Veterinary Immunology and Immunopathology 180 (2016) 1–8

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Immunoproteomic characterization of a *Dermatophagoides farinae* extract used in the treatment of canine atopic dermatitis

Raquel Moya[a], Jerónimo Carnés (PhD)[a,*,1], Nuria Sinovas[a], Laura Ramió[b], Pilar Brazis[b,1], Anna Puigdemont[c,1]

[a] R&D Department, Laboratorios LETI, S.L. C/del Sol 5, 28760 Tres Cantos, Madrid, Spain
[b] UNIVET, SL. Avd. Cerdanyola 92, 08173 Sant Cugat del Vallès, Barcelona, Spain
[c] Dept. Farmacologia,Terapeùtica i Toxicologia, Universitat Autònoma de Barcelona, Spain

ARTICLE INFO

Article history:
Received 31 October 2015
Received in revised form 28 April 2016
Accepted 3 August 2016

Keywords:
Immunotherapy
Allergen sensitization
Mite allergy
Allergens
Canine atopic dermatitis

ABSTRACT

Background: Canine atopic dermatitis is a pruritic allergic skin disease. House dust mites have been identified as the main non-seasonal responsible agent. Unlike in human allergic patients, groups 1 and 2 antigens have been described as minor allergens in dogs, while groups 15 and 18 are considered the major allergens. Despite these differences, allergic dogs have traditionally been treated using extracts intended for human immunotherapy.
Objectives: To investigate the immunological characteristics and the allergen reactivity of dogs with atopic dermatitis using a *Dermatophagoides farinae* commercial extract.
Methods: Eighteen dogs diagnosed with atopic dermatitis and 3 healthy control dogs from the Iberian Peninsula were included in the study. All the animals were older than 12 months, from both sexes and different breeds and showed positive specific IgE against *D. farinae* (>2500 ELISA Absorbance Units). The *D. farinae* allergenic extract used in this study was manufactured and characterized. The allergenic profile of the dogs was investigated by immunoblot and specific IgE, IgG, IgG1 and IgG2 measured by direct ELISA. Allergen identity was confirmed by immunoblot inhibition and mass spectrometry analyses.
Results: The results confirmed the relevance of groups 15 and 18 antigens, but also groups 1, 2 and other medium molecular weight allergens in the sensitization of dogs with atopic dermatitis. Immunoblot inhibition and mass spectrometry assays confirmed these results. Relevant allergens were quantified by scanning densitometry (Der f 1: 17 μg/mg, Der f 2: 20.3 μg/mg, Der f 15: 18.1 μg/mg and Der f 18: 9.4 μg/mg). Concerning immunoglobulins profile, differences in IgE and IgG1 levels were observed between non-atopic and atopic dogs.
Conclusions: The commercial *D. farinae* extract characterized in this study contains the major allergens involved in the sensitization of dogs with atopic dermatitis, representing a suitable candidate for its use in the diagnosis and immunotherapy of mite allergic dogs.

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1. Introduction

Canine atopic dermatitis (CAD) is a genetically predisposed inflammatory and pruritic allergic skin disease, associated to the production of IgE antibodies against environmental allergens, mainly mites and pollens (Lian and Halliwell, 1998; DeBoer and Hillier, 2001; Hill and DeBoer, 2001; Jackson et al., 2002; Farmaki et al., 2010; Mueller et al., 2016).

House dust mites (HDM) have been identified as the main cause of non seasonal canine and human allergies (Nuttall et al., 2006). In Western countries, *Dermatophagoides farinae* is accountable for more than 70% of CAD cases identified (Codner and Tinker, 1995; Weber et al., 2003). Contrary to what happens in humans, dogs show lower positive responses against *Dermatophagoides pteronyssinus*, even in locations where this mite is more prevalent than *D. farinae* (Jackson et al., 2005; Nuttall et al., 2006; Farmaki et al., 2012; Fernández-Caldas, 2013).

Significant differences between the human and canine HDM allergens profile have also been reported. Whereas the major allergens for humans are proteins of relatively low molecular weight

Abbreviations: CAD, canine atopic dermatitis; HDM, house dust mites; LMW, low molecular weight; HMW, high molecular weight; AD, atopic dermatitis; EAU, ELISA Absorbance Units; MW, molecular weight; OD, optical density; ASIT, allergen specific immunotherapy.

* Corresponding author at: R&D Director (Research & Development Department), Laboratorios LETI, S.L Calle del Sol no. 5, 28760 Tres Cantos, Spain.
E-mail address: jcarnes@leti.com (J. Carnés).

[1] These authors have equally contributed to the elaboration of this project.

http://dx.doi.org/10.1016/j.vetimm.2016.08.004
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(LMW) (Thomas and Smith, 1999), this is not the case in dogs. The most important allergens of *D. farinae* in atopic dogs are included in groups 15 and 18, which belong to the high molecular weight (HMW) fractions of HDM extracts (Nuttall et al., 2001). Preliminary immunological studies have shown that Der f 15 presents two IgE-binding capacity bands at 98 kDa and 109 kDa, whereas Der f 18 presents as a single IgE-binding band of about 60 kDa (McCall et al., 2001; Weber et al., 2003; Hou et al., 2008). Based on the published studies, the major allergens described in allergic human patients, Der f 1 and Der f 2 with IgE binding to 25 kD and 14 kD bands respectively, are recognized by less than 50% of atopic dogs, and therefore should be considered as minor allergens for this species (Bard and Esch, 1992; Noli et al., 1996; Masuda et al., 1999; Thomas et al., 2002; Goicoa et al., 2008).

Several hypotheses have been postulated to explain the cause of the discrepancies observed between dogs and humans, including genetic, immunologic and metabolic theories (Fernández-Caldas, 2013). One of these hypotheses is based on the differences in T-cell epitopes and subsequent T-cell responses (Nuttall et al., 2006). Other authors blame this discrepancy on the route of sensitization to mite allergens (Marsella et al., 2006), that, in dogs (unlike in humans) could be partially the ingestion route due to the contamination of dog food with mites (Brazis et al., 2008). In spite of these important differences in allergen recognition between species, *D. farinae* allergens included in allergen specific immunotherapy (ASIT) used to desensitize both human and canine patients, are usually the same. Normally, *D. farinae* immunotherapy is standardized regarding Der f 1, to determine activity and potency.

Major mite allergens for humans have been intensively studied, however, the information of major mite allergens for dogs available is more limited (Noli et al., 1996; Masuda et al., 1999; McCall et al., 2001; Hou et al., 2008). Further research of mite allergic dogs is required to clarify remaining questions such as the role of mite allergens in the development of CAD or the quality of mite extracts used for the diagnosis and treatment of these animals.

The objectives of this study were to characterise a commercial *D. farinae* extract, identifying key allergens involved in the sensitization of dogs suffering from CAD, in order to evaluate whether *D. farinae* extracts normally used in canine immunotherapy contain these allergens, and to investigate the allergenic profile of sensitization and the immunological characteristics of dogs suffering from atopic dermatitis (AD). For this objective, different immunological assays were performed with sera from both healthy and atopic dogs using a commercially available *D. farinae* extract.

2. Materials and methods

2.1. Canine serum samples

Serum samples were collected following standard veterinary procedures from 18 dogs clinically diagnosed with AD by private veterinarians. Selected animals fulfilled the following inclusion criteria: (1) Dogs older than 12 months of age, of both sexes and any breed, with a clinical diagnosis of non-seasonal AD according to the criteria of Favrot et al. (2010); (2) Dogs with a positive serological test (ELISA test from Greer Laboratories [Lenoir, NC; USA]) for detection of allergen specific IgE against *D. farinae*, evidencing ELISA Absorbance Units (EAU) higher than 2500.

None of the following exclusion criteria were fulfilled by any of the animals included in the study: pregnancy or lactation, seasonal signs of AD involving flea infestation or food allergens (based on response to treatment for flea infestation and to an elimination diet [at least 6 weeks]), presence of bacterial or *Malassezia* sp. skin infections or other infestations causing pruritus (e.g. mites), and having been treated with glucocorticoids or oral corticosteroids within 4 weeks of enrolment and/or injectable glucocorticoids within 8 weeks of enrolment.

Negative control samples were collected from 3 healthy dogs with no dermatological lesions and showing less than 50 EAU after assessing *D. farinae* specific IgE determination by serological ELISA test.

The characteristics of the patient population are described in detail in Table 1.

2.2. Characterization of *D. farinae* extract

D. farinae extract was prepared according to internal manufacturing procedures (Laboratorios LETI, Madrid, Spain). Briefly, the extract was prepared using semipurified mite bodies (≥80% mite bodies) in 0.01 mol/L of phosphate buffered saline (1:20 wt/volume) and extracted for 24 h. Afterwards the solution was centrifuged, and the supernatant collected, dialyzed, filtered, and freeze-dried. The extract was characterized as follows.

2.2.1. Protein content

The protein content was determined using the Bradford method (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

2.2.2. Protein profile

Protein profile of the extract was investigated by SDS-PAGE under reducing conditions following two different strategies: (1) 15%T acrylamide:bis-acrylamide gels, with LMW range standard for the identification of proteins in a range between 10 and 70 kDa; (2) Any kD™ Mini-PROTEAN® TGX™ Gels (BioRad, Hercules, CA, USA) with a HMW range for the characterization of proteins between 45 and 200 kDa. In both cases gels were Coomassie stained. Gel images were analysed using the ImageQuant TL 2005 software (GE Healthcare, Uppsala, Sweden).

Concentration of each individual band compared to the whole protein composition was calculated by scanning densitometry, to quantify the content of individual allergens compared to the whole protein concentration.

2.3. Allergenic profile

Allergenic profile of each serum sample was determined by immunoblot using *D. farinae* extract in solid phase. Briefly, 50 μg of allergen extract/serum sample were run in a gel. After electrophoresis, gels were electrotransferred onto a Trans-Blot® Turbo™ Transfer Pack (Bio-Rad). Membranes were blocked with 5% skimmed milk in PBS 0.01 mol/L; Tween 0.1% for 1 h and incubated overnight with individual sera (diluted 1/10 in PBS 0.01 mol/L). After washing, the membranes were incubated with goat anti dog IgE:HRP diluted 1:10000 (AbD Serotec, Bio-Rad). Finally, membranes were developed with luminol solution (Clarity™ Western ECL Substrate, Bio-Rad) and detected by chemiluminescence (ChemiDoc XRS, Bio-Rad). The molecular weight (MW) of the detected bands was determined using Image Quant TL 2005 (GE Healthcare).

2.4. ELISA assays

Specific IgE, IgG, IgG1 and IgG2 to *D. farinae* were measured in the individual serum samples by direct ELISA. Briefly, microplates (Immulon IV; Thermo Scientific) were coated with *D. farinae* extract (20 μg/ml) and incubated overnight at room temperature. Plates were blocked for 1 h with 1% BSA in PBS 0.01 mol/L; Tween 0.1%. Serum samples were serially diluted and incubated for 2 h. After washing, the secondary antibody (diluted 1:10000) consisting on goat anti dog IgE:HRP, sheep anti dog IgG:HRP, goat anti dog

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