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Natural anti-insulin autoantibodies in cats: Enzyme-linked immunosorbent assay for the determination of plasma anti-insulin IgG and its concentrations in domestic cats

Satoshi Takashima [a], Naohito Nishii [a,b], Tatsuyuki Hachisu [c], Masaaki Kojima [c], Megumi Kigure-Hoshino [c], Shizuko Ogawa [b], Takafumi Suzuki [a], Atsushi Iwasawa [b], Yasunori Ohba [a,b], Hitoshi Kitagawa [a,b,*]

[a] Department of Veterinary Medicine, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Faculty of Applied Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[c] Shibayagi Co., Ltd., 1062-1 Ishihara, Shibukawa City, Gunma 377-0007, Japan

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ABSTRACT

Anti-insulin immunoglobulin G (IgG) has been found in the sera of healthy cats. To determine the concentrations of these antibodies, an enzyme-linked immunosorbent assay (ELISA) for anti-insulin IgG was developed. ELISA maintained the linearity of a standard concentration line between 67.5 and 2160 ng/ml. The coefficients of variances (CVs) of intra-assays in two different plasma samples were 4.0% and 3.7%, respectively. The inter-assay CVs in two different plasma samples were 5.1% and 6.9%, respectively. The dilution curves of two samples were rectilinear. Anti-insulin IgG was detected in all 84 of the healthy cats that were tested. Plasma anti-insulin IgG concentrations ranged from 80 to 1578 μg/ml, with a median concentration of 221 μg/ml, and this value correlated positively with total plasma IgG concentrations ($r = 0.383$, $p < 0.01$). In an intravenous glucose tolerance test, plasma anti-insulin IgG concentrations did not alter, even with changes in plasma glucose and insulin concentrations. The ELISA that was developed was able to determine plasma anti-insulin IgG in domestic cats, and confirmed that all healthy cats had plasma anti-insulin IgG. Determining the plasma concentrations of anti-insulin IgG in cats with various pathological conditions might clarify the role of anti-insulin IgG.
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1. Introduction

Anti-insulin antibodies have been described in human patients with type 1 diabetes involving insulitis (Palmer et al., 1983), as well as in humans (Reeves and Kelly, 1982; Rendell et al., 1981) and dogs (Davison et al., 2008) with diabetes mellitus treated with insulin products derived from other species. These antibodies have also been reported in human patients with autoimmune insulin syndrome, in which hypoglycaemia is induced through the unexpected dissociation of the insulin-antibody complex (Burch et al., 1992). Recently, in the process of developing enzyme-linked immunosorbent assay (ELISA) for feline insulin concentrations, we found anti-insulin IgG in the sera of 10 clinically healthy adult cats (Nishii et al., 2010). However, the physiologic and pathologic functions of anti-insulin IgG in healthy cats are unknown, and the role of anti-insulin IgG in cats might be different from that in diseased humans and dogs.

There have been no reports describing the peripheral anti-insulin IgG concentrations in domestic cats. The determination of plasma anti-insulin concentrations could be the first step in elucidating the role of these antibodies in cats. The present study describes the development of an ELISA to determine plasma anti-insulin IgG concentrations in cats and to determine reference values in healthy cats and in cats subjected to conditions that alter insulin and glucose concentrations.

2. Materials and methods

2.1. Materials

For ELISA, pure insulin was isolated from the pancreas of a cat, and pure anti-insulin IgG, as a standard substance, was separated from the serum of a healthy adult cat. These substances were confirmed as pure in a previous study (Nishii et al., 2010). As a captured antibody, rabbit anti-cat IgG, labeled by horseradish peroxidase (R1308 HRP, Acris Antibodies GmbH, Herford, Germany) was used.

* Corresponding author. Address: Laboratory of Veterinary Internal Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. Tel.: +81 58 293 2950; fax: +81 58 293 2964.
E-mail address: hkitagaw@gifu-u.ac.jp (H. Kitagawa).

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2.2. Animals and blood collection

The present study used 84 adult domestic cats (six intact males, 38 neutered males, nine intact females and 31 neutered females) that were diagnosed as clinically healthy through physical examination, complete blood counts, blood biochemical analyses, and urinalysis. The ages of the cats ranged from 1 to 13 years old, with a median of 6 years old. Body condition scores (BCS) were recorded using a nine-point scale, ranging from 2 to 9 with a median of 5. Cats were classified as intact or as neutered males and females. Blood samples were collected from the cephalic vein at fasting for 12 h and were placed into tubes containing EDTA-2Na and aprotinin to prevent the deterioration of insulin. Plasma separated by centrifugation (4 °C, 1000*g*, 15 min) was stored at −80 °C until determination within three months. This study was conducted in a manner consistent with the Gifu University Guidelines for Animal Experimentation (approval numbers: 09044 and 09075).

2.3. Anti-insulin IgG assay

Initially, each well of a 96-well microplate (NUNC MaxiSorp, Thermo Fisher Scientific, NY, USA) was coated with feline insulin (500 ng/well) at room temperature (RT) for 2 h. The plate was then washed with a buffer solution (10 mM PBS containing 0.025% Tween20). Blocking was performed with PBS containing 10 mg/ml of bovine serum albumin (A8022, Sigma, St. Louis, MO, USA) at RT for 18 h. After washing, plasma samples or standard solution was added to the wells. After incubation and re-washing, diluted rabbit anti-cat IgG, labeled by horseradish peroxidase, was added. After incubation at RT for 2 h and then washing, a substrate solution, containing 3,3′,5,5′-tetramethylebenzidine (Dako, Produktionsvej, Glostrup, Denmark), was added. After incubation for 40 min, a stop solution (2 N sulfuric acid) was added. The optical density was measured at 450 to 620 nm with a microplate reader (Model 680, Bio-Rad, Hercules, USA).

For the validation analysis of the precision of ELISA, a dilution test for the linearity and recovery study was performed. To test for the linearity of ELISA, serial dilutions in PBS (undiluted, 1:1, 1:2 and 1:4) of two plasma samples derived from clinically healthy cats were created and then assayed. A recovery study was performed to assess the sensitivity of the assay by adding known amounts of anti-insulin IgG to the plasma samples at varying concentrations. The determination of intra-assay variability was performed four times on the same plate using two plasma samples. The determination of inter-assay variability was performed for four different assays with two plasma samples.

The determinations of anti-insulin IgG were used for frozen plasma within three months after sampling. To eliminate the effects of freezing on the components, we confirmed the frozen stability of plasma sample kept at −80 °C by repeated measurements following 0 and 3 months of storage. Measurements of preserved samples were performed in triplicate using two plasma samples.

2.4. Determination of plasma total IgG and insulin concentration

Plasma total IgG concentrations were determined with validated commercial sandwich ELISA, according to the manufacturer's instructions (Cat IgG ELISA Quantitation Set, Bethyl Laboratories, Montgomery, TX, USA) and according to Campbell et al. (2004). Plasma insulin concentrations were determined in plasma using a validated sandwich ELISA kit (Feline insulin measurement kit, Morinaga Institute of Biological Science, Inc., Yokohama, Japan), which could determine feline insulin concentrations in ranges from 0.1 to 3.6 ng/ml (Nakaya et al., 2009).

2.5. Protocol of intravenous glucose tolerance test

An intravenous glucose tolerance test was undertaken in three healthy cats with BCS 5/9. After collection of blood samples, glucose solution (50%, w/v, 1 g/kg body weight) was administered intravenously in a bolus. Blood samples were collected at 10, 30, 60, 90 and 120 min post-glucose injection. Plasma glucose concentrations were determined with dry-slide technology, using a multilayered analytical film (Fuji DRI-CHEM, Tokyo, Japan).

2.6. Statistical analysis

The data are expressed as medians and ranges. To confirm the frozen stability, the differences in the means of concentrations between immediately after sampling and three months after sampling were evaluated by the paired *t*-test, following the normality test. Regarding the plasma anti-insulin IgG concentrations in cats categorized by sex, the differences in means among the four groups on intact or neutered males and females were analyzed by the Kruskal–Wallis test, following the recognition of non-normal distribution. The significance of correlations between the plasma anti-insulin IgG concentrations and age, BCS and total IgG or insulin concentration was evaluated by Spearman's correlation coefficient or by rank test. On the glucose tolerance test, regarding the plasma concentrations of glucose, insulin and anti-insulin IgG each time after glucose intravenous administration, Bartlett's test was used for comparison of the variances. The differences in plasma concentrations between pretreatment (0 min) and after each glucose intravenous administration were analyzed by the single-factor ANOVA and Dunnett test. A p value below 0.05 was considered to be statistically significant.

3. Results

3.1. Analytical validation of anti-insulin IgG ELISA in cats

Fig. 1 depicts the representative standard curve of ELISA for anti-insulin IgG concentrations. The assay generated a linear calibration curve from 33.8 ng/ml to 2160 ng/ml. Two plasma samples were assayed either undiluted or after serial dilution in PBS, and the anti-insulin IgG concentrations were linear between 40 and 783 ng/ml (Fig. 2). The recovery of authentic anti-insulin IgG added to plasma was high. The volumes recovered from plasma were 92.2, 91.9, and 92.0%, respectively, for standards that read 45.3, 129.5, and 200.9 ng/ml in the assay. The intra-assay CVs (n = 4)

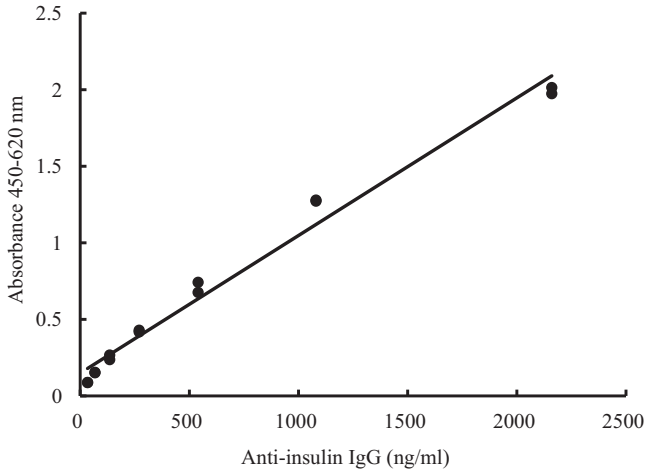

Fig. 1. A typical standard curve of anti-insulin IgG concentration for the ELISA.

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