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Research paper

A longitudinal study of autoantibodies against cytochrome P450 side-chain cleavage enzyme in dogs (*Canis lupus familiaris*) affected with hypoadrenocorticism (Addison's disease)

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ABSTRACT

Autoantibodies directed against the P450 side chain cleavage enzyme (P450scc) have been recently described in dogs affected with hypoadrenocorticism, consistent with an immune-mediated pathogenesis of this endocrinopathy. In human autoimmune Addison's disease, autoantibodies may have a predictive value, being detectable before clinical signs developing, and have been shown to persist for a period of time after diagnosis. Furthermore, an autoantibody positive status post-diagnosis has been associated with successful remission of Addison's disease following B-cell depletion, suggesting active immunopathology in these cases. The current study was designed to investigate changes in serum P450scc autoantibody status over time in dogs diagnosed with spontaneous hypoadrenocorticism. P450scc autoantibodies were measured using a species-specific radioimmunoprecipitation assay in an initial cohort of 213 dogs, indicating a prevalence of 24%. Thirty two of these dogs had repeat samples (n = 80 in total) available for analysis. Five dogs were consistently P450scc autoantibody positive in all samples, for up to 425 days following first sampling. Three dogs were initially autoantibody positive, then became seronegative at later time points. One dog, a 1 year old female entire standard poodle, was initially negative for P450scc autoantibodies, but seroconverted 18 months after diagnosis. The remaining 23 dogs with multiple samples available were consistently P450scc autoantibody negative. Persistence was not associated with sex (p = .673). This study demonstrates persistence of P450scc autoantibodies in a subset of dogs affected with hypoadrenocorticism and seroconversion over one year post-diagnosis. P450scc autoantibody reactivity in human autoimmune Addison's disease has been associated with sex, with females having a higher prevalence, possibly due to P450scc expression in the ovary acting as an additional source of antigenic stimulation. However, there was no sex difference in autoantibody persistence in the dogs affected with hypoadrenocorticism. Autontibody persistence in dogs with hypoadrenocorticism might represent persistent pathology, due to residual antigenic stimulation and autoimmune inflammation in the adrenal gland.

1. Introduction

Canine hypoadrenocorticism (Addison's disease), caused by deficiency of corticosteroid hormones production by the adrenal gland, has important health and welfare implications (Scott-Moncrieff, 2015) (Summers et al., 2010). The condition can be challenging to diagnose due to the waxing and waning clinical signs that are not pathognomonic, including lethargy, anorexia, polyuria/polydipsia, vomiting and diarrhoea (Hughes et al., 2007; Peterson et al., 1996; Thompson et al., 2007). Hypoadrenocorticism is diagnosed by ACTH stimulation test, demonstrating a deficiency in cortisol secretory capacity (Scott-Moncrieff, 2015).

Adrenal glands histopathology from affected dogs indicates that a lymphocytic adrenalitis is present, followed by atrophy in end-stage

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disease (Adissu et al., 2010; Boujon et al., 1994; Frank et al., 2013; Hadlow, 1953; Schaer et al., 1986). These changes suggest an autoimmune disorder analogous to human autoimmune Addison's disease (AAD) (Betterle et al., 2002; Mitchell and Pearce, 2012). Genetic evidence supports an autoimmune pathogenesis for canine hypoadrenocorticism, with susceptibility linked to immune response genes including MHC class II, *CTLA4* and *PTPN22* (Boag and Catchpole, 2014; Chase et al., 2006; Hughes et al., 2011; Hughes et al., 2010; Massey et al., 2013; Short et al., 2013; Short et al., 2014).

Circulating autoantibodies are regarded as an important indicator of autoimmune disease (Blizzard and Kyle, 1963; Lleo et al., 2010; Rose and Bona, 1993), autoantibodies in human AAD patients have long been recognised (Anderson et al., 1957). The primary autoantigen in human AAD is 21-hydroxylase (21-OH), with specific autoantibodies present in 90% of patients at diagnosis and in approximately 50% of patients with longer standing disease (Bednarek et al., 1992; Betterle et al., 2005; Husebye and Løvås, 2009). A case report of a hypoadrenocorticoid dog with evidence of serum 21-OH autoantibodies has been recently published (Cartwright et al., 2016), although 21-OH autoantibodies were not identified in a larger cohort of affected dogs (Boag et al., 2015). In contrast, antibodies against the cytochrome P450 side-chain cleavage enzyme (P450scc) have been described in 24% of dogs affected with hypoadrenocorticism (Boag et al., 2015), implicating an autoimmune-mediated pathogenesis in at least a proportion of cases. Autoantibodies against P450scc have also been described in human AAD, with the highest prevalence in female patients also affected with premature ovarian failure (POF), due to suspected autoimmune oophoritis (Betterle et al., 2005; Betterle et al., 1999; De Carmo Silva et al., 2000; Falorni et al., 2002; Pra et al., 2003).

The presence of adrenal autoantibodies has been shown to have a significant predictive role in a multivariate analysis of risk factors for development of human AAD (Betterle et al., 2005). In healthy humans that are positive for 21-OH autoantibodies, around 15% will subsequently develop AAD within a six year period (Husebye and Løvås, 2009). Similarly, in a cohort of 234 thyroglobulin autoantibody (TGAA) positive dogs, 20% progressed within one year to demonstrate clinicopathological evidence of thyroid dysfunction (Graham et al., 2001). Autoantibodies are also associated with continued inflammatory or autoimmune processes and their persistence may indicate active disease in the adrenal gland. Autoantibody persistence has been shown to be associated with a worse clinical outcome and progressive disease in humans with latent autoimmune diabetes of adults (Huang et al., 2016) and with an increased risk of progressing to Type 1 diabetes mellitus in children, when compared to those with reversion of autoantibodies (Vehik et al., 2016). Use of autoantibody status as a biomarker of active disease has been highlighted in a study showing clinical remission following B cell depletion, in a small open label pilot trial in humans with AAD, where one of the six patients was able to discontinue steroid therapy and had improved dynamic cortisol tests up to 27 months later (Pearce et al., 2012).

Given the potential for autoantibody testing for diagnosis and monitoring of canine hypoadrenocorticism, the aim of the present study was to document P450scc autoantibody status in a cohort of dogs affected with hypoadrenocorticism, where repeat blood sampling had been undertaken.

2. Materials and methods

2.1. Study population

The study population consisted of 213 dogs affected with hypoadrenocorticism (Boag et al., 2015). Residual serum samples were obtained following completion of diagnostic testing, undertaken either by the Royal Veterinary College (RVC) Diagnostic Service (Hatfield, UK) or NationWide Laboratories (Poulton-le-Fylde, UK). All sera included in the current study were from NationWide Laboratories.

The Royal Veterinary College Institutional Ethics and Welfare Committee approved the use of residual blood samples, taken for diagnostic purposes, for research with informed owner consent. NationWide Laboratories has approval for utilising residual clinical samples for development of diagnostic assays, provided that UK data protection legislation is observed.

2.2. Radioimmunoprecipitation assay

The autoantibody assay was carried out as previously described (Boag et al., 2015). Briefly, radiolabelled recombinant P450scc protein (20,000 CPM radioactivity; 20 µL per reaction) diluted in IMP buffer (10 mM HEPES, 150 mM NaCl, 20 mM methionine, 10 mM benzamidine, 0.01% BSA, 2.5 mL 0.5% Triton X-100; all Sigma–Aldrich, Poole, UK) was mixed with 10 µL serum in triplicate wells and incubated in V bottomed 96-well plates at 4 °C for ~18 h. Opaque microtitre filter plates were blocked overnight with 100 µL/well of 2 mg/mL BSA in phosphate-buffered saline (PBS) and washed twice with IMP buffer prior to use. Protein A sepharose (Sigma–Aldrich, Poole, UK) was added (10 µL/well) and incubated with agitation for 20 min. The immunoprecipitate was transferred to wells of the filter plate and washed with IMP buffer. Following drying, 100 µL/well MicroScint™ (Perkin Elmer, Cambridge, UK) was added and a Chameleon™ V plate reader (Hidex, Turku, Finland) used to quantify the amount of radioimmunoprecipitate. P450scc autoantibodies have been shown at both higher and lower dilutions and inter-assay coefficient of variation does not differ substantially with sample dilution; a representative example of dilutional parallelism is shown in Supplementary Fig. 1.

Relative autoantibody reactivity was calculated to allow inter-assay normalisation of data as follows: $(CPM_{sample} - CPM_{negative_standard})/(CPM_{high_standard} - CPM_{negative_standard}) \times 100$. The threshold value for autoantibody positivity was set at the mean + 3 × SD of controls ($n = 30$) as previously described (Boag et al., 2015).

2.3. Statistical analysis

Statistical analyses were performed using SPSS© Statistics for Windows, version 20.0 (IBM Corp, Armonk, NY, USA). GraphPad Prism version 6.02 (GraphPad Software Inc., CA, USA) was used for construction of graphs. Categorical data were analysed using contingency tables, with Chi squared or Fisher's exact test used for comparisons. Continuous data was tested for normality by manual inspection of histograms, Q-Q plots and Shapiro-Wilk (Razali and Wah, 2011). For normally distributed data, comparisons were made using two-sided unpaired Student's *t*-tests, or ANOVA with post-hoc Bonferroni correction for multiple comparisons. Data not normally distributed were analysed using the Mann-Whitney *U* test or Kruskal-Wallis H test. Significance was accepted at $p < 0.05$.

3. Results

Samples from 213 dogs were previously screened for P450scc autoantibodies, with a prevalence of 24% (Boag et al., 2015). Within this cohort, 32 dogs had between two and six repeat blood samples, with 80 samples available overall; collected between two and 787 days from the first sample being taken. The mean time from diagnosis for all samples from dogs with a known date of diagnosis, was 200 days.

Five dogs were consistently autoantibody positive in all samples analysed, up to 425 days between sampling times, these comprised one male and four female dogs, and 3 dogs (a 3 y 11 month old male entire bull terrier, a 4 y 5 month old female neutered beagle and a 2 y female entire crossbreed dog) were initially autoantibody positive, then became seronegative at later time points (Fig. 1). One dog, a 1 y 8 month old female entire standard poodle, initially negative for P450scc autoantibodies, seroconverted 18 months after diagnosis (Fig. 1). The remaining 23 dogs with multiple samples were consistently autoantibody

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