



Assessment of autoantibodies to glutamate decarboxylase and anti-insulin in spontaneous diabetic dogs and experimental diabetic rat model

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ABSTRACT

The aim of the study was to determine autoantibodies to GAD and insulin in diabetic dogs and diabetes induced wistar rat model. Eight diabetic dogs were used for the study. While, eight clinically healthy dogs were used as controls. Six rats were injected with streptozotocin 40 mg/kg body weight intraperitoneally for 5 consecutive days to induced diabetes. Another six rats without streptozotocin served as healthy controls. Dogs and wistar rats were screened for autoantibodies to GAD and insulin by using radioimmunoassay. All diabetic dogs shown anti-GAD antibody and while auto-antibody to insulin was shown by only 37.5% of diabetic dogs. None of the diabetic rat revealed auto-antibodies to GAD and insulin. The current study clearly indicates that assessment of anti-GAD antibody could be a novel predictive of diabetes in dogs. Concurrently, non-reactivity of antibodies to GAD and insulin in induced diabetic rats indicates incompatibility of this model to diabetes in dogs.

Key words-: Autoantibody, diabetes; dog; glutamate decarboxylase, insulin; rat

I. Introduction

Spontaneous diabetes mellitus has been manifested in several mammals including dogs (Hoenig, 2002) as well as experimental animals such as non-obese diabetic (NOD) mouse (Kachapati et al., 2012). Owing to reproducible detection of autoantibodies directed against biochemically defined target antigens such as GAD65 (glutamate decarboxylase), IA-2 and insulin, autoantibodies are most widely used as predictive markers for insulin-dependent diabetes mellitus (IDDM) (Bingley, 2010; Wenzlau et al., 2013). In human patients, glutamate decarboxylase (GAD) is a major target for autoantibodies and autoreactive T cells with IDDM. Radio-binding based measurement of antibodies to GAD and insulinoma antigen -2A has been demonstrated to achieve high sensitivity and specificity in almost all diagnostic laboratories of diabetes mellitus (Törn et al., 2008). Positive correlation between human IDDM and canine diabetes is well established (German et al., 2010). Recently, possible similarity between canine diabetes and latent autoimmune diabetes of adult human (LADA) has been hypothesized (Catchpole et al., 2005). Nevertheless, the role of auto-immune reaction in occurrence of canine diabetes is still controversial. Davison et al. (2008a) demonstrated complete absence of humoral autoimmunity in diabetic dogs, while Falorni and Calcinaro (2002) demonstrated the presence of anti insulin antibody in 12.5% diabetic dogs.

In monitoring the development and progress of IDDM, evidence of autoantibody to GAD is expected to be a valuable indicator. In this context, the role of serum autoantibody to GAD in diabetic dogs has not been explored in detail and available literature is scanty in nature. Moreover, experimentally streptozotocin induced diabetic rat model is commonly used to underpin the patho-

biology and remedial of spontaneous diabetes of dogs and human being. But, the compatibility of this model to spontaneous diabetes in dogs is yet not well defined. Therefore, the present investigation was aimed to evaluate the status of autoantibodies to GAD65 and insulin in serum of spontaneous diabetic dogs and well as experimentally induced diabetic rat model.

II. Materials and Methods

2.1 Animals and sample collection

The study was conducted in dogs aged more than five years in and around Bareilly district of Uttar Pradesh, India. Wistar male rats with experimentally induced diabetes by multiple low dose streptozotocin protocol (40 mg/kg of streptozotocin in freshly prepared citrate buffer with a pH of 4.5 ip for 5 days) were also examined for autoantibodies. Male wistar rats of about 100-150 gm procured from the Laboratory Animal Resource section of IVRI, Izatnagar used in the present study. They were maintained in standard environmental conditions of temperature ($25\pm 20^{\circ}\text{C}$), relative humidity ($55\pm 10\%$) and 12 hrs dark/light cycle. They were fed with standard diet and water ad libitum. The present animal experimentation was in compliance with the guidelines laid out by the Institutional Animal Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted under the directives of Ministry of Social Justice and Empowerment, Govt. of India.

Approximately 5 ml of blood samples was collected from each dog and used for the study. One millilitre of blood was transferred to vials containing sodium fluoride as anticoagulant used for estimation of blood glucose and another 1ml was taken in EDTA containing tubes for determination of glycosylated haemoglobin (HbA1c). Three ml of blood was allowed to clot at room temperature for 30 minutes and were transported in an ice box to the laboratory and thereafter were centrifuged at $1300 \times g$ for 10 min. Serum samples were harvested and stored at -20°C .

2.2 Screening of diabetic dogs

Random and fasting blood glucose level after 10-12 hrs of fast was measured by glucose oxidase peroxidase method (Kaplan, 1984). Dogs showing fasting blood glucose level above 120 mg/dl were subjected to further study. Glycosylated haemoglobin (HbA1c) in these animals was estimated by ion exchange chromatography method of Trivelli et al. (1971). Serum fructosamine was estimated by NBT reduction method (Sahu and Sarkar, 2008). Urine glucose was estimated by Benedict's test.

2.3 Grouping of animals

Eight dogs with fasting blood glucose level above 200 mg/dl, glycated haemoglobin above 8% and serum fructosamine above 4.5 mmol/L with clinical symptoms of polydipsia, polyuria, polyphagia, glycosuria, and emaciation/obesity were taken as diabetes positive animals (Group II). Healthy dogs with fasting blood glucose level below 120mg/dl, glycated haemoglobin below 6.5% and serum fructosamine below 2.7 mmol/L were selected to serve as healthy controls (Group I). Six wistar male rats of about 100-150 gm weight were experimentally induced diabetes by injecting streptozotocin 40 mg/kg body weight in freshly prepared citrate buffer of pH 4.5 ip for 5 consecutive days (Group III). Sex and weight matched six wistar rats served as healthy controls (Group IV). After 72 hrs of streptozotocin injection, the tail vein blood was collected to determine fasting blood glucose level. Only rats with fasting blood glucose above 250 mg/dl were considered diabetic and included in the experiments. About 1 ml of blood samples were collected on days 14 and 21 after induction of diabetes from all rats from inner canthus of eye by using capillary tubes and serum was separated for estimation of autoantibodies to GAD and insulin.

2.4 Estimation of autoantibodies to GAD 65 and insulin

Anti-GAD65 and ant-insulin antibodies were assayed by using immune radiometric assay kit (Immunotech, France).

2.4.1 Anti-GAD 65 antibody

Briefly, 125I-labeled recombinant human GAD65 was used as a tracer. For the immune precipitation assay, 20 µl of undiluted test sera and 50 µl of the tracer reagent 125I-labeled GAD solution were mixed in a 12×75-mm plastic test tube, and incubated for 2 h at room temperature, then 50 µl of precipitation reagent containing Protein A was added. After incubation of this mixture for 1 h at room temperature, 1 ml of assay buffer was added, the precipitate was pelleted by centrifugation at 1500×g for 30 min at 4°C, and the supernatant was aspirated off. The precipitate was then placed in a gamma counter and its radioactivity was measured. The titre of GAD antibody in each sample was determined by comparison with a calibration curve plotted from titres of standard serum; 0, 0.09 1.0, 3, 10, 30 12 and 300 U/ml. The intra- and inter assay coefficients of variation (CVs) were 3.7 and 7% respectively. Intra assay CVs using this kit in samples with low (6.4U/ml) and high titres (42.7U/ml) of GAD antibodies to be 4.9 and 7%, respectively. The detection limit of the assay was determined to be 0.09 U/ml. Serum samples in dogs and wistar rats were considered positive if they contained GAD antibodies at concentrations over 0.17 U/ml which exceeded 3 S.D. above the control mean.

2.4.2 Anti -insulin antibody

In this assay, 125I-labeled recombinant human insulin was used as a tracer. For the immune precipitation assay, 20 µl of test sera, calibrator, control and 25 µl of the tracer reagent 125I-labeled insulin solution were mixed in a 12×75-mm plastic test tube, and incubated for 24 h at room temperature, then 100 µl of precipitation reagent was added. After incubation of this mixture for 1 h at 2-80C, 2 1 ml office cold assay buffer was added, the precipitate was pelleted by centrifugation at 1500×g for 20 min at 4°C, and the supernatant was aspirated off. The precipitate was then placed in a gamma counter and its radioactivity was measured. The titre of insulin antibody in each sample was determined by comparison with a calibration curve plotted from titers of standard serum; 0, 0.4 1.0, 10 and 50 U/ml. The inter assay coefficients of variation (CVs) were 5.8 and 3% respectively. Intra assay CVs using this kit in samples with low (0.73 U/ml) and high titres (11.1 U/ml) of insulin antibodies to be 6.7 and 4.2%, respectively. The detection limit of the assay was determined to be 0.2 U/ml. Serum samples in dogs were considered positive if they contained insulin antibody at concentrations over 0.4 U/ml which exceeded 3 S.D. above the control mean and rats were considered positive if insulin antibodies above 0.23U/ml that exceeds 3 SD above the control mean.

2.5 Statistical analysis

Values obtained were expressed as mean ±S.E. Statistical software package SPSS 10.0 for windows (SPSS Inc., Chicago) was used for statistical analysis. The data was analyzed using one-way analysis of variance.

III. Results

Eight dogs from each group (group I, II) were screened for auto-antibodies to GAD 65 and insulin. Of these eight dogs in healthy controls, three dogs revealed reactivity to antibody to GAD 65, but cocentration was lower than the standard positive level (0.17 U/ml). Mean GAD antibody concentration in this group was 0.126±0.008 U/ml (Table 1). GAD antibody concentration above 0.17 U/ml was taken as standard positive. All eight dogs from diabetes group (group II) (100%) revealed posisive reactivity to GAD antibody and their mean GAD antibody concentration was 0.72±0.04 U/ml.

The concentration of GAD 65 antibody in diabetic dogs (Group II) was significantly higher ($P \leq 0.05$) in comparison with the healthy controls (group I).

The mean anti-insulin antibody concentration in healthy control dogs was 0.22 ± 0.02 U/ml. All healthy dogs revealed reactivity to anti-insulin antibody but concentration of anti-insulin antibody in these dogs was of below the standard positive level (0.4 U/ml). While in diabetic dogs (group II), three dogs (37.5%) revealed positive reactivity to anti-insulin antibody and titres were 0.42U/ml, 0.47U/ml and 0.54 U/ml respectively. The mean titre of anti-insulin antibody in this group was 0.41 ± 0.03 U/ml. The concentration of anti-insulin antibody in diabetic dogs (group II) was significantly higher ($P \leq 0.05$) in comparison with the healthy controls.

IV. Discussion

GAD 65, which is an intracellular pancreatic β -cells antigen, is considered as a valuable early predictive biomarker of diabetes mellitus. Its association with insulin dependent diabetes mellitus (Tilz et al., 2011) and latent autoimmune diabetes in human adults (Rosario et al., 2011) has been documented. The appearance of autoantibodies to one or several autoantigens for instance GAD65, IA-2 and insulin indicates an autoimmune pathogenesis of beta-cell destruction (Pihoker et al., 2005). The present study showed detectable level of anti-GAD antibody and anti-insulin antibody in diabetic dogs. This is in agreement with (Davison et al., 2008a) who demonstrated serological reactivity to GAD 65 and IA2 in a proportion of newly diagnosed diabetic dogs and circulating antibodies to insulin (Davison et al., 2008b). Present study suggests that antibodies to GAD are likely to be a very useful indicator in monitoring the development and progress of diabetes in dogs.

Experimentally streptozotocin induced diabetic rat model is commonly used to underpin pathobiology and remedials of spontaneous diabetes of dogs and human being. The multiple low-dose injections of streptozotocin (40 mg/kg for five days) could induce a gradual, autoimmune destruction of β cells instead of the rapid destruction induced by a single high-dose injection (Howarth et al., 2005; Kim et al., 2006; Sotnikova et al., 2006). Nevertheless the present study failed to demonstrate autoantibodies to GAD and insulin in sera samples of experimentally induced diabetic wistar rats by multiple dose protocol. This is in agreement with findings of Mackay et al. (1996) who demonstrated the autoantibody level in sera samples of diabetes prone BB rats or NOD mice did not exceed the levels in control rats or mice and suggested no autoimmune serological reactions in rodent model of insulin dependent diabetes. Therefore, the incompatibility of this rat model to spontaneous diabetes in dogs cannot be overlooked when interventional therapy for human and canine IDDM is warranted (Mackay et al., 1996).

V. Conclusion

Autoantibody to glutamate decarboxylase could be valuable early predictive biomarkers of spontaneous diabetes in dogs. Additionally, experimentally streptozotocin induced diabetic rat model seems to be an incompatible model for the studies on spontaneous IDDM of dogs.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. The funding bodies had no involvement in the study.

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