PRECLINICAL DETECTION OF HYPOADRENOCORTICISM IN DOGS

By

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ABSTRACT

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Approximately 70-90% of hypoadrenocorticism cases in human medicine result from immune-mediated adrenalitis. Although clinical signs are not present until ~90% of the adrenal cortex is destroyed, several different anti-adrenal autoantibodies can be identified in serum. In human medicine, presence of these antibodies constitutes the primary criteria for an early diagnosis of adrenalitis. Such a diagnostic tool is not currently available in veterinary medicine. However, from preliminary studies, we hypothesized that as in human hypoadrenocorticism, anti 21-hydroxylase antibody production occurs in canine hypoadrenocorticism. The antibody production precedes clinical disease, and is more prevalent in dog breeds susceptible to developing hypoadrenocorticism. The specific aims of the proposed research are to: 1) determine whether anti-adrenal autoantibodies are present in dogs with hypoadrenocorticism, 2) establish a diagnostic test to detect canine anti-adrenal autoantibodies, and 3) determine whether development of anti-adrenal autoantibodies has breed, sex, and age based predispositions.

The canine 21-hydroxylase was expressed in *E. coli*, using standard techniques. The protein was purified and two rabbits and two dogs were immunized with the purified protein. The obtained positive control sera from these animals were used to establish an enzyme-linked

immunosorbent assay (ELISA) for the detection of 21-hydroxylase autoantibodies in dogs. The preliminary data obtained with this ELISA showed that approximately 30 % of dogs with naturally occurring primary Addison's disease produce antibodies against 21-hydroxylase. What remains unknown is whether autoantibody production precedes clinical disease, implying a role in the pathogenesis of the disease; if it is then the presence of 21-hydroxylase autoantibodies would be expected to be more prevalent in dog breeds susceptible to developing hypoadrenocorticism. Further development of the ELISA will enable epidemiologic studies to address specific aim three.

DEDICATION

To my parents, Erhard and Brigitte Rick,

for their support throughout this lengthy study.

To Dr. Tina Andrea Mueller,

for her dedication to help me succeed.

"Everything should be made as simple as possible, but not simpler."

Albert Einstein (1879-1955)

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone, corticotropin, adrenocorticotrophic hormone
AHDL	Animal Health Diagnostic Laboratory
AIDS	Acquired immune deficiency syndrome
AIRE	Autoimmune suppressor or autoimmune regulator gene
APS	Autoimmune polyendocrine syndrome
BCA	Bicinchoninic acid
BV	Bed-volume
CAPS	3-[cyclohexylamino]-1 propane sulfonic acid
CD4	Cluster of differentiation 4
CHAPS	(3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)
CI	Confidence interval
CO_2	Carbon dioxide
cpm	Counts per minutes
CRH	Corticotropin releasing hormone
DCPAH	Diagnostic Center for Population and Animal Health
DMSO	Dimethyl sulfoxide
DOCA	Desoxycorticosterone acetate
DOCP	Desoxycorticosterone pivalate
DTT	Dithiothreitol

EB	Equilibration buffer
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELB	Elution buffer
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead box P3
FPLC	Fast-protein liquid chromatography
FR	Flowrate
GI	Gastro-intestinal
GST	Glutathione S-transferase
H&E	Hematoxylin and eosin stain
His	Hexa histidine-tag
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HR	High risk dog group
HRP	Horseradish peroxidase
IFN-γ	Interferon-gamma
IgG	Immunoglobulin gamma
IgGFc	Immunoglobulin gamma Fc region
IHC	Immunohistochemistry
iIFA	Indirect immunofluorescence technique

IL-2	Interleukin-2
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
LR	Low risk god group/ negative (healthy) control samples
MALDI	Matrix-assisted laser desorption/ ionization
MES	2-(N -morpholine)-ethanesulfonic acid
MHC	Major histocompatibility complex
MICA	MHC class I chain related A
MSU	Michigan State University
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NSB	Non-specific binding
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline, containing 1 % Tween
PCR	Polymerase chain reaction
PDS	Polydioxanone
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
RT	Room temperature
SD	Sick dog group
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SN	Supernatant
TBS	Tris buffered saline
TCR	T cell receptor
TFA	Trifluoroacetic acid
TgAA	Thyroglobulin autoantibodies
TGF-β	Transforming growth factor beta
Th17	T helper 17
TMB	3,3’,5,5’-tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TSH	Thyroid stimulating hormone
WB	Wash buffer
WB	Western blot

INTRODUCTION

The clinical characteristics of primary hypoadrenocorticism in the dog have been well documented in the veterinary literature[1-6]. However, clinical signs in a dog are nonspecific and can mimic those of other conditions[7]. The clinical signs are caused by deficiencies of glucocorticoids and mineralocorticoids and are very similar in dogs and humans. In detail, the lack of glucocorticoids causes anorexia, vomiting, weakness, abdominal pain, diarrhea, lethargy, fasting hypoglycemia from impaired energy metabolism, and makes it difficult for an individual to handle stress. Mineralocorticoid insufficiency on the other hand might not cause clinical signs, if the electrolyte balance is not disturbed. However, if hyperkalemia and hyponatremia occur, above mentioned clinical signs worsen and cardiac abnormalities occur due to hyperkalemia. Hyponatremia causes hypovolemia, hypotension, and reduces cardiac output. Hypoperfusion exacerbates hyperkalemia and leads to many other clinical features including elevation of liver and kidney enzymes, azotemia, low urine specific gravity, shaking, trembling and muscle weakness.

More than one-third of dogs that are affected with Addison's disease are presented in a life-threatening condition because the disease goes unrecognized until there is an acute adrenal crisis[8,9]. Many dogs die during such an acute crisis. If treatment is possible, emergency treatment in these situations is intensive, expensive and time-consuming[8]. An inexpensive screening tool for preclinical adrenalitis used in predisposed dogs and in animals displaying

vague clinical signs, therefore, has the potential to be both life- and money- saving, especially since maintenance therapy in a dog with hypoadrenocorticism with an early diagnosis is relatively straight forward[8]. In human medicine, the importance of such a diagnostic tool is already recognized because clinical signs and changes in laboratory values are not observed before about 90 % of the adrenal cortex is destroyed[10]. We suspect the same to be true in dogs and expect an anti-adrenal antibody test to be valuable in the detection of early immune-mediated adrenalitis in dogs.

So far, several reports describe characteristic bilateral atrophy of the adrenal cortices with mononuclear cell infiltration and fibrosis of the capsule in dogs with hypoadrenocorticism[11,12]. Anti-adrenal autoantibodies have been isolated from two dogs with naturally occurring hypoadrenocorticism and from two of six beagles that developed hypoadrenocorticism after inhalation of aerosols of plutonium-238 dioxide[11,13]. In a retrospective study, 24 adrenal gland tissue slides obtained during diagnostic necropsies performed at AHDL (Animal Health Diagnostic Laboratory)/ DCPAH (Diagnostic Center for Population and Animal Health) were examined (Rick, Williams, unpublished data). Normal adrenal glands were used as negative controls. The severity of inflammation was subjectively graded as mild (1), moderate (2), or severe (3) by two independent observers depending on the amount of inflammation observed (Rick and Williams). Ten cases were classified as grade 1, eight as grade 2, and five as grade 3. One adrenal gland did not show inflammation. However, in this case the adrenal cortex was completely collapsed and fibrotic. The number of lymphocytes seemed to be inversely correlated to the number of macrophages. It also

appeared that sections with lesser adrenal cortical atrophy contained higher numbers of lymphocytes and lesser macrophages. Sections with more cortical atrophy on the other hand contained more macrophages and fewer lymphocytes. None of the 12 control adrenal glands showed inflammation. Immunohistochemistry identified both T (CD3) (cluster of differentiation) and B (CD79a) cells in the infiltrate, although the majority of cells were T cells.

Before the mid-1950's, the primary causes of hypoadrenocorticism in human beings were tuberculosis or cases were classified as idiopathic[14,15]. Approximately 70-90% of recent hypoadrenocorticism cases in human medicine are known to be the result of immune-mediated adrenalitis[14-19]. Evidence supporting such a pathogenesis in humans includes 1) lymphocytic infiltration of the adrenal cortex[20-22], 2) the presence of circulating autoantibodies reactive to the adrenal cortex and to 21-hydroxylase[18,23-26], 3) an association with immune-response genes in the HLA-DR region[27,28], 4) the frequent occurrence of other putative autoimmune diseases in the same individual or in family members[29-32], 5) the presence of Ia-positive T lymphocytes ("activated" T lymphocytes) at diagnosis[33], and 6) other cell-mediated abnormalities[34]. The measurement of several different anti-adrenal autoantibodies, especially the measurement of 21-hydroxylase autoantibodies, the major antigen in human-immune-mediated hypoadrenocorticism[18,23-26], is used by physicians for the laboratory diagnosis of adrenalitis prior to the onset of clinical hypoadrenocorticism.

Since there are so many similarities between these two species in regards to Addison's disease, we therefore hypothesized that the same is true in dogs: "As in human hypoadrenocorticism, anti-21-hydroxylase antibody production occurs in naturally occurring primary canine hypoadrenocorticism. The antibody production precedes clinical disease, and is more prevalent in dog breeds susceptible to developing hypoadrenocorticism."

Hypoadrenocorticism is relatively common in the dog. The prevalence has been estimated to range from 0.6 to 3.1 per 1000 dogs[35]. Affected dogs may be of all ages, breeds and sexes[8]. Recent studies indicate a higher incidence for young to middle-aged female dogs[8]. Some breeds have been reported to have a 10-50 % higher-than-average risk for hypoadrenocorticism[36]. Reported breeds are Bearded Collie, West Highland White Terrier, Standard Poodle, Portuguese Water Dog, Leonberger, Great Dane, Airedale Terrier, Basset Hound, Wheaten Terrier and Rottweiler. Breeds at lower risk include Boston Terriers, Lhasa Apsos, Yorkshire Terriers and mixed-breed dogs[7,8,37-39]. We therefore believe, that the outcomes of the proposed study will improve the health and welfare of dogs with adrenal disease by improving the understanding of its pathogenesis and the value of early detection. Hypoadrenocorticism is perceived as a significant problem, especially in the purebred dog, with a large genetic component in several breeds, but progress towards understanding and elimination of the disorder has been hampered by lack of diagnostics for the condition before onset of sometimes catastrophic signs. The techniques arising of this study could therefore also assist in breeding programs and could provide a strong basis for future molecular genetic research of the pathogenesis and inheritance of canine hypoadrenocorticism.

In this dissertation, the preliminary research that was done to derive this hypothesis is described. Then, the expression, purification, and evaluation of recombinant canine 21-hydroxylase as an antigen during diagnostic testing are explained in detail. The specific aims of this study are 1) to establish a diagnostic test to detect canine anti-adrenal autoantibodies; 2) to determine whether anti-adrenal autoantibodies are present in dogs with hypoadrenocorticism; 3) to determine whether development of anti-adrenal autoantibodies has breed, sex, and age-based predispositions.

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Chapter I: Literature review

Physiology of the adrenal glands and their hormones

"For a long period I had from time to time met with a very remarkable form of general aneamia, occurring without any discoverable cause whatever. The disease presented in every instance the same general character, pursued a similar course, and, with scarcely a single exception, was followed, after a variable period, by the same fatal result. The appetite is impaired or entirely lost; the whites of the eyes become pearly; the pulse small and feeble. The body wastes, slight pain or uneasiness is from time to time referred to the region of the stomach, and there is occasionally actual vomiting, which in one instance was both urgent and distressing; Neither the most diligent inquiry, nor the most careful physical examination, tends to throw the slightest gleam of light upon the precise nature of the patient's malady; But with a more or less manifestation of the symptoms already enumerated, we discover a most remarkable, and, so far as I know, characteristic discoloration taking place in the skin[1]." This was the description of 10 patients that Thomas Addison, a remarkable physician and scientist at Guy's Hospital in London, recorded in 1855[2]. Even though this original citation is 156 years old and medicine has advanced tremendously, Addison's description of his namesake disease is still accurate. Addison's disease, which is also called adrenal insufficiency, hypocorticolism,

hypocorticism or hypoadrenocorticism, remains a diagnostic challenge in humans and veterinary medicine. If the diagnosis is missed or delayed, it is fatal.

Addison's disease is defined by the following: "The bilateral destruction or dysfunction of the adrenal cortex gives rise to primary adrenocortical insufficiency, or Addison's disease, and the biochemical pattern of this condition is a deficient production of glucocorticoids, mineralocorticoids and androgens, associated with high levels of both ACTH (adrenocorticotropic hormone, corticotropin, adrenocorticotrophic hormone) and plasma renin activity[3]."

Adrenal glands consist of the outer adrenal cortex and the inner adrenal medulla (figure I.1). The medulla synthesizes and secrets catecholamines. The cortex consists of three layers. The outer layer is the zona glomerulosa. This layer synthesizes and secretes mineralocorticoids, which are steroid hormones and also provides precursor cells to the inner layers, the zona fasciculata and the zona reticularis. The zona fasciculata synthesizes and secretes glucocorticoids, which are also steroid hormones. The inner layer, the zona reticularis, synthesizes and secretes androgens, another form of steroid hormones. Cholesterol is a precursor for these hormones and can be rapidly converted into these upon stimulation, therefore storage of steroid hormones is not necessary. The biochemical pathways of the steroidogenesis are summarized in figure I.2. The two most important glucocorticoids and mineralocorticoids are cortisol and aldosterone, respectively. The adrenal cortex has a distinctive and very important exclusive role, much more than the adrenal medulla. If the cortex is absent, there is a total lack of synthesis and secretion of steroids with fatal

consequences whereas when the medulla is absent, catecholamines can still be produced by the autonomic nervous system[4].

Cortisol synthesis and secretion is under the control of ACTH, which is secreted by the anterior pituitary gland. The secretion of ACTH is controlled by CRH (corticotropin releasing hormone) from the hypothalamus. There is a negative feedback inhibition loop in place in which cortisol inhibits the secretion of CRH and ACTH (figure I.3). ACTH secretion follows a circardian rhythm in humans, whereas the secretion in dogs is non-circardian[5-8]. Even though only ACTH can directly stimulate cortisol secretion, stress (pain, mental stress, illnesses, etc.) can stimulate CRH secretion, and therefore can indirectly increase cortisol secretion[6]. Aldosterone on the other hand is controlled by the renin-angiotensin system with angiotensin II being the most important direct stimulator[6]. The goal of the renin-angiotensin system is to maintain the extracellular fluid volume secondary to sodium retention[9]. The renin-angiotensin system is activated by a reduction in the extracellular fluid, by a decrease in blood pressure or when the sodium concentration in the renal filtrate decreases[10]. Further, aldosterone secretion is stimulated by an increase in extracellular potassium, by hyponatremia or by a decrease in extracellular pH. Aldosterone secretion is suppressed by a decrease in extracellular potassium, dopamine, and atrial natriuretic peptide. A rise in blood pressure will also decrease aldosterone secretion. ACTH also has some stimulating effect on aldosterone secretion[6,8,10]. In addition,

cortisol and aldosterone both seem to be affected by cytokines, ion concentrations and many other factors[4].

Cortisol affects almost all tissues and is involved in a variety of processes, including energy metabolism, mineral homeostasis, immune functions and many other cellular functions by gene regulation, all depending on its concentration: It helps to maintain blood pressure, water balance, vascular volume, vascular tone, vascular permeability, endothelial integrity and stimulates erythrocytes. It increases the vascular response to catecholamines, lipolysis and gluconeogenesis and decreases glucose utilization during hunger in order to maintain normoglycemia. It suppresses inflammation, counteracts stress, and it has a catabolic effect on connective tissue, muscle and bone[5,6,8,9,11].

Aldosterone's function is unique to the control of mineral homeostasis[12]. It primarily works in the kidneys, but also, to a lesser extent, in the intestinal mucosa, salivary and sweat glands[6]. It also increases reabsorption of sodium and chloride and excretion of potassium and hydrogen. In the kidneys, it inserts sodium channels in the luminal surface of the cortical collecting duct cells and stimulates the sodium-potassium ATPase pump, which is located on the basolateral side of these cells. It also raises the number of open potassium channels in the luminal membrane[9,10,13]. The water homeostasis is therefore maintained secondary to retention of sodium. Both cortisol and aldosterone are necessary to maintain life. In animal models in which the hypothalamic-pituitary-adrenal axis has been experimentally interrupted, as well as in human and veterinary patients, it has been shown that an interruption of the

hypothalamic-pituitary-adrenal axis most certainly ends in death if appropriate therapy is not initiated immediately[14,15].

Addison's disease in human medicine

In this part of the literature review, I will cover human Addison's disease, including its pathophysiology, symptoms, diagnosis, treatment, and long-term outcome.

Introduction

Addison's disease is a rare, chronic endocrine disorder in which the adrenal glands produce insufficient steroid hormones, as described previously. The prevalence of Addison's disease in people in Western Europe is estimated to be 110-144 cases per million people[16-19]. The incidence is estimated to be 4.4-6 new cases per million population a year[18], whereas in the 1960s the prevalence was only estimated to be between 40 to 70 cases per million people[20,21]. The main cause of hypoadrenocorticism in surveys of human beings in the first half of the last century was tuberculosis, although a few cases were classified as idiopathic[22]. Although tuberculosis is now less common in developed countries, the frequency of hypoadrenocorticism increasing, thus it is reasonable to assume that the proportion of autoimmune adrenal insufficiency has increased[23]. Currently approximately 70 to 90% of cases

of Addison's disease in adult human beings are considered to be the result of immune-mediated adrenalitis[16,24]. However, two other risk groups have been identified recently. AIDS (acquired immune deficiency syndrome) patients are at high risk to develop adrenal insufficiency (up to 20%) and patients with head trauma develop pituitary insufficiency frequently[25-27]. These and causes such as other infections, congenital deficiencies of steroidogenic enzymes, infiltrative diseases, primary and secondary cancers, drugs or surgery-induced and tuberculosis account for the remaining 10 to 30%[16]. The situation is different in pediatric patients in which 72% result from congenital adrenal hyperplasia, 6% result from other genetic diseases and only 13% are the result of an immune-mediated destruction of the adrenal glands; the remainder is of unknown origin[28].

Addison's disease is divided into two groups, primary adrenal insufficiency and secondary adrenal insufficiency. In primary cases, the end result is usually a destruction of the adrenal cortex, resulting in a total lack of glucocorticoids, mineralocorticoids, and adrenal androgens. In some cases, the medulla gets destroyed as well. Secondary cases usually only result in glucocorticoid deficiency, which means that mineralocorticoids remain unaffected. It is therefore unlikely to see patients with secondary hypoadrenocorticism presenting in an acute Addisonian crisis.

Primary causes include autoimmune diseases, as well as adrenal infections and inflammation due to tuberculosis, fungal disease and end-stage AIDS, bilateral adrenal metastasis, bilateral adrenalectomy, adrenal enzyme deficiency, adrenal hemorrhage or

necrosis due to sepsis or coagulation disorders, idiopathic, drug-induced and adrenoleukodystrophy and other congenital disorders. Secondary causes include pituitary or hypothalamic tumors, pituitary irradiation, pituitary surgery, pituitary (brain) trauma, infections or inflammatory/ autoimmune disorders in the pituitary region, pituitary necrosis or bleeding and acute interruption of prolonged pharmacologic glucocorticoid therapy. Patients after adrenal ectomy, with adrenal hemorrhage or necrosis, after pituitary surgery, with pituitary trauma, necrosis or bleeding and after acute interruption of prolonged glucocorticoid therapy show an increased risk for developing signs of an acute adrenal crisis[4].

Recently, another form of adrenal insufficiency has been described. There is recognition of a relative adrenal insufficiency in patients with severe non-adrenal or pituitary disease. These people secrete an inappropriate amount of cortisol to cope with a stressful situation and supplementation with exogenous glucocorticoids can improve the outcome of the disease[12,29,30].

The autoimmune form of Addison's disease, the main focus of this dissertation, is a chronic disease, separated into five stages, called 0 to 4[16]. The only abnormality in stage 0 is the presence of adrenal cortex and/ or 21-hydroxylase antibodies; ACTH, cortisol, plasma renin activity and aldosterone levels remain normal. Patients with Addison's disease stage 1 show an increased plasma renin activity. Stage 2 includes the same abnormalities, but the cortisol response to ACTH stimulation is decreased. As the capacity for cortisol is further decreased, stage 3 is characterized by addition of a compensatory increase of ACTH production. It appears

that the zona glomerulosa, the outer layer of the adrenal glands that produces aldosterone, might be more susceptible to the autoimmune attack and therefore may get injured first, hence the increase in renin activity to counteract the decrease in aldosterone secretion in stage 1 alone. The zona fasciculata might be protected longer due to the local production of cortisone, followed by the progressive decrease in cortisol in stages 2 through 4. Very low cortisol levels, a very high ACTH concentration and clinical signs of the disease define stage 4. Stage 0 is a potential Addison's disease status, 1 to 3 are considered to be subclinical Addison's disease and stage 4 represents clinical Addison's[31-35]. The disease has a very long asymptomatic phase in which the only abnormality is the presence of circulating adrenal cortex antibodies, that recognize steroidogenic enzymes. It was shown in 1983 that the presence of this autoantibody has a predictive value for disease development, when 4 out of 9 antibody positive patients developed clinical Addison's disease within 1 to 31 months of follow-up[36]. However, not all autoantibody positive patients develop Addison's disease and a mathematical risk stratification model for the development of Addison's disease has been developed. The model predicts the risk of developing Addison's disease as low, medium, and high, based on five important variables: patient's age, autoantibody titers, adrenal function status at time of detection of adrenal autoantibodies, patient gender and types of other autoimmune diseases present[37].

Autoimmune Addison's disease occurs in conjunction with other autoimmune diseases as part of an APS (autoimmune polyendocrine syndrome). Neufeld and Blizzard classified four types of APSs[38,39]. APS type 1 consists of chronic candidiasis, chronic hypoparathyroidism and

Addison's disease; APS type 2 consists of Addison's disease, thyroid autoimmune diseases and/ or type 1 diabetes mellitus; APS type 3, the only one that does not contain Addison's disease consists of thyroid autoimmune diseases and other autoimmune diseases without Addison's disease; and APS type 4 consists of a combination of two or more autoimmune diseases that do not fall into any of the other three categories (table I.1). There is also the form of Addison's disease as a single immune-mediated endocrine deficiency.

APS type 1 is the early onset form (mean age in the Betterle study was 14 years[16]). APS type 1 is caused by a mutation in the AIRE (autoimmune suppressor or autoimmune regulator) gene, which is located on chromosome 21q22.3. Sixty mutations within AIRE have been discovered so far that can cause APS type 1. Eighty % of people with these mutations develop Addison's disease and are at increased risk for mucocutaneous candidiasis, hypoparathyroidism and type 1 diabetes mellitus[40-43].

If Addison's disease develops in adults, it is either related to APS type 2 or occurs in isolation[40]. APS type 2 is a polygenic syndrome that is related to a polymorphism of the HLA (human leukocyte antigen) system, with genotypes HLA-DR3-DQ2 and/or DR4-DQ8 haplotypes shown to increase the risk to develop Addison's disease. Further, a reduction of DR1 and DR13 might also increase the risk for the disease[44-46]. There also seems to be an association between Addison's disease and a mutation in allele 5.1 of a MICA (MHC (major histocompatibility complex) class I chain related A) gene. This gene is independent of the DR or DQ gene polymorphism[47]. Besides developing Addison's disease, patients with APS type 2 may also

develop hypothyroidism, type 1 diabetes and many other autoimmune diseases like pernicious anemia, vitiligo, alopecia, celiac disease[48] and gonadal insufficiencies[47]. Autoimmune Addison's disease connected to APS type 4 is extremely rare and not a lot is known about it[16]. The influence of environmental factors on disease development is not well understood. Stressful situations certainly can trigger an Addisonian crisis if an autoimmune process has damaged adrenal glands.

Betterle, a researcher from Italy who's main interest is Addison's disease, looked at 501 human patients in 2001 and published the following epidemiologic data (figure I.4): the mean age of the 501 patients dealing with autoimmune Addison's disease was 31 years, females were almost twice as often affected than males (1.7 to 1) and adults were five times more commonly affected than children. APS type 2 was most common (301 cases, representing 62 % of the diseased population). Patients with APS type 2 were on average 35 years old, the female to male ratio was 2.3 to 1, and adults were 15 times more likely to be affected than children. The second most common form of autoimmune hypoadrenocorticism was isolated Addison's disease (90 cases, 18 %). The mean age of this mostly adult group (ratio 4 adults to 1 child) was 32 years and males were more likely affected than females (female to male ratio 0.7 to 1). APS type 1 was represented by 65 cases (13 %). The mean age of this group was 14 years, with a child to adult ratio of 16 to 1. The female to male ratio was 1.9 to 1. APS type 4 was the least common (36 cases, 7.2 %) form. Children were 8 times more likely to be affected than adults were, and the female to male ratio was 0.9 to 1[16]. Of the 82 % of patients with autoimmune Addison's disease that also had one or more subclinical, clinical or latent autoimmune disease,

62 % had thyroid autoimmune disease, 40 % had gastric autoimmunity, 39 % had premature ovarian failure, 24 % had type I diabetes mellitus, 11 % had chronic hypoparathyroidism, 10 % had chronic candidiasis, 8 % vitiligo, 5 % had either celiac disease or alopecia, 4 % had autoimmune hepatitis, 3 % had cancer and 1 % had intestinal malabsorption or Sjögren's syndrome[16]. The findings of a nationwide Norwegian study were similar. In this population, autoimmune Addison's disease was accompanied by another clinical autoimmune disease in 61 % of patients. When subclinical cases were included, this percentage rose to 88 %. Of the 61 % of patients with autoimmune Addison's disease in the Norwegian population, 47 % had concurrent autoimmune thyroid disease, 12 % had diabetes mellitus type 1, 11 % had vitiligo, 10 % had pernicious anemia, and 4 % had alopecia[49].

Pathophysiology

Over time, it has become more and more clear that most cases of human Addison's disease are caused by autoimmune destruction of the adrenal glands. Adrenal cortex antibodies were first identified in 1957[50]. For the next 30 years, antibodies directed against a microsomal autoantigen located in the cytoplasm of the zona glomerulosa, zona fasciculata and zona reticularis were identified via indirect immunofluorescence technique in 51 % to 61 % of patients with autoimmune Addison's disease and in close to 90 % of patients with newly diagnosed (as opposed to patients on long-term treatment) autoimmune Addison's disease. Two % of patients with Addison's disease due to tuberculosis were antibody positive[51-53].

Then, in 1992, three groups independently described that the adrenal cortex antibody recognizes a major enzyme of the steroid-pathway, 21-hydroxylase[54-56]. In order to test for antibodies, an immunoprecipitation assay with ^{35}S-methionine labeled recombinant 21-hydroxylase was developed in 1995, and another using recombinant human 21-hydroxylase produced in yeast and labeled with ^{125}I was described in 1997[57]. With these assays, it was discovered that 78 % of patients with autoimmune Addison's disease tested positive for autoantibody, including more than 90% of newly diagnosed patients. Positivity was also found in 1.9 % of the general population[53]. In addition to 21-hydroxylase antibodies, 17-hydroxylase antibodies and cholesterol side chain-cleaving enzyme antibodies can be identified in serum. 21-hydroxylase antibodies are by far the most common, while cholesterol side chain-cleaving antibodies are less commonly found. 17-hydroxylase antibodies are the least commonly found antibodies[58].

Addison's disease has been successfully induced in mice and rats by injecting adrenal cortex extracts. These animals showed clinical signs of adrenal insufficiency and circulating antibodies against adrenal tissue were demonstrated. Upon autopsy, the adrenal cortices were infiltrated with mononuclear cells[59,60]. Non-obese diabetic mice that spontaneously develop diabetes mellitus type 1 may also develop mononuclear cell infiltration in the adrenal glands, but these animals do not show signs of adrenal insufficiency[61]. Dogs and cats can develop autoimmune adrenal insufficiency (experimentally induced or naturally occurring); however, it

has been proven difficult and unreliable to identify the circulating adrenal antibodies in these species[62-71].

Adrenal glands are small in human patients with Addison's disease. The cortex is markedly atrophied with various amounts of fibrosis and infiltrated with lymphocytes, plasma cells and macrophages, sometimes located in germinal centers. Lymphocytes are usually T cells and the CD4 (cluster of differentiation 4) to CD8 ratio is 5 or 6 to 1. Only 5 % are B cells, of which half are positive for class II HLA. If there are adrenocortical cells left, they are usually hyperplastic. However, the adrenal medulla remains unchanged[72]. It is not clear how the adrenal cortex gets destroyed in patients with autoimmune Addison's disease, but it appears that antibodies do not mediate the disease[73] for the following reasons: The antigens involved are all located intracellularly and localized in the endoplasmic reticulum. Since plasma membranes are not permeable for immunoglobulins, destruction of the antigens can only take place after the destruction of the tissue[58]. Even though antibodies against 21-hydroxylase can block the enzymatic activity of this enzyme in vitro[74], a passive transfer from mother to fetus of 21-hydroxylase antibodies does not cause disease in the baby[75] and the presence of ACTH receptor-blocking antibodies could not be verified[76,77]. Antibodies might activate the cytolytic complement cascade though, which ultimately would lead to the lysis of the cell or might initiate antibody-dependent cell-mediated immunity, which would lead to phagocytosis[58] and complement fixing antibodies have been identified in a number of patients[31,78,79]. On the

other hand, patients with autoimmune Addison's disease have mononuclear cells that can be stimulated by 21-hydroxylase to secrete IFN-γ (interferon-gamma) and IL-2 (interleukin-2). In vitro, it appeared that especially amino acids 342 to 361 of the 21-hydroxylase enzyme were able to stimulate these mononuclear cells. This area of the 21-hydroxylase may comprise a disease epitope, represented by cells carrying genotype HLA-DRB1*0404[80]. Patients also have circulating Ia-positive T cells[79], a proliferative T cell response to an adrenal-specific protein[81] and a defect in the suppressor of the CD4+/CD25+ regulatory T cell. The latter has only been identified in patients with APS type 2[82]. These findings made it plausible to believe that the disease is caused by cell-mediated immunity, but further studies are needed[16].

Symptoms of adrenal insufficiency

Symptoms of Addison's disease in humans are non-specific and likely to not appear until after a long subclinical phase. Since Addison's disease in humans is mostly of immune mediated etiology, most patients have measurable circulating adrenal cortex and/ or 21-hydroxylase antibodies at time of diagnosis[16]. Patients with autoimmune adrenal disease go through a long preclinical phase of disease in which antibodies are present, but clinical signs are not. Most of these patients also have other concurrent autoimmune diseases as mentioned above. Clinical signs then usually appear when at least 90 % of the glands are destroyed, or earlier if an extremely stressful event occurs[45]. Symptoms and signs of chronic primary and secondary

adrenal insufficiency include fatigue, weakness, lethargy, loss of energy, anorexia, weight loss, postural hypotension, nausea, vomiting, myalgia, general malaise, diffuse abdominal pain/ gastric pain, joint pain, dizziness, salt craving and in women loss of libido and loss of axillary and pubic hair (due to pronounced androgen deficiency)[4,16]. Cutaneous and mucosal hyperpigmentation can result from an excess of opiomelanocortin and melanocyte stimulating hormone. Also, ACTH is similar in structure to melanocyte stimulating hormone, causing it to react with its receptor[83]. Sodium (in all patients), chloride and bicarbonate concentrations are low at time of diagnosis and potassium concentrations are elevated (in 50 to 70 %). Ten to 20 % of patients show hypercalcemia[3] and many show fasting hypoglycemia. The combination of the two should make the physician highly suspicious of adrenal insufficiency[4]. Even though it is not understood why, 40 to 50 % of patients are anemic, with 10 to 15 % having eosinophilia and lymphocytosis[16]. The acute patient shows the same biochemical abnormalities, but presents with hypotension or hypotensive shock due to mineralocorticoid deficiencies and the resulting hyponatremia and plasma volume depletion. The circulation is further decreased by an elevation of prostaglandine E and a decreased responsiveness to norepinephrine and angiotensin II. On top of that, whatever caused the acute adrenal crisis, for example sepsis, surgery or trauma, might mask it[4]. Because symptoms are very nonspecific, patients are commonly misdiagnosed with other conditions with potentially serious consequences[84,85].

Diagnosis of adrenal insufficiency

The most useful diagnostic tests for Addison's disease in humans are blood tests. Cortisol in circulation is bound to cortisol binding globulin (also called transcortin), and its level can be decreased due to chronic liver or kidney disease or because of an exposure to estrogens. Measured low total cortisol concentrations therefore should not be used to confirm a diagnosis of hypoadrenocorticism. Rather free cortisol levels should be measured or calculated based on total cortisol and transcortin measurement[86,87]. To diagnose adrenal insufficiency, it is necessary to differentiate between screening tests and confirmatory tests. As a screening test, early morning basal cortisol/ free serum cortisol should be measured. Levels greater than 20 µg/ dL in a patient not in an acute stress situation rule out a differential diagnosis of adrenal insufficiency. If transcortin concentrations are normal, then the normal morning cortisol level ranges between 10 to 20 µg/ dL. Levels less than 10 µg/ dL necessitate more workup and levels less than 3 µg/ dL make adrenal insufficiency very likely. Furthermore, plasma basal ACTH in the normal range or below 100 pg/ mL rule out a differential diagnosis of primary hypoadrenocorticism. Normal to low renin and normal aldosterone further make adrenal insufficiency unlikely. However, elevated renin concentrations are one of the earliest changes seen with primary adrenal insufficiency. If the screening test (measurement of early morning basal cortisol/ free serum cortisol) result is equivocal and a patient needs more workup, a stimulation test should be performed. A stimulation test should also be performed if patients are positive for adrenal antibodies but do not show clinical signs yet. The gold standard simulation test is to take a baseline blood sample for cortisol and aldosterone measurement

from the patient followed by an administration of 1 or 250 µg of corticotropin. Cortisol and aldosterone should be measured again 30 and 60 minutes later. Cortisol concentrations of greater than 20 µg/ dL at any time rule out a differential diagnosis of primary adrenal insufficiency, but do not rule out the presence of secondary adrenal insufficiency. Other screening tests include stimulation of the pituitary-adrenal axis by insulin-induced hypoglycemia or stimulation with corticotropin-releasing hormone. The first test is especially useful if there is a suspicion of pathology in the hypothalamic-pituitary axis. A dose of 0.1 U of regular insulin is administered intravenously and basal, 30, 60 and 90 minute levels of cortisol, corticotropin and sometimes of growth hormone in cases of suspected multiple pituitary hormone deficiencies are measured. A normal cortisol response would exceed 20 µg/ dL. The latter test is helpful to differentiate between hypothalamic and pituitary origin of adrenal insufficiency[4].

Different tests should be performed during acute illness in order to initiate therapy immediately. The physician should be suspicious of adrenal insufficiency in patients that show unexplained hypotension and that are at high risk to develop adrenal insufficiency. This group includes patients with AIDS, patients that just discontinued glucocorticoid therapy, patients with autoimmune diseases, patients with hyperpigmentation, patients that are chronically tired or patients that show acute clinical signs of an adrenal insufficiency as mentioned above[4].

Immediate tests that should be performed include simple diagnostic tests that give immediate results and that are rapidly available, including measurement of sodium, potassium, bicarbonate and cortisol. A plasma sample for measurement of corticotropin, renin and

aldosterone should be obtained. A short corticotropin stimulation test, in which a post corticotropin sample for the measurement of cortisol 30 min post injection is taken, should also be performed. Once samples are obtained and tests are performed, therapeutic intervention should be started until results are available[4]. Untreated cortisol deficiency could also lead to a modest elevation in thyroid stimulating hormone in 30 % of patients, because cortisol usually inhibits TSH (thyroid stimulating hormone) secretion[16,88]. If adrenal insufficiency can be confirmed, it is recommended to identify the etiology of the disease in order to address the underlying issue, screen for other autoimmune diseases or make sure that relatives are followed up with and monitored appropriately.

Imaging, including ultrasound and x-rays, only appear to be important if a patient does not have adrenal autoantibodies[89]. Enlargement of the adrenal glands with and without calcification can occur with tuberculosis, cancer, infections and infiltrative or vascular diseases[3].

Treatment of adrenal insufficiency

For treatment purposes, it is necessary to differentiate between the acute phase, and the chronic, maintenance phase of hypoadrenocorticism. The acute phase is life threatening and therefore depends primarily on the patient's clinical condition, but treatment should be initiated immediately after diagnostic samples have been taken. Treatment in the acute phase includes electrolytes, fluid and hydrocortisone at a dose of 150-300 mg/ d. The hydrocortisone

is given either intravenously or intramuscularly until the patient is stable[4]. At such a high dose, which is also called 'physiologic stress dosage', hydrocortisone activates the mineralocorticoid receptor directly and additional mineralocorticoid supplementation is therefore not necessary. In physiologic concentrations, cortisol does not activate the mineralocorticoid receptor, even though it has the same affinity to it as aldosterone has. This is related to the sudden intrarenal inactivation of cortisol to cortisone via 11-β-steroid dehydrogenase in mineralocorticoid-responsive tissues. Total inactivation only occurs when physiologic concentrations of cortisol are present[4]. Once the patient is stable, the dosage can be gradually decreased. During acute phase, a lower hydrocortisone dosage could yield same results, but no clinical studies have been conducted to address this question[4]. Further, the mineralocorticoid effect of the high dosage of hydrocortisone is desirable.

Maintenance replacement therapy of glucocorticoids and mineralocorticoids is given orally. The goal is to give the lowest dosage of hydrocortisone that is necessary to avoid symptoms of adrenal insufficiency and to mimic physiologic cortisol production. Good follow-up hormone tests, like they exist with thyroxine replacement therapy for hypothyroidism, are not defined for treatment of hypoadrenocorticism with glucocorticoids. The evaluation of the clinical response therefore is extremely important[90,91]. However, some authors believe that measurement of 24-hour free cortisone in urine (urinary free cortisol) is a way to determine adequacy of cortisone replacement therapy[92,93]. Daily cortisol production is estimated to be at 8 mg/ m^2/ d, measured via isotope dilution methodology[4]. Cortisol secretion follows a diurnal

rhythm in humans. Over time, cortisol replacement therapy has been decreased from 30 mg/ d of hydrocortisone or even more to 15 to 25 mg/ d of which 15 to 10 mg are given after waking up and 5 to 10 mg are given in the afternoon or early evening to mimic the diurnal rhythm. Twenty five to 50 mg of cortisone acetate per day divided in two or three doses can also be used. This dosage is still slightly above the physiologic concentration but is probably needed to offset the inactivation of cortisol into cortisone after oral intake. Patient and family education is extremely important, as is that the patient carries a medical emergency alert card with him at all times. Even though replacement therapy has been gradually decreased over time from 30 mg or more of hydrocortisone plus fludrocortisone per day, patients seldom suffer from an acute adrenal crisis, partly because they are taught to increase the dosage by 50 to 300 % during acute disease or extreme stressful situations, including but not limited to illness, injury, vomiting, surgical procedures, pregnancies, etc.[4,90]. To maintain electrolyte homeostasis, 50 to 200 µg fludrocortisone per day is given[47]. A diet rich in sodium might be beneficial as well[16]. In theory, excess salt is especially needed during summer, because the sweat of a person suffering from adrenal insufficiency contains more salt as compared to a healthy individual. Mineralocorticoid dosage can be easily monitored by measurement of blood pressure, plasma renin activity, and electrolyte levels[94]. Women with adrenal insufficiency might show additional benefit from replacement with dehydroepiandrosterone to compensate for the inability to produce androgens[4].

Long-term outcome of adrenal insufficiency

One might think that people with Addison's disease, once diagnosed, have a normal life expectancy. However, even when patients are diagnosed and treated correctly, retrospective studies have shown that the standardized mortality ratios of both women and men with primary adrenal failure are more than 2-fold increased, compared to the general population. A recent study estimates these standardized mortality rates at 2.9, 95 % CI (confidence interval) 2.7-3.0 and 2.5, 95 % CI 2.3-2.7, respectively. The risk seems to be especially high when patients are diagnosed during childhood. Also, patients with APS 1 have a higher standardized mortality rate compared to patients with APS 2, 4.6, 95% CI 3.5-6.0 and 2.1, 95% CI 1.9-2.4[95,96]. The exact reasons for that are not fully understood.

Addison's disease in dogs

In this part of the literature review, I will cover Addison's disease in dogs, including its pathophysiology, symptoms, diagnosis, treatment, and long-term outcome.

Introduction

Addison's disease is a chronic endocrine disorder in which the adrenal glands produce insufficient steroid hormones, as described previously. Hypoadrenocorticism is not uncommon in the dog. The average small animal veterinarian sees 1400 to 1600 dogs/ year and combined with the estimated incidence rate, a full-time veterinarian would be expected to diagnose 0.17 to 0.53 dogs with hypoadrenocorticism per year. The average two veterinarian practice in which each veterinarian sees around 1500 dogs/ year should therefore expect to diagnose one case per year[97].

Several breeds have been reported to have a 10-50 % higher-than-average risk for hypoadrenocorticism. These breeds include the Bearded Collie, West Highland White Terrier, Standard Poodle (and other Poodles), Portuguese Water Dog, Leonberger, Great Dane, Airedale Terrier, Basset Hound, Wheaten Terrier, Rottweiler, Springer Spaniel and Nova Scotia Duck Tolling Retriever. Breeds at lower risk include Boston Terrier, Lhasa Apso, Yorkshire Terrier and mixed-breed dogs[5,97-102]. Excluding the studies of Kelch and Peterson, no one takes into account the breed distribution in the overall population and studies might therefore be

misleading. Kelch points out however that "results are remarkably consistent in that in no case was a breed found to be at increased risk in one dataset and at decreased risk in another[103]." These two studies find that Great Danes, Poodles (see table I.2 for more details on Poodles) and West Highland White Terriers are at increased risk[99,103]. Portuguese Water Dogs, Rottweilers, and Soft Coated Wheaten Terriers have been reported by Peterson to be at increased risk, which has not been confirmed by Kelch[99]. A decreased risk among Golden Retrievers, Lhasa Apsos and Yorkshire Terriers has been reported by Peterson[99]. Kelch confirms the decreased risk in the latter two, but cannot confirm it for the Golden Retriever[103]. Kelch's and Peterson's findings are summarized in tables I.2 and I.3. In certain breeds that are at higher risk to develop primary hypoadrenocorticism, the condition is considered to be heritable with a possible familial predisposition in the Portuguese Water Dog, Leonberger, Standard Poodle, Bearded Collie and Nova Scotia Duck Tolling Retriever[5,102,104]. An autosomal recessive mode of inheritance has been suggested in the Nova Scotia Duck Tolling Retriever, the Standard Poodle and, most likely, in the Portuguese Water Dog. This mode of inheritance has not been confirmed for the Bearded Collie[100-102,105].

The duration of the disease, from time of diagnosis to death, related or unrelated to Addison's disease, is approximately 4.9 years with adequate medical replacement therapy. In a steady-state, the prevalence equals the incidence times duration of disease (P=I*D). In his dissertation from 1996, Kelch analyzed three different groups of dogs with hypoadrenocorticism and reported the following: the first group consists of 244 cases that were

found in the veterinary literature; the second group (n=266) of dogs enrolled in a clinical study to evaluate the microcrystalline drug desoxycorticosterone pivalate; and the third group consists of 376 cases obtained from colleges of veterinary medicine and other veterinary referral hospitals. The prevalence estimates in these three groups are 0.6/ 1000 dogs, 1.7/ 1000 dogs and 0.32/ 1000 dogs, respectively, with a point estimate of 1.8/ 1000 dogs[103].

Different studies in veterinary medicine throughout the world reveal similar results concerning incidence, prevalence, age of onset, breed and gender distribution[97,103,106-110]. However, the most current study was done in France between 1997 and 2001 and was presented during the meeting of the European Society of Veterinary Internal Medicine in Spain in 2004[109]. It revealed a lower prevalence of 0.003/ per 1000 dogs than previously reported[109], but the diagnosis was based on low cortisol and aldosterone concentrations post administration of ACTH, whereas other studies only required low plasma cortisol concentrations post ACTH administration[109]. The study also excluded dogs of unknown status and dogs that weren't followed-up three years post diagnosis, leading to a decrease in total number of cases and therefore a smaller prevalence. I therefore believe that this study is misleading rather than helpful.

The most useful epidemiologic data in dogs is extractable from the Kelch and Peterson study[99,103]. As mentioned above, most studies do not take into account the prevalence of a breed distribution in the overall population and are therefore misleading. Kelch and Peterson however calculated odds ratios from the number of cases in a particular breed compared with

the number of that breed seen overall[97,99,103]. Peterson's reference population consists of n=3512 dogs that were examined at the Animal Medical Center in New York City between January 1979 and January 1993 that did not have hypoadrenocorticism. Included dogs were randomly selected from all examined dogs, and each individual dog was only included once[99]. Kelch's control group consisted of patients from 1000 randomly selected veterinarians from the American Veterinary Medical Association's list of small animal practitioners. A questionnaire was sent to these veterinarians asking for data on dogs diagnosed with hypoadrenocorticism and for controls which were defined as the next and the fifth next dog in the veterinarian's database that were owned by a different owner. The response rate was 56 %; 9 % were excluded. The number of active dog patients per veterinarian was also reported. Data (age of onset, female to male ratio, mean body weight at onset) from different studies are summarized in table I.4. The average age of diagnosis of the summarized studies was 4.8 years, with the average in these studies ranging from 4.0 to 6.0 years. The average body weight at onset was 20.5 kg (range: 10.2 to 28.5 kg) with a female to male ratio of 65 to 35.

The point estimate for age in the three different groups defined by Kelch is 4.9 years (range 5 weeks to 15 years)[103]. Other studies obtained similar results (range 4 weeks to 16 years with an average age of onset of 4 to 5 years), reinforcing that the disease can be diagnosed in very old dogs or in very young dogs. The average reported age of onset for the Nova Scotia Duck Tolling Retriever is only 2.6 years[8,97-102]. Kelch calculated odds ratios that show that age is associated with the occurrence of the disease. The probability increases with age and peaks in the age group 4 to 7 years (1.9, $p<.0001$)[103] (table I.5).

All studies approximate the risk for females developing hypoadrenocorticism compared to males as close to 2 to 1 (except Bearded Collies, Portuguese Water Dogs and Standard Poodles in which males are as much affected as females are) and results are remarkably similar, even though most of the studies do not take into account the sex distribution in the entire dog population[5,97,99-103,105-107,110-112]. Peterson does consider the sex distribution and concludes that females are more likely to develop disease than males[99]. Sex-specific incidence estimates from Kelch's data sets (veterinary referral hospitals, mail survey from Ciba Animal Health veterinarians, and from randomly selected veterinarians) conclude that ovariohysterectomized females are more likely to be affected than intact females, that castrated males are more likely to be affected than intact males, and that neutered dogs in general are more likely to develop hypoadrenocorticism than intact ones (table I.6)[97,103].

This data contradicts Peterson's study, which reports that intact females had an increased probability of disease compared to ovariohysterectomized females. Peterson calculated odds ratios of developing hypoadrenocorticism for gender. Relative to sexually intact males which had the lowest risk, intact females had the highest risk (5.55, $p<0.001$) (table I.7)[99]. The association between spaying and hypoadrenocorticism could be influenced by the age at which dogs are ovariohysterectomized, which can explain the different outcome. Dogs are usually spayed at a very young age.

Pathophysiology

It has been reported that up to 10 % of dogs with Addison's disease might present with 'atypical' primary Addison's disease, in which serum electrolytes (and sometimes aldosterone levels) are normal at diagnosis[5,111]. However, it is suspected that these dogs develop electrolyte abnormalities later on during progression of disease and a discrepancy of specialists opinions remains on whether this condition should be named or treated differently than the classic form of Addison's disease.

Naturally occurring primary Addison's disease, with a lack of both glucocorticoids and mineralocorticoids results from destruction or atrophy of all layers of the adrenal cortex. The primary cause for this destruction is hypothesized to be immune-mediated, and bilateral adrenocortical atrophy with mononuclear infiltrates has been described[71,113]. Also, when assessed, a small proportion of dogs showed anti-adrenal antibodies[71,114]. Studies however were difficult to replicate. Other, mostly rare causes in the dog for primary Addison's disease are infiltrative diseases including fungal infections (Histoplasma, Blastomyces, Coccidioides, Cryptococcus), neoplastic diseases, amyloidosis, trauma, coagulopathy, and iatrogenic due to mitotane (Lysodren, o-p'-DDD; Bristol-Myers-Squibb, Princeton, New Jersey, USA) and trilostane (Vetoryl, Dechra Veterinary Products, Overland Park, Kansas, USA) use[5,7,111].

Iatrogenic cases of primary hypoadrenocorticism are relatively common and result from oversuppression of the adrenal glands during treatment of hyperadrenocorticism (Cushing's syndrome). Whereas most humans with Addison's disease have other autoimmune diseases,

autoimmune polyglandular syndrome in the dog is uncommon. Out of 187 dogs that were diagnosed with primary hypoadrenocorticism, only 28 (15%) had at least one other confirmed autoimmune disease[5]. Other autoimmune diseases present were hypothyroidism in 16 dogs, insulin-dependent diabetes mellitus in 14 dogs, hypoparathyroidism in three dogs and two had azoospermia[5].

Naturally occurring secondary hypoadrenocorticism is related to insufficient ACTH secretion because of a pituitary lesion, such as neoplasia, trauma or inflammation[5,111], resulting in atrophy of the zonae fasciculata and reticularis. Aldosterone secretion and serum electrolytes are maintained[5,6]; clinical signs therefore result from a lack of cortisol. This condition is sometimes very difficult to diagnose since blood work and other tests can remain normal. The exact incidence of this condition is not known, but it is rare- estimates range from 4 % to 24 %[5,111] of all dogs with hypoadrenocorticism.

Iatrogenic secondary hypoadrenocorticism is the most common form, and is caused by a sudden withdrawal of chronic use of injectable, oral, ophthalmic, otic or topical exogenous steroids[5]. The use of exogenous steroids suppresses ACTH secretion, which in turn causes the zona reticularis and zona fasciculata to atrophy. Hypoadrenocorticism then occurs when exogenous steroids are withdrawn too quickly.

Tertiary hypoadrenocorticism is caused by a lack or decrease of CRH secretion, which in turn causes a decrease in ACTH secretion. The clinical manifestation is then the same as in secondary hypoadrenocorticism, which results from a lack of ACTH[5].

Symptoms of adrenal insufficiency

For the purpose of this research and review, I will from now on focus on dogs with the classic form of primary Addison's disease.

The clinical characteristics of primary hypoadrenocorticism in the dog have been well documented in the veterinary literature[5,99,104,108,110,115-120]. Clinical signs that are observed and reported by the owner are caused by deficiencies of glucocorticoids and mineralocorticoids and are very similar in dogs and humans. The lack of glucocorticoids causes gastrointestinal signs including anorexia (88 % to 95 %), vomiting and regurgitation (68 % to 75 %), diarrhea (35 %), and abdominal pain (8 %); renal signs including polyuria and polydipsia (17 % to 25 %); neurologic signs including shaking, shivering and tremors (17 % to 27 %), and collapse (10 %); and non-specific systemic signs including lethargy and depression (85 % to 95 %), weakness (51 % to 75 %), and weight loss (40 % to 50 %). Also noted can be fasting hypoglycemia from impaired energy metabolism, hematemesis, hematochezia, melena, ataxia, seizures, difficulty breathing, hair loss, muscle cramps in front and back limbs, prior response to nonspecific fluid- and glucocorticoid therapy and statements that a dog has a hard time handling stressful situations. Clinical signs are non-specific, and mimic these of gastrointestinal disease, renal

failure or neurological disease, which makes finding a diagnosis difficult. Clinical signs may also only appear episodically or so weakly that they remain unnoticed in 25 % to 43 % of cases[5,99]. It might therefore take up to 52 weeks before a dog is diagnosed correctly[5,99,111]. Some dogs become sick acutely and very severely[5]. Such an acute phase can be triggered by stress, including boarding, traveling, grooming or changes in routine or habitat[5,11].

Mineralocorticoid insufficiency might not cause clinical signs if the electrolyte balance is not disturbed. However, if hyperkalemia and hyponatremia occur, above mentioned clinical signs worsen and cardiac abnormalities occur due to hyperkalemia. Hyperkalemia causes decreased myocardial excitability, slowed conduction and finally cardiac arrest[5,8,9,13]. Hyponatremia causes hypovolemia and hypotension as well as reduces cardiac output. Hypoperfusion exacerbates hyperkalemia and leads to many other clinical features including elevation of liver and kidney enzymes, azotemia, low urine specific gravity, shaking, trembling and muscle weakness.

Diagnosis of adrenal insufficiency

Diagnosing hypoadrenocorticism in dogs can be difficult, because its clinical signs are nonspecific and can mimic those of other conditions[99]. Upon physical examination by a trained veterinarian, dogs present with lethargy and depression (87 %), a thin physique (82 %), weakness (66 % to 69 %), dehydration (42 %), shock and collapse (24 % to 29 %), hypothermia

(15 % to 34 %), bradycardia (22 % to 25 %), weak pulse (22 %), melena and hematochezia (17 %), and abdominal pain (7 %)[5,11,99,104,119,120]. More than one-third of affected dogs are presented in a life-threatening condition because the disease goes unrecognized until there is an acute adrenal crisis[97,107]. Emergency treatment in these situations is intensive, expensive and time-consuming[97]. Other diagnostic procedures that should be performed include laboratory tests, diagnostic imaging and an electrocardiogram[5].

Up to 95 % of dogs with primary hypoadrenocorticism are hyperkalemic, up to 86 % are hyponatremic, 40 % are hypochloremic and up to 95 % show a decreased sodium to potassium ratio of less than 27 to 1[5,111,119]. These changes can happen isolated or together, are not pathognomonic for the disease, but the clinician should be suspicious of primary Addison's disease if present. If changes are absent however, primary hypoadrenocorticism still remains a possibility. Sometimes the sodium to potassium ratio drops below 15 to 1, which should increase the suspicion of hypoadrenocorticism. Hyperkalemia might lead to cardiac arrest, first decreasing the myocardial excitability and slowing conduction and then, if more severe, finally causing the heart to stop[5,8,9,13]. Differential diagnoses for these electrolyte changes include gastrointestinal disease (vomiting, diarrhea, duodenal perforation, gastric torsion), urinary tract disease (renal failure, post-obstructive diuresis), liver failure, parasitic infection (pseudohypoadrenocorticism due to whipworms), congestive heart failure, chylothorax, metabolic acidosis (diabetic ketoacidosis, respiratory acidosis) or pregnancy. Electrolyte

changes could also be a laboratory artifact related to suboptimal sample quality (lipemia, hemolysis, thrombocytosis and/or leukocytosis)[5,10,121].

Sixty-six % to 95 % of dogs with primary hypoadrenocorticism have pre-renal azotemia, caused by a decrease in renal perfusion and glomerular filtration rate[5,8,9,104,111,119,120]. Even though pre-renal azotemia usually causes an increase in urinary specific gravity, 60 % to 88 % of dogs with primary Addison's disease show a decrease in urinary specific gravity, most likely caused by chronic urinary sodium loss, which consequently leads to renal medullary washout, loss of the normal medullary concentration gradient and hence a dilute urine[5,8,9,104,111,119]. This decrease and the most likely presence of elevation of BUN and creatinine due to gastrointestinal bleeding, renal hypoperfusion or hypovolemia, usually respond well to fluid therapy (if adequate), whereas dogs with renal failure do not. Sixty-six % to 95 % are hyperphosphatemic, most likely a result from the decreased glomerular filtration rate and decreased renal excretion of phosphorus[119,120].

Approximately 50 % of dogs develop metabolic acidosis, because of the inability of the kidneys to excrete hydrogen ions without the presence of aldosterone. Hypovolemia, hypotension and hypoperfusion further exacerbate the metabolic acidosis, which ranges from mild to severe (in less than 10 %)[5,6,8,9,111,120]. The metabolic acidosis causes potassium to move from the intracellular space into the extracellular space in exchange for hydrogen ions. This then causes the hyperkalemia to worsen[5,8,9,13].

Elevation of total calcium occurs in up to 30 % of dogs with primary hypoadrenocorticism, even though ionized calcium might remain within the normal range. Most authors hypothesize that many factors contribute to the development of hypercalcemia, including hemoconcentration, decreased glomerular filtration rate, decreased calcium secretion, development of hyperkalemia and dehydration. Hypercalcemia in an Addisonian dog usually does not cause clinical signs and resolves quickly with adequate rehydration and glucocorticoid replacement therapy. Special therapy to treat hypercalcemia is not required[5,104,119,120]. Differential diagnoses for hypercalcemia include neoplasia, primary hyperparathyroidism, vitamin D toxicity and granulomatous disease processes[5,104,119].

Hypoglycemia develops in 22 % of dogs with primary hypoadrenocorticism and is related to the lack of glucocorticoid-mediated gluconeogenesis and mobilization of glycogen stores. Also, cortisol antagonizes the action of insulin, and the decreased cortisol concentration further intensifies hypoglycemia. Clinical signs caused by hypoglycemia however remain very uncommon. Differential diagnoses causing hypoglycemia include sepsis, liver disease, starvation or maldigestion, hunting dog hypoglycemia, insulinomas, or excessive use of exogenous insulin. Improper samples or sample handling can also cause an artificially decreased glucose concentration[5,104,111,120].

Seventeen % to 39 % of affected dogs develop moderate to severe hypoalbuminemia. Hypoalbuminemia in dogs with hypoadrenocorticism is caused by anorexia, gastrointestinal loss

and decreased synthesis. Differential diagnoses include liver disease, gastrointestinal disease or kidney disease[5,104,119].

Alanine aminotransferase and aspartate aminotransferase are mildly to moderately elevated in 30 % to 50 % of patients. It is not yet understood what causes this elevation, but contributing factors include poor cardiac output, hypotension, poor perfusion and concurrent immune-mediated hepatopathy[5,104,111,119,120]. Eighteen % of dogs with primary hypoadrenocorticism present with hypocholesterolemia[5].

The lack of glucocorticoids causes bone-marrow suppression, which may result in mild anemia. Gastrointestinal bleeding can exacerbate the anemia, and mild to moderate normocytic, normochromic, nonregenerative anemia is present in 21 % to 25 % of patients. The anemia might be masked by hemoconcentration[5,111,119,122].

The white blood cell count might range from low-normal to mildly increased, related to concurrent infection. Ten % to 20 % of dogs with primary hypoadrenocorticism are eosinophilic, and absolute lymphocytosis exists in 10 % to 13 % of patients[5,104,111,119,120].

The use of diagnostic imaging or measurement of blood pressure in dogs with primary hypoadrenocorticism is of questionable merit for routine diagnosis. Hypotension most likely is present in the dog with hypoadrenocorticism, but there is insufficient data in the literature[5]. Diagnostic imaging is usually included in the work-up of a critically ill patient and might reveal changes that are related to hypovolemia, including microcardia, reduced vena cava size,

pulmonary hypoperfusion, and microhepatica. Megaesophagus has been reported as a concurrent disease in dogs with hypoadrenocorticism[5,99,104,111,119]. A recent study compared adrenal gland size measured via ultrasound. Enrolled were 14 healthy dogs and 28 dogs with hypoadrenocorticism. Adrenal glands are significantly smaller in dogs with Addison's disease that also show mineralocorticoid insufficiency, compared to healthy dogs. The difference in the comparison of healthy dogs with dogs with hypoadrenocorticism without mineralocorticoid insufficiency approaches significance[123].

Changes seen on electrocardiogram (ECG) are related to hyperkalemia, and, even though not completely correlated to rising potassium levels, get more significant when levels increase. Performing an ECG enables the clinician to initiate immediate and potentially lifesaving therapy. Continuous monitoring helps assessing the response to therapy. Changes seen on an ECG result in changes in cell membrane excitability and depress the heart's conduction system due to hyperkalemia, and might also be affected by hyponatremia and metabolic acidosis[124,125]. Bradyarrhythmias, tachyarrhythmias, conduction disturbances, sinoventricular rhythm, ventricular fibrillation, and asystoles can be identified in 46 % of dogs with primary Addison's disease[124]. In a different study, 47 % of dogs showed atrial standstill, 29% bradycardia, 6% atrial or ventricular extrasystoles, and 5 % a 2^{nd} or 3^{rd} degree heart block[99].

It is not sufficient to measure baseline cortisol levels alone to diagnose Addison's disease, as dogs with non-adrenal illness sometimes have very low baseline cortisol concentrations, which could result in a misdiagnosis. The negative predictive value is high though, in cases where the baseline cortisol concentration is ≥ 55.2 nmol/ L in dogs that have not received exogenous steroids, mitotane, ketoconazole or trilostane, and the dog is unlikely to have hypoadrenocorticism[126]. Baseline cortisol measurement alone has 100 % sensitivity and 98 % specificity for hypoadrenocorticism if the level is ≤ 27.6 nmol/ L and 100 % sensitivity but only 78 % specificity if the level is ≤ 55.2 nmol/ L[126]. An ACTH stimulation test, should always be performed when baseline cortisol concentrations are between 27.6 nmol/ L and 55.2 nmol/ L or in case the baseline cortisol concentration is ≥ 55.2nmol/ L, but the primary differential diagnosis is hypoadrenocorticism[126].

Since all of the above-mentioned laboratory abnormalities, and most of the clinical signs, are non-specific or not precise enough, the gold standard test, the ACTH stimulation test, has to be performed whenever primary hypoadrenocorticism is suspected in order to confirm or to rule out the diagnosis[5]. The ACTH stimulation test is easy to perform, safe, rapid, reliable, and is usually easy to interpret as long as prior administration of chronic exogenous steroids can be excluded (suppression of pituitary-adrenal axis) as well as prior administration of prednisone, prednisolone or hydrocortisone, which cross-react in immunoassays[5,104,127]. The principle of the ACTH stimulation test is as follows: synthetic ACTH, which contains the active portion of the hormone (amino acids 1 to 24), or a gel preparation from porcine pituitary

extract which contains the 39- amino acid full-length hormone, are administered after a baseline blood sample has been taken[128]. A one-hour post stimulation sample (synthetic ACTH) or a two-hour post stimulation sample (ACTH gel) is then obtained. The administered ACTH, like the endogenous ACTH from the pituitary gland, stimulates the zona fasciculata and zona reticularis to produce cortisol, and the zona glomerulosa to produce aldosterone. The stimulation test therefore assesses the ability of the adrenal cortices to respond to maximum stimulation and therefore gives information on how much responsive adrenal cortex is present. Cortisol is measured in both samples. The additional measurement of aldosterone might help to differentiate between primary, secondary and tertiary hypoadrenocorticism, but might not yield additional information in a dog with primary hypoadrenocorticism and the assay is not readily available. If a dog with primary Addison's disease does not have any functional adrenal cortex, no stimulation of either cortisol or aldosterone post-ACTH administration can be seen. A typical dog with primary hypoadrenocorticism would have pre and post cortisol results of ≤55.2 nmol/ L (normal range pre-ACTH 13.8 to 137.9 nmol/ L, post ACTH 151.8 to 469 nmol/ L)[5,111,126,128,129].

To perform an ACTH stimulation test, either synthetic ACTH or ACTH gel are recommended and yield very similar results. It is recommend to inject 2.2U/ kg ACTH gel intramuscularly, with the post sample taken 2 hours post injection[5,128]. ACTH gel needs to be kept refrigerated at all times. The use of compounded ACTH gel is not recommended because of concerns with consistent bioavailability[128,130]. There are two protocols available on how

much synthetic ACTH is necessary to cause maximum stimulation. The classic test uses one vial of synthetic ACTH (cosyntropin, Cortrosyn; Amphastar Pharmaceuticals, Rancho Cucamonga, California, USA) (250 µg), given intravenously or intramuscularly with the post stimulation sample taken one hour later. If an animal is severely dehydrated, absorption of intramuscularly given ACTH might be prolonged, and rather the intravenous route should be used instead[99,119,131]. The alternative protocol recommends the use of 5 µg/ kg given intravenously, with a maximum dose of 250 µg[5,131-134]. A prospective crossover study comparing the two dosages in dogs with clinical signs of Addison's disease did not reveal statistically significant differences between the two dosages[133]. The clinician should therefore be encouraged to use the lower dosage since synthetic ACTH is extremely expensive and sometimes hard to come by. An open vial of synthetic ACTH remains effective when refrigerated for 21 days or up to six months when kept at -20°C in a non self-defrosting freezer[128,135,136].

Treatment of adrenal insufficiency

As in human medicine, treating a dog with acute primary Addison's disease is different from treating a dog with chronic adrenal insufficiency.

The acute patient is usually presented in an emergency, with severe dehydration, hypovolemia, hypotension, electrolyte abnormalities including hyperkalemia and hypoglycemia,

and metabolic acidosis. Counteracting these changes immediately on top of sufficient corticosteroid replacement therapy is important[5,111,137].

An animal in an Addisonian crisis requires aggressive intravenous fluid replacement therapy to correct the hypovolemia, which, if uncorrected, is the main cause of death in an Addisonian crisis. It has been shown that intraperitoneal or subcutaneous fluid therapy is not adequate in such a situation. A larger-gauge, short catheter in a peripheral vein can be placed quickly and allows for infusion of larger volumes than a jugular catheter. After placing the catheter, if necessary via venous cut-down, a baseline blood sample should be collected for diagnostic purposes to rule in or out the diagnosis, before starting fluid therapy at a shock-rate of 60 to 90 mL/ kg/ h for one to two hours with 0.9 % sodium chloride solution. Sodium chloride solution has the benefit of replacing the sodium loss, reducing the hyperkalemia and bettering the metabolic acidosis without providing more potassium. If necessary, lactated Ringer's or Normosol-R can be used, as using these fluids is still far better than not giving fluids at all[5,111,137]. By giving fluids, hyperkalemia usually improves because of hemodilution, increased renal perfusion, and in turn increased urine output, and by shifting potassium from the extracellular space to the intracellular space as metabolic acidosis gets better[5,137]. If life-threatening bradyarrhythmia is present, additional therapy is indicated to counteract hyperkalemia immediately. 0.2 Units/ kg of regular insulin, given intravenously, rapidly lowers serum potassium levels by driving potassium into the cells. The effect however only lasts for up to 30 minutes. Dextrose should be given at the same time and blood glucose levels have to be monitored carefully- ideally every 30 to 60 minutes- to avoid hypoglycemia. Another option to

decrease hyperkalemia is to use bicarbonate. Bicarbonate does not take effect for an hour after administration, but lasts for several hours. 1 to 2 mEq/kg is given slowly intravenously. Hydrogen leaves the cells and potassium is thereby driven into the cells[111,137]. Bicarbonate can also be used to correct metabolic acidosis, especially if the acidosis does not resolve with fluid therapy alone. The goal when using bicarbonate to counteract and correct acidosis is not to completely correct the acidosis, but rather to increase pH to 7.2 and the HCO_3 to 12 mmol/ L[137]. The formula 0.3 * body weight (kg) * (24-patient's HCO_3) can be used to estimate the bicarbonate deficit[137]. Calcium gluconate also decreases hyperkalemia: 0.5 to 1 mL/ kg or 2 to 10 mL/ dog of a 10 % calcium gluconate solution given as a slow infusion over 15 minutes antagonizes the effects of hyperkalemia on cell membranes, therefore allowing normal action potentials. The effect is very sudden, but also does not last for more than 30 minutes. Further, it is recommended to monitor the ECG while giving the calcium gluconate infusion. If bradycardia worsens, S-T segment elevates, or Q-T shortens, the infusion should be stopped immediately. Clinicians usually prefer the use of insulin or calcium gluconate if fluid therapy is not enough to lower potassium concentrations. While giving these, fluid therapy has to be continued[136,137].

Dextrose should be used to treat hypoglycemia. Either a bolus of 0.5 mL to 1 mL of 50% dextrose (given diluted to prevent phlebitis) if clinical signs of hypoglycemia exist or 2.5 % to 5 % added to the fluids if no clinical signs of hypoglycemia exist, should be given[137].

The second most important treatment besides fluid therapy includes glucocorticoid supplementation. Recommended products are 0.5 to 4 mg/ kg of rapid acting dexamethasone sodium phosphate given intravenously, 15 to 20 mg/ kg of prednisolone sodium succinate (over 3 minutes) given intravenously, or 5 mg/ kg bolus (over 5 minutes, followed by 1 mg/ kg every 6 hours) or 0.3mg/ kg/ h intravenously given hydrocortisone sodium succinate. Glucocorticoids should be given repeatedly every 2 to 6 hours, which approximates 3 to 10 times the physiological requirements. Prednisolone and hydrocortisone interfere with cortisol-immuno-assays via cross-reaction with the assay antibodies. ACTH stimulation tests should therefore be completed prior to administration or dexamethasone has to be used, which does not cross-react in the cortisol assays. Hydrocortisone also has some mineralocorticoid activity. The goal is to improve vascular and GI (gastro-intestinal) integrity and to help improve blood pressure and circulation volume[137].

Supplementation with mineralocorticoids during an acute crisis is of lesser benefit[5,104,111,119,120]. The two mineralocorticoid products currently on the market are the long acting, injectable desoxycorticosterone pivalate and the short acting oral fludrocortisone. In the acutely vomiting patient, oral medication might not get absorbed properly. Once DOCP is injected, it lasts approximately 25 days. However, DOCP is a very safe drug and appears to not have any side effects, even if administered to a completely healthy animal. Each individual clinician should therefore decide on his or her own on whether to administer it during acute crisis or not[5,119,136,138,139].

Fluid therapy and glucocorticoid therapy are most important in the treatment of an acute Addisonian crisis. Other symptoms that should be addressed (treatment suggestions in parentheses), depending on severity, include anemia due to ulceration (transfusion), GI ulceration (GI protectants including sucralfate, H-2 blockers, proton-pump inhibitors, broad spectrum antibiotics in case of sepsis), and vomiting (antiemetics- prochlorperazine is contraindicated though)[5]. These treatment suggestions are guidelines and need to be adjusted depending on treatment response and results during hourly monitoring and they should be continued as long as necessary. Once hyperkalemia reaches the non-life-threatening level of < 6.5 mmol/L, electrolyte and venous gas do not have to be rechecked hourly, but rather every 6 to 8 hours. Care should also be taken that sodium concentrations do not rise more than 12 mmol/L per day, as a faster rise could lead to pontine myelinolysis, which is a non-inflammatory neurological disorder with clinical signs including lethargy, weakness, dysphagia, trismus, and ataxia progressing to hypermetria and spastic quadriparesis. This syndrome and its signs are rare in veterinary medicine, but if present, they could take months to resolve[137,138,140-142]. Most patients are completely stable within 24 to 48 hours and should be weaned off emergency treatment, switched over to chronic treatment as described in the following paragraph and monitored for reoccurrence of clinical signs prior to discharge[129].

A dog with primary Addison's disease needs to be supplemented with glucocorticoids and mineralocorticoids for the rest of its life. Oral prednisone should be initially given at a physiologic dosage of 0.22 mg/ kg twice daily and then tapered down to the lowest dose necessary to maintain well-being of the patient without seeing unwanted side effects such as

polyuria, polydipsia, panting, and polyphagia. Just as a human patient would be instructed to do, the owner of a dog with hypoadrenocorticism should be instructed to increase the dose by 2 to 10 times the physiologic dose whenever a stressful situation occurs or is foreseen. The dose should only be adjusted every 6 weeks to see how a patient is doing on a lower dose before attempting to decrease it further[5].

For mineralocorticoid supplementation, there are currently two choices available, DOCP and fludrocortisone acetate, as mentioned above. DOCP is a long-acting preparation. The short acting DOCA (desoxycorticosterone acetate) was developed in the 1940's, but is no longer available. DOCP is the long acting ester of DOCA and DOCP has no glucocorticoid activity. For veterinary use, it is formulated in a microcrystalline suspension, which has to be given intramuscularly and lasts for 25 days. The initial dose is 2.2 mg/ kg. It starts working within 1 h post injection. It is a very safe drug and very well tolerated. However, DOCP is not inexpensive, therefore there are many prospective studies on what is the most economical dosage to use and what might be more cost-effective, for example giving a higher than recommended dose and then going for a longer interval or the other way around. Clinicians at this point remain uncertain. The manufacturer recommended protocol is as follows: injecting 2.2 mg/ kg intramuscularly on day 0, then recheck (electrolytes and physical examination) at day 12 and day 25. The peak effect of DOCP is at day 12. If hyperkalemia and/ or hyponatremia exist at recheck day 12, the DOCP dose should be increased by 5 to 10 %, or decreased by 5 to 10 % in case of hypokalemia and/ or hypernatremia. The frequency of injection should be shortened by one day or prolonged by one day if these changes occur during recheck at day 25[5,119,136]. The

second mineralocorticoid choice for managing a dog with primary hypoadrenocorticism is oral fludrocortisone acetate, which is a synthetic glucocorticoid with predominantly mineralocorticoid activity. The starting dose is 0.01 mg/ kg given twice daily; however, this dose usually needs to be increased within the first year of treatment. Treatment goal is to maintain normal electrolyte concentrations. Since fludrocortisone acetate does exert glucocorticoid activity, up to 50 % of dogs do not require additional prednisone supplementation. However, the fludrocortisone dose that is necessary to maintain normal electrolyte concentrations is often times so high that many dogs start showing signs of hypercortisolism. Finding the right dosage with this drug is therefore very difficult, and it should be recommended to switch these patients to DOCP. Even though a diet rich in salt might be beneficial for humans, the feeding of table salt to these dogs remains controversial[5,119].

In my opinion, it is necessary to teach the owner of a dog diagnosed with primary hypoadrenocorticism about the disease, and that certain clinical signs have a huge impact on how to adjust the glucocorticoid and mineralocorticoid dose. In brief, vomiting, diarrhea, lethargy, or inappetence suggest that the glucocorticoid dose needs to be increased. Polyuria and polydipsia suggests that the glucocorticoid dose needs to be decreased. However, the dosage of mineralocorticoids should not be changed without the evaluation of serum electrolytes.

Long-term outcome of adrenal insufficiency

Kintzer reports that more than 80 % of dogs have a good to excellent response to therapy and a fair response to treatment is seen in 13 %[106]. Median survival time of these dogs was 4.7 years[106]. Kelch estimates a comparable 4.9 years as average disease duration[103]. There appears to be no difference in survival time between dogs treated with fludrocortisone versus DOCP. Of the dogs that died during the study, 97% died of reasons other than hypoadrenocorticism[103].

Addison's disease in human and veterinary medicine

Overall, it is apparent that there are many similarities between hypoadrenocorticism in dogs and humans. The major clinical signs and laboratory changes of hypoadrenocorticism in dogs and humans are similar. Females are more affected than males (average of all cited studies: dogs: 64 % to 36 %; humans 53 % to 47 %), and in both species it is a disease of the younger to middle aged individual (dogs: 4.7 years (range 0.1 to 15 years); humans: 36 years (range 5 to 79 years)).

Incidences and prevalences are higher in dogs, which might be related to the smaller genetic pool in dogs and degree of inbreeding (dogs: 13 to 60/ 100,000 dogs/ year incidence,

600 to 3200/ 1,000,000 dogs prevalence; humans: 0.5 to 0.83/ 100,000 people/ year incidence, 5 to 140/ 1,000,000 people prevalence).

The main difference, the unknown etiology of Addison's disease in dogs, versus immune-mediated in humans, is the basis for the work in this dissertation. The autoimmune etiology of primary Addison's disease in dogs has not been well defined, and up to date, most cases are still classified as idiopathic.

APPENDIX

Appendix

Figure I.1: Schematic showing the cellular zonation of the adrenal cortex and blood flow through the cortex to the collecting veins in the medulla (retrieved on 07/02/2011 from http://www.hakeem-sy.com/main/files/adrenal.jpg). (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation).

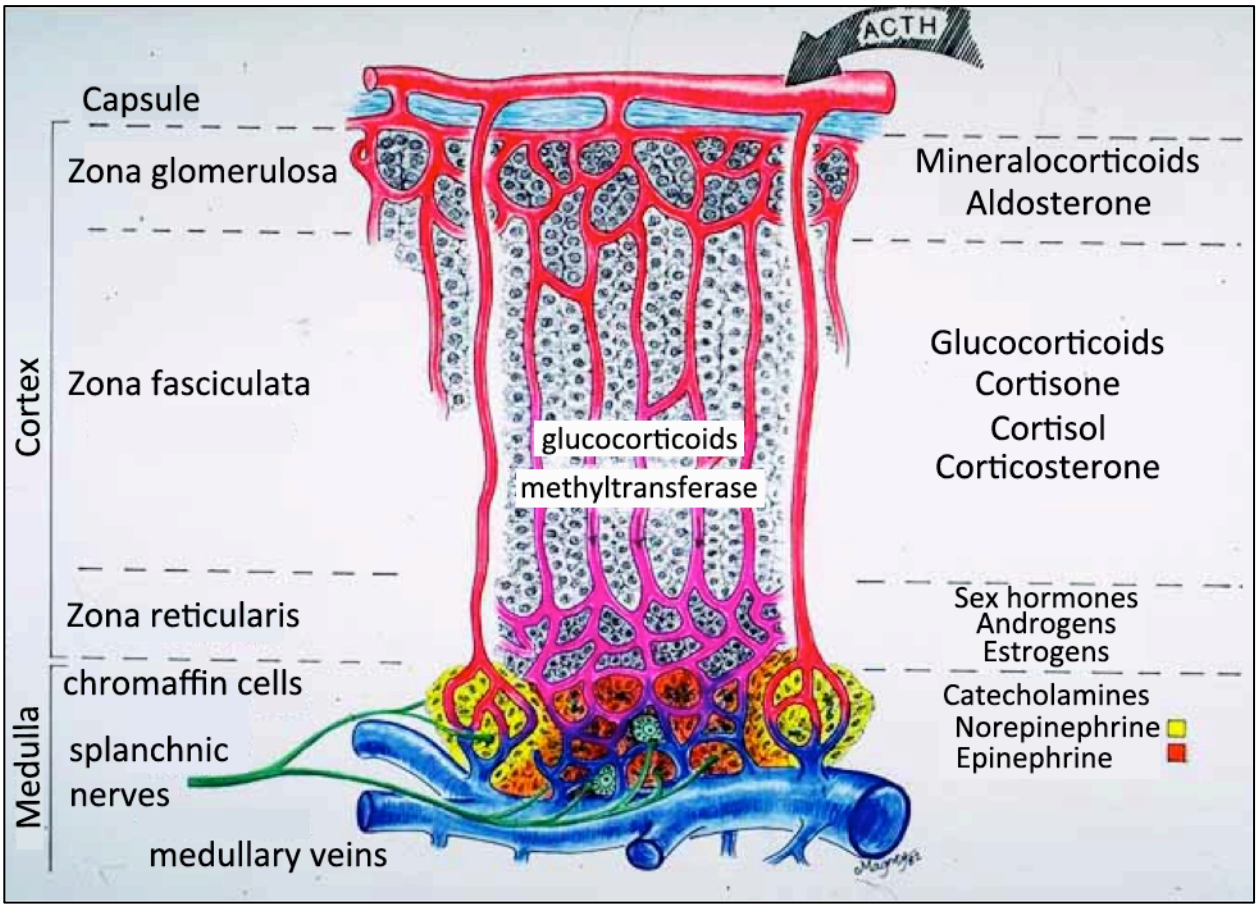

Figure I.2: Steroidogenesis (from: Annual Rev Physiology (2001) 63:193); Cholesterol is either obtained from the diet or synthesized from acetate by a CoA reductase enzyme. The adrenal cortex (also ovaries and testes) use cholesterol to produce a range of steroid hormones, including aldosterone, cortisol and testosterone/estradiol.

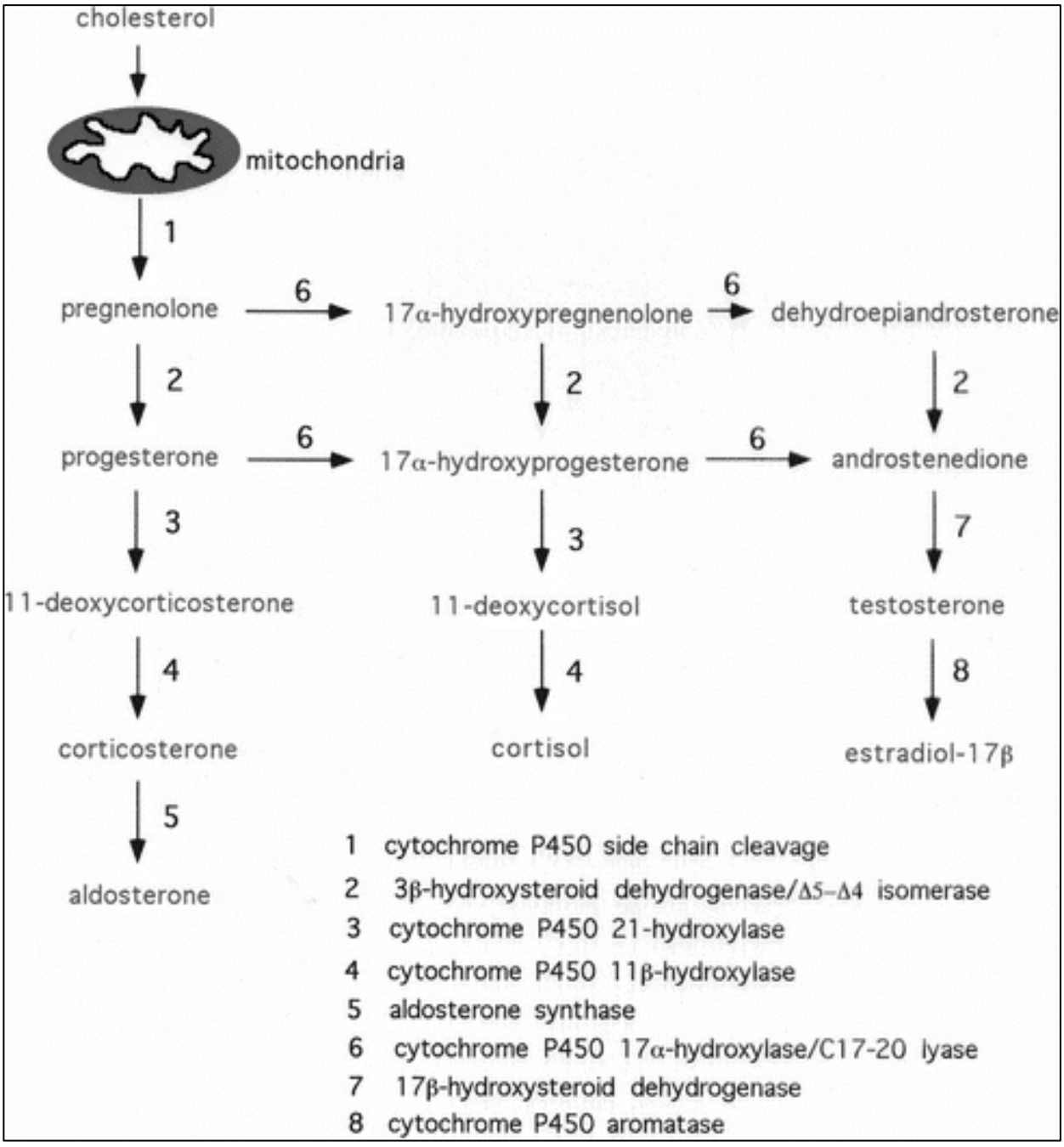

Figure I.3: The hypothalamic-pituitary-adrenal axis (from: Addison's Disease in the Dog, Catherine Scott-Moncrieff, 05/15/2011, retrieved from: http://www.vetgrad.co.uk/show10MinuteTopUp.php?type=&Entity=10MinuteTopUps&Entity=10MinuteTopUps&ID=51 on 09/09/2011).

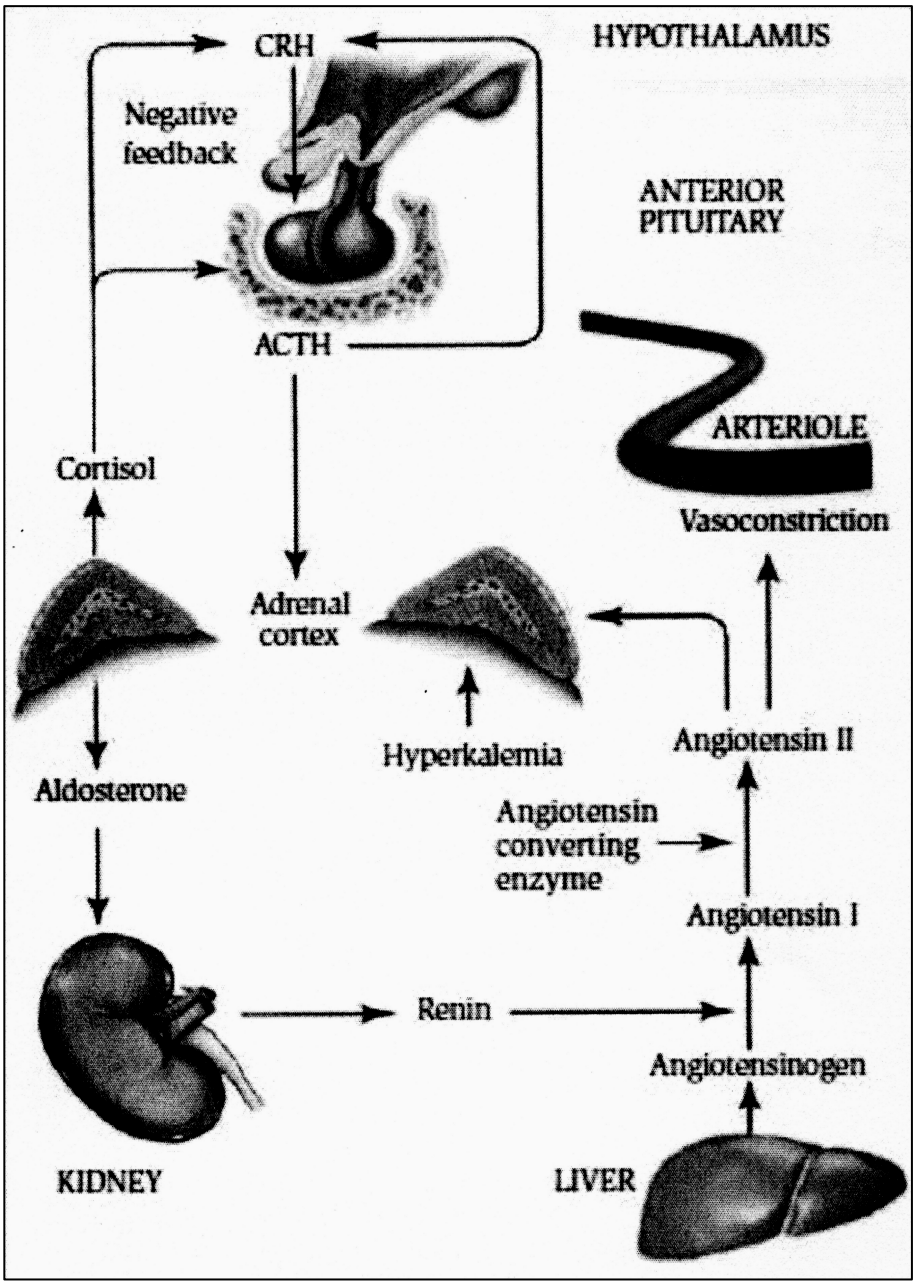

Table I.1: Classification of autoimmune polyendocrine syndrome (APS) in humans (adapted from Betterle Endocr Dev 2011;20:161-72).

APS type 1	Chronic candidiasis + chronic hypoparathyroidism + Addison's disease
APS type 2	Addison's disease + thyroid autoimmune diseases and/ or type 1 diabetes mellitus
APS type 3	Thyroid autoimmune diseases + other autoimmune diseases (excluding Addison's disease)
APS type 4	Combinations of two or more autoimmune diseases not falling into the above categories (i.e. Addison's disease and autoimmune gastritis, or vitiligo, or alopecia or celiac disease)

Figure I.4: Main features of autoimmune Addison's disease and their subtypes in humans (adapted from Betterle Endocr Dev 2011;20:161-72).

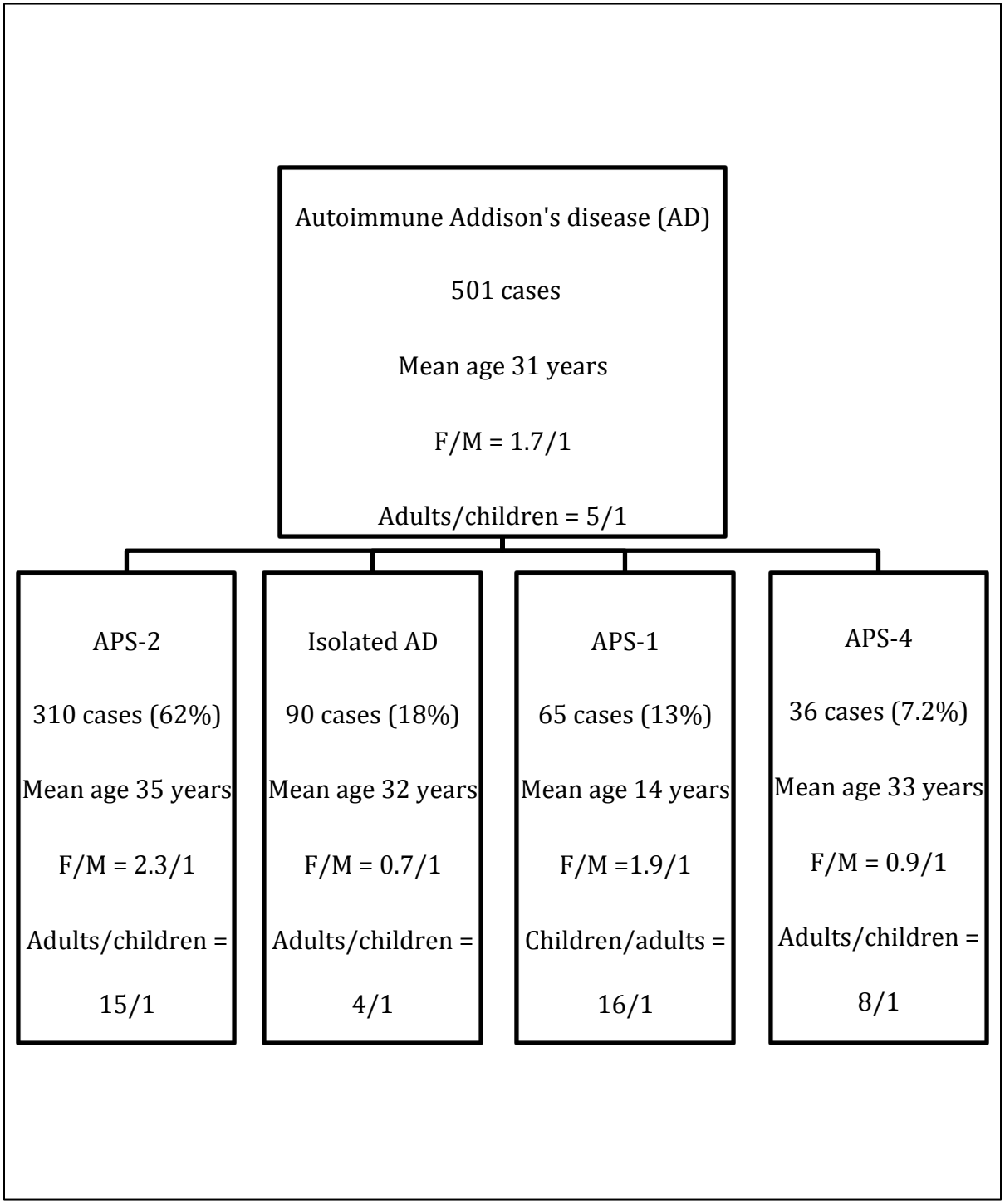

Table I.2: Breeds that are found to be at increased risk of developing hypoadrenocorticism (CI=confidence interval, N/A=data not available)

Breed of dog	Kelch[103]			Peterson[99]		
	Odds ratio	P value	95% CI	Odds ratio	P value	95% CI
Airedale Terrier	2.61	<.05	1.06-6.56	N/A	N/A	N/A
Basset Hound	3.38	<.001	1.50-7.79	3.90	.060	0.94-12.22
Bearded Collie	4.19	<.01	1.20-18.36	N/A	N/A	N/A
German Shorthaired Pointer	2.52	<.05	1.06-6.06	3.90	.060	0.94-12.22
Great Dane	7.63	<.0001	2.50-31.09	11.98	<.001	4.65-29.94
Poodle (Standard, Miniature, Toy)	3.55	<.0001	2.55-4.93	N/A	N/A	N/A
Poodle (Standard)	N/A	N/A	N/A	8.90	<.001	4.43-17.32
Portuguese Water Dog	N/A	N/A	N/A	46.66	.002	3.73-245.9
Rottweiler	1.25	>.05	0.71-2.21	2.6	.014	1.22-5.05

Table I.2 (cont'd):

Springer Spaniel	2.54	<.01	1.29-5.04	5.85	.051	0.99-24.56
West Highland White Terrier	5.93	<.0001	2.69-13.46	11.42	<.001	4.69-26.97
Soft Coated Wheaten Terrier	N/A	N/A	N/A	6.68	.039	1.11-29.49

Table I.3: Breeds that are found to be at decreased risk to develop hypoadrenocorticism (CI=confidence interval; N/A=data not available)

Breed of dog	Kelch[103]			Peterson[99]		
	Odds ratio	P value	95% CI	Odds ratio	P value	95% CI
Boxer	.12	<.05	.00-.77	N/A	N/A	N/A
Chihuahua	.83	>.05	.22-2.67	.18	.058	.00-1.04
Cocker Spaniel	.51	<.01	.30-.85	.31	.097	.04-1.15
Dalmatian	.00	<.01	.00-.50	N/A	N/A	N/A
Golden Retriever	.77	>.05	.49-1.21	.00	.001	.00-.49
Labrador Retriever	1.00	>.05	.72-1.38	N/A	N/A	N/A
Lhasa Apso	.22	<.01	.04-.75	.00	.036	.00-.92
Mixed Breed	.76	.072	.56-1.04	N/A	N/A	N/A
Pit Bull Terrier	.00	<.05	.00-.84	.35	.103	.00-1.26
Pomeranian	.12	<.05	.00-.77	N/A	N/A	N/A
Schnauzer (Giant, Standard, Miniature)	.73	>.05	.33-1.55	.00	.068	.00-1.07

Table I.3 (cont'd):

Shetland Sheepdog	.15	<.001	.03-.47	N/A	N/A	N/A
Shih Tzu	.25	<.05	.05-.83	N/A	N/A	N/A
Yorkshire Terrier	.14	<.01	.02-.58	.00	.003	.00-.56

Table I.4: Mean age and body weight, female to male ratio in dogs with hypoadrenocorticism (female intact (FI), male intact (MI), female spayed (FS), male neutered (MN), SD=standard deviation, SEM=standard error of the mean, N/A=data not available)

	Mean age of onset in years (SD, SEM, range)	Female: Male (Fi: mi: fs: mn) [%]	Mean bodyweight (SD, SEM, Range) [kg]
Kelch (244)[103]	4.3 (N/A, N/A, 0.2-12)	65:35 (17:22:48:13)	N/A
Kelch (262)[103]	4.9 (3.0, 0.2, 0.3-13)	68:32 (6:13:62:19)	23.4 (13.6, 0.8, 2.7-72.7)
Kelch (376)[103]	5.4 (3.2, 0.2, 0.1-15)	68:32 (14:13:54:19)	N/A
Peterson[99]	4.0 (N/A, N/A, 0.3-14)	71:29 (16:16:55:13)	20 (N/A, N/A, 2.3-64.5)
Melián[107]	4.5 (N/A, N/A, 1-12)	67:33 (12:10:55:24)	10.2 (2.5-46.1)
Willard[110]	4.2	68:32	N/A
Lifton[143]	6.0 (N/A, N/A, 2.8-11)	56:44	28.5 (3.2-43)
Average (Range)	4.8 (4.0-6.0)	66:34	20.5 (10.2-28.5)

Table I.5: Hypoadrenocorticism and age (CI=confidence interval)

Odds of a dog developing hypoadrenocorticism based on age (Kelch study[103])			
Age	Odds ratio	P value	95% CI
0-1	0.12	<.0001	0.06-0.22
1-4	1.01	>.05	0.78-1.29
4-7	1.90	<.0001	1.49-2.42
7-10	1.51	<.01	1.16-1.98
>10	1.06	>.05	0.78-1.43

Table I.6: Hypoadrenocorticism incidence and gender

Estimated incidences by gender (Kelch Study[103])	
Gender	Estimated cases/1000/year
All dogs of all genders	0.6
Females	0.7
Ovariohysterectomized females	1.0
Intact females	0.3
Males	0.4
Castrated males	0.6
Intact males	0.2

Table I.7: Odds ratios for hypoadrenocorticism and gender

Odds of a dog developing hypoadrenocorticism based on gender (Relative to intact males) (Peterson study[99])			
Sex	Odds ratio	P value	95% CI
Ovariohysterectomized females	1.44	0.158	0.88-2.36
Castrated males	1.83	0.026	1.07-3.11
Intact females	5.55	<0.001	3.75-8.37

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Chapter II: Pathogenesis of autoimmune diseases

Introduction

Autoimmune diseases occur when an organism fails to recognize its own cellular components as self, and therefore mounts an immune response against its own cells and tissues. Another, more precise definition defines an autoimmune disease as a "clinical syndrome caused by the activation of T cells or B cells, or both, in the absence of an ongoing infection or other discernible cause"[1]. Autoimmune diseases affect five percent of the population in Western countries; the most common autoimmune diseases include Celiac disease, diabetes mellitus type 1, systemic lupus erythematosus, Sjögren's syndrome, Hashimoto's thyroiditis, Graves' disease, idiopathic thrombocytopenic purpura, rheumatoid arthritis, and hypersensitivity.

For many years, it was believed that autoreactive T cells and B cells were eliminated during clonal deletion, leaving only immune cells specific for foreign antigen recognition. It is now understood and accepted that a low level of autoreactivity is normal and physiologic and vital for normal immune function and to enhance an immune response in its early stage when the availability of foreign antigen is limited[2].

Stefanova et al. demonstrated that self-MHC identification maintains the responsiveness of CD4+ (cluster of differentiation 4) T cells in the absence of antigens[3]. To

prove this, an anti-MHC (major histocompatibility complex) Class II antibody was injected into mice. This antibody temporarily prevented CD4+ T cell-MHC interaction. Harvested naïve CD4+ T cells from these mice showed decreased responsiveness to the presentation of an antigen, thus they were able to show that self-MHC recognition maintains the responsiveness of CD4+ T cells when foreign antigen is not present[3]. Further, a low level of autoimmunity might help CD8+ T cells in the recognition of cancerous cells, therefore reducing the incidence of certain cancers.

Autoantigens help to form the repertoire of mature lymphocytes[1], and B and T cells in the periphery need constant exposure to autoantigens in order to survive[4,5]. Some have speculated that lymphocytes are able to distinguish self from non-self. However, there is no central difference in foreign antigens and self antigens, and it appears that the microenvironment is important for development of an immune response, and that this usually only occurs in the presence of inflammatory cytokines[6]. For this physiologic autoreactivity transition to a pathologic reaction, additional stimuli are necessary. The most common insults include: 1) loss of immunologic tolerance, 2) genetic susceptibility, 3) environmental and internal triggers and changes in pathologic processes during autoimmune-disease progression, and/ or 4) different mechanisms of tissue injury[1]. The fact that certain insults are necessary, and a genetic predisposition by itself does not automatically result in the development of an autoimmune disease, is clearly illustrated by the at best 50 % penetrance of autoimmune diseases in identical twins raised in the same environment. Certain factors are necessary that

create a perfect storm of events leading to autoimmune disease. This is also demonstrated in the Venn diagram of autoimmune diseases, as further discussed at the end of this review. The four above-mentioned potential mechanisms that contribute to the development of autoimmune diseases are discussed in the following paragraphs. At the end, I will also briefly discuss the function of the relatively recently discovered Th17 (T helper 17) cells.

Immunologic tolerance

Most autoimmune diseases are related to the loss of immunological tolerance, resulting in T cells and antibody-producing B cells that recognize self-antigens as non-self or foreign[7,8]. Although the exact mechanisms of immunologic tolerance remain unclear, different but not mutually exclusive theories have been proposed, and if any cells escape any of these processes, an autoimmune disease may be triggered. Below, several mechanisms that promote or facilitate immunologic tolerance are briefly described.

Clonal deletion: In 1960, Frank M. Burnet and Peter B. Medaware received the Nobel Prize in Physiology for their "discovery of acquired immunological tolerance". They discovered that self-reactive lymphoid cells are destroyed during development of the immune-system[9]. During development, T and B lymphocytes interact with self antigens. If their unique antigen receptor recognizes self antigens, these cells are then deleted through apoptosis or programmed cell death. For example, T cells developed in the thymus originally express neither CD4 nor CD8. Later, they acquire both CD4 and CD8 and express low levels of αβ T cell receptor

(TCR). These cells are positively selected after interacting with class I or class II MHC molecules. Cells with low affinity for MHC are positively selected- unselected cells die by apoptosis, a process called "death by neglect". The elected T cells are presented with self-peptides presented in the context of MHC molecules by dendritic cells. Any T cells with high affinity receptors for MHC + self-peptide undergo clonal deletion. Any T-cells that escape this process may cause an autoimmune disease, and genetic mutations that disrupt T cell deletion in the thymus can result in catastrophic autoimmunity. For example, a genetic mutation in the autoimmune regulator (AIRE) is seen in patients with autoimmune polyendocrinopathy syndrome type 1 (APS-1), with clinical manifestation of hypoparathyroidism, primary adrenocortical failure and chronic mucocutaneous candidiasis. A transgenic mouse knock-out has been created to study the exact mechanisms[10]. AIRE, a human transcription factor that is expressed in the medulla of the thymus, is important in the inhibition of autoimmune diseases. AIRE initiates the transcription of many organ specific genes in the thymus. The epitopes of proteins, which are usually only expressed in the periphery, then bind to T cells. The 'auto-reactive' T cells that bind these proteins are then eliminated via the process of negative selection- thereby reducing the occurrence of autoimmunity later on. By this mechanism, maturing thymocytes become tolerant towards peripheral organs or self-proteins. One of these proteins for example is insulin. Its' transcription is initiated by AIRE in the thymus. AIRE is also expressed in many other tissues[11,12]. During B-cell development, a similar strategy as described for T cells is used to eliminate self-reactive B cells.

Clonal anergy: Anergy is another process that induces tolerance, modifying the immune

system to prevent self-destruction. Clonal anergy was first described by Gustav Nossal for B lymphocytes and later on, in very similar fashion, by Ronald Schwartz and Marc Jenkins for T lymphocytes[13-19]. Self reactive T- or B-cells are inactivated and therefore cannot increase an immune reponse[16]. If an antigen-presenting cell does not have co-stimulatory molecules CD80 or CD86 when presenting an antigen to a T cell, the T cell becomes anergic (nonresponsive). Further, when T-cells are activated through CTLA4, Interleukin (IL)-2 production is inhibited and they become anergic. When B cells are exposed to large amounts of soluble antigen, surface IgM is down-regulated, surface Fas molecules are up-regulated and they become anergic. Fas-ligand bearing T cells interact with these, resulting in their death via apoptosis. The clinical significance of clonal anergy, beyond reducing autoimmune diseases, is seen in some infectious diseases, including HIV (human immunodeficiency virus). HIV seems to utilize the immune system's use of tolerance induction and is therefore able to evade the immune system. Anergy might however also be significant for therapeutic uses, for example after organ transplants or to induce activated lymphocytes to become unresponsive with autoimmune diseases like diabetes mellitus, multiple sclerosis, and rheumatoid arthritis.

Idiotype network and anti-idiotype antibody: During a process called tolerization, antibodies are produced against specific antibody-idiotypes. Such an antibody might prevent the B cell receptor from interacting with its specific antigen. Naturally existing antibodies further are able to build a network and are able to neutralize self-reactive antibodies[20,21].

Clonal ignorance: Host immune responses ignore self-antigens. T cells reactive to self-antigen mature and migrate to the periphery, where they might never meet the appropriate antigen. These cells might then die, because a stimulus is missing. B-cells that escape deletion might never meet their specific antigen or the specific antigen and might also die because lack of stimulus[22].

Peripheral tolerance: If T and B cells do not undergo clonal deletion, they could cause an autoimmune response once they reach lymphoid organs in the periphery. Therefore, the immune system has additional peripheral checkpoints to delete the cells that did not undergo deletion[23].

Activation-induced cell death: Besides producing cytokines when activated with an antigen, T cells also express FasL, which binds to Fas, which in turn triggers apoptosis via activation of caspase-8. Mice with mutations in Fas or FasL develop severe autoimmune diseases and usually die within the next 6 months. In humans, similar mutations cause a lymphoproliferative disease called autoimmune lymphoproliferative syndrome[24].

Regulatory T cells: Normal CD4+ T cells express CD25, but regulatory T cells, which have recently been discovered, also express the forkhead family transcription factor Foxp3 (forkhead box P3), by which they can be differentiated from normal CD4+ T cells. Foxp3 is required for regulatory T cell development and function, although the mechanism by which regulatory T cells suppress other T cells is not fully understood. It has been suggested that one mechanism includes the production of cytokines such as TGF-β (Transforming growth factor beta) and IL-10,

which have an immunosuppressive action. The fact that a mutation in humans in Foxp3 causes a deadly autoimmune syndrome (IPEX: immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) proves that regulatory T cells play an important role in preventing autoimmune disease[25,26].

Genetic susceptibility

The deletion or overexpression of genes predisposes mice to autoimmune diseases and more than 25 genes have been identified so far. This clearly shows the effect of gene mutations on development of autoimmune diseases. The genes that were modified encode for cytokines, antigen-coreceptors, elements of the cytokine- or antigen-signaling cascade, costimulatory molecules or molecules that promote apoptosis or inhibits it, and molecules that clear antigens or antigen-antibody complexes[1]. However, in these experiments, genetic engineering of one single gene did not automatically cause autoimmune diseases, but it rather predisposed the animal to develop one. This means that disease development overall depends on the total genetic background of the host. Further, some genetic mutations predisposed the mice to develop more than one autoimmune disease, showing that the development of certain autoimmune diseases may have the same pathway[1]. Findings in human medicine confirmed the results of these studies; however, it was shown that a genetic locus is more likely to cause an increased susceptibility to an autoimmune disease rather than a single gene mutation alone,

as opposed to the case with autoimmune proliferative syndrome and polyglandular endocrinopathy. Certain loci also seem to be responsible for causing more than just one autoimmune disease. A single mutation can increase susceptibility, but the development of disease phenotype depends on other genes. Oftentimes autoimmune diseases are multigenic, with many susceptibility genes working together to cause disease phenotype. The genetic background also influences the severity of disease as some people do not show disease and have a normal immune function, besides carrying the polymorphism. Some genes also posses a much higher risk than others as it is the case with especially the ones that are related to the major histocompatibility complex, T cell receptors or immunoglobulins. And then there are certain HLA alleles that protect against disease, even if the susceptibility allele is present[27,28]. Further, it has been observed that more than one autoimmune disease appears within a family, suggesting that some genes at certain loci predispose patients to more than one disease[27-30]. For some time it was hard to understand why some people with the same serologic abnormalities did not necessarily have the same tissue changes. However, an explanation has been found in an animal model, demonstrating that the vulnerability of an organ to an immune-mediated destruction is genetically determined. The animal model demonstrated the presence of a variable threshold to cardiac and renal damage[31,32].

Further underlying the importance of genetic factors on the development of autoimmune diseases are the results of many epidemiologic studies, which have demonstrated familial clustering, the higher rate of similar autoimmune diseases in monozygous versus dizygous twins, and the fact that almost 75 % of the more than 23.5 million Americans that are

affected with autoimmune diseases are women[33,34]. The exact reason why women are overrepresented is not well understood and could be related to genetics, but reasons could also include that they mount a larger inflammatory response when stimulated than men, the involvement of sex hormones, history of pregnancy and therefore exposure to antigens of the baby, imbalanced X chromosome inactivation or other complex X-linked susceptibility mechanisms[35].

Environmental triggers, internal triggers and changes in pathologic processes during autoimmune-disease progression

Epidemiologic studies of twins (lower-than-expected rate of same autoimmune diseases among monozygotic twins[36]) and genetically similar populations living in different locations show that an environmental exposure is necessary to trigger autoreactivity, even if a genetic predisposition is present[37,38], as shown by the change of incidence of certain autoimmune disease when people move away (for example with type 1 diabetes, multiple sclerosis, and pemphigus foliaceus). Besides an environmental exposure, a change in internal environment or infections could also trigger disease. Unfortunately, the trigger for most autoimmune diseases is unknown. For example, certain chemicals (for example Procainamide) can cause drug-induced lupus erythematosus, estrogens may exacerbate it, cigarette smoking increases risk for incidence and severity of rheumatoid arthritis, most likely due to increased citrullination of

proteins, penicillin and cephalosporin can bind to the red-cell membrane, causing production of an antibody which causes hemolytic anemia, and the blockade of TNF-α (Tumor necrosis factor alpha), used in patients with Crohn's disease or rheumatoid arthritis, may induce antinuclear antibodies, systemic lupus erythematosus and multiple sclerosis[39].

Microbial agents can trigger an autoimmune disease, related to polyclonal activation, release of previously sequestered antigens, or molecular imitation (or mimicry). Polyclonal activation is believed to be the reason for the increased incidence of autoimmune diseases in rodents that are exposed to microbes[40]. The microbes can cause inflammation, and a release of sequestered antigens, which then can result in autoimmunity[40,41]. Similarities exist in humans. In humans, it has been shown that inflammation (in the absence or presence of infection) can cause polyclonal activation and autoreactivity[42]. The activation of B cells by T cells can be bypassed and B cells can secrete large amounts of antibodies without activation. Polyclonal activation of B cells can happen by directly binding to the β-subunit of the T cell receptor in a non-specific fashion. Interesting on the other hand is that some studies have found that parasitic infections in humans are actually associated with reduced activity of autoimmune diseases[43,44]. The parasite might influence the hosts immune response in order to protect itself, probably by secreting anti-inflammatories or by interfering with the hosts signaling mechanism.

With molecular imitation, it has been speculated that an infection causes the initial activation of lymphocytes that mediate the disease. Antibodies that are produced during initial

infection cross-react with autoantigens (antigenic-cross reactivity), which sustain the activation even after the foreign antigen is eliminated[1]. This mechanism has been speculated to cause rheumatic fever (due to streptococcal infection)[45-47], Guillain-Barré syndrome (Campylobacter jejuni)[48], autoimmune diabetes (coxsackievirus)[40,49], and multiple sclerosis (Epstein-Barr, influenzavirus type A, human papillomavirus)[50].

Further, regulatory cells carry an importance in the development of autoimmune diseases. Some regulatory cells mature in the thymus, and others must be activated by autoantigens in the periphery. Important autoreactivity-controlling regulatory cells include CD1 restricted T cells, T cells with γ/δ receptors, CD4+CD25+ T cells, and T cells that produce cytokines, which in turn suppress autoreactive cells[51]. The importance of these regulatory cells in the development of autoimmunity was demonstrated in epidemiologic studies. It was shown that monozygotic twins that are discordant for diabetes have different levels of CD1 restricted T cells, where the affected twin has much lower levels than the unaffected twin[52]. The exact mechanisms in which antigens activate regulatory T cells are not clearly understood[1].

Epitope spreading occurs during disease progression, that is when the disease progresses from activation to chronic and more autoantigen attacked by T cells and antibodies becomes available. Both autoreactive B and T cells add to epitope spreading. Autoreactive B cells not only present antigen to T cells, but they also make novel peptides of proteins and protein complexes and present these to naïve T cells. This causes a cascade to start, with T cells

activating more autoreactive B cells, and B cells then presenting even more, additional self-epitopes. The result is increased autoreactivity to many more autoantigens. Further, different cells contribute to chronic disease compared to initial activation, including cytokines and other inflammatory mediators, which also contributes to epitope spreading[53-57].

A T cell- B cell discordance has been recognized in for example Celiac disease. B cells, which recognize transglutaminase, are receiving assistance from T cells that recognize gliadin. It is hypothesized that a B cell that is specific for IgGFc (immunoglobulin gamma Fc region) can recruit and get help from a T cell that responds to an antigen co-endocytosed with IgG by B cells.

Autoimmune diseases in humans are mostly restricted to a few antigens, including some that are known to have signaling roles in the immune reaction. Spontaneous autoimmunity may occur when abnormal signals are sent to parent B cells through membrane bound ligands (including B cell receptor for antigen, IgG Fc receptors, CD21, Toll-like receptors 9 and 7), after binding of an antibody to certain antigens. This idea, together with the idea of T cell B cell discordance, shaped the basis for the hypothesis of self-perpetuating autoreactive B cells[58]. It is hypothesized that these cells survive due to subversion of both the feedback signal via the B cell receptor and of the T cell help pathway. Negating the negative signals causing B cell self tolerance, without automatically losing T cell self tolerance. This is called aberrant B cell receptor-mediated feedback.

Antigenic epitopes that are found in the antigen-binding portion of an immunoglobulin molecule are called idiotypes. If an idiotype on an antiviral antibody and a host cell receptor for the virus in question cross-react, autoimmunity can occur. The host cells are then attacked by the anti-idiotype antibodies, because the host-cell receptor is seen as the virus[59].

Cytokines either promote function of helper T cells type 1 or helper T cells type 2. The cytokines that promote function of helper T cells type 2 appear to have a role in prevention of exaggeration of pro-inflammatory immune responses of type 1 T cells. A few cytokines that are in this group are IL-4, IL-10, and TGF-β. This phenomenon is called 'cytokine dysregulation'.

Dendritic cells present antigens to active lymphocytes. If the dendritic cells are defective in apoptosis, it could result in an inappropriate systemic activation of lymphocyte apoptosis and as a consequence a decline in self-tolerance[60].

Over time, the effector cells and inflammatory mediators of an autoimmune disease might change, which makes it very difficult for treatment. A treatment that is beneficial early on could lose its effectiveness, or might even be harmful later on. These changes have been demonstrated in studies in animals, but can also be experienced during treatment of many autoimmune diseases in humans, of which the most common are Crohn's disease, and rheumatoid arthritis. Blockade of TNF-α in patients with either disease is of huge benefit, but induces production of antinuclear antibodies in 10% of treated patients and systemic lupus erythematosus in a few. The question remains whether naïve cells or memory cells cause progression of disease[1,39].

Further under investigation in the pathogenesis of autoimmune diseases is the role of regulatory T cells, natural killer cells, and γδ T cells.

Autoimmune mechanisms of tissue injury

The effector mechanisms of the immune system may be directed against self-antigens, as described previously. Since the adaptive immune system is not able to remove an autoantigen, the immune response persists and might get stronger, because of the constant supply of new autoantigen. T cells and autoantibodies cause tissue damage during an autoimmune disease. T cells induce cytolysis through perforin-induced necrosis or through granzyme B-induced apoptosis. Type 1 and type 2 helper T cells cause tissue damage through the production of cytokines and through recruitment of inflammatory cells and mediators[61-64].

IgE, IgM, and IgG appear to be of importance during autoimmune diseases, even though IgE does not seem to have a major role. They have been identified in some autoimmune diseases, for example in autoimmune vasculitis, an inflammatory disease of blood vessels that is also known as Churg-Strauss vasculitis. However, it has not been proven that the IgE antibodies mediate the disease. Autoimmune diseases that cause tissue damage by means similar to a type II hypersensitivity response are very common though. The IgG or IgM response is against autoantigens that are located on cell surfaces or the extracellular matrix, which then causes injury. Injury related to autoimmunity similar to a type II response involves the formation of

immune complexes, containing antibodies to soluble autoantigens. These autoimmune diseases are usually systemic and are characterized by autoimmune vasculitis.

Th17 cells

Th17 cells, a subset of T helper cells, were first described in the literature between 2005 and 2007[65-67]. The T helper cell differentiation process and their protective and harmful role are shown in figure II.1. Cytokines transforming growth factor beta (questionable in humans), IL-1β, IL-6, IL-21, and IL-23 contribute to their formation in humans and mice[68,69], as well as other proteins including signal transducer and activator of transcription 3, retinoic-acid-receptor-related orphan receptors alpha and gamma[68]. Interferon gamma, IL-4, and IL-12 have been shown to negatively regulate Th17 differentiation in man, as well as IL-2, IL-25, IL-27, and IL-35 in mice (see figure II.2).

Th17 cells are highly inflammatory in nature, and excessive amounts of Th17 have a key role in the development of autoimmune diseases and have been linked to multiple sclerosis, psoriasis, autoimmune uveitis, juvenile diabetes, rheumatoid arthritis, inflammatory bowl disease, arthritis and Crohn's disease[70-75], most likely by causing inflammation and tissue injury. Their physiologic role is to defend[76] against extracellular pathogens at epithelial and mucosal barriers, including Klebsiella and Citrobacter bacterial species[77] by producing cytokines

(effector cytokines are IL-17 (especially IL-17A and F), IL-21, and IL-22[78]), which stimulate epithelial cells to produce anti-microbial proteins. Especially IL-17A and IL-17F recruit, activate, and are involved in the recruitment of neutrophils. A lack of Th17 would leave a host vulnerable to opportunistic infections. The role of Th17 cells in carcinogenesis remains under investigation[79].

Conclusions

In order to develop superior treatments for autoimmune diseases, it will be necessary to better understand the exact mechanisms that control development of autoimmune disease phenotype. A model for the development of autoimmune diseases needs to include genetic predispositions, environmental factors and immune regulations or dysreglations, as schematized in figure II.3, and as described previously. Besides other markers of predisposition to autoimmune diseases, and other genes that are important in the regulation of an immune response, MHC molecules regulate and form the specificity of the adaptive immune response and have been associated with the development of autoimmune diseases. The genetic makeup of humans determines how the immune system reacts to antigenic confronts from the environment. Further, it is responsible for staying tolerant towards self-antigens. Under certain conditions, an infection, an inappropriate immune response to cross-reactive self-antigens, and/ or the failure of regulatory mechanisms, may cause tissue damage. Severe tissue damage

can further strengthen the immune response, and a clinical manifest autoimmune disease results.

APPENDIX

Appendix

Figure II.1: Heterogeneity in helper T cell fates. The helper T cell differentiation process is initiated by signaling from dendritic cell to T cell in the lymph node, resulting in division and differentiation. The mature helper T cells and their signature transcription factors are illustrated. Cytokines play a critical role in the induction or repression of the lineages. The different helper T cell subsets have distinct protective and pathological roles. Host defense is orchestrated by the three major fates, Th1, Th2, and Th17. Adaptive regulatory T (aTreg) cells can downregulate immune responses, although a physiological role in vivo is yet uncertain. The mature helper T cell progeny must eventually exit the lymph node and migrate to infected tissue to exert their function in host defense. Some of the mature progeny may, instead, migrate to B cell follicles to promote antibody subclasses that will suit the particular immune response (from: Reiner, S. L., Development in Motion: Helper T Cells at Work, Cell 129, April 6, 2007, pages 33 – 36).

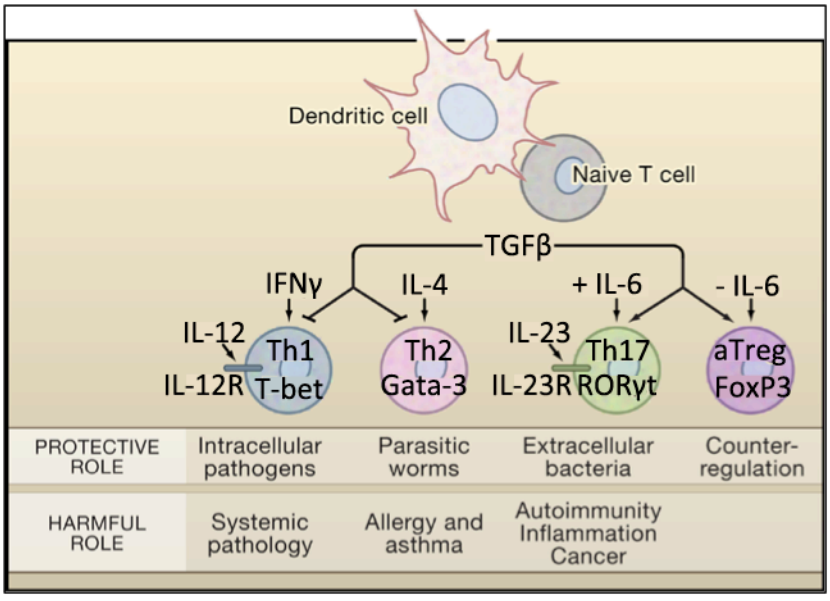


Figure II.2: Th17 differentiation in mice and man (from: *Gut* 2009;**58**:1152-1167)

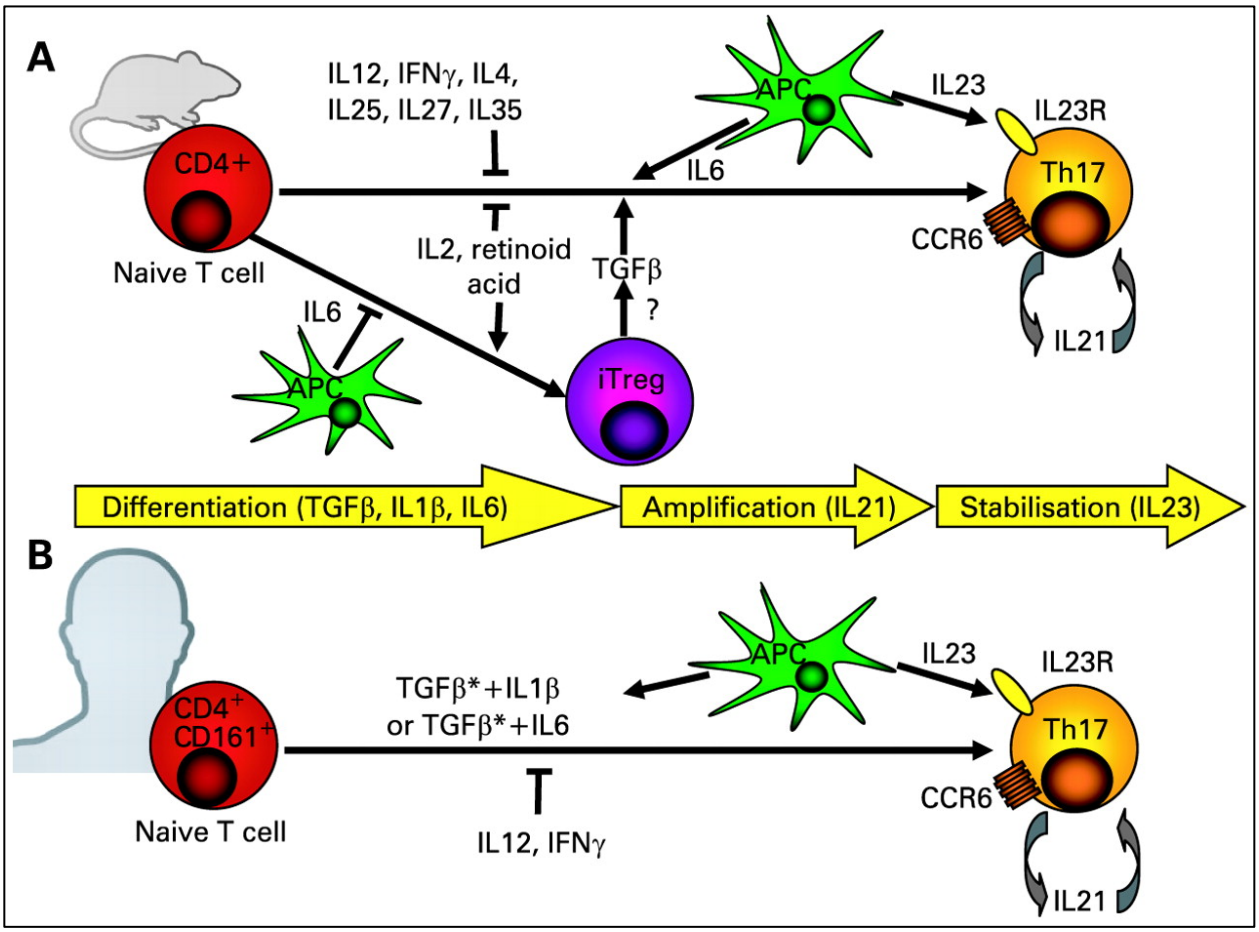
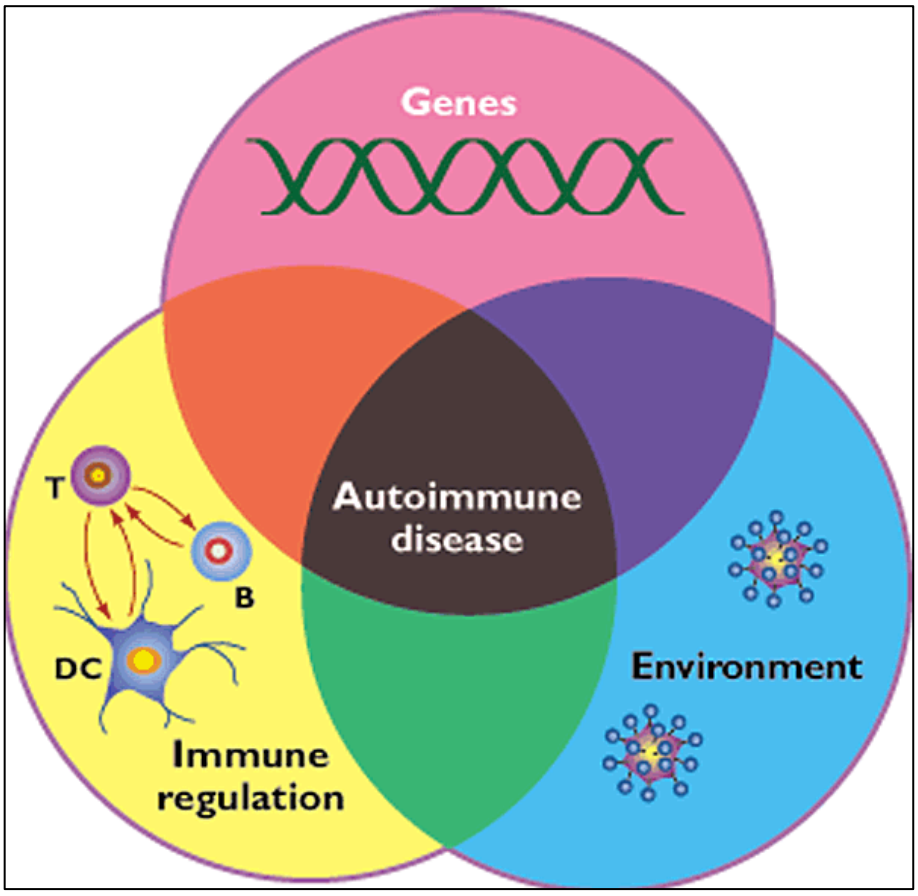


Figure II.3: Requirements for the development of an autoimmune disease. The immune response of a genetically predisposed individual to an environmental pathogen, in association with defects in immunoregulatory mechanisms, can lead to the development of an autoimmune disease. The importance of the single components represented in this Venn diagram may vary between individuals and diseases. However, the appearance of an autoimmune disease requires the convergence of all three components. T, T cell; B, B cell; DC, dendritic cell (from: Focus on Autoimmunity, Nature Immunology, Sept 2001 (Vol. 2)).



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Chapter III: Preliminary research leading to my hypothesis

Retrospective study: histology and histopathology of normal canine adrenal glands and adrenal glands from Addison's disease dogs

Twenty-four adrenal gland tissue slides from dogs diagnosed with adrenal insufficiency were stained with hematoxylin and eosin stain (H&E) and examined. Normal adrenal glands were obtained during senior student surgery labs and were used as negative controls. In the Addison's disease adrenal glands, the severity of inflammation was subjectively graded as mild (1), moderate (2), or severe (3) by two independent observers depending on the amount of inflammation observed (Rick and Williams). Ten cases were classified as grade 1, eight as grade 2, and five as grade 3 (figures III.1 and III.2). One adrenal gland did not show inflammation; however, in this case the adrenal cortex was collapsed and atrophied. As would be expected in immune-mediated disease, the inflammation was primarily lymphocytic early in the course of the disease, and became mostly macrophagic coincident with the destruction and loss of gland parenchyma. None of the 12 control adrenal glands had any evident inflammatory cells present within. Immunohistochemistry was utilized to identify the lymphocytes as either T cells (CD3+) (cluster of differentiation 3+) or B cells (CD79a+). Again, as would be expected for an immune-mediated disease, the majority of cells are T cells (figure III.3).

Detection of anti-adrenal autoantibodies using Western blot analysis

I followed the protocol of the Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University (MSU), East Lansing, MI, USA, Western blot (WB) PARASOP.0026.01 (Standard operating procedure for detection of Sarcoystic neurona by WB). Freshly collected adrenal glands were homogenized in 50 mM Tris, containing 0.4 mM KI, and 1 mM EDTA, and centrifuged at 4°C and 1,000 x g for 10 minutes. The antigen preparations were used immediately or stored at -80°C. Following SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), antigens were transferred to nitrocellulose sheets. Subsequently, the sheets were incubated with negative and suspect positive sera or plasma (normal or slightly lipemic samples only), and antigen-antibody complexes were visualized by incubating with a peroxidase-conjugated goat anti-canine IgG (immunoglobulin gamma), heavy and light chain, and a substrate-chromogen mixture.

I analyzed a total of 17 samples (14 affected dogs, three healthy control dogs). Out of the 14 affected dogs, five showed a distinct band around 20 kDa (figure III.4). None of the three negative control samples showed this band. However, human microsomal autoantigen 21-hydroxylase weighs 55 kDa. In humans that have been recently diagnosed with hypoadrenocorticism, there is a proliferative T cell response to an 18 kDa to 24 kDa molecular weight protein fraction[1,2]. This protein fraction is adrenal specific and corresponds in weight to the band that I observed in this preliminary experiment. WB is a reliable and sensitive diagnostic test for the detection of antibody in serum. However, it is also very sensitive to slight

changes in conditions. Further, antigen preparations should be purified to decrease nonspecific binding.

Detection of 21-hydroxylase antibodies with a commercially available radioimmunoassay for the detection of human 21-hydroxylase antibodies

A commercially available radioimmunoassay for the identification of 21-hydroxylase autoantibodies in human serum is distributed by Kronus® (Boise, Idaho, USA). This assay was used according to the manufacturer recommendations and three different dog populations were analyzed. First, canine serum or plasma samples from dogs with a confirmed diagnosis of hypoadrenocorticism, based on ACTH (adrenocorticotropic hormone) stimulation test, and no history of chronic exogenous steroid, Lysodren, trilostane or ketoconazole use were used, representing the sick dog group (SD). Second, serum or plasma samples from breeds that are reported to have a higher incidence of hypoadrenocorticism compared to the general dog population, or from affected lines of dogs were used, and represented a group of dogs at higher risk to develop the disease compared to the general dog population (HR). Serum or plasma samples from healthy dogs sent to us for thyroid evaluation for registration by the Orthopedic Foundation for Animals were used as negative (healthy) control samples (LR). The samples were stored at -18°C prior to analysis. By definition, positive samples contained >1 U/mL antibodies which is the same cut-off established for human samples. Results are summarized in table III.1.

Non-parametric statistical analyses showed a difference among groups (Kruskal-Wallis, p=0.001) and Mann-Whitney with Bonferroni correction showed differences between individual groups:

- Significant difference between SD to LR (p=0.0003) and HR to LR (p=0.0013)
- No significant difference between SD to HR (p=0.8338)

The 21-hydroxylase antibody activity was reduced in two out of two positive samples after incubation with an excess amount of unlabeled 21-hydroxylase indicating that they contained specific 21-hydroxylase antibodies.

Unfortunately, this assay gave very poor replication and the differences in raw counts per minutes (cpm) between supposedly positive and supposedly negative samples were less then 200 cpm. Although preliminary data with this strategy were promising, I concluded that this method did not provide adequate specificity for detecting canine 21-hydroxylase antibodies.

Detection of 21-hydroxylase antibodies with radioactively labeled amino acid capture probe

The protein sequence for canine 21-hydroxylase is published. There is 78 % homology of amino acid sequence between canine and human 21-hydroxylase. The autoantibody epitope is well described in the human literature and is reported to be located at amino acids 298-356. N-terminal deletions up to 280 had no effect on binding. C-terminal deletions 281-494 on the other hand showed a marked effect on antibody binding. However, a conformational epitope

might exist. All antibodies reacted to full length 21-hydroxylase that was expressed in vitro[3-5]. According to this information, a 21 amino acid peptide (YKDRARLPLLNATIAEVLRLR) was designed. The iodination was performed according to standard protocol. We iodinated 2 µg of protein, over 50 % of the iodine was bound to the protein and we saved a total of 0.3 mCi of iodinated protein.

Analysis of 54 samples from dogs with hypoadrenocorticism and 10 control samples was performed. The assay procedure was the same as recommended for the radioimmunoassay for the identification of human 21-hydroxylase antibodies in human serum commercially available through Kronus® (see above). Results are summarized in Table III.2.

Mann-Whitney statistical analysis with Bonferroni correction showed a significant difference in counts between the two individual groups:

- Significant difference between SD to LR (p=0.0343)

The biggest problem in this experiment was the presence of a nonspecific binding raw count of greater than 2000/ min. I tried different incubation times, different sample and tracer volumes, different dilutions, and different capturing mechanisms, utilizing protein A, protein G, and N6. I also added various amounts of protein from different species to the reaction buffers. Results remained confusing and not explainable, and I concluded that there must be some kind of matrix interferences between molecules in the serum or plasma samples with the tracer.

Detection of adrenal autoantibodies with indirect immunofluorescence technique

Normal to slightly hemolyzed, previously assayed canine serum or plasma samples, with confirmed diagnosis of hypoadrenocorticism based on ACTH stimulation test and no history of chronic exogenous steroid, Lysodren, trilostane or ketoconazole use, were used as positive control samples. Normal to slightly hemolyzed serum or plasma samples from healthy dogs sent to us for thyroid evaluation for registration by the Orthopedic Foundation for Animals were used as negative control samples. The samples were stored at -18°C prior to analysis. Adrenal tissue from healthy beagle dogs were collected and immediately frozen and stored at -80°C until used for preparing cryostat sections. None of the adrenal tissues had been stored longer than six months at the time of this experiment. Working temperature for cryo-sectioning was approximately -20°C. The frozen tissues were transferred onto slides and air-dried for 30 minutes. Then, standard indirect immunofluorescence procedures were performed with both unfixed and acetone fixed (10 minutes) tissues. I was not able to differentiate supposedly positive from supposedly negative serum samples. After consultation with Dr. Ludek Vajner, who has considerable experience with indirect immunofluorescence technique (iIFA), especially with thyroid tissues, we used his protocol to detect thyroglobulin autoantibodies (TgAA) in sera on frozen canine thyroid cryostat sections. Thyroid glands were harvested from healthy beagle dogs and immediately frozen in liquid nitrogen and stored at -80°C until they were used for this experiment. Serum plasma samples positive or negative for TgAA were identified using a

commercially available ELISA (Enzyme-linked immunosorbent assay) test (Oxford Biomedical Research, Rochester, MI, USA) in our laboratory. Using iIFA, I was able to differentiate TgAA positive serum samples from TgAA negative serum samples (figure III.5).

Using the same technique for adrenal antibody analysis, I was not able to differentiate supposedly positive from supposedly negative samples because of severe background. The use of different blocking agents including 5% non-fat dried milk and serum from different species did not decrease background.

It became apparent, that it is difficult to establish and find a proper working technique without having a confirmed positive serum or plasma sample available. I decided not to repeat this experiment until the creation or finding of a known positive control sample.

APPENDIX

Appendix

Figure III.1: Adrenal gland histology, 2.5x magnification hematoxylin and eosin stain.

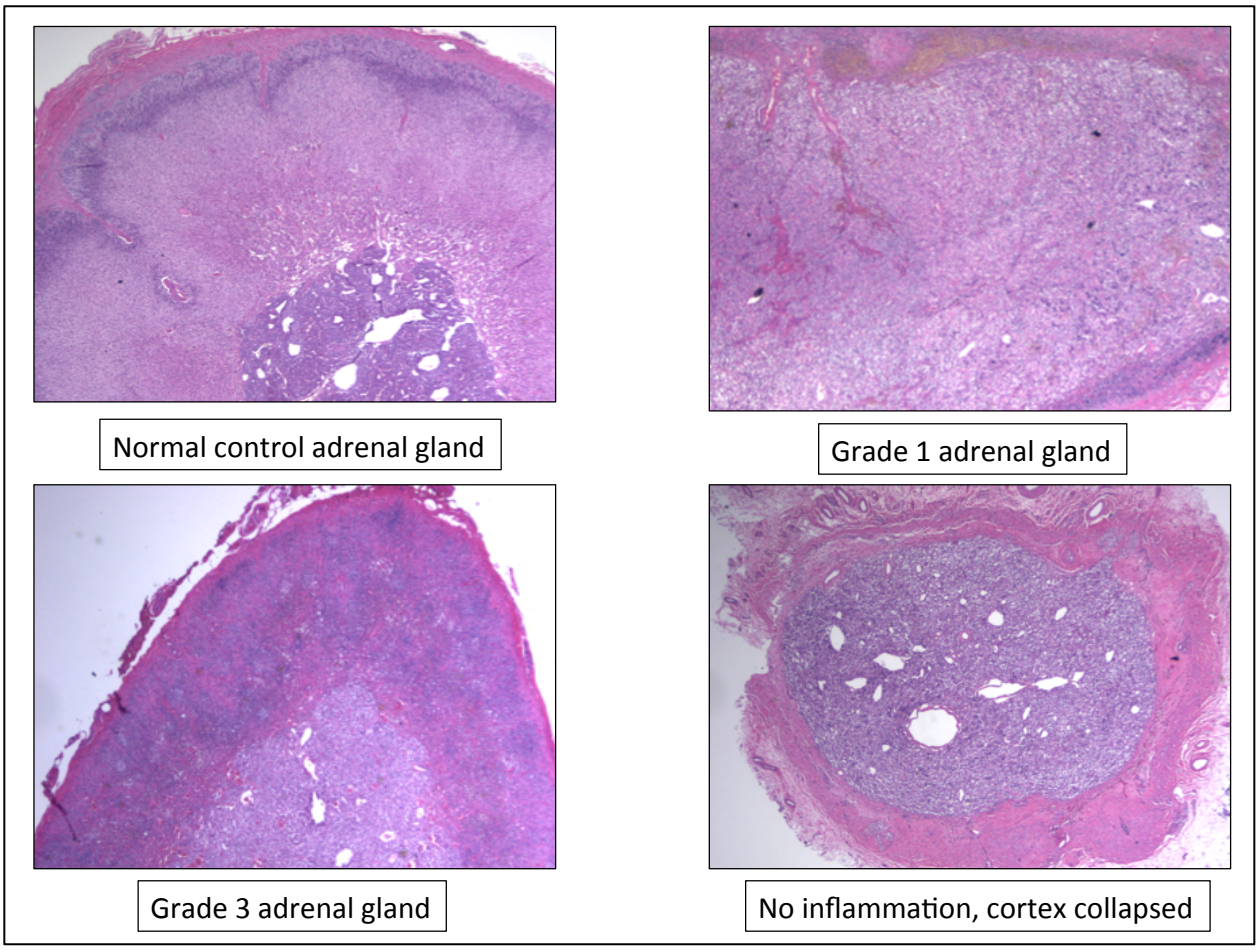


Figure III.2: Adrenal gland histology, 20x magnification, hematoxylin and eosin stain.

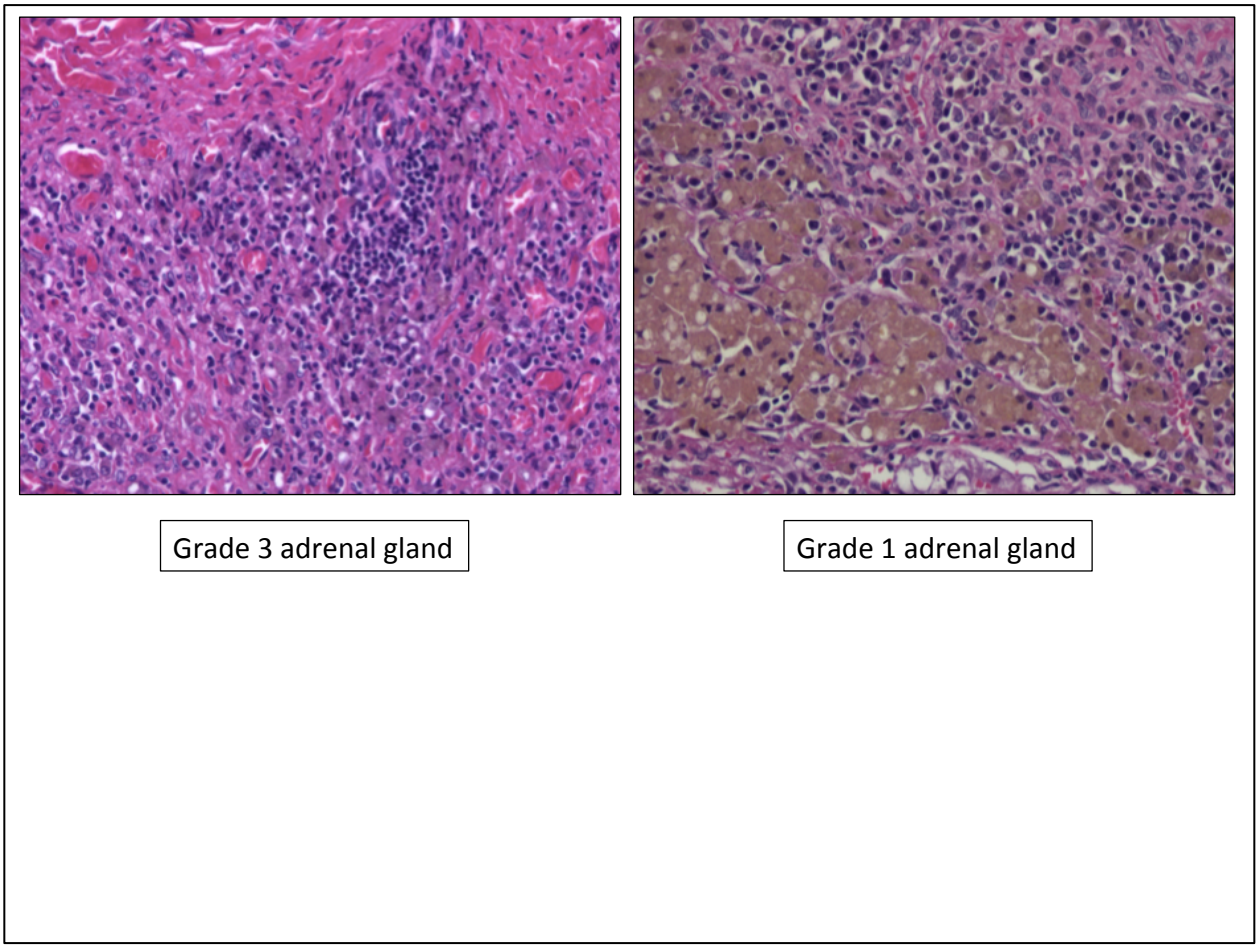


Figure III.3: Adrenal gland histology, 20x magnification, CD3 and CD79a immunohistochemistry.

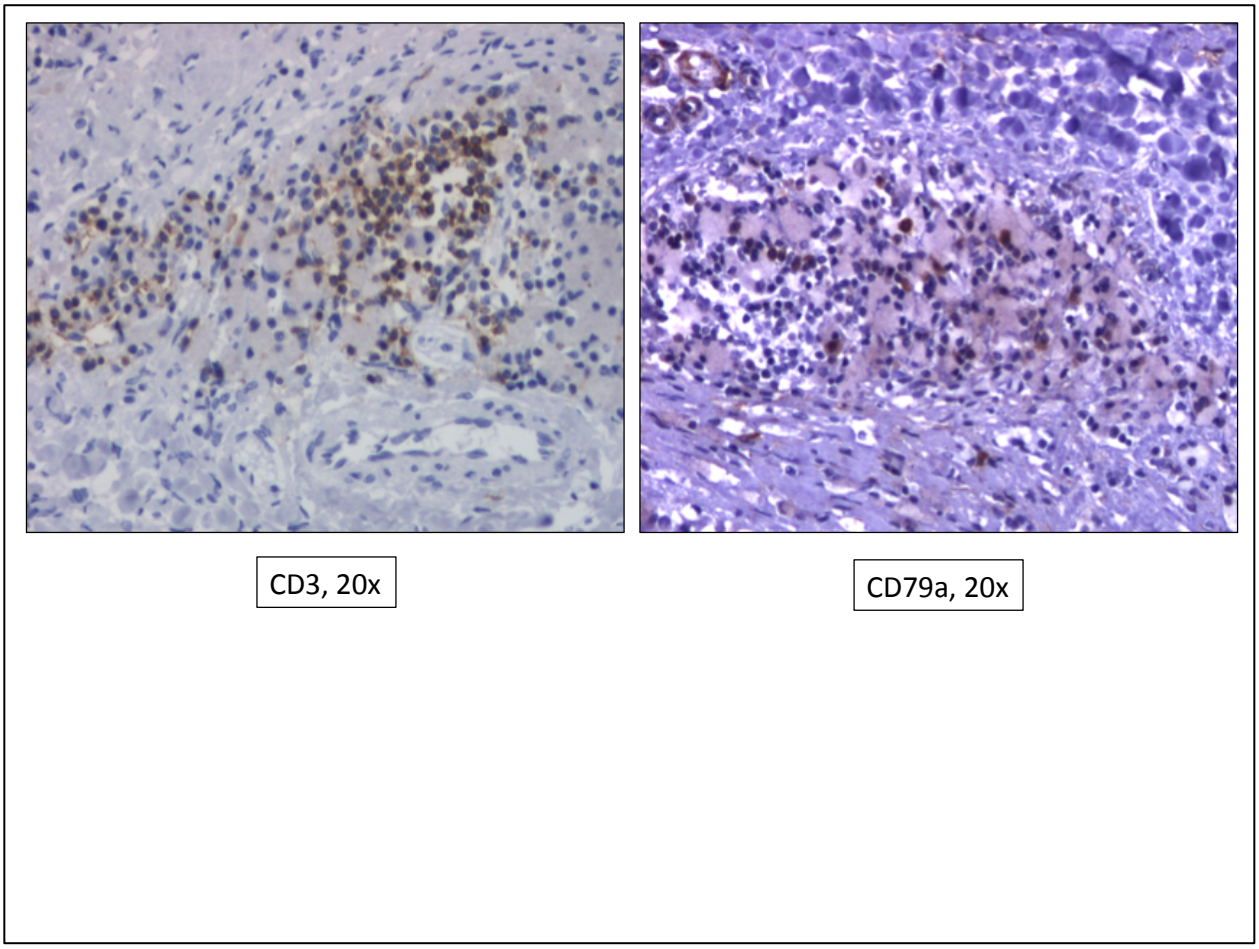


Figure III.4: Detection of anti adrenal antibodies with Western blotting.

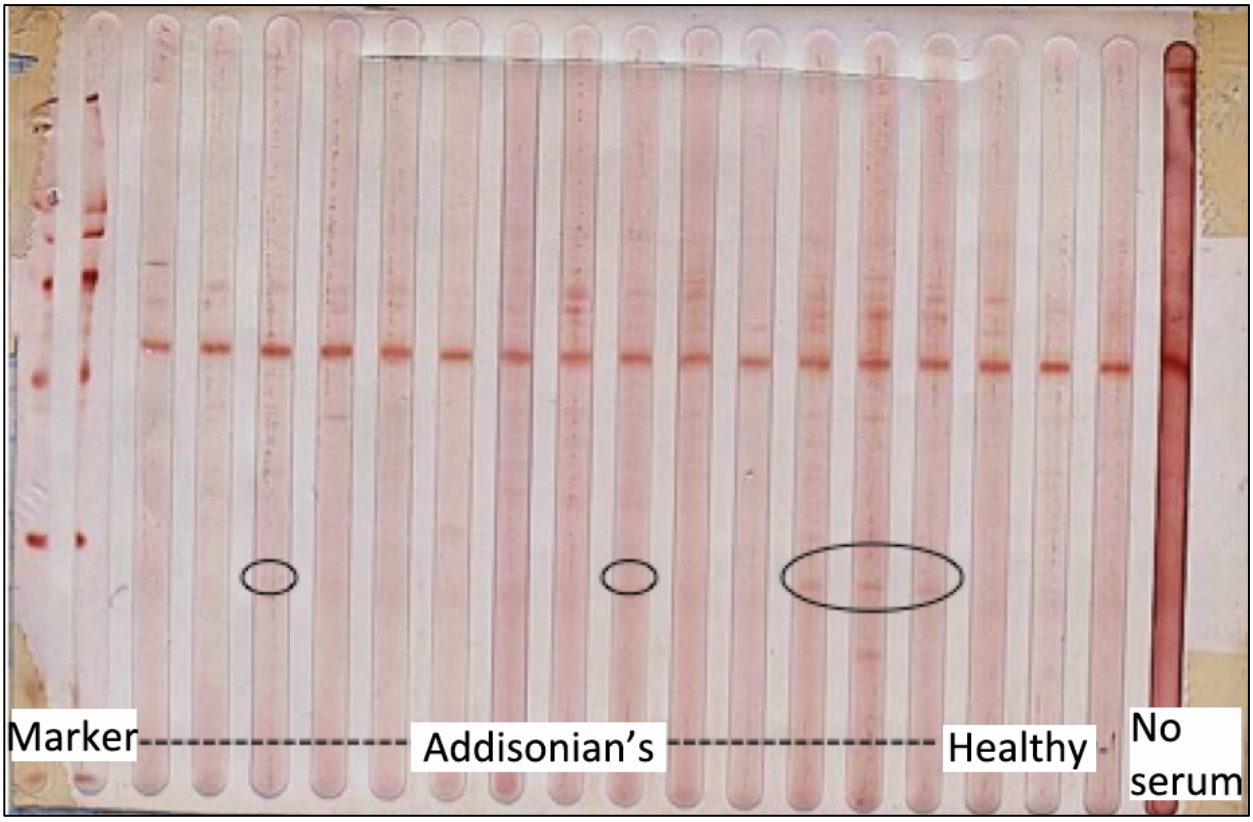


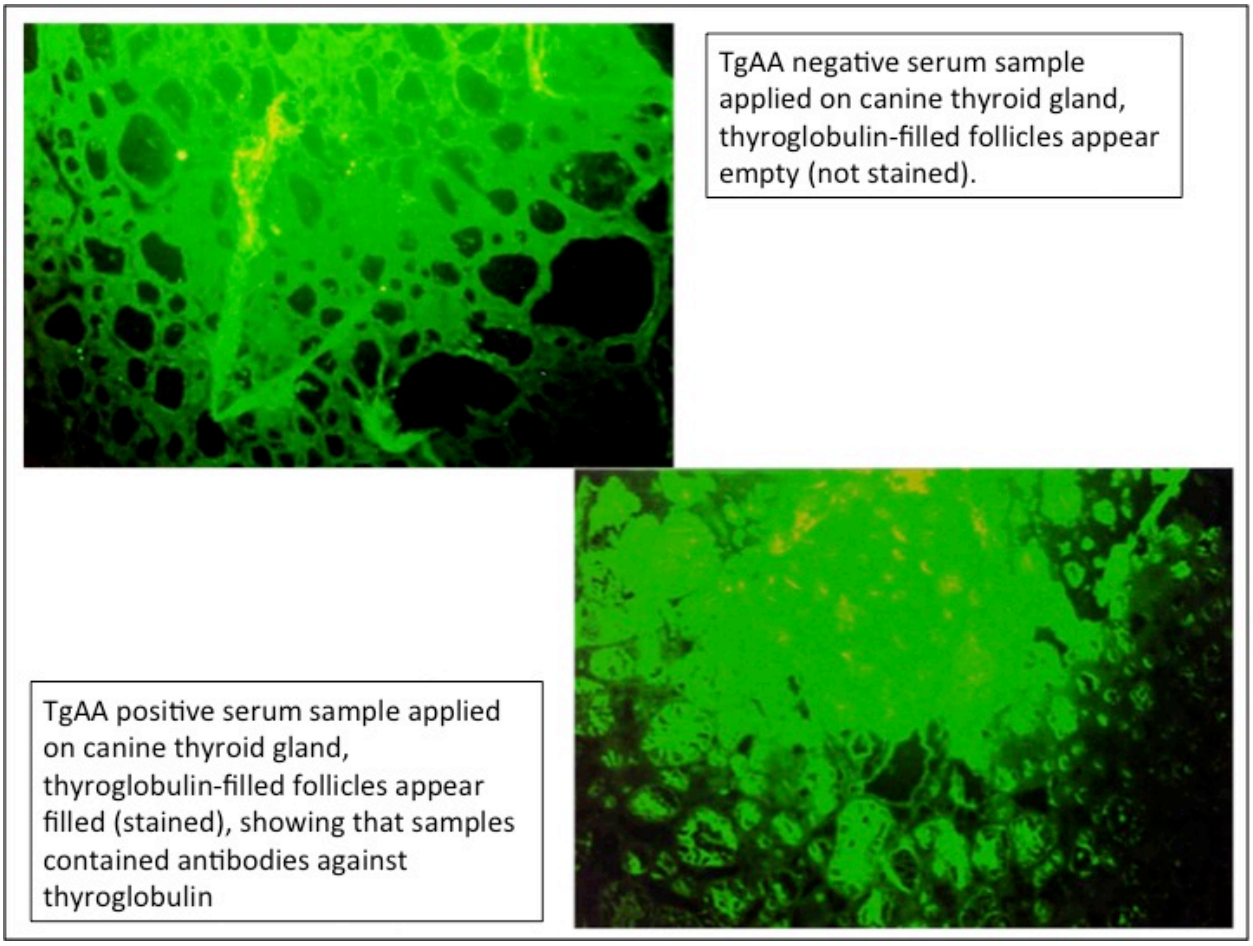
Table III.1: Results with a commercially available radioimmunoassay for the detection of 21-hydroxylase autoantibodies in human serum.

	Population	Positive	Median [U/mL]	25% percentile [U/mL]	75% percentile [u/mL]
Sick dogs (SD)	53	18 (34%)	0.8	0.7	1.1
High risk dogs (HR)	21	9 (43%)	0.8	0.6	1.2
Low risk dogs (LR)	13	0 (0%)	0.5	0.5	0.7

Table III.2: Results with radioactively labeled amino acid capture probe.

	Population	Median raw counts	Median [U/mL]	25% percentile [U/mL]	75% percentile [U/mL]
Sick dogs (SD)	54	6166.5	0.8	0.7	1.1
Low risk dogs (LR)	10	4587.5	0.5	0.5	0.7

Figure III.5: Detection of adrenal autoantibodies with indirect immunofluorescence technique.



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Chapter IV: Hypothesis and specific aims

Based on what is known about Addison's disease in humans and the results of the preliminary experiments, I formulated the following hypothesis and specific aims:

Hypothesis

As in human hypoadrenocorticism, anti-21-hydroxylase antibody production occurs in naturally occurring primary canine hypoadrenocorticism. The antibody production precedes clinical disease, and is more prevalent in dog breeds susceptible to developing hypoadrenocorticism.

Specific aims

1) To establish a diagnostic test to detect canine anti-adrenal autoantibodies;

2) To determine whether anti-adrenal autoantibodies are present in dogs with hypoadrenocorticism;

3) To determine whether development of anti-adrenal autoantibodies has breed, sex, and age-based predispositions.

Chapter V: The expression of canine 21-hydroxylase

Introduction

The overall amino acid homology between canine and human 21-hydroxylase is 78 %. As described in preliminary research, a radioimmunoassay for the detection of 21-hydroxylase antibodies in humans was tried for use in dogs. Results were unsatisfactory. Therefore, our strategy was to express recombinant canine 21-hydroxylase for use as an antigen to detect autoantibodies by either Western blot (WB) or by enzyme-linked immunosorbent assay (ELISA).

To get a feeling and get used to molecular techniques, I decided to do the following preliminary approach, which will not be covered in detail in this dissertation: I obtained canine adrenal glands within 30 sec of euthanasia and quick-froze them in liquid nitrogen. Total RNA was extracted using TRIZOL according to the instructions provided by the manufacturer and then reverse-transcribed via Superscript III® enzyme, using random hexamers. Primers were designed in the 5' and 3' untranslated region of the canine CYP21A2 gene and the whole transcript was amplified using TaKaRa LA PCR Kit version 2.1. NotI and BamHI restriction sites were added by PCR mutagenesis 5' of the translational start codon and 3' of the termination codon, respectively. The sequence was then compared to the published sequence and no mutations were detected. A GST (glutathione S-transferase) fusion protein expression vector was digested with the same restriction enzymes, and the cDNA fragment subcloned. This fused the entire canine 21-hydroxylase cDNA in frame 3' of GST. This expression construct was

transformed into BL21 (DE3) cells and expression of the 21-hydroxylase-GST fusion protein was induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside). Expression of the recombinant fusion protein was confirmed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), analyzing both soluble and insoluble fractions of the bacterial pellet. Only a minimal amount of 21-hydroxylase was expressed and found primarily in the insoluble fraction (figure V.1). Since the yield was unsatisfactory, I changed my approach.

The new approach, namely the generation of a panel of different fusion proteins (GST and His (hexa histidine) tags) generated according to the instructions provided by Arase and Waterman[1] will be discussed here. In short, the membrane anchor and basic region of P450c21 was deleted and replaced with MAKKTSSKGK from CYP2C3[2] and chaperone proteins were co-expressed to increase solubility and yield.

Materials and methods

Constructs: Cloning and construction of the modified canine 21-hydroxylase fusion proteins was done according to published methods[3]. In order to facilitate expression of the 21-hydroxylase, the membrane anchor and basic region of P450c21 was replaced with MAKKTSSKGK from CYP2C3 as indicated in the primers by small letters. A nested PCR (polymerase chain reaction) was done using forward primer 5'-acatcatctaaaggtaagCTCCCACCTCTTGTCCCT -3' and reverse primer 5'-GCGGCCGCTCATGGGTGCTGGCCACGT -3' in the first reaction and forward primer 5'-

GGGAGGGATCCCCatggctaaaaagacatcatctaaaggtaag -3’ (the underline indicating the BamHI site) and reverse primer 5’- GCGGCCGCTCATGGGTGCTGGCCACGT -3’ (the underline indicating the NotI site) in the second reaction, using the previously obtained template and TaKaRa LA PCR Kit version 2.1 (TaKaRa Bio Inc., Otsu, Shiga, Japan). The reverse primer contained a stop codon immediately following the NotI site. The PCR product was isolated (Qiagen PCR Purificarion Kit, Qiagen, Valencia, CA, USA) and ligated into the TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequencing using M13 forward- and M13 reverse primers, as well as internal forward primer 5’- gccatcatctgtcacctcac -3’ and internal reverse primer 5’- cgaatcccgtacagagaccc -3’. The sequence was compared with the modified canine 21-hydroxylase derived from the unmodified canine 21-hydroxylase (GenBank accession number BAB79541.1). Plasmid DNA was then extracted with standard miniprep and digested with BamHI and NotI. The resulting digest was gel purified using a gel purification kit (Qiagen) and ligated into vectors pET28c and pGEX-5X-3, respectively, digested with the same restriction enzymes, thus creating the modified canine 21-hydroxylase/ pET28c and the modified canine 21-hydroxylase/ pGEX-5X-3 construct. Both ligation products were then transformed into *E. coli* DH5α maximum efficiency cells. In order to replace the BamH1 site with NdeI and to remove the stop codon at the 3’ end, the modified canine 21-hydroxylase/ pGEX-5X-3 DNA was used as a template for PCR with forward primer 5’- GGTCGTGGGATCCATATGGCTAAAAAGACA -3’ (the underline indicating the NdeI site) and reverse primer 5’- CGATGCGGCCGCTAATGGGTGCTGGCC -3’ (the underline indicating the NotI site). The PCR product was isolated (Qiagen PCR Purificarion Kit) and ligated into the TOPO vector (Invitrogen) for sequencing as described above. After sequence analysis, the NdeI/NotI

fragment was cut out and subcloned into pET42b, yielding the expression plasmid modified canine 21-hydroxylase/ pET42b.

Chaperone vector pG-KJE8 (TaKaRa) was transformed into BL21 (DE3) Gold cells (Agilent Technologies Inc., Santa Clara, CA, USA) and C41(DE3) cells (Lucigen Corporation, Middletown, WI, USA), which were made competent using a standard $CaCl_2$ protocol[4]. The three previously obtained constructs were then transformed into these BL21 (DE3) Gold, and C41 (DE3) cells, respectively, both containing the chaperone expression plasmid pG-KJE8, and screened on LB plates supplemented with the appropriate antibiotic. For an overview of the final expression systems see table V.1.

Small-scale induction: From the three expression systems described above in the two different hosts, a 2.5 mL Lysogeny broth (LB) culture with the appropriate antibiotics was grown overnight at 37°C under constant shaking. The next day, this culture was transferred into 250 mL of LB, again containing the appropriate antibiotics. The culture was shaken constantly at 37°C. Once an OD between 0.4 and 0.6 was reached (with some selected conditions, expression was not initiated until the OD reached 1.0), a negative control, of 1.5 mL culture was taken and spun down at 4°C at 12,000 rpm for 1 min and the pellet was kept at -80°C until further analysis. The culture was allowed to cool down to the temperature at which the expression was done. To each culture, 1 mM δ-aminolevulinic acid (precursor of heme biosynthesis) was added. 21-hydroxylase fusion protein expression was then induced with 1 mM IPTG. In addition to the 1 mM IPTG and 1 mM δ-aminolevulinic acid, the following additions were tested in separate

cultures: 5 ng/ mL Tetracycline (for the induction of chaperone proteins GroES, and GroEL); 4 mg/ mL of Arabinose (for the induction of chaperone proteins DnaK, DnaJ, and GrpE), 5 ng/ mL Tetracycline, and 4 mg/ mL of Arabinose; 5 ng/ mL Tetracycline, and 2 % ethanol (to increase solubility). To evaluate optimal temperature expression conditions, each condition was also grown at three different temperatures (16°C, 27°C, and 37°C). All these conditions resulted in 90 different set-ups (indeed, I was that crazy!). All these cultures were shaken for another 72 hours, taking 1.5 mL samples at 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 48, and 72 h post induction. These samples were spun down as described previously and kept at -80°C until analysis. At the end of the experiment, the remainder was spun down at 4,400 x g for 30 min at 4°C and the pellets were kept at -80°C until further analysis. Most of the 1350 different samples were analyzed for expression levels, and solubility of fusion protein.

Determination of cellular location of the 21-hydroxylase fusion proteins by cell fractionation: First, the cell pellets that were obtained from the 1.5 mL mini-cultures were suspended in 150 µL of freshly prepared solution of lysozyme (1 mg/ mL), 20 % sucrose, 30 mM Tris HCl (pH 8.0), 1 mM EDTA (Ethylenediaminetetraacetic acid) (pH 8.0), and placed on ice for 10 min. Cells were then recovered by centrifugation in a tabletop microcentrifuge at 12,000 rpm for 1 min at 4°C. Second, the resulting pellet was resuspended in 400 µL of 0.1 M Tris HCl (pH 8.0) and cells were broken open by freezing and thawing for three times in liquid nitrogen. The suspension was spun down for 5 min at 12,000 rpm at 4°C. Third, membrane proteins were solubilized by incubating the pellet in 500 µL 1 % Triton X-100 for 10 min at 4°C. The solution

was spun again for 5 min at 12,000 rpm at 4°C and all obtained fractions, including the remaining pellet, were loaded onto SDS-PAGE for analysis.

Large-scale expression of fusion proteins 21-hydroxylase: For electro-elution, the modified canine 21-hydroxylase in pET42b/ pGKJ-E8 and the modified canine 21-hydroxylase in pGEX-5X-3/ pGKJ-E8 were chosen. From each construct, a 10 mL LB culture with the appropriate antibiotics was grown overnight at 37°C under constant shaking. The next day, this culture was transferred into 1000 mL of LB, again containing the appropriate antibiotics. The culture was shaken constantly at 37°C. Once the OD reached between 0.4 and 0.6, the culture was allowed to cool down to 27°C. To each culture, 1 mM δ-aminolevulinic acid was added. 21-hydroxylase fusion protein expression was induced with 1 mM IPTG and the culture was shaken for another 38 hours. For electro-elution, the cultures were split into 100 mL aliquots and spun down at 4°C for 30 min at 4,400 x g and the pellets were kept at -80°C until further analysis. Fractionation was done as described previously, increasing the buffer volumes of the first and second step to 10 mL, and of the third to 15 mL.

For ELISA development, pET42b/ pGKJ-E8 was used and the whole 1 L bacteria culture was centrifuged at 4,400 x g (F10S rotor) for 30 min at 4°C and pellets were kept at -80°C until needed.

Results

Constructs: The translated sequence was compared with the modified canine 21-hydroxylase and 7 silent mutations were detected (T659C, G1011A, A1143G, A1278C, C1386T, C1539T, A1581G). The constructs were therefore used during the remainder of this study.

Small-scale induction and determination of cellular location of the 21-hydroxylase fusion proteins by cell fractionation: The layout of the experiment enabled me to confidently identify the expressed 21-hydroxylase fusion proteins (21-hydroxylase-GST: 75 kDA, 21-hydroxylase-His: 50 kDa) as well as chaperone proteins DnaK (70 kDa), DnaJ (40 kDa), GrpE (22 kDa), GroES (10 kDa), and GroEL (60 kDa), by comparing the uninduced cultures and the different conditions with one another, respectively. It also became clear that induction of DnaK made identification of 21-hydroxylase-GST difficult. Protein fractionation enabled me to determine the exact location of the expressed modified canine 21-hydroxylase. The first fraction represents the periplasmic, the second the cytoplasmic, and the third fraction the membrane bound protein. The Laemmli buffer resuspended left-over pellet represents the inclusion body protein. In short, analysis by SDS-PAGE showed that most of the expressed 21-hydroxylase remained in the insoluble fraction, independent of construct, expression temperature, addition of ethanol, OD at induction, and the co-expression of chaperone proteins. The host cell did not have an effect on expression yield, either. Optimal conditions for expression were established to be 38 hours at 27°C without the induction of chaperone proteins. The expression system containing the modified canine 21-hydroxylase in pET42b and pGKJ-E8 resulted in more expression of the 21-hydroxylase-His than the expression system

with the modified canine 21-hydroxylase in pET28c and pGKJ-E8. In general, protein expression was higher in BL21 (DE3) Gold cells than C41 (DE3) cells.

Large-scale expression of fusion proteins 21-hydroxylase: With the previously established conditions, I was able to repeatedly express robust levels of 21-hydroxylase-His and 21-hydroxylase-GST.

Discussion

Protein production in *E. coli* enables the researcher to produce a large amount of the protein of interest for further studies. The most extensively used expression systems for the expression of recombinant proteins are the GST- and His-tag fusion protein systems.

Unfortunately, it is very difficult to produce recombinant P450 enzymes in *E. coli*. It has been shown that modifications and/ or the co-expression of chaperone proteins are necessary. For example, successful expression of 21-hydroxylase in *E. coli* depends on N-terminal modification and co-expression with chaperone proteins GroEL and GroES[1]. The use of molecular chaperones GroES and GroEL has also been shown to be essential for the expression of this class of enzymes such as mitochondrial vitamin D3- hydroxylase CYP27B1[5,6]. In the CYP2B subfamily, it has been shown that expression levels are increased by truncation of the N-terminal anchor[7]. The same has been confirmed for several other P450 enzymes[1]. Other groups replaced the basic region of difficult to express P450s with ones that can be efficiently

expressed. The basic regions of CYP2C11 and CYP17 have been used to increase the expression of the aromatase CYP19[8,9] and that of CYP2E1 has been used for the overproduction of CYP2C8 and CYP2A6[10]. Arase and Waterman used the modified sequence of the basic region MAKKTSSKGK from CYP2C3 for the successful expression of bovine P450c21 upon co-expression with chaperones GroES and GroEL[1]. This modified sequence had previously been used for the expression of soluble and monomeric forms of P450s[11].

Besides these difficulties, key points in the production of soluble, intact fusion proteins are to optimize several factors including selection of host strain, growth temperature, cell density at time of induction, length of expression, addition of ethanol to growth media, and co-expression of chaperone proteins[12]. In both above-mentioned systems, the expression of inserts is under the control of the IPTG-inducible tac promoter. Both the pGEX vector, as well as the used pET vectors, have an internal *lacI* gene, whose product binds to the operator region of the tac promoter. This binding prevents expression until induction by IPTG. Even though this represents a tight control over expression of insert, basal levels of expression (leaky expression) might take place, making it more difficult to identify the protein of interest in the expression sample compared to the uninduced control. However, my controls allowed me to clearly identify the protein of interest.

A wide variety of *E. coli* host strains can be used for cloning and expression with pGEX and pET vectors. Specially engineered strains, which are more suitable for expression of fusion proteins, are preferred. An example of such a host is *E. coli* strain BL21 (DE3), a strain defective

in OmpT and Lon protease production[13-16]. In this work, strains BL21 (DE3) and C41 (DE3) were compared. The C41 (DE3) is a mutant host strain of the BL21 (DE3), which was selected for growing to high saturation density and for the continuous production of proteins without toxic effects when compared to the regular BL21 (DE3) cells[17]. C41 (DE3) host strain is frequently used to overcome the toxicity associated with overexpressing recombinant proteins that use the bacteriophage T7 RNA polymerase expression system[17]. Often times sufficient protein expression can not be achieved in BL21 (DE3) cells because of bacterial cell death caused by the foreign protein. My studies have shown that the regular host strain BL21 (DE3) was superior.

High-level expression of fusion proteins might result in the formation of an insoluble product, called inclusion body. An inclusion body is the formation of a dense precipitated aggregate in which the expressed fusion protein is complexed with RNA, making it very difficult to get into solution. To avoid formation of inclusion bodies, a variety of growth parameters should be investigated: lowering the temperature to between 16°C and 30°C, inducing for a shorter time, inducing at a higher cell density, adding ethanol to the growth media, the co-expression of chaperone proteins, and increasing aeration. All possibilities were evaluated, but the 21-hydroxylase fusion proteins primarily remained in the insoluble fraction. Chaperone proteins have been shown to increase solubility of expressed fusion proteins. The formation of inclusion bodies can be a result of improper folding of expressed proteins and chaperone proteins are involved in the proper protein folding process.

Since none of the suggested techniques worked, I decided to use the formation of inclusion bodies as a means to purify the protein of interest, even though the solubilization

later on proved to be difficult, too.

APPENDIX

Appendix

Figure V.1: The 21-hydroxylase-GST fusion protein is primarily found in the insoluble fraction. (-): uninduced; (+): induced with IPTG.

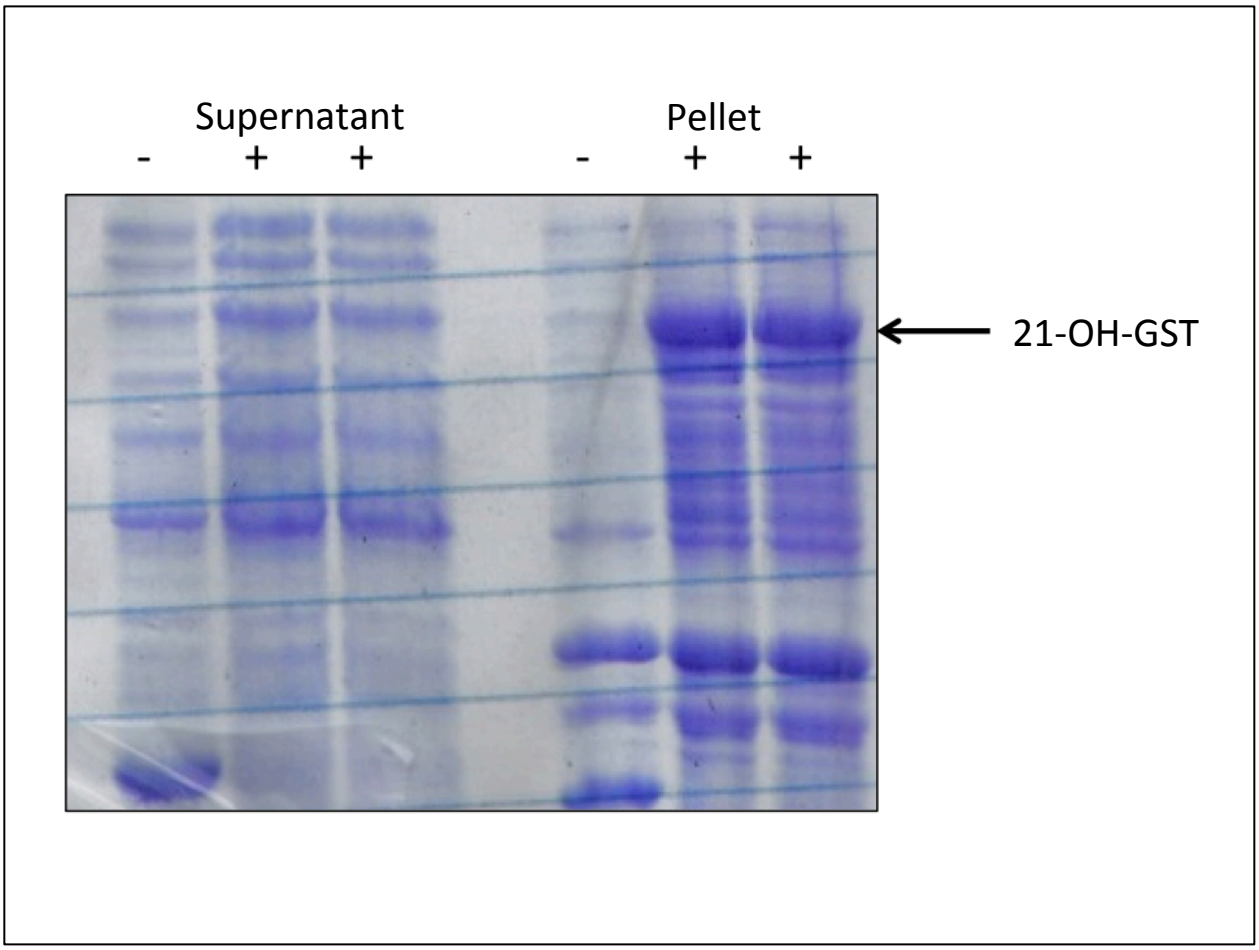


Table V.1: Overview of the final expression systems.

Plasmids	Tag	Protein (inducer)	Resistant marker
• Modified canine 21-hydroxylase in pGEX-5X-3 • pGKJ-E8	• N-terminal glutathione S-transferase (GST) • None	• Modified canine 21-hydroxylase (IPTG) • DnaK-DnaJ-GrpE (L-Arabinose) • GroES-GroEL (Tetra)	• Ampicillin • Chloramphenicol
• Modified canine 21-hydroxylase in pET28c • pGKJ-E8	• N-terminal 6x histidine • None	• Modified canine 21-hydroxylase (IPTG) • DnaK-DnaJ-GrpE (L-Arabinose) GroES-GroEL (Tetra)	• Kanamycin • Chloramphenicol
• Modified canine 21-hydroxylase in pET42b • pGKJ-E8	• C-terminal 6 x histidine • None	• Modified canine 21-hydroxylase (IPTG) • DnaK-DnaJ-GrpE (L-Arabinose) GroES-GroEL (Tetra)	• Kanamycin • Chloramphenicol

Figure V.2: The soluble proteins expressed in pET42b/ pGKJ-E8, clearly showing that hardly any 21-hydroxylase-His is in this fraction. Different conditions were tested (IPTG: 1 mM IPTG; AA: 1 mM δ-aminolevulinic acid; Neg: no IPTG; Tetra: 5 ng/ mL Tetracycline; Arab: 4 mg/ mL of Arabinose), which enabled me to identify the 21-hydroxylase and the chaperone proteins, and determine their location.

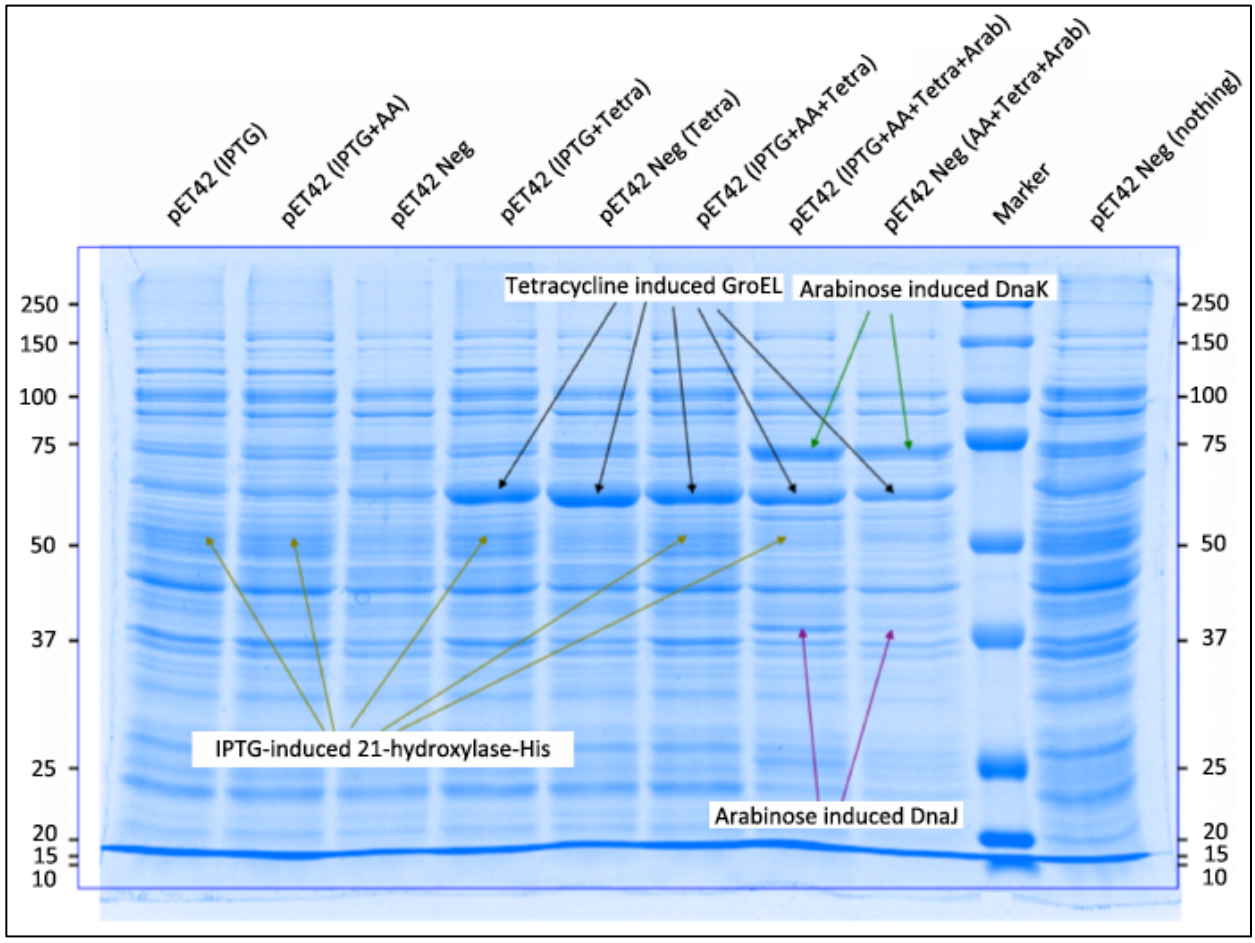


Figure V.3: Inclusion body proteins expressed in pET42b/ pGKJ-E8, showing that the 21-hydroxylase-His is primarily in this fraction. Different conditions were tested (IPTG: 1 mM IPTG; AA: 1 mM δ-aminolevulinic acid; Neg: no IPTG; Tetra: 5 ng/ mL Tetracycline; Arab: 4 mg/ mL of Arabinose), which enabled me to identify the 21-hydroxylase and the chaperone proteins, and determine their location.

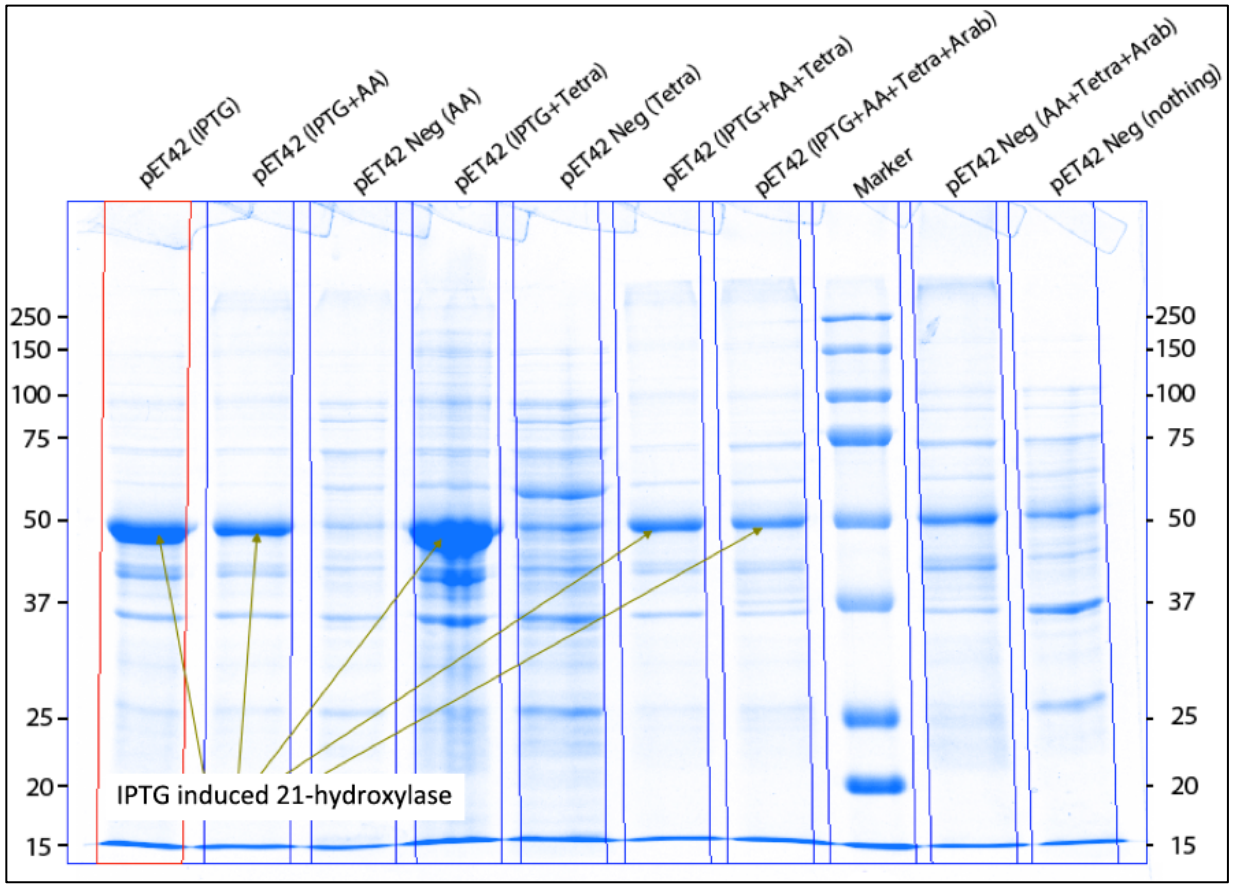


Figure V.4: Fractionation of pET42b/ pGKJ-E8, demonstrating that the 21-hydroxylase-His fusion protein is expressed into inclusion bodies (induced with IPTG to the left, without IPTG to the right).

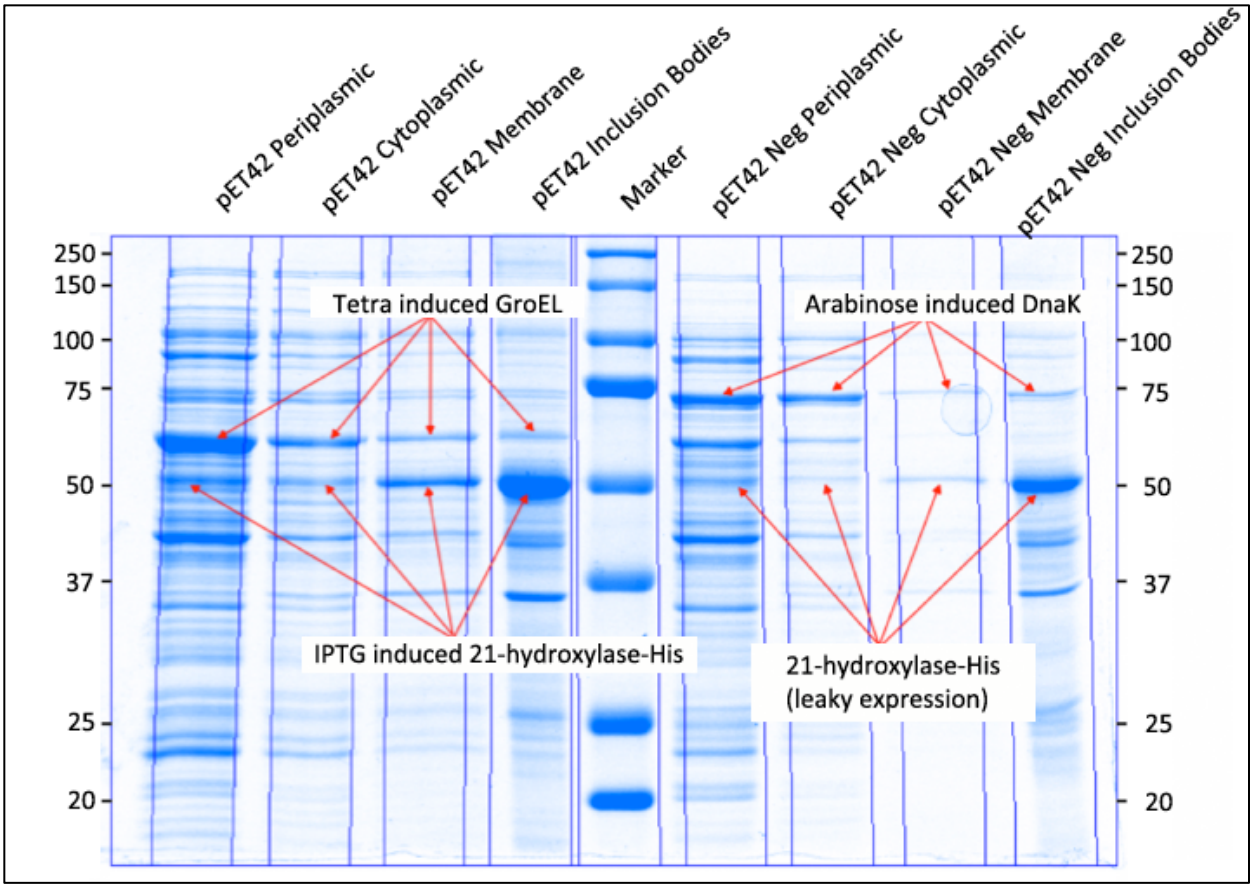
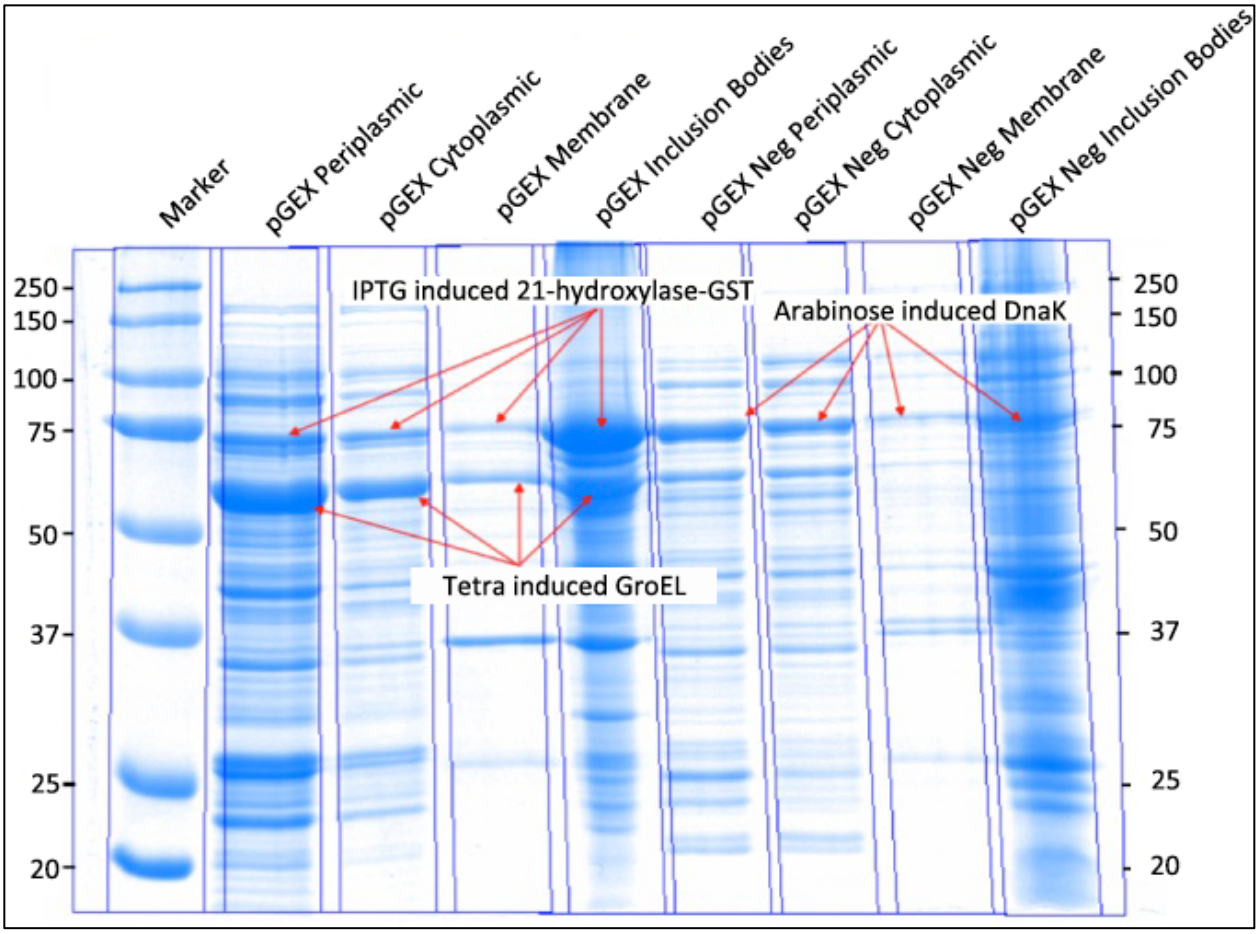


Figure V.5: Fractionation of pGEX-5X-3/ pGKJ-E8, demonstrating that the 21-hydroxylase-GST fusion protein is expressed into inclusion bodies (induced with IPTG to the left, without IPTG to the right).



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Chapter VI: Purification of fusion proteins 21-hydroxylase-glutathione S-transferase (21-hydroxylase-GST) and 21-hydroxylase-6xhistidine (21-hydroxylase-His)

Introduction

The purification of the 21-hydroxylase fusion proteins remained very difficult, because most of the expressed protein was in the insoluble inclusion bodies, even after optimization of expression conditions as discussed previously. Pure fusion proteins were required, however, for immunization of dogs and rabbits for antibody production (21-hydroxylase-GST) (21-hydroxylase-glutathione S-transferase), for running controls (control by size) during SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (21-hydroxylase-GST and 21-hydroxylase-His) (21-hydroxylase-hexa histidine) and for ELISA (enzyme-linked immunosorbent assay) development (21-hydroxylase-His). Since the requirements and the amount needed for these pure fusion proteins were different, different techniques were applied to purify them. A small-scale SDS-PAGE purification was performed, followed by a passive elution of the protein out of the gel-matrix for proof of concept. If successful, the plan was to scale up this method and to do an active elution out of a bigger gel to purify more fusion protein for the running control (by size) and as the antigen source for immunization. The immunization will be discussed in the following chapter (chapter VII). To create enough protein for ELISA

development, fast-protein liquid chromatography (FPLC) was used after solubilization of the 21-hydroxylase-His inclusion bodies. The 21-hydroxylase-GST was chosen for injection, because the GST tag has a size of 220 amino acids (roughly 25 kDa), which increases the immunogenicity of the 21-hydroxylase. The 21-hydroxylase-His was chosen as the antigen for ELISA development, since it is easier and much more economical to purify His-tagged fusion proteins in a larger scale than GST-tagged proteins.

Materials and methods

Elution of the fusion proteins out of SDS gel-matrix: For the passive small-scale gel purification, 100 mL of each 21-hydroxylase-GST and 21-hydroxylase-His induced bacteria cultures were fractionated as described before. The remaining pellets were dissolved in 3000 µL of 2x SDS-PAGE Laemmli sample buffer. From each mixture, 100 µL were run on a 1 mm thick 10 % SDS-PAGE gel, using BioRads Protean Mini system (BioRad, Hercules, CA, USA) at constant 180 V for 60 minutes. Three protein staining techniques were compared: gels were stained (1) in 0.25 M KCl solution, (2) in Coomassie, and (3) in Coomassie, where only a cut-off side strip of the gel was stained, followed by aligning this stained strip with the rest of the gel. After the fusion proteins were located, the 21-hydroxylase-GST and the 21-hydroxylase-His bands were excised with a clean razor blade. When the bands were cut out, the remaining gels were stained in Coomassie to determine the accuracy of the excision. All cut out bands were then carefully cut-up into small pieces and transferred into 1.5 mL microcentrifuge tubes containing 500 µL elution buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1mM EDTA (ethylenediaminetetraacetic acid);

pH 7.5) and incubated overnight on a rotary shaker at 30°C. The next day, the tubes were centrifuged at 12,000 rpm for 10 minutes and the supernatants were carefully pipetted into clean microcentrifuge tubes. An aliquot of 20 µL of each supernatant was tested for the presence of each fusion protein via mini 10 % SDS-PAGE electrophoresis, followed by staining with Coomassie blue.

For the active, scaled-up gel purification, 100 mL of each 21-hydroxylase-GST and 21-hydroxylase-His induced bacteria culture were fractionated as described before. The remaining pellets were dissolved in 3000 µL of 2x SDS-PAGE Laemmli sample buffer and the total volume was loaded onto a 3 mm thick 6 % SDS-PAGE gel, prepared using BioRad's Protean II XL system (gel size: W x H = 18.3 cm x 20 cm), as shown in figure VI.1. Usually the gels were loaded as follows: molecular marker, small volume 21-hydroxylase preparation, protein sample, and again a small volume of 21-hydroxylase preparation. For the small volume of the 21-hydroxylase preparation, only 100 µL of the protein solution were loaded, to make the identification and excision of the band of interest easier by getting a sharper band. The gels were then run at constant 210 V for 5 hours with cooling with cold water. The gels were stained in Coomassie blue for 30 minutes and then destained with Coomassie destain solution until the bands of interest were easily identified. The bands were then excised with a clean razor blade and transferred into Coomassie destain solution and kept at 4°C until further use.

For the electro-elution, the following two buffers were prepared: soaking buffer (2% SDS in 0.4 M NH_4HCO_3), and elution buffer (0.1 % SDS in 0.05 M NH_4HCO_3). For details on the

electrophoretic elution tank and the electrophoretic elution cell, manufactured by C.B.S. Scientific Corporation, Del Mar, California, USA, see figures VI.2 and VI.3. A 15 kDa cut-off Spectra 6 dialysis membrane was soaked in 0.1 % NaN_3 and inserted into the electrophoretic elution cell. The previously excised bands were briefly rinsed with soaking buffer and cut-up into 1 mm x 1 mm cubes. The cubes were then soaked for 5 minutes in soaking buffer to remove any remaining Coomassie destain solution and the buffer was removed by suction and blotting. The pieces were transferred into the gel loading well of the electrophoretic elution cell and covered with soaking buffer. This mixture was stirred until no more bubbles would escape and DTT (dithiothreitol) was added to a final concentration of 0.1 % (total volume of the gel loading well from the bottom of the cell to the bottom of the cross passage is 2 mL) and incubated for 30 minutes. After that, the gel pieces in soaking buffer were carefully overlaid with elution buffer just to the top of the cross passage, which also filled up the sample collection well. The electrophoretic elution cell was then inserted into the elution tank and elution buffer was added to the elution tank to a level just above the drain ports in each electrode chamber and another 75 mL of elution buffer was added to the mixing chamber. A two-channel peristaltic pump was used to move buffer (3 mL/ min per line) from the mixing chamber to the electrode chamber. In order to avoid an extraneous current path, care had to be taken that the buffer was dripping rather than streaming out of and into the electrode chambers. Air bubbles were removed from underneath the elution cell caps by using a bent Pasteur pipette and a constant 50 V was applied for 24 hours, cathode near the gel loading well. Throughout these 24 hours, the air bubbles underneath the elution cell caps needed to be removed on a more or less regular basis. After 24 hours, the voltage was reversed for 30 sec to

loosen any protein that was stuck to the dialysis membrane and the liquid from the gel loading well and the cross passage was carefully removed and stored independently. After that the liquid from the sample collection well was pipetted up and down for 10 times to further loosen any protein that was stuck to the dialysis membrane, transferred into a clean microcentrifuge tube, and kept at 4°C until further analysis and use. The gel pieces were again covered with soaking buffer, containing 0.1 % DTT, carefully overlaid with elution buffer to the top of the cross-passage and re-inserted into the elution tank, containing fresh elution buffer. The elution was repeated for another 24 hours. During optimization of the electro-elution, I also used a potential difference of 75 V to increase protein yield. At the end of both runs, all fractions were collected and analyzed via 10 % SDS-PAGE, loading 1 µL, 5 µL, and 10 µL, all including 1x Laemmli sample buffer, and the optical density was compared to a four-point BSA standard curve (0.1 µg, 1 µg, 5 µg, and 10 µg) to estimate protein concentrations, using the BioRad ChemiDoc XRS+ and the manufacturer provided software Image Lab.

To confirm the presence of 21-hydroxylase-GST and 21-hydroxylase-His fusion proteins in the electro-eluted samples, the following three experiments were done: First, the actual size of the eluted protein was compared with the calculated size of each fusion protein on SDS-PAGE; second, a standard Western blot (WB) with both fusion proteins was carried out, using 1/10 of the amount of protein that was easily visible on a Coomassie stained gel, and the manufacturer recommended concentrations of anti-GST (1:200) or anti-His (1:3000) as the primary antibody in 1 % (w/V) instant nonfat dry milk in PBS-T (1xPBS (phosphate buffered saline), containing 1 % Tween), and a horseradish-peroxidase labeled anti-mouse IgG

(immunoglobulin gamma) antibody in 1 % (w/V) instant nonfat dry milk in PBS-T (1:5000) as the secondary antibody; and third, the samples were analyzed using Matrix-assisted laser desorption/ionization (MALDI).

SDS-PAGE was done according to Laemmli[1]. The WB technique, immediately following SDS-PAGE, in detail: Polyvinylidene fluoride (PVDF) membranes were cut to an approximate size of the SDS-PAGE gel. Two Whatman papers per each PVDF membrane were cut to a size slightly bigger than the PVDF membrane. The PVDF membrane was then soaked in 100 % methanol. Transfer buffer was prepared (900 mL of 10 mM 3-[cyclohexylamino]-1 propane sulfonic acid (CAPS) plus 100 mL 100 % methanol), which was poured into a glass form, the running apparatus (BioRad) and a little bit into a weight boat. The PVDF membranes were then equilibrated in the weight boat containing the transfer buffer. The sandwich cast was then inspected for cracks and breaks and put into the glass form containing the transfer buffer, the black side facing down. The two foam pads were also put into this glass dish for soaking. The SDS-PAGE gel was disassembled, the stacking gel was removed with a razor blade and the remaining gel was cut on both sides at the spacer with a razor blade. One of the soaked foam pads was then put on the black side of the cast, followed by a Whatman paper, followed by the SDS-PAGE gel. At this point, everything was centered and any air bubbles were removed. The PVDF membrane was then removed with forceps from the transfer buffer and placed on top of the gel. The second soaked Whatman paper was then placed on top of the membrane and the whole sandwich was streaked out with a cut-off pipette to increase contact and to remove air-bubbles. The second pad was put on top, and the cast was closed. The sandwich was then

placed into the transfer apparatus, the black side facing the black side of the apparatus. Each apparatus could hold two sandwiches. An ice-pack was then added and the whole apparatus was filled with transfer buffer, so that the whole sandwich was submerged in it. The transfer was done using constant 0.51 A for 60 min. For incubation with the primary antibody, 1 L of 1xPBS-T, was prepared. In 50 mL, 0.5 g of instant nonfat dry milk was dissolved, and the antibody was diluted in it according to manufacturer recommendations to a total volume of 1 mL per membrane. A glass plate was covered with stretched parafilm. 1 mL of each antibody-containing buffer was then pipetted onto this wrapped glass plate and one membrane was placed onto one antibody-containing buffer, using forceps, paying attention not to introduce any air-bubbles. This was then closed with an old culture-dish lid and transported into the cold room and incubated overnight at 4°C. The next day, the membrane was washed three times for 15 min on a shaker with 1xPBS-T in a weight boat. The horseradish peroxidase labeled secondary antibody was then diluted in 1xPBS-T containing 1 % (w/v) instant nonfat dry milk according to manufacturer recommendations to a total volume of 10 mL and the washed membrane was incubated in it on a shaker for one hour at room temperature. After that, the membrane was washed three times as described previously. The chemiluminescence solution was then prepared, mixing one vial of luminol (50 µL of 0.44 g of luminol dissolved in 10 mL of dimethyl sulfoxide (DMSO)), one vial of coumaric acid (25 µL of 0.15 g of coumaric acid in 10 mL DMSO), 10 mL of 0.1 M Tris (pH 8.5), and 5 µl of H_2O_2. The membrane was then placed into this solution for 60 sec and kept in the dark, before pictures were taken on the ChemiDoc XRS+.

For MALDI, 50 µl of the electro-eluted 21-hydroxylase-His and 50 µl of the electro-eluted 21-hydroxylase-GST were digested with 1 µl of 0.1 µg/ µl trypsin for 2 h at 37°C. The digested protein was then mixed with 6 µl of 1 % Trifluoroacetic acid (TFA) and 8.33 µl of 6 M guanidine-HCl. Three µl of 50 % acetonitrile (ACN) in 0.1 % TFA in MilliQ H_20 was pipetted into a clean microcentrifuge tube. The C18 ZipTip (Millipore/Fisher) was wetted twice with 100 % ACN, the ACN was discarded, and the ZipTip was equilibrated twice with 0.1 % TFA in MilliQ H_20, again discarding the 0.1 % TFA in MilliQ H_20. The protein mixture from above was then bound to the tip by pipetting up and down 10 times, without the introduction of air. After that, the tips were washed three times with 0.1 % TFA in MilliQ H_20, by taking up the solution and discarding it. Elution of the protein out of the ZipTip was then done by pipetting up and down four times, without the introduction of air, the 50 % ACN in 0.1 % TFA in MilliQ H_20 that has previously been dispensed into a clean microcentrifgue tube. This cleaned-up sample was then submitted to be analyzed with MALDI at Michigan State University (MSU), East Lansing, MI, USA, and the resulting data were compared with a theoretical trypsin digest of the protein.

Purification of 21-hydroxylase-His for ELISA development: For ELISA development, the 21-hydroxylase-His fusion protein inclusion bodies were solubilized. The following solubilization conditions were tested: (1) 8 M urea in TBS (tris buffered saline) (50 mM Tris HCL, 150 mM NaCl (pH 8.0), (2) 8 M urea and 5 mM DTT in TBS, (3) 8 M urea, 5 mM DTT, and 5% Triton X-100 in TBS, (4) 8 M guanidine and 5 mM DTT in TBS, (5) 50 mM CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) in TBS, (6) 5 % TWEEN-20 in TBS, and (7) 5 %

polyoxyethylene-9-laurylether, 5 mM DTT, 0.05 mg/mL deoxyribonuclease, and 0.2 mg/mL lysozyme in TBS. Prior to use, 0.1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to each solution. Seven bacterial pellets from 100 ml of induced 21-hydroxylase in pET42 BL21 (DE3), in which the chaperone proteins were not induced, were fractionated and spun down as described previously. The supernatants were discarded. The resulting pellets were washed twice with 20 mL TBS-T (0.05% Tween in TBS). Each wash was followed by centrifugation at 20,000 x g (F21-B rotor) at 4°C for 15 min and the supernatants were discarded. The third wash was done with TBS without the addition of Tween. After centrifugation and discard of the supernatants, 5 mL of one the above-mentioned solutions was added to the pellet, which was then dissolved by brief sonification. A stir bar was then added to each and the samples were slowly stirred at room temperature for 24 hours. A 200 µL aliquot from each sample for analysis on SDS-PAGE was collected every 30 to 60 min. All aliquots were spun down for 4 min at 15,000 rpm in a table-top microcentrifuge. All supernatants but the ones containing guanidine were mixed with Laemmli sample buffer to a final concentration of 1x. The pellets that did not contain guanidine were dissolved in 200 µL 2x Laemmli sample buffer. To 100 µL of the supernatants of the guanidine containing samples, 900 µL of ice cold ethanol was added and the samples were kept overnight at -20°C. The guanidine containing pellets were dissolved in 450 µL of ice cold ethanol and were also kept overnight in the freezer. The next day, these samples were spun down at 15,000 rpm for 15 min at 4°C. The resulting pellets were washed with 800 µL of 90 % ice cold ethanol and spun at 15,000 rpm for 15 min at 4°C. After that, pellets were allowed to air dry and were dissolved in 100 µL 2x Laemmli sample

buffer. 5 µL of each sample was then loaded onto SDS-PAGE. As a size control, I included the previously purified and electro-eluted 21-hydroxylase-His on all gels. After analysis of results, these additional solubilization conditions were tested: (1) 8 M guanidine and 10 mM DTT in TBS, (2) 8 M guanidine and 4 mM DTT in TBS, and (3) 8 M guanidine in TBS. The experiment was performed in triplicate, comparing the effect of the inclusion body wash on yield and purity of protein as well as the effect of the previously described fractionation of the protein on yield and purity. Otherwise, the experiment was done the same way as described above. Results again were analyzed. The experiment was repeated once again, using 8 M guanidine and 4 mM DTT in TBS, taking an aliquot every 20 minutes over 5 hours. After that, a pull-down was performed as proof of concept and to evaluate the effect of DTT on the resin, using HisPur Cobalt resin (Thermo Scientific, IL, USA). 600 µL slurry was pipetted into 2 microcentrifuge tubes and washed three times with 1 mL of H_2O. Each wash step was followed by centrifugation at 1,200 rpm at room temperature in a table-top microcentrifuge and discard of the supernatant. The resin was then washed three times with TBS, including 8 M guanidine, and the other tube with TBS, including 8M guanidine and 4 mM DTT, again, setting the supernatant aside after each centrifugation. 500 µL of the solubilized protein plus 500 µL of either TBS with 8 M Guanidine or TBS with 8 M Guanidine and 4 mM DTT were then applied and the tubes were transferred onto a rotary shaker in the cold room where they were left for an hour. After an hour, the tubes were centrifuged as mentioned above and the supernatants were set aside. The resin was then washed three times with the same buffers that were used before, this time however also containing 20 mM imidazole. The protein was then eluted from the resin, again using the same buffers as before, this time however containing 200 mM imidazole. The

different fractions (SN (supernatant) post incubation, wash fractions post incubation, elution fractions) were analyzed on SDS-PAGE post ethanol precipitation.

The sonification and solubilization condition that were superior to the others and which were then repeated multiple times, now using 1 L of induced 21-hydroxylase in pET42 BL21 (DE3), in which the chaperone proteins were not induced, are summarized in detail: 1 L of induced bacteria cell cultures was centrifuged at 4,400 x g (F10S rotor) for 30 min at 4°C and the supernatant was discarded. The resulting pellet was kept at -80°C until further use. The pellet was then sonicated on ice in 90 mL Buffer A (50 mM potassium phosphate (pH 7.4), 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, 500 mM sodium acetate, 1.5 % sodium cholate, and 1 % Tween), including freshly added 0.1M PMSF, three times for 2 min at 3.5 output and 50 % duty cycle. The sample was allowed to cool down on ice for 6 minutes in between each sonification step. The resulting lysate was spun down in an ultracentrifuge at 45,000 x g at 4°C for 60 min. The supernatant was discarded and the pellet was frozen at -80°C until further use. The pellet was then resuspended in ice-cold 40 mL TBS, containing 8 M guanidine, 4 mM DTT, and 0.1 mM freshly added 0.1 mM PMSF and stirred at room temperature for 2 h 20 min, spun down at 23,500 x g (SS34 rotor) at 4°C for 30 min. The resulting supernatant was diluted 1:4 in TBS, containing 8 M guanidine, and 0.1 mM freshly added PMSF, before being purified on FPLC. The pellet was discarded.

For FPLC, the following buffers were prepared: Equilibration buffer (EB) (8 M guanidine in TBS), wash buffer (WB) (8 M guanidine, and 10 mM imidazole in TBS), and elution buffer

(ELB) (8 M guanidine, and 500 mM imidazole in TBS). To all buffers but the ELB, 0.1 mM PMSF was added prior to use. The HisPur Cobalt resin column (1.5 cm diameter) was prepared the day before use, containing 6 mL bed-volume (BV) of HisPur Cobalt resin, and washed with 5 BV H_2O at a flowrate (FR) of 0.75 mL/ min. The day of use, the column was equilibrated with 3 BV EB at a FR of 0.75 mL/ min. The diluted, solubilized sample was then loaded onto the column at a FR of 0.25 mL/ min. During this time, the column was closely watched to avoid compression. If the agarose beads compressed, the sample was further diluted with EB or the FR was slightly decreased. After loading of the sample, the column was washed with 6 BV WB, increasing the FR from an initial 0.25 mL/ min ever 6 mL by 0.25 mL/ min to a final FR of 0.75 mL/ min, again closely looking for compression of the agarose beads. The 21-hydroxylase-His was then eluted from the column with 5 BV of ELB at a FR of 0.75 mL/min. During the whole column-purification, all fractions were collected and were analyzed on SDS-PAGE gel, after protein precipitation with ethanol as described above. After each purification run, the resin was washed (up to three times) with 10 BV H_2O (FR 1 mL/min), followed by 10 BV MES buffer (regeneration MES Buffer: 20 mM 2-(N -morpholine)-ethanesulfonic acid, 0.1 M sodium chloride (pH 5.0)) (FR 1 mL/min), followed by 10 BV H_2O (FR 1 mL/min), followed by 5 BV of a solution of 20 mM sodium phosphate supplemented with 0.5 M NaCl, and 50 mM EDTA (pH 7.0) (FR 1 mL/ min), followed by 5 BV H_2O (FR 1 mL/ min), followed by 5 BV 0.1 M cobalt (II) sulfate heptahydrate (FR 1 mL/ min), and finally 5 BV H_2O (FR 1 mL/ min). The eluates were treated in different ways: (1) a salt exchange against TBS, using pre-packed Sephadex PD-10 Desalting Columns (GE Healthcare,

Piscataway, NJ, USA), following the manufacturers instructions, was performed. Further, 10 mL of the eluates were dialyzed in the cold room for 12 h against 1 L of (2) TBS, (3) 50 mM phosphate buffer (pH 7.4), 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 1 % sodium cholate, (4) 100 mM sodium carbonate, (5) TBS containing 1 M guanidine, (6) TBS containing 1 M urea, (7) TBS containing 4 M guanidine, and (8) TBS containing 4 M urea, with a buffer exchange after 6 h; against 1 L for 12 h at room temperature with a buffer exchange after 6 h against (9) TBS containing 2 % SDS, 1 % glycerol, and 1 % 2-mercaptoethanol, and (10) TBS containing 2% SDS, 1 % glycerol, and 5 mM DTT. Lastly, (11) the eluate was diluted 1:2 with TBS. The dialysates were spun down for 15 min at 15,000 rpm in a tabletop centrifuge and analyzed on SDS-PAGE gel (ethanol precipitation in guanidine containing samples as described previously) for protein content in the supernatants versus pellets. The salt exchanged sample and the one that was diluted 1:2 were also analyzed on SDS-PAGE gel, followed by WB to confirm the presence of 21-hydroxylase-His. Some samples were further analyzed for suitability for ELISA plate coating, which will be described in chapter VIII.

Results

Elution of the fusion proteins out of SDS gel-matrix: The small-scale purification and passive elution was successful to purify small amounts of 21-OH-GST and 21-OH-His. Figure VI.4 shows the SDS-PAGE gel from which the 21-hydroxylase-GST was excised. Figure VI.5 shows the purified 21-hydroxylase-GST and 21-hydroxylase-His after passive elution out of the gel-matrix. The three staining techniques were compared in order to see what condition best conserved

the protein and did not cause any protein degradation. There was no difference between the three different staining techniques and in all cases the band of interest could be easily and precisely identified. Therefore, identification of the correct band during the large-scale purifications was done using a standard Coomassie staining protocol. The large-scale electro-elution was also successful. The BioRad Protean SDS-PAGE gel of the 21-hydroxylase-GST, from which the band was cut-out is shown in VI.6. The excision of the 21-hydroxylase-GST band could be done precisely. The presence of fusion proteins 21-hydroxylase-GST and 21-hydroxylase-His in these samples was confirmed using SDS-PAGE, WB, and MALDI as described in materials and methods (figures VI.7 to VI.11). The sizes of the fusion proteins were as expected, and there were strong signals in WB with the anti-His antibody and the 21-hydroxylase-His, and the anti-GST antibody and the 21-hydroxylase-GST, respectively. With MALDI, the resulting spectra of both the 21-hydroxylase-GST and 21-hydroxylase-His clearly showed that the most abundant protein in our samples was 21-hydroxylase. The procedure was suitable to purify sufficient amount of 21-hydroxylase-GST to use as the antigen source for antibody production in both rabbits and dogs. The average estimated protein concentration of our purified samples was 1.27 µg/ µL, with a range of 0.32 to 3.21 µg/ µL. There was no difference in the amount of the 21-hydroxylase-GST compared to the 21-hydroxylase-His. Increasing the electro potential to 75 V increased the yield of protein, but unfortunately caused the protein to degrade (figure VI.12).

Purification of 21-hydroxylase-His for ELISA development: For the purification of 21-hydroxylase-His for ELISA development, the inclusion bodies needed to be solubilized and the purification was done under denaturing conditions. The protein precipitation, using ice-cold ethanol, was suitable to remove the guanidine out of the samples to be able to analyze them on

SDS-PAGE. Guanidine containing samples can otherwise not be analyzed on SDS-PAGE. After analyzing all SDS-PAGE gels, it became apparent that with TBS containing 8 M guanidine and 5 mM DTT, some of the inclusion body protein was solubilized (figure VI.13) whereas none of the other conditions was successful. As a representative example, figure VI.14 shows the negative attempt to solubilize the 21-hydroxylase-His with TBS containing 8 M urea, 5 mM DTT, and 5% Triton X-100. The effect of different DTT concentrations was then tested, using TBS containing 8 M guanidine and 0 mM DTT, 4 mM DTT, and 10 mM DTT. These three conditions were tested in triplicates, also testing the need to fractionate the protein and to wash the inclusion bodies prior to solubilization. The extra condition with 4 mM DTT was included, because HisPur Cobalt resin, which was needed for the large-scale column purification, is very sensitive to reducing agents, and only concentrations up to 5 mM can be used. The data showed that it was not necessary to fractionate the protein or to wash the inclusion bodies prior to solubilization, but including DTT in the solubilization buffer was necessary to solubilize the 21-hydroxylase-His and 4 mM was an adequate concentration (figure VI.15). The optimal length of the solubilization step was established to be 2 h 20 min.

In order to find the best conditions for the column purification, a pull-down with the same resin was performed. In particular, the binding between the solubilized 21-hydroxylase-His and the HisPur Cobalt resin and the effects of DTT were examined. The results showed that the addition of extra DTT during pull-down was not necessary (figures VI.16). Having determined the best conditions, the purification of 21-hydroxylase-His was scaled up. During FPLC, unfortunately some 21-hydroxylase-His did not stick to the column and was lost during the loading phase. However, most protein was bound to the HisPur Cobalt resin and washing of

the resin removed most contaminating proteins, resulting in an apparently pure 21-hydroxylase-His protein upon elution as visualized by SDS-PAGE after ethanol precipitation of samples (figures VI.17 and VI.18). Unfortunately, the purified 21-hydroxylase-His precipitated and buffer conditions for storage had to be determined. The use of PD-10 Desalting columns resulted in protein loss. Nine different dialysis conditions were therefore tested, as described in materials and methods. After 12 hours of dialysis, the samples were collected and analyzed. All samples but those that were dialyzed against TBS containing 4 M urea or TBS containing 4 M guanidine were cloudy, showing that at least some of the protein was precipitated. All samples were spun down and the protein concentration in the pellets and supernatants were compared to each other. The macroscopic observations were confirmed, meaning that most of the protein was in the pellet except for the samples dialyzed against TBS containing 4 M urea or the TBS containing 4 M guanidine (figures VI.19 and VI.20). Alternatively, the guanidine samples were diluted 1:2 with TBS, resulting in 4 M guanidine and 250 mM imidazole as final concentrations. No precipitation occurred, indicating that the protein remained in solution. Looking at the intensity of bands on SDS-PAGE, this condition appeared to have given the highest amount of purified fusion protein 21-hydroxylase-His. Further, this condition is also suitable for a protein concentration assay (Bradford or bicinchoninic acid (BCA)), which is necessary for estimation of protein concentration for ELISA plate coating. Further, it is acceptable to use protein mixtures that contain 4 M guanidine and 250 mM imidazole for ELISA plate coating. Protein concentration measurement and coating of ELISA plates will be discussed in chapter VIII. Based on all findings, the following 21-hydroxylase-His preparations were submitted to Oxford Biomedical Research for evaluation for ELISA: 21-hydroxylase-His in TBS (salt exchanged), 21-

hydroxylase-His in TBS containing 4 M urea (dialyzed), 21-hydroxylase-His in TBS containing 4 M guanidine (dialyzed), and 21-hydroxylase-His in TBS containing 4 M guanidine and 250 mM imidazole (diluted).

Discussion

Usually, SDS-PAGE is used as an analytical tool to assess protein purification. In this experiment, SDS-PAGE was used as an active step in the purification process, because of its excellent ability to resolve individual components of complex mixtures. After resolving the individual components of the complete protein mixture via SDS-PAGE, the proteins of interest were identified by negative stain with 0.25 mM KCl, side strip staining with Coomassie blue stain, or stain of the entire gel with Coomassie. The purified proteins were then passively and actively eluted from the gel matrix. The resulting purified proteins were identified by size, WB, and MALDI, and the 21-hydroxylase-GST fusion protein was prepared for antibody production in two dogs and two rabbits. The 21-hydroxylase-GST was chosen for injection, because of its size, which increases the immunogenicity of the fusion protein. GST has been shown to induce an antibody response in experimental animals, sometimes to such a strength where it provides partial protection against a number of parasitic worms, including Schistosoma and Fasciola[2]. In addition, the 21-hydroxylase-GST and the 21-hydroxylase-His fusion proteins were used as standards during SDS-PAGE. The 21-hydroxylase-His was chosen as the antigen for further assay development, since it is easier and more economical to purify His-tagged fusion proteins in a

larger scale. The histidine tag consists of six histidine residues linked to the protein and was invented by Roche[3]. Polyhistidine tags are a common method for affinity purification of recombinant proteins expressed in *E. coli*[4]. Affinity columns contain bound metal ions such as nickel or cobalt, to which the polyhistidine tag binds with high affinity. The resin is washed with buffers containing low concentrations of imidazole to remove non-specifically bound proteins and the protein of interest is then eluted with a buffer containing high imidazole concentrations. The main advantage of His-tag affinity purification is the purity of the protein especially when expressed in a prokaryotic organism. The purification from an expression system in a eukaryotic organism may require a tandem affinity purification with two tags[5]. Using polyhistidine tagged proteins is also the option of choice for purifying recombinant proteins under denaturing conditions, because its mode of action is dependent only on the primary structure of proteins, whereas GST purification requires the protein to be properly folded.

Cholesterol is the parent compound from which all steroid classes are produced. The process is complex and is mediated by enzymes from the cytochrome P450 family. P450 enzymes are membrane bound, contain heme and serve as terminal oxidases in electron-transfer chains originating in NADPH (nicotinamide adenine dinucleotide phosphate), or the hydroxysteroid dehydrogenase superfamily[6]. The predominant glucocorticoid and mineralocorticoid in several mammals, including the dog, are cortisol and aldosterone, respectively. These are synthesized by the adrenal cortex and characterized by a hydroxyl group

at position C21 of the steroid ring. The microsomal steroid 21-hydroxylase cytochrome P450c21 enzyme catalyzes the hydroxylation of progesterone or deoxycorticosterone at position C21. The deficiency of this enzyme in humans is of great clinical importance and is called congenital adrenal hyperplasia. It has also been recognized that this enzyme is the main target in the development of autoimmune Addison's disease in humans. To prove whether this enzyme plays the same important role in Addison's disease in dogs, the canine 21-hydroxylase needed to be cloned, overexpressed in *E. coli* and purified. The canine 21-hydroxylase is structurally closely related to the P450c21 from other species and is only found in the adrenals[7]. Because of the structural similarities to the bovine enzyme, we expected that the modifications suggested by Arase and Waterman[8] would yield similar results for the expression of canine 21-hydroxylase in *E. coli*. In this study, the anchor and basic region of the 21-hydroxylase was replaced and the protein was co-expressed with chaperone proteins significantly increasing the overproduction[8].

Unfortunately, the same strategy did not result in increased overexpression of the canine 21-hydroxylase. In short, the results remained unsatisfactory and the protein was primarily found in the insoluble fraction independent of the tag and the condition used. P450 enzymes are known to be notoriously difficult to overexpress[8-16]. For assay development, it is not absolutely necessary to obtain a protein in its native form, even though sometimes preferred for ELISAs. I therefore decided to solubilize the protein from the inclusion bodies and purify it under denaturing conditions. To solubilize the inclusion bodies, a variety of anionic, cationic, and zwitterionic detergents, as well as ethoxylates were tested, and only 8 M

guanidine in the presence of 4 mM DTT gave the desired results. After the optimal conditions were found, the purification was relatively straight-forward, even though very time-consuming and expensive.

For the FPLC purificartion, HisPur Cobalt resin was chosen rather than a Ni(2+)-based resin, because although Ni(2+) chelate resins achieve high protein yields, proteins from *E. coli* can bind indiscriminantly, resulting in suboptimal purity and the requirement of additional cleanup steps[4], whereas cobalt maximizes protein purity without sacrificing protein yield. HisPur Cobalt Resin binds fewer contaminating proteins, such as FKBP-type peptidyl prolyl isomerase from *E. coli*[3]. Cobalt also displays less metal leaching and enables less stringent elution conditions than nickel resins. However, the present purification scheme is time- and labor consuming because the column has to be constantly watched during the purification to avoid damages due to overpressure. The viscosity of the solubilized inclusion bodies required dilution of the sample by at least 1:4 in connection with a FR of no more than 0.25 mL/min, which meant that the loading of the protein onto the column alone took at least 10.5 h. Minor compression of the resin was reversible and could be counteracted with further dilution of the sample or with decreasing the FR. If the compression got too severe however, the resin was damaged, dramatically decreasing its binding capacity. The addition of 8 M guanidine to all buffers adds to the costs and has to be taken into account when scaling the procedure up. Approximately 500 g of guanidine for every 1 L of induced bacteria culture was needed.

To summarize, the protocol developed here results in an apparently pure canine-21-hydroxylase, is reproducible and produced sufficient amounts for the development of an ELISA.

APPENDIX

Appendix

Figure VI.1: BioRad's Protean II XL system

Figure VI.2: Electrophoretic elution tank. (a) Top view; (b) side view; (c) end view; (d) port connector for pump tubing. A, terminal lug; B, platinum wire electrode; C, baffle plate; D, separation plate for electrode chambers; E, slot for elution cell; F, drain trough; G, mirrored surface; H, to pump tubing; I, to tank chamber; J, set screw. Plexiglas covers for tank chambers are not shown. (from: Methods in Enzymology, Volume 91, Enzyme Structure Part 1, 1983, page 229).

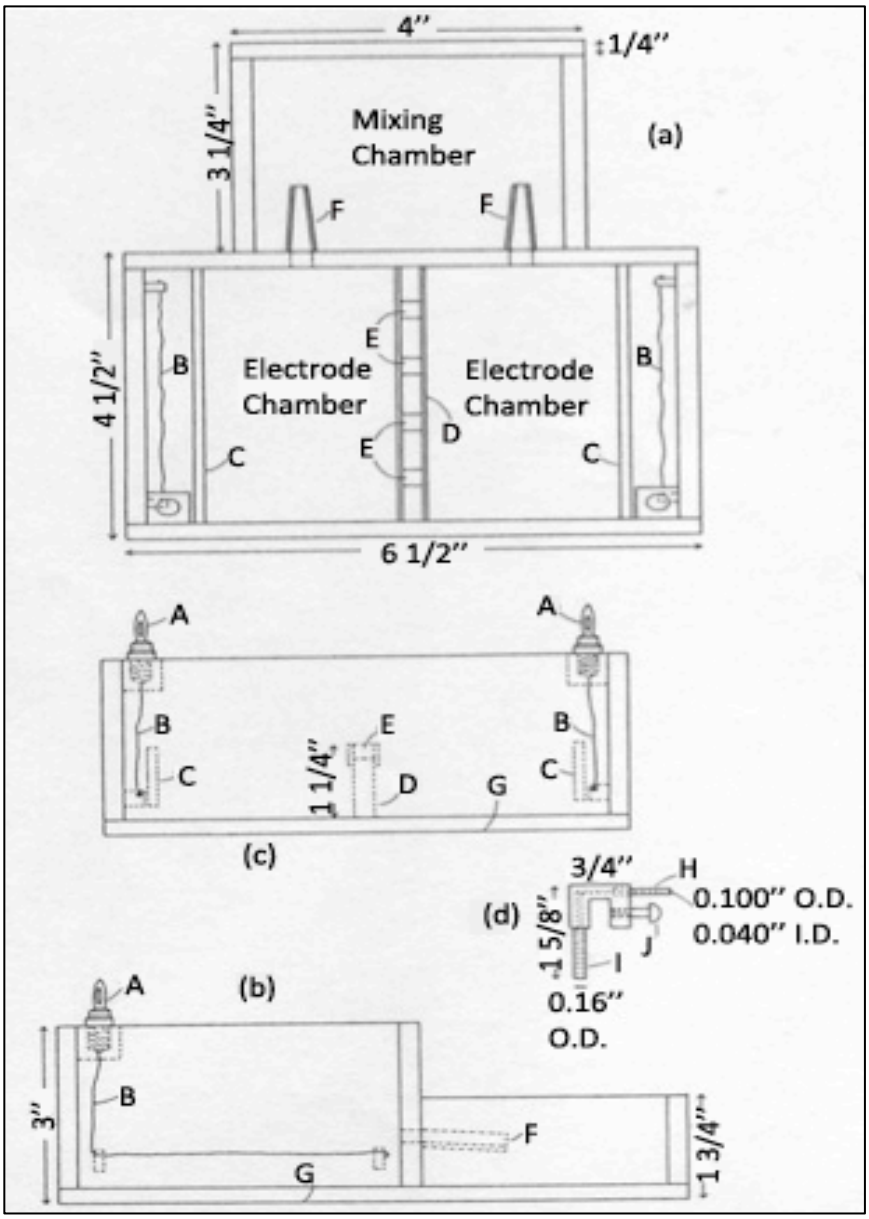


Figure VI.3: Electrophoretic elution cell. A, gel loading well; B, sample collection well; C, Spectra/Por disk; D, silicon rubber washer; E. screw cap with open top; F, peg for holding slot in elution tank; G, cross passage. (from: Methods in Enzymology, Volume 91, Enzyme Structure Part 1, 1983, page 229).

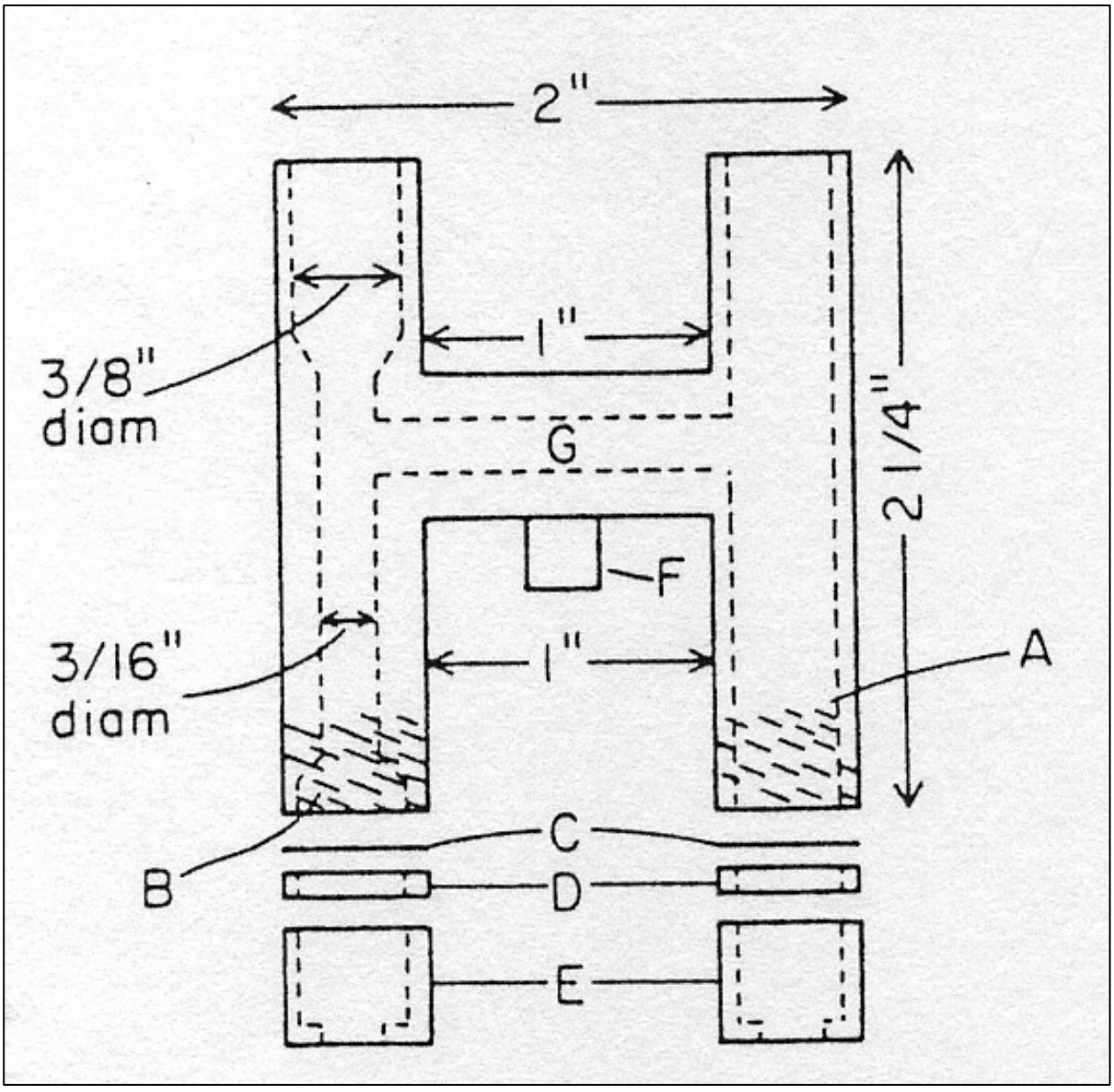


Figure VI.4: SDS-PAGE, showing the 21-hydroxylase-GST prior to gel-purification and small scale passive elution.

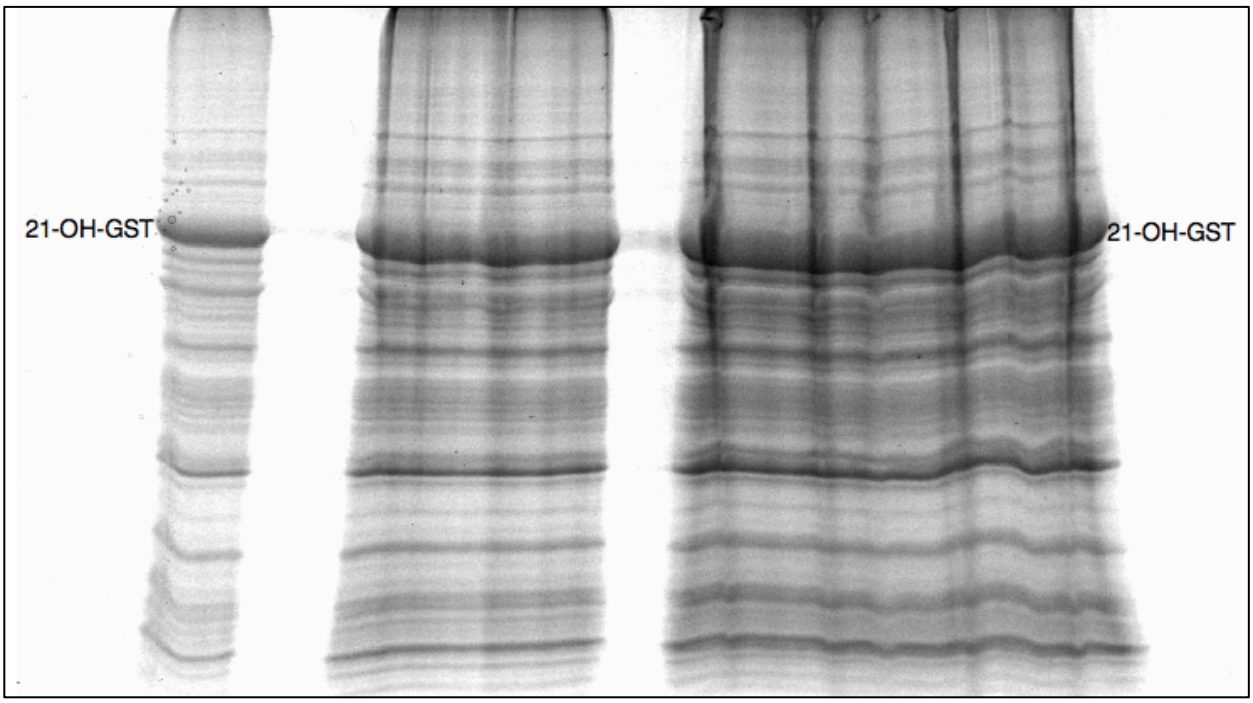


Figure VI.5: SDS-PAGE, showing the purified 21-hydroxylase-His and 21-hydroxylase-GST post gel purification and passive elution out of the gel-matrix.

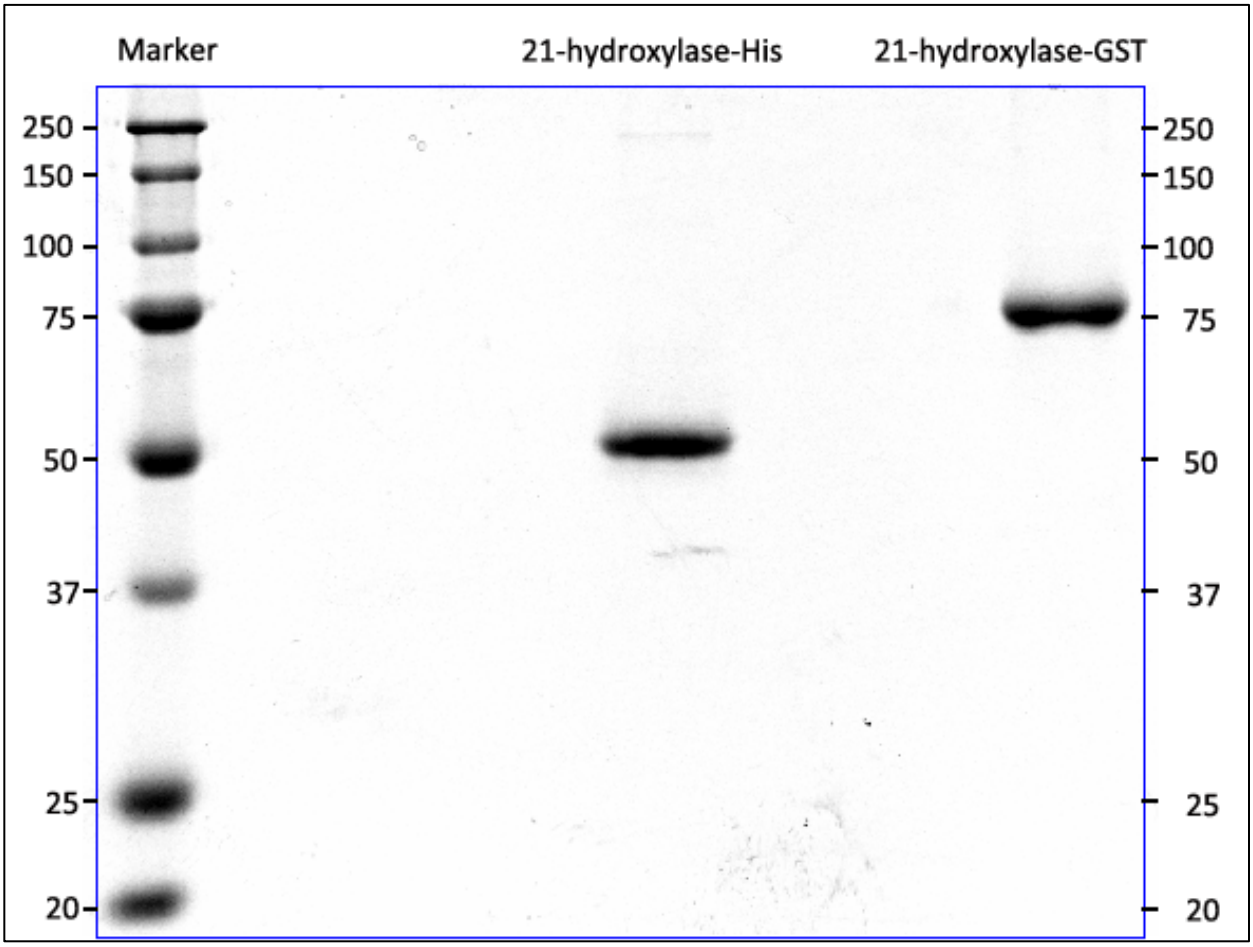


Figure VI.6: SDS-PAGE maxi gel, after the 21-hydroxylase-GST was excised. Using Coomassie stain, it was possible to accurately identify the band of interest.

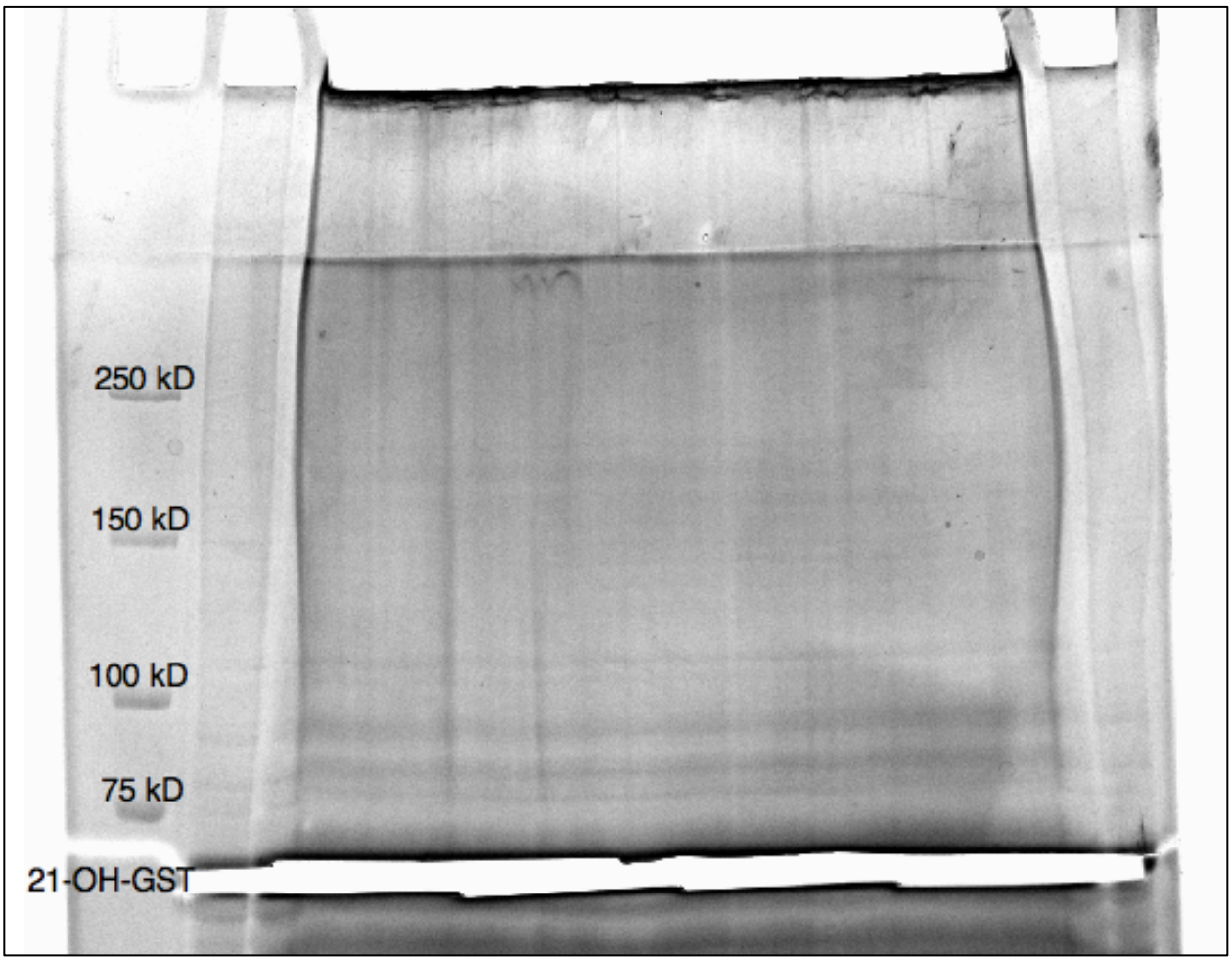


Figure VI.7: SDS-PAGE, showing the gel-purified and electro-eluted 21-hydroxylase-His in comparison to BSA. The known concentrations of BSA were used to estimate the concentration of the electro-eluted 21-hydroxylase-His.

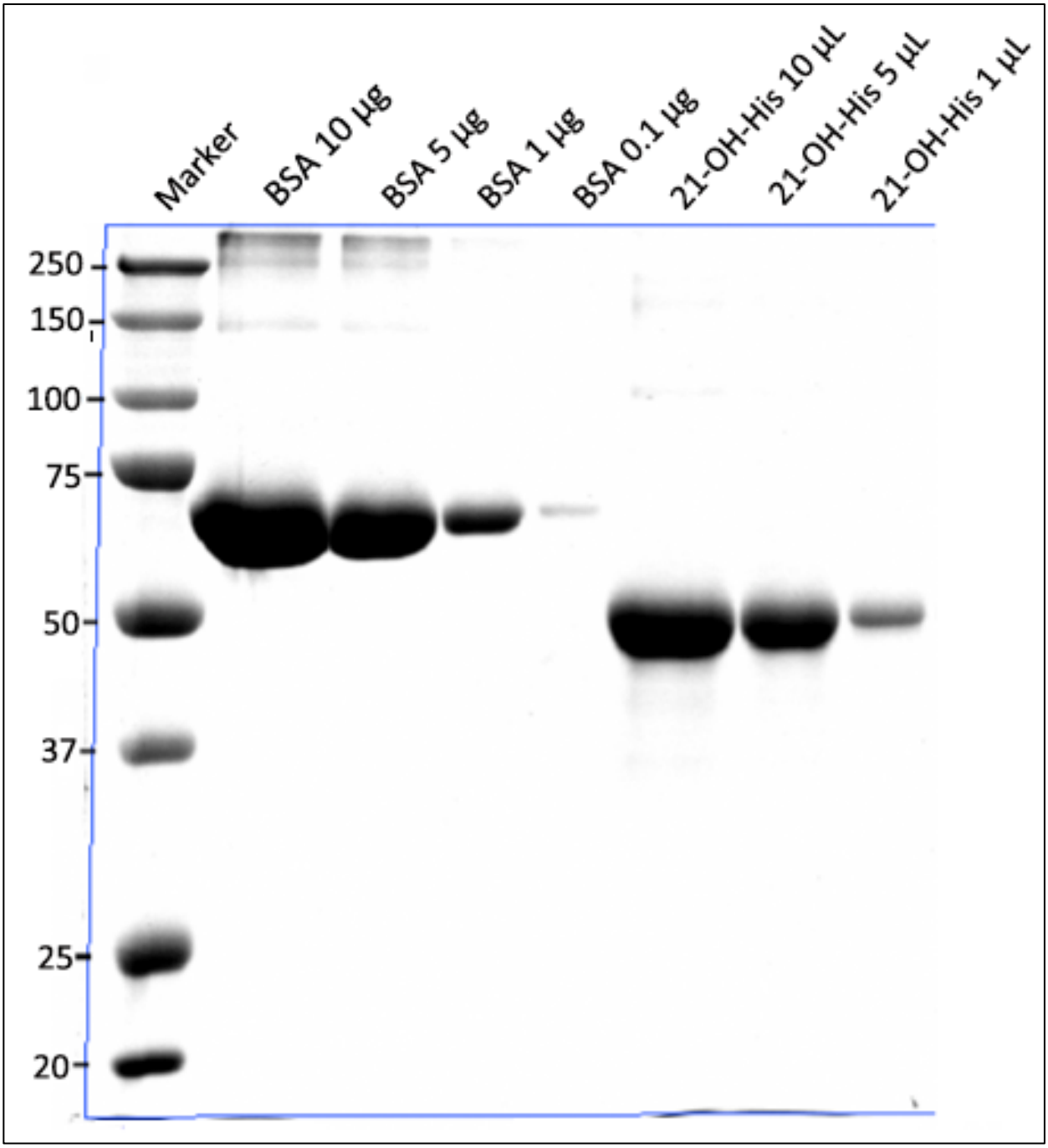


Figure VI.8: SDS-PAGE, showing the gel-purified and electro-eluted 21-hydroxylase-GST in comparison to BSA. The known concentrations of BSA were used to estimate the concentration of the electro-eluted 21-hydroxylase-GST.

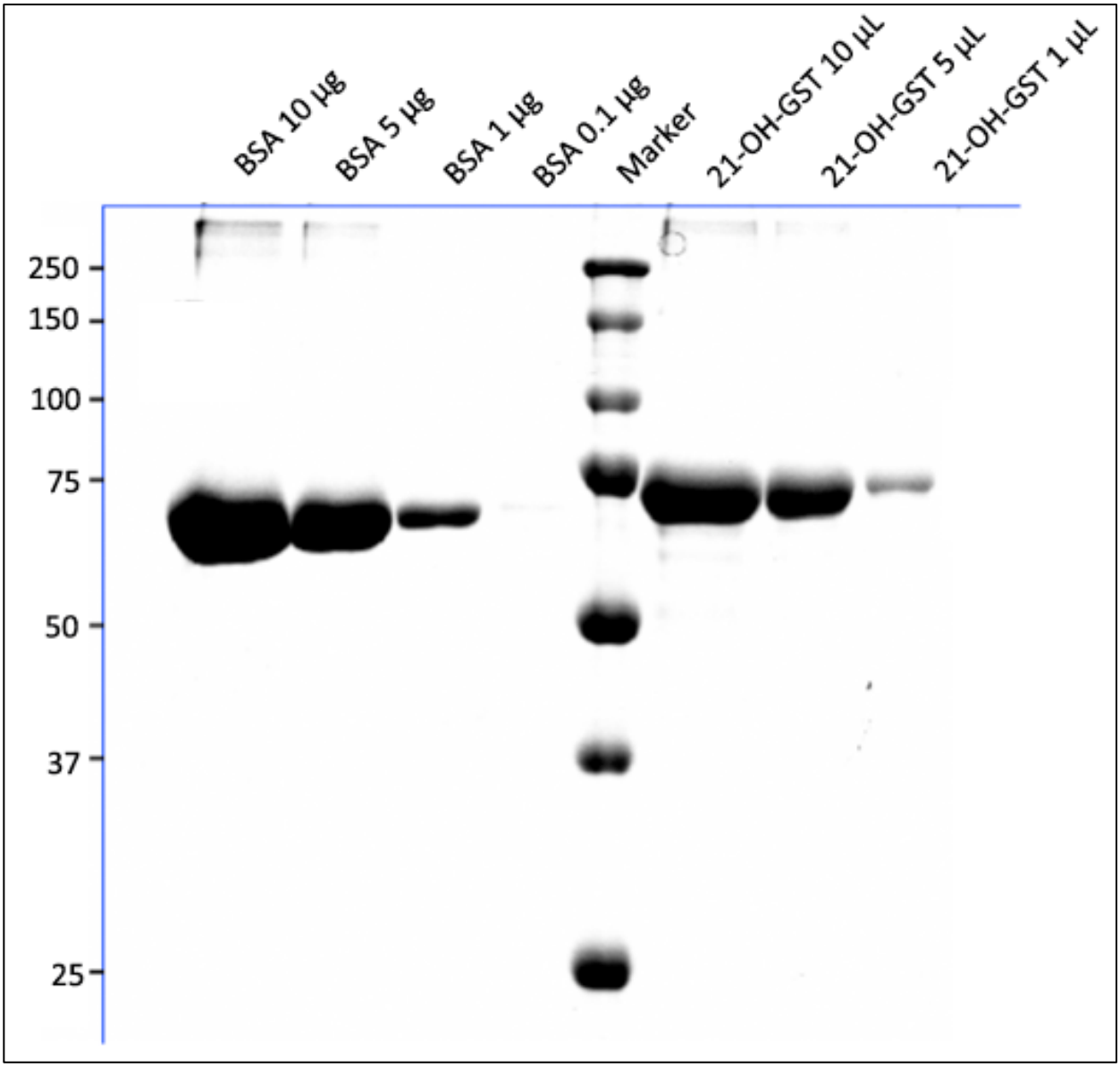


Figure VI.9: WB of the electro-eluted 21-hydroxylase-His, showing that the purified protein reacts strongly with an antibody against His. (From left to right: lane 1: marker, lanes 2 through 5: 21-hydroxylase-His from different electro-elution runs).

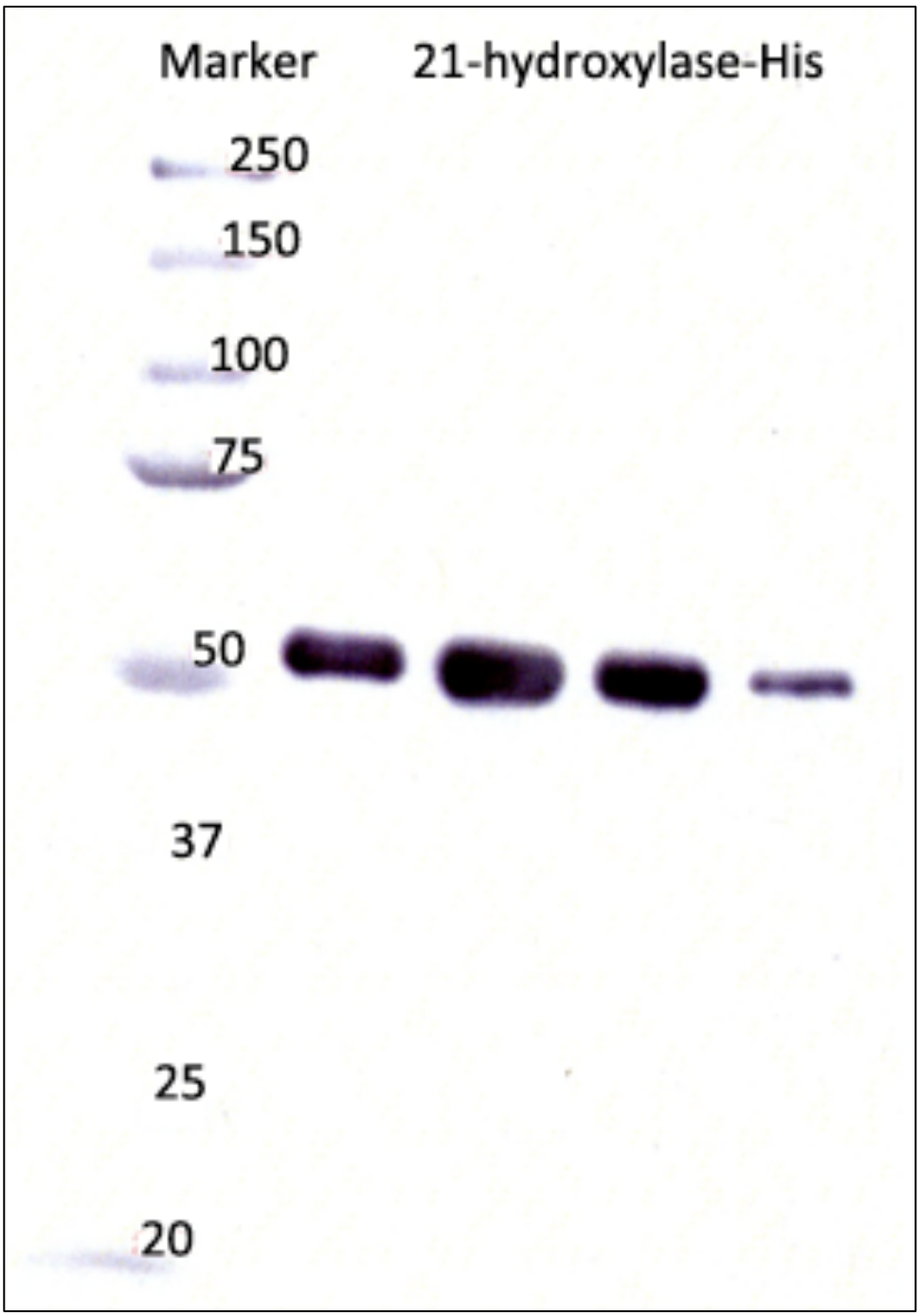


Figure VI.10: WB of the electro-eluted 21-hydroxylase-GST, showing that the purified protein reacts strongly with an antibody against GST. (From left to right: lane 1: Marker, lanes 2 through 4: 21-hydroxylase-GST from different electro-elution runs).

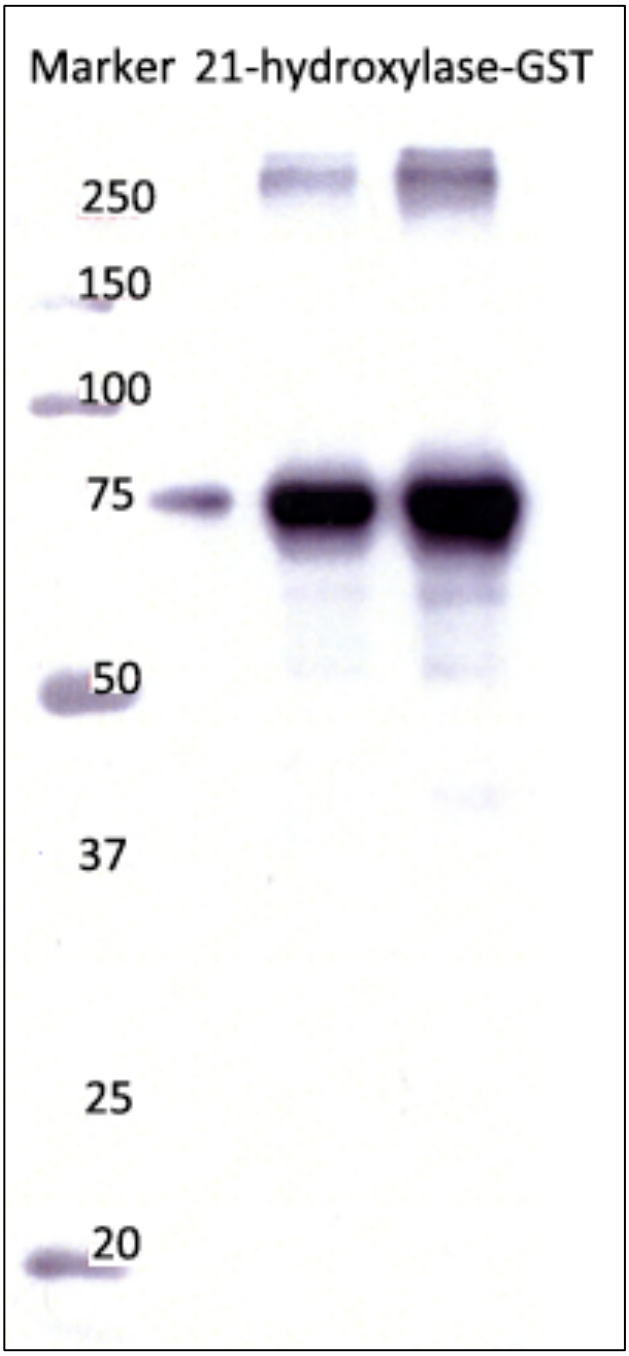


Figure VI.11: MALDI spectra of the 21-hydroxylase-GST. The peaks are similar to the ones of the theoretical trypsin digest of the 21-hydroxylase-GST.

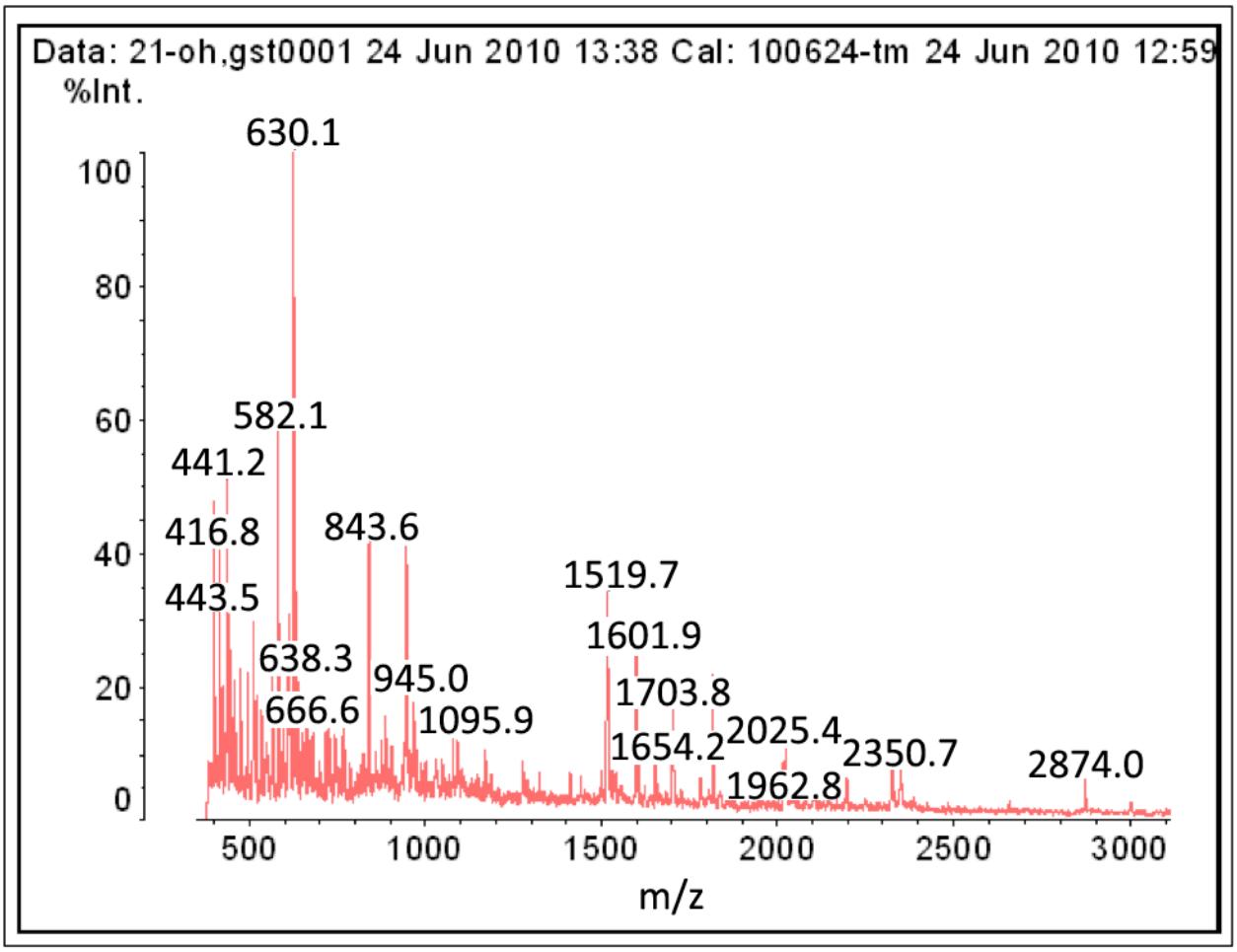


Figure VI.12: SDS-PAGE, showing the gel-purified and degraded electro-eluted 21-hydroxylase-GST, after increasing the electro-potential during electro-elution from 50 V to 75 V.

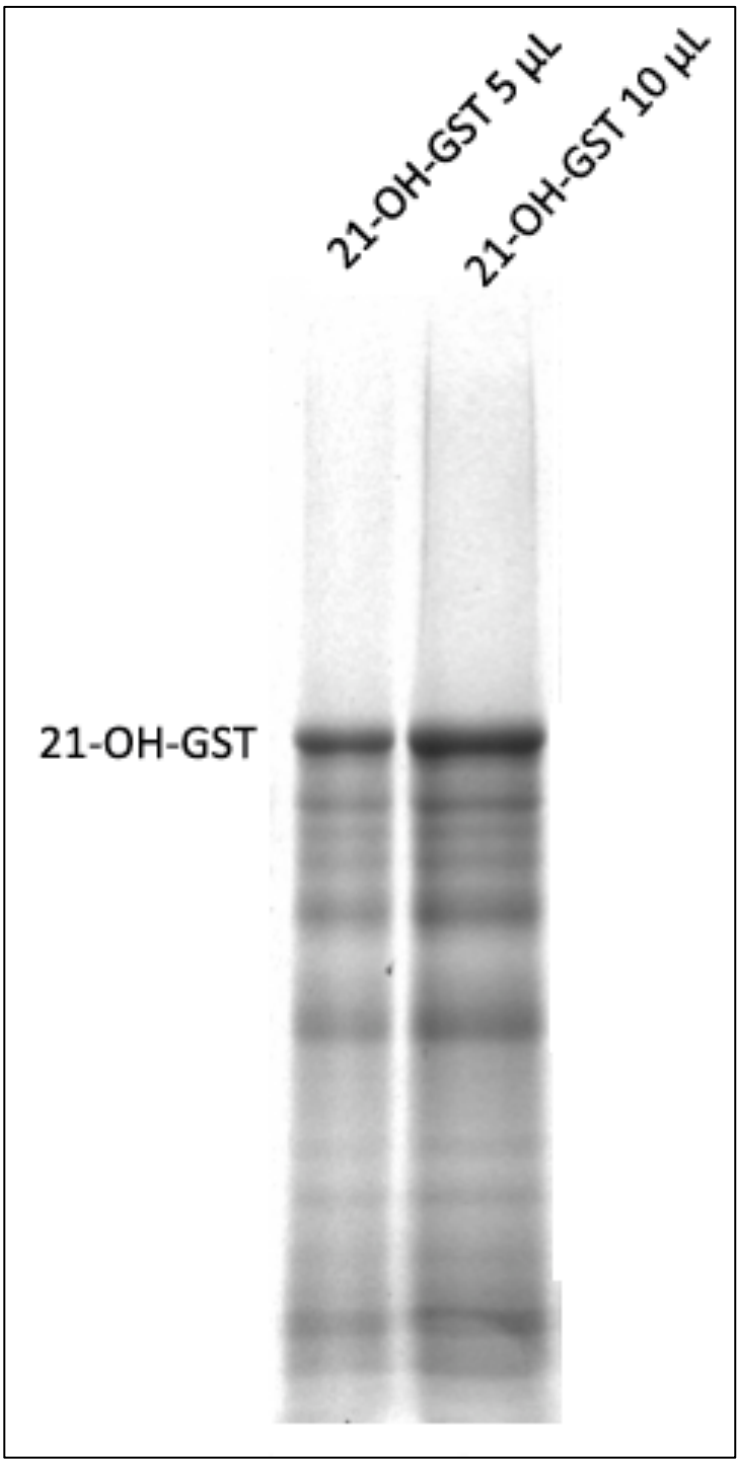


Figure VI.13: Solubilization of 21-hydroxylase-His inclusion bodies with TBS containing 8 M guanidine, and 5 mM DTT over 24 hours. Some of the 21-hydroxylase-His is found in the supernatant. (SN: supernatant; P: pellet).

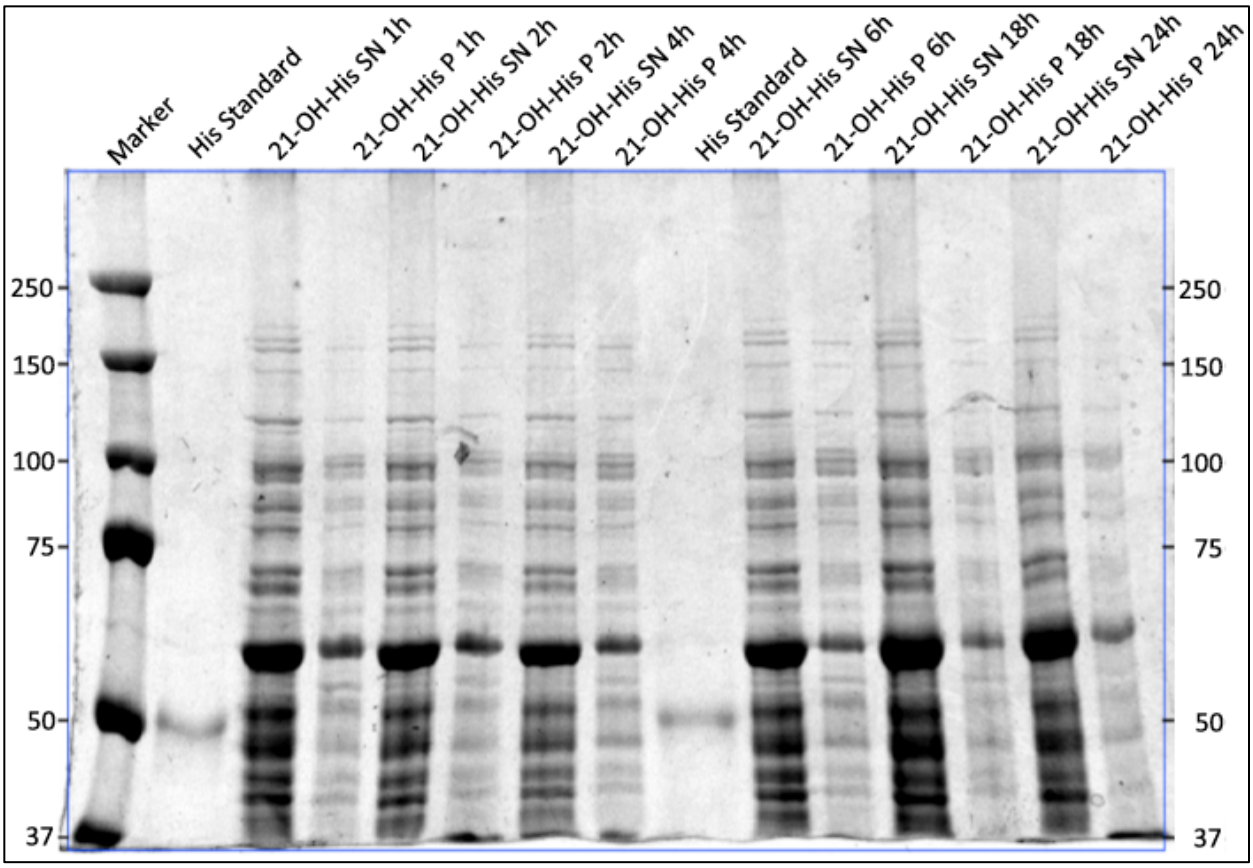


Figure VI.14: Solubilization of 21-hydroxylase-His inclusion bodies with TBS containing 8 M urea, 5 mM DTT, and 5% Triton X-100 over 24 hours. The 21-hydroxylase-His remains in the pellet. (SN: supernatant; P = pellet).

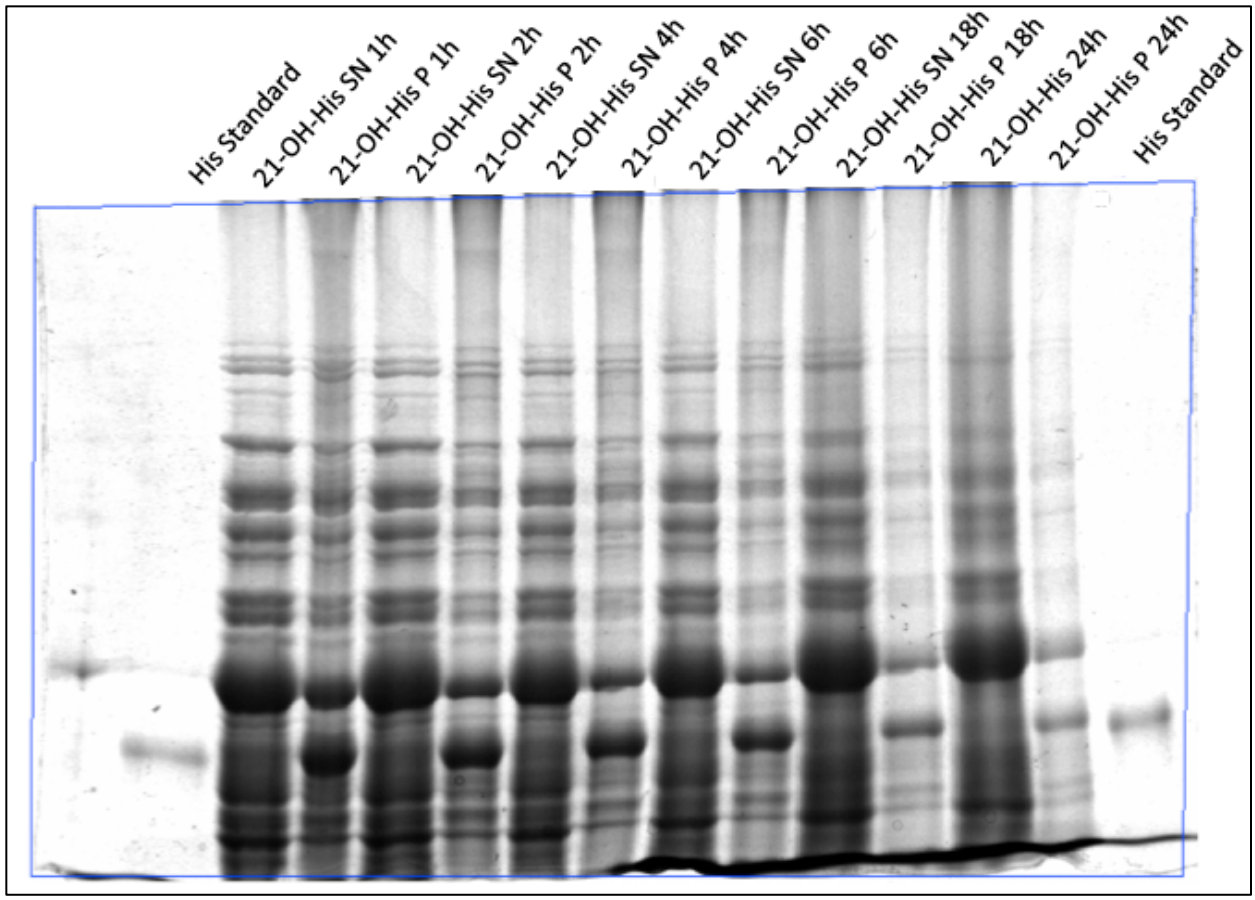


Figure VI.15: Solubilization of 21-hydroxylase-His inclusion bodies with TBS containing 8 M guanidine versus TBS containing 8 M guanidine, and 4 mM DTT after 2 h 20 min. The yield of 21-hydroxylase-His in the supernatant is increased with DTT. (SN: supernatant; P: pellet).

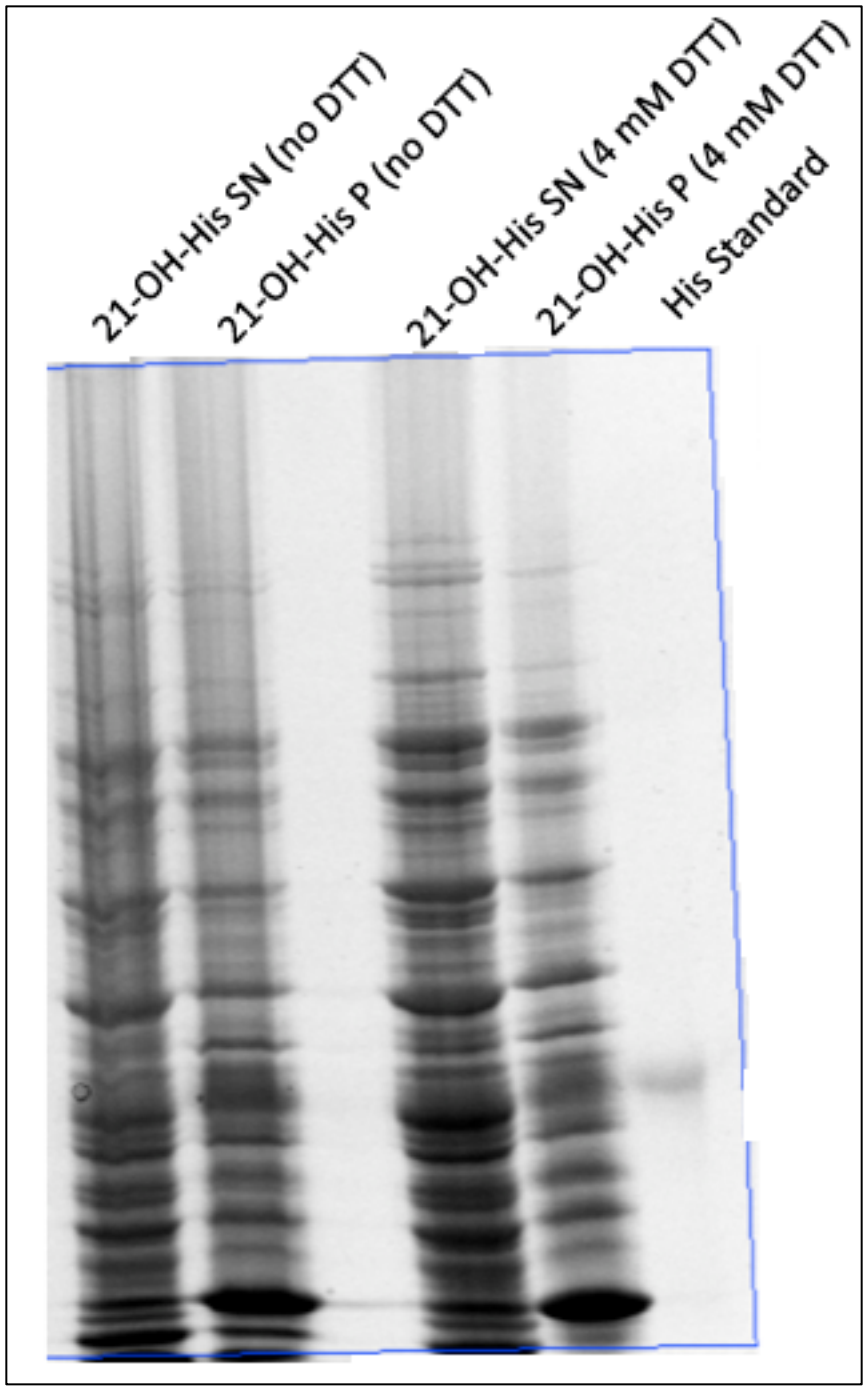


Figure VI.16: Pulldown of solubilized 21-hydroxylase-His in TBS containing 8 M guanidine with HisPur cobalt resin, without the addition of additional DTT. The 21-hydroxylase-His was solubilized in TBS, containing 8 M guanidine and 4 mM DTT. The sample was diluted 1:2 with TBS prior to pulldown, decreasing the final DTT concentration to 2 mM. (SN: supernatant (unbound protein post incubation with HisPur resin); W: wash fractions; E: elution fractions).

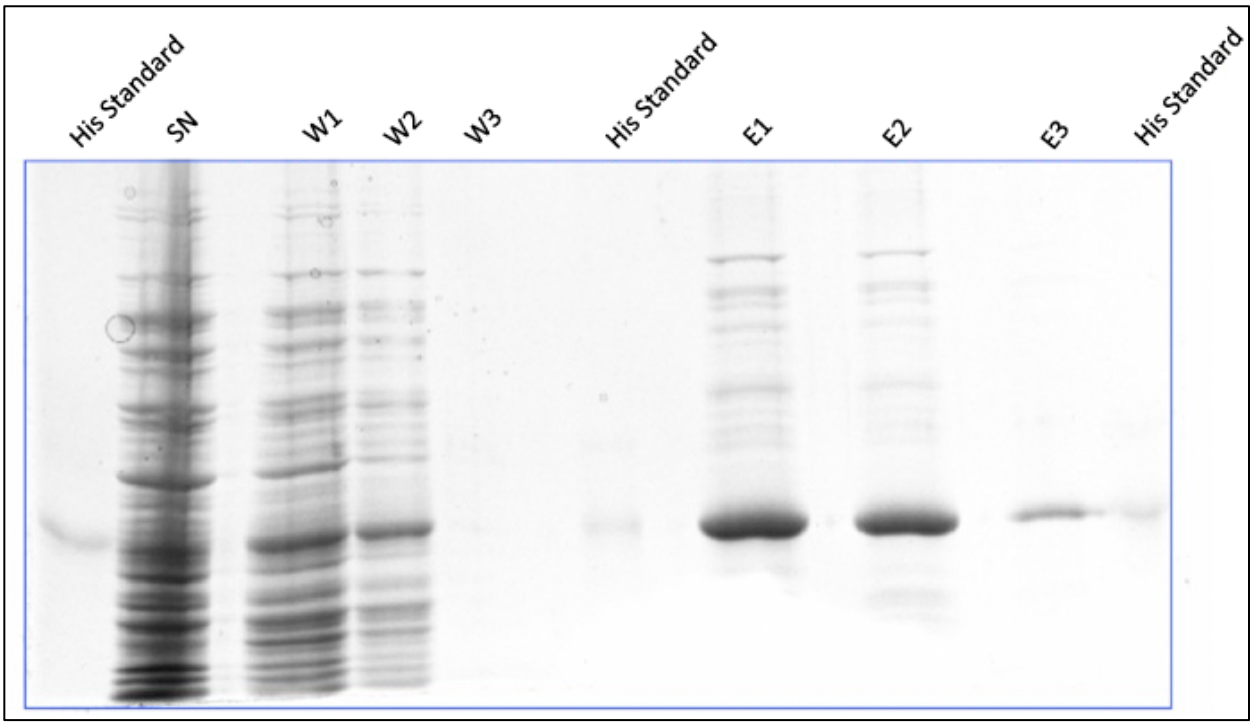


Figure VI.17: FPLC of 21-hydroxylase-His, loading and wash fractions. Contaminating proteins do not bind to the HisPur cobalt resin and are washed off during washing. Some of the 21-hydroxylase-His however is lost during loading and washing. (SN, induced: induced bacteria culture post sonification and ultracentrifugation, supernatant; P, uninduced: uninduced bacteria culture post sonification and ultracentrifugation, pellet (negative control); P, induced: induced bacteria culture post sonification and ultracentrifugation, pellet (positive control); L: loading fraction, number corresponds to the collected fraction number; W: wash fraction, number corresponds to the collected fraction number).

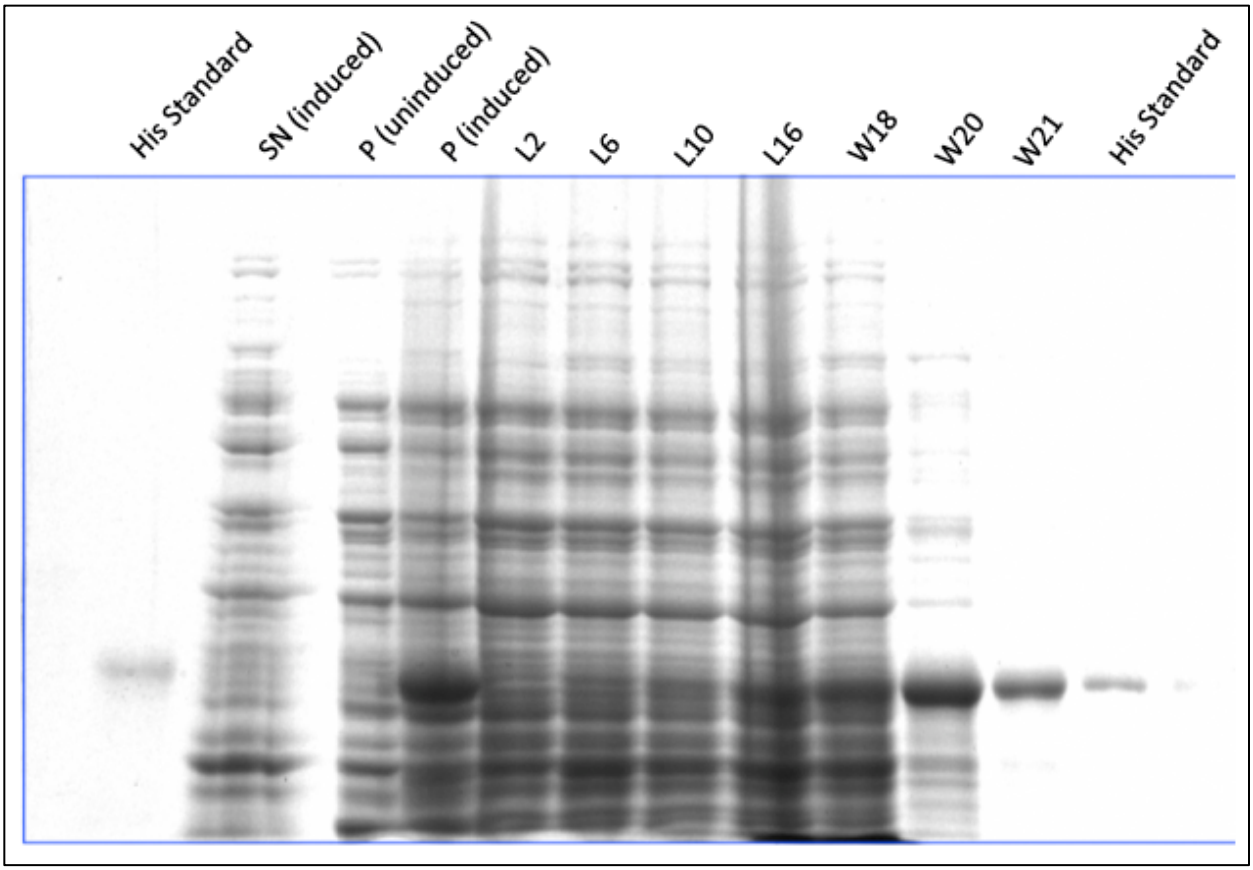


Figure VI.18: FPLC of 21-hydroxylase-His, wash and elution fractions. Some of the 21-hydroxylase-His was lost during washing, but most was eluted with high imidazole concentration. (W: wash fraction, number corresponds to the collected fraction number; E: elution fraction, number corresponds to the collected fraction number).

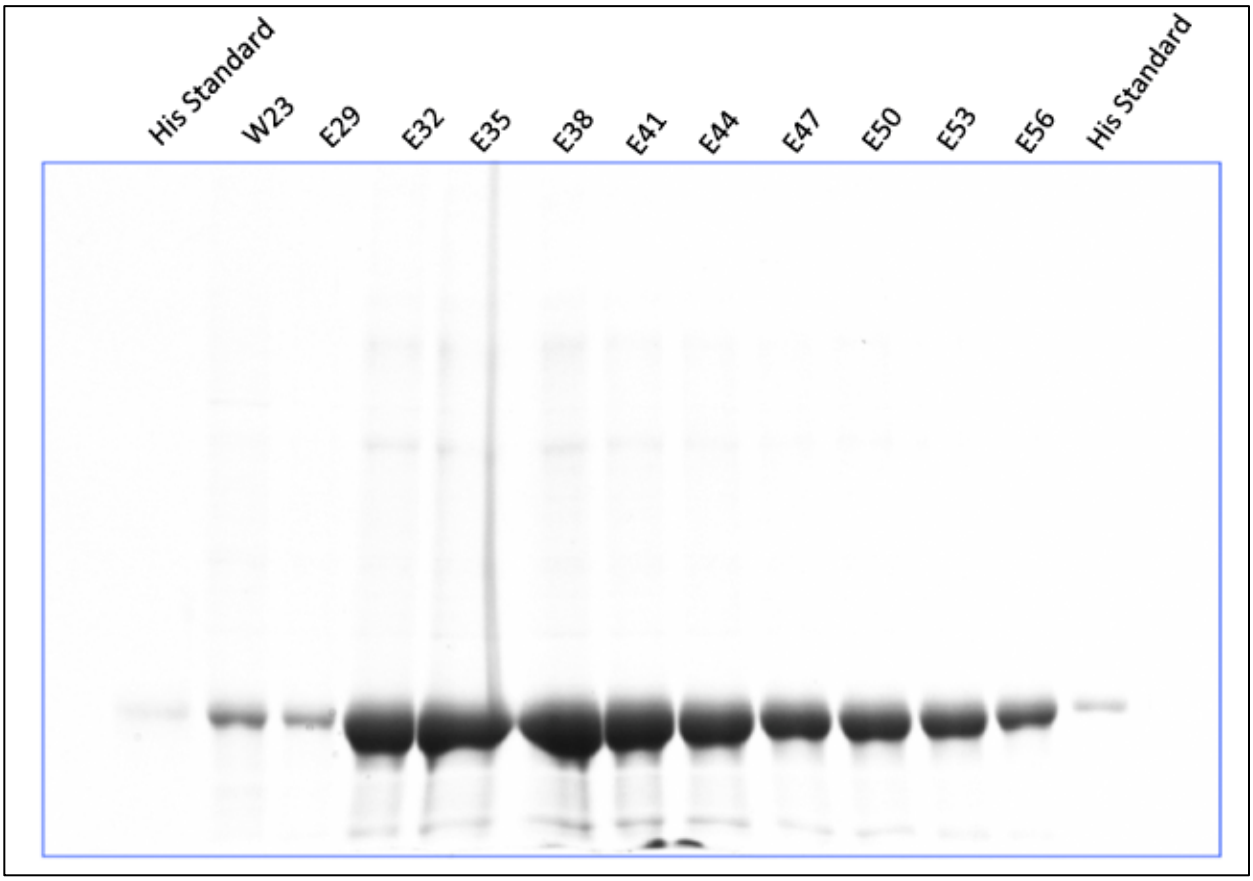


Figure VI.19: Localization of the purified 21-hydroxylase-His post dialysis in 7 different conditions. Most of the 21-hydroxylase-his remains in the pellet, except for TBS containing 4 M guanidine, TBS containing 4 M urea, and TBS containing 2 % SDS, 1 % glycerol, and 1 % 2-mercaptoethanol. 'Pool' is the pool of fractions that were used for the dialysis. (P: pellet post centrifugation of the dialysates).

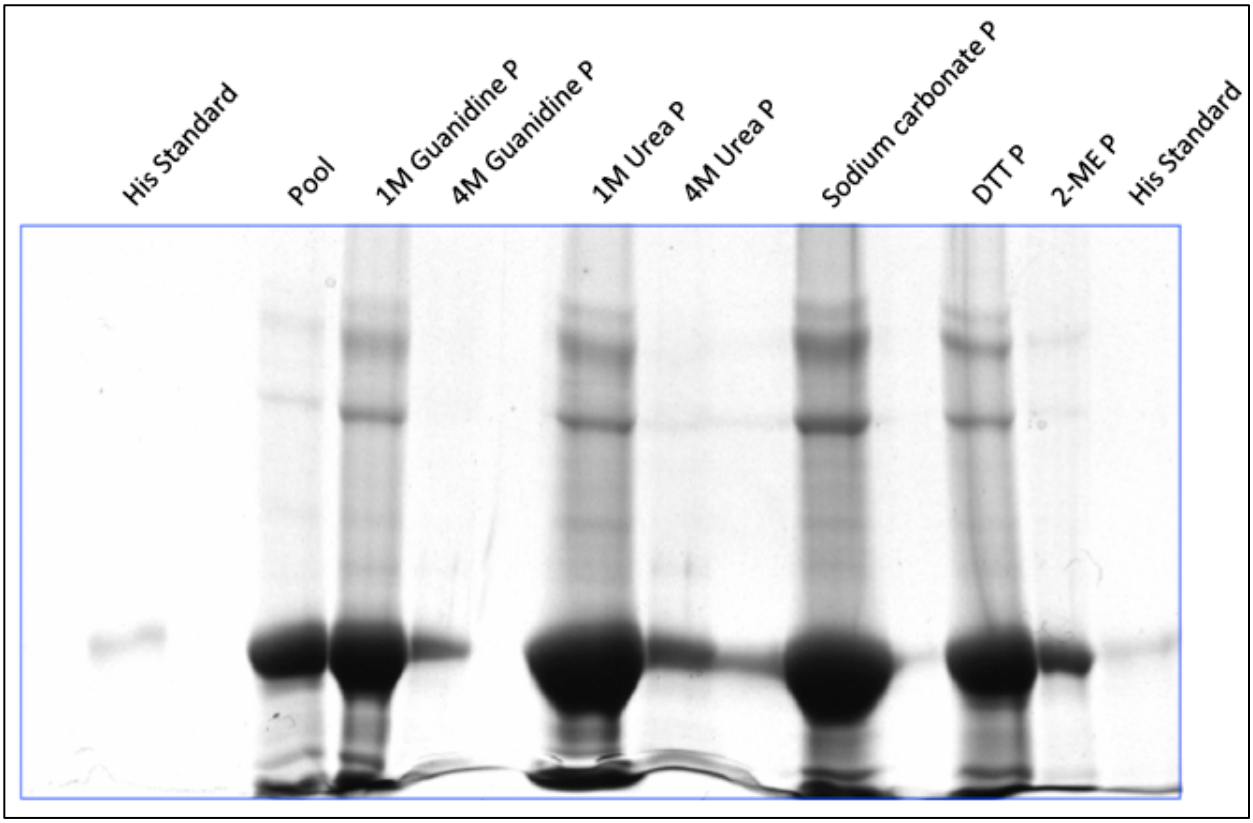
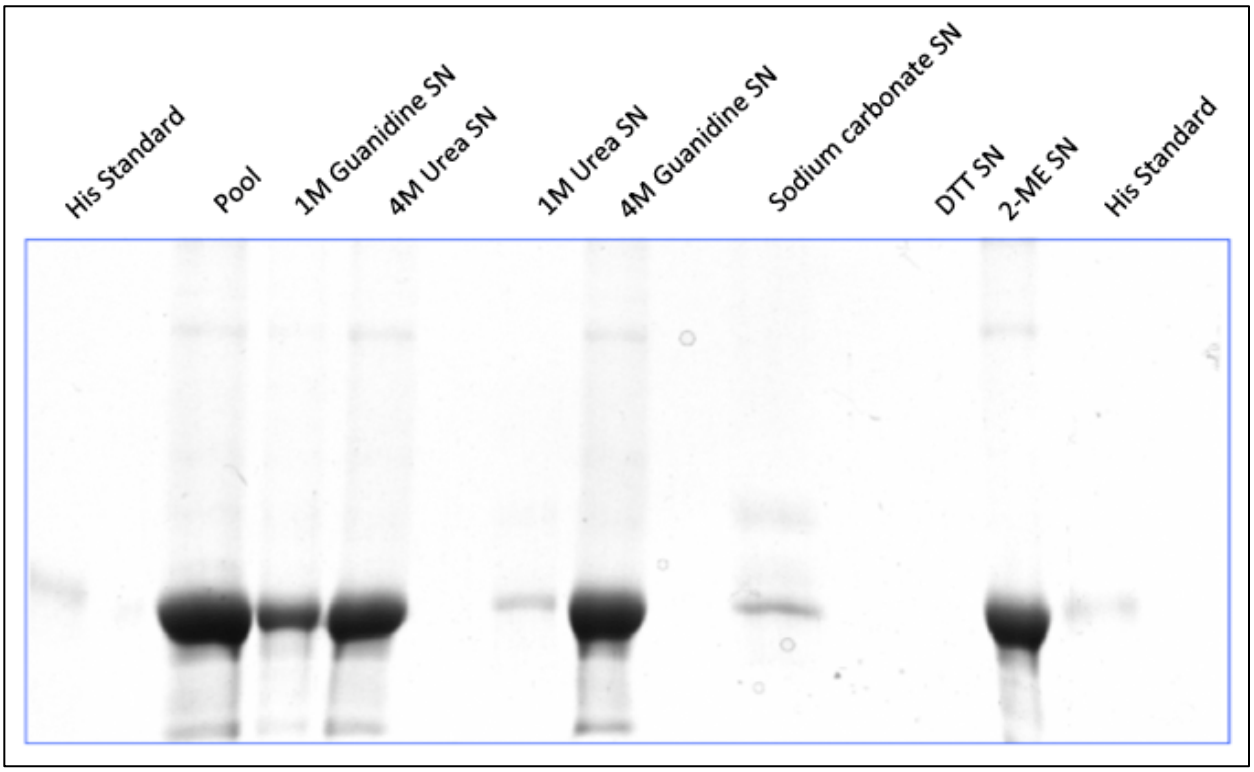


Figure VI.20: Localization of the purified 21-hydroxylase-His post dialysis in 7 different conditions. Most of the 21-hydroxylase-his remains in the pellet, except for TBS containing 4 M guanidine, TBS containing 4 M urea, and TBS containing 2 % SDS, 1 % glycerol, and 1 % 2-mercaptoethanol, in which the majority was found in the SN. 'Pool' is the pool of fractions that were used for the dialysis. (SN: supernatant post centrifugation of the dialysates).



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Chapter VII: Creation of positive control sera for assay development

Introduction

As demonstrated during preliminary experiments, progress was impeded by the lack of positive control serum for assay development. Two dogs were therefore immunized with modified canine 21-hydroxylase, which was expressed in *E. coli*. I was also attempting to induce autoimmune-induced adrenal inflammation and dysfunction, which would be demonstrated via ACTH (adrenocorticotropic hormone) stimulation tests and histopathology of the adrenal glands at the end of this experiment. The Institutional Animal Care and Use Committee at Michigan State University (MSU), East Lansing, MI, USA, approval number #02/08-017-00, approved this part of the project. Protein was also provided to Oxford Biomedical Research, Rochester, MI, USA, who outsourced the immunization of two rabbits. The immunization of these two rabbits was done using standard procedures and will not be further discussed. The decision to immunize two rabbits was made because of the uncertainty that the two dogs would effectively mount an immune response. The obtained pre- and post- immunization sera however were analyzed for antibody content, which will be discussed in the following chapter.

Materials and methods

Three healthy dogs were obtained from other researchers at MSU and housed at the Vivarium at the College of Veterinary Medicine at MSU. The three dogs had access to ad libitum water, were fed a commercially available diet once a day in the morning, and were dewormed and vaccinated on a regular basis. Blanket, an intact male Beagle-Briard-Corgi mix, was born in January of 2007. His weight throughout the study was approximately 17 kg. Lola, a spayed female Beagle, was born in July of 2006. Her weight throughout the study was approximately 8 kg. Lucy, another spayed female Beagle, was the negative control dog that did not get immunized with recombinant modified canine 21-hydroxylase-GST. She was born in February of 2006. Her weight throughout the study was approximately 7 kg. A control dog that did not get immunized with 21-hydroxylase-GST was included to rule out biopsy-induced inflammation and dysfunction of the adrenal glands.

The timeline of this complete experiment is summarized in table VII.1. The different steps done are described in detail below. In order to monitor antibody production, progression of adrenal inflammation and potential development of hypoadrenocorticism, we collected adrenal biopsies and were performing ACTH stimulation tests.

Blood collection: Early experiments by Meek and Eyster in 1921, testing acute hemorrhage in dogs, determined that loss of 2% of body weight (400 mL for a 20 kg dog), which is about 25% of blood volume, "usually causes no serious physiologic effect[1]". Experiences of veterinarians collecting blood from blood donors in the literature and at MSU have also been

helpful. They report that dogs weighing > 27 kg have donated 450 mL of blood at 3 week intervals for over a 2 year period without adverse effects[2]. Our dogs weight was between 6 kg and 17 kg, and no more than 50 mL of blood were collected from the two smaller dogs and no more than 100 mL of blood from the larger dog at any given time. Blood was collected in the morning, after an overnight fast. Blood was collected via venipuncture from the jugular veins, into 10 mL vacutainer tubes. Prior to collection, capillary refill time and mucous membranes were checked. The tubes were left at room temperature for 30 minutes to allow for clotting, before being spun down in a blood-centrifuge for 20 minutes. The serum was then separated and frozen at -80°C until use.

A final blood collection was done under deep standard anesthesia prior to euthanasia. Blood was collected from the jugular vein as well, and we collected approximately 5% of body weight of blood before the dogs were euthanized. Blood was then treated as described above.

ACTH stimulation test: Approximately 10 ml of blood was collected as described above and 5 µg/ kg of synthetic ACTH was administered intravenously. Exactly one hour later, another blood sample was collected and cortisol and aldosterone concentrations were measured via commercially available and validated test at the Endocrinology Laboratory, Diagnostic Center for Population and Animal Health (DCPAH), MSU. The ACTH stimulation test was performed to demonstrate adrenal function and was always done between 9 am and 11 am.

Immunization: Lola and Blanket were immunized once with 125 µg of the previously described purified recombinant modified canine 21-hydroxylase-GST fusion protein, mixed with

complete Freund's adjuvants, and four additional times with the same protein, but mixed with incomplete Freund's adjuvants, over a time of approximately 4 months. The complete and incomplete adjuvants were mixed 1 to 1 (v/v) with the antigen and the final volume for each injection was then brought up to 1 mL with sterile 0.9 % NaCl (sodium chloride) solution. The mixture was injected subcutaneously and the inoculation sites were separated between each injection (first injection: left dorsal ribcage; second injection: right dorsal ribcage; third injection: left dorsal abdomen; fourth injection: right dorsal abdomen; fifth injection: left ventral abdomen). Lucy, the negative control dog, did not get immunized. Over the next two weeks post each injection, the injection site was observed daily for swelling, and the dogs overall clinical well-being was observed and recorded.

Laparoscopic adrenal biopsy: Prior to surgery, the dogs were induced with intravenous thiopental. The dogs were then intubated and anesthesia maintenance was achieved with Isoflurane in a semi closed circle system. The anesthetized dogs were placed in dorsal recumbency and the ventral abdomen was clipped from the xiphoid to the pubis and each lateral flank. The exposed skin was cleaned with alternating skin prep agents (alcohol and dilute chlorhexidine surgical scrub). Standard sterile draping techniques were employed. A 6 mm skin incision was made immediately caudal to the umbilicus on midline. Using blunt dissection, the linea alba was then identified. Two stay sutures were placed in the linea alba and a sharp tipped 5 mm trocar/ cannula was inserted into the peritoneal cavity. A Storz CO_2 (carbon dioxide) insufflating device was connected to the 5 mm cannula and CO_2 was infused into the abdomen at a rate of 1 L/ minute until intraabdominal pressure was 12 mmHg. A 5 mm 0 degree rigid

telescope was then placed into the midline cannula and the abdomen was explored. Under direct visualization of the endoscopic camera, two 5 mm instrument cannulas were placed on ventral midline 3-5 cm cranial and caudal to the first cannula. To place these portals, a 2-3 mm stab incision into the skin was made using a sharp trocar tip to penetrate into the peritoneal cavity. Once all three portals were in place and secured, the animal was placed in right lateral recumbency.

A 5 mm blunt-tipped fan retractor was then used to displace the spleen and stomach to the right side of the abdomen; this exposed the left kidney and left adrenal gland. Once the left adrenal gland was identified, a 5 mm laparoscopic punch biopsy instrument was inserted into the abdomen and a biopsy of the adrenal gland was taken immediately caudal to the phrenicoabdominal vein. Hemorrhage was controlled by direct pressure from a 5 mm blunt probe, and a hemostatic agent (Gelfoam) was placed on the biopsy site. Once any hemorrhage was controlled, the patient was turned into left lateral recumbency and a fan retractor was used to displace the caudate lobe of the liver and the proximal duodenum in a cranial-left-lateral direction to expose the right kidney and right adrenal gland. Once the right adrenal gland was identified, the same biopsy technique was employed.

The patient was then placed back into dorsal recumbency and the pneumoperitoneum was released by removing all three portals. Once the CO_2 was evacuated, two small sutures of 3-0 PDS (polydioxanone) were used in an interrupted pattern to close the 5 mm defects in the linea alba and 3-0 Nylon was used to close the skin.

Flank approach adrenal biopsy: Dogs were induced with intravenous Propofol. The dogs were then intubated and anesthesia maintenance was achieved with Isoflurane in a semi-closed circle system. The anesthetized dogs were then placed in dorsal recumbency and the ventral abdomen was clipped from the xiphoid to the pubis and along each flank. The exposed skin was then cleaned with alternating antiseptics (alcohol and dilute chlorhexidine surgical scrub). For left adrenal biopsy, the dog was positioned in right lateral recumbency. Standard sterile draping techniques were employed. A 10 cm incision was made caudal to the last rib, extending through skin and subcutaneous tissues. The abdominal musculature was separated with sharp and blunt dissection to allow entry of the peritoneal cavity. Gelpi or Nelson retractors were used to improve exposure through the incision. The left adrenal gland was then identified on the cranial pole of the left kidney. Adhesions from previous adrenal biopsy procedures were removed with electrocautery and an adrenal biopsy sample was obtained with 5 mm laparoscopic punch biopsy forceps. Hemorrhage from the biopsy site was controlled with direct pressure, electrocautery, a topical hemostatic sponge (Gelfoam), and vascular ligatures as needed. The incision was closed with 3-0 PDS suture in three layers (body wall, subcutaneous tissue, and skin). For right adrenal biopsy, the same technique was employed with the dog in left lateral recumbency.

Necropsy: Dogs were induced with intravenous Propofol. Dogs were then intubated and anesthesia maintenance was achieved with Isoflurane in a semi-closed circle system. After collecting blood as described above, the dogs were euthanized using an overdose of pentobarbital. After opening the dogs up in midline, both adrenal glands, and a section from the thyroid glands, spleen, kidneys, and liver were harvested and immediately immersed into

10% neutral buffered formalin for fixation. The tissues were submitted to the Histology Laboratory at the DCPAH, MSU, where they were embedded in paraffin and routinely processed and stained with hematoxylin and eosin for evaluation. Additional sections of the right and left adrenal glands were assessed for the presence of lymphocytes, using immunohistochemistry to identify T cells and B cells using antibodies directed against cell type-specific antigens (CD3- (cluster of differentiation 3) T cells; CD79a- B cells).

Results

Blood collection: The harvested serum was clear and amber in color. There were no negative side effects for the dogs from the blood collection. The serum was aliquoted and stored at -80°C until further analysis as described in chapter VIII.

ACTH stimulation test: The cortisol and aldosterone results are summarized in tables VII.2 and VII.3. The cortisol and aldosterone concentrations post ACTH are graphed in figures VII.1 through VII.4. Given only two treatment animals and one control animal, it was not appropriate to perform statistical tests for differences over-time or by treatment. Since there is variation in the baseline levels, the decision was made to graph post-ACTH cortisol and post-ACTH aldosterone percentage difference over time so that the control dog (biopsies but no injections) could be compared to the treated dogs on the same basis. The data indicates that there are no differences attributable to the immunizations when compared to the control dog. There did not appear to be a consistent effect over time (multiple biopsies) although

subjectively cortisol response may have been lower on the final day 01/18/2011 and aldosterone response reduced on 11/14/2011. Almost all values remained within the reference range, and Lola's slightly decreased aldosterone concentrations pre-ACTH on 11/14/2010, 12/5/2010 and 1/18/2011 are of questionable clinical significance, based on the normal aldosterone response post-ACTH. The slightly increased cortisol concentrations pre-ACTH on 10/23/2010 and 10/23/2010, 11/14/2010, and 12/5/2010 in Lola and Lucy, respectively, again are of questionable clinical significance and are most likely related to stress.

Immunization: Other than minor swelling at the injection site for an average of 8 days, there were no negative side effects for the dogs from the immunizations. Each immunization was given in a small final volume of 1 ml, injected into five different sites on the flank of the dogs. Many of the undesirable side effects of Freund's adjuvant were eliminated through refinements in its use. Special care was taken in mixing the adjuvants with the protein preparation to get an emulsion, which decreases side effects.

Laparoscopic adrenal biopsy: The first laparoscopic adrenal biopsies went well and a biopsy sample from all adrenal glands from all three dogs was obtained. The dogs recovered well and there were no complications. During the second attempt, significant adhesions and scar tissues were encountered. When Lola was biopsied a second time via laparoscopic surgery, no sample was obtained from the left adrenal gland. The right adrenal gland was adhered underneath the vena cava and retroperitoneal fat and no sample was obtainable. Laparoscopic adrenal biopsy was therefore only performed in all dogs to obtain baseline histopathology. The adrenal glands are small, vascular structures located near the vena cava, aorta, renal veins, and

lie dorsal to the phrenicoabdominal veins. When anatomy cannot be adequately visualized in this area, minimally invasive biopsy methods risk damage to surrounding vascular structures, potentially causing life-threatening hemorrhage. For subsequent adrenal biopsies, the Animal Use Form was amended to do flank approach adrenal biopsies. This approach provides direct access to the adrenal glands through a smaller incision than that necessary with a more conventional midline incision, allowing excellent access to the glands for sampling, ease of releasing adhesions, and better control of hemostasis with postoperative discomfort similar to that with a laparoscopic procedure.

The results of the histopathology will be discussed together with the biopsies obtained during flank approach and during necropsy later on in this chapter.

Flank approach adrenal biopsy: Obtaining adrenal biopsies via flank approach as described in materials and methods was very easy and straightforward. The dogs recovered quickly and there were no complications. Adrenal biopsies from both adrenal glands from all three dogs were obtained. The results of the histopathology will be discussed together with the biopsies obtained during laparoscopic approach and during necropsy later on in this chapter.

Necropsy: The adrenal gland biopsies taken throughout the experiment were histologically normal and no adrenal inflammation could be demonstrated. The adrenal glands of the study dogs were indiscernible from the control dog adrenal glands, and no infiltration of T (CD3 +) or B lymphocytes (CD79a) were identified immunohistochemically.

The zona glomerulosa, which lies just beneath the adrenal capsule, was composed of small clusters and short trabeculae of relatively small, well defined cells. These cells had less

cytoplasm than the other cortical cells. The zona glomerulosa accounted for approximately 15 % of the cortical volume. The zona fasciculate was formed by a broad band of large cells with distinct membranes that were arranged in cords, usually two cells wide. The cytoplasm had numerous small lipid vacuoles, which sometimes indented the central nucleus and resembled lipoblasts. The zona fasciculata accounted for approximately 75 % of the cortical volume. The zona reticularis was grossly browner than the other two layers. Cells, smaller than the cells of the zona fasciculata, were randomly arranged, with granular and eosinophilic cytoplasm with lipofsucin but minimal lipid. This layer was thinner than the zona glomerulosa or zona fasciculata.

Discussion

Throughout this experiment, adrenal inflammation was not observed by the injection of the purified 21-hydroxylase. The histology of the adrenal glands remained completely normal, adrenal function remained within the physiologic limit and no treatment effect was found. Based on this, I concluded that hypoadrenocorticism was not induced. These findings were not unexpected since previous studies showed that the immunization of animals with homologous adrenal did not result in histopathologic changes; whereas only the immunization with heterologous adrenal resulted in adrenalitis with predominantly polymorphonuclear cells in some glands and round cells in others[3-6].

Because of the use of adjuvants, I was still expecting that the two injected dogs developed antibodies against 21-hydroxylase, which will be tested and discussed in the following chapter. The rabbit sera were tested as well and also discussed in the following chapter.

In order to obtain a strong immune response to the injected 21-hydroxylase-GST fusion protein, I used Freund's adjuvants. Freund's adjuvant is an immunopotentiator and is named after Jules T. Freund. Freund's complete adjuvant contains inactivated and dried *Mycobacterium tuberculosis* in mineral oil, whereas the incomplete form only contains water in mineral oil. Due to its painful reactions and potential for tissue damage including necrosis, the use of Freund's adjuvants is strictly regulated and only subcutaneous or intraperitoneal injections are allowed. Freund's adjuvant has been investigated in the prevention of juvenile diabetes and in an animal model of Parkinson's disease[7-10]. Even though other adjuvants are nowadays available, Freund's adjuvant was chosen for my experiment since the production of antibodies in dogs is best described using Freund's adjuvants[11-15].

An adjuvant may stimulate the immune system without having any specific antigenic effect itself. An adjuvant is defined as "any substance that acts to accelerate, prolong, or enhance antigen-specific immune responses when used in combination with specific antigens"[16]. An adjuvant stimulates cell-mediated immunity and leads to the potentiation and production of immunoglobulins. The potentiation is accomplished by mimicking sets of evolutionary conserved molecules (PAMPs), including liposomes, lipopolysaccharide, molecular

cages for antigen, components of cell walls, aluminum salts, and endocytosed nucleic acids[17]. These specific antigenic moieties are recognized by the immune system, and the recognition in conjunction with another antigen greatly increases the innate immune response to the antigen by augmenting the activities of dendritic cells, lymphocytes, and macrophages by mimicking a natural infection. Since there are many links between the innate immune system and the adaptive immune system, an adjuvant-enhanced innate immune response results in an enhanced adaptive immune response. Specifically, a recent study showed that adjuvants may exert their immune-enhancing effects by five immune-functional activities[18]: First, adjuvants help in the translocation of antigens to the lymph nodes. In the lymph node, they can be recognized by T cells, ultimately resulting in greater T cell activity. Second, adjuvants physically protect antigens, which increases the exposure duration, upregulating the production of B and T cells. Third, adjuvants cause local reactions at the injection site, inducing a greater release of chemokines, which in turn increases the release of helper T cells and mast cells. Fourth, adjuvants induce the release of inflammatory cytokines, which recruit B and T cells, and increase transcriptional events, leading to a net increase of immune cells as a whole. Fifth, adjuvants increase the innate immune response to the antigen by interacting with pattern recognition receptors, including Toll-like receptors.

My hope was to achieve a robust immune response in the injected animals. This would enable me to use their sera as positive and negative (Lucy) controls during assay development. Positive and negative controls are needed during ELISA development to eliminate alternate explanations of test results.

APPENDIX

Appendix

Table VII.1: Immunization timeline.

Antibody production schedule:		
6/20/2010	ACTH stimulation	#1- Baseline ACTH stimulation
6/21/2010	Bleed	#1- Pre-immune bleed
6/28/2010	Laparoscopic adrenal biopsy	#1- Baseline adrenal biopsy
7/4/2010	Bleed	#2- Production bleed
7/11/2010	Immunization	#1- Complete Freund's Adjuvant
8/1/2010	Immunization	#2- Incomplete Freund's Adjuvant
8/8/2010	Bleed	#3- Production bleed
8/20/2010	ACTH stimulation	#2- ACTH stimulation
8/20/2010	Laparoscopic adrenal biopsy	(unsuccessful, see text)
8/30/2010	Immunization	#3- Incomplete Freund's Adjuvant.
9/5/2010	Bleed	#4- Production bleed
9/19/2010	Immunization	#4- Incomplete Freund's Adjuvant.

Table VII.1 (cont'd):

9/26/2010	Bleed	#5- Production bleed
10/23/2010	ACTH stimulation	#3- ACTH stimulation
10/27/2010	Adrenal biopsy through flank	#2- Adrenal biopsy
11/7/2010	Immunization	#5- Incomplete Freund's Adjuvant.
11/14/2010	Bleed	#6- Production bleed
11/14/2010	ACTH stimulation	#4- ACTH stimulation
12/5/2010	Bleed	#7- Production bleed
12/5/2010	ACTH stimulation	#5-ACTH stimulation
1/18/2010	Bleed	#8- Production bleed
1/18/2011	ACTH stimulation	#6-ACTH stimulation
1/20/2011	Euthanasia and necropsy	#7-Production bleed; #3- Adrenal biopsy

Table VII.2: ACTH stimulation test, cortisol concentrations.

Cortisol (normal): pre: 15-110 nmol/L post: 220-550 nmol/L	Baseline	8/20/2010	10/23/2010	11/14/2010	12/5/2010	1/18/2011
Blanket pre:	27	10	35	40	89	92
post:	352	316	326	342	340	286
Lola pre:	61	37	164	81	70	91
post:	480	334	486	488	444	245
Lucy pre:	120	52	294	167	128	51
post:	450	335	459	449	378	314

Table VII.3: ACTH stimulation test, aldosterone concentrations.

Aldosterone (normal): pre: 14-957 nmol/L post: 197-2103 nmol/L	Baseline	8/20/2010	10/23/2010	11/14/2010	12/5/2010	1/18/2011
Blanket pre:	55	32	65	39	134	67
post:	499	308	514	286	535	431
Lola pre:	38	109	22	0	5	11
post:	475	392	489	223	439	280
Lucy pre:	156	135	459	77	257	151
post:	897	851	792	469	704	611

Figure VII.1: Cortisol concentrations post ACTH [% of baseline].

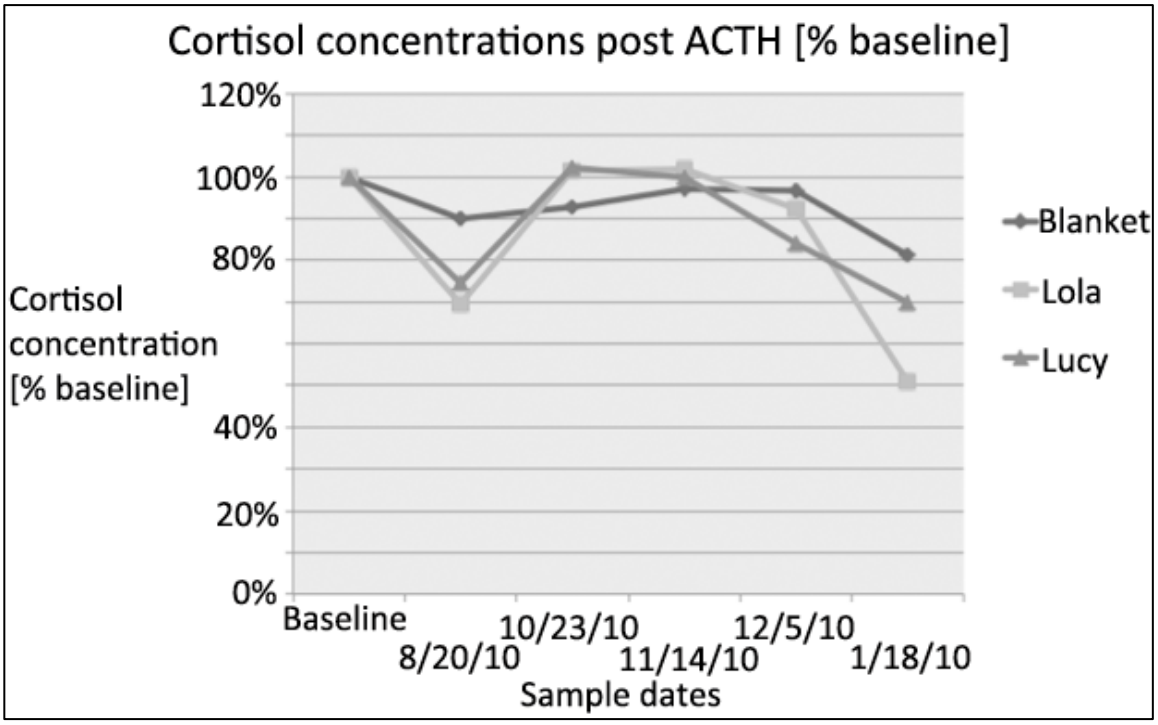


Figure VII.2: Cortisol concentrations post ACTH.

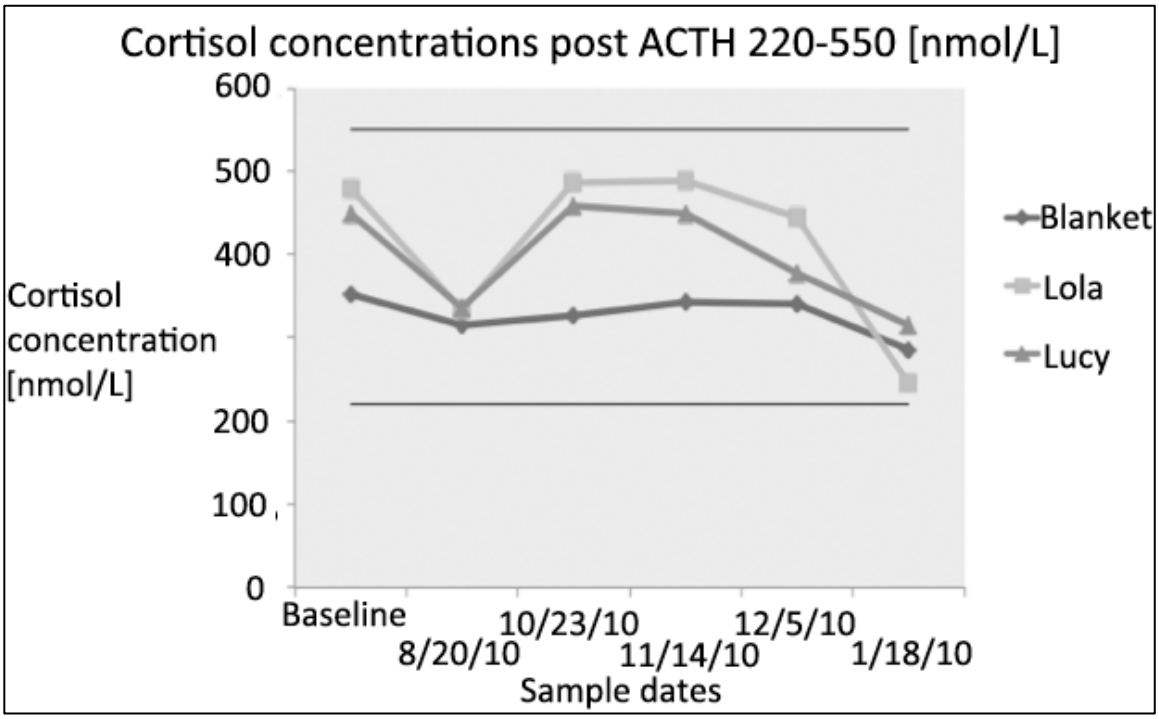


Figure VII.3: Aldosterone concentrations post ACTH [% of baseline].

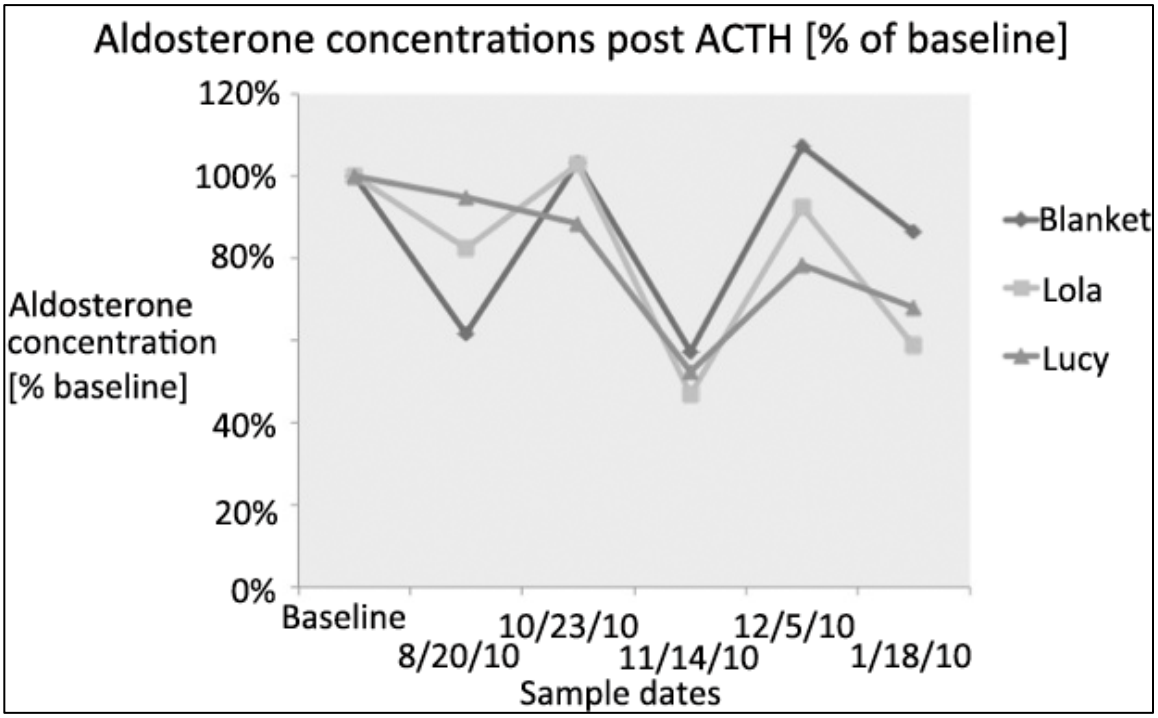
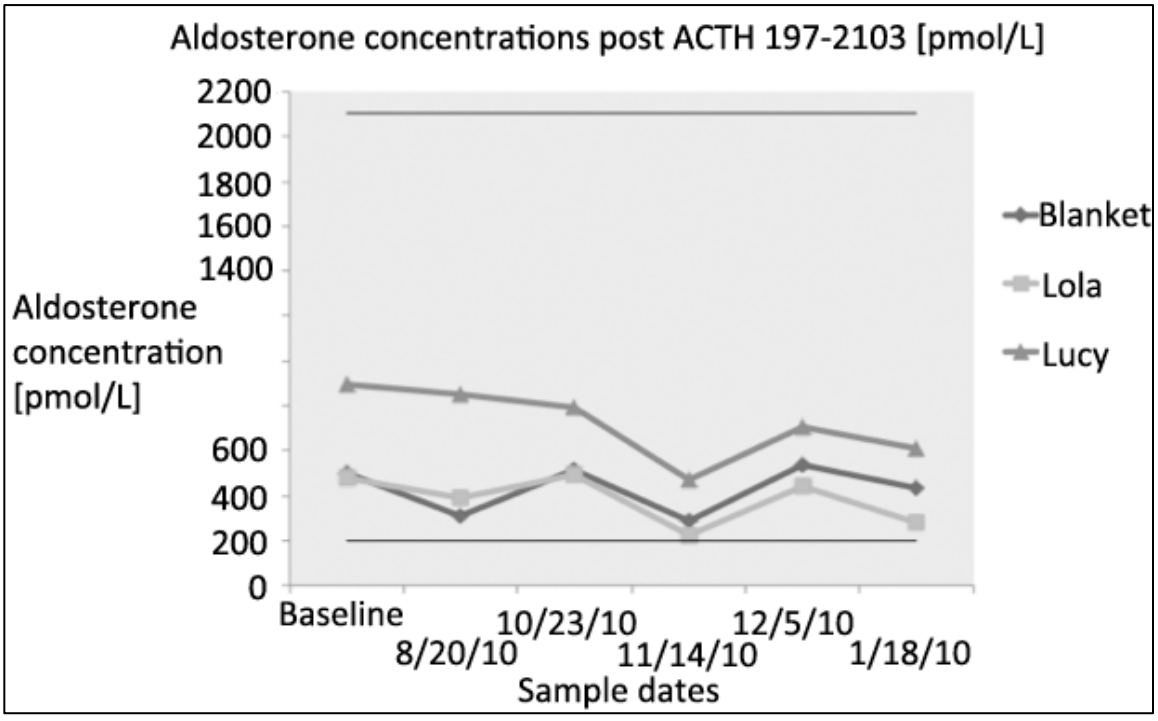


Figure VII.4: Aldosterone concentrations post ACTH.



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Chapter VIII: Evaluation of immunoreactivity of canine and rabbit sera with the purified 21-hydroxylase-His

Introduction

In this part of the project, the immunoreactivity of the dog and rabbit sera pre- and post-immunization against the purified 21-hydroxylase-His was evaluated using Western blot (WB). The immunoreactivity of sera from dogs with Addison's disease (collected at the Endocrinology Section, Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University (MSU), East Lansing, MI, USA) against the purified 21-hydroxylase-His was also evaluated using WB. Further analyzed were study dog sera pre and post immunization with equine adrenal glands using immunohistochemistry (IHC). Lastly, the immunoreactivity of the dog sera pre- and post-immunization and sera from dogs with Addison's disease (collected at the Endocrinology Section, DCPAH, MSU) against purified 21-hydroxylase-His was analyzed using enzyme-linked immunosorbent assay (ELISA).

Materials and methods

For the three proposed techniques, the protein concentration of the 21-hydroxylase-His in TBS, the 21-hydroxylase-His in TBS containing 4 M urea, the 21-hydroxylase-His in TBS containing 4 M guanidine, and the 21-hydroxylase-His in TBS containing 4 M guanidine, plus 250 mM imidazole was measured via bicinchoninic acid (BCA) protein assay, following the

manufacturer instructions. The appropriate amount of urea, guanidine and/ or imidazole was added to prepare the standards for the BCA assay to adjust for interferences from these chemicals. The optical density was then read at 562 nm and the concentrations were calculated.

Western Blot: Approximately 200 ng of 21-hydroxylase-His was run on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride (PVDF) membranes in transfer buffer (10 mM CAPS (3-[cyclohexylamino]-1 propane sulfonic acid), pH 11 containing 10% methanol) for one hour at 0.51 A. The membranes were then incubated with the primary antibody (canine and rabbit serum) in 1xPBS (phosphate buffered saline), containing 1 % Tween (PBS-T), and 1 % (w/V) instant nonfat dry milk milk at 4°C overnight at a dilution of 1:1,000. The membranes were washed three times with PBS-T for 15 min each at room temperature and incubated with the secondary antibody (anti-canine or anti-rabbit IgG/ horseradish peroxidase, 1:5,000 in PBS-T, containing 1 % (w/V) instant nonfat dry milk). After the wash step, chemiluminescence solution was added for about one minute and the signals detected using the ChemiDoc XRS+.

Immunohistochemistry: Normal equine adrenal gland collected served as control tissue to test for the presence of 21-hydroxylase autoantibody. 5-6 µm paraffin sections were placed on glass slides. To perform the IHC, the sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. Non-specific protein binding sites were blocked using bovine serum albumin in PBS. Serum from the experimental dogs (pre- and post-vaccination) as well as known positive serum from a human Addison's disease patient and normal human serum served as positive and negative controls. All serum samples (primary antibody) were applied to

the equine adrenal sections at 1:50, 1: 1,000, and 1:10,000 concentrations. Negative controls for the primary antibody consisted of PBS substitution for the primary antibody or application of normal mouse serum (nonsense control). The secondary antibodies consisted of horseradish peroxidase labeled rabbit anti-canine IgG (immunoglobulin gamma) and rabbit anti-human IgG. Nova red served as the chromagen substrate for the horseradish peroxidase catalyzed reaction at sites of antibody binding. Each slide was lightly counterstained with hematoxylin.

ELISA: Coating buffer was prepared (0.1 M Na_2CO_3; pH 9.6) and the wells of a 96-well high-binding microplate were coated with 100 µL of 21-hydroxylase-His in TBS, 21-hydroxylase-His in TBS containing 4 M guanidine, and 21-hydroxylase-His in TBS containing 4 M urea, diluted in coating buffer at a concentration of 0.01 mg/ mL. Each well contained 1 µg of antigen obtained from three different preparations as described in chapter VI. A second plate was coated with just coating buffer to measure non-specific binding (NSB) of the samples. The plates were then incubated at 4°C overnight (approx. 16-20 hours).

Upon removal from refrigeration, the wells were blocked by adding 300 µL of blocking buffer (obtained from Oxford Laboratories, Rochester, MI, USA) to each well. The blocking buffer was added directly over the coating solution and allowed to incubate at room temperature (RT) for one hour.

After the blocking incubation, the plate was washed using the following procedure: 1) decant the contents of the plate; 2) add 300 µL of wash buffer (Oxford Laboratories) to each well. Let stand for 2 minutes at RT, then decant. This was repeated two more times for a total

of three washes; and 3) the plates were inverted and blotted to remove any residual moisture from the wells.

To perform the assay, canine serum was diluted 1:100 in enzyme immunoassay (EIA) buffer (Oxford Laboratories) and 100 µL of the diluted serum in duplicate wells of both antigen-coated and NSB plates was added. The plates were incubated at RT for 2 hours and then washed as described in steps 1-3 above.

100 µL of affinity purified horseradish peroxidase (HRP)-conjugated goat anti-Dog IgG (heavy and light chain) (Kirkegaard & Perry Laboratories, Maryland, USA) (final dilution of 1:70,000 in EIA buffer) was added to wells (antigen-coated and NSB) and incubated at RT for one hour. The plates were then washed as in steps 1-3 above.

150 µL of TMB (3,3′,5,5′-tetramethylbenzidine) (obtained from Oxford Laboratories) substrate was transferred to each well and incubated at RT for 30 minutes or until an appreciable blue color had formed. Color development was terminated by adding 50 µL of 3N H_2SO_4 to the wells. Plates were read on a spectrophotometric plate reader at 450 nm.

In the first experiment, three different antigen preparations of the purified 21-hydroxylase-His were compared with one another, using serum from the two study dogs (Lola and Blanket) pre- and post-immunization, from our control dog (Lucy), and from two young and healthy dogs. In one well, only buffer was added. In the second experiment, the 21-hydroxylase-His in TBS was used for plate coating, and sera from our three dogs, from 10 healthy dogs, and from 12 dogs diagnosed with hypoadrenocorticism were evaluated for immunoreactivity with the purified 21-hydroxylase-His.

Results

After one FPLC (fast-protein liquid chromatography) purification run, the elution fractions were pooled. After that, an aliquot was either salt-exchanged against TBS, using PD-10 desalting columns (GE Healthcare, Piscataway, NJ, USA), dialyzed against TBS containing 4 M guanidine or TBS containing 4 M urea, or diluted 1:2 with TBS. The concentration of the 21-hydroxylase-His was then measured as described in materials and methods. The results are shown in table VIII.1, clearly showing that the highest amount of purified 21-hydroxylase-His was present in the sample that was diluted 1:2 with TBS. The sample that was salt exchanged, using a PD-10 desalting column had the lowest concentration.

Western Blot: The samples from the immunized dogs and rabbits showed immunoreactivity with the 21-hydroxylase post-immunization, but not before (figure VIII.1 and figure VIII.2). Out of 13 analyzed samples from dogs with hypoadrenocorticism, four tested positive (31%) using WB.

Immunohistochemistry: No specific labeling for 21-hydroxylase could be identified in the equine adrenal glands using either the pre- or post-vaccination canine serum or the two different (known positive and known negative) human sera. There was extensive diffuse background labeling of all of the equine adrenal gland sections, including the negative controls, thus I could not identify the presence of 21-hydroxylase auto-antibodies using immunohistochemistry.

ELISA: Results of experiment 1 are shown in figure VIII.3. The three compared 21-hydroxylase-His preparations gave similar results. The optical density is significantly increased in the two immunized dogs post-immunization (2 to more than 3 fold), demonstrating

immunoreactivity between the purified 21-hydroxylase-His and the sera from these animals. In contrast, Lucy (our negative control), Aerdenbouts, and Partydoll are considered to be healthy and all have a low optical density. In the second experiment, 3 out of the 12 dogs that have been diagnosed with hypoadrenocorticism showed a high optical density, as well as did Blanket and Lola post immunization (figure VIII.4). Due to time-constraints of our collaborators, the 21-hydroxylase-His in TBS containing 4 M guanidine, and 250 mM imidazole has not yet been tested.

Discussion

The results of WB are encouraging, showing that the immunized dogs mounted a measurable immune response against the 21-hydroxylase-GST (21-hydroxylase-glutathione S-transferase). The serum of the immunized dogs could therefore be used for adjusting IHC techniques and for ELISA development. The results of IHC remain unsatisfactory, having to deal with an immense amount of background staining. The preliminary results of ELISA are very promising, even though further work needs to be done. Ideally, more clinical samples as well as samples from certain breeds that are known to be at high risk to develop hypoadrenocorticism should be analyzed. Up to this point, some dogs clearly show immunoreactivity with the purified 21-hydroxylase-His, which is promising, but not all analyzed dogs with Addison's disease show this kind of immunoreactivity. Further, some outwardly healthy dogs, including Blanket pre-immunization, have a relatively high result in ELISA. The fact that some healthy dogs have a high result in ELISA was repeatable and cannot be explained.

Non-specific reactions in ELISAs are a known problem, and from my own experience at

the Endocrinology Laboratory at the DCPAH, MSU, especially tricky when used with canine serum. In order to decrease non-specific reactions and to increase specificity and sensitivity of an ELISA, primarily two steps should be taken. First, care should be taken during the purification of the 21-hydroxylase-His to have an as pure as possible protein as an antigen source. This is the most important factor during ELISA development to minimize background reactions or nonspecific reactions[1-3]. Second, the optimal working conditions for each component of an ELISA need to be found to increase specificity and decrease background readings, including but not limited to the determination of antigen concentrations, test sera dilutions, blocking agents, incubation times and washing steps. As an example, if the antigen concentration is too high, it might inhibit the binding of the antibody through steric inhibition. Further, it may increase stacking or layering of antigen, which may lead to an unstable interaction of subsequent reagent. In contrast, low-density binding of antigen may result in an unevenly coated plate, and unoccupied spaces might then bind sera protein or other contaminating proteins[1,4]. The conditions of the developed ELISA in its current form have not been optimized yet and further work needs to be done. In the literature, especially the use of Tween 20 in the washing buffers and the use of bovine serum albumin as a blocking agent in any buffers have been shown to inhibit nonspecific reactions[4].

Even not optimized, the ELISA in its current form is highly reproducible, showing minimal intra and inter-assay variation of less than 10%. Because of the low number of repeated testing as of yet, statistical analysis could not be performed. Even though the interpretation of the less than 10% of intra- and inter-assay variation should be done cautiously

because of the low numbers of testing, both values are clearly within the standard repeatability criterion for ELISA validation[5].

Another critical step in the standardization of an ELISA and the determination of diagnostic usefulness of a serologic test is the determination of the cutoff value to separate positive from negative results. At present, different methods are used to set these cutoff values[6-9]. The most commonly used method for cutoff determination is adding two or three, and occasionally, especially for in-house ELISAs, four standard deviations to the mean of the negative control sera[10]. Depending on the purpose of the test, two or three standard deviations might be more appropriate, with two preferred for screening purposes and three appropriate for diagnostic purposes[11]. Once the cutoff has been set, the specificity, sensitivity, and reproducibility can be assessed. Therefore, future evaluation and optimization of the ELISA is needed.

For further discussion of results obtained in this chapter, also see chapter IX.

APPENDIX

Appendix

Table VIII.1: Comparison of the 21-hydroxylase-His concentrations in the four different preparations, clearly showing that the highest concentration was found in TBS, containing 4 M guanidine, and 250 mM imidazole.

21 OH in	Concentration [mg/ml]	Total amount of 21-OH-His from 2.5 mL starting volume [mg]
TBS	0.09	0.315
TBS, containing 4 M guanidine	0.18	0.45
TBS, containing 4 M urea	0.16	0.4
TBS, containing 4 M guanidine, and 250 mM imidazole	0.15	0.75

Figure VIII.1: Western blotting of the rabbit sera, clearly showing immunoreactivity with the expressed 21-hydroxyalse-His after immunization, but not before.

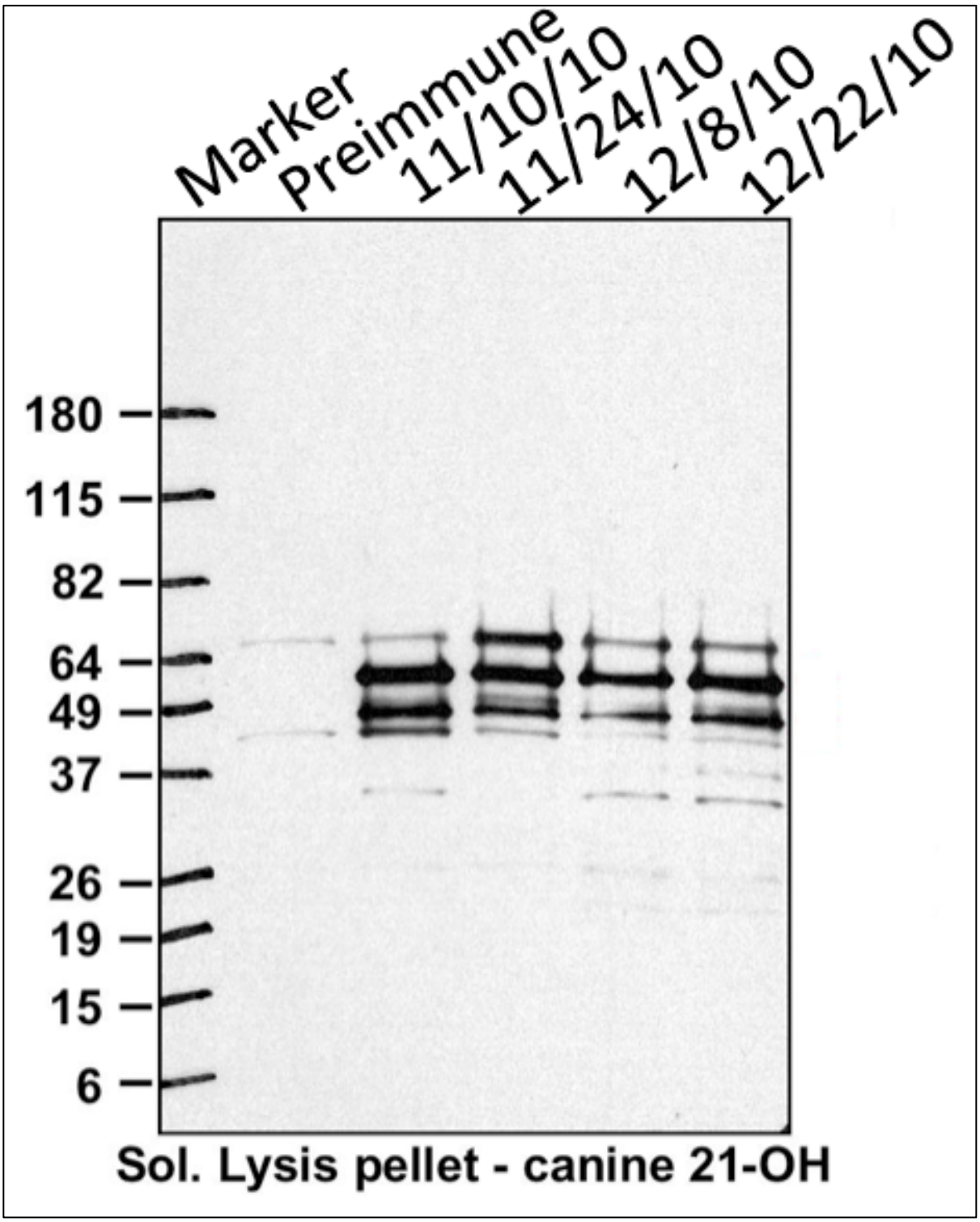

Figure VIII.2: Western blotting of the dog sera, clearly showing immunoreactivity between the expressed 21-hydroxylase-His and 21-hydroxylase-GST and the two study dogs post immunization, but not before. Lucy's serum does not show immunoreactivity.

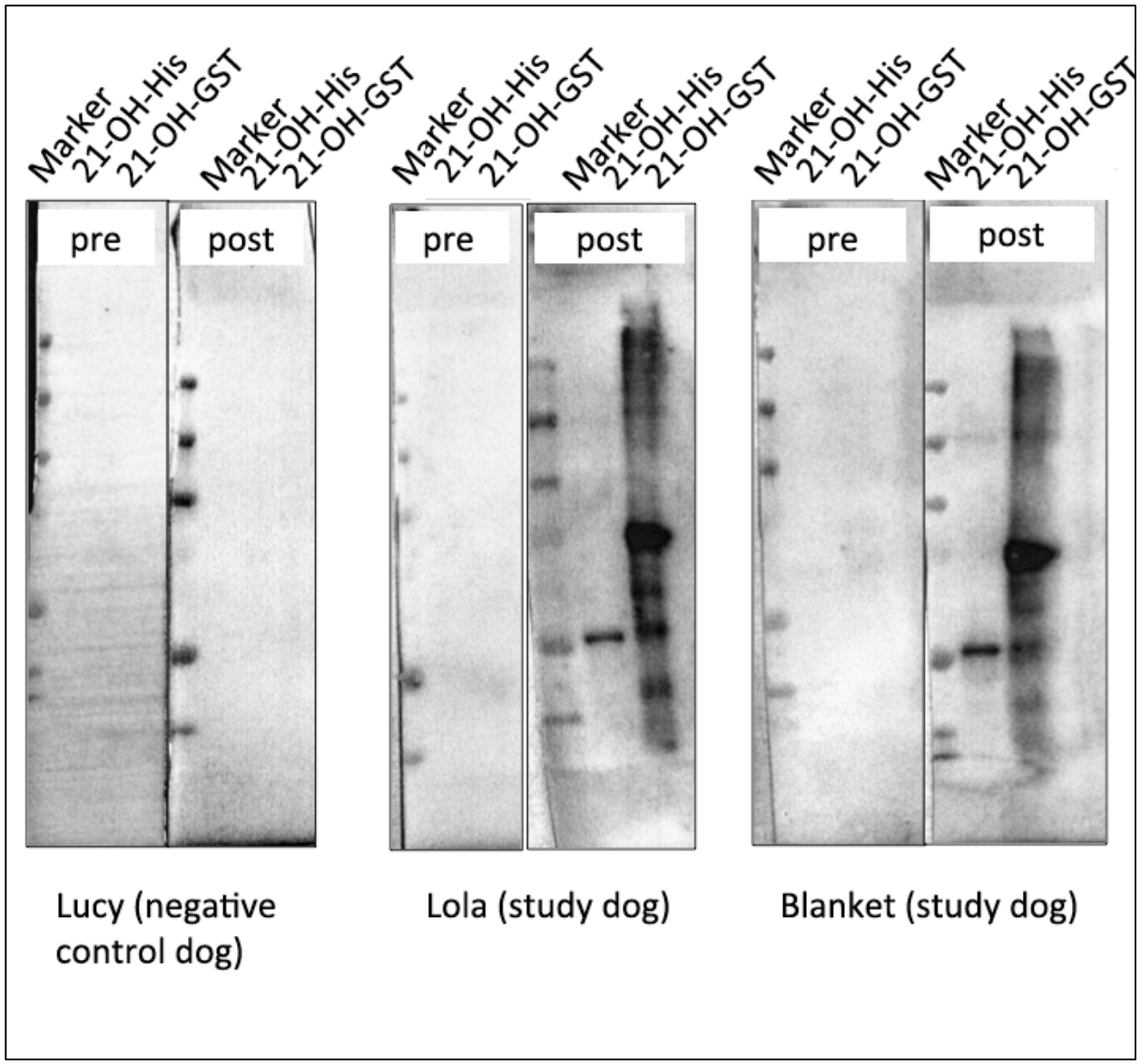

Figure VIII.3: Comparison of the three 21-hydroxylase-His preparations in ELISA, using sera from the study dogs, two healthy dogs, and buffer alone. All three preparations gave similar results. The optical density is significantly increased in the two immunized dogs post-immunization (2 to more than 3 fold), demonstrating immunoreactivity between the purified 21-hydroxylase-His and the sera from these animals.

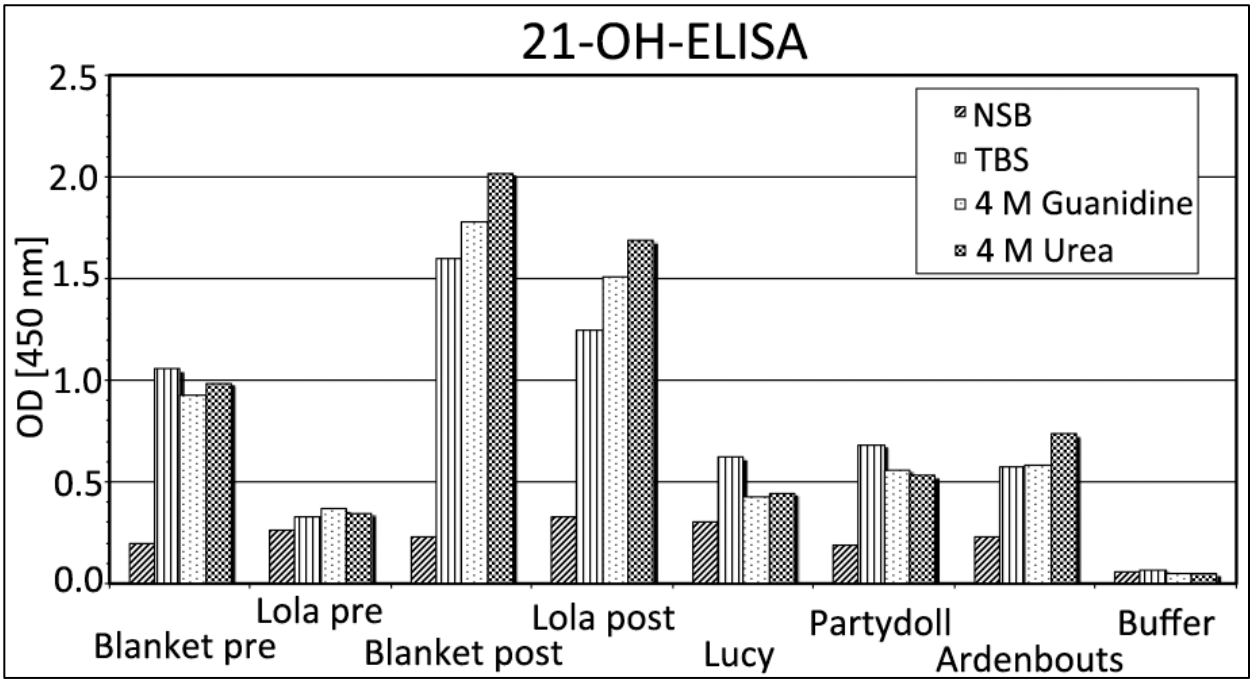

Figure VIII.4: Results of the second ELISA experiment, testing canine serum from healthy dogs, dogs diagnosed with Addison's disease, and from our two positive control study and dogs. (1: Blanket pre immunization; 2: Blanket post immunization; 3: Lola pre immunization; 4: Lola post immunization; 5 to 14: healthy dogs; 15 to 26: dogs diagnosed with hypoadrenocorticism (see table VIII.2 for details)). Three out of the 12 dogs that have been diagnosed with hypoadrenocorticism show a high optical density, as well as Blanket and Lola post immunization.

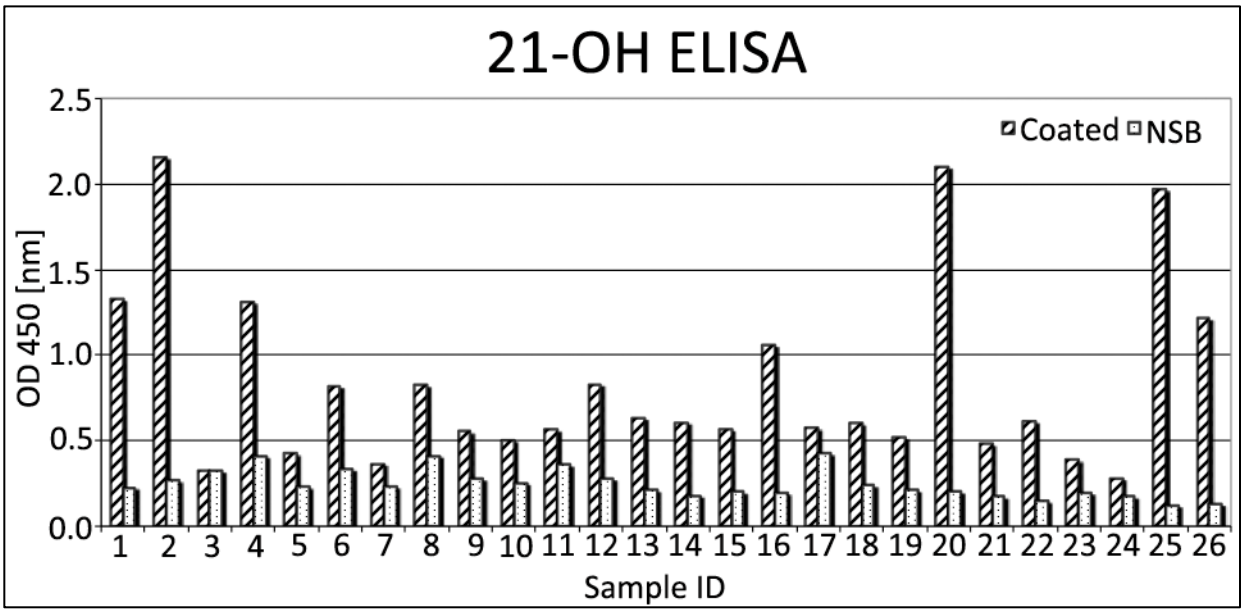

Table VIII.2: Information on the Addisonian dogs that were tested using the newly developed ELISA. A German Shepherd Mix, a Rottweiler, and a Jack Russell tested positive for the presence of 21-hydroxylase antibodies.

Sample ID	Age [years]	Sex	Sample type	Breed
15	1.8	MC	Plasma	German Shorthaired Pointer
16	12	FS	Serum	Chow Mix
17	5	MC	Plasma	Australian Terrier
18	2	FS	Plasma	Golden Retriever
19	2	FS	Plasma	West Highland White Terrier
20	5	M	Plasma	German Shepherd Mix
21	4	FS	Plasma	Schnoodle
22	2	FS	Plasma	English Mastiff
23	1	FS	Serum	West Highland White Terrier
24	3	MC	Plasma	Shih Tzu
25	2	MC	Plasma	Rottweiler
26	7	FS	Plasma	Jack Russell Terrier

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Chapter IX: Discussion and future research

The overarching goal of my dissertation research was to develop a diagnostic test to facilitate early diagnosis of hypoadrenocorticism in dogs before they develop more serious life-threatening clinical signs. The test, when developed, will also help to better understand the pathogenesis and epidemiology of the disease and has the potential to assist in future molecular genetic research.

The data showed that dogs with naturally occurring primary Addison's disease produce 21-hydroxylase antibodies. This is consistent with the human form of the disease. What remains unknown is whether autoantibody production precedes clinical disease, implying a role in the pathogenesis of the disease; if it is, then the presence of 21-hydroxylase autoantibodies would be expected to be more prevalent in dog breeds susceptible to developing hypoadrenocorticism. The specific aims were to establish a diagnostic test to detect canine anti-adrenal autoantibodies, and to determine whether such autoantibodies are present in dogs with hypoadrenocorticism; both aims were successfully established. Development of the test to detect autoantibodies would enable epidemiologic studies to address specific aim 3 "to determine whether development of anti-adrenal autoantibodies has breed, sex, and age-based predispositions".

The canine 21-hydroxylase was expressed in *E. coli*, using standard techniques. First, RNA was obtained from freshly harvested canine adrenal glands. The expression sequence of the 21-hydroxylase gene was then cloned into expression vectors and 21-hydroxylase-fusion protein production was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG).

Unfortunately, even though the anchor of the protein was modified to make it more soluble, the protein was almost exclusively expressed into inclusion bodies. The purification of the protein was therefore very difficult and had to be done under denaturing conditions. Since the purification was so difficult and time-intensive, I decided to describe it in more detail than what is usually expected in a dissertation. My goal was that anybody who would be interested in optimizing the purification of the 21-hydroxylase-fusion proteins would be able to do so with this dissertation, and would know exactly what had been tried before. I also strongly believe that purification conditions could further be optimized to increase protein yield.

The results of Western blotting (WB) suggested that the immunized dogs mounted an immune response to the denatured 21-hydroxylase-glutathione S-transferase (GST) when injected with adjuvants. We therefore were able to use their sera for the development of an ELISA (enzyme-linked immunosorbent assay) that would be able to detect antibodies in clinical samples. Using the same conditions that gave us positive signals in WB with the serum of our immunized dogs, an array of sera from dogs with and without Addison's disease was also examined. Out of 13 analyzed samples from dogs diagnosed with hypoadrenocorticism, four tested positive (31%). The results strongly suggested the presence of circulating 21-hydroxylase antibodies in dogs that were diagnosed with hypoadrenocorticism.

Using the positive control sera, an ELISA was developed and partially optimized. Thus far 12 clinical samples from dogs with hypoadrenocorticism have been tested. Of the 12 samples tested, three (25%) tested positive for the presence of 21-hydroxylase antibodies. As a control, 10 healthy samples were tested. None of 10 healthy control samples tested positive. Taken

together with the results from WB, I have shown that 21-hydroxylase antibodies are present in Addison's disease in dogs.

In humans, up to 90 % of newly diagnosed patients with Addison's disease have detectable circulating 21-hydroxylase autoantibodies. The relatively low percentage of antibody positive samples obtained from dogs with Addison's disease compared to humans may be explained by several different mechanisms. First, other antibodies, for example 17-hydroxylase antibodies or antibodies against cholesterol side chain-cleaving enzyme may play a prominent role in the pathogenesis of hypoadrenocorticism in dogs. Second, antibody titers might disappear once a dog develops clinical signs. In humans, it has been observed that only 78% of individuals with chronic Addison's disease remain antibody positive. This phenomenon is also observed in dogs that develop autoimmune hypothyroidism; many dogs, over time and once they receive adequate thyroid replacement therapy, become thyroglobulin antibody negative even though they tested positive upon or prior to diagnosis. In addition, clinical Addison's disease does not develop until at least 90 % of the adrenal gland is destroyed. Once the organ is completely destroyed, the offending antigens disappear and the antibody titer many become undetectable.

Third, the conditions for the ELISA need to be optimized. Different blocking and incubation conditions for the ELISA need to be evaluated. Luckily, our collaborator throughout this study, Oxford Biomedical Research, Rochester, MI, USA, has extensive expertise in the development of ELISAs for the use in dogs. ELISAs appear to be very sensitive to non-specific binding of serum of dogs; this is a common problem when working with canine serum in such

laboratory settings. This characteristic of canine serum may have contributed to the unusually high background. Currently, Oxford Biomedical Research is the only company that markets a useful enzyme immunoassay for the detection of thyroglobulin autoantibodies (TgAA) in dog serum. The TgAA is a marker for the early development of hypothyroidism in dogs, long before clinical signs appear. Many other companies tried to develop a similar assay, but their success was hampered by non-specific binding of canine serum. Oxford Biomedical Research developed a proprietary blocking buffer that took care of this non-specific binding. Their expertise will help us in the further development of the 21-hydroxylase-antibody assay. On the other hand, the ELISA format might not be the preferred method to detect 21-hydroxylase antibodies, as currently all of the commercially available test kits to detect 21-hydroxylase antibodies in humans are radioimmunoassays. An iodination of the purified 21-hydroxylase-His should therefore be considered and evaluated.

Lastly, the low percentage of detected dogs might be related to the fact that the purification of the 21-hydroxylase-His was done under denaturing conditions and the purified denatured 21-hydroxylase might not react with the 21-hydroxylase antibody present in dogs with Addison's disease.

At this point, we have only tested a small number of samples from dogs with confirmed Addison's disease, i.e. dogs with advanced disease. More clinical samples need to be analyzed, especially samples from currently healthy dogs from pedigrees or breeds that are known to be at high risk to develop hypoadrenocorticism (Bearded Collies, West Highland White Terriers, Standard Poodles, Portuguese Water Dogs, Leonbergers, Great Danes, Airedale Terriers, Basset

Hounds, Wheaten Terriers, Rottweilers, Springer Spaniels, Great Pyrenees, and Nova Scotia Duck Tolling Retrievers). I would like to attend breed shows to collect serum samples from these breeds of dogs. These and individual dogs should be followed in long-term prospective studies to ascertain changes in 21-hydroxylase autoantibody levels over time as dogs go on to develop clinical Addison's disease. Further, samples that are sent to the laboratory at the Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University (MSU), East Lansing, MI, USA, for the thyroid registration through the Orthopedic Foundation for Animals should be analyzed for the presence of 21-hydroxylase antibodies. Received are samples from healthy, purebred dogs. The submitting veterinarian has to sign a statement that the dog is healthy at the time the sample was drawn. Samples are only accepted if they were shipped to the laboratory on ice within two days, which means they are very fresh and high in quality. Collecting data from these two groups will help us validate and improve the test and to better understand the epidemiology and pathogenesis of the disease.

In summary, the 21-hydroxylase-autoantibody ELISA, which was developed during this study will prove to be a useful tool for the early detection of Addison's disease in dogs. The test will improve the health and welfare of dogs with adrenal disease by improving our understanding of its pathogenesis and the value of early detection. Hypoadrenocorticism is perceived as a significant problem, especially in the purebred dog population, with a large genetic component in several breeds, but progress towards understanding and elimination of the disorder has been hampered by lack of diagnostics for the condition before onset of clinical signs. Diagnostic tests currently exist to confidently document the presence of hypoadrenocorticism only after adrenal function is compromised and clinical signs are present,

but not before. The technique developed in this study is likely to be both life- and money-saving by making an early diagnosis, and could conceivably assist in making decisions about which dogs to breed in susceptible breeds, and provide a framework around future studies into the molecular pathogenesis of this important canine disease. Tests to document preclinical hypothyroidism, for example, have been well received by breeders of dogs and are used on a regular basis. As a long-term outcome, the assay for the detection of 21-hydroxylase antibodies can be used to calculate the annual progression rate from preclinical hypoadrenocorticism to clinical hypoadrenocorticism and will help to assess the value of early detection of adrenal disease.

Overall, I am satisfied with the obtained results and very optimistic that the 21-hydroxylase antibody ELISA will be a valuable tool for the early discovery of Addison's disease in dogs.