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Inverse association between endotoxin exposure and canine atopic dermatitis

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ABSTRACT

The development of canine atopic dermatitis (CAD) may be related to exposure to mite allergens, bacterial endotoxin and/or fungal glucans. In this study, indoor exposure levels of house dust mite allergens, endotoxins and fungal glucans were measured to determine their possible association with CAD. A case-control study including adult Labrador retrievers with ($n = 28$) and without (controls; $n = 65$) CAD was conducted. Dust samples were collected from the living room floor and the bedding and coat of the dog and these were analyzed for house dust mite allergens Der p1 and Der f1, endotoxin and (1 → 3)- β -D-glucan levels. Dog owners were also required to return a questionnaire regarding their home characteristics.

The endotoxin exposure level in the coats of dogs was significantly inversely associated with CAD (odds ratio 0.38; 95% confidence interval 0.15–0.97; $P < 0.05$). No significant difference was found in exposure levels to house dust mite allergens and fungal glucans. The results indicated that endotoxin exposure is inversely associated with CAD, suggesting a protective effect of high indoor endotoxin exposure towards the development of the condition.

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Introduction

The rise of human atopic disease (AD) in Western countries in recent years has been described by several large-scale epidemiological studies. There are currently no data available to show whether the phenomenon also occurs in pets exposed to the same indoor living environment. AD naturally develop in dogs and cats, and canine atopic dermatitis (CAD) in particular shows a remarkable similarity in disease pathogenesis and clinical symptoms to its human counterpart (Olivry and Hill, 2001b; Nuttall et al., 2002; Marsella et al., 2006; Pucheu-Haston et al., 2008).

CAD is a genetically predisposed, inflammatory and pruritic allergic skin disease with characteristic clinical features associated most commonly with the production of IgE antibodies to environmental allergens. As the result of pruritus, dermatitis is commonly noticed at the feet, face, ears and axillae. Approximately 75% of dogs show the first symptoms before the age of 3 years. About 10% of the canine population is affected with CAD, and it is more common in certain breeds including Labrador retrievers (Hillier and Griffin, 2001; Shaw et al., 2004).

Exposure to environmental allergens is crucial for the development of CAD. Most frequently sensitization to house dust mite

(HDM) allergens is reported (Hill and DeBoer, 2001; Nuttall et al., 2006), in particular allergens from the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. Although it is clear that exposure to environmental allergens is a prerequisite for sensitization, the relationship between the level of exposure and sensitization and development of AD remains elusive. It has been shown that there is a positive relationship between level of HDM exposure and atopic eczema in children (Kramer et al., 2006). In addition, some found this effect only with sensitization to HDM and without development of AD (Ricci et al., 1999), whereas other studies failed to determine a clear relationship (Cullinan et al., 2004; Harris et al., 2007). Several environmental factors other than HDM allergens are also commonly associated with sensitization and clinical atopy in humans or, in contrast, with an inhibitory effect on disease development.

Endotoxin is a strong pro-inflammatory response-inducing agent produced from the cell walls of Gram-negative bacteria. High exposure to endotoxin in early life is associated with low incidence of atopic eczema and other AD in children with a parental history of asthma or allergies (Gehring et al., 2001; Park et al., 2001; Braun-Fahrlander et al., 2002; Phipatanakul et al., 2004; Douwes et al., 2006). Depending on timing and dose of exposure, mould components (such as fungal glucans) may have adjuvant or protective effects on atopy (Schram et al., 2005; Schram-Bijkerk et al., 2005).

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In this case-control study, Labrador retriever owners were asked to collect dust samples from the living room floor and from the bedding and coat of the dog to explore the possible association between CAD and exposure to HDM allergens, bacterial endotoxin, and fungal glucans.

Materials and methods

Study population

We analyzed dust samples from households with an adult Labrador retriever with ($n = 28$) or without (controls; $n = 65$) CAD. The diagnosis of CAD was based on standard criteria such as pruritus, early age of onset, presence of a corticosteroid-responsive dermatitis (at least on feet, face, and pinnae), immediate skin test reactivity to environmental allergens including *D. pteronyssinus* or *D. farinae*, and the presence of allergen-specific IgE in serum (Willemse, 1986; Favrot et al., 2010). Control dogs were healthy and did not have a history or clinical signs compatible with CAD. The adult retrievers were randomly selected from the CAD database of the university hospital (cases) or from among members of the Dutch Labrador Retriever Kennel Club (controls).

Dust collection

Dust samples were collected from the owners' living rooms and from the dogs' bedding and coats. All samples were collected between March and October 2007. Dust samples were collected using nylon sampling socks (Allied Filter Fabrics) and a vacuum cleaner according to the protocol used in the PARSIFAL study (Schram-Bijkerk et al., 2005). Briefly, for the collection of dust samples, sampling time and area depended on the type of floor covering of the room: for fully carpeted floors, 1 m² was vacuumed for 2 min; for smooth floors with a rug larger than 4 m², 1 m² of rug was vacuumed for 2 min; for smooth floors with no rug or with a rug smaller than 4 m², 4 m² of smooth floor was vacuumed for 4 min. The surface of the dogs' bedding was vacuumed for 2 min. For the dust sample from the dogs' coats, the whole dog was evenly vacuumed for 1 min. After collection, dust samples were sealed in plastic bags and stored at -20°C for 0–5 months until extraction.

Dust extraction and analysis

Dust was transferred from the nylon filter bag into a pyrogen-free Greiner tube, weighed, and then extracted in a volume of 5–40 mL of pyrogen-free water with 0.05% Tween20 (<0.5 g in 5 mL, 0.5–1.0 g in 10 mL, 1.0–2.0 g in 20 mL, and >2.0 g in 40 mL). Endotoxins, allergens, and glucans were extracted sequentially (Schram et al., 2005). For endotoxin, suspensions were incubated using an end-over-end roller for 1 h at room temperature (RT), and after centrifugation (15 min at 1000 g), dust-free supernatant was removed and stored at -20°C . For allergen extraction, the removed volume supernatant was replaced with exactly the same volume 10x concentrated phosphate-buffered saline (PBS). After re-suspending the dust sediment, the tubes were incubated at RT for 1 h followed by centrifugation (15 min at 3000 g). The supernatant was transferred and stored at -20°C . For glucan extraction, the removed supernatant was replaced with the same volume of PBS-Tween (0.05%). After re-suspending the settled dust, the tubes were placed on an end-over-end roller for 15 min at RT, followed by 1 h heat incubation in an autoclave (1 bar, 120°C). Thereafter, tubes were again placed for 15 min on an end-over-end roller at RT and centrifuged (15 min at 1000 g). The dust-free supernatant was collected and stored at -20°C .

Concentrations of HDM allergens (Der p1 and Der f1; ng/mL) were measured with a sandwich enzyme immunoassay (EIA) using a dust mite ELISA kit (Indoor Biotechnologies; van Strien et al., 2002). The quantitative kinetic chromogenic Limulus amoebocyte lysate (LAL) assay (BioWhittaker) was used to determine the amount of endotoxin in the dust samples. Concentrations were expressed as endotoxin units per mL. Levels of $(1 \rightarrow 3)\text{-}\beta\text{-D-glucans}$ ($\mu\text{g/mL}$) were measured with an inhibition EIA (Douwes et al., 1996). Measured levels of HDM allergens and microbial agents were converted to concentrations per g of collected dust and per m² of surface area.

Questionnaire data

All participants completed a questionnaire concerning household characteristics such as type of housing, type of floor covering, age of carpeting/rug, floor level, ventilation system, isolation of floors and windows, temperature and use of heating, dampness of the house, and number and type of indoor pets. The questionnaire also contained questions about the dogs' health (allergic symptoms, allergies diagnosed by veterinarian) and housing conditions.

Statistical analyses

Differences between cases and controls in the general data of the study population and home characteristics were determined using the Mann-Whitney *U* test and Chi-square test. Dust samples that had undetectable amounts of allergen,

endotoxin, or $(1 \rightarrow 3)\text{-}\beta\text{-D-glucan}$ were given a value of two-thirds of the detection limit. These substituted values were included in the statistical analyses in order to prevent the loss of data and not to overestimate the values above the detection level (Baccarelli et al., 2005). The detection limit for HDMs was 10 ng Der p1/g of dust and 22 ng/g for Der f1; endotoxin and $(1 \rightarrow 3)\text{-}\beta\text{-D-glucan}$ levels were detected above 1159 EU/g and 77 $\mu\text{g/g}$, respectively. Data were not normally distributed even after *ln*-transformation, and therefore the Mann-Whitney *U* test was used to compare the means between cases and controls.

Logistic regression analyses were performed to calculate associations between CAD and exposure to HDM allergens, endotoxin, or $(1 \rightarrow 3)\text{-}\beta\text{-D-glucans}$. The odds ratios (OR) with 95% confidence intervals (CI) were calculated with and without adjustment for age and sex. Logistic regression analyses on continuous exposure data did not reveal any interpretable results. Further logistic regression analyses were performed after dichotomizing exposure data at the median or at the limit of detection when more than half the samples yielded no detectable results. Division was made at the detection level to have an as much as possible equal distribution between the cells. Correlation between exposure levels of samples taken from the living room and the dogs' bedding and coat were analyzed using the Spearman rank correlation test. All statistical analyses were performed with SPSS statistical software (version 16.0 for Windows, SPSS Inc.).

Results

The characteristics of the study population are shown in Table 1. Dogs with CAD were older, more often male than control dogs, and were washed more frequently. Home characteristics regarding the number of dogs, the presence of other pets (such as cats, rodents, and birds), the age of the house, and the living room conditions

Table 1
Characteristics of the study population and home characteristics.

Characteristics	Cases ($n = 28$)	Controls ($n = 65$)
Age (years), median (range) ^a	7.0 (2.5–13.0)	3.5 (1.0–13.5)
Age at diagnosis (years), median (range)	3.6 (1.6–8.0)	–
Male (%) ^b	18 (64)	15 (23)
Wash frequency		
0–3 times/year ^c	13 (46)	57 (88)
4–52 times/year ^c	12 (43)	2 (3)
Unknown	3 (11)	6 (9)
Season of sampling (%)		
Spring ^d	0 (0)	14 (21)
Summer ^d	18 (64)	20 (31)
Autumn	10 (36)	31 (48)
Household		
Number of dogs	1.5	1.9
Other pet present	7 (25)	19 (29)
House built		
Before 1920	3 (11)	7 (11)
1920–1975	10 (36)	24 (37)
After 1975	14 (50)	33 (51)
Date moved into present house	1996	1995
Type of home (%)		
Single-family home	23 (82)	56 (86)
Ground-floor flat	0 (0)	2 (3)
Upper-floor flat ^e	4 (14)	2 (3)
Farmhouse	0 (0)	5 (8)
Type of floor cover in living room (%)		
Smooth floor	16 (57)	38 (58)
With rug >4 m ² or carpet	11 (39)	27 (42)
Age of carpet/rug (years)	9.2	8.1
Double-glazed windows in living room (%)	27 (96)	62 (95)
Indoor temperature in winter ($^{\circ}\text{C}$)	20	20

^a Significant difference between the age of cases and controls tested with Mann-Whitney *U* test ($P < 0.05$).

^b Significant difference in gender between cases and controls tested with Chi-square ($P < 0.01$).

^c Significant difference in wash frequency tested with Chi-square ($P < 0.01$).

^d Significant difference between the season in which samples were collected, tested with Chi-square ($P < 0.05$).

^e Significant difference between cases living in an upper-floor flat compared to controls, tested with Chi-square ($P < 0.05$).

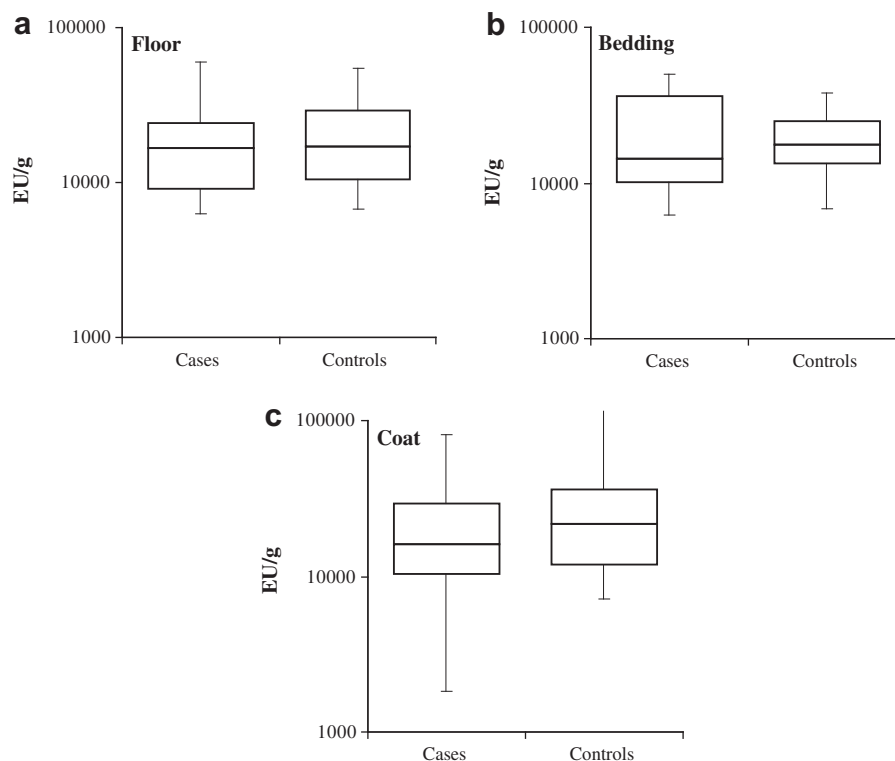


Fig. 1. Exposure levels of endotoxin expressed as endotoxin units (EU) per g of dust from the floor in the living room (a) and from the bedding (b) and coat of the dog (c). Box plots showing median and interquartile range (box); the whiskers indicate 10th and 90th percentiles.

(such as type of floor covering, temperature, and type of windows) were not significantly different between the two groups. About 50% of the houses were built after 1975, and it was observed that the majority of dogs had lived their entire lives in their present homes. The type of home in which the dog lived was usually a single-family home with more cases living in an upper-floor flat.

The number of samples with a detectable amount was higher for Der p1 than Der f1, and samples taken from the coat of the dog were more frequently below the level of detection compared to samples from the living room floor or from dogs' bedding. Almost all dust samples contained detectable amounts of endotoxin and (1 → 3)-β-D-glucans.

The endotoxin exposure levels in dust from the living room floor, bedding, and coat of the dog are shown in Fig. 1. The median endotoxin exposure was higher for controls than cases with the largest difference found in coat samples. However, these differences were not significant. There were no significant differences in exposure levels of Der p1 or Der f1 and (1 → 3)-β-D-glucans between cases and controls (data not shown) in samples from all three sample locations. The concentration of Der p1 measured in all samples of house dust was significantly higher than Der f1 ($P < 0.01$), with 42 ng/g being the median concentration for Der p1, whereas the median concentration for Der f1 was below the detection level (data not shown). The same distribution was found when exposure levels in the three different sample locations were analyzed per m² (data not shown). Exposure levels were stratified by type of floor (smooth floor, carpet/rug) and season, and analysis of these stratified data did not show significant differences (data not shown).

Owing to the high number of samples below the detection level, HDM exposure levels were divided into samples below and above the detection level. No significant relationship between HDM exposure in each of the three sample locations and CAD was found (Table 2), and all CI contained 1 for both unadjusted (cOR) models

Table 2

Exposure of Der p1 and Der f1 per g of dust for cases and controls.

	Cases	Controls	cOR ^a (95% CI)	aOR ^a (95% CI)
<i>Der p1 floor (ng/g)^b</i>				
<LOD	15	29	0.70	0.57
10–32,886	13	36	(0.29–1.70)	(0.19–1.67)
<i>Der p1 bedding (ng/g)^b</i>				
<LOD	15	30	0.74	0.59
10–25,919	13	35	(0.31–1.81)	(0.20–1.76)
<i>Der p1 coat (ng/g)^b</i>				
<LOD	21	45	0.64	0.23
10–3362	6	20	(0.22–1.83)	(0.05–1.02)
<i>Der f1 floor (ng/g)^b</i>				
<LOD	20	47	1.04	1.93
22–1767	8	18	(0.39–2.79)	(0.56–6.59)
<i>Der f1 bedding (ng/g)^b</i>				
<LOD	21	52	1.09	0.44
22–4010	6	13	(0.37–3.24)	(0.10–1.85)
<i>Der f1 coat (ng/g)^b</i>				
<LOD	21	55	1.51	1.65
22–1301	6	10	(0.51–4.87)	(0.39–6.91)

^a Crude Odds Ratios (cOR) and adjusted OR (aOR) with 95% confidence intervals (CI) are shown. ORs are adjusted for age and sex.

^b Exposure levels are dichotomized at the level of detection (LOD) to generate nominal data.

and those adjusted for age and sex (aOR). In contrast to HDMs, endotoxin exposure measured in the coat (Table 3) was found to be significantly and inversely associated with CAD (cOR 0.38; 95% CI 0.15–0.97; $P < 0.05$) indicating that high (above median) endotoxin levels are less likely to be found in the coats of dogs with CAD. After adjusting for age and sex the association between endotoxin exposure and CAD was not significant (aOR 0.45; 95% CI 0.15–1.40; $P = 0.17$). Endotoxin exposure levels from both the floor

Table 3
Exposure of endotoxin and (1 → 3)-β-D-glucan per gram of dust for cases and controls.

	Cases	Controls	cOR ^a (95% CI)	aOR ^a (95% CI)
<i>Endotoxin floor (EU/g)^b</i>				
773–17,104	14	32	0.90	0.75
17,237–625,049	13	33	(0.37–2.21)	(0.24–2.28)
<i>Endotoxin bedding (EU/g)^b</i>				
773–17,354	16	30	0.64	0.73
17,433–148,916	12	35	(0.26–1.57)	(0.25–2.13)
<i>Endotoxin coat (EU/g)^b</i>				
773–18,345	18	28	0.38 [*]	0.45
19,205–1,616,531	9	37	(0.15–0.97)	(0.15–1.41)
<i>Glucan floor (μg/g)^b</i>				
51–1220	12	34	1.46	1.54
1222–10,706	16	31	(0.60–3.57)	(0.53–4.51)
<i>Glucan bedding (μg/g)^b</i>				
51–1382	16	30	0.64	0.39
1385–5047	12	35	(0.26–1.57)	(0.13–1.19)
<i>Glucan coat (μg/g)^b</i>				
51–706	14	32	0.90	0.76
766–7960	13	33	(0.37–2.21)	(0.25–2.29)

^a Crude Odds Ratios (cOR) and adjusted OR (aOR) with 95% confidence intervals are shown. ORs are adjusted for age and sex.

^b Exposure levels are dichotomized at the median to generate nominal data.

^{*} $P < 0.05$.

in the living room and bedding were not significantly associated with CAD. No relationship was found for (1 → 3)-β-D-glucan exposure in all three sample locations and CAD. A post hoc power analysis on the non-significant results showed that this could be due to low power (<50%) instead of a small effect of indoor exposure.

Discussion

In this case-control study, we have shown that low endotoxin levels in the coats of dogs were associated with CAD. Our findings indicate that environmental exposure to endotoxins may be protective against the development of CAD. This is the first study to address the association between endotoxin exposure and AD in dogs. No associations were found between CAD and exposure to HDM allergens or fungal glucans.

Our observations are supported by several human studies demonstrating that high exposure to endotoxins may have a protective effect against allergic diseases. Endotoxin levels found in dust from living rooms or mattresses were shown to be inversely related to the occurrence of atopic dermatitis, atopic asthma, or atopic sensitization in children (Gehring et al., 2001, 2007; Braun-Fahrlander et al., 2002; Phipatanakul et al., 2004; Schram et al., 2005; Schram-Bijkerk et al., 2005; Douwes et al., 2006).

The suggested protective effect of endotoxins may be explained by their immunoregulatory capabilities. Exposure to Gram-negative bacteria as a source of endotoxins, especially during early childhood, will augment the stimulation of helper T cells type 1 (Th1). These Th1 cells will in turn inhibit the generation of helper T cells type 2 (Th2), which play an important role in the development of allergic diseases (Gereda et al., 2000). Besides the classical Th1–Th2 balance, regulatory T cells are also stimulated by endotoxin exposure and may suppress the allergic inflammatory reaction (Caramalho et al., 2003; Romagnani, 2007).

Interbreed variations may be an important form of bias, especially for determining environmental exposure levels in the coat. For this reason, we included only Labrador retrievers in this study. Another potential confounding factor could be how often dogs are washed. We have shown that dogs with AD were washed more frequently than healthy dogs. However, there were no associations between washing frequency and exposure levels to mites, endo-

toxins, and fungal glucans or with the amount of dust collected in the coat samples (data not shown). Only adult dogs were selected to reduce the proportion of false negative selection in the control group as allergy develops within the first years of a dog's life. One of the potential limitations of the case-control study design has been that only present exposure is determined and not exposure before sensitization. However, the majority of dogs in this study lived their entire lives in the same houses, and it has been shown by others that indoor exposure levels are relatively stable (Heinrich et al., 2003). Therefore the measurements in our study may be representative of exposure levels in early life.

We saw no clear correlation between samples taken from the floor of the living room and from the bedding and coats of the dogs (data not shown). Levels of endotoxin were significantly higher in the coat of a dog living in a house with a smooth floor in the living room, whereas no differences in endotoxin levels were found between smooth and carpeted floors. For carpeted floors, we found significantly higher levels of Der p1 and glucans compared to smooth floors, as has also been reported in other studies (van Strien et al., 1994; Wouters et al., 2000), but no higher levels of these two compounds were found in the coats and bedding of dogs living in carpeted living rooms.

It has been assumed that the major route of allergen exposure is through the epidermis (Olivry and Hill, 2001a), and therefore we would expect that coat samples give a good representation of exposure at the level of the epidermis. The lack of correlation between sample locations indicates that it is advisable for both human and veterinarian epidemiological studies to obtain dust samples from different sample locations to get an overall picture of actual exposure. It also shows that efforts to reduce indoor mite allergen exposure by removing carpets in the living area do not result in lower allergen exposure levels in the coat of the dog.

Conclusions

Endotoxin exposure in the coats of dogs is inversely associated with CAD. This finding suggests that reduced endotoxin exposure may play a role in the pathogenesis of atopic dermatitis in dogs.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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