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SPONTANEOUSLY ARISING DISEASE

Humoral and Cellular Immune Response in Canine Hypothyroidism

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Summary

Hypothyroidism is one of the most common endocrine diseases in dogs and is generally considered to be autoimmune in nature. In human hypothyroidism, the thyroid gland is destroyed by both cellular (i.e. autoreactive helper and cytotoxic T lymphocytes) and humoral (i.e. autoantibodies specific for thyroglobulin, thyroxine and triiodothyronine) effector mechanisms. Other suggested factors include impaired peripheral immune suppression (i.e. the malfunction of regulatory T cells) or an additional pro-inflammatory effect of T helper 17 lymphocytes. The aim of this study was to evaluate immunological changes in canine hypothyroidism. Twenty-eight clinically healthy dogs, 25 hypothyroid dogs without thyroglobulin antibodies and eight hypothyroid dogs with these autoantibodies were enrolled into the study. There were alterations in serum proteins in hypothyroid dogs compared with healthy controls (i.e. raised concentrations of α-globulins, β2- and γ-globulins) as well as higher concentration of acute phase proteins and circulating immune complexes. Hypothyroid animals had a lower CD4:CD8 ratio in peripheral blood compared with control dogs and diseased dogs also had higher expression of interferon γ (gene and protein expression) and CD28 (gene expression). Similar findings were found in both groups of hypothyroid dogs. Canine hypothyroidism is therefore characterized by systemic inflammation with dominance of a cellular immune response.

Keywords: autoimmune disease; dog; hypothyroidism; T lymphocyte

Introduction

Although hypothyroidism is one of the most common endocrine diseases in dogs, its pathogenesis remains unclear. According to the literature, canine hypothyroidism is generally considered to reflect an autoimmune destruction of the thyroid gland with histological evidence of lymphocytic thyroiditis (Day and Shaw, 2008; Mooney, 2011) and has many similarities with Hashimoto's thyroiditis in man (Beierwaltes and Nishiyama, 1968). In human patients, the thyroid gland is destroyed by both cellular (i.e. autoreactive helper and cytotoxic T lymphocytes) and humoral (i.e. autoantibodies specific for thyroglobulin, thyroxine and triiodothyronine) immune effector mechanisms and there is increasing evidence for an underlying deregulation of cellular immunity (i.e. a T helper [Th]1/Th2 imbalance) in the development of the disease (Phenekos *et al.*, 2004). Other possible immunological mechanisms involved in disease pathogenesis are the pro-inflammatory effect of Th17 lymphocytes (Qin *et al.*, 2012) and reduced function of regulatory T cells (Marazuela *et al.*, 2006; Glick *et al.*, 2013). Regardless of the underlying cause, the final effect of the process is cellular infiltration of the gland and production of autoantibodies, which are probably not directly responsible for tissue pathology, but may contribute to the cytotoxic effect via antibody-dependent cell-mediated cytotoxicity (Ben-Skowronek *et al.*, 2013).

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Less is known about the pathogenesis of canine hypothyroidism. Affected thyroid glands are infiltrated by plasma cells and lymphocytes and there is follicular loss (Choi *et al*., 2006; Day and Shaw, 2008) in addition to the presence of autoantibodies specific for thyroglobulin (Tg) and/or triiodothyronine (T3) and thyroxine (T4) in the serum (Nachreiner *et al*., 2002; Patzl and Möstl, 2003). Increased expression of Th1-type cytokines (i.e. interleukin [IL]-15, IL-18 and interferon [IFN]-γ) was detected in peripheral blood mononuclear cells (PBMCs) during the development of induced canine autoimmune thyroiditis (Choi *et al*., 2006), although the results differed between animals.

The aim of the present study was to explore further the range of immunological changes occurring in dogs with spontaneously arising hypothyroidism.

Materials and Methods

Case Selection

The diagnosis of hypothyroidism was based on clinical observation and laboratory findings, including the results tests for measurement of free T4 (fT4), T4 and, in some cases, thyroid-stimulating hormone (TSH). All animals had been treated with L-thyroxine for at least 4 months. Thirty-three hypothyroid dogs were divided into two groups, based on the presence of thyroglobulin autoantibodies (TgA). The first group consisted of eight dogs with confirmed presence of antibodies (TgA^+). The second group consisted of 25 hypothyroid dogs with no serum autoantibodies (TgA^-). The control group included 28 healthy dogs.

Blood Samples

Peripheral blood samples were collected into heparinized vials and serum tubes by cephalic venipuncture. Heparinized blood was used for whole blood culture and for density gradient isolation of PBMCs. Some of the isolated cells ($1-2 \times 10^6$) were frozen in Fenozol™ (A&A Biotechnology, Gdynia, Poland) and stored at −80°C for subsequent RNA isolation. Circulating immune complexes (CICs) were measured in fresh serum and the remaining serum was stored at −20°C.

Serum Proteins

Total serum protein concentration was determined by use of the biuret test. Serum protein electrophoresis was performed using standard paper electrophoresis. Serum (15 μl) was applied as a narrow band across the centre of the strip and electrophoresis (300V, 16A) was carried out for 4 h in 0.05 M veronal buffer (pH 8.6). Strips were dried for 20 min at 80°C, stained with 1.3% amido black 10B for 10 min and decolourized in a solution of 96% ethanol, 99.5% acetic acid and water (10:10:1). Stained proteins were cut from the strips, placed in separate tubes and covered (12 ml for the albumin fraction, 6 ml for each globulin fraction) with an elution buffer (4% sodium carbonate, 96% ethanol and water at 10:10:1). After 12 h the absorbance of each fraction was read at 590 nm. The total absorbance was designated as 100% and the concentration of each fraction was determined based on the total protein concentration measured by the biuret method.

Plasma fibrinogen concentration was measured by use of a heat precipitation test (Millar *et al*., 1971). In brief, a capillary tube was filled with EDTA-anticoagulated blood, centrifuged for 5 min at 16,900 g, heated to 56°C and again centrifuged for 5 min. Fibrinogen concentration in this method is considered equivalent to the length of the heat precipitate.

Serum samples were analysed for haptoglobin concentration by a modification of the method of Jones and Mould (1984) based on the measurement of peroxidatic activity of the haemoglobin (Hb)—methaemoglobin (MetHb) complex. The test was performed in flat bottomed microplates. Three serum dilutions (1 in 25, 1 in 5 and 1 in 2) in buffered saline were used. Bovine serum albumin solution (1%) was used as the control sample. The standard curve was determined using human haptoglobin standards (20—200 μl/ml; Sigma Aldrich, St. Louis, Missouri, USA). A 50 μl volume of each sample was mixed with 50 μl of 0.05% equine MetHb and incubated for 10 min at room temperature (RT). Following this, 150 μl of guaiacol reagent (0.08 M guaiacol in a buffer containing 0.2 M acetic acid/sodium hydroxide, pH 4.0) and 50 μl of 0.02 M H_20_2 were added. After 5 min, the absorbance of the samples was measured at 492 nm.

Circulating Immune Complexes

The concentration of CICs was estimated by a modification of the method of Levinson and Goldman (1983). Briefly, 750 μl of 3.3% polyethylene glycol was added to 250 μl fresh serum and incubated for 18 h at 4°C. After incubation, the samples were centrifuged (15 min, 25,155 g, at 4°C). The sediment was resuspended in 1 ml of 2.5% polyethylene glycol (weight:volume). A second centrifugation step was followed by resuspension in 2.5 ml of 0.1% NaOH. The optical density was measured at 280 nm in a