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Characterisation of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions

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ABSTRACT

The immunopathogenesis of cutaneous adverse food reactions (CAFRs) in dogs is unknown. Since the clinical manifestations in the skin are like those found in canine atopic dermatitis (AD), this study investigated the similarity in T cell phenotypes and gene expression of cytokines and transcription factors in CAFRs. In addition, the influence of an elimination diet on these parameters was tested.

In the skin of canine CAFRs, a predominant presence of $CD8^+$ T cells and increased expression of the *IL-4*, *IL-13*, *Foxp3* and *SOCS-3* genes were observed. *IFN-γ* gene expression was increased in lesional compared to non-lesional skin. The predominance of $CD8^+$ T cells indicates that the immunopathogenesis of CAFRs is different from that of canine AD. The elimination diet relieved clinical signs, but did not influence T cell phenotypes or expression of the cytokine and transcription factor genes in the skin of dogs with CAFRs, indicating a continuously pre-activated immune status in dogs sensitised to food constituents.

Introduction

Skin responses to dietary components in dogs are generally referred to as cutaneous adverse food reactions (CAFRs), because the underlying immunological response patterns are unknown (Hillier and Griffin, 2001). Although food allergen exposure occurs first in the digestive tract, only 20–30% of CAFR dogs have gastrointestinal signs (Hillier and Griffin, 2001) whereas the majority of the dogs only show cutaneous symptoms.

Signs of CAFRs can occur at the same predilection sites and may be clinically indistinguishable from atopic dermatitis (AD). In the most detailed retrospective study, 63% of dogs diagnosed with food hypersensitivities based on dietary elimination and provocation tests exhibited cutaneous lesions and pruritus suggestive of AD (Chesney, 2002). However, CAFRs may also manifest as papular abdominal rash, otitis, seborrhoea or recurrent superficial pyoderma (White, 1986; Carlotti et al., 1990; Harvey, 1993; Rosser, 1993; Leistra et al., 2001).

Currently, it is unknown how exposure to food leads to adverse reactions in the skin. In contrast, cell subsets and cytokine production in the skin of dogs with AD have been investigated extensively. In AD, inflammation is characterised by an influx of $CD4^+$ and $CD8^+$ T cells in the lesional skin (Olivry et al., 1997; Sinke et al., 1997) and a mixed cytokine profile with predominant expression of *IL-6*, *TARC*, *IL-4* and *IL-13* genes in the early stage followed by *IFN-γ*, *IL-12* and *IL-18* later on (Olivry et al., 1999; Nuttall et al., 2002; Marsella et al., 2006). Whereas most dogs with AD have circulating allergen-specific IgE (DeBoer and Hillier, 2001), there is controversial evidence for a similar reaction phenomenon in dogs with spontaneous manifestations of CAFRs (Jackson et al., 2003; Pucheu-Haston et al., 2008; Puigdemont et al., 2006). Increasing our insight in the immunopathogenesis of CAFRs may facilitate the development of novel modalities to treat them.

Since the clinical manifestations of CAFRs are comparable to those of AD, we hypothesised that the cutaneous reaction pattern of dogs with CAFRs is comparable to that of dogs with AD. To test this hypothesis, the inflammatory response (T cells and expression of cytokines and transcription factors representative for Th1, Th2 and regulatory T cells; reviewed in Wilson et al. (2009); Ozdemir et al. (2009)) in the skin of dogs with CAFRs was investigated after a provocation diet with the animals' original food and a subsequent elimination diet.

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Materials and methods

Animals and experimental design

Ten client-owned CAFR dogs without intestinal symptoms (five females, five males; age 10 months–8 years, median 3.5 years) were referred to the Utrecht University Faculty of Veterinary Medicine. The group consisted of four Labrador retrievers and one of each of the following breeds: shorthaired Dachshund, a German Shepherd, English Bulldog, English Cocker spaniel, West Highland White terrier, and a mixed-bred dog. There is no known breed predisposition for CAFRs. The seven healthy control dogs (three females, four males; age 4–10 years, median 8 years) included in this study were three Beagles and four mixed-bred dogs. The study fulfilled the requirements set by the Utrecht University Animal Experiments Committee.

Prior to dietary testing, flea bite hypersensitivity was ruled out by skin testing, IgE serology and flea control measures with adulticides and insect growth inhibitors at 1-month intervals. Other parasitic disorders were excluded by routine diagnostic methods. Secondary infections (staphylococcal pyoderma, *Malassezia* spp. dermatitis) were treated, if applicable, before and throughout the trial. The CAFR dogs were included based on the following dietary testing procedure. First, a full reduction in pruritus and associated symptoms had to be achieved after an elimination diet using a novel protein (ostrich, turkey, horse or goat meat) home-cooked diet for at least 8 weeks. Second, a provocative test with the original food had to result in reappearance of the pruritus (T0). Third, after a second elimination diet the pruritus had to disappear for a second time (T1). The healthy control dogs underwent a similar dietary regimen for 8, 2 and 2 weeks, being the average periods necessary for dietary responses in CAFR dogs.

Skin samples

Skin biopsies from both lesional (LS) and non-lesional skin (NLS) were taken using a 6-mm disposable punch (Kai Industries) under general anaesthesia (medetomidine 20 μg/kg and propofol 1–3 mg/kg IV on effect) within 10 days following the onset of signs due to the dietary provocation (T0) and after the disappearance of signs resulting from the second elimination diet (T1). Since clinical signs vanished after the elimination diet (T1) and LS was no longer visible, biopsies were taken at the same locations as at T0. Skin biopsies of the healthy control dogs were collected from the front legs and the thorax. Skin samples were snap frozen in liquid nitrogen for immunohistochemistry and RNA isolation, or fixed in 4% neutral buffered formaldehyde for routine haematoxylin and eosin (HE) staining.

RNA isolation

Frozen LS and NLS biopsies were immersed in TRIzol at 4 °C (Invitrogen), cut in smaller pieces and homogenised using an Ultra-Turrax disperser (T8, IKA Labortechnik). Isolation of total RNA and removal of genomic DNA was performed as previously described (Schlotter et al., 2009) and consisted of a combination of TRIzol and RNeasy mini kit (Qiagen) procedures.

Primer design and quantitative polymerase chain reaction (Q-PCR)

Oligonucleotide primers (Eurogentec) were designed for Th1-related genes (*IL-12p35*, *IL-18*, *IFN-γ*, *STAT-4*, *SOCS-5*, *TNF-α*), Th2-related genes (*IL-4*, *GATA-3*, *STAT-6*, *SOCS-3*, *IL-13*, *TARC*) and tolerance-related genes (*IL-10*, *TGF-β*, *Foxp3*) based on the sequences described in the ensemble project[1] using the Primer-3 software. Primer sequences and optimum annealing temperatures are shown in Table 1.

cDNA synthesis and Q-PCR conditions were as previously described (Brinkhof et al., 2006) with the following modifications. The Q-PCR programme included a 5 min polymerase activation step and continued with 40 cycles consisting of a denaturing step at 95 °C for 30 s, an annealing step for 30 s and an elongation step at 72 °C for 30 s with a final extension for 2 min at 72 °C. All PCR reaction efficiencies were between 95% and 105% and product melting curves showed single products and absence of a product in the negative controls (data not shown). If the expression of a gene was below the detection limit, the lowest detectable value was used for statistical analysis. The results of each sample were normalised to the average amounts of the endogenous reference genes (*HPRT* and *RPS19*) of the same sample (Vandesompele et al., 2002; Schlotter et al., 2009): the relative gene expression. *RPS5* was used as control gene for statistical analysis.

Immunohistochemistry

Biopsies were mounted in Tissue-Tek (Sakura Finetek Europe) and 6 μm cryostat sections were placed on Superfrost Plus slides (Menzel-Glaser), dried and stored at −70 °C until use. After thawing, the tissue sections were fixed in 100% acetone for 10 min at room temperature (RT). Endogenous peroxidase was eliminated by 20 min incubation in Tris-buffered saline (TBS: 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.5) supplemented with 0.3% hydrogen peroxide, followed by washing in TBS. Sections were incubated for 25 min in blocking reagent (TBS with 10% inactivated normal dog and normal goat serum) and subsequently for 1 h at RT with primary unlabelled antibodies diluted in 1% blocking reagent (Table 2). After washing, sections were incubated for 30 min with horseradish peroxidase (HRP)-labelled antibodies (Table 2), washed and blocked for 30 min with unlabelled rabbit anti-mouse antibodies (Table 2) to cover any unbound primary antibodies.

After further washing, the sections were incubated for 45 min with the second primary unlabelled antibodies, washed and incubated for 30 min with alkaline phosphatase (AP)-labelled antibodies (Table 2). Staining was developed with 3-amino-9-ethyl-carbazole (AEC, Sigma–Aldrich) resulting in a red-brownish colour (HRP) and thereafter with Fast Blue (AP), resulting in a blue colour. Double stained cells appeared purple. Cells in the epidermis and dermis from two biopsies were counted using light microscopy. Average numbers of positive cells/mm^2 were used for statistical analysis.

Table 1
Quantitative PCR primer sequences and annealing temperature.

Gene	Primer sequences 5′–3′	Annealing temperature (°C)
	Forward (F) and Reverse (R)	
IL-4	F: ccaaagaacacaagcgataaggaa	61
	R: gtttgccatgctgctgaggtt	
IL-10	F: cccgggctgagaaccacgac	63
	R: aaatgcgctcttcacctgctccac	
TARC	F: ggagccattcctatcagcag	64.5
	R: ggtcggaacagatggacttg	
IL-12p35	F: taatggatcccaagaggcag	62.5
	R: tcaagggaggatttctgtgg	
IL-18	F: gaggatatgcccgattctga	56
	R: tccggaggactcatttctg	
IFN-γ	F: agcgcaaggcgataaatg	55.8
	R: gcggcctcgaaacagatt	
TGF-β	F: caaggatctgggctggaagtgga	65
	R: ccaggaccttgctgtactgcgtgt	
GATA-3	F: tacgtccccgaatacagctc	64
	R: actccctgccttctgtgct	
STAT-4	F: actggaagaggcgacaacag	59
	R: gccttctgagttggaacagg	
STAT-6	F: aactgcagcggctctatgtc	64
	R: catgttgcagcagaaggtgt	
SOCS-5	F: tctgccgtgcagtaatctgt	61
	R: gccttgactggttctcgttc	
TNF-α	F: ccccgggctccagaaggtg	64
	R: gcagcaggcagaagagtgtggtg	
SOCS-3	F: acaccagcctgcgcctcaagacct	63
	R: cgcctcgccgcccgtca	
IL-13	F: gaggagctggtcaacatca	59
	R: tgcagtcggagacattga	
Foxp3	F: caaatggtgtctgcaagtgg	59
	R: gtgctctgcccttctcatct	
HPRT	F: agcttgctggtgaaaaggac	56
	R: ttatagtcaagggcatatcc	
RPS19	F: ccttcctcaaaaagtctggg	61
	R: gttctcatcgtagggagcaag	
RPS5	F: tcactggtgagaaccccct	62.5
	R: cctgattcacacggcgtag	

Statistical analysis

The relative gene expression and immunohistochemistry results were analysed with the SPSS version 15 software. A linear mixed model (West et al., 2007) was done to analyze the relative gene expression as outcome variable. The relative expression of all genes together was analyzed in one model. The explanatory factors in the model are gene, LS/NLS/Control group, T0/T1 group and three two-way interactions between the three factors. The subject variable dog was used as the random factor to account for the repeated measurements within the dogs and was assumed to have a normal distribution. *RPS5* was used as the control gene. Residuals were studied to check the validity of the model. Multiple comparisons were corrected with the False Discovery Rate implying that $P \leqslant 0.05$ was considered statistically significant.

Since the results for immunohistochemistry were discontinuous values, non-parametric statistical tests were used. For the comparison between skin sections of CAFRs and control dogs, the Mann–Whitney *U* test (non-parametric analogue of independent *t* test) was used and for the comparison of skin sections between T0 and T1 and between LS and NLS skin, the Wilcoxon Signed Ranks test (non-parametric analogue of the paired *t* test) was used.

[1] See: http://www.ensembl.org/, version v.32.