

Short communication

Lack of detection of circulating skin-specific IgE autoantibodies in dogs with moderate or severe atopic dermatitis

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Abstract

Human patients with atopic dermatitis (AD) commonly exhibit IgE reactivity to cutaneous self-antigens. The presence of serum IgE autoantibodies appears to correlate with disease severity, and it is suspected to reflect or contribute to tissue damage. The objective of this study was to determine whether IgE autoantibodies specific for cutaneous antigens could be detected in the serum of dogs with AD. Serum was collected from 19 dogs with untreated moderate to severe AD and four specific-pathogen free (SPF) dogs. Indirect immunofluorescence was performed using normal canine skin collected at four different locations (concave ear, nose, medial thigh and lateral thorax), while Western immunoblotting was done using normal canine ear pinna epidermal and dermal extracts and reducing conditions. In both methods, IgE was detected using a monoclonal antibody specific for heat stable epitopes of canine IgE. At 1:10 dilution, specific IgE autoantibodies against cutaneous autoantigens were not detected, with either method, in AD and SPF canine sera. Either IgE autoreactivity is not associated with moderate to severe AD in dogs, or the methods employed herein were not sensitive enough to permit IgE autoantibody detection.

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1. Introduction

After the seminal discovery, in 1991, of the existence of similarity in structure between exogenous allergens (e.g. profilins) and human proteins (Valenta et al., 1991), the systematic search for IgE autoantibodies began in earnest in human patients with atopic dermatitis (AD). Presently, there are five human autoallergens – named Hom s 1–5 – officially recognized by the International Union of Immunolo-

gical Societies (Table 1) (<http://www.allergen.org>). The relevance of such IgE autoimmunity to the pathogenesis of human AD is highlighted by several observations: the capability of autoantigen-specific IgE to cause basophil and mast cell degranulation, the discovery of T-lymphocyte recognition of such allergens and the apparent correlation between the level of serum IgE against these autoallergens and the severity of clinical signs in human individuals with chronic AD (reviewed in (Mittermann et al., 2004)). Additionally, the presence of IgE autoreactivity in humans with AD recently was shown to be significantly associated with the persistence of eczema during infancy, the presence of recurrent bacterial and viral skin infections, food allergy, pruritus, dry and scaly skin and abnormal sweating (Mothes et al., 2005). Moreover, children with detectable IgE

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Table 1
Recognized *Homo sapiens* autoallergens

Autoallergens ^a	MW (kDa)	Biochemical name	Function
Hom s 1	55–60	Squamous cell carcinoma antigen SART1	Carcinoma antigen
Hom s 2	~30	Alpha subunit of nascent polypeptide-associated complex	Sorting and translocation of intracellular proteins
Hom s 3	22–23	BCL7B	Oncogen
Hom s 4	54	Atopy-related autoantigen CALC	Calcium-binding protein
Hom s 5	60	Type II keratin 6a (K6a)	Intermediate filament protein

^a Only autoallergens with official recognition by the International Union of Immunological Societies (IUIS) were added in this table.

autoantibodies tended to have higher serum total IgE levels compared to those without these antibodies, and the appearance of such autoreactivity appeared to be one of the first detectable, as it could be seen in the first years of life (Mothes et al., 2005).

Whether such IgE autoimmunity precedes or follows AD-induced skin damage is best answered using animal models. It is with interest, therefore, that allergic sensitization of mice to Hom s 2 – the alpha chain of the nascent polypeptide associated complex – or its murine analog was found to induce atopic asthma-like manifestations (Bunder et al., 2004).

Finally, IgE autoreactivity does not appear to be limited to the five allergens officially recognized for *Homo sapiens*. Indeed, human IgE has been shown to also recognize numerous environmental allergens that crossreact with human homologues. Examples of such allergens include albumins, milk proteins, profilins or fungal cyclophilins and manganese superoxide dismutase (<http://www.allergome.org>). The clinical relevance of such observations remains uncertain, however.

In dogs, AD has been redefined recently as “a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens” (Halliwell, 2006). It must be differentiated, principally, from atopic-like dermatitis (ALD), a clinically similar disease in which IgE antibodies cannot be documented (Halliwell, 2006). To look for allergen-specific IgE antibodies, and hence help differentiate AD from ALD, veterinarians have recourse to either intradermal or serological testing, methods that help determine whether mast cell bound or circulating IgE antibodies specific for environmental allergens are present (DeBoer and Hillier, 2001b; Hillier and DeBoer, 2001). Whenever dogs present with signs of AD but tests fail to reveal IgE hypersensitivity, clinicians are left with a conundrum: determine whether the patient is affected with ALD, or whether

IgE antibodies specific for other but yet untested allergens do exist. If IgE specific for cutaneous self-antigens were to be present and relevant to the pathogenesis of AD in dogs, this phenomenon would logically explain the dilemma highlighted above: such dogs would present with clinical signs of AD, but IgE autoantibodies would be undetectable using conventional in clinic intradermal or commercially available serological methods.

The objective of this study was to determine whether IgE specific for cutaneous antigens could be detected, using routine immunofluorescence and immunoblotting techniques, in the serum of dogs with moderate or severe AD.

2. Methods

2.1. Characterization of atopic dogs

Serum samples were obtained from *nineteen dogs with nonseasonal AD* diagnosed based on standard guidelines (clinical diagnosis, exclusion of resembling diseases and demonstration of hypersensitivity to common aeroallergens by either intradermal testing positive immediate reactions or high levels of serum allergen-specific IgE) (DeBoer and Hillier, 2001a). These sera had been obtained at the time of enrollment in three clinical trials (Olivry et al., 2003a, 2002, 2003b), and per trial protocol, dogs could not have been treated recently with glucocorticoids or calcineurin-inhibitors prior to blood collection.

Study subjects belonged to six pure breeds (six Labradors, four retrievers, two Jack Russell terriers, one west highland white terrier, one boxer, one walker hound), and there were four crossbred dogs (Table 2). We included seven males and 12 females, and ages at time of collection ranged from 1.5 to 11 years (median and mean: 5 years). Dogs were selected because they exhibited moderate to severe skin lesions of AD, this being reflected by their trial's baseline Canine Atopic

Table 2
Characterization of atopic dog sera

#	Breed	Sex	Age	CADESI v.2
1	Labrador	MC	2.0	86
2	Labrador	FS	6.0	70
3	Labrador	MC	5.0	50
4	Jack Russell terrier	FS	4.0	55
5	Jack Russell terrier	MC	4.0	252
6	Labrador	MC	5.0	54
7	Labrador	FS	6.0	77
8	Retriever	FS	7.5	87
9	Shepherd crossbred	FS	11.0	107
10	Shepherd crossbred	FS	2.0	66
11	Retriever	M	3.0	77
12	Retriever	MC	8.0	85
13	Crossbred	FS	9.0	52
14	Boxer	FS	2.0	57
15	Labrador	FS	1.5	75
16	Walker hound	FS	5.0	60
17	West highland white terrier	MC	3.0	124
18	Retriever	FS	2.0	66
19	Crossbred	FS	8.0	54

Abbreviations: FS: female spayed; M: male; MC: male castrated.

Dermatitis Extent and Severity Index (CADESI version 2) superior to 50 (Olivry et al., 2002). In all, CADESI-2 values ranged from 50 to 252 (mean: 70, median: 82). Serum samples from four specific-pathogen free (SPF) dogs served as negative controls. All sera had been kept frozen at -80°C between times of collection and analysis.

2.2. Indirect immunofluorescence

Samples from concave ear, philtrum (rostrum-ventral nose), medial thigh and lateral thorax were collected after death from two adult pound dogs without skin lesions. Frozen skin sections were blocked with 1% newborn calf serum and 5% normal dog serum for 20 min before incubation with canine AD and SPF sera diluted at 1:10 for 30 min. Serum IgE was detected by successive incubations with mouse monoclonal antibody 5.91 generated by one of us (Hammerberg et al., 1997) against canine monoclonal IgE (5 $\mu\text{g}/\text{mL}$ for 30 min; P115, Bethyl Labs, Montgomery, TX). This antibody is specific for an heat stable epitope between amino-acids 356–374 of dog IgE (US Patent Application No. 11/585,712; Publication No. US-2007-0161066-A1). This first step was followed by incubation with goat antimouse IgG-fluorescein (1:200 for 20 min; F 5387, Sigma–Aldrich, St. Louis, MO). Vectashield-DAPI was used as a mounting medium (H-1200, Vector, Burlingame, CA). Sections of normal dog spleen served as positive

controls. Saline in lieu of canine sera was used as negative control.

Epidermal and follicular keratinocytes and fibroblasts were examined in each immunostained section for the presence or absence of fluorescence signifying IgE autoantibody binding. Whenever observed, the intensity of fluorescence was to be recorded as low or high.

2.3. Immunoblotting

Large samples from nonlesional concave ear skin were obtained from two adult dog cadavers that did not exhibit visible skin lesions. This sampling area was selected because of preliminary experiments showing that the lack of hair follicles in this localization did not prevent adequate separation of epidermis from dermis and it allowed appropriate isolation of extracts. Moreover, in humans with AD, IgE autoantibodies are present in epidermal or dermal cells irrespective of the source (i.e. location) of the specimens used. In our samples, the epidermis was separated from underlying dermis using 1 M sodium chloride by gentle rubbing as described previously (Olivry et al., 1998). Epidermal and dermal specimens were deposited in 20 μL of homogenization buffer (20 mM of Tris base, 10 mM of EDTA, 2 mM of EGTA) to which were added 20 μL of Triton X and 1 mg of leupeptin (Olivry et al., 1998). After adding 0.1 M of phenylmethylsulphonyl fluoride, the samples were homogenized and centrifuged. The supernatant (e.g. cytosol fraction) was aliquoted and frozen until used. Samples of dermal or epidermal supernatants were combined with 4 $\times$ sample buffer, nuclease free water and 10 $\times$ reducing agent (dithiothreitol) (Invitrogen, Carlsbad, CA) and separated using a 10% NuPage gel (Invitrogen) for 35 min before being transferred to nitrocellulose membranes. After initial blocking with 1% dry milk for 1 h, membranes were incubated overnight with 1:10 dilutions of the dog sera at room temperature before rinsing three times for 10 min in Tris phosphate buffered saline. The second incubation was with biotinylated mouse anticanine monoclonal antibody 5.91 (1 $\mu\text{g}/\text{mL}$ for 2 h; same source as for immunofluorescence). After three other rinses, the strips were incubated with NeutrAvidin-alkaline phosphatase at (2 $\mu\text{g}/\text{mL}$ for 30 min; #31002, Pierce, Rockford, IL), rinsed further and finally stained with an appropriate chromogen. The positive control consisted of monoclonal canine IgE clone 2.39 (Gebhard et al., 1995) that was separated by electrophoresis and detected as indicated above.

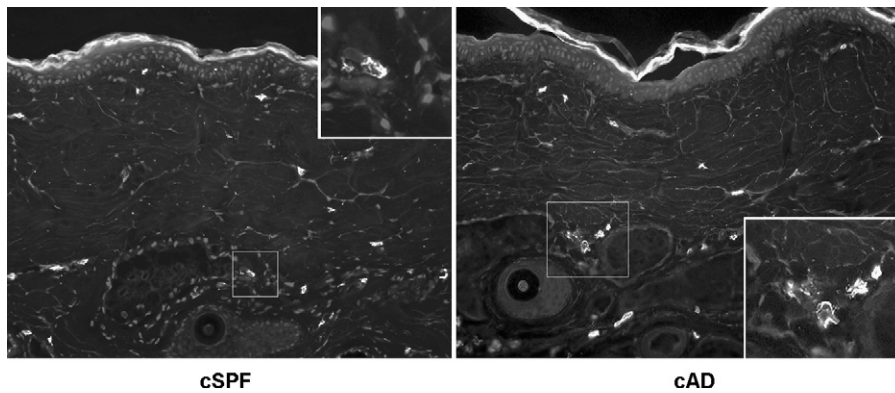


Fig. 1. Indirect immunofluorescence. Mast cells (inset) with membrane and/or cytoplasmic IgE were visualized using both categories of canine sera (SPF and AD) as well as with saline negative control (not shown).

3. Results and discussion

3.1. Indirect immunofluorescence

Using both categories of sera (SPF and AD) – or even when saline replaced dog serum – indirect immunofluorescence revealed specific IgE deposition on dermal mast cells (Fig. 1, inset). Variable staining of the stratum

corneum was also seen with all sera but not with the negative control. There were no specific fluorescence of either epidermal keratinocytes of stratum basale, spinosum or granulosum, hair follicle cells from anagen hair bulbs, inferior segment, isthmus or infundibulum, or of dermal fibroblasts. Altogether, differences in fluorescence patterns between AD or SPF canine sera were not apparent. This visualization of IgE on the

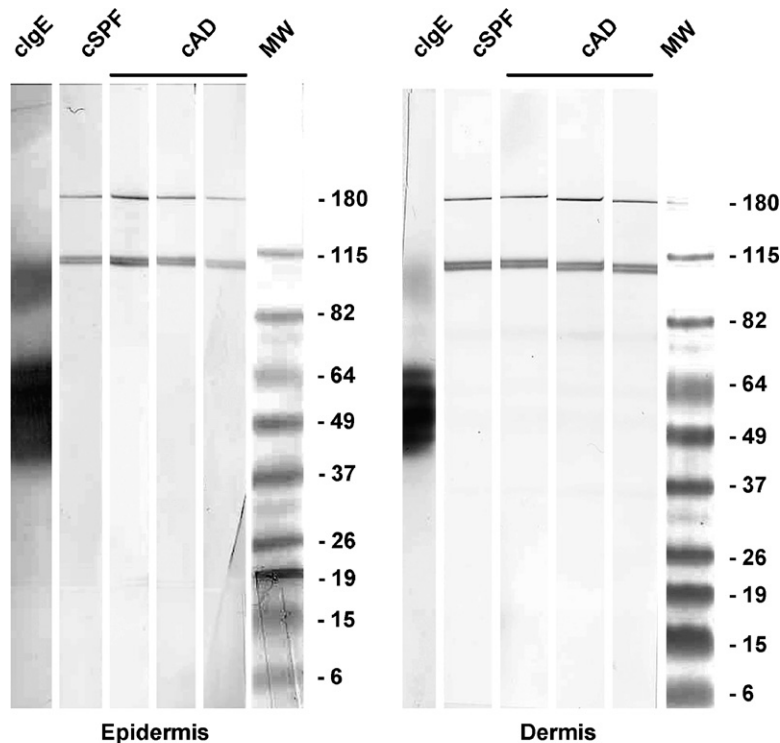


Fig. 2. Immunoblotting. Using both epidermal and dermal extracts, serum IgE specific for AD autoantigens were not detected differently between canine SPF and AD sera. Antigens of 180 and 110 kDa were revealed using all sera, irrespective of their category. Dog IgE served as positive control (leftmost lane of each panel).

membrane – and sometimes cytoplasm – of dermal mast cells is consistent with previous reports documenting the presence of such IgE⁺ cells in the skin of normal and atopic dogs (Halliwell, 1973; Olivry et al., 1996). Of particular interest was our lack of detection of IgE binding to the membrane or cytoplasm of epidermal or follicular keratinocytes or dermal fibroblasts using a 1:10 dilution of sera of affected dogs, even those with severe AD (i.e. CADESI-2 greater than 100). This inability of detection is in contrast with results from a study using sera from human patients with AD in which cytoplasmic and/or membrane staining of keratinocytes was visualized using both indirect immunofluorescence and flow cytometry (Altrichter et al., 2005).

3.2. Immunoblotting

Using epidermal and dermal extracts obtained from whole skin samples processed as done previously (Olivry et al., 1998), our immunoblotting method did not permit the identification of IgE autoantibodies specific for canine AD. We observed, however, that all AD and SPF dog sera contained IgE that recognized antigens of 180 and 110 kDa in epidermal and dermal extracts (Fig. 2). Even though the heavier and lighter bands are of molecular weights comparable to those of whole IgE and two epsilon chains, respectively, the reducing conditions and the use of IgE as positive control, which separated at different areas—reduced the possibility that anticanine IgE recognized tissue-bound IgE. Additionally, the antigens recognized are of a weight different from that of the two known IgE receptors (FcεRI: 50/60 kDa; FcεRII/CD23: 27/45 kDa). As a result, the nature of these two antigens remains unknown. Disappointingly, we could not detect IgE autoantibodies against low molecular weight epithelial antigens (e.g. 20–60 kDa) in our canine AD sera—even in those from dogs with severe AD and a CADESI greater than 100 ($n = 3$). This lack of detection is in contrast with the findings that approximately one-fourth of human patients with AD exhibit serum IgE autoantibodies against epithelial cell-derived antigens (Mothes et al., 2005). The prevalence of circulating IgE autoantibodies is even markedly higher (80%) in children with high total serum IgE levels (Mothes et al., 2005). A major difference between our study and that by Mothes and colleagues resides, however, in the nature of the substrate used for immunoblotting. It is conceivable that the whole epidermis and dermis extracts that we employed could have a lower concentration of putative target antigens compared to the keratinocyte cell line used in the study by Mothes et al. (2005).

In summary, using both immunofluorescence and immunoblotting techniques employing conventional methods and substrates isolated from cadaver dog skin, serum IgE autoantibodies specific for AD were not identified in dogs with moderate to severe disease. In all, either IgE autoreactivity is not associated with active AD in dogs, or the strategy employed herein was not sensitive enough to permit IgE autoantibody detection. Future studies will be performed to determine whether IgE autoantibodies can be found with extracts of canine and human keratinocyte cultures, and whether such autoantibodies could be present in dogs in which conventional intradermal and serological methods have failed to reveal IgE hypersensitivity to environmental and dietary allergens (e.g. dogs with ALD).

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