgE B [kU/l]	IgE D [kU/l]	IgE D/B
Group ASIT (*n*=28)	**12.0*** [0.1-42.0] 90%, 26	9.8 [0.1-53.0] 90%, 26	1.0 [0.3-9.5]	0 [0.0-0.0] 0%	0 [0.0-0.4] 3%, 1	1.0 [1.0-4.0]
Group ASIT+ (*n*=24)	9.1 [0.3-33.0] 100%, 30	7.5 [0.1-40.0] 93%, 28	1.0 [0.1-5.1]	0 [0.0-9.7] 17%, 5	0 [0.0-0.0] 0%	1.0 [0.2-1]
Group CTRL (*n*=32)	**5.7*** [0.1-33.0] 81%, 26	--	--	0 [0.0-0.0] 0%	--	--

Table 4. *Summary of IgE changes against D. farinae and Betula before and during treatment*. Group ASIT, therapy with ASIT no corticosteroid; Group ASIT+, therapy with ASIT and corticosteroids; Group CTRL, dogs without dermatological signs and without therapy. B, before therapy; D, during therapy; D/B, ratio of IgE during and before therapy. EU: ELISA Unit.
First line: results expressed as median with [range]. Second line: percentage and number of dogs with detectable antibody levels. All zero levels were arbitrary defined as 0.1EU.
*****, significant difference with Kruskal-Wallis One-Way ANOVA not corrected for Ties ($p<0.01$) between Group ASIT and Group CTRL.