DEVELOPMENT OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST FELINE PROINSULIN AND C-PEPTIDE

by

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(Under the Direction of Margarethe Hoenig)

ABSTRACT

The common spontaneous form of diabetes mellitus seen in cats closely resembles that of human type 2 diabetes. Similar risk factors for the disease are seen in cats and people, such as age and obesity. Progression to type 2 diabetes is marked by increased resistance to insulin action in peripheral tissues, especially skeletal muscle and adipose tissue, and decreased function of the ß-cell in insulin production and/or secretion. Early detection of ß-cell failure is critical in determining therapeutic routes to counter the disease. Recently, in human studies, it has been found that the earliest stages of ß-cell function loss can be observed as an increase in the proinsulin/insulin ratio in serum during fasting states. This study has produced antibodies whose specificity for feline insulin, proinsulin, and C-peptide may prove useful in the development of highly specific assays for determining serum concentrations of intact feline proinsulin and insulin.

INDEX WORDS: Feline, diabetes, obesity, insulin, proinsulin, C-peptide, immunoassay, antibodies

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B.S., Berry College, 2000

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2006

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May 2006

ACKNOWLEDGEMENTS

I would like to thank Dr. Margarethe Hoenig and Dr. Duncan Ferguson for their guidance and support. I would also like to thank all of my lab mates that have come and gone, especially Erin Tittle for all of her hard work which helped so much. But most of all I would like to thank my wife, Kerry. Even though I was almost as stressed as you and a lot more annoying, you didn't kill me! Your support and your love got me through this adventure. To many years to come. Thank you.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

The common spontaneous form of diabetes mellitus seen in cats closely resembles that of human type 2 diabetes. Similar risk factors for the disease are seen in cats and people, such as age and obesity. Progression to type 2 diabetes is marked by increased resistance to insulin action in peripheral tissues, especially skeletal muscle and adipose tissue, and decreased function of the ß-cell in insulin production and/or secretion. Early detection of ß-cell failure is critical in determining therapeutic routes to counter the disease. Recently, in human studies, it has been found that the earliest stages of ß-cell function loss can be observed as an increase in the proinsulin/insulin ratio in serum during fasting states. Proinsulin has been difficult to measure in healthy individuals because proinsulin concentrations are typically only 1-3% that of fasting insulin concentrations. With ß-cell failure, proinsulin secretion increases and reaches a higher percent of the fasting insulin concentration. Increasing the difficulty of measuring proinsulin in serum is the high rate of similarity between insulin and proinsulin. Since proinsulin is the precursor to insulin, the insulin molecule is resident within proinsulin. Immune reagents generated against either peptide, typically have high cross-reactivity with the other peptide. It is the goal of this study to develop sensitive and specific immune reagents to certain portions of the feline proinsulin molecule that would limit cross-reactivity with feline insulin. Such immune reagents would be necessary for development of a specific assay for measuring intact proinsulin in serum.

CHAPTER 2

LITERATURE REVIEW

Insulin Biosynthesis.

Insulin is a metabolic hormone most notably secreted in response to changes in blood glucose concentrations. At the cellular level, insulin binds to insulin receptors which become autophosphorylated and initiate intracellular signaling pathways (White 1997). One of the most important is the activation of glucose transporters which ultimately leads to glucose uptake into cells through cell surface glucose transporters (White and Myers 2001).

Insulin is a heterodipeptide consisting of two dissimilar peptide chains covalently bound by two intermolecular disulfide bonds and one intramolecular disulfide bond. These two constitutive peptides are known as the A-chain and the B-chain. Insulin is synthesized in the ß-cells within the Islets of Langerhans of the endocrine pancreas as the precursor proinsulin (Steiner et al 1967). Proinsulin is a single pro-peptide consisting of the two chains of insulin (A-chain and B-chain) and an additional peptide sequence known as the connecting peptide (C-peptide). mRNA for insulin encodes four distinct regions which are translated into the rough endoplasmic reticulum (RER): signal sequence, B-chain, C-peptide, and the A-chain, respectively. This translated peptide is known as preproinsulin. The signal sequence directs the translated protein into the RER as translation is completed. The signal sequence is rapidly cleaved by a signal peptidase as translation of the remainder of the proinsulin continues into the lumen of the RER. The resultant proinsulin molecule is packaged into vesicles which translocate

from the exterior surface of the RER to the *cis*-Golgi network. The C-peptide is thought to provide the proper spacing and orientation in order for proinsulin to fold properly and for the formation of cysteine-cysteine disulfide bonds to stabilize the tertiary structure. Proinsulin is translocated from the cis-Golgi to the trans-Golgi where it is then packaged into clathrin-coated immature secretory granules along with prohormone convertase 1/3 (PC1/3), prohormone convertase 2 (PC2), and carboxypeptidase H (CPH) (Steiner et al 1974, Davidson and Hutton 1987, and Davidson et al 1984). Within the immature secretory granule PC1/3 readily acts on intact proinsulin cleaving the junction between the B-chain and the C-peptide at Arg_{32} and Glu_{33} in most mammalian species. PC2 has a higher affinity for des-31,32-proinsulin than for intact proinsulin suggesting the PC2 mediated cleavage of the C-peptide-A-chain junction at Arg_{65}-Gly_{66}, or similar position in species other than human, occurs secondary to the PC1/3 mediated conversion of the B-chain-C-peptide junction (Rhodes et al 1992, Furuta et al 1998, and Zhu et al 2002). Rapid cleavage of the two C-terminal basic residues is mediated by CPH. This cleavage reaction results in free C-peptide and intact insulin. Maturation of the secretory granule consists of clathrin disassociating after formation of the immature secretory granule and is thought to take with it the prohormone converting enzymes, purging the cell of these proteases to prevent further digestion of insulin and C-peptide (Molinete et al 2001).

Insulin self-associates by forming hexamers around 2 Zn atoms which bind to His_{10} of the B-chain. These hexamers condense into a crystalline structure within the mature secretory granule until secretion. Blundell *et al* (1972) suggest that association of the insulin molecule with Zn initiates in the proinsulin form. The self association of the proinsulin molecule positions the C-peptide portion of the molecule on the external surfaces of the hexamer structure possibly assisting in the PC1/3 and PC2 mediated conversion of proinsulin to insulin (Steiner et al 1974).

Following conversion of proinsulin, the C-peptide remains freely soluble within the secretory granule until secretion (Neerman-Arbez and Halban 1993). Converted insulin remains associated with Zn molecules in hexamer form and condensed into a crystalline structure which stabilizes storage of insulin within the secretory granules (Halban and Iminger 2003). Upon exocytosis of the secretory granule insulin and C-peptide are both released into the extracellular space in equimolar ratio. Although insulin and C-peptide have different half-lives within the bloodstream, C-peptide is still considered a good marker for ß-cell health especially in those patients undergoing insulin replacement therapy, and its measurement is preferred in the diagnosis of insulinoma (Berzins et al 1987, Chammas et al 2003).

Normal Insulin Secretion.

The primary physiological stimulator of insulin secretion is glucose. In healthy individuals, pancreatic ß-cells secrete insulin at a rate which maintains glucose concentrations in the bloodstream within a very narrow range. Stimulation of insulin secretion by glucose occurs through the [Ca^{2+}] dependent triggering pathway and the amplification pathway, which increases insulin secretion by increasing the effect of [Ca^{2+}] on the exocytosis machinery (Henquin 2000). The triggering pathway involves the increase in intracellular Ca^{2+} by the following steps. Glucose is transported into the ß-cells by facilitated diffusion and undergoes oxidative glycolysis, resulting in an increase in the ATP/ADP ratio within the cell. Increasing ATP closes ATP-sensitive K^+ channels. Closure of K^+_{ATP} channels causes depolarization of the cell membrane and opening of voltage sensitive Ca^{2+}channels. Influx of Ca^{2+} through these channels activates the exocytosis machinery causing storage granules to release insulin into the bloodstream (Henquin 2000, Henquin 2002). The amplification pathway is marked by the increased efficacy of Ca^{2+} for activation of the exocytosis machinery, rather than a change in the

[Ca^{2+}] within the cell. Some hormones (Glucagon-like peptide I, cholecystokinin, glucose-dependent insulinotropic hormone, growth hormone, glucocorticoids, prolactin, placental lactogen, and the sex hormones) and neurotransmitters (acetylcholine) as well as increased metabolism (specifically high activity of protein kinases A and C) resultant from increased intracellular glucose within the ß-cell are known to activate this amplification pathway (Polonsky et al 2001, Henquin 2002, Gilon et al 2002, Wollheim et al. 1996, Bratanova-Tochkova et al 2002).

Since the 1960's it has been known that the insulin response to rapid and sustained glucose is biphasic (Cerasi and Luft 1967, Curry et al 1968, Porte et al 1969) A rapid peak in insulin secretion (Phase I) is followed by a nadir and then a slowly rising second peak (Phase II) (Henquin 1994). It is thought that this ability to rapidly respond to elevations in blood glucose is important for maintaining glucose homeostasis (Henquin 1994).

Exocytosis of Secretory Granules.

Secretion of insulin from the pancreatic ß-cells follows the regulated, rather than the constitutive pathway. Large pools of insulin are stored in secretory granules and released in response to glucose. The rate of release is a function of the concentration of glucose, the rate of change of the glucose concentration, and the presence of additional secretagogues which may amplify the signal for release of insulin granules (Polonsky et al 2001, Henquin 1994, Burgoyne and Morgan 2003). Upon initial stimulation, up to 0.3-1% of the total stored insulin are released from these internal stores (Henquin 2002, Burgoyne and Morgan 2003, Bratanova-Tochkova et al 2002). Researchers have suggested that internal stores of insulin are separated into two distinct pools of secretory granules: small, readily available pools representing the first phase of insulin secretion and larger, less readily available pools which feed into the smaller pools over

time representing the slower, longer acting second phase of insulin secretion (Porte et al 1969, Grodsky 1972). Popular consensus today is that these two pools are reserve granules and readily releasable granules. Readily releasable granules are already docked with the plasma membrane and are spatially prepared for exocytosis. The readily releasable granules can be further divided into two additional groups: those ready for immediate release and those in various stages of preparation for release (Bratanova-Tochkova et al 2002). Docking of secretory granules refers to the formation of a core complex of syntaxin and synaptosomal-associated protein 25 (SNAP-25) emanating from the plasma membrane, and the vesicle-associated membrane protein 2 (VAMP-2)/ synaptabrevin 2 emanating from the vesicular membrane (Bratanova-Tochkova et al 2002). Priming, or preparation for release, and exocytosis of the docked granule are induced by [Ca^{2+}] and ATP-dependent steps which are as of yet, not fully elucidated (Bratanova-Tochkova et al 2002). The priming step has been shown to require ATP as well as submicromolar amounts of Ca^{2+} (Wollheim et al 1996). This priming step is not fully understood but is thought to involve alterations of granule associated phospholipids as well as N-ethyl-maleimide (NEM)-sensitive fusion protein (Wollheim 1996). It has been firmly established that heterotrimeric GTPases are involved in the regulation of exocytosis as well as intracellular trafficking; however, little evidence is available to support their exact areas of action and the degree of control they wield over secretion and intracellular trafficking (Wollheim 1996).

Insulin Resistance.

Secretion of insulin elicits many metabolic effects. Upon binding to the insulin receptor, a tyrosine kinase, the cytosolic tail of the receptor autophosphorylates tyrosine residues initiating a series of varied reactions (White 1997). Autophosphorylation of the insulin receptor activates the insulin receptor substrates (IRS), a group of phosphotyrosine phosphatases (PTPases) which

then activate a multitude of downstream signaling pathways (Myers and White 1993). The primary reaction of interest is the activation of phosphatidylinositol 3-kinase (PI-3-kinase) which in turn activates many downstream signaling pathways, one of which is protein kinase B (PKB). PKB activates additional kinases that stimulate glucose transport, protein and glycogen synthesis, and cellular proliferation and survival (Alessi and Cohen 1998). Glucose transport is accomplished by facilitated diffusion through glucose transport proteins (GLUT). GLUT1 is expressed universally throughout most cell types and highly expressed in the liver. GLUT4, in contrast, is expressed in skeletal muscle and adipose tissue (White and Myers 2001). Insulin increases glucose transport by instigating the translocation of GLUT4 from intracellular vesicles to the cell membrane (White and Myers 2001). It has no effect on GLUT1.

Insulin resistance is the state at which insulin receptors are no longer partially or fully activated upon insulin binding. The causes of insulin resistance are not fully understood. One theory suggests that over-active serine kinases phosphorylate regulatory sites on the intracellular portion of the insulin receptor inhibiting activation upon binding of insulin (Virkamaki 1999). Another theory centers on the link between obesity and insulin resistance and diabetes. It suggests that insulin resistance in obese states is linked to cytokines, specifically TNF-α and leptin (Cohen et al 1996, Hotamisligil and Peraldi et al 1996). Leptin may reduce insulin secretion resulting in impaired ß-cell function (Wang and Koyama et al 1998). During hyperinsulinemia and states of increased body mass index, adipose tissue secretes increased levels of TNF-α. TNF-α acts locally to increase serine phosphorylation of insulin receptor substrates (IRS). This decreases insulin induced tyrosine kinase activity, thereby inhibiting insulin related intracellular signaling (Hotamisligil and Peraldi et al 1996). While the root cause of insulin resistance is not fully comprehended, it has been shown that insulin resistance alone

does not lead to type 2 diabetes. Insulin resistance alone is typically compensated for by increased ß-cell secretion of insulin. In this state, patients are still considered glucose tolerant, while having peripheral insulin resistance. Insulin resistance must be accompanied by impaired insulin secretion for type 2 diabetes to occur (White and Myers 2001, Kahn 2001).

Impaired Insulin Secretion.

Impaired insulin secretion can take the form of reduced insulin response to glucose and other secretagogues due to changes in the pulsatile and/or oscillatory secretion of insulin, and changes in the efficiency of conversion of proinsulin to insulin (Kahn 2001). Healthy non-diabetic subjects have characteristic pulsatile insulin secretion that manifests in two ways: a short spontaneous oscillation every 8-10 min and superimposed over the smaller oscillations are longer oscillations lasting approximately 120min (Matthews and Lang et al 1983, Polonsky et al 1988). These natural oscillations are no longer present in diabetic subjects (O'Meara and Sturis et al 1993). They appear to be spontaneous and continue within isolated cultured islets (Bergstrom et al 1989). It is unknown what causes the loss of oscillations in diabetics. It is hypothesized that these oscillations are replaced by more constant secretion. This may induce insulin resistance by constant activation of the insulin receptor inducing deactivation of the tyrosine kinase (Kahn 2001).

Most significant in relation to our study is the reduction in efficiency of the cleavage of proinsulin to insulin associated with type 2 diabetes. Recent studies have confirmed that fasting proinsulin concentrations, and more specifically the proinsulin/insulin ratio, are sensitive, early markers for insulin resistance in type 2 diabetes (Wareham et al 1999, Røder et al 1999, Hanley and D'Agostino et al 2002, Pfützner and Kunt et al 2004, Pfützner and Kann et al 2004, Langenfield et al 2004, Pfützner and Standl et al 2005). The conversion of proinsulin to insulin

with the secretory granule of the ß-cell is accomplished by the specific cleavage of the basic dipeptide B-C chain and C-A chain linkages by prohormone convertase 1/3 (PC1/3) and prohormone convertase 2 (PC2), respectively (Rhodes et al 1992). Proinsulin is initially cleaved by PC1/3 yielding des-31,32-proinsulin and subsequently cleaved by PC2 yielding insulin and C-peptide. Is has been shown that PC2 preferentially cleaves des-31,32-proinsulin over intact proinsulin (Rhodes et al 1992). This is believed to be the reason that circulating proinsulin levels consist mainly of des-32,33-proinsulin and intact proinsulin, with des-64,65-proinsulin not being present at all in circulation even during later stages of type 2 diabetes (Pfützner and Kann et al 2004). The degree of incomplete processing of proinsulin increases with the disease progression of diabetes. Over time, the proinsulin/insulin ratio increases until onset of glucose intolerance and hyperglycemia (Pfützner and Kann et al 2004). It is for this reason that a sensitive and specific assay for feline proinsulin would be a valuable diagnostic tool for early diagnosis and accurate monitoring of the development and progression of type 2 diabetes in the cat.

Radioimmunoassays for human proinsulin have been around since the 1970's (Duckworth and Kitabchi et al 1972, Heding 1977, Rainbow et al 1979). Early on, problems arose in proinsulin measurement due to cross-reactivity of partially cleaved and intact proinsulin. Gray et al (1984) first analyzed the differences in immunoreactivity between intact proinsulin and its cleavage intermediates by utilizing biosynthetic human proinsulin as well as purified native human proinsulin. This disparity raised awareness that more than one form of proinsulin may be secreted from the ß-cells. Gray et al (1984) measured immunoreactivity of limited and completely trypsinized biosynthetic proinsulin using a sandwich radioimmunoassay pairing an insulin specific capture antibody with a C-peptide specific screening antibody. This particular method was found to be very useful in measurements of total values of partially cleaved

proinsulin; however, the cross-reactivity with intact proinsulin was not useful for specific measurement of intact proinsulin (Gray et al 1984).

The first non-radiometric assay for proinsulin was described in 1986 (Hartling et al 1986). Hartling et al (1986) utilized a polyclonal-polyclonal antibody sandwich in microtiter wells, pairing an anti-insulin guinea pig polyclonal antibody with a peroxidase labeled and were able to demonstrate a limit of detection down to 1.2pmol/l. While this was a sensitive assay, due to the nature of polyclonal antibodies, it can not be considered specific detection of intact and/or intermediate proinsulin cleavage products. One additional draw-back of polyclonal antibodies is the limitation of the supply.

In 1987 Gray et al described the development of 4 monoclonal antibody lines directed against biosynthetic proinsulin. These monoclonal antibodies were shown to have varied cross-reactivities to intact and cleavage intermediate forms of proinsulin. The specific epitope of each antibody could be roughly assessed based on cross-reactivate patterns of the various forms of proinsulin. The first immunoassay for intact proinsulin was an ELISA method first described in 1998 (Houssa et al). Two monoclonal antibodies, one specific for the B-C chain junction of proinsulin and another specific for the C-A chain junction were used in a two-site immunoassay to measure intact proinsulin in both a sensitive and a specific method (Houssa et al 1998). One additional advantage of using a pair of monoclonal antibodies for such an assay is the unlimited supply of the immunochemicals.

CHAPTER 3

MATERIALS AND METHODS

Antigen Preparation

Feline C-peptide and N-terminal tyrosine-feline C-peptide were acquired through Syn-Pep, Inc. (Dublin, CA) and were manufactured via synthetic peptide synthesis. The sequences of feline C-peptide (FCP) and tyrosine-feline C-peptide (yFCP) are as follows:

FCP: **EAEDLQGKDAELGEAPGAGGLQPSALEAPLQ**

yFCP: **YEAEDLQGKDAELGEAPGAGGLQPSALEAPLQ**

Feline proinsulin was expressed in BL21(de3) E.*coli* (Novagen, Madison, WI) and purified as described in detail (Hoenig et al 2005). Feline C-peptide was prepared as keyhole limpet hemocyanin (KLH) conjugate, bovine serum albumin (BSA) conjugate, glutaraldehyde aggregate, and as multiple antigenic peptides (MAPs), and feline proinsulin was prepared as glutaraldehyde conjugate and MAPs as described below.

KLH-C-peptide conjugate was prepared as follows. Before conjugation of feline C-peptide to KLH, the peptide was allowed to react with N-succinimidyl-S-acetylthioglycolic acid (SATA; Pierce, Rockford, IL) to introduce protected sulfhydryl functional groups to primary amines. Ten 1mL aliquots of synthetic feline C-peptide were prepared by diluting the C-peptide to 180µg/mL in 10ml of SATA reaction mixture (50mM sodium phosphate, 1mM EDTA, pH 7.5). Thirteen to 15mg of SATA were dissolved in 1ml dimethylsulfoxide (DMSO). Ten µL of the SATA solution was combined with each C-peptide aliquot in a borosilicate glass test tube,

sealed with parafilm, and vortexed. The reaction was incubated at room temperature for 30min. The reaction was then applied to a Bio-Gel P2 gel filtration column equilibrated in reaction buffer (Bio-Rad; 2 x 25cm). The flow rate was 1mL/min. Peak elution was monitored at 280nm and the first peak was collected. The collected peak was separated into 1mL aliquots in 12x75mm borosilicate glass tubes. To each aliquot, 100µL of deacetylation solution was added (50mm Sodium phosphate, 25mM EDTA, 0.5 M hydroxylamine HCL, pH 7.5; Pierce, Rockford, IL). Each tube was sealed with parafilm and allowed to incubate at room temperature for 2 hours. The reaction was then applied to a Bio-Gel P2 gel filtration column equilibrated in 1:10 diluted reaction buffer and monitored at 280nm. The flow rate was 1ml/min. The first peak was collected and pooled. The pooled fractions were snap frozen in a dry ice-ethanol bath and lyophilized overnight. A 2mg aliquot of Pierce's (Rockford, IL) maleimide activated KLH (mcKLH) was reconstituted in 200µl double-distilled (dd) H_2O for a final concentration of 10mg/mL. The lyophilized C-peptide fraction was reconstituted in 500µl ddH_2O and immediately added to the reconstituted mcKLH. This reaction was incubated at room temperature for 2 hours and then applied to a D-Salt polyacrylamide desalting column (Pierce #43240, Rockford, IL) equilibrated in purification buffered salts (Pierce #77159, Rockford, IL; 3-5 column volumes). The conjugation reaction was eluted in 0.5mL fractions with purification buffered salts. Each fraction was analyzed for UV_{280} absorbance and those fractions incorporating the first peak were pooled, sterile filtered, and stored at -20°C until used for injections.

C-peptide-BSA conjugate was prepared using Pierce's Imject Immunogen EDC Conjugation kit (Pierce #77123, Rockford, IL). One 2mg vial of Imject BSA was dissolved in 200µl of ddH2O resulting in a 10mg/mL solution. Synthetic feline C-peptide was reconstituted

in Pierce conjugation buffer (0.1M MES, 0.9M NaCl, 0.02%NaN_3, pH 4.7) for a final concentration of 4 mg/mL. Five-hundred µL of this C-peptide solution was combined with 200µL of the reconstituted BSA and added to one vial of EDC (1-Ethyl-3-[3-Dimethylaminopropyl]carbodiimide Hydrochloride, 10mg). The reaction was gently mixed and allowed to react for 2 hours at room temperature. The final reaction was applied to a D-Salt dextran desalting column (Pierce #1856112) equilibrated with purification buffer salts supplied by the manufacturer. Elution aliquots (0.5 ml each) were collected and analyzed using a Bradford protein assay (Bio-Rad; Hercules, CA). The protein peak was pooled and stored at -20°C.

Glutaraldehyde Aggregated Proinsulin was prepared as follows. A 2mg sample of lyophilized feline proinsulin was reconstituted in 400µl of PBS, pH 7.4, and transferred to a 2ml eppendorf tube. Four hundred µl of 0.2% glutaraldehyde in PBS, pH 7.4, was added drop wise with constant stirring. The eppendorf tube was sealed and the reaction was allowed to incubate at room temperature for 1 hour with stirring. Two hundred µl of a 1M glycine solution in PBS, pH 7.4, was added to the reaction and incubated for another hour at room temp to quench the remaining glutaraldehyde. This aggregation reaction was centrifuged at 16,000 x g for 5 minutes at room temperature and the supernatant was applied to a D-Salt dextran desalting column equilibrated with purification buffer salts (Pierce, Rockford, IL). Elution aliquots (0.5 ml each) were collected and analyzed using a Bradford protein assay (Bio Rad, Hercules, CA). The protein peak was pooled, sterile filtered, added to the previous pellet, and stored at -20°C.

MAP Resins preparation. For purposes of screening for the linkage regions between the B-chain-C-peptide and the C-peptide-A-chain regions of feline proinsulin as well as the C-peptide, Multiple-Antigenic Peptides (MAPs) consisting of 15-20 amino acid residues from these linkage regions were synthesized (Sigma-Genosys, St. Louis, MO). The F_{24}-Q_{38} construct spans

the dipeptide linkage of $R_{31}R_{32}$ between the B-chain and C-peptide of feline proinsulin. The S_{56}-Q_{70} construct spans the dipeptide linkage of $K_{64}R_{65}$ between the C-peptide and A-chain of feline proinsulin. The C-E_1-A_{15} construct is a 15-mer representing the N-terminal region of feline C-peptide. The sequence of these three peptides is as follows (dipeptide linkages are underscored):

F_{24}-Q_{38}: **FFYTPKARREAEDLQ**

S_{56}-Q_{70}: **SALEAPLQKRGIVEQ**

C-E_1-A_{15}: **EAEDLQGKDAELGEA**

MAP peptides are prepared by linking four peptides via C-terminal covalent linkage to a backbone of 4 lysine residues.

ELISA Screening Method

Plate Coating

A) Antibody capture assays

Thermo Electron Corp's Immulon 4HBX high binding 1x12 removawell strips (Waltham, MA) were used for protein binding. All plates, for use in antibody capture assays, were prepared in a similar manner. Well antigens (1.0µg or 0.1µg) were prepared in phosphate buffered saline (PBS) coating buffer (0.15M NaCl, 0.01M Na_2HPO_4, pH7.4) per 50µl or 100µl. Wells were coated with 50µl or 100µl of antigen in coating buffer at room temperature for 2 hours with rotary shaking, and then overnight at 4°C. Wells were then washed 3 times with 350µl of PBS (pH 7.4) and tapped dry. Wells were then blocked with 250µl of blocking buffer (Coating buffer with 2% Bovine Serum Albumin, 5% Sucrose) at room temperature for 2 hours with rotary shaking. Wells were washed again with 350µl of PBS and tapped dry. These coated wells were stored at 4°C in desiccated containers.

B) Antigen capture and sandwich assays

Wells for use in antigen capture and sandwich assays were coated with 2.0µg of antibody in 200µl of PBS per well. Subsequent coating protocol was the same as above.

ELISA Screening of Polyclonal Serum

ELISA screening of mouse and rabbit polyclonal serum occurred as follows. All serum dilutions were made with immunoassay buffer (25mM Tris-Base, 0.15M KCl, 10mM EGTA, 0.2% BSA, pH 7.5). Initial screenings for each antigen varied. For screening of KLH-C-peptide antisera, the following antigens were used and the signal compared to a buffer control: feline-C-peptide, BSA, BSA-C-peptide, KLH-C-peptide, and mcKLH. For screening of glutaraldehyde aggregated feline proinsulin antisera, the following antigens were used: feline proinsulin, BSA-C-peptide, bovine insulin, F_{24}-Q_{38}, and S_{56}-Q_{70}. For screening of S_{56}-Q_{70} MAP antisera, the following antigens were used: S_{56}-Q_{70} MAP and feline proinsulin. The pre-immune bleed (PI) for each rabbit and non-immune BALB/c serum were used as controls in serum assays.

Initial antibody capture screenings of mouse and rabbit polyclonal sera were performed at 1:200 dilutions using the relevant antigen coated wells as stated above. After confirmation of an immunogenic response, polyclonal sera from subsequent boost bleed cycles was screened at further dilutions, from 1:10 to 1:100K, in order to characterize the immunogenic response of each animal. Fifty µl of diluted serum was added to each coated well and incubated at room temperature with rotary shaking for 2 hours or overnight at 4°C. Each well was washed with 350µl of immunoassay wash buffer (20mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Tween, pH 7.0) 3 times and tapped dry. The secondary antibody was diluted into immunoassay buffer to 1:5,000 for goat-anti-mouse-HRP antibody (Sigma, A-4416, St. Louis, MO) and to 1:10,000 for goat-anti-rabbit-HRP antibody (Sigma, A-6667, St. Louis, MO). Fifty µl or 100µl of diluted secondary antibody was added to each well and incubated at room temperature with rotary

shaking for 1 hour. Each well was then washed 3 times with immunoassay wash buffer and tapped dry. Fifty µl or 100µl of TMB substrate (Sigma, T-8665, St. Louis, MO) was added to each well and allowed to incubate for 10-60 minutes dependent upon the strength and speed of reaction. At the endpoint, 25µl or 50µl of either 1M H_2SO_4 or 1M HCl was added to stop the reaction. The absorbance of each well at 450nm was then determined on an EL_X800 Universal Micro plate Reader (Bio-Tek Instruments, Inc., Winooski, VE).

^{125}I-Streptavidin Radioimmunoassay

Coating of Tubes

Nunc-Immuno™ Tubes were coated with 1.5µg of antibody in 300µl PBS overnight at 4°C (Cat. # 82-470319, Nalge Nunc International, Rochester, NY). Coated tubes were washed once with PBS and tapped dry. Tubes were then blocked with 500µl of blocking buffer overnight at 4°C. Blocked tubes were then washed twice with PBS and tapped dry.

Iodination of Streptavidin

Ten µg of streptavidin (Sigma, S-0677, St. Louis, MO) in 100µl of ddH_2O were added to one IODO-GEN® pre-coated iodination tube (Pierce Biotechnology, Inc., Rockford, IL) followed by 1 mCi $Na^{125}I$ (MP Biomedicals, Irvine, CA) in 10µl of 0.1N NaOH. The reaction was incubated at room temperature for 15 min with occasional agitation of the reaction tube. The reaction was then applied to a 10ml Sephadex G-75 (Amersham Biosciences, Piscataway, NJ) column equilibrated in PBS with 0.2% BSA. Forty elution aliquots of 0.5ml each were collected. Five µl of each collected fraction was counted on a PerkinElmer 1470 Wizard Gamma counter (Wellesley, MA). Fractions 10 through 15 contained the first and largest peak representing the ^{125}iodine incorporated into streptavidin. These fractions were pooled and stored at 4°C. The relative activity of ^{125}I-streptavidin was 224.08µCi/ml.

Tube based Sandwich RIA

Various concentrations of feline proinsulin standard ranging from 0-10ng/ml were prepared in 50µl of 3X serum immunoassay buffer (25mM Tris Base, 0.15M KCl, 2mM EDTA, 10 mM HEPES, 0.5% Tween 20, 0.1% BSA, pH 7.5). Biotinylated monoclonal and polyclonal antibodies were prepared by diluting the antibody to 5-20ng/50µl in 3X serum immunoassay buffer. Assays were performed by adding 50µl proinsulin standard to coated assay tubes followed by 50µl of biotinylated monoclonal or polyclonal antibody. Two-hundred µl of either ddH2O or filtered lamb serum were then added to each tube. The reaction tubes were allowed to incubate overnight at room temperature with rotary shaking. Each tube was then washed 3 times with 2ml immunoassay wash buffer. ^{125}I-Streptavidin was diluted into 1X serum immunoassay buffer at 100,000cpm per 300µl. Three hundred µl of diluted ^{125}I-Streptavidin was added to each tube and incubated at room temperature with rotary shaking for 1 hour. Tubes were then washed 3 times with immunoassay wash buffer. Tubes were then counted on a PerkinElmer 1470 Wizard Gamma counter for 60 seconds each (Wellesley, MA).

Polyclonal Antibody Development

Polyclonal antibody development in rabbits was performed by the animal resources facility of the Department of Biochemistry and Molecular Biology at the University of Georgia. Development of polyclonal antibodies was attempted against the following antigens: aggregated feline proinsulin, S_{56}-Q_{70} MAP, and KLH-C-peptide. Development protocols were similar in each experiment. Two white SPF New Zealand rabbits (Myrtle's Rabbitry, Thompson Station, TN) were immunized with 0.5 ml each of 200µg/mL of antigen subcutaneously (SQ) with 0.5ml of Complete Freund's Adjuvant (CFA). 3 weeks after the initial immunization, the first boost injection was performed with 0.5ml of 200µg/ml antigen and 0.5ml Incomplete Freund's

Adjuvant (IFA) SQ. An initial test bleed was performed at 1 week post boost. Serum was screened via antibody capture assay against relevant antigens. Rabbit IgG coated wells (0.1µg/well) were used as a positive controls. Bovine Serum Albumin wells (BSA, 0.1µg/well) were used as negative controls. Antibody was detected using GAR-HRP and TMB substrate (KPL, Gaithersburg, MD). Subsequent similar boosts were performed at 4 or 6 weeks intervals with tests bleeds following at 1 week post boosts. Boost and bleed schedules were continued until titers remained stable. Large exsanguination bleeds were then performed yielding approximately 60mls of polyclonal serum per rabbit.

Affinity Purification of Polyclonal Antibodies from Rabbit Serum

Protein A antibody affinity purification was performed on polyclonal rabbit serum using either 1 ml Affinity-Pak Protein A columns or 3 ml ImmunoPure Immobilzed Protein A columns poured in house (Pierce #20356, #20334, Rockford, IL). Protein A is a bacterial cell wall component protein which binds the Fc region of many immunoglobulins. Purifications were performed as per Pierce's protocols using ImmunoPure Binding buffer (Pierce # 21001) and ImmunoPure Elution buffer (Pierce #21004, Rockford, IL). One-half mL or 1.0mL elutions were collected directly into 0.1 volumes of Tris-Base buffer (1.0M Tris, pH 9.5). Pre-column, flow-through, wash, and elution fractions were screened via antibody capture ELISA at 1:500 dilutions. Those elution fractions with absorbances greater than 3 times background were pooled and dialyzed 1:1000 2 times into PBS, pH 7.4, at 4°C for >8 hours each. Dialysis was performed in Spectra/Por 7 3,500 MWCO dialysis tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Antibody concentrations were determined using the Bradford method with bovine γ-globulin used as standards (Bradford 1976).

Biotinylation of Antibodies.

Antibodies intended for use in sandwich assays were biotinylated using Pierce Biotechnology's EZ-Link Sulfo-NHS-LC-Biotin as per protocol (Pierce #21335, Rockford, IL). Two mg in 1ml of PBS were reacted with 26.6µl of freshly prepared 10mM Sulfo-NHS-LC-Biotin in ddH_2O for 30min at room temperature. After the reaction was completed, the biotinylated antibody was dialyzed 1:500 into PBS using 10K MWCO Spectra/Por 7 dialysis tubing for 8 hours at 4°C. The dialysis buffer was exchanged and allowed to dialyze for another 8 hours at 4°C.

Monoclonal Antibody Development

Monoclonal antibody development was performed by the Monoclonal Antibody Facility, College of Veterinary Medicine, University of Georgia, Athens, GA. Development of monoclonal antibodies was attempted against the following antigens: KLH-C-peptide, aggregated feline proinsulin, aggregated-C-peptide, & C-E_1-A_{15} MAP. Two BALB/c mice (Harlan, Indianapolis, IN) per antigen were given an SQ initial injection of 100µg of antigen in a volume of 50µl of PBS and 50µl of CFA (designated M2 & M3). One BALB/c mouse per antigen was given an initial intraperitoneal (IP) injection of 100µg of antigen in a volume of 50µl of PBS and 50µl of Ribi's adjuvant (designated M1, ImmunoChem Research, Inc., Hamilton, MT). Subsequent boosts were all performed IP with 50µg of antigen and 50µl of Incomplete Freund's Adjuvant (IFA, M2 & M3) or Ribi's adjuvant (M1) at 4-6 weeks interval. Five to 7 days post boost, each animal was bled via the tail vein. Approximately 50µl of neat serum was provided from each mouse for screening purposes.

Splenic cell fusions were performed on selected mice whose specific response at a 1:200 dilution in ELISA screenings was greater than 3 times the background. Fusion of splenic cells

with myeloma cells (SP2/0) was accomplished by the polyethylene glycol-aided fusion technique. All fusions were performed by the Monoclonal Antibody Facility. Hybridomas were raised in 96 well plates on macrophage feeder cell layers. Hybridoma supernatants were screened without dilution using the same method for ELISA as discussed above. Supernatants with responses greater than 3 times that of background were cloned by serial dilution in 96 well plates. Hybridomas were cloned by limiting dilution to form monoclonal hybridomas. Monoclonal cell lines were isotyped by the monoclonal antibody facility using Sigma's ISO2 Mouse Monoclonal Antibody Isotyping Reagents (ISO2-1KT, St. Louis, MO). Monoclonal cells were grown to confluency in 1L flasks. Monoclonal antibodies were pelleted by ammonium sulfate precipitation, reconstituted in 0.05 volumes of PBS and dialyzed against PBS to remove excess ammonium sulfate. Precipitated antibodies were purified by protein A affinity purification as described above.

All statistical analyses were performed using Graphpad Prism software (San Diego, CA).

CHAPTER 4

RESULTS

Monoclonal Antibodies Against KLH-C-peptide Antigen.

Three BALB/c mice were immunized with feline C-peptide conjugated to Keyhole limpet hemocyanin (KLH-C-peptide). After 1 initial immunization and 4 subsequent boosts at 3 week intervals, Mouse 3 (M3) reached our set threshold for fusion, a specific signal ≥3 times background when screened against feline C-peptide conjugated to bovine serum albumin (BSA-C-peptide) at a dilution of 1:200 of polyclonal sera into assay buffer. Mouse 1 (M1) reached the threshold for fusion after 1 initial immunization and 8 subsequent boosts at 4-6 week intervals. Fusion of M3 yielded three 4 mouse-anti-feline C-peptide monoclonal antibodies: 7G7, 5D2, 2F1, and 9G3. All 4 antibodies were determined to be IgM isotypes. One liter cultures of the two monoclonal antibodies (MAb) with the strongest signals, 7G7 and 9G3, were grown and harvested by ammonium sulfate precipitation. Each MAb was screened against wells coated with BSA-C-peptide at dilutions from 1:10-1:10K. 7G7 and 9G3 showed very low titers against our target protein, Log EC_{50}= -2.114 and -1.876, respectively (Figure 1 A-B). The fusion of M1 yielded 17 monoclonal antibody lines, however, none had affinity for BSA-C-peptide (Figure2).

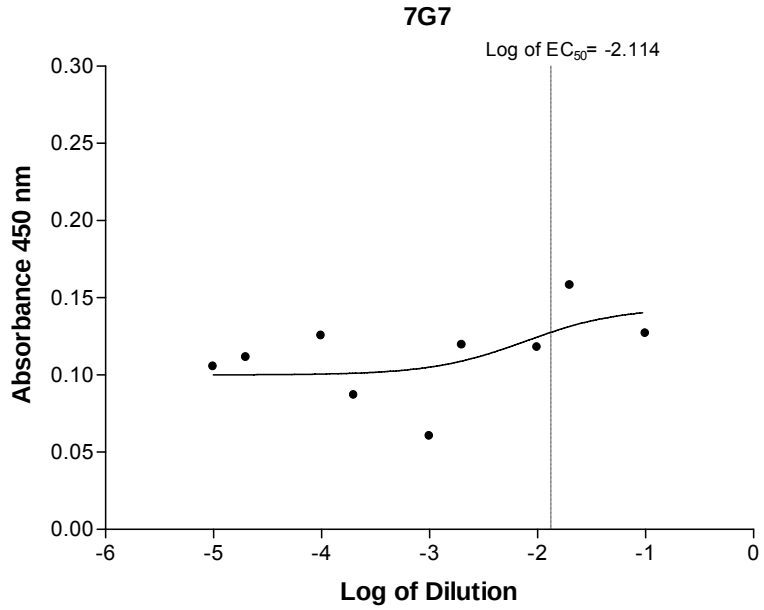

Figure 1A. Absorbance 450 nm values for Enzyme-linked immunosorbent assay (ELISA) for the 7G7 monoclonal antibody produced from the fusion of KLH-C-peptide mouse 3. Specific data, N=1.

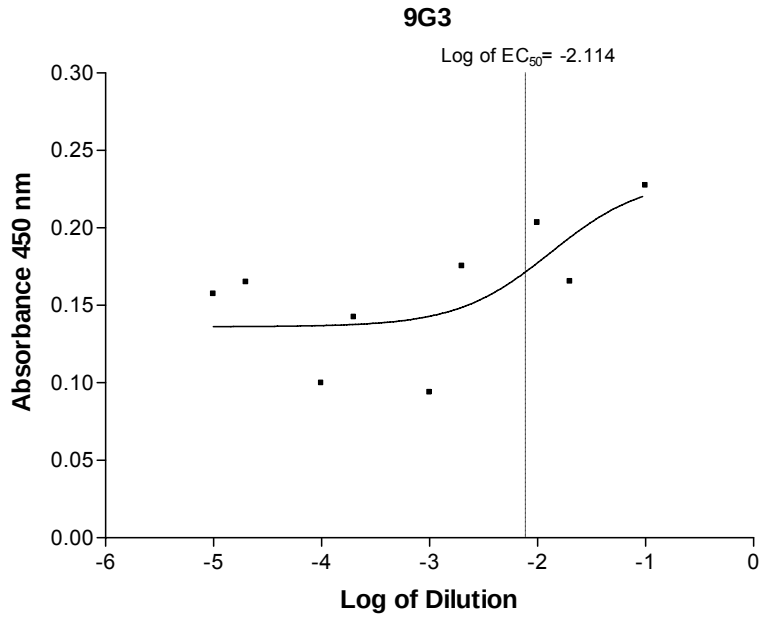

Figure 1B. Absorbance 450 nm values for Enzyme-linked immunosorbent assay (ELISA) for the 9G3 monoclonal antibody produced from the fusion of KLH-C-peptide mouse 3. Specific data, N=1.

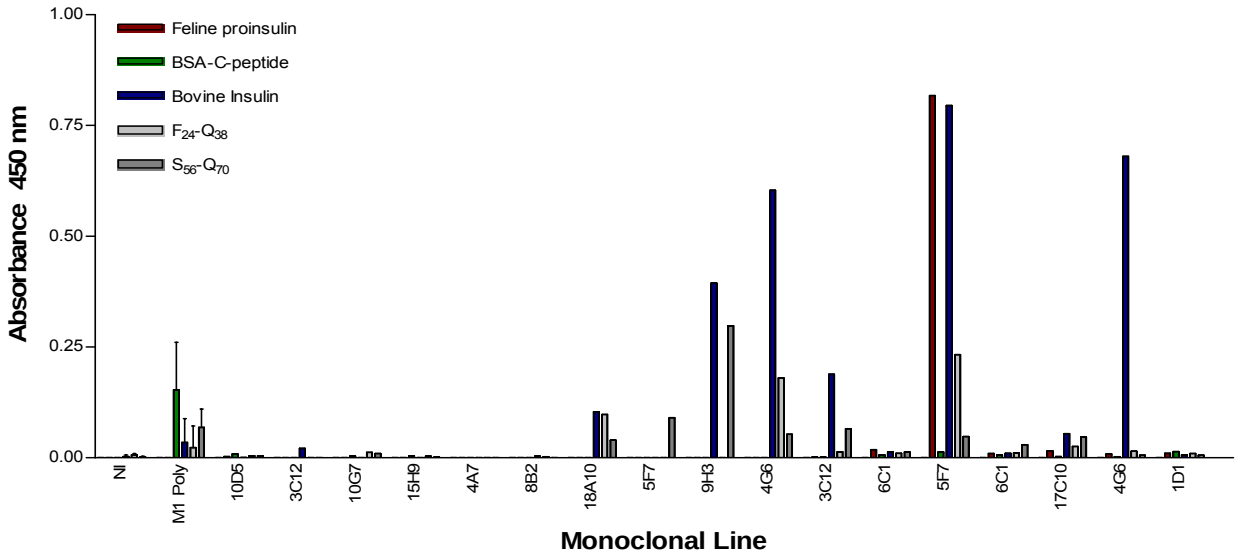

Figure 2. Absorbance 450 nm values for ELISA for the M1 monoclonal lines developed against KLH-C-peptide. Specific data, N=1. NI= Non-immune BALB/c mouse serum, M1 poly= polyclonal serum from mouse 1.

Polyclonal Antibodies Against KLH-C-peptide Antigen.

Two Rabbits (R726 & R731) were immunized using feline C-peptide conjugated to KLH. One initial immunization and 4 boosts were performed at 4-6 week intervals. Rabbit serum from each post boost bleed was screened at a 1:200 dilution against BSA-C-peptide wells. Serum response peaked after the second boost. The serum titer began to fall after the second boost, suggesting that the rabbits were developing tolerance to the antigen. R726 had a response ~50% greater than that of R731 following the fourth and final boost, absorbance at 450nm of 0.938 verses 0.616. R726 was terminated and an exsanguination bleed was performed yielding ~60ml of rabbit-anti-feline C-peptide anti-sera. R731 was terminated. The R726 anti-sera was screened against BSA-C-peptide wells using dilutions from 1:10 to 1:100K into immunoassay buffer

(Figure 3). Log EC_{50} for binding of R726 anti-feline C-peptide polyclonal sera to BSA-C-peptide is -1.761.

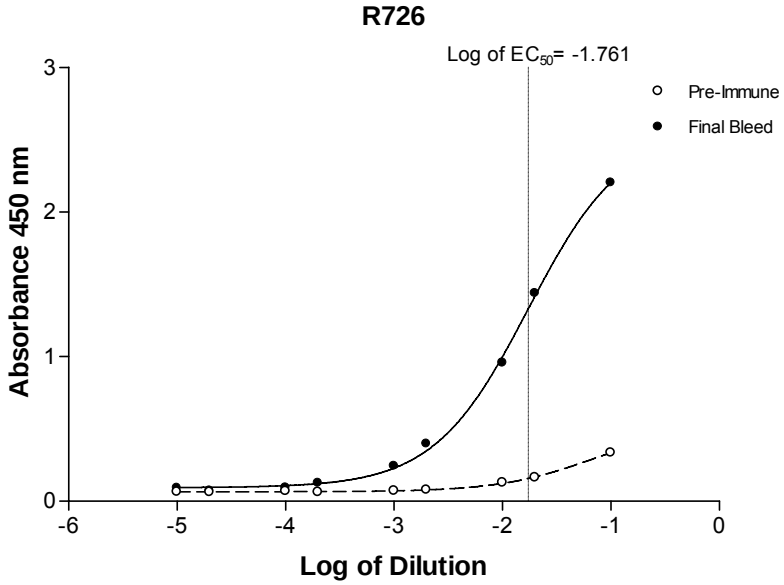

Figure 3. Absorbance 450 nm values for Enzyme-linked immunosorbent assay (ELISA) for the polyclonal anti-sera generated against KLH-C-peptide in rabbit R726. N=2.

Monoclonal Antibodies Against Glutaraldehyde Aggregated Feline Proinsulin.

Three BALB/c mice (M1, M2, & M3) were immunized with glutaraldehyde aggregated recombinant feline proinsulin. After 1 initial immunization and 3 subsequent boosts at 3 week intervals, all three mice had developed very high titers against feline proinsulin (Figure 4). M1 had the greatest response and was fused. The fusion of M1 was very prolific and yielded 3 batches of monoclonal antibodies representing 52 cell lines (Figure 5).

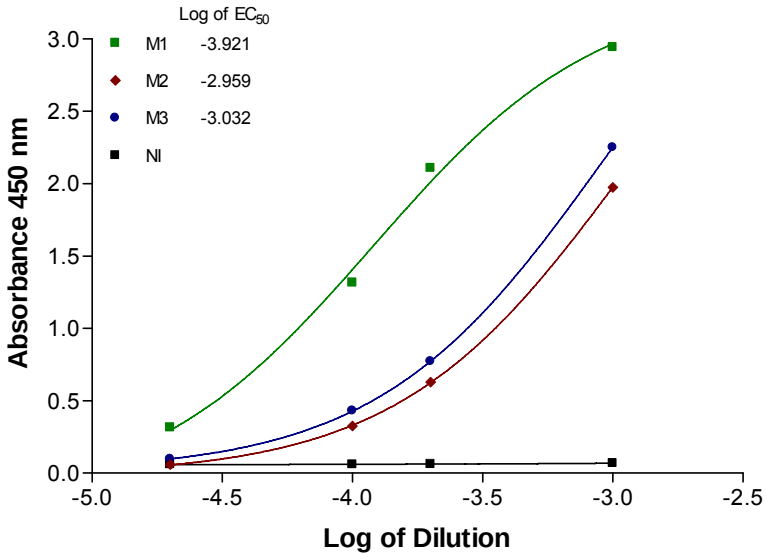

Figure 4. ELISA assay showing the polyclonal mouse serum titer after the third boost with aggregated recombinant feline proinsulin.

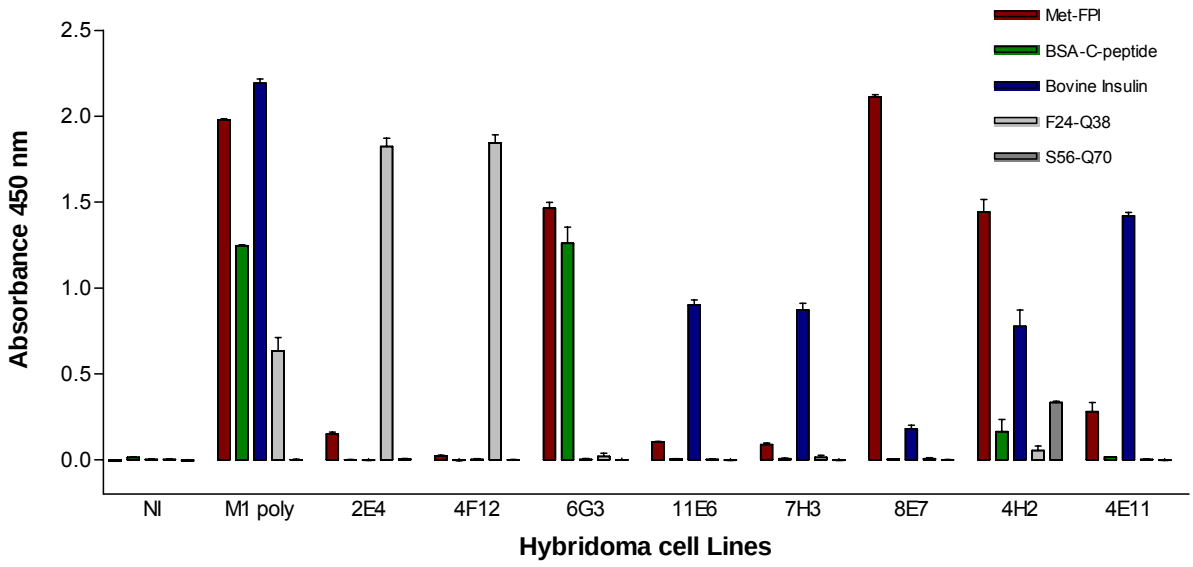

Figure 5. Absorbance 450 nm values for ELISA for the M1 monoclonal lines developed against recombinant feline proinsulin. 8 selected hybridoma lines from 52 total cell lines.

Eight hybridoma cell lines were determined to be monoclonal by limited dilution and were aliquoted and stored on liquid nitrogen: 2E4, 4F12, 6G3, 11E6, 7H3, 8E7, 4H2, 4E11. The cross-reactivities of the various monoclonal lines are shown in table 1.

Table 1. Cross-reactivities of monoclonal cell lines generated against recombinant feline proinsulin. Cross-reactivities are presented as percent of maximal binding.

Monoclonal Line	Met-FPI	BSA-C-peptide	Bovine Insulin	F24-Q38	S56-Q70
M1 poly	90.3 %	56.9 %	100.0 %	28.9 %	0.0 %
NI	0.0 %	0.6 %	0.1 %	0.1 %	0.0 %
2E4	8.2 %	0.0 %	0.0 %	100.0 %	0.2 %
4F12	1.2 %	0.0 %	0.1 %	100.0 %	0.0 %
6G3	100.0 %	86.1 %	0.1 %	1.4 %	0.0 %
11E6	11.4 %	0.4 %	100.0 %	0.2 %	0.0 %
7H3	10.2 %	0.7 %	100.0 %	1.6 %	0.0 %
8E7	100.0 %	0.2 %	8.6 %	0.3 %	0.0 %
4H2	100.0 %	11.4 %	53.9 %	3.7 %	23.1 %
4E11	19.7 %	1.1 %	100.0 %	0.1 %	0.0 %

Each monoclonal antibody was isotyped using Sigma-Aldrich's ISO2 mouse monoclonal Isotyping kit. The isotypes of each monoclonal antibody are shown in table 2.

Table 2. Isotypes of saved monoclonal antibodies.

Monoclonal Line	Isotype
2E4	IgG_1
4F12	IgG_1
6G3	IgG_1
11E6	IgG_1
7H3	IgG_1
8E7	IgG_{2b}
4H2	IgG_{2b}
4E11	IgG_1

One liter cultures of monoclonal hybridomas were grown for each monoclonal. Antibodies were precipitated out of the media using saturated ammonium phosphate, and subsequently dialyzed against PBS. Each precipitated monoclonal line was purified by protein A affinity purification as per manufacturer's protocol (Pierce, Rockford, IL). Figure 6 shows a representative elution profile for the affinity purification of each monoclonal antibody.

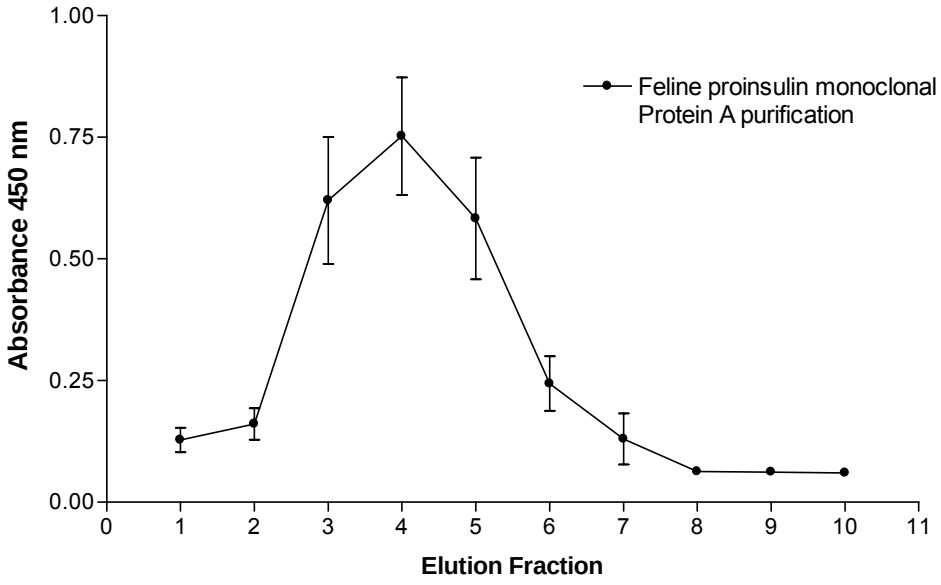

Figure 6. Representative graph of elution profile for protein A purification of monoclonal antibodies generated against recombinant feline proinsulin, screened using antibody capture ELISA. (0.5 ml fractions)

Polyclonal Antibodies Against Recombinant Feline Proinsulin.

Two rabbits (R754 & R755) were immunized using glutaraldehyde aggregated recombinant feline proinsulin. One initial immunization and 7 boosts were performed at 4-6 week intervals. At this point the immune response of R754 and R755 had reached a plateau having a Log of EC_{50} of -2.471 and -2.954, respectively (Figure 7 A-B).

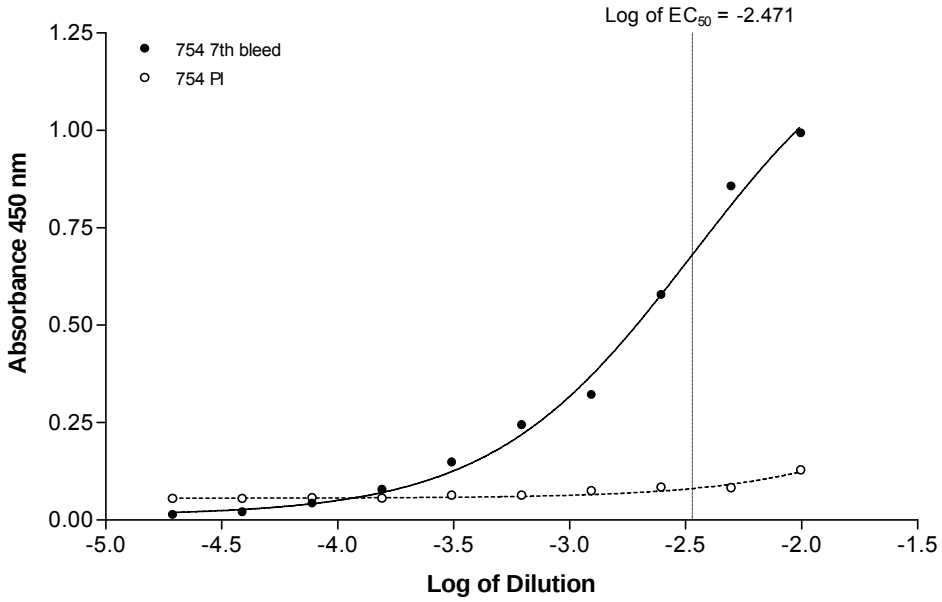

Figure 7A. ELISA assay showing the immune response to recombinant feline proinsulin in R754 after 7 boosts. N=2.

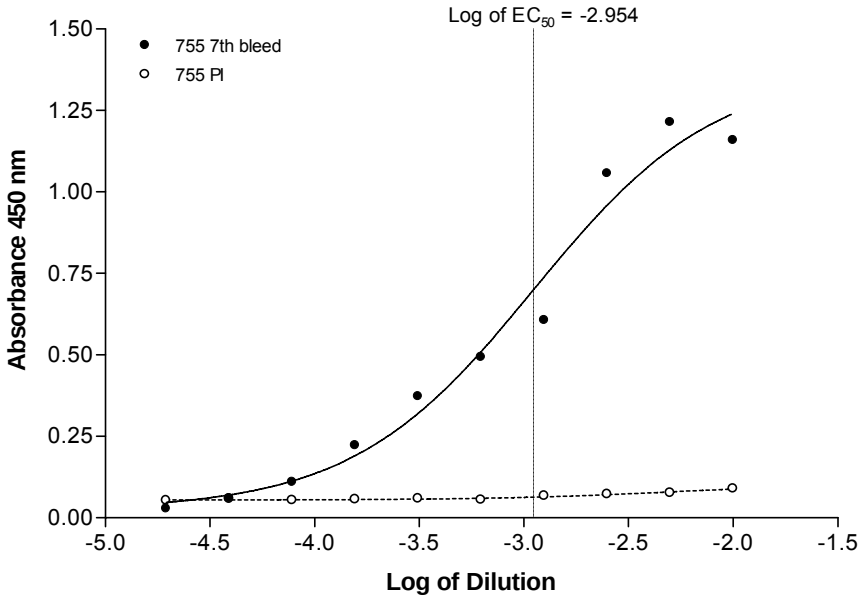

Figure 7B. ELISA assay showing the immune response to recombinant feline proinsulin in R755 after 7 boosts. N=2.

An additional 4 boost at 4-6 week intervals were followed by production bleeds at 1 week post yielding ~15ml of anti-sera per rabbit per boost. Polyclonal antibodies from the rabbit anti-sera were purified by protein A affinity purification as per manufacturers protocols (Pierce, Rockford, IL). Figure 8 is representative of the elution profile of the protein A affinity purification when screened in an antibody capture ELISA using wells coated will recombinant feline proinsulin.

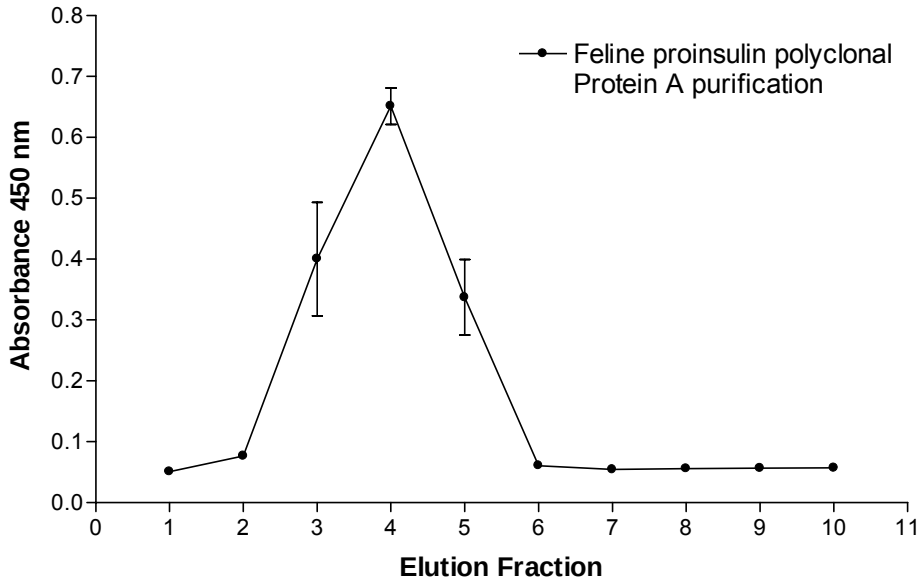

Figure 8. Representative graph of elution profile for protein A purification of R754 and R755 polyclonal antibodies screened using antibody capture ELISA. (0.5 ml fractions) n=10.

Table 3 is a listing of the cross-reactivities of R754 and R755 polyclonal antibodies.

Table 3. Cross-reactivities of polyclonal antibodies generated against recombinant feline proinsulin. Cross-reactivities are presented as percent of maximal binding. (PI = Pre-immune)

Polyclonal Line	Met-FPI	BSA-C-peptide	Bovine Insulin	F24-Q38	S56-Q70
R754 PI	0.0 %	2.5 %	0.5 %	0.5 %	0.0 %
R754	100.0 %	80.9 %	0.3 %	2.8 %	11.1 %
R755 PI	0.0 %	0.6 %	0.0 %	0.0 %	0.0 %
R755	100.0 %	49.0 %	9.8 %	2.5 %	15.7 %

Polyclonal Antibodies Against S_{56}-Q_{70} Multiple Antigenic Peptide.

Two rabbits (R1 & R2) were immunized using the S_{56}-Q_{70} multiple antigenic peptide (MAP). One initial immunization and 5 boosts were performed at 4-6 week intervals. After the second boost, the antibody response to S_{56}-Q_{70} wells was very strong in R1 having a Log of EC_{50} = -4.064 and R2 having a Log of EC50 = -4.021 (Figure 9 A-B).

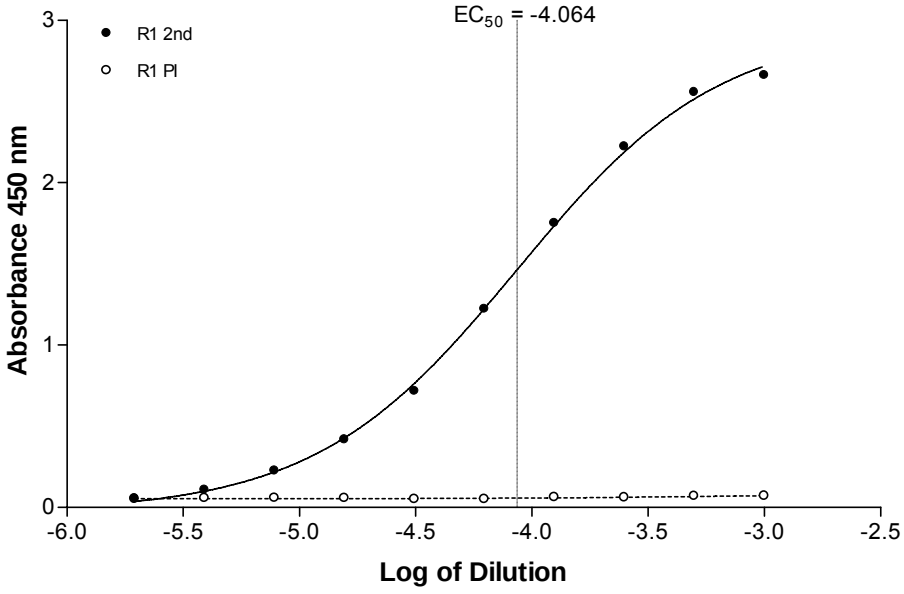

Figure 9A. ELISA assay showing the serum response of R1 after 2 boost with S_{56}-Q_{70} antigen. N=3.

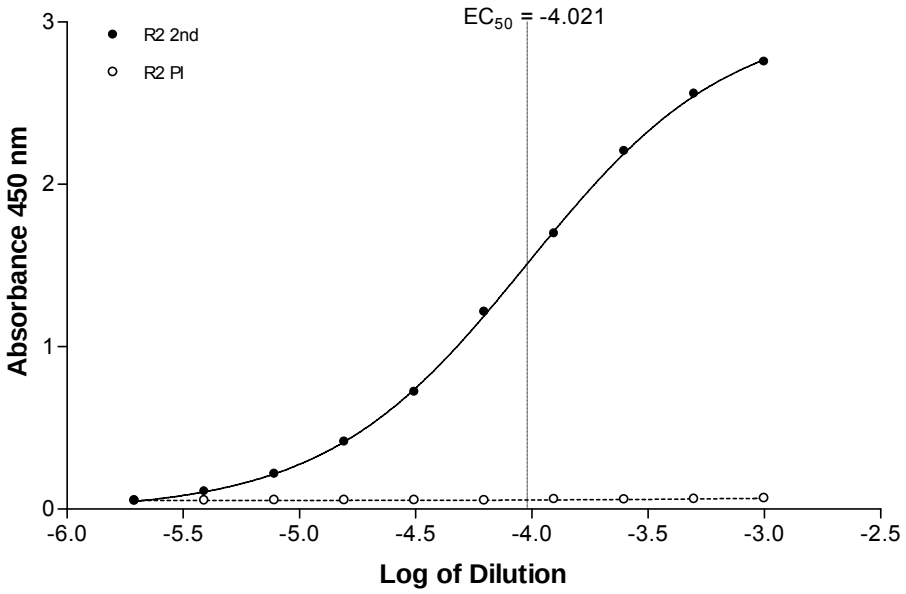

Figure 9B. ELISA assay showing the serum response of R2 after 2nd boost. N=3.

Five subsequent boosts were performed followed by production bleeds yielding ~15mls of anti-sera per rabbit per bleed. Both rabbits were terminated and exsanguination bleeds of both yielded ~60mls of anti-sera per rabbit. Polyclonal antibodies were purified from the anti-sera by protein A affinity purification (Figure 10). Cross-reactivity of R1 and R2 polyclonal serum was determined by antibody capture ELISA at a dilution of 1:500 (Table 4).

Table 4. Cross-reactivities of polyclonal antibodies generated against S_{56}-Q_{70}. Cross-reactivities are presented as percent of maximal binding. (PI = Pre-immune)

Polyclonal Line	Met-FPI	BSA-C-peptide	Bovine Insulin	F24-Q38	S56-Q70
R1 PI	0.0 %	1.7 %	0.9 %	3.1 %	0.0 %
R1	53.4 %	0.3 %	26.5 %	0.0 %	100.0 %
R2 PI	0.0 %	1.9 %	0.0 %	1.5 %	0.0 %
R2	36.3 %	13.6 %	0.0 %	0.0 %	100.0 %

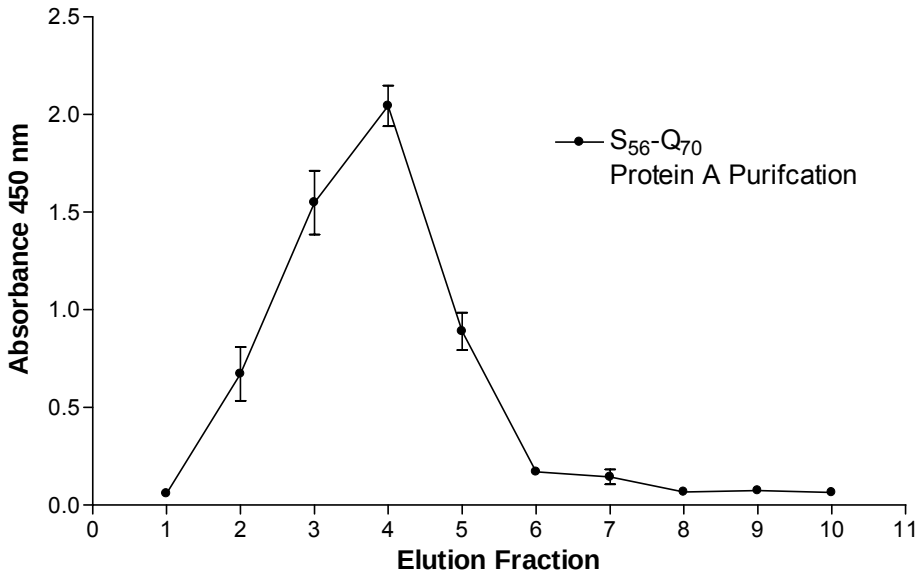

Figure 10. Representative graph of elution profile for protein A purification of R1 and R2 polyclonal antibodies screened using antibody capture ELISA. (0.5ml fractions) N=10.

Development of a Feline Proinsulin Assay.

For the purpose of developing a two-site immunometric (sandwich) assay, all polyclonal and monoclonal antibodies were biotinylated. This allows for an easy, universal detection system with the potential for moving from a plate ELISA sandwich assay to a tube based, sandwich radioimmunoassay while utilizing the same immunochemicals. Two mg of each antibody were biotinylated as per manufacturer's protocols (Pierce, Rockford, IL). Monoclonal-monoclonal and monoclonal-polyclonal antibody pairs were assayed for their ability to work together in an assay and to bind to feline proinsulin. Table 5 displays the results from pairing all monoclonals as well as monoclonals with polyclonals in a one step sandwich plate based ELISA.

Table 5. ELISA screening of antibody pairs in search for good sandwich assay candidates. Capture antibodies were coated on the plate at 2.0 μg / well. Twenty ng / well of biotinylated

antibody were used for screening. The concentration of feline proinsulin was 10ng/ml. All values represent absorbance 450 nm with background using only buffer, no hormone, subtracted out. Highlighted pairs were selected for their ability to be used together in an assay as well as the area of proinsulin to which they bind.

Capture Antibody	Screening Antibody										
	8E7	2E4	11E6	4F12	7H3	6G3	R1	R2	R726	R754	R755
8E7	0.000	0.000	2.574	0.000	1.653	0.008	0.061	0.012	0.062	0.139	0.514
2E4	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.004	0.012
11E6	1.738	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.043	0.380
4F12	0.118	0.013	0.031	0.040	0.000	0.011	0.000	0.012	0.004	0.013	0.071
7H3	1.066	0.004	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.025	0.158
6G3	0.034	0.000	0.000	0.000	0.000	0.007	0.021	0.037	0.000	0.066	1.624

The 4 pairs highlighted above were screened again in ELISA assay using dilutions of feline proinsulin ranging from 0.1 to 10 ng/ml. Figure 11 A-D shows the result of these 4 pairings.

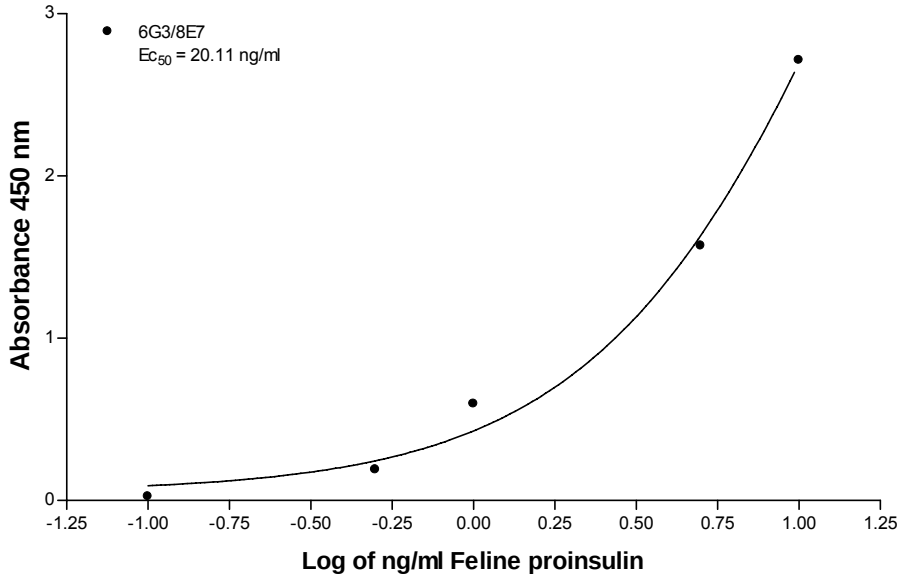

Figure 11A. 6G3 capture antibody, 8E7-Biotin screening antibody, FPI dilutions. Specific data. N=2.

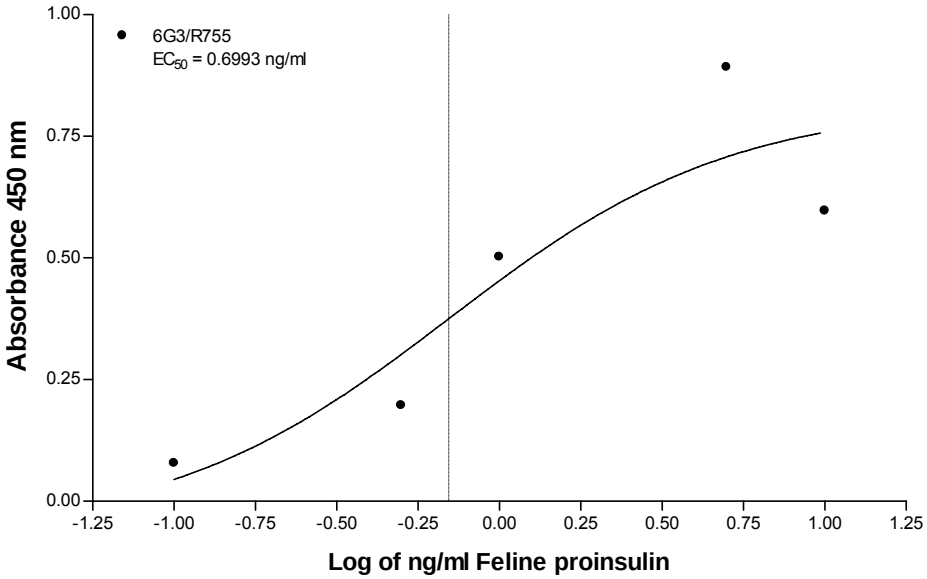

Figure 11B. 6G3 capture antibody, R755-Biotin screening antibody, FPI dilutions. Specific data. N=2.

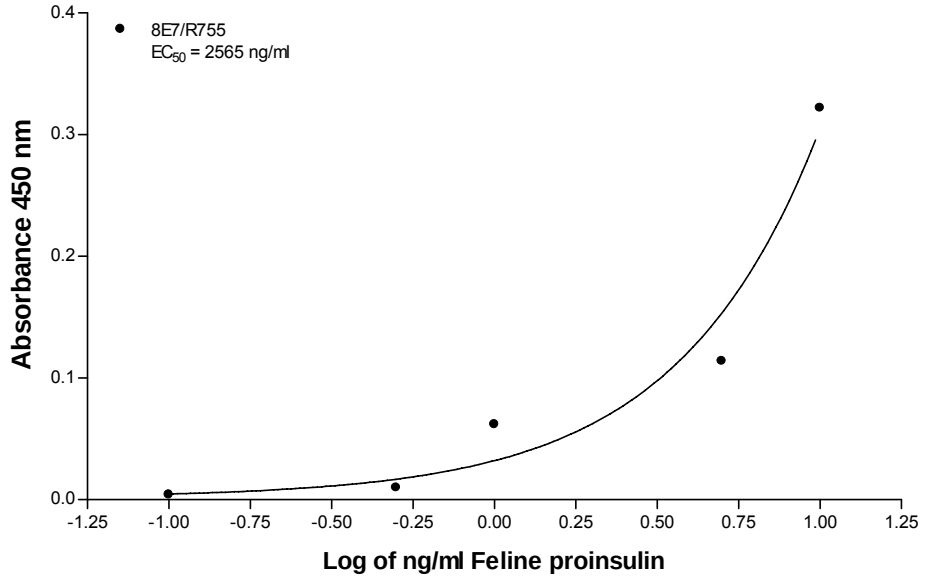

Figure 11C. 8E7 capture antibody, R755-Biotin screening antibody, FPI dilutions. Specific data. N=2.

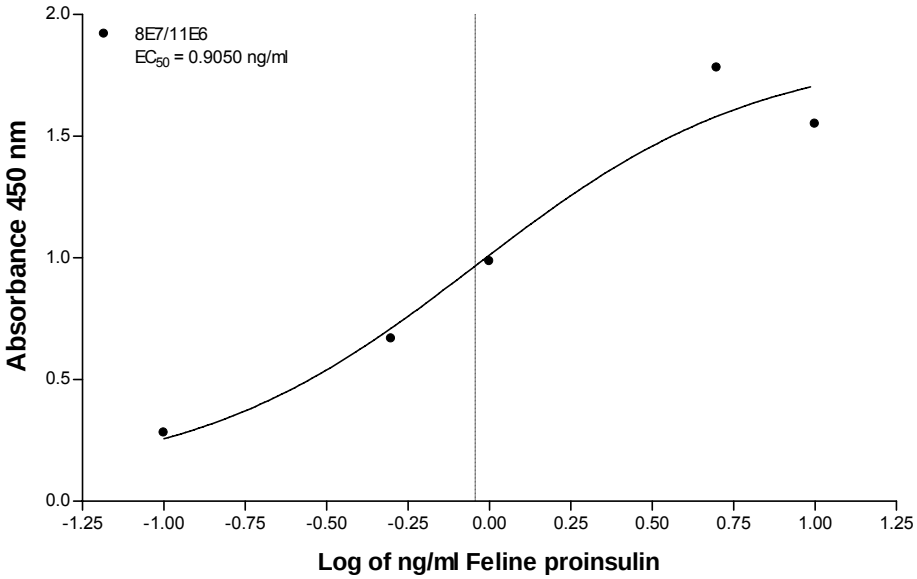

Figure 11D. 8E7 capture antibody, 11E6-Biotin screening antibody, FPI dilutions. N=2.

In an attempt to gain sensitivity, tube based assays were performed using similar pairings as was performed in plate based ELISA's. 6G3 and 8E7 were used as capture antibodies. 8E7 was paired with either 7H3-Biotin or 11E6-bioitin. 6G3 was paired with 8E7-Biotin. Each pairing was screened with 0.02-0.3 ng/ml feline proinsulin. In all three pairs, r2 for linear regression analysis ≥ 0.98 indicating a good fit for that hormone range (Figure 12).

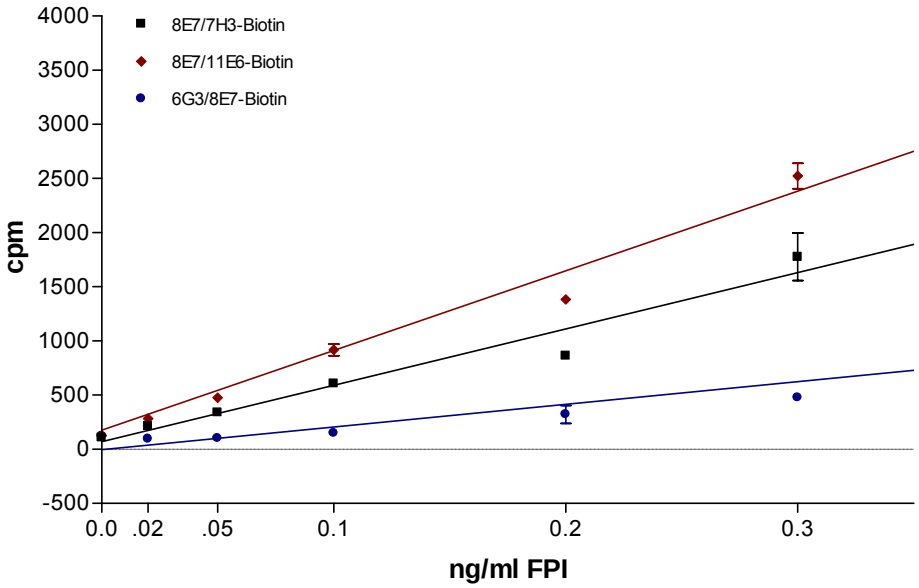

Figure 12. Tube based two-site radioimmunoassay using 125I-streptavidin as tracer. N=1.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In the search for sensitivity and specificity in immune reagents, the epitopes for those immunochemicals need to be as narrowly determined as possible in order to have a greater understanding of the degree of expected cross-reactivity. In this project, all antibody development was screened against several different molecules representing different regions of feline proinsulin. The hope was to find at least two antibodies which recognize intact proinsulin at two sites far enough apart that they can both bind the hormone simultaneously. This has proved to be difficult when working with a highly soluble hormone that is present in most animal species.

Our research yielded one polyclonal antibody generated against feline C-peptide, two polyclonal antibodies generated against the entire feline proinsulin molecule, and two polyclonal antibodies generated against the C-A junction of feline proinsulin. Our research also produced 7 monoclonal antibody lines generated against feline proinsulin. The apparent specificities of these monoclonals are as follows: 2 lines specific for the B-C junction of proinsulin, 3 lines specific for insulin, 1 line specific for intact proinsulin, and 1 line which reacts almost equally with feline C-peptide and intact feline proinsulin.

Our initial goal for a specific assay involved the use of one antibody against the B-C junction and pairing it with and antibody specific for the C-A junction in a two-site immunometric assay. Unfortunately, pairing the R1 and R2 polyclonal antibodies with the 2E4 and 4F12 monoclonal antibodies in a two-site sandwich assay did not prove to be feasible.

However, due to the fact that the R1 and R2 polyclonal antibodies were developed against a small peptide fragment of the feline proinsulin molecule, the tertiary structure of the epitopes recognized by these antibodies may not be accessible in intact feline proinsulin. These two antibodies may prove very useful in screening for proinsulin cleaved between the B-C junction because that cleavage would expose the internal portion of the C-A chain junction which contains the S_{56}-Q_{70} sequence. This possibility becomes very intriguing when you consider that it is generally accepted that the predominant secreted proinsulin molecule is the des-32,33-proinsulin, an intermediate cleavage form.

Our preliminary assays show that several monoclonal and polyclonal antibodies seem to be capable of pairing up for screening of intact proinsulin. Two predominant monoclonals, 6G3 and 8E7, appear to be the most probable for use as capture antibodies due to their low cross-reactivities with insulin. A capture antibody which binds sufficiently to insulin would Interfere with proinsulin measurement by allowing insulin to compete with proinsulin for binding to the capture antibody. However, capturing with either 6G3 or 8E7 would limit interference by insulin competition due to their low cross-reactivities with insulin. However, an anti-insulin antibody could be used for screening if used in conjunction with a capture antibody having little or no cross-reactivity with insulin. Unbound insulin would be washed away during multiple wash steps.

While our preliminary studies show that screening of proinsulin is possible almost to the level predicted in the literature for fasting healthy individuals, much work is still needed to optimize the use of antibody pairs for measurement of proinsulin in serum samples.

Figure 13 shows our estimates for location of epitopes for each antibody.

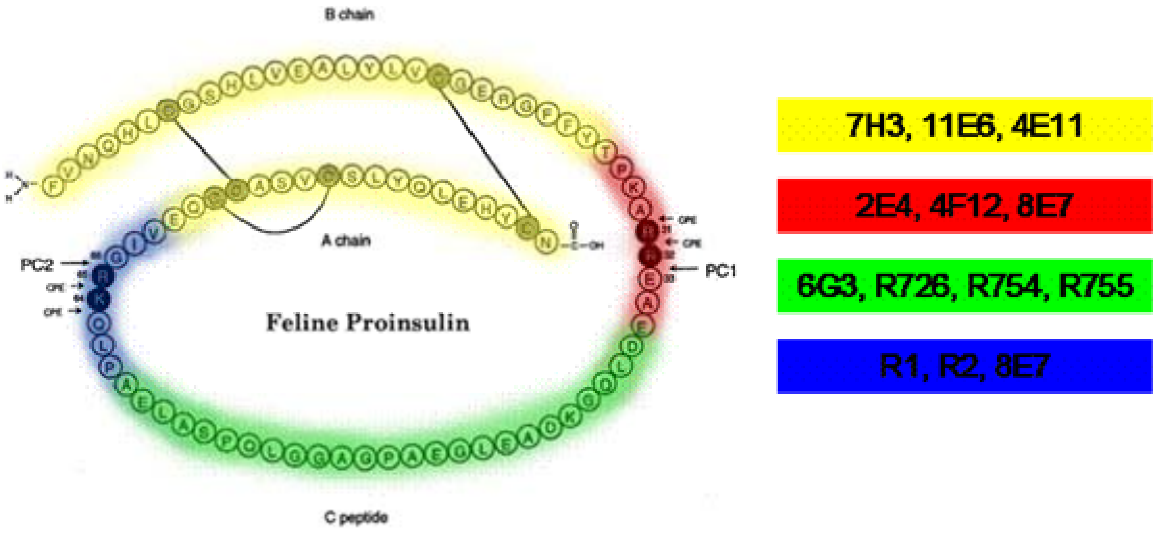

Figure 13. Possible location of epitopes recognized by each antibody.

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