**FULL PAPER** *Biochemistry*

# Ligand-binding characteristics of feline insulin-binding immunoglobulin G

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ABSTRACT. Polyclonal immunoglobulin (Ig) G autoantibodies against insulin have been identified in sera of healthy cats. We purified and fractionated insulin-binding IgGs from cat sera by affinity chromatography and analyzed affinity of insulin-binding IgGs for insulin and their epitopes. Following the passing of fraction A, which did not bind to insulin, insulin-binding IgGs were eluted into two fractions, B and C, by affinity chromatography using a column fixed with bovine insulin. Dissociation constant (KD) values between insulin-binding IgGs and insulin, determined by surface plasmon resonance analysis (Biacore™system), were $1.64e^{-4}$ M for fraction B (low affinity IgGs) and $2e^{-5}$ M for fraction C (high affinity IgGs). Epitope analysis was conducted using 16 peptide fragments synthesized in concord with the amino acid sequence of feline insulin by an enzyme-linked immunosorbent assay. Fractions B and C showed higher absorbance (affinity) of the peptide fragment of 10 amino acid residues at the carboxyl-terminal of the B chain (peptide No. 19), followed by peptide fragments of 6 to 15 amino acid residues of the B chain (peptide No. 8). Fraction C showed a higher absorbance to 7 to 16 amino acid residues of the B chain (peptide No. 5) compared with the absorbance of fraction B. Polyclonal insulin-binding IgGs may form a macromolecule complex with insulin through the multiple affinity sites of IgG molecules. Feline insulin-binding IgGs are multifocal and may be composed of multiple IgG components and insulin.
KEY WORDS: antibody, binding characterization, feline, insulin

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Anti-insulin antibody is found in human patients with type 1 diabetes mellitus (DM) [14] treated with insulin preparations [17] and those with insulin autoimmunity syndrome [4]. The anti-insulin antibody induces autoimmune insulitis, insulin resistance or sudden hypoglycemia [1, 18]. The anti-insulin antibody adversely affects blood glucose concentrations in humans. On the other hand, a recent study [16] reported the presence of anti-insulin antibodies (IgG and IgM) in healthy humans with normal blood glucose levels. IgM molecules with affinity to insulin have been detected in healthy C57BL/6 mice [15]. Both the humans and mice did not develop any signs of DM or spontaneous autoimmune insulitis, and the influence of anti-insulin antibodies have not been known on blood glucose control.

Nishii *et al.* [11] reported the presence of insulin-binding IgG in sera of normal cats. In this study, they conducted the Scatchard plot analysis for insulin-binding IgGs and suggested the presence of 2 types of IgG molecules with different affinities for insulin, from the curvilinear line determined. Furthermore, Takashima *et al.* [23] detected insulin-binding IgG molecules in plasma of all 84 healthy cats determined, and the concentrations correlated significantly with total plasma IgG concentrations but not with plasma insulin concentrations. As seen above, information on insulin-binding IgGs in cats has been limited.

Studies on endogenous hormone-binding proteins have been mainly performed on thyroid hormone-binding proteins in humans [12, 19, 22]. In the circulation, thyroid hormones combine with albumin, thyroxine-binding globulin and thyroxine-binding prealbumin [13]. In these studies, each binding protein was purified, and then, the molecular features were characterized [13, 21]. After purifications of the hormone-binding proteins with electrophoresis and chromatography, the affinity for the hormone was evaluated, and the maximum binding capacity of the protein purified was calculated. The study of binding characteristics of the insulin-binding IgGs can contribute to elucidate the physiological role of the insulin-binding IgGs on insulin action. In the present study, we purified and fractionated the insulin-binding IgG molecules from cat sera according to the previous studies on thyroid hormone. Then, we evaluated the affinity for insulin and conducted epitope analysis of the fractionated insulin-binding IgGs.

## MATERIALS AND METHODS

*Blood samples*: Peripheral blood was collected from 8 clinically healthy adult cats (4 males and 4 females) that received no treatments with insulin preparations. After separation by centrifugation, serum samples were commingled,

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and a total 150 m*l* of sera was stored separately at −80°C until analysis.

*Extraction of total IgG content including insulin-binding IgGs*: Total IgG content, including insulin-binding IgG, was extracted from sera using the Melon$^{TM}$ Gel Chromatography Cartridge (Thermo Fisher Scientific, Inc., Rockford, IL, U.S.A.) according to the manufacturer's instructions. Total IgG content was recovered as the passing fraction under conditions to maintain molecular activity [7].

*Removal of endogenous insulin from the IgGs*: Endogenous insulin was removed from insulin-IgG complexes by the method of Gerbitz [6] with modification. Briefly, 150 $\mu l$ of 1 N HCl was added to 1 m*l* of the Melon Gel-extracted solution, followed by ultrafiltration using a centrifugal filter membrane with molecular weight cut-off of 30,000 Da (Vivaspin 20; GE Healthcare, Uppsala, Sweden). After centrifugation at 3,500 rpm for 30 min, 20 m*l* of phosphate-buffered saline (PBS) was added to the concentrated sample, and the solution was recentrifuged until the total volume was reduced to approximately 1 m*l*. The insulin removal ability of the filter was confirmed by SDS-PAGE stained with Coomassie brilliant blue (Supplementary Fig. 1). The concentrated insulin-binding IgGs were purified and fractionated by affinity chromatography.

*Insulin-immobilizing affinity chromatography*: Bovine insulin (I5500, Sigma-Aldrich Corporation, St. Louis, MO, U.S.A.), which has been confirmed to bind with feline insulin-binding IgGs, was fixed on a Hitrap NHS (n-hydroxysuccinimide)-activated HP column (17-0716-01; GE Healthcare). Bovine insulin differs from feline insulin by one amino acid residue on the A chain. The histidine at position 18 of the A chain of feline insulin is replaced by an asparagine within the bovine insulin sequence. Competitive radioimmunoassay using $^{125}$I-bovine insulin was conducted by Nishii *et al.* [11]. They indicated the binding of feline insulin-binding IgGs with bovine insulin by the evidence that the $^{125}$I-bovine insulin was displaced by unlabeled bovine insulin. The column was equilibrated with binding buffer (3 M NaCl, 1.5 M glycine, pH 8.5). The concentrated sample of purified IgGs without endogenous insulin was diluted with an equivalent volume of binding buffer solution and then added to the column. After incubation for 1 hr at room temperature, insulin-binding IgGs were fractionated by affinity for bovine insulin using a high-performance liquid chromatography system (PX-8010; Toso Co., Ltd., Tokyo, Japan) equipped with a binary pump (CCPM; Toso Co., Ltd.), a microvacuum degasser (Gastorr BG-12, Flom, Tokyo, Japan), a UV detector (UV-8010, Toso Co., Ltd.) and a data analyzer ($\mu$7 Data Station; System Instrument Co., Ltd., Tokyo, Japan).

Various elution conditions with different concentrations of NaCl and glycine, and pH values were tested to determine the optimal conditions for IgG elution. Finally, IgG molecules were clearly separated under the following elution conditions. After washing the column with 3 M NaCl and 1.5 M glycine at pH 8.5 for 20 min, IgGs were eluted with a linear gradient from 100% 3 M NaCl and 1.5 M glycine at pH 8.5 to 50% 0.05 M phosphate buffer solution for 10 min, followed by 50% 0.05 M phosphate buffer (for 5 min) and then 50% 3 M NaCl, 1.5 M glycine pH 8.5 to 100% 0.05 M phosphate buffer for 10 min. Finally, the column was washed with 0.05 M phosphate buffer for 15 min. The flow rate of the eluent was 0.5 m*l*/min. The total run-time was 60 min, and the eluted proteins were detected by monitoring of UV absorption at λ=280 nm.

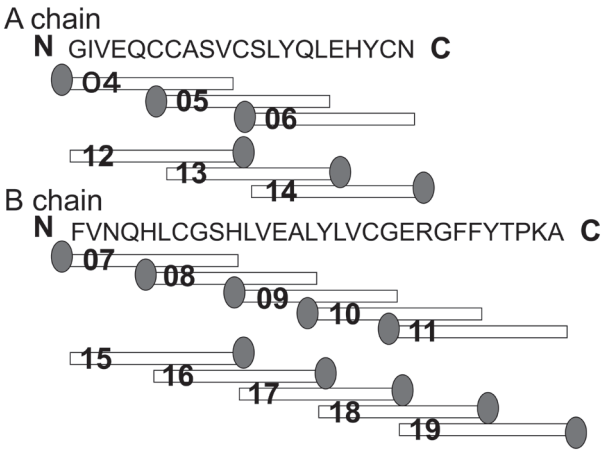


Fig. 1. Biotinylated peptides. Amino acid sequences of feline insulin and 16 synthesized peptides consisting of feline insulin proteins. Each peptide contained biotin (filled ellipse) at the end of the molecule.

To confirm the presence of eluted IgGs, a sample of serum IgGs with endogenous insulin removed and fractions separated by the affinity chromatography and bovine insulin were electrophoresed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% polyacrylamide gels for IgG, followed by staining with Coomassie Brilliant Blue.

*Interactive surface plasmon resonance analysis*: Real-time analyses of association and dissociation of the insulin-binding IgGs and insulin were performed with a surface plasmon resonance system (Biacore J; Biacore International AB, Uppsala, Sweden). The analyte was bovine insulin (Sigma-Aldrich Corporation), and the solvent was HBS-N buffer (GE Healthcare) containing 0.01 M HEPES, 0.2 M NaCl, 0.05% Tween20 and 6.6 mM HCl (pH 3.5). The analyte was injected at a flow rate of 10 $\mu l$/min at 25°C. Using dual channel detection of the surface plasmon resonance signal, a dextran layer of a sensor chip was activated by injection of 140 $\mu l$ of a mixture of 0.2 M n-ethyl-n′-(dimethylaminopropyl) carbodiimide (Amine Coupling Kit; GE Healthcare) and 0.05 M n-hydroxysuccinimide (Amine Coupling Kit), followed by 180 $\mu l$ of goat anticat IgG (30 $\mu$g/m*l*) (Southern Biotechnology Associates, Inc., Birmingham, AL, U.S.A.) diluted in 10 mM sodium acetate (pH 5.0), were immobilized to the dextran matrix of a CM3 sensor chip (GE Healthcare). Furthermore, the uncoupled activated dextran of the sensor chip was blocked by injection of 80 $\mu l$ of 1.0 M ethanolamine-HCl at pH 8.5 (Amine Coupling Kit). Finally, using a single channel, 40 $\mu l$ of the fractionated insulin-binding IgGs (20 $\mu$g/m*l*) diluted in buffer solution were immobilized to goat anticat

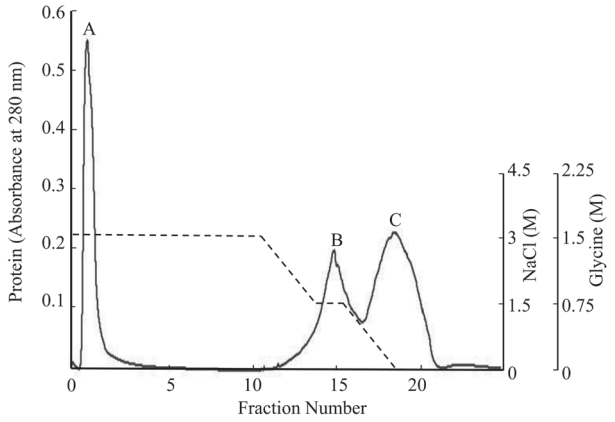


Fig. 2. Affinity chromatography. The continuous line represents absorbance at 280 nm, and the broken line represents the concentration of NaCl and glycine. Fraction B was eluted with 1.5 M NaCl and 0.75 M glycine, while fraction C was eluted with 0 M NaCl and 0 M glycine. Fraction A was the passing fraction and did not bind to insulin fixed in the column.

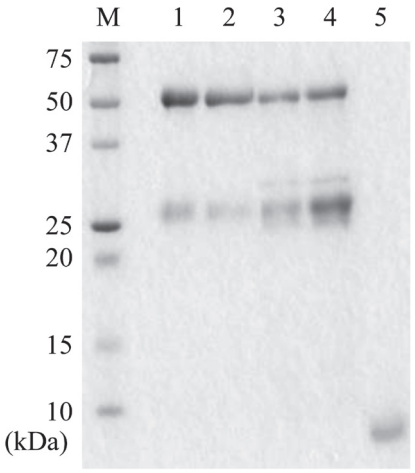


Fig. 3. SDS-PAGE for total IgG content, fractions obtained from affinity chromatography and bovine insulin. SDS-PAGE showed biphasic bands of 25 and 50 kDa, which was consistent with IgG. Lane 1: Sample with endogenous insulin removed after extraction from sera of cats using a Melon$^{TM}$ Gel Chromatography Cartridge. Lane 2: fraction A. Lane 3: fraction B. Lane 4: fraction C. Lane 5: bovine insulin.

IgG on the sensor chip. Bovine insulin was diluted in buffer solution (50 or 100 $\mu$g/m*l*), and 80 $\mu l$ of diluted insulin solution was injected over the sensor surface. Following completion of the association phase, dissociation was monitored in buffer solution for 4 min at the same flow rate. At the end of each cycle, the surface was regenerated using 80 $\mu l$ of 10 mM glycine-HCl buffer solution at pH 1.7. The data were analyzed with a 1:1 Langmuir binding model using Biacore J evaluation software (Biacore International AB). With this system, kinetic rate constants for the association and dissociation of insulin-binding IgGs were obtained, and the KD values were calculated from the 2 kinetic rate constants.

*Epitope analysis*: Biotinylated peptides composed of feline insulin fragments (Fig. 1) were used for epitope analysis (Operon Biotechnology, Tokyo, Japan). Because of biotin modification, the peptides were bound in a particular orientation on Immobilizer 96-well microplates (Nunc A/S Plastfabrikation, Roskilde, Denmark). Microplates were coated with each peptide at a concentration of 50 nM and blocked with Block Ace solution (DS Pharma Biomedical, Osaka, Japan) diluted to 4-fold of the liquid concentrate for 2 hr to reduce nonspecific binding of insulin-binding IgGs. Serum samples were diluted 100-fold with an incubation buffer (Can Get Signal; Toyobo Co., Ltd., Osaka, Japan), transferred to the coated plate at a volume of 100 $\mu l$/well and then incubated for 30 min at room temperature. The fractionated insulin-binding IgGs were diluted to 1:10 in an incubation buffer and transferred to the coated plate. After washing, horseradish peroxidase-labeled goat anticat IgG-Fc detection antibody (Cat IgG ELISA Quantitation Set; Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.) diluted to 1:20,000 in PBS containing 0.05% Tween20 was allowed to react for 1 hr (100 $\mu l$/well). After washing, 100 $\mu l$ of tetramethylbenzidine substrate solution (Cat IgG ELISA Quantitation Set) was added. After 10 min, the reaction was stopped by adding 100 $\mu l$ of 1 M sulfuric acid, and the absorbance of insulin-binding IgGs against each peptide, indicating binding affinity, was measured at 450 nm.

*Examination approval*: The study protocol was approved by the Gifu University Guidelines for Animal Experimentation (approval number: 09044).

## RESULTS

*Affinity chromatography*: Three clear peaks (A, B and C) were fractionated by affinity chromatography (Fig. 2). Fraction A was the passing fraction, which did not bind to insulin fixed in the column. Fractions B and C were insulin-binding IgGs that were eluted with a solution of 1.5 M NaCl with 0.75 M glycine and 0.05 M phosphate buffer solution, respectively. SDS-PAGE showed biphasic bands of 25 and 50 kDa (Fig. 3), which indicated the presence of IgG molecules consisting of light and heavy chains in the fractions produced by affinity chromatography.

*Binding affinities between insulin-binding IgGs and insulin*: The observed interactions between insulin-binding IgGs and insulin indicated single-phase association and dissociation kinetics (Fig. 4). The association and dissociation rate constants of fraction B were approximately 4-fold smaller and 1.8-fold larger than that of fraction C, respectively (Table 1). The KD values (Kd/Ka) were $1.64e^{-4}$ M for fraction B and $2e^{-5}$ M for fraction C.

*Epitope analysis of insulin-binding IgGs*: A background absorbance of non-specific binding between goat anti-cat IgG-Fc antibody and peptide was deducted for all peptides. In the ELISA, insulin-binding IgGs (fractions B and C) showed the greatest absorbance, indicating the greatest affinity with peptide No.19, a fragment of 10 amino acid residues at the

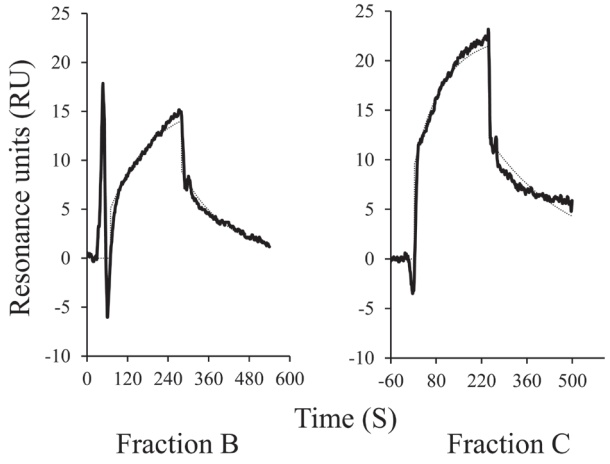


Fig. 4. Interactive analysis of insulin-binding IgGs using the Biacore™ system. Insulin-binding IgGs were immobilized on the sensor chip, and insulin from the bovine pancreas was injected over the sensor surface. The continuous line indicated that insulin-binding IgGs bound to insulin on the sensor chip, and the broken line is a fitted curve using nonlinear least squares method.

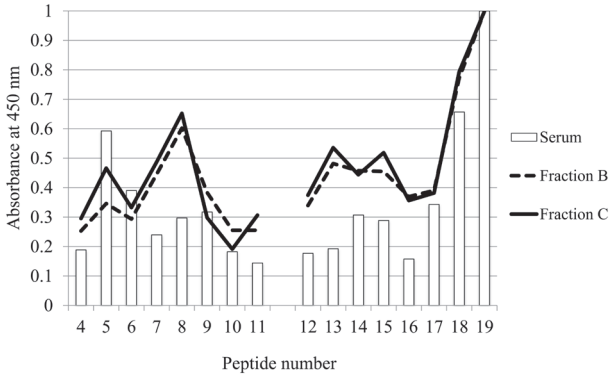


Fig. 5. Epitope analysis of insulin-binding IgGs. Binding of whole IgGs contained in serum and insulin-binding IgGs to the synthesized peptides of insulin as determined with a sandwich ELISA using a plate coated with the 16 peptides and labeled anti-IgG antibodies. Among the synthesized peptides, No. 19 showed the greatest binding. The relative values for the peptide with the highest absorbance in each sample of serum and fractions B and C are shown. Fractions B and C showed high relative absorbance to peptide No. 8 compared with serum.

Table 1. Kinetic parameters of interactive analysis

| Fraction | Ka (1/Ms) | Kd (1/s) | KD (M) |
|---|---|---|---|
| B | 45.9 | $7.38e^{-03}$ | $1.61e^{-04}$ |
| C | 202 | $4.04e^{-03}$ | $2.00e^{-05}$ |

carboxyl-terminal of the B chain of feline insulin (Fig. 5). Figure 5 shows the relative absorbance values for each peptide to that for peptide No. 19. Fractions B and C showed higher absorbance values to peptide No. 8 (6 to 15 amino acid residues of the B chain) in comparison with whole IgGs. Besides, fractions B and C showed lower absorbance values to peptide No. 5 (7 to 16 amino acid residues of the A chain) than whole IgGs. A difference in relative absorbance between fractions B and C was observed in the peptide No. 5; the absorbance was lower in fraction B than fraction C.

## DISCUSSION

Gradient-eluted affinity chromatography separated insulin-binding IgGs into 2 fractions, B and C. The KD values for bovine insulin were $1.64e^{-4}$ M (low affinity IgGs) and $2e^{-5}$ M (high affinity IgGs) in fractions B and C, respectively, and the KD value of fraction C was 10-fold smaller than that of fraction B. Kure *et al.* [9] reported the affinity of insulin for anti-insulin antibodies observed in human patients with diabetes determined with the Biacore™ system. In their report, the affinities of anti-insulin antibodies were divided into two categories according to the presence or not presence of a hypoglycemic episode. Patients with the episode had higher KD values ($5.44 \pm 10.3e^{-5}$ M) than those without the episode ($3.73 \pm 6.91e^{-8}$ M). The mean KD value of human patients with the episode of hypoglycemia ($5.44 \pm 10.3e^{-5}$ M) was similar to that of fraction C (high affinity IgGs, KD value was $2e^{-5}$ M) of insulin-binding IgGs in cats determined in the present study. Previous studies [2, 3, 9] reported anti-insulin antibodies with relatively higher affinities for insulin were observed in human patients with DM who received insulin preparations and those with poor blood sugar control, such as hyperglycemia. On the other hand, antibodies with relatively lower affinities may cause hypoglycemia through the dissociation of insulin/antibody complexes. The affinities for insulin of fractions B and C in cats were comparable with those for human anti-insulin antibodies with lower affinities to easily dissociate insulin. However, it is unclear whether insulin-binding IgGs dissociate from insulin in cats, because healthy cats do not show signs of hypoglycemia. Previous reports showed the Scatchard plot analyzed by the 2-binding-site model. The binding capacities of insulin-binding IgGs for binding sites 1 and 2 were $1.93 \pm 2.29$ nM and $227.46 \pm 171.68$ nM for insulin [11]. In addition, a concentration of insulin-binding IgGs and insulin were 0.5–10.8 $\mu$M and 0.05–0.9 $\mu$M, respectively [23]. These ratios indicated that most of insulin secreted into circulation combined with insulin-binding IgGs. All insulin may be transported to the receptors in the binding state with insulin-binding IgGs in the circulation. The binding affinity of each insulin-binding IgG to insulin is low, but avidity of insulin-binding IgG to insulin may influence the binding of insulin with receptors.

Results of epitope analysis showed that the highest antigenic activity was detected in the carboxyl-terminal fragment of the B chain (the B20 to B30 amino acid residues, peptide No. 19), and the second highest antigenic activity was detected in the 6 to 15 amino acid residues of the B chain (peptide No. 8) among the synthesized peptides both in fractions B and C of insulin-binding IgGs. The receptor-binding domains of insulin with the insulin receptor were amino acid residues A2, A3 and A19 of the A chain, and common anti-

genic sites are residues of B23 and B24 of the B chain [5]. Furthermore, the insulin B-chain C-terminal contributes to the mechanism of a long-proposed conformational switch in insulin upon receptor engagement [10]. These results indicate the possibility that insulin-binding IgGs combine with the receptor-binding domains of insulin in blood and competitively inhibit the binding of insulin and insulin receptors.

In addition, both fractions B and C showed affinities to 6 to 15 amino acid residues (peptide No. 8) of the B chain and the fragment other than the part of C-terminus of the B chain, indicating that insulin contained multiple antigenic epitopes for insulin-binding IgGs in cats. The insulin-binding IgGs of cats should be polyclonal [11]. If the polyclonal insulin-binding IgGs bind to multiple insulin epitopes, insulin-binding IgG may crosslink with insulin and form a macromolecule complex of insulin and IgGs. Reports by Keilacker *et al.* [8] and Shechter *et al.* [20] described the possibility that the macromolecule complex of polyclonal anti-insulin antibodies and insulin induced aggregation of receptors on the cell membrane, and the complex might enhance the bioactivity of insulin through the combination with many receptors. However, the effect of formation of a macromolecule complex on bioactivity of insulin is unknown in cats.

In conclusion, feline insulin-binding IgGs could be separated into two fractions with low and multifocal affinities to insulin. Further studies are necessary to evaluate, if feline insulin-binding IgGs compose multiple IgG components with insulin. IgG molecules may influence the action and stability of insulin, but it remains to be elucidated.

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