

Inoculation of dogs with a recombinant ACTH vaccine

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Objective—To determine whether inoculation of healthy dogs with a recombinant peptide containing 3 copies of ACTH would result in the production of antibodies against ACTH and whether this would affect pituitary-adrenocortical function.

Animals—8 healthy dogs.

Procedures—A recombinant peptide consisting of 3 copies of ACTH fused to a T-helper cell epitope was produced in *Escherichia coli*. The protein was inoculated into 4 dogs at 4-week intervals (total of 3 inoculations/dog). Four control dogs received inoculations of PBS solution mixed with adjuvant. Blood samples were collected for determination of antibody titers against ACTH and for measurement of basal and ACTH-stimulated plasma cortisol concentrations.

Results—Inoculation with the ACTH vaccine resulted in production of anti-ACTH antibodies in all 4 dogs. Titers were initially high but declined by 15 weeks after the initial inoculation. Basal cortisol concentrations were unaffected by inoculation with the ACTH vaccine. Plasma cortisol concentrations in response to ACTH stimulation were reduced at 12 weeks, but not at 15 weeks, after the first inoculation.

Conclusions and Clinical Relevance—Inoculation of dogs with a recombinant ACTH vaccine resulted in the production of antibodies against the hormone. Anti-ACTH titers were initially high but were not sustained. The only detectable endocrine effect in treated dogs was a reduction in cortisol concentration in response to ACTH stimulation in 2 of 4 dogs at 12 weeks after the first inoculation. The effect of vaccine administration on the pituitary-adrenal system was subtle and transient. (*Am J Vet Res* 2013;74:1499–1505).

Immunization with self-peptide hormones and the subsequent production of antibodies against them can suppress activity of a variety of endocrine systems. An established example involves use of GnRH vaccination to cause immunocastration, reduction of aggression in males, suppression of boar taint, and enhancement of growth rates in livestock.^{1–5} Several other hormonal peptides have been used as immunogens in an attempt to regulate endocrine physiology. For example, immunization with angiotensin I and II has been used in an attempt to reduce blood pressure,⁶ and parathyroid hormone immunization has been used to control hypercalcemia in patients with parathyroid carcinoma.^{7,8} Active immunization with ACTH has been used in lambs and pigs to lower circulating cortisol concentrations and therefore potentially improve growth and meat quality.^{9–11} These procedures have met with variable success, although immunization with ACTH conjugates usually results in at least transient reductions in basal

ABBREVIATIONS

| | |
|-------------------|------------------------------------|
| BSA | Bovine serum albumin |
| GnRH | Gonadotropin-releasing hormone |
| LB | Luria broth |
| OD ₆₀₀ | Optical density measured at 600 nm |

or stress-induced concentrations of cortisol. The reasons that immunization with some peptide hormones is more effective at suppressing endocrine activity than for other hormones is unknown, but potential factors include the magnitude of the antibody response, affinity of the antibodies produced, peptide epitopes recognized by the antibodies, circulating concentrations of the target hormone, and compensatory responses in the endocrine axis.¹²

Pituitary-dependent hyperadrenocorticism is a common endocrinopathy in dogs.¹³ The disease is usually caused by a microadenoma of corticotroph cells in the anterior pituitary gland that oversecrete ACTH. Support for the central role for ACTH in mediating the disease is derived from several lines of evidence. Circulating concentrations of ACTH are generally higher in dogs with pituitary-dependent hyperadrenocorticism than are concentrations in healthy dogs.¹⁴ In addition, concentrations of corticotropin-releasing hormone (the hypothalamic peptide that regulates secretion of ACTH) are reduced in CSF of dogs with pituitary-dependent

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hyperadrenocorticism, compared with concentrations in healthy control dogs,¹⁵ which supports the idea that the disease originates in the pituitary gland rather than in the hypothalamus or higher brain centers (ie, elevated concentrations of cortisol in the body suppress synthesis and release of corticotropin-releasing hormone). Finally, hypophysectomy has been used as an effective treatment for the disease.¹⁶

Despite the fact that the disease likely originates in the pituitary gland, treatments currently used most frequently (mitotane and trilostane) are directed at the adrenal glands to suppress excessive secretion of cortisol.¹⁷ Although these treatments are effective, they are not ideal, have potentially adverse effects, and require expensive monitoring. In addition, there can be relapses or the development of hypoadrenocorticism with these treatments.

The purpose of the study reported here was to examine the feasibility of inoculation with ACTH to affect (suppress) adrenal gland secretion of cortisol in dogs. The objectives were to determine whether such inoculation would result in an immune response to the hormone and whether antibodies against ACTH would affect adrenocortical function, as assessed by measurement of plasma cortisol concentrations in the basal state and in response to a stimulatory injection of ACTH.

Materials and Methods

Animals—Eight sexually intact American Foxhounds (5 males and 3 females) were used in the study. The females did not display estrus behavior during the time of the study, and progesterone concentrations were < 1 ng/mL in all females at the start of the study. The dogs were 1 to 5 years old and weighed 15 to 24 kg. All were considered to be healthy on the basis of results of physical examination, a CBC, biochemical analysis, and heartworm occult antigen testing. Each dog was vaccinated against canine distemper virus with a modified live-virus vaccine^a administered 1 month before the start of the study. The dogs were housed in standard kennels at a university facility, fed dry adult maintenance food, and provided ad libitum access to water. The protocol was approved by the Auburn University Institutional Animal Care and Use Committee.

Procedures—The dogs were allocated to 2 groups (4 dogs/group) by use of a randomization procedure (each dog was assigned a number on a card, and cards were mixed and chosen blindly from a box). An initial ACTH response test was performed on all dogs. Seven days later, the ACTH immunogen (2 mg; 94 nmol/injection) was inoculated IM into the muscles in the lumbar region of the 4 treatment dogs, whereas the control dogs received an inoculation consisting of an equivalent volume of the adjuvant^b mixed with PBS solution; day of the first inoculation was designated as day 0. Booster inoculations were given 4 and 8 weeks later. A venous blood sample (3 mL) was collected from each dog 1 week before the first inoculation, 2 weeks after each inoculation, and at 4 and 7 weeks following the final inoculation and used for measurement of anti-ACTH antibody titers. The ACTH response test was repeated 4 weeks after the final booster inoculation (12 weeks

after the initial inoculation) and again 3 weeks later (15 weeks after the initial inoculation). Dogs were returned to the College of Veterinary Medicine's research colony after conclusion of the study.

Synthesis of ACTH immunogen—The immunogen was a recombinant peptide (188 amino acids) produced in *Escherichia coli* by use of an expression vector.^c Beginning at the N-terminus, the expressed peptide consisted of the 8-amino acid strep-tag II, followed by a 17-amino acid sequence of canine distemper envelope glycoprotein (ie, p35), then 3 copies of canine ACTH, and finally a 10-amino acid histidine tag. These segments of the molecule were separated from each other by various numbers of amino acids. The strep-tag II and histidine tag sequences were encoded in the commercially available expression vector and assisted in purification and detection of the recombinant peptide. The p35 peptide sequence reportedly acts as a T-helper cell epitope¹⁸ and, when fused to GnRH to produce a synthetic immunogen, can result in high titers of anti-GnRH antibodies and evidence of suppression of reproductive function in male dogs.^{19,20} Vaccination with the modified live canine distemper virus vaccine exposed the immune system of each dog to the p35 peptide sequence to potentially facilitate subsequent antibody production in response to the ACTH immunogen. The sequence encoding the p35 peptide sequence was cloned initially into a plasmid by use of another vector^d by designing complementary oligonucleotides that code for the sequence and that had overhangs compatible with *Hind*III and *Eco*R1 restriction sites. The plasmid was treated with *Hind*III and *Eco*R1, and the oligonucleotide was ligated into this vector by use of T4 DNA ligase. Three sequential copies of the DNA sequence for canine ACTH were inserted into the p3XFlag-CMV-10-p35 vector with the *Bam*HI-*Bgl*II method for directional cloning of multiple repeat sequences.²¹ The cDNA for canine ACTH was obtained by use of a reverse-transcription PCR assay. The pituitary gland was obtained from a dog immediately after that dog had been used in the veterinary student surgery laboratory and euthanized; RNA was collected from the pituitary gland by use of a kit.^e Primers used to amplify the ACTH sequence were designed on the basis of GenBank sequence AY024339, which is a partial sequence of canine proopiomelanocortin. The resultant plasmid contained the p35-3XACTH DNA sequence and was used as a template for the PCR assay and ligation-independent cloning to create a DNA fragment that was subsequently inserted into the pET-51b(+) Ek/LIC expression vector. The final plasmid used for production of the recombinant peptide was sequenced to confirm that the p35-3XACTH fragment was in frame and that no mutations had been created during the cloning process.

Production of the recombinant peptide—Production of the recombinant peptide was accomplished in accordance with standard methods for protein expression and production in *E coli*. Briefly, the expression plasmid pET-51b(+) p35-3XACTH was transformed into an expression host *E coli*, BL21(DE3), and colonies were produced by culturing on LB agar plates

containing ampicillin. A colony was transferred into a small volume of LB with ampicillin and amplified with vigorous shaking at 37°C. Growth was monitored by spectrophotometric determination of the OD₆₀₀. Once the OD₆₀₀ reached approximately 0.5, the contents were added to a flask containing a larger volume of LB-ampicillin, and this flask was vigorously shaken at 37°C for several hours (until the OD₆₀₀ reached approx 1 to 1.2). At that time, isopropyl-β-D-thiogalactopyranoside was added to provide a final concentration of 1mM. Three hours later, the bacterial mixture was centrifuged at 10,000 × g for 10 minutes, and the supernatant then was discarded. Bacteria were lysed with a mild detergent^f in conjunction with an endonuclease.^g The recombinant peptide was in the insoluble fraction. The insoluble paste was first treated with lysozyme to further digest bacterial cell walls and was then washed repeatedly by suspending it in a diluted solution (1:10 dilution with water) of the mild detergent,^f which was followed by centrifugation.

Further purification was performed by solubilizing the cleaned pelleted material in a 6M guanidine solution (that contained 100mM NaH₂PO₄ and 10mM Tris-Cl [pH, 8.0]). This solubilization solution was then loaded onto a nickel-nitrilotriacetic metal-affinity matrix^h by use of a low-pressure chromatography system. This allowed for binding of the 6xhistidine tag on the recombinant peptide to the nickel-nitrilotriacetic metal affinity matrix. The bound peptide was extensively washed in 6M guanidine solution and then in a solution containing 8M urea. The urea content was reduced from 8M to 0M with a gradient system. The peptide was eluted from the matrix with a gradient of imidazole solutions (that contained 50mM NaH₂PO₄ and 300mM NaCl [pH, 8.0]) ranging from 0 to 500nM imidazole. The fractions containing the peptide were pooled and dialyzed against PBS solution (pH, 7.4) by incubation overnight. After dialysis was completed, the peptide appeared to precipitate from solution and form a suspension. This suspension was centrifuged (10,000 × g for 20 minutes), and SDS-PAGE analysis confirmed that virtually all the recombinant peptide was in the insoluble fraction. This insoluble fraction was suspended in a small volume (5 mL) of PBS solution to which Triton X-114 was added to achieve a final concentration of 1% (vol:vol).²² The mixture was incubated at 4°C for 30 minutes with stirring; it then was transferred to a 37°C water bath and incubated for 10 minutes, which was followed by centrifugation at 20,000 × g for 10 minutes at 25°C to achieve separation of the aqueous and organic layers. The upper aqueous layer contained the peptide. This procedure was repeated 2 more times. This method for separation has proven effective for removal of endotoxin from recombinant proteins.²² Endotoxin concentrations were assayedⁱ in the peptide solution after separation, and they were essentially undetectable, whereas endotoxin was detected in the peptide solution before the separation procedures. Thus, treatment with the detergent resulted in solubilization of the recombinant peptide (in PBS solution) and removal of substantial amounts of endotoxin. The final peptide solutions obtained from 4 bacterial preparations were pooled and filtered through a 0.22-μm sterilization filter. An ali-

quot of this solution was removed for evaluation with an ACTH ELISA²³ and SDS-PAGE followed by staining with Coomassie G-250 stain.^j

ACTH response testing and blood sample collection—For the ACTH response test, a blood sample (3 mL) was collected from the jugular vein of each dog for determination of the basal cortisol concentration before administration of a stimulatory dose of ACTH. This was followed immediately by injection of synthetic ACTH^k (5 μg/kg, IV). A blood sample was collected from the contralateral jugular vein 1 hour later. Blood samples were collected into tubes containing EDTA; tubes were gently inverted to ensure proper mixing and then placed in an ice bath until centrifugation. Blood samples were centrifuged within 1 hour after collection; plasma was harvested and stored at -20°C until analysis. Blood samples for determination of antibodies against ACTH were collected in a similar manner, except the samples were collected into anticoagulant-free glass tubes and allowed to clot for 1 hour. Samples were then centrifuged and the serum harvested.

Measurement of hormone concentrations and antibodies against ACTH—Plasma concentrations of cortisol were determined with a validated assay.²⁴ Measurement of circulating anti-ACTH antibodies was performed by first coating wells of a 96-well plate^l with 100 ng of human ACTH1-39/well diluted in coating concentrate solution^m and incubation overnight at 4°C. Wells then were washed 3 times in PBS solution containing 0.05% Tween-20 (all subsequent washing steps were identical) and then blocked by the addition of 300 μL of PBS solution-Tween-20 containing 2% BSA. The following day, the wells were again washed, and then serum was added to each well (100 μL/well) at various dilutions (dilutions were created with PBS solution-Tween-20 containing 2% BSA). Plates were incubated for 2 hours at room temperature (22°C), and the wells then were washed. Goat-origin anti-canine IgG conjugated to horseradish peroxidaseⁿ (100 μL, diluted 1:5,000 in PBS solution-Tween-20 containing 2% BSA) was added to each well, and plates were incubated for 1 hour at room temperature. After incubation, wells were washed, 100 μL of peroxidase substrateⁿ was added to each well, and the plates were incubated at room temperature with gentle agitation for 20 to 30 minutes to allow color development. Finally, 100 μL of 1 N HCl was added, and the absorbance of each well at 450 nm was determined with a microplate reader. Each serum sample was serially diluted; antibody titer was expressed as the reciprocal of the highest dilution that had an absorbance value 3 times as high as the mean absorbance for 6 wells that contained all assay components except canine serum.²⁰

Statistical analysis—Data were analyzed with a 2-way ANOVA for repeated measures or a repeated-measures ANOVA on ranks (basal plasma cortisol concentrations); analysis was performed with commercial statistical software.^o The 2 main variables were treatment (control vs ACTH immunogen) and time. Basal cortisol concentrations and concentrations after ACTH administration were analyzed separately. When signifi-

cant differences were detected, post hoc comparisons were made with the Student-Newman-Keuls method. Values of $P < 0.05$ were considered significant.

Results

The final pooled recombinant peptide solution was assayed with an ELISA to determine the ACTH concentration, and the concentration was 4.1 mg/mL. Analysis with SDS-PAGE revealed that $> 90\%$ of the peptide migrated as a single band of the expected molecular size (21 kDa; data not shown).

No obvious adverse effects associated with inoculation with the recombinant ACTH peptide were detected. Inoculation resulted in detectable concentrations of anti-ACTH antibodies in serum of all treated dogs 2 weeks after the initial inoculation (Figure 1). Titers increased in 3 of 4 dogs in response to the first booster inoculation, but a further increase was evident in only 1 of 4 dogs after the second booster inoculation. All 4 dogs had a clearly declining, but detectable, titer at the conclusion of the study (15 weeks after the initial inoculation). Anti-ACTH antibodies were not detected in serum obtained from any dogs prior to the first inoculation or in control dogs after inoculations.

Basal plasma cortisol concentrations were not affected by inoculation with the peptide (Figure 2). Results of a 2-way ANOVA for repeated measures indicated that there were significant time and treatment-by-time interactions for plasma cortisol concentrations after injection of a stimulatory dose of ACTH (Figure 3). Post hoc analysis indicated that plasma cortisol concentrations before and after ACTH administration did not differ significantly between treated and control dogs before the first inoculation. However, plasma cortisol concentrations after ACTH injection were significantly reduced in treated dogs, compared with concentrations in control dogs, at 12 weeks after the initial inoculation. No differences were detected in plasma cortisol

concentrations before or after ACTH injection at 15 weeks after the initial inoculation. Anti-ACTH antibody titers had declined by week 12 in all dogs and were even lower at week 15.

Examination of results of plasma cortisol concentrations after ACTH injection at 12 weeks after initial

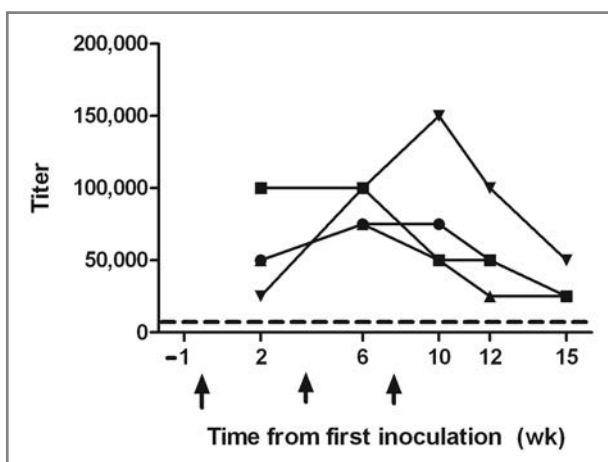


Figure 1—Anti-ACTH antibody titers in 4 dogs inoculated with a recombinant ACTH vaccine. Each symbol represents results for 1 dog. Inoculations were administered at 4-week intervals (arrows); day of the first inoculation was designated as day 0. The dashed line indicates the lower limit of detection of the assay; all inoculated dogs had titers below the lower limit prior to the first inoculation. Results for 4 control dogs that received adjuvant mixed with PBS solution at 4-week intervals were also below the lower limit of the assay at each time point (data not shown).

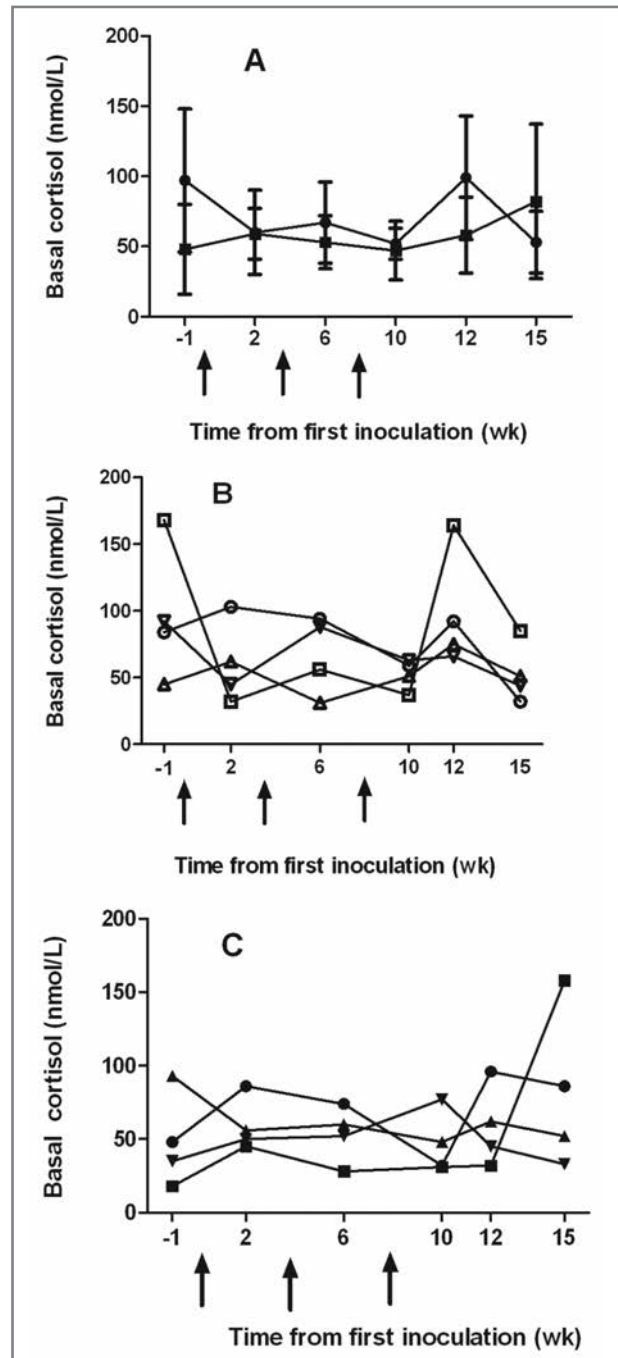


Figure 2—Mean \pm SD basal plasma cortisol concentrations in 4 dogs that received inoculations of ACTH vaccine (treated [squares]) and 4 dogs that received inoculations of adjuvant mixed with PBS solution (control [circles]; A) and basal plasma cortisol concentrations in each of the control (B) and treated (C) dogs. In panels B and C, each symbol represents results for 1 dog. Basal plasma cortisol concentrations did not differ significantly ($P \geq 0.05$) between the 2 groups at any time point. See Figure 1 for remainder of key.

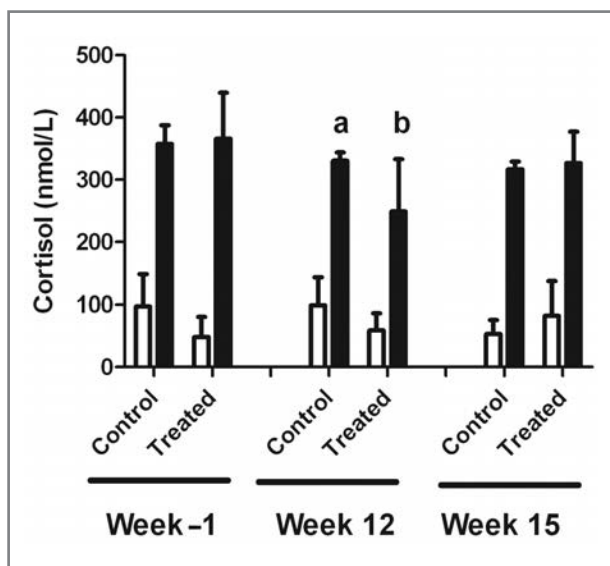


Figure 3—Mean \pm SD plasma cortisol concentrations before (white bars) and after (black bars) injection of ACTH (5 μ g/kg, IV) for 4 dogs receiving an inoculation of a recombinant ACTH vaccine (treated) and 4 dogs receiving an inoculation of adjuvant mixed with PBS solution (control). Response tests were performed 1 week before and 12 and 15 weeks after the initial inoculation; day of first inoculation was designated as day 0. ^{a,b}Values with different letters differ significantly ($P < 0.05$).

inoculation revealed that concentrations were clearly lower in 2 treated dogs (130 and 247 nmol/L, respectively), compared with the mean of the control dogs (330 nmol/L). By week 15, plasma cortisol concentrations after ACTH injection in these 2 dogs had increased to 325 and 290 nmol/L, respectively. In terms of anti-ACTH antibody titers, the first of these 2 dogs (a female) had an intermediate titer, whereas the second of these 2 dogs (a male) had the highest titer of all 4 treated dogs (Figure 1).

Discussion

The purpose of the present study was to determine whether inoculation of dogs with a modified totally synthetic form of canine ACTH would result in an immune response and production of antibodies against the immunogen. In turn, these antibodies conceivably would bind to, and sequester, each inoculated dog's endogenous ACTH. As a consequence, sequestration of sufficient amounts of endogenous ACTH would reduce the ability of the hormone to bind to its receptors on adrenocortical cells. The lack of ACTH binding and receptor activation would potentially result in reduced secretory function or atrophy (or both) of the zona fasciculata and reticularis of the adrenal cortex. It is known that these zones of the adrenal cortex are dependent on exposure to ACTH to maintain function, and loss of ACTH (such as that resulting from long-term glucocorticoid administration) will lead to atrophy of the adrenal cortex.²⁵ Such atrophy is associated with a reduction in cortisol secretion and in particular a decline in the ability of the zona fasciculata and reticularis cells to respond to subsequent exogenous ACTH challenge exposure with an increase in cortisol secretion.

The present study clearly indicated that immunization with a recombinant peptide containing 3 copies of canine ACTH resulted in circulating anti-ACTH antibodies in all treated dogs. Furthermore, immunization was associated with a significant reduction in secretion of cortisol in response to a stimulatory injection of ACTH in 2 of 4 treated dogs. However, immunization did not affect basal concentrations of cortisol. Also, the effect on cortisol concentrations after ACTH injection was transitory because post-ACTH cortisol concentrations in the 2 affected dogs returned to values similar to those of the control dogs 3 weeks later.

Immunization with various hormones has been used in several species in an attempt to induce an immune response and suppress the physiologic action of that hormone. In particular, immunization with GnRH has been studied, and commercial vaccines are marketed for use of this immunogen to suppress reproductive activity in males and females.^{26,27} Perhaps GnRH is an ideal candidate for these purposes because it circulates in relatively low concentrations and is central to reproductive processes in both males and females. The GnRH immunogens have been produced by various means, including chemical conjugation of the peptide to a large foreign carrier protein²⁷ and a technique similar to that used in the present study to produce a recombinant peptide.^{19,20} Several other reproductive hormones have been used in a similar manner, usually for contraception, including use in humans (eg, immunization with human chorionic gonadotropin)¹² and other species, including companion animals.²⁸ In addition to attempts to suppress reproductive activity, immunization with conjugates of peptide hormones have successfully been used for parathyroid hormone to lower calcium concentrations in patients with parathyroid carcinoma,^{7,8} and angiotensin I and II vaccines have been used to reduce blood pressure in humans.⁶ Review of the results for such an approach indicates that there is variation in the overall effectiveness and the duration of a physiologic response is inconsistent. Factors such as the titer and affinity or avidity of the produced antibodies are likely important to the success or failure.^{7,12}

The ACTH immunogen used in the present study was a synthetic construct designed on the basis of previous experiments that involved GnRH in dogs wherein peptide synthesis was used to create an immunogenic fusion of the GnRH hormone with a 17-amino acid peptide sequence (p35) chosen from the sequence of the fusion protein of the Morbillivirus canine distemper virus.¹⁸ The latter peptide sequence was identified with a lymphocyte proliferation assay that involved the use of blood mononuclear cells from dogs inoculated with canine distemper virus.¹⁸ The p35 peptide was stimulatory to proliferation of cells obtained from dogs of various breeds and ages and, when fused to the GnRH sequence and inoculated into dogs, resulted in high antibody titers against GnRH.¹⁸⁻²⁰ Therefore, the p35 peptide sequence was selected as an epitope for the induction of T-helper cells needed for the disruption of tolerance and generation of antibody responses to small self-peptides. The GnRH synthetic vaccines used in those studies were made with a peptide synthesizer; the ACTH immunogen in the present study was too

large to manufacture in such a manner, so recombinant methods were used. Three copies of canine ACTH were incorporated into the immunogen on the basis of data that indicated an increased immune response correlating positively with the number of copies of GnRH incorporated in synthetic GnRH vaccines.^{29,30} It is possible that incorporation of even more copies of ACTH into a vaccine may improve the immune response; 3 copies of the molecule were chosen for this study because it was believed that inclusion of more than 1 copy would improve the immune response, compared with the response to a single ACTH molecule. The dose of ACTH immunogen (94 nmol/injection) was approximately 2-fold that of the p35-GnRH synthetic immunogen used in dogs to induce anti-GnRH antibodies and suppress testosterone concentrations.²⁰ The immune and endocrine responses may be improved in future studies by testing a variety of doses of the immunogen.

Another approach for construction of vaccines against self-peptides involves chemical conjugation of the peptide to a large foreign protein, such as keyhole limpet hemocyanin²⁷ or tetanus toxoid,¹² and subsequent use of this fusion product as an immunogen. Disadvantages for such an approach include heterogeneity in the product with each conjugation reaction, induction of a strong antibody response to the carrier, and the potential for carrier-induced epitope suppression.²⁰ Therefore, totally synthetic immunogens are more attractive in that the exact chemical structure is known and alterations in the base molecule can easily be generated to optimize performance.

Results of the present study indicated that inoculation with a synthetic peptide containing ACTH elicited an immune response in that the dogs rapidly produced antibodies against ACTH. Interestingly, the response to repeat inoculation resulted in an increase in titers in some but not all dogs. In 2 of 4 dogs, evidence of a physiologic effect of the inoculation on adrenal gland function was indicated by the finding of a reduction in plasma cortisol concentration after ACTH injection. The anti-ACTH antibody titer in one of these dogs was the highest measured, whereas the other dog had an intermediate titer. This finding suggested that it would be feasible to affect (suppress) adrenal gland function in dogs through immunization against ACTH. However, the effect on adrenal gland responsiveness to ACTH was observed in only 2 of 4 treated dogs. Furthermore, the effect was transient (post-ACTH plasma cortisol concentrations were not different from those concentrations in control dogs 3 weeks later). It should be mentioned that at the time of the ACTH response test 12 weeks after the initial inoculation, anti-ACTH antibody titers were declining in all dogs, and they continued to decrease in 3 of 4 dogs through week 15. Consequently, ACTH response testing at earlier times may have revealed a greater difference between treatment groups because the higher concentrations of anti-ACTH antibodies in the circulation may have been sufficient to bind and sequester endogenous ACTH and thus affect adrenal gland responsiveness. It is possible that more subtle changes in the adrenal axis were present in treated dogs but were undetected by the evalua-

tions used. For example, basal cortisol concentrations change during the day in dogs³¹ so that infrequent sampling (as was used in the present study) may not detect subtle differences. Lower doses of ACTH (lower than the 5 µg/kg dose used) may have provided a more sensitive means to detect mild adrenal gland suppression or atrophy.²⁴ Endogenous ACTH concentrations were not determined because of the potential confounding influence of anti-ACTH antibodies. Even though mild changes in pituitary-adrenocortical activity may not have been detected, analysis of the results indicated that the influence of vaccination with this ACTH immunogen on the adrenal axis was, at best, mild and inconsistent.

Although most dogs with hyperadrenocorticism are treated with drugs (mitotane and trilostane) designed to act on the adrenal gland and suppress cortisol synthesis and secretion, medical treatments directed at suppressing ACTH secretion have also been used. In the past, serotonergic and dopaminergic agents such as cyproheptadine and bromocriptine were used, but with limited success.³² Newer drugs such as the somatostatin analogue pasireotide have been used in human patients and have yielded positive results.³³ Retinoic acid was successfully used to suppress ACTH and cortisol concentrations in dogs with hyperadrenocorticism, which resulted in improvement in clinical signs.³⁴ The drug appeared to exert its effects by suppressing proopiomelanocortin gene expression and additionally by inhibiting proliferation of corticotrophic tumor cells. Other molecules located in pituitary and corticotroph cells that have been identified as potential targets for suppressing oversecretion of ACTH include peroxisome-proliferator activation receptor- γ ,³⁵ the orphan nuclear receptor TR4,³⁶ epidermal growth factor receptor,³⁷ and inhibitors of cyclin-dependent kinase.³⁸ The exact mechanisms involved in corticotrophic tumor formation have yet to be identified; elucidation of the pathways involved in the process will likely be important for designing specific treatments capable of a reduction in excessive secretion of ACTH that is characteristic of the disease.

A number of potential modifications in the ACTH immunogen could be used to potentially improve the immune response and thus cause a more profound effect on pituitary-adrenocortical activity. Adjuvants substantially influence the immune response, and the addition of CpG oligodeoxynucleotides could be explored.^{6,39} Modifications of ACTH, including use of multiple antigenic peptides that have a polylysine core,^{7,8} inclusion of ACTH sequences from species other than dogs,^{7,8} and altering the numbers or arrangement of the ACTH peptide sequences could be attempted. Testing different (or multiple) T-helper cell epitopes would provide another method worthy of examination.²⁰ For example, the immune and endocrine response (titers against GnRH and suppression of testosterone and progesterone concentrations) after vaccination with various T-helper peptide-GnRH constructs had wide variation when responses were compared among dogs.²⁰ However, inoculation with a pool of T-helper peptides fused to GnRH resulted in higher anti-GnRH antibody titers, greater consistency in titers among dogs of different

breeds, and a more effective suppression of hormone concentrations. It is possible that such an approach would improve the immune and endocrine suppressive response to the ACTH immunogen used in the present study. Analysis of results of these previous studies also indicates that the immune and endocrine responses to vaccination with synthetic self-peptides differs among dogs and therefore will likely have greater effectiveness in a subset of animals.

- a. DA2PPVL, Merck Animal Health, Summit, NJ.
- b. Emulsigen BCL, MVP Laboratories, Omaha, Neb.
- c. pET-51b(+) Ek/LIC, EMD Millipore, Billerica, Mass.
- d. p3XFLAG-CMV-10, Sigma-Aldrich, St Louis, Mo.
- e. RNeasy Plus mini kit, Qiagen Inc, Valencia, Calif.
- f. BugBuster, EMD Millipore, Billerica, Mass.
- g. Benzoinase, EMD Millipore, Billerica, Mass.
- h. Ni-NTA agarose, Qiagen Inc, Valencia, Calif.
- i. Pyrogen gel clot LAL assay, Lonza, Walkersville, Md.
- j. GelCode Blue, Fisher Scientific, Pittsburgh, Pa.
- k. Cortrosyn, Organon, Rancho Cucamonga, Calif.
- l. Immuno-module, Nalge Nunc International, Rochester, NY.
- m. Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.
- n. SureBlue Reserve TMB solution, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.
- o. SigmaStat 3.1 for Windows, Systat Software, Point Richmond, Calif.

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