DISSERTATION

BIOMARKERS OF SYSTEMIC DISEASE IN DOGS AND CATS

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JACQUELINE CHRISTINE WHITTEMORE ENTITLED BIOMARKERS OF SYSTEMIC DISEASE IN DOGS AND CATS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

BIOMARKERS OF SYSTEMIC DISEASE IN DOGS AND CATS

Microalbuminuria assays were performed and compared with clinical diagnoses recorded within 3 months of urinalyses in dogs without overt proteinuria and in cats. A Western blot immunoassay was developed and validated for detection of antibodies to Crandell Rees Feline Kidney (CRFK) cell proteins in sera. Immunodominant CRFK proteins were identified. Antibody temporal appearance patterns were determined for cats inoculated with CRFK proteins and cats receiving commercially available FVRCP vaccines. The Western blot immunoassay was adapted to detect CRFK antigens in feline and murine tissues. Associations between CRFK antibodies and vaccination were determined. Associations between CRFK antibodies and biochemical abnormalities were determined in 1,477 privately-owned cats.

Dogs positive by the quantitative microalbuminuria assay were 2.3 times as likely to have systemic disease as dogs without microalbuminuria and 2.7 times as likely to have neoplasia. Quantitative microalbuminuria assay results were also significantly associated with age, BUN, and hematuria. Dogs with positive semiquantitative microalbuminuria tests were 4.8 times as likely to have systemic disease and 8.1 times as likely to have neoplasia. Semiquantitative microalbuminuria assay results were also significantly associated with age, BUN, and creatinine.

Cats with positive quantitative microalbuminuria results were 6.7 times as like to have underlying disease as cats without microalbuminuria. Quantitative microalbuminuria results were also significantly associated with BUN, pyuria, and hematuria. Cats with positive semiquantitative microalbuminuria assay results were 2.4 times as likely to have underlying disease. Positive semiquantitative microalbuminuria

results were significantly associated with serum creatinine, age, pyuria, and hematuria.

Gender was not significantly associated with results of either microalbuminuria assay. The urine albumin:creatinine ratio had very poor sensitivity for systemic disease in both species.

Crandell Rees Feline Kidney cell protein inoculation was associated with autoantibody development measurable by Western blot immunoassay. Cats receiving commercially available FVRCP vaccines according to a standard vaccination schedule developed antibodies measurable using the validated Western blot immunoassay. Antibody development occurred as early as four weeks after vaccination. The number of antibody bands and density of those bands was greater after one year booster inoculation. Antibody bands developed against antigens 47kD, 40kD and 38kD in size.

Immunodominant CRFK antigens were identified as α-enolase, annexin A2, and macrophage capping protein. Wide tissue distributions for α-enolase and annexin A2 were found in cats, consistent with the human literature. Our studies also documented a wide tissue distribution for macrophage capping protein.

Significant associations were found between anti-CRFK antibody levels and biochemical abnormalities. There was a significant positive correlation between anti-CRFK antibody levels and serum bilirubin levels. There were significant negative associations between anti-CRFK antibody levels and serum creatinine, ALP activity, and glucose.

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INDEX WORDS: Biomarker, Microalbuminuria, Albuminuria, Albumin:creatinine ratio, Protein:creatinine ratio, Crandell Rees Feline Kidney (CRFK) cell line, Autoimmune disease, Vaccine-associated disease, Autoantibody, FVRCP vaccine, Western blot immunoassay, Enzyme-linked immunosorbent assay, α-enolase, Macrophage capping protein, Annexin A2

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The most exciting phrase to hear in science, the one that heralds the most discoveries, is not 'Eureka!' (I found it!), but 'That's funny...'

-Isaac Asimov

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INTRODUCTION

Increasing diagnostic sophistication in human and veterinary medicine has been associated with earlier diagnosis of disease and better outcome. One of medicine's greater ironies remains the fact that doctors have the best chance in achieving positive outcomes for diseases if they are identified before patients become symptomatic. Unfortunately, such early identification depends upon doctors being aware of the risk of and having a means for detecting the disease that is independent of the development of clinical signs. Generally, the latter requires identification and validation of an appropriate biomarker, defined by the NIH as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." In addition to decreasing short and long-term morbidity and mortality through earlier intervention, by incorporating biomarker data into clinical prediction rules, more aggressive interventions can be focused on 'at-risk' populations, balancing potential side effects of interventions against risk of disease complications to individuals.

A biomarker of general risk that has gained prominence in human medicine is microalbuminuria. Microalbuminuria is defined as the presence of albumin in the urine in quantities greater than the reference limit, but below the limit of detection of standard urine dipstick assays. Microalbuminuria has been associated with many different diseases in humans including hypertension, oxidative damage, inflammation, endothelial disease, and renal disease. Its sensitivity for inflammation and underlying disease has led to its incorporation into routine office screening as well as clinical prediction rules.

Semiquantitative and quantitative microalbuminuria assays have been validated for both dogs and cats. In dogs, microalbuminuria has been associated with increasing age, neoplasia, heartworm disease, and hereditary nephropathies. In cats, microalbuminuria has been associated with azotemia, hypertension and increased all-cause mortality. Based on the human literature, microalbuminuria may be an early warning sign of disease, allowing earlier disease diagnosis and intervention, but its usefulness as a predictor of systemic disease in veterinary medicine has not previously been evaluated. Thus, the first major objective of the work described in this dissertation was to evaluate the usefulness of microalbuminuria as a biomarker of systemic disease in dogs and cats. Two prospective studies were performed comparing the presence and types of systemic disease with microalbuminuria levels in dogs (Chapter 2) and cats (Chapter 3).

Widespread vaccination of feline patients has been associated with dramatic decreases in morbidity and mortality from a variety of diseases. The vaccine against feline herpesvirus, calicivirus, and panleukopenia virus (FVRCP) remains one of the most commonly administered feline vaccinations and for the most part, is very effective with minimal side-effects. The viruses included in this vaccine are cultivated using the Crandell Rees Feline Kidney (CRFK) cell line, a cell line developed from naïve feline kidney tissue. Previous work has documented CRFK antibody development post-vaccination and has hinted at a potential temporal association with interstitial nephritis in hyperinoculated cats. However, associations between antibody development and disease development in cats have not been studied extensively and the identities of the immunodominant antigens were unknown. Thus, the other major objective of the work described in this dissertation was to evaluate potential associations between antibodies to the CRFK cell line and biochemical markers of systemic disease in cats.

To accomplish this objective, a Western blot immunoassay was developed and optimized for detection of CRFK antibodies in sera (Chapter 4). Immunodominant CRFK

antibody bands were identified in the sera of hyperinoculated cats and a hyperinoculated rabbit. Sera from cats vaccinated with FVRCP vaccines were evaluated for the presence and the number of these bands. The polyclonal α-CRFK rabbit serum was then utilized in an optimized protein A column technique to purify CRFK cell lysate. Bands corresponding to the previously identified immunodominant antibodies were harvested and sequenced for protein identification (Chapter 4). The Western blot analysis was modified, using polyclonal rabbit anti-CRFK serum, to detect CRFK immunodominant antigens in tissues from unowned cats and a mouse to determine the distribution of CRFK immunodominant antigens throughout the body (Chapter 5). Finally, a previously validated CRFK antibody ELISA was used to characterize antibody development patterns in cats vaccinated with one of five different commercially available vaccines (Chapter 6). The ELISA was also run on sera from 1,477 cats and compared with biochemical data to assess for associations between CRFK antibodies and biomarkers of systemic disease (Chapter 6).

LITERATURE REVIEW

Once considered tertiary members of the household or property, companion animals have gained prominence as primary family members. In a 2001 census, 46.1% of cat-owners considered their cats family members, 52.1% considering them pets or companions and only 1.8% considering them property.[1] Similar attitude shifts have been documented for dogs. These changes have correlated with increased provision of advanced medical procedures like kidney transplants, increased use of more basic veterinary care like vaccines, and increased utilization of geriatric wellness programs.

Earlier disease diagnosis leading to earlier therapeutic intervention, often while the patient is still asymptomatic, has been associated with improved survival for a wide variety of conditions. In human medicine, meta-analyses have also demonstrated financial benefits with implementation of screening and wellness programs. The benefits of occult disease diagnosis have shifted the focus of routine medical practice from utilization of clinical signs for disease diagnosis to reliable and predictive biomarkers of asymptomatic disease.

A biomarker is defined by the NIH as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."[2] Biomarkers generally must be reliable and have high sensitivity and specificity to be useful as markers of occult disease. Useful commonly utilized biomarkers in veterinary medicine include serum creatinine concentration for renal dysfunction, trypsin-like

immunoreactivity for exocrine pancreatic insufficiency, and serum fructosamine for diabetes mellitus. Additional proportional biomarkers for general conditions, like inflammation, that affect a number of diseases and biomarkers for specific diseases continue to be sought.

Microalbuminuria

Renal proteinuria has historically been classified in one of three categories: functional renal, pathologic glomerular, and pathologic tubular.[3] Functional renal proteinuria generally results from alterations in glomerular permselectivity or renal vasoconstriction and is usually transient. Pathologic tubular proteinuria results from decreased tubular resorption of normal quantities of urinary protein due to decreased nephron numbers or decreased tubular function due to local inflammation. Although tubular proteinuria may be large, generally the degree of protein loss is mild. Pathologic glomerular proteinuria is associated with persistent increased protein loss through the glomerulus due to altered glomerular permselectivity. This protein loss overwhelms the normal resorptive capacity of the tubules, leading to measurable proteinuria. Protein loss due to glomerular proteinuria varies in severity from mild to profound.

Protein concentrations greater than 40 mg/dl in urine can be detected using routine urine dipstick analysis. Because these measurements can be influenced by a number of factors including urine specific gravity and presence of inflammation or hematuria, false positive results are common. Although sulfasalicylic acid testing is less susceptible to false readings, it is not commonly performed in primary care clinics and has the same limitation with regard to specific gravity. Due to these limitations, dipstick positive test results are often confirmed by performing urine protein:creatinine ratios (UPC). In contrast to the urine dipstick, the UPC seems to have few false positive results

and is also semi-quantitative. Until recently, a cut-off value of 0.5-1.0 has been used to identify cases with clinically significant proteinuria.[3]

Recently, the focus in human medicine has shifted to detection of previously occult proteinuria, referred to as microalbuminuria (MALB). Microalbuminuria is defined as the presence of abnormal amounts of protein in the urine at a quantity below the limit of detection of previous tests, approximately 40 mg/dl. It consistently precedes the development of overt proteinuria in humans and is interpreted as a warning sign of glomerular disease.[4-6] Microalbuminuria can be measured via multiple techniques, including 24-hour urinary albumin excretion or the urinary albumin excretion rate, untimed single-sample urine albumin concentration, and standardization of one time albumin measurement by use of the albumin:creatinine (UAC) ratio. The UAC has been determined to be clinically and statistically superior to other methods of measurement in human medicine, where it has gained prominence as a biomarker of disease. Interpretation of UAC results in humans, however, can be complicated by differences in urine creatinine excretion between genders and among races.[7-8] Because creatinine excretion is not affected by gender in dogs, the UAC may have better predictive values than microalbuminuria measurement in dogs. Conflicting data exists regarding the role of gender on creatinine excretion in cats.

Human diseases in which microalbuminuria is diagnostically useful can be divided into those that do and do not cause true nephropathy. The former category includes diabetes mellitus[4,9-15] and essential hypertension.[4,9-10,16-18] Numerous studies have documented the usefulness of microalbuminuria in identifying diabetic patients at high-risk for diabetic nephropathy, increased morbidity, and increased mortality. The strength of these associations has led to incorporation of microalbuminuria into prediction rules for diabetes patients to maximize the benefits of therapeutic intervention while minimizing complications and optimizing resource allocation.[14-15] Similarly,

microalbuminuria testing has increased early identification and treatment of patients with essential hypertension, leading to decreases in both morbidity and mortality. Microalbuminuria screening is now considered standard practice for patients at risk of developing either condition. In addition, the benefits of early identification of at-risk patients have led to recommendations by some investigators to use microalbuminuria as a screening tool for nephritic disease in the general population.[19-20]

Management of critical care cases, sepsis, myocardial infarction, and ischemic stroke are examples of situations that do not consistently involve nephropathies but for which microalbuminuria evaluation is helpful.[21-27] For diseases in this category, microalbuminuria probably serves as a sentinel for underlying systemic inflammation. The development of microalbuminuria is highly correlated with C reactive protein levels, establishing a role for microalbuminuria as a marker of systemic inflammation.[28]

Microalbuminuria assays, both semiquantitative and quantitative, have been developed and validated for use with both canine and feline urine.[a-c] In both assays, albumin measurement is standardized by normalization of values to a urine specific gravity of 1.010. Advantages associated with the use of microalbuminuria testing over other measures of urine protein loss in animals include higher sensitivity and specificity for detection of urinary protein loss and decreased influences of exercise, inflammation of the lower portion of the urinary tract, and hematuria on interpretation of results.[29-30] Results of preliminary investigation suggest that semiquantitative microalbuminuria testing yields diagnostic data equivalent to determination of the UAC in cats.[d] Similar studies are lacking in dogs.

A variety of studies have been performed evaluating microalbuminuria in dogs and cats. In a study that evaluated over 3,000 dogs owned by veterinary hospital personnel, the prevalence of microalbuminuria in dogs increased with age.[e] Follow-up data[31] was provided for 572 of the 751 microalbuminuric dogs, from which it was

determined that 56% had underlying infectious, inflammatory, neoplastic, or metabolic diseases that could be associated with alterations in glomerular permeability or secondary glomerular injury. An additional 31% (177) of those dogs had renal disease, 12.1% (69) had no specific disease diagnosis, and the remainder had either multiple diagnoses or a disease process not thought to be associated with proteinuria. The prevalence of systemic disease in dogs that were not microalbuminuric was not determined; therefore, the predictive value of those results is unknown. Other studies have documented the development of microalbuminuria prior to development of overt proteinuria in dogs with experimentally induced heartworm disease[f] and hereditary nephropathies.[g] Increased urinary albumin loss has also been reported in dogs with neoplasia but not in those with orthopedic disease.[h]

In a pilot study[i] performed in our laboratory, urine samples from 105 dogs were assessed by means of urinalysis, UPC ratio determination, and testing for microalbuminuria. Infectious, inflammatory, or neoplastic diseases that have been associated with proteinuria were diagnosed within 3 months of initial urine collection in 31 (56.3%) of the dogs that had positive results for a microalbuminuria assay but negative results of dipstick testing and 6 (26%) dogs that had negative results for both a microalbuminuria assay and dipstick test. Although the difference between groups was not significant, there was high likelihood of type-2 error in that study as a result of the small study sample size.

Microalbuminuria has also been evaluated in differing cat populations. Cats were documented to have increasing prevalence of microalbuminuria with age in one cross-sectional analysis.[32] However, when healthy cats were excluded, the prevalence of microalbuminuria was more consistent across all age groups. These data would suggest that increasing microalbuminuria prevalence with age in cats is not strictly a consequence of aging but may partially reflect increasing prevalence of underlying

diseases with age. Results from additional studies evaluating an independent association between age and proteinuria in cats conflict.[33-34] Significant associations have been demonstrated between the UAC and the presence of hypertension and azotemia.[33] The UAC has been shown to be a predictor of mortality in healthy cats[j] as well as cats with azotemia and hypertension.[33] In these studies, ROC curves identified cut-off values for increasing mortality that are much lower than conventional reference ranges, suggesting that reference intervals may need to be refined as more data becomes available.

It is clear that microalbuminuria is more sensitive for detection of urinary protein loss than previous methods of detection in both dogs and cats. It remains to be determined whether the increased sensitivity of microalbuminuria has clinical utility for screening dogs and cats for underlying disease or directing interventional strategies. Identification of a screening tool to separate patients with better versus poorer prognoses earlier may lead to earlier interventions in at-risk patients and more appropriate allocation of resources.

Autoimmunity

Development of autoimmune disease requires persistent exposure to an autoantigen combined with failure of a variety of immune system fail-safes. It is important to recognize that persistent exposure to an autoantigen is not, by itself, sufficient to cause autoimmune disease.[35-36] In the presence of a persistent autoantigen, the immune system may respond in one or more ways. First, the immune system may eliminate autoreactive Th0 cells during intrathymic development. It may also simply suppress the activity[37] of autoreactive T cells and return to a non-autoimmune state.

Persistent exposure to an autoantigen may activate Th0 cells to differentiate along a Th2 pathway and produce antibody. The production of autoantibody may or may

not be associated with the development of disease.[36] Whether autoantibodies are associated with disease is often determined by how accessible the autoantigen is to the immune system.[36] In some cases, antibody may persist without disease until unrelated or incidental damage leads to antigen exposure, at which time clinical signs or disease may develop.[35-36] Th2 cell-associated tissue damage may result from an antibody-associated lymphocyte attack on affected cells, cellular death secondary to sublytic complement binding, antibody-antigen complex deposition or a combination of these mechanisms. Recognition of autoantigens by Th2 cells instead of Th1 cells can even confer protective benefits against autoimmune disease,[38] as has been demonstrated in experimental models for multiple sclerosis and diabetes mellitus.

Finally, the immune system may mount primarily a Th1 response to an autoantigen with damage mediated by cytotoxic T cell infiltrations. Although antibody may still be produced, antibody levels may or may not be clinically relevant. A good example is human thyroiditis where neither the presence nor the severity of disease correlates with antibody levels.[39] In addition, roughly 15% of dogs that are positive for thyroglobulin antibodies remain euthyroid and revert to an antibody negative state.[40] In some cases, antibody levels may peak at the time of the onset of clinical signs of disease and then disappear thereafter. This has been demonstrated for humans affected by insulin dependent diabetes mellitus (IDDM).[36]

Autoreactive cytotoxic T cells exist harmlessly in many individuals, sometimes controlled even while in direct contact with their target tissues.[37] Changes in antigen-presenting cell tissue distribution, endothelial adhesion molecule expression, and the priming and homing potential of T cells secondary to unrelated infection have all been implicated as causes of T cell release from regulatory control with subsequent disease development.[37] Even after activation of cytotoxic cells, individuals may exist in a state of controlled inflammation without progression to clinical disease as is seen in murine

models of IDDM.[37] In transgenic diabetic mouse models, the transition to disease has been convincingly associated with shifts in the pathogenic capabilities and regulatory balance of infiltrated lymphocytes, possibly tied to changes in inhibitory cell signaling, and accessory cell populations. Cytotoxic lymphocyte-associated disease may progress with intermittent inflammation or relapsing signs, as constant antigen exposure maintains a hypersensitive state that is boosted from time to time by further immunization, bursts of increased antigen exposure, or unrelated immunologic stimulation.

Development of autoimmunity requires appropriate conditions of exposure as well as a complex interplay of environmental and genetic factors. Concurrent administration of adjuvants and infectious agents with self-proteins has been shown to be effective in stimulating the development of autoimmunity in laboratory animals.[35] Oral immunization of antigen has been shown to consistently result in a Th2 response, while injectable inoculation can lead to either Th1 or Th2 responses.[38] A series of experiments has demonstrated that low level exposure to autoantigens or exposure to antigens of low avidity during thymic development may allow escape of high-affinity autoreactive T cells during intrathymic selection, while exposure to high levels of autoantigens inhibits the development of autoreactive Th0 cells.[41-42] Though propagation of autoreactive T cells is not sufficient by itself to cause autoimmune disease, concomitant or subsequent inflammation or infection may 'break' tolerance, leading to autoimmune disease. It has been postulated that development of a high affinity autoreactive Th0 cell population may also stimulate a shift toward a Th1 phenotype, leading to greater destructive potential.[39]

Individual host factors have been shown to play a large role in the development of autoimmune disease in humans. The association between MHC susceptibility alleles, specifically HLA alleles, and the development of IDDM[39,41-45] is probably the most compelling example of the importance of genetic factors. Similar associations have been demonstrated for a wide variety of human autoimmune diseases.[44] Genetically mediated

aberrant cytokine expression has been associated with development of autoimmune disease in transgenic mice.[45-46] Similar associations may exist for naturally occurring autoimmune disease. It is likely that genetic factors play a similar role in feline autoimmune disease.

Vaccination and autoimmunity

Proving associations between autoantibodies and disease is an incredibly difficult task, and proving a specific association between vaccination and a given disease is even more onerous. There are a number of known and posited links between vaccinations and systemic disease in humans and companion animals. In humans, vaccination has been associated with Guillain-Barré syndrome, immune-mediated thrombocytopenia and hemolytic anemia, diabetes mellitus, and reactive arthritis,[47-49] though the mechanisms of most of these processes remain unknown. Vaccine contaminants specifically have been associated with reactions including urticaria, eczema, granuloma formation, and macrophagic myofasciitis.[49-50]

Temporal associations have been identified between vaccination and immune-mediated thrombocytopenia and hemolytic anemia in dogs[51] though studies exist with conflicting results.[52] Nephritis has been documented secondary to canine adenovirus (CAV)-1 and CAV-2 vaccination in dogs.[53-54] Both vaccines have been associated with two distinct renal processes: persistent renal viral shedding and focal interstitial nephritis that may stem from a Type 4 hypersensitivity. The former has not been associated with reversion to pathogenic strains or development of renal disease in client-owned animals. It is therefore an unlikely contributor to autoimmune disease in animals. The latter occurs independently of persistent infection and is consistent with an immune-mediated process. The potential for vaccine-associated nephritis was broached in a paper characterizing Lyme nephritis but the lack of follow-up data precluded evaluation.[55]

Development of immune reactions against vaccine contaminants has also been documented in animals.[56-57] For example, anti-thyroglobulin antibody production has been documented in dogs after administration of vaccines that were thought to be contaminated with bovine thyroglobulin.[57] Other links have been found between vaccination and injection-site fibrosarcomas in cats,[58-59] dogs,[60] and ferrets.[61] Further links will probably be identified as research in the area of vaccine-associated disease continues.

Vaccination and disease in cats

Indicators suggest that the percentage of owned cats receiving initial and booster vaccinations is increasing, just as the total owned cat population has increased over the past several decades. According to census data, there were 30 million owned cats in 1970, 60 million in 1990, and roughly 70 million in 2001.[1,62] Although the current population is not known for certain, it is estimated to be between 70 and 80 million. Reported vaccination rates have increased from 64.4% in 1991 to 70.5% in 2001 for cats presented to veterinarians.[1]

The increase in vaccination at least partially reflects a shift in the role of the cat in the American household. Once considered a tertiary member of the household, the cat has gained prominence as a primary family member. In the 2001 census, 46.1% of cat-owners considered their cats family members, 52.1% considering them pets or companions and only 1.8% considering them property.[1] This increase has correlated with increased provision of advanced medical procedures like kidney transplants, as well as increased use of more basic veterinary care like vaccines and geriatric wellness programs. In addition, this trend is supported by the emergence of feline specialty practices. The increased use of vaccinations in cats means that the cat population is also more susceptible to the negative effects of over-vaccination.

Simultaneously, over the last three decades, a variety of inflammatory diseases of cats have been identified, some with increasing frequency based on prevalences in both the Colorado State University Veterinary Medical Center (CSUVMC) database and the Veterinary Medical Database (VMDB). These include interstitial nephritis and cystitis, inflammatory bowel disease, pancreatitis, and cholangiohepatitis. Prevalences have increased for renal disease (CSUVMC 6.6 times; VMDB 1.5 times), inflammatory bowel disease (CSUVMC 2.3 times, VMDB 1.3 times), pancreatitis (CSUVMC 13.1 times, VMDB 5.0 times), and cholangiohepatitis (CSUVMC 83 times, VMDB 39 times). Although part of the increase can be attributed to increased diagnostic sensitivity for some of the listed diseases, much of the increase remains unexplained.

Crandell Rees Feline Kidney cell line

The Crandell Rees Feline Kidney (CRFK) cell line was produced in the 1960s by homogenization of feline renal tissue.[63] In the 1970s, the CRFK line was determined to be useful for the growth of feline viruses, including the viruses in FVRCP vaccines–feline herpesvirus 1, calicivirus, and panleukopenia virus.[64-65] Since that time, most FVRCP vaccines have been cultivated using the CRFK cell line.

Whole virus vaccines are generated by harvesting the viruses from host cells and purifying them. Because the purification process is incomplete, small quantities of host cell proteins are incorporated into the produced vaccines. The presence of feline cell proteins in FVRCP vaccines was inadvertently discovered during generation of recombinant antigen ELISAs for monitoring vaccination immunity in cats.[66] Follow-up study has confirmed that CRFK proteins of varying concentrations can be isolated from many market-leading FVRCP vaccines (unpublished data).

After inadvertent discovery of feline cell proteins in FVRCP vaccines, antibody development as measured by polyclonal CRFK ELISA was evaluated in cats

hyperinoculated with CRFK proteins and receiving market-leading FVRCP vaccines.[67] For the study, 14 age-matched eight week old cats were divided into pairs with each pair receiving either: one of three subcutaneous (SQ) vaccines, an intranasal vaccine, or one of three CRFK injection protocols. Cats were vaccinated according to a standard vaccination protocol while cats inoculated with CRFK protein received more frequent inoculations in order to simulate the cumulative effect of lifetime vaccination.

Significant anti-CRFK antibody development was documented in the cats that were hyperinoculated with CRFK proteins. Anti-CRFK antibody development was also confirmed in cats receiving injectable FVRCP vaccinations on a standard vaccination schedule. Cats receiving intranasal FVRCP vaccines did not develop detectable anti-CRFK antibodies as measured by ELISA, although they had equivalent or greater FHV-1 cell mediated immune responses, concanavalin A induced lymphoblast transformation responses, and FHV-1, FCV, and FPV serological responses than cats administered SQ FVRCP vaccines.[68] Antibody development, measured using a validated polyclonal ELISA, against a lysate of homogenized feline renal tissue was also confirmed in many of these cats. Nephritis was not identified on histopathology six weeks after vaccines were boosted at one year of age.

Since publication of the initial findings, cats from the CRFK inoculation groups and the intranasal vaccination group were re-biopsied two weeks after booster inoculations at two years of age. Lymphoplasmacytic inflammation was noted for one cat from each CRFK hyperinoculated pair when samples were evaluated in a blinded fashion;[69] neither cat receiving intranasal vaccinations developed nephritis. One of the cats with histologic evidence of nephritis on follow-up biopsies has since died of chronic renal failure (unpublished data). On necropsy, severe interstitial nephritis with lymphocytic-plasmacytic and histiocytic inflammation was documented.

The presence of inflammation two weeks after inoculation without inflammation six weeks after biopsy suggests that vaccination may lead to recurrent waxing and waning nephritis. It is currently unknown whether this inflammation, over a number of years, is sufficient to generate clinically relevant renal dysfunction. The presence of inflammation in each CRFK dose group suggests that, if real, the development of CRFK-associated nephritis is a multi-factorial process and that host factors may play an important role in its development.

Footnotes

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ASSOCIATION OF MICROALBUMINURIA AND URINE ALBUMIN:CREATININE RATIO WITH SYSTEMIC DISEASE IN DOGS

Whittemore J.C., Gill V.L, Jensen W.A., Radecki S.V., and Lappin M.R. *Journal of the American Veterinary Medical Association* 2006;229:958-963.

Objective—To evaluate semiquantitative and quantitative assays for microalbuminuria and determination of the urine albumin:creatinine (UAC) ratio in detection of systemic disease in dogs without overt proteinuria.

Design—Prospective study.

Sample population—408 dogs.

Procedures—Urine samples that had been obtained from dogs for which a complete medical record was available and in which results of a dipstick test for urine protein were negative were evaluated. Urine protein:creatinine ratios (cutoff values, 0.5 and 0.1), semiquantitative and quantitative microalbuminuria values (cutoff, value, 1 mg/dL), and UAC ratios (cutoff values, 100 and 200 mg/g) were determined. Clinical diagnoses rendered within 3 months of enrollment in the study were recorded. Sensitivity and specificity were determined with disease status serving as the standard. Associations with clinical diagnosis, sex, age, BUN and serum creatinine concentrations, blood pressure, results of bacterial culture of urine, temperature, pyuria, hematuria, and bacteriuria were evaluated by use of logistic regression analysis.

Results—Forty-eight dogs were healthy and 360 had at least 1 disease. Significant associations were detected between age, presence of disease, presence of neoplastic disease, BUN and serum creatinine concentrations, and hematuria and results of 1 or both of the microalbuminuria assays.

Conclusions and Clinical Relevance—Microalbuminuria was associated with underlying disease. The sensitivity and specificity of the semiquantitative microalbuminuria test for detection of systemic disease were superior to those of other tests. Microalbuminuria testing in conjunction with other screening procedures may increase diagnosis of subclinical disease, but a prospective study in which the predictive values of screening tests are evaluated, with and without microalbuminuria determination, is needed.

ABBREVIATIONS

UAC	Urinary albumin:creatinine
UPC	Urinary protein:creatinine
$MALB_E$	Microalbuminuria assay (semiquantitative)
$MALB_Q$	Microalbuminuria assay (quantitative)

The presence of albumin in the urine in quantities greater than the reference limit, but below the limit of detection of standard urine dipstick assays, is defined as microalbuminuria. Microalbuminuria consistently precedes development of overt proteinuria in humans and is interpreted as a warning sign of glomerular disease.[1-3] In addition, systemic inflammation is associated with protein leakage across endothelia and development of microalbuminuria, making microalbuminuria an excellent marker of underlying disease and a predictor of morbidity and mortality rates in humans with underlying or primary diseases.[4-9] The benefits of early identification of at-risk patients have led to recommendations by some investigators[10] to use microalbuminuria as a screening tool for nephritic disease in the general population.

Microalbuminuria can be measured via multiple techniques, including 24-hour urinary albumin excretion or the urinary albumin excretion rate, untimed single-sample urine albumin concentration, and standardization of 1-time albumin measurement by use of the UAC ratio. Two microalbuminuria assays (a semiquantitative[a], and a quantitative[b] microalbuminuria assay) have been validated for use in dogs. In both assays, albumin measurement is standardized by normalization of values to a urine specific gravity of 1.010. Advantages associated with the use of microalbuminuria testing over urine dipstick tests for protein and determination of the UPC ratio include higher sensitivity and specificity for detection of urinary protein loss and decreased influences of exercise, inflammation of the lower portion of the urinary tract, and hematuria on interpretation of results.[c-e,11] Results of preliminary investigation in cats suggest that semiquantitative microalbuminuria testing yields prognostic data equivalent to determination of the UAC ratio, whereas in humans, UAC ratio determination is considered more accurate for detecting microalbuminuria.[f] Interpretation of UAC results in humans, however, can be complicated by differences in urine creatinine excretion between genders and among

races.[12,13] Because creatinine excretion is not affected by sex in dogs, the UAC ratio may have better predictive values than microalbuminuria measurement in dogs. To our knowledge, this hypothesis has not been tested.

In a recent study[g] in which > 3,000 dogs owned by veterinary hospital personnel were evaluated, the prevalence of microalbuminuria in dogs increased with age. When follow-up data were obtained for 572 of the 751 dogs that were microalbuminuric, it was determined that 322 (56.3%) had underlying infectious, inflammatory, neoplastic, or metabolic diseases that could be associated with alterations in glomerular permeability or secondary glomerular injury.[14] An additional 177 (31%) of those dogs had renal disease, 69 (12.1%) had no specific disease diagnosis, and the remainder had either multiple diagnoses or a disease process not thought to be associated with proteinuria. The prevalence of systemic disease in dogs that were not microalbuminuric was not determined; therefore, the predictive value of those results is unknown.

In another study,[h] 105 dogs were evaluated by means of urinalysis, UPC ratio determination, and testing for microalbuminuria. Infectious, inflammatory, or neoplastic diseases that have been associated with proteinuria were diagnosed within 3 months of initial urine collection in 31 (56.3%) of the dogs that had positive results of a microalbuminuria assay but negative results of dipstick testing and 6 (26%) dogs that had negative results of both a microalbuminuria assay and dipstick test. Although the difference between groups was not significant, there was a high likelihood of type-2 error in that study as a result of the small study sample size. Data obtained by comparing clinical disease findings in dogs with and without microalbuminuria are needed to assess the diagnostic utility of the tests.

The objective of the present study was to evaluate semiquantitative and quantitative assays for microalbuminuria and determination of the UAC ratio in detection of systemic disease in dogs without overt proteinuria.

Materials and Methods

Patient selection—Six hundred dogs that were examined serially at the Colorado State University Veterinary Teaching Hospital from March 3, 2003, to August 20, 2003 for which urine samples were available were initially evaluated and prospectively enrolled in the study. Case numbers were recorded, and urine was frozen. After a period of 3 months, records were reviewed and data were extracted. Of the initial 600 dogs enrolled, results of a dipstick test for urine protein were negative and the medical record was complete in 408 dogs. Records were reviewed by 1 of 2 authors (JCW or VLG) and clinical diagnoses entered within 3 months of the time of urine collection were recorded. At the time of data collection, urinalysis results were not available to the investigators. Clinical diagnoses included healthy; neoplasia (excluding lipomas); infectious, immune-mediated, or inflammatory disease; urinary tract disease; and other. Rectal temperature, systolic blood pressure, diagnostic tests performed, and medications dogs were receiving were also recorded.

Urine assays—Urine samples collected by free catch, catheterization, or cystocentesis were included. Urinalysis was performed by technicians in the Clinical Pathology Laboratory at the veterinary teaching hospital and samples were frozen at -20°C until the other assays were performed. Technicians unaware of the urinalysis results performed the $MALB_E$ according to the manufacturer's instructions. Briefly, the sample was normalized to urine specific gravity of 1.010 by dilution with distilled water in a sample dilution tube. The test device was inserted into the diluted sample for a minimum of 3 minutes, and results were determined by comparing the intensity of 2 colored bands in the test window.

After the $MALB_E$ was performed, samples were refrozen for transport and submitted to a commercial laboratory[i] where the $MALB_Q$, urine total protein assay, and

urine creatinine assay were performed by technicians unaware of the urinalysis results. The $MALB_Q$ is an immunoturbidimetric assay in which results are generated from quantitative measurement of agglutination caused by the reaction of polyclonal anti-albumin antibodies and albumin in the urine sample. Turbidity was measured at 340 nm and 700 nm on an analyzer. The albumin concentration in the sample was determined by comparing sample absorbance to a multipoint calibration curve, and results were normalized to a urine specific gravity of 1.010. Evaluation of earlier (unpublished) data indicated that trace quantities of albumin in canine urine samples did not degrade with repeated freeze-thaw cycles. The UPC and UAC were calculated by dividing the total protein or albumin concentration by the creatinine concentration of each sample prior to standardization for specific gravity.

Statistical analysis—All $MALB_E$ and $MALB_Q$ results $\geq$ 1 mg/dL were considered positive. Cutoff UAC values of 100 (UAC_{100}) and 200 (UAC_{200}) mg/g were evaluated; those values were chosen on the basis of human data and data obtained in cats.[15] Cutoff values of UPC $\geq$ 0.1 ($UPC_{0.1}$) and $\geq$ 0.5 ($UPC_{0.5}$) were also evaluated.

Diagnostic sensitivity and specificity of the $MALB_Q$ and $MALB_E$ assays and for the $UPC_{0.1}$, $UPC_{0.5}$, UAC_{100}, and UAC_{200} ratios were calculated using χ^2 analysis with presence or absence of systemic disease designated as the independent variable. Backward-selection logistic regression was performed to evaluate associations between disease status, sex, age, results of bacterial culture of urine, rectal temperature, pyuria, hematuria, bacteriuria, or the interaction between disease status and age, and results of $MALB_Q$, $MALB_E$, $UPC_{0.1}$, and $UPC_{0.5}$ tests. Disease status was designated as healthy or diseased and was included in the statistical analysis as a dichotomous explanatory variable. All other explanatory variables (e.g., age, results of bacterial urine culture, rectal temperature, pyuria, hematuria, and bacteriuria) were included in the model as continuous explanatory variables. Dogs with disease were suballocated into various

disease categories, and logistic regression was performed on those disease subpopulations. However, in a number of the subpopulations, the model-fitting procedure failed to converge, probably as a result of insufficient sample sizes. Results of subpopulation evaluations where model-fitting procedures converged are summarized below. Values of $P < 0.05$ were considered significant. Odds ratios were determined where possible. Data were analyzed by use of commercially available software packages.[j]

Results

Of the 408 dogs included in analyses, 56 had > 1 disease. Of the remaining 352 dogs, 48 (14%) were classified as healthy; 81 (23%) had a diagnosis of neoplasia; 67 (19%) had infectious, immune-mediated, or inflammatory disease; 45 (13%) had urinary disorders; and 111 (32%) had other diseases (Table 1). The distribution of values for microalbuminuria obtained via $MALB_Q$ was not the same among those categories (**Figure 1**). Eight urine samples were obtained by use of an indwelling urinary catheter, whereas the other samples were obtained by free catch or cystocentesis. The distribution of dogs with positive results of $MALB_Q$, $MALB_E$, $UPC_{0.1}$ ratio, $UPC_{0.5}$ ratio, UAC_{100} ratio, and UAC_{200} ratio by diagnostic code was summarized (**Table 1**). The sensitivity and specificity of the $MALB_Q$, $MALB_E$, $UPC_{0.1}$ ratio, $UPC_{0.5}$ ratio, UAC_{100} ratio, and UAC_{200} ratio for distinguishing healthy from non-healthy dogs were determined (**Table 2**). When results were calculated with and without dogs with multiple diagnoses, no significant differences were detected.

Logistic regression could not be performed for $UPC_{0.5}$, UAC_{100}, or UAC_{200} ratio determination because of the low number of dogs with positive test results. Significant associations between older age, presence of disease, high BUN and serum creatinine concentrations, and hematuria and positive results of certain urine tests were detected

(**Table 3**). There was no significant association between sex, blood pressure, bacterial urine culture results, rectal temperature, pyuria, or bacteriuria and results of $MALB_Q$, $MALB_E$, or the $UPC_{0.1}$ ratio. The only disease subpopulations in which there were enough samples with positive results for logistic regression analysis were neoplasia, infectious-inflammatory-immune, urinary system, and other.

Discussion

The etiologies of proteinuria may be classified as prerenal, functional renal, pathologic tubular, or pathologic glomerular.[16] Overt proteinuria (i.e., UPC > 1.0) is commonly accepted as abnormal and is an indication for further diagnostic testing. The clinical importance of low-grade proteinuria remains unknown. Data from dogs, cats, and humans suggest that the finding of microalbuminuria may be an indication for further evaluation, because it may be associated with higher morbidity and all-cause mortality rates.[4-10,17,k,l] The development of a quantitative assay for accurate measurement of microalbuminuria has been pivotal to this discovery in human medicine.

There are 2 major clinical uses for results of assays for microalbuminuria. Because the assays are more specific than dipstick assays for urine protein loss, they can be used to confirm positive dipstick test results. Because microalbuminuria assays are more sensitive than the dipstick and UPC tests, they can be used to detect urine protein loss below the limits of detection with urine dipsticks. Increased urine albumin concentrations have been reported in dogs with neoplasia, but not in those with orthopedic disease.[m] To the authors' knowledge, there are no published studies in which an association between microalbuminuria and systemic disease in dogs without overt proteinuria. The best combination of sensitivity and specificity for disease were observed with the $MALB_E$ and the $MALB_Q$ tests. Exclusion of samples with positive results of a dipstick test for urine protein from this study likely resulted in decreased values for

sensitivity and specificity of the microalbuminuria tests, compared with results of previous analyses. Given their low specificity and sensitivity, respectively, both the $UPC_{0.1}$ and $UPC_{0.5}$ ratios appeared to have limited value for screening dogs without overt proteinuria for systemic disease. These findings should also be considered in assessment of results of the UPC ratio with a positive cutoff value of 1.0.

The poor diagnostic usefulness of UAC_{100} and UAC_{200} ratios for screening dogs for systemic disease was unexpected. In humans, significant associations exist between the UAC ratio and idiopathic hypertension[3] and diabetic nephropathy[18-20]; determination of the UAC ratio has also been more accurate than microalbuminuria assay results in some studies[12,13]. In a previous investigation[15] in cats, there was a significant association between the UAC ratio and both hypertension and azotemia. The UAC ratio is a predictor of death in healthy cats as well as in cats with those conditions.[15,I] Similar results would be anticipated in dogs, but this was not supported in the present study. The UAC_{100} and UAC_{200} ratios had poor sensitivity in the present study and thus appeared to be of little use for detection of occult systemic disease in dogs with negative results of dipstick tests for urine protein. Further studies are recommended before the diagnostic usefulness of UAC_{100} and UAC_{200} ratios is completely discounted. However, the results of the present study suggest that if dogs with negative dipstick test results are to be further assessed, the $MALB_E$ or $MALB_Q$ should be used.

The association between low levels of proteinuria and increasing age in dogs of this study supports results of previous studies.[g] To our knowledge, persistence of this association, independent of disease status, has not been previously reported and contrasts with findings from previous reports[15,21] in cats. The association between low-grade proteinuria and disease as indicated by the $MALB_E$ was striking. The association between azotemia and MALB supports the use of microalbuminuria testing for early identification of dogs with renal disease. However, the lack of association between the

urinary tract disease category and microalbuminuria appears to conflict with the results. We feel that this was not an unexpected finding, because that diagnosis category included dogs with any urinary tract problem, including inappropriate elimination or urinary incontinence, whereas values for BUN and serum creatinine concentration better reflect the subset of dogs with true renal disease.

The association between neoplasia and positive results of microalbuminuria testing is of potential clinical importance because occult neoplasia can be difficult to diagnose and is not typically identified on routine health screening tests such as hemograms and serum biochemical analyses. Identification of late-stage neoplasia in an apparently healthy animal is a common source of frustration for clients and practitioners. Should testing for microalbuminuria prove to be a means of identifying animals with an increased likelihood of occult neoplastic disease, it may be a useful adjunct to other screening tests in geriatric veterinary patients. Many of the dogs in the "other" disease subcategory had systemic diseases that were likely inflammatory in nature, but the dogs had not been evaluated extensively enough to be grouped in a given subcategory (e.g., liver disease). Therefore, the association between that disease subcategory and $MALB_E$ results was not surprising.

The present study had a number of limitations. To prevent bias or operator variability, samples were analyzed en masse. This approach helped to limit diagnostic work-up bias because urine test results were not available to the dogs' attending clinicians. The downside of this approach was that clinicians did not have the opportunity to use urine test results in assessment of their patients. Because the study was observational, another important limitation was that some of the dogs enrolled were undergoing ongoing management during the time of the study. Some of those dogs were in remission for incurable disease, and some were almost recovered from previously diagnosed disease, a fact that may limit applicability of these results to a naïve

population. Given the high prevalence and the continuum of disease in geriatric dogs, we feel that this concern is of limited importance. The nonrandomized enrollment of dogs at the teaching hospital and the disease characteristics of dogs examined at a tertiary care facility resulted in a disparity between the number of healthy and unhealthy animals. This distribution inequality may have skewed interpretation of the statistical analyses, because the number of dogs with positive results was not similar to the number of dogs with negative results. Finally, the inclusion of dogs with diseases not known to be associated with proteinuria (e.g., dermatologic disease) in the diseased category for logistic regression analysis may have biased the analysis against identifying significant correlations. Such dogs were included because other options (e.g., censoring those cases or categorizing them as healthy) would have decreased applicability of the study results to the general practice population. In addition, such choices would have required prospective judgment of which diseases should or should not be associated with microalbuminuria without the benefit of objective supporting data.

Results of this study support the recommendation to evaluate geriatric animals for occult proteinuria even when the results of urine dipstick testing are negative.[16] Point-of-care tests are used frequently in veterinary practices. In the present study, sensitivity and specificity of the $MALB_E$ for detection of systemic disease were superior to those of the other tests, and the $MALB_E$ can be performed in the veterinary clinic. We conclude that there is benefit for the use of the $MALB_E$ or $MALB_Q$ in conjunction with other routine geriatric screening tests (e.g., signalment, history, physical examination, CBC, serum biochemical analysis, urinalysis, and blood pressure) to increase the likelihood of identifying occult disease. A prospective study in which the positive and negative predictive values of other geriatric screening tests are evaluated with and without a MALB assay is needed to further validate this recommendation.

Footnotes

a. E.R.D.-HealthScreen® Canine Urine Test, Heska Corporation, Loveland, CO.
b. ERD™ Test, Heska Corporation, Loveland, CO.
c. Jensen WA, Andrews J and Simpson D. Prevalence of microalbuminuria in dogs (abst). *J Vet Intern Med* 2001;15:300.
d. Grauer GF, Moore LE, Smith AR et al. Comparison of conventional urine protein test strip method and a quantitative ELISA for the detection of canine and feline albuminuria (abst). *J Vet Intern Med* 2004;18:127.
e. Gary AT, Cohn LA, Kerl ME et al. The effects of exercise on microalbuminuria in dogs (abst). *J Vet Intern Med* 2003;17:229.
f. Syme HM and Elliott J. Comparison of urinary albumin excretion normalized by creatinine concentration or urine specific gravity (abst). *J Vet Intern Med* 2005;19:240.
g. Radecki S, Donnelly R, Jensen WA et al. Effect of age and breed on the prevalence of microalbuminuria in dogs (abst). *J Vet Intern Med* 2003;17:110.
h. Whittemore JC, Jensen WA, Prause L et al. Comparison of microalbuminuria, urine protein dipstick, and urine protein creatinine ratio results in clinically ill dogs (abst.). *J Vet Intern Med* 2003;17:234.
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Table 1—Distribution of urine samples with positive results of $MALB_Q$ and $MALB_E$ and UPC and UAC ratios by clinical diagnosis and in all dogs as a group (n = 352) that were healthy or had a single disease and in which results of a dipstick test for urine protein were negative. Data are given as percentage (number) of dogs.

Clinical diagnosis	$MALB_Q$	$MALB_E$	$UPC_{0.1}$	$UPC_{0.5}$	UAC_{100}	UAC_{200}	All dogs*
Healthy	6 (7)	4 (4)	16 (39)	0 (0)	0 (0)	0 (0)	14 (48)
Neoplastic	31 (34)	30 (34)	22 (54)	38 (5)	52 (15)	45 (5)	23 (81)
Infectious/immune/ inflammatory	20 (22)	21 (24)	18 (45)	15 (2)	21 (6)	27 (3)	19 (67)
Urinary system	14 (16)	13 (15)	12 (29)	15 (2)	3 (1)	0 (0)	13 (45)
Other	29 (32)	32 (37)	33 (84)	31 (4)	24 (7)	27 (3)	32 (111)
Total	100 (111)	100 (114)	100 (251)	100 (13)	100 (29)	100 (11)	100 (352)

*All dogs with 1 diagnosis code by diagnosis category.

$UPC_{0.1}$ = Urine protein:creatinine ratio with cutoff value of 0.1. $UPC_{0.5}$ = Urine protein:creatinine ratio with cutoff value of 0.5. UAC_{100} = Urine albumin:creatinine ratio with cutoff value of 100. UAC_{200} = Urine albumin:creatinine ratio with cutoff value of 200.

Table 2—Sensitivity and specificity of urine tests for detection of systemic disease in 408 dogs with 1 disease, multiple diseases, or without disease and in which results of a dipstick test for urine protein were negative.

Variable	$MALB_Q$	$MALB_E$	$UPC_{0.1}$	$UPC_{0.5}$	UAC_{100}	UAC_{200}
Sensitivity	35.6	36.9	71.1	4.5	10	3.6
Specificity	85.4	91.7	18.8	100	100	100

See Table 1 for remainder of key.

Table 3—Results of logistic regression for associations between positive results of microalbuminuria assays or $UPC_{0.1}$ ratio and disease status in dogs.

Disease category	$MALB_Q$		$MALB_E$		$UPC_{0.1}$	
	P value	OR (CI)	*P* value	OR (CI)	*P* value	OR (CI)
Healthy (n = 48 dogs) or with 1 disease (304 dogs)						
Age	< 0.001*	1.148 (1.079-1.221)	< 0.001*	1.129 (1.061-1.200)	0.047*	1.065 (1.001-1.133)
Health status (diseased vs. healthy)[+]	0.051	2.331 (0.998-5.443)	0.004*	4.845 (1.680-13.971)	0.021*	2.086 (1.115-3.901)
BUN	0.005*	1.039 (1.012-1.066)	< 0.001*	1.049 (1.021-1.078)	> 0.10	—
Serum creatinine concentration	0.056	0.595 (0.350-1.013)	0.029*	0.550 (0.322-0.941)	> 0.10	—
Hematuria	0.002*	1.479 (1.159-1.887)	0.092	1.225 (0.967-1.551)	0.006*	1.874 (1.196-2.936)
Neoplasia (81 dogs)						
Having this disease subcategory	0.046*	2.690 (1.017-7.113)	< 0.001*	8.070 (2.609-24.958)	0.004*	3.186 (1.460-6.949)
Age	0.047*	1.148 (1.002-1.315)	> 0.10	—	> 0.10	—
Hematuria	0.082	1.413 (0.957-2.087)	> 0.10	—	> 0.10	—
BUN	> 0.10	—	0.025*	1.058 (1.007-1.112)	> 0.10	—
Serum creatinine concentration	> 0.10	—	0.095	0.391 (0.130-1.179)	> 0.10	—
Infectious, inflammatory, and immune-mediated (67 dogs)						
Having this disease subcategory	> 0.10	—	> 0.10	—	0.015*	1.048 (1.009-1.089)

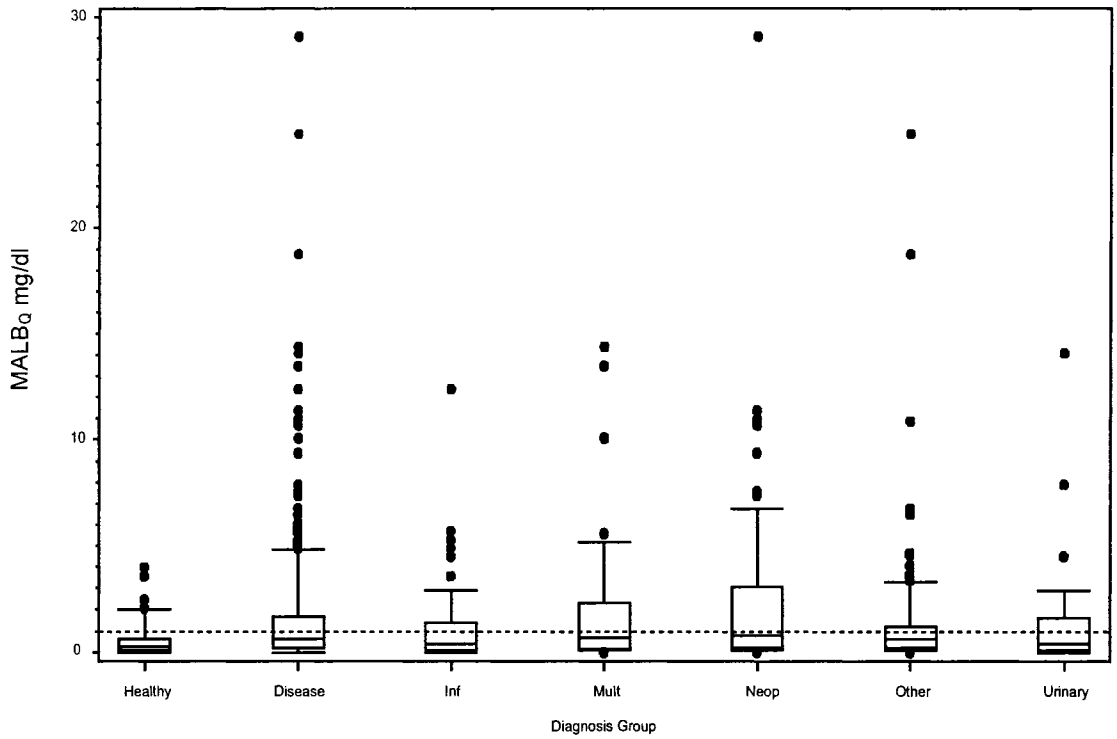
Urinary systems (45 dogs)						
Having this disease subcategory	> 0.10	—	> 0.10	—	> 0.10	—
Age	0.078	1.143 (0.985-1.327)	> 0.10	—	> 0.10	—
Urine bacterial culture	0.009*	2.717 (1.278-5.779)	> 0.10	—	> 0.10	—
Hematuria	0.074	1.814 (0.945-3.483)	0.018*	2.230 (1.145-4.343)	> 0.10	—
BUN	> 0.10	—	0.018*	1.062 (1.010-1.117)	> 0.10	—
Bacteria	> 0.10	—	< 0.001*	2.497 (1.499-4.161)	> 0.10	—
Temperature	> 0.10	—	> 0.10		0.063	0.480 (0.221-1.041)
Other (90 dogs)						
Having this disease subcategory	> 0.10	—	0.018*	1.060 (1.010-1.113)	0.005*	1.048 (1.015-1.083)
Age	0.001*	1.221 (1.083-1.377)	< 0.001*	1.236 (1.096-1.394)	> 0.10	—
Serum creatinine concentration	0.005*	0.214 (0.073-0.626)	0.018*	0.271 (0.092-0.798)	0.079	0.441 (0.177-1.099)
Urine bacterial culture	> 0.10	—	> 0.10	—	0.018*	0.209 (0.058-0.761)

*Significant ($P < 0.05$) value. [+] Compared with a health status of 'healthy'.

OR = Odds ratio. CI = Confidence interval. — = Not calculable because of the lack of significance.

See Tables 1 and 2 for remainder of key.

Figure 1—Distribution of $MALB_Q$ values for microalbuminuria by diagnosis category in 408 dogs with or without disease and with negative results of a dipstick test for urine protein.



Healthy = Dogs that had no disease conditions. Disease = Dogs that were not categorized as healthy. Inf = Dogs with infectious, immune-mediated, or inflammatory disease. Mult = Dogs with > 1 diagnosis. Neop = Dogs with neoplastic disease. Other = Dogs with 1 disease that did not fall into one of the other primary disease categories. Urinary = Dogs with urinary tract disease. The dotted line represents the cutoff value of 1 mg/dL. The line in each box represents the median $MALB_Q$ value for that diagnosis category, the box encompasses the 25^{th} to 75^{th} percentiles, whiskers are the 10^{th} and 90^{th} percentiles, and individual dots represent outliers.

ASSOCIATION OF MICROALBUMINURIA AND THE URINE ALBUMIN:CREATININE RATIO WITH SYSTEMIC DISEASE IN CATS

Whittemore J.C., Miyoshi Z., Jensen W.A., Radecki S.V., and Lappin M.R. *Journal of the American Veterinary Medical Association* 2007;230:1165-1169.

Objective—Determine the diagnostic usefulness of the semiquantitative and quantitative microalbuminuria assays and urine albumin:creatinine (UAC) ratio for detecting disease in cats.

Design—Prospective study.

Sample Population—441 cats evaluated at a veterinary teaching hospital.

Procedure—Urine samples from cats for which a complete medical record was available were included. Urine dipstick results, urine protein:creatinine ratios (cutoffs, 0.1 and 0.4), semi-quantitative and quantitative microalbuminuria values (cutoffs, 1 mg/dL), and UAC ratio values (cutoffs, 100 and 200 mg/gm) were determined. Clinical diagnoses determined within 3 months of enrollment were recorded. Sensitivity and specificity were determined with disease status used as the standard. The influences of clinical diagnosis, sex, age, serum BUN and creatinine concentrations, blood pressure, bacterial urine culture results, temperature, pyuria, hematuria, and bacteriuria were evaluated by means of logistic regression.

Results—Of 441 cats that were eligible for inclusion, 40 were healthy and 401 had ≥ 1 disease. Results of logistic regression indicated that significant associations existed for age, presence of disease, presence of urinary tract disease, azotemia, hematuria, and pyuria and results of one or both of the microalbuminuria tests.

Conclusions and Clinical Relevance—Microalbuminuria is associated with underlying disease. Sensitivity and specificity of the microalbuminuria tests for detection of systemic disease were superior to those of other tests. Microalbuminuria testing in conjunction with other screening procedures may increase identification of occult disease. A prospective study evaluating the predictive values of screening tests with and without microalbuminuria determination is needed to validate this recommendation.

ABBREVIATIONS

UAC	Urinary albumin:creatinine
UPC	Urinary protein:creatinine
$MALB_E$	Microalbuminuria assay (semiquantitative)
$MALB_Q$	Microalbuminuria assay (quantitative)

The presence of albumin in the urine in a quantity that is greater than normal, but below the limit of detection of standard urine dipsticks, is defined as microalbuminuria. In addition to being a warning sign of glomerular disease, microalbuminuria is associated with endothelial protein leakage and systemic inflammation. Preliminary data in cats suggest that microalbuminuria may be an indication for further evaluation given its possible associations with increased morbidity and mortality rates.[1,a]

Microalbuminuria can be measured by use of various techniques. Two microalbuminuria assays (a semiquantitative[b] and a quantitative[c] assay) have been validated for use in cats. In both assays, albumin measurement is standardized by normalizing values to a urine specific gravity of 1.010. Onetime albumin measurement may also be adjusted for glomerular filtration rate by use of the UAC ratio. Advantages associated with use of microalbuminuria testing over urine protein dipstick analysis and UPC ratio determination in dogs include higher sensitivity and specificity for detection of urinary protein loss and less influence from inflammation of the lower portion of the urinary tract and hematuria[d,2-3] on interpretation of results. Results of 1 study[e] suggested that the $MALB_E$ yields results equivalent to the UAC ratio in cats.

A recent investigation in cats revealed a significant association between UAC ratio and hypertension and azotemia.[1] The UAC ratio can be a predictor of fatality in healthy cats[a] as well as in cats with hypertension or azotemia.[1] In another study involving cats, the prevalence of microalbuminuria increased with age.[4] However, when only cats with disease were evaluated, the prevalence of microalbuminuria was more consistent across all age groups. These data suggest that the increasing microalbuminuria prevalence associated with increasing age is not entirely a consequence of aging, but may partially reflect an increasing prevalence of underlying disease with age. This is consistent with results of another study[1] in which microalbuminuria was not significantly associated with age. It remains to be determined

whether detection of microalbuminuria leads to earlier disease identification, more aggressive therapeutic intervention, or better outcome.

The objectives of the present study were to determine the prevalence of various clinical diagnoses in cats with and without microalbuminuria and to determine the diagnostic usefulness of a semiquantitative microalbuminuria kit, a quantitative microalbuminuria assay, and determination of the UAC ratio for detecting systemic disease in cats.

Materials and methods

Selection of cats—Six hundred eleven cats that were evaluated serially at the Veterinary Medical Center at Colorado State University from March 3, 2003, to May 6, 2005, and for which a urine sample was available were initially evaluated. After elimination of repeated submissions from the same cat and cats without a complete medical record, 441 cases remained. The medical record for each cat was reviewed by 1 of 2 authors (JCW or ZM) who were blinded with regard to urinalysis results at the time of data collection. Rectal temperature, systolic blood pressure, diagnostic tests performed, current medications, and clinical diagnoses entered within 3 months of the time of urine collection were recorded. Clinical diagnoses included healthy; and neoplastic; infectious, inflammatory, or immune-mediated; urinary and renal; endocrine; and other diseases.

Urine assays—Urine samples collected by free catch, catheterization, or cystocentesis were included. Urinalyses were performed by technicians in the clinical pathology laboratory at the veterinary teaching hospital, and samples were frozen at -20°C until the other assays were performed. Technicians blinded to the urinalysis results performed the $MALB_E$; samples were refrozen for transport and submitted to an outside laboratory[f] where the $MALB_Q$, urine total protein assay, and urine creatinine assay were performed

by technicians blinded to the urinalysis results. The UPC and UAC ratios were calculated by dividing the total protein or quantitative microalbuminuria concentration by the creatinine concentration in each sample prior to standardization for specific gravity. All assays were performed according to methods that have been described.[5]

Statistical analysis—Urine protein dipstick results of trace or greater were considered positive. Microalbuminuria test results ($MALB_E$ or $MALB_Q$) ≥ 1 mg/dL were considered positive. Urine albumin:creatinine ratio cutoff values of 100 (UAC_{100}) and 200 (UAC_{200}) were evaluated; these values were chosen on the basis of data from humans and cats.[1] Urine protein:creatinine ratio cutoff values ≥ 0.1 ($UPC_{0.1}$) and ≥ 0.4 ($UPC_{0.4}$) were evaluated. Diagnostic sensitivity and specificity of the $MALB_Q$ and $MALB_E$, $UPC_{0.1}$ and $UPC_{0.4}$ ratios, UAC_{100} and UAC_{200} ratios, and dipstick protein results were calculated using X^2 analysis with the presence or absence of systemic disease designated as the independent variable.

Stepwise backward-selection logistic regression was performed to evaluate the association between disease status, sex, age, BUN, serum creatinine concentration, results of urine bacterial culture, systolic blood pressure, rectal temperature, pyuria, hematuria, or bacteriuria, and results of the $MALB_Q$, $MALB_E$, or dipstick protein tests. Logistic regression was performed on subpopulations of various disease categories. Results of subpopulation evaluations where model-fitting procedures converged were summarized. Values of $P < 0.05$ were considered significant. Odds ratios and confidence intervals were determined where possible. All statistical analyses were performed according to previously described methodology by use of commercially available software.[g]

Results

Of 441 cats for which medical records were available and included in analyses, > 1 disease process was identified in 117. Of the remaining 324 cats, 40 (12%) were classified as healthy; 53 (16%) had neoplasia; 52 (16%) had infectious, inflammatory, or immune-mediated diseases; 61 (19%) had urinary tract disease; 52 (16%) had endocrine disease; and 66 (20%) had other diseases **(Table 1)**. The distribution of microalbuminuria values among the diagnosis categories was not the same **(Figure 1)**. Four urine samples were obtained from indwelling urinary catheters, and the other samples were obtained by either free catch or cystocentesis. Distribution of cases with positive results via $MALB_Q$ and $MALB_E$, $UPC_{0.1}$ and $UPC_{0.4}$, UAC_{100} and UAC_{200}, and dipstick protein by diagnostic code was summarized. The sensitivity and specificity of the $MALB_Q$ and $MALB_E$, $UPC_{0.1}$ and $UPC_{0.4}$, UAC_{100} and UAC_{200}, and dipstick protein for distinguishing healthy from non-healthy cats were determined **(Table 2)**. Results calculated with and without cats with multiple diagnoses were not significantly different.

All cats had positive results when the 0.1 cutoff value for UPC ratio was used, so the $UPC_{0.1}$ test was not evaluated further. The small number of cats with positive results via $UPC_{0.4}$ (n = 16), UAC_{100} (16), and UAC_{200} (7) precluded statistical evaluation of those tests. The number of samples with available blood pressure or rectal temperature measurements was insufficient to assess for association with urine test results. Factors significantly associated with positive $MALB_Q$ results were health status, presence of urinary disease, azotemia (BUN or serum creatinine concentration, depending on subgroup), pyuria, and hematuria. Factors significantly associated with positive $MALB_E$ results were health status, presence of urinary disease, age, azotemia (BUN or serum creatinine concentration, depending on subgroup), pyuria, and hematuria. The only factor significantly associated with dipstick protein status was hematuria. Neoplasia and urinary tract disease were the only disease subcategories for which there were enough

cases to perform subgroup regression analysis. Significant associations were summarized **(Table 3)**.

Discussion

Proteinuria may be classified as pre-renal, functional renal, pathologic tubular, or pathologic glomerular in etiology. Overt proteinuria (UPC ratio > 1.0) is commonly accepted as abnormal and generally indicates a need for further diagnostic testing. Data from studies in dogs,[5,h] cats,[1,a,i] and humans[6,7] suggest that subtle proteinuria, manifested as microalbuminuria, may be an indication for further evaluation because it may be associated with systemic disease, morbidity, and higher all-cause mortality rates.

Given the low specificity and sensitivity, respectively, of $UPC_{0.1}$ and $UPC_{0.4}$, these indices appear to have limited value for screening cats without overt proteinuria for systemic disease. The low sensitivity of the UPC ratio using a cutoff of 0.4 was consistent with results of recent studies[1,a] and suggests that an even higher cutoff for a positive result of 1.0, as is currently used in practice, may be too high for use in cats. The low prevalence of proteinuria, determined on the basis of a UPC ratio > 0.4, in this and the previously cited studies, contrasts markedly with the prevalences of proteinuria (defined by a UPC ratio > 0.5) of 34% in diseased cats and 5% in healthy cats reported in another recent study.[8] Differences in study population, the prevalences of various disease categories, and the methodologies used to measure the UPC ratio may be associated with the differing results.

The absence of association between dipstick protein results and pyuria was unexpected given results of previous reports[2] on the specificity of the MALB assays versus dipstick testing for diagnosis of urinary tract inflammation. However, to date, most animals with pyuria that were assessed with microalbuminuria assays and dipstick

protein analysis were dogs. Although the explanation for this discrepancy between cats and dogs is unknown, our results suggest that in cats, false positive dipstick protein results associated with pyuria are unlikely to occur.

The poor usefulness of UAC_{100} and UAC_{200} ratios for screening cats for systemic disease was unexpected. Previous work in cats revealed a significant association between the UAC ratio and both hypertension and azotemia.[1] The UAC ratio is also a predictor of fatality in apparently healthy cats as well as cats with those conditions.[1,a] There are several possible explanations for this apparent conflict in results. It may be that the UAC ratio is not sensitive enough to function as a marker for disease despite the association between increasing values of UAC and disease. In the previously mentioned studies,[1,a] an endpoint of death and a repeated sampling technique were used, whereas in the present study, cats were evaluated for only 3 months and single time point sampling was used. It may be that the shorter time frame and single time point sampling used in the present study was not sufficient to evaluate this relationship. Although differences in the study population may have affected the results, we would have anticipated a higher prevalence of cats with positive results on the basis of the UAC ratio in this population because of the small number of healthy cats. Urine handling and storage conditions were not different among the various studies and should not have affected recovery of albumin from urine. Finally, sampling bias cannot be ruled out as a factor. Clinicians may have had more difficulty obtaining urine from cats with more severe illness, resulting in a bias toward sampling more stable sick cats with less severe disease.

The differences in the association between explanatory variables and individual microalbuminuria tests were not unexpected, given the potential differences in the performance of these 2 tests. The association between BUN, serum creatinine concentration, and presence of urinary disease and positive microalbuminuria results

was not surprising, and was consistent with findings from a previous study.[1] The lack of a sufficient number of cats to analyze for an association between microalbuminuria and hypertension was unfortunate, because results are conflicting in current scientific literature.[1,8] The absence of an association between microalbuminuria and neoplasia differed from the results we obtained in dogs, where the odds ratios for neoplasia were 2.7 and 8 for positive $MALB_Q$ and $MALB_E$ results, respectively.[5] A potential explanation is that the neoplasms in cats of the present study were less inflammatory than those affecting dogs in the dog study. We consider this to be unlikely, because a large proportion (17/53 [32%]) of the cats with neoplasia in the present study had vaccine-associated sarcomas, which are considered to be highly inflammatory.[9] Alternatively, immune complexes and other inflammatory mediators may interact differently in dog and cat glomeruli. Unfortunately, because only those 2 categories had a sufficient number of cats for the statistical model to converge, further conclusions about associations between specific disease categories or types of disease and proteinuria cannot be drawn.

This study had a number of limitations. To prevent potential bias or operator variation, samples were analyzed en masse. This approach helped limit diagnostic work-up bias, because urine test results were not available to the attending clinicians involved with the cats. The disadvantage of this approach was that clinicians did not have the opportunity to use urine test results in clinical evaluation of cats. Because this study was observational in nature, another important limitation was that cats with ongoing disease were included in case enrollment. Some of these cats were in remission for incurable disease, and some were nearly recovered from previously identified disease. This may limit application of these results to a naïve population. Given the high prevalence of systemic disease in geriatric cats and the continuum of disease in cats when initially evaluated, we feel that this concern has limited validity. Sequential enrollment of cats

and the disease characteristics of cats evaluated at a tertiary care facility led to disparity between the number of healthy and unhealthy cats and the relative prevalence of certain conditions. This distribution inequality may have skewed interpretation of the statistical results because the number of positive results was not similar to the number of negative results. Data generated early in the study indicated that degradation of albumin secondary to repeated freeze-thaw cycles affected approximately 10% of urine samples. To avoid introduction of bias, no change was made in the urine-handling protocol. It is possible that degradation of albumin in some samples may have negatively affected sensitivity, specificity, and regression analysis results for the $MALB_E$, $MALB_Q$, UAC_{100}, and UAC_{200} tests. Finally, inclusion of cats with diseases not known to be associated with proteinuria (e.g., dermatologic disease) in the diseased category for logistic regression analysis may have biased the analysis against identifying significant correlations. Such cats were included because other options (censoring these cats or categorizing them as healthy) would have decreased applicability of the study results to general practice populations. In addition, such choices would have required prospective judgment of which diseases should or should not be associated with microalbuminuria without the benefit of objective supporting data.

In the present study, UAC ratios were not useful in identifying underlying disease because of poor sensitivity. In contrast, 1 or both of the MALB assays was associated with the presence of underlying disease, the presence of renal disease, and age. Conflicting results relative to previously reported studies may reflect the complex and multifactorial nature of processes that cause microalbuminuria. In the present study, sensitivity and specificity of the microalbuminuria assays for detection of systemic disease were superior to those of the other tests. It appears that there is benefit for the use of the microalbuminuria assays in conjunction with other screening tests (e.g., signalment, history, physical examination, CBC, serum biochemical analysis, urinalysis,

and blood pressure) to increase detection of occult disease. Further prospective studies in which the positive and negative predictive values of other screening tests are evaluated, with and without a microalbuminuria assay, are needed to further validate this recommendation.

Footnotes

a. Walker D, Syme HM, Markwell P, et al. Predictors of survival in healthy, non-azotaemic cats (abst.). *J Vet Intern Med* 2004;18:123.
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c. ERD™ Test, Heska Corporation, Loveland, Colo.
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f. HESKA Veterinary Diagnostic Laboratory, Loveland, Colo.
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h. Pressler BM, Proulx DA, Williams LE, et al. Urine albumin concentration is increased in dogs with lymphoma or osteosarcoma (abst.). *J Vet Intern Med* 2003;17:101.
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Table 1—Distribution of urine samples with positive $MALB_Q$, $MALB_E$, UPC ratios, and UAC ratio results by clinical diagnosis in 324 cats that did or did not have a single disease.

Clinical diagnosis	$MALB_Q$	$MALB_E$	$UPC_{0.1}$	$UPC_{0.4}$	UAC_{100}	UAC_{200}	DpP	All cats
Healthy	2 (2)	6 (7)	11 (31)	0 (0)	0 (0)	0 (0)	9 (10)	12 (40)
Neoplastic disease	11 (9)	13 (14)	17 (48)	13 (2)	6 (1)	0 (0)	19 (21)	16 (53)
Infectious-inflammatory-immune-mediated disease	20 (17)	15 (17)	18 (51)	19 (3)	25 (4)	14 (1)	18 (20)	16 (52)
Urinary system disease	33 (28)	29 (32)	18 (51)	31 (5)	31 (5)	57 (4)	22 (25)	19 (61)
Endocrine disease	16 (14)	16 (18)	15 (43)	19 (3)	31 (5)	29 (2)	17 (19)	16 (52)
Other	18 (15)	20 (22)	21 (61)	19 (3)	6 (1)	0 (0)	16 (18)	20 (66)
Total	**100 (85)**	**99 (110)**	**100 (285)**	**101 (16)**	**99 (16)**	**100 (7)**	**101 (113)**	**99 (324)**

Data are given as percentage (no.) of cats.

$UPC_{0.1}$ and $UPC_{0.4}$ = Urine protein:creatinine ratios with cutoff values of 0.1 and 0.4, respectively. UAC_{100} and UAC_{200} = Urine albumin:creatinine ratios with cutoff values of 100 and 200, respectively. DpP = Urine dipstick test with a cutoff value of trace.

Table 2—Sensitivity and specificity of urine tests for detection of systemic disease in 441 cats that did or did not have systemic disease.

	$MALB_Q$	$MALB_E$	$UPC_{0.1}$	$UPC_{0.4}$	UAC_{100}	UAC_{200}	DpP
Sensitivity (%)	36.96	43.14	100	6.7	7.82	3.63	36.91
Specificity (%)	93.55	82.5	0	100	100	100	75

Results represent the full study sample set, including cats with multiple diagnoses.

See Table 1 for key.

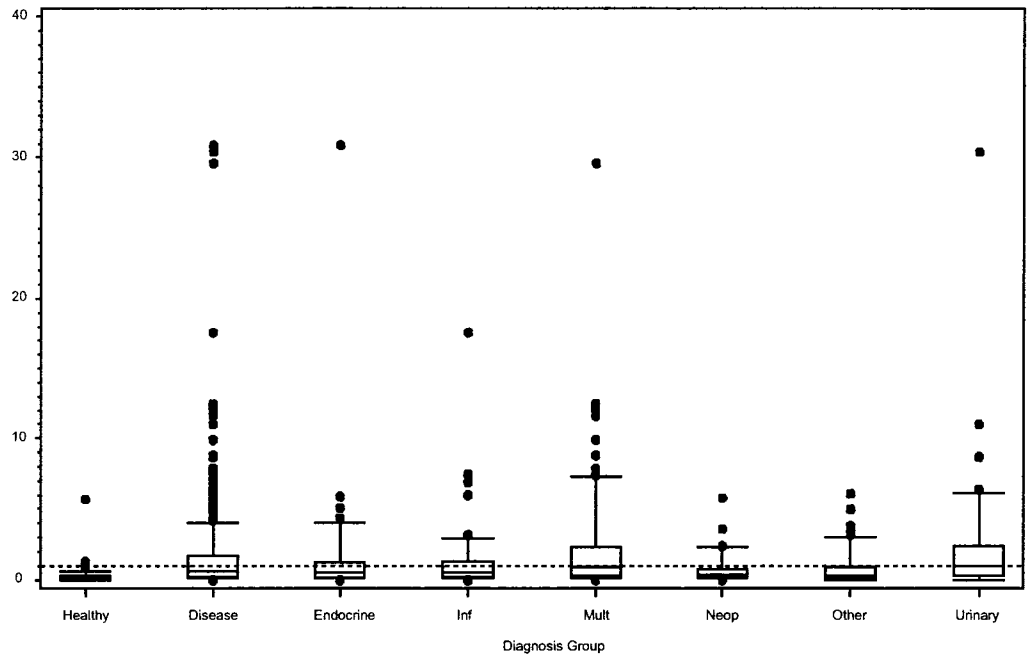
Table 3—Results of logistic regression analysis for associations between positive microalbuminuria assay or dipstick protein results and disease status in cats.

Disease status	$MALB_Q$		$MALB_E$		Dipstick protein test	
	P value	OR (CI)	*P* value	OR (CI)	*P* value	OR (CI)
All cats healthy (n = 40) or with disease (401)						
Health status (diseased vs healthy)*	0.011†	6.685 (1.544-28.946)	0.048†	2.414 (1.009-5.776)	> 0.10	—
Age	> 0.10	—	0.001†	1.078 (1.030-1.129)	> 0.10	—
Sex (female versus male)	0.091	0.673 (0.425-1.065)	> 0.10	—	> 0.10	—
BUN	<0.001†	1.027 (1.014-1.040)	> 0.10	—	> 0.10	—
Serum creatinine concentration	> 0.10	—	<0.001†	1.466 (1.182-1.818)	> 0.10	—
Pyuria	0.002†	2.172 (1.341-3.520)	0.004†	1.934 (1.229-3.042)	0.077	1.335 (0.969-1.840)
Hematuria	0.007†	1.242 (1.062-1.452)	<0.001†	1.321 (1.138-1.532)	<0.001†	1.287 (1.120-1.478)
Neoplasia: n = 53 cats						
Having this disease subcategory	> 0.10	—	> 0.10	—	> 0.10	—
Age	> 0.10	—	> 0.10	—	0.049†	1.140 (1.000-1.299)
Serum creatinine concentration	> 0.10	—	> 0.10	—	0.056	0.428 (0.179-1.023)
Pyuria	0.019†	6.230 (1.345-28.848)	> 0.10	—	0.084	3.465 (0.848-14.166)
Hematuria	<0.001†	2.555 (1.507-4.331)	0.017†	1.482 (1.074-2.044)	0.028†	1.431 (1.040-1.970)

Urinary systems: n = 61 cats						
Having this disease subcategory	<0.001†	1.623 (1.226-2.148))	0.008†	1.296 (1.070-1.570)	> 0.10	—
BUN	> 0.10	—	0.043†	1.027 (1.001-1.053)	> 0.10	—
Serum creatinine concentration	0.025†	1.713 (1.070-2.743)	> 0.10	—	> 0.10	—
Pyuria	> 0.10	—	0.034†	3.013 (1.087-8.352)	> 0.10	—
Hematuria	0.025†	1.606 (1.063-2.428)	0.018†	1.528 (1.075-2.171)	0.003†	1.543 (1.160-2.050)
Urine bacterial culture	0.096	0.460 (0.184-1.147)	0.046†	0.316 (0.102-0.979)	> 0.10	—

*Compared with a health status of healthy. †Significant ($P < 0.05$) difference. OR = Odds ratio. CI = Confidence interval. — = Not calculable because of lack of significance. *See* Table 1 for remainder of key.

Figure 1—Distribution of $MALB_Q$ values by diagnosis category in 441 cats with or without disease. Values of MALB > 50mg/dL (n = 3 cats) have been removed for clarity (2 in cats with urinary disease and 1 cat with multiple diseases).



Healthy = Cats that had no disease conditions. Disease = All cats not categorized as healthy. Endocrine = Cats with endocrinologic disease. Inf = Cats with infectious, immune-mediated, or inflammatory disease. Mult = Cats with > a 1 diagnosis. Neop = Cats with neoplastic disease. Other = Cats with 1 disease that did not fall into one of the other primary categories. Urinary = Cats with urinary system disease. The dotted line represents the cutoff value of 1 mg/dL. The line in each box is the median $MALB_Q$ value for that diagnosis category, boxes encompass the 25^{th} to 75^{th} percentiles, whiskers are the 10^{th} and 90^{th} percentiles, and individual dots represent outliers.

ISOLATION AND IDENTIFICATION OF IMMUNODOMINANT CRANDELL REES FELINE KIDNEY (CRFK) CELL LINE ANTIGENS AND DETERMINATION OF THEIR TEMPORAL RECOGNITION PATTERNS IN CATS

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Objective—Develop a Western blot immunoassay for Crandell Rees Feline Kidney (CRFK) cell proteins, determine the temporal antigen recognition patterns in cats, and identify the immunodominant antigens.

Sample Population—Hyperinoculated rabbit. 14 age-matched unvaccinated kittens divided into seven groups.

Procedure—Each group of kittens was given one of three CRFK inoculation protocols, a commercially available FVRCP vaccine for IN administration, or one of three commercially available FVRCP vaccines for SQ administration. Kittens were administered CRFK lysate inoculations every two to four weeks for 11 injections. Kittens were administered vaccines on weeks 0, 3, 6, and 50. Sera collected from the kittens at multiple time-points were assayed using a Western blot immunoassay. Immunodominant antigens were determined using banding patterns of the hyperinoculated rabbit and kittens. Antigens were purified from CRFK lysates using a protein A column and were sequenced.

Results—Immunodominant antigens were 47 kD, 40 kD, and 38 kD in size. Serum of all 14 kittens recognized at least one of the immunodominant antigens after inoculation. Maximal band numbers were recognized in weeks 50 through 56. Protein isolation and sequencing revealed three of the immunodominant CRFK proteins to be α-enolase, annexin A2, and macrophage capping protein.

Conclusions and Clinical Relevance—This study validated the use of Western blot analysis for detection of CRFK protein antibodies and identified three immunodominant CRFK proteins. In humans, anti-α-enolase antibodies are directly nephritogenic; anti-α-enolase and anti-annexin A2 antibodies have been associated with autoimmune diseases. Further research will be necessary to determine the clinical significance of these findings.

Introduction

Some feline vaccinal viruses, including feline herpesvirus 1, calicivirus, and panleukopenia virus, are grown on Crandell Rees Feline Kidney (CRFK) cells prior to incorporation in vaccines.[1-2] The CRFK cell line was initially created by homogenization of naïve feline kidney tissue and is used because many feline viruses rapidly replicate in the cells.[1-3] When purification of the viruses is performed, some CRFK proteins persist and contaminate FVRCP vaccines. Previous work from our research group documented development of anti-CRFK antibodies and anti-renal tissue antibodies as measured by ELISA in cats receiving injectable FVRCP vaccines according to a standard vaccination schedule and in cats hyperinoculated with CRFK lysates.[4] There was no association with nephritis noted in biopsies performed six weeks after one-year booster inoculations. One year later, CRFK lysates were administered one more time to the CRFK hyperinoculated cats; renal biopsies obtained two weeks after the injection documented lymphocytic plasmacytic nephritis in three of the six cats.[5] Because the vaccinated cats were not available for repeat biopsy, a definitive link between FVRCP vaccination and disease has not been made. In addition, the identities of the immunodominant antigens are unknown.

The objectives of this study were to validate a Western blot immunoassay for detection of anti-CRFK antibodies in serum, to determine the immunodominant CRFK antigens and antigen recognition patterns in cats hyperinoculated with CRFK proteins and in a group of vaccinated cats, and to isolate and identify the immunodominant antigens.

Materials and Methods

Preparation of CRFK cell extract—Crandell Rees Feline Kidney cells from a continuous cell line were suspended in fresh 5% Fetal Bovine Serum[a] in MEM NEAA

Earle's Salts medium[a] and plated in two 175 cm^3 tissue culture flasks.[b] Cells were amplified in 175 cm^3 flasks for one week and harvested by washing the cell layers with 1X Trypsin EDTA solution.[a] Harvested cells were washed three times in sterile filtered phosphate buffered saline solution (PBS) to remove residual FBS. Cells were centrifuged at 1000 rpm for 5 minutes. The cell pellet was suspended in PBS and sonicated with 20-1 minute bursts at 35% power while cooled in an ice bath. The lysate was centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant was assayed for protein content then stored in multiple aliquots at -70°C until used in the Western blot immunoassay.

Generation of polyclonal anti-CRFK antibodies—Serum from a rabbit hyperinoculated with CRFK lysate was collected for Western blot analysis. The rabbit received 500 µg of CRFK protein mixed with complete Freund's adjuvant on day 1 SQ after 10-20 mL blood was harvested. On days 14, 28, 42, and 54, the rabbit received 500 µg of CRFK protein mixed with incomplete Freund's adjuvant IM after 2 mL blood was harvested. On day 70, after 2 mL blood was harvested, the rabbit was adopted by a private individual. The collected serum was filtered[c] per manufacturer instructions to remove particulate. Immunoglobulins were purified and concentrated using a Protein A purification kit[d] per manufacturer's instructions. The experimental design was reviewed and approved by the Colorado State University Animal Care and Use Committee.

Gel Electrophoresis and transfer—Two different experiments were performed to determine optimal assay characteristics. In experiment 1, CRFK antigens were separated under reducing conditions on pre-cast 4-12% Bis-Tris mini-gels[e] and a discontinuous buffer system according to manufacturer instructions. A total protein concentration of 65 µg CRFK antigen was mixed with the appropriate amount of sample buffer[f] and reducing agent,[g] denatured and loaded on the gel along with a molecular weight standard[h] and electrophoresed at 200V constant until the dye front reached the

bottom of the gel. Once electrophoresis was complete, polyvinylidene difluorure (PVDF) membranes[j] were prepared by premoistening with anhydrous methanol for 30 seconds, rinsing with ddH_2O and soaking in transfer buffer,[i] prepared according to manufacturer's instructions with 20% methanol. The proteins were transferred to a PVDF membrane using the XCell II Blot Module[j] at 30V constant for 90 minutes. The membranes were air dried, placed in plastic bags and stored at -20°C. In experiment 2, CRFK antigens were separated under reducing conditions using the protocol previously described with the exception that pre-cast 10% Bis-Tris mini-gels[e] with MOPS under reducing conditions were used.

Protein staining—Two protein stains were used in this experiment. An 0.1% amido black protein stain[k] was used to stain electrophoresed CRFK cell lysates on PDVF membranes per manufacturer's instructions. The membranes were destained with 5% acetic acid and dried. A Coomassie blue protein staining kit[l] was used to stain and destain Bis-Tris gels per manufacturer's instructions.

Western Blot Immunoassay—Membranes were thawed at 20°C (room temperature), premoistened with anhydrous methanol, rinsed with ddH_2O and blocked with 1X NEH (250mM HEPES, 25mM EDTA, 750mM NaCl, 0.05%Triton X-100, 0.25% Gelatin, 3% BSA) for one hour on a rocker at room temperature. After the block was complete, the membranes were placed in the Miniblotter 16[m] and incubated for two hours at room temperature in serum diluted 1:10 (feline) or 1:50 (rabbit) in 0.01M Tris Base, 0.15M NaCl, 0.5% Tween 80 and 0.001M phenylmethyl-sulfonyl fluoride (PMSF) plus 10% nonfat milk (TNTP+10% milk). Following the primary antibody incubation, the membranes were washed three times for five minutes each in PBS-0.5% Tween 80 and incubated for one hour in horseradish peroxidase labeled goat anti-feline or anti-rabbit IgG (H only)[n] diluted 1:250 in TNTP+10% milk. After incubation with the secondary antibody, the membranes were washed as described previously. The membranes were

incubated in a 1:10 dilution of 4-chloro-1-naphthol/3,3'diamino benzidine tetrahydrochloride in peroxide substrate buffer (CN/DAB)[o] until color reaction was visible. The reaction was terminated by rinsing the membranes with ddH_2O.

Molecular mass estimation —Once dry, membranes were photographed using an image capture system.[p] Bands were plotted on a semi-log regression line of standards with known molecular weights to estimate their molecular masses.

Western blot immunoassay validation—Samples known to be negative and positive using a previously validated ELISA[4] were used for sequential comparison of varied antigen concentrations, two different antigen preparation methods, multiple buffers, blocks, and secondary antibody solutions to achieve a preparation with minimal reactivity in the negative samples but the highest level of reactivity in the known positives. Pooled sera from known positive and negative samples were run in five separate assays to evaluate inter-assay precision. Pooled positive and negative sera were run in five sample lanes each in two separate assays to evaluate intra-assay precision. Bands were consistent across assays for both intra- and inter-assay precision.

Determination of immunodominant antigens—Sera from 14 age-matched mixed sex unvaccinated cats divided into pairs that received either vaccinations on a standard vaccination schedule or CRFK protein injections as described in a previously reported experiment[4] were utilized. The sera had been stored at -70°C until utilized in this experiment. Cats in the CRFK group (Group C) received either 10 µg of CRFK protein SQ; 50 µg of CRFK protein SQ; or 50 µg of CRFK protein plus an aluminum adjuvant SQ. Cats in the vaccination group (Group V) received either a FVRCP vaccine for intranasal administration,[q] or one of three FVRCP vaccines for SQ administration.[r-t] Cats receiving CRFK proteins were inoculated every two weeks for two months, then monthly for four months, then every other month for six months for a total of eleven injections. Cats receiving vaccines were inoculated every three weeks for three inoculations and

then were boostered one year later. Samples were collected at 0, 4, 8, 16, 24, 50, and 54 weeks following initial injection for analysis by Western blot. The experimental design was reviewed and approved by the Colorado State University Animal Care and Use Committee.

After validation of the Western blot assay, immunodominant protein bands were determined by comparison of an amido black stain of a CRFK protein blot, the CRFK hyperinoculated rabbit Western blot, and the pre and post-inoculation Group C Western blots. Once immunodominant bands had been defined, the Group V blots were analyzed for their presence.

Isolation of immunodominant CRFK antigens—The CRFK cell lysate was incubated with polyclonal anti-CRFK immunoglobulin for one hour at 20°C. The incubated sample was then run through the protein A column as previously described and the eluent collected. The eluted sample was electrophoresed using blot technique 2, transferred to a PDVF membrane, and stained with 0.1% amido black as described above. Multiple titrations were performed until a 1:1 ratio of antigen to antibody was achieved in the final lysate purification. Coomassie blue staining of the purified CRFK lysate was performed on a 10% Bis-Tris gel as described above. After the membrane was destained, protein bands were aseptically harvested for digestion and sequencing. After initial sequencing results were received, the lysate was further concentrated by lyophilization. The lysate was again separated as described above and protein bands were reanalyzed.

In-gel protein digestion (In-gel)—Excised bands were cut into small pieces of approximately 1 mm^2 and placed in Eppendorf tube. Gel pieces were washed for 15 minutes with 100 μl 100mM NH_4HCO_3/50% ACN, spun down, and the supernatant discarded. This process was repeated until the dye was removed. After removal of the final wash solution, 100 μl of 100% CAN was added and incubated at 20°C for five minutes. The ACN was carefully removed and the sample air-dried at 20°C for five

minutes. The sample was suspended in 100 μL of DTT solution (10mM in 100 mM NH_4HCO_3) and incubated at 60°C for 30 minutes. The sample was then centrifuged, the supernatant discarded and 100 μL of IAA (55mM in 100 mM NH_4HCO_3) added before incubation in a dark room at 20°C for 30 minutes. The sample was centrifuged, supernatant removed, and the sample washed for five minutes with 100 μl 100mM NH_4HCO_3/50% ACN. The sample was then centrifuged, the supernatant discarded and 100 μl of 100% ACN added before incubation at 20°C for five minutes. The ACN was carefully removed and the sample air-dried again at 20° C for 5 minutes.

Six μl trypsin solution and 34 μl 100 mM NH_4HCO_3 were added to achieve a final trypsin concentration of 15 ng/μl. Samples were checked after 20 minutes and additional 100 mM NH_4HCO_3 was added as necessary to keep the gel pieces covered. Samples were then incubated overnight at 37°C. The sample was centrifuged, the supernatant removed and transferred to a clean Eppendorf tube (collection tube). Forty μl of extraction solution (50% ACN, 0.1% TFA) was added to the pellet and the sample incubated 20 minutes with gentle vortexing. The sample was centrifuged, the supernatant removed and added to the collection tube. Extraction was repeated once more. The sample was concentrated until almost dry at which point it was resuspended in 15 μl of 5% ACN, 0.1% acetic acid solution.

Mass spectrometry and database analysis—Five μl of purified sample was injected onto a reverse phase capillary column (1100 Agilent HPLC, 75 μmID x 15cm column). Peptides were eluted directly into the mass spectrometer using a 40 minute linear gradient from 2%-60% buffer B (80% ACN, 0.1% acetic acid) at a flow rate of 5 μl/min. Spectra were collected over a m/z range of 200-2000 Da[u] using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Bioworks 3.0 software[v]

with an intensity threshold of 5,000 and 1 scan/group. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot[w] and Sequest.[x] Mascot was set up to search the NCBInr_20070216 database (selected for .Dog, 34163 entries) assuming the digestion enzyme trypsin. Sequest was set up to search the NCBInr_dog.fasta.hdr database (42859 entries) also assuming trypsin. Mascot and Sequest were searched using average mass, with a fragment ion mass tolerance of 0.80 Da, a parent ion tolerance of 1.8 Da, and only allowing for 1 missed cleavage. Oxidation of methionine and iodoacetamide derivative of cysteine (carbamidomethylation) were specified in Mascot and Sequest as variable modifications.

Criteria for protein identification—Scaffold[y] was used to combine MS/MS based peptide and protein identifications from the individual Sequest and Mascot database searches. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required at least ion minus identity scores of greater than 30 and ion scores of greater than 40. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.5, 2.0, 2.5 and 3.5 for singly, doubly, triply, and quadruply charged peptides. Protein identifications were accepted if they contained at least two unique identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Results

Protein stains of the electrophoresed CRFK cell lysate revealed a large number of bands (Figure 1). These bands spanned the gel's entire size range of three to 188 kD and were of variable density. Western blot of the polyclonal anti-CRFK rabbit sera had fewer bands, but a number of bands of variable sizes were still identified (Figure 2). When these blots were compared with the samples from Group C cats, four bands were

identified consistently in the post-inoculation samples that were not present in the pre-inoculation samples. These bands were in the following molecular mass groupings: 46-49 kD, 41-43 kD, 37-39 kD, and 35-37 kD (Figure 3). Because these bands were so closely related in size, it was not possible to determine their exact sizes or evaluate tandem repeats using the 4-12% Bis-Tris gels.

Presence of at least one of these bands was identified in at least one post-inoculation sample from all of the Group C cats (Table 1). For cats where more than one of these bands were identified, band numbers and density were greater in samples from weeks 50 and 54. Samples from the Group V cats were then analyzed for presence of these bands (Table 2). Four of the Group V cats had faint bands in the pre-vaccination samples. These bands were less dense than bands that developed following vaccination. There was one cat in this group that did not develop any of the target bands at any time point.

When the week 50 and 54 samples were analyzed using 10% Bis-Tris gels, the 35-37 kD band was not present. The remaining detectable bands had apparent molecular masses of 47 kD (n = 18), 40 kD (n = 11), and 38 kD (n = 9). In addition, there was a trend toward an increased number of significant bands per sample. All cats positive for bands using the 4-12% Bis-Tris gel also were positive for bands using the 10% Bis-Tris gel. The cat that had had no significant bands detected with the 4-12% Bis-Tris gel had two detectable bands in both the week 50 and 54 samples using the 10% Bis-Tris gel.

Initial protein isolation and sequencing identified two proteins matching the CRFK immunodominant band sizes: α-enolase and macrophage capping protein (MCP). A third protein, annexin A2, was identified using the lyophilized lysate preparation. Although there is no published cat database, all sequenced proteins had very high predicted probabilities of correct identification based on database size, sample characteristics, and

percent sequence overlap. Alpha-enolase was identified with a 100.00% protein identification probability based on two unique peptides with 6.75% sequence coverage (Tables 3 and 4). Macrophage capping protein was identified with a 99.8% protein identification probability based on two unique peptides with 7.2% sequence coverage (Tables 5 and 6). Annexin A2 was identified with a 100.00% protein identification probability based on five unique peptides with 17% sequence coverage (Tables 7 and 8).

Discussion

This study documented the development of a Western blot immunoassay for anti-CRFK antibodies in serum. The presence of faint bands in some of the pre-inoculation samples is not surprising as the CRFK cell line is composed of feline cells and so it might be expected that some of the cats would have mild autoreactivity.

Initial analysis using the 4-12% Bis-Tris gel results revealed the 46-49 kD, 41-43 kD, 37-39 kD, and 35-37 kD antigens to be immunodominant. The 10% Bis-Tris gel was used in the second experiment to further clarify the target bands for a more accurate assessment of the molecular masses. As the band sizes were clarified to be 40 kD and 38 kD, it is likely that previously defined 37-39 kD antigen that was no longer detected reflected of a combination of laddering or shadow banding and drift of the slightly larger 40 kD band smaller 38 kD bands. The presence of false negative bands in one cat on the first gel was most likely due to misclassification of nearby bands due to similar wandering.

Alpha-enolase is a glycolytic enzyme of approximately 47 kD that is widely distributed throughout the body but present in greatest concentrations in the kidney and thymus.[6] In addition to its important roles in the glycolytic pathway, α-enolase functions as a membrane-bound plasminogen receptor in a variety of tissues.[6-8] Alpha-enolase is

considered a shock protein and its expression is upregulated by hypoxia in certain cells.[6-7] In human medicine, α-enolase autoantibodies have been associated with a wide variety of autoimmune diseases, including systemic lupus erythematosus (SLE), autoimmune-mediated retinopathy, autoimmune hepatitis, severe asthma, premature ovarian failure, and Hashimoto's encephalopathy.[6-13] Anti-α-enolase antibodies are not diagnostic for a particular autoimmune disease in humans, but they are associated with increasing mortality and so have prognostic value.[6,11] It is currently unknown why humans develop anti-α-enolase antibodies.

In our previous work, we documented interstitial nephritis in some cats hyperinoculated with the CRFK lysates used here.[5] In people, anti-α-enolase antibodies are also widely recognized as nephritogenic with direct pathogenic effects on the kidney.[7] The mechanism of anti-α-enolase antibody associated damage remains unclear, however immune-complex mediated cellular attack, complement mediated sublytic damage, inhibition of plasminogen binding leading to derangement of the fibrinolytic system, and direct induction of apoptotic processes may all be contributors.[7-8] Human studies regarding anti-α-enolase antibodies have been somewhat hampered by the lack of a realistic animal model. Given how highly conserved α-enolase is, it may be possible to utilize human monoclonal antibodies to improve the sensitivity and specificity of future studies evaluating anti-α-enolase antibody associated disease in cats. In addition, this discovery suggests that the cat may be a viable animal model for human studies.

Annexin A2, a member of the annexin family, is a conserved calcium-binding and phospholipid-binding protein with both intracellular and membrane-bound distributions.[14] Its published size ranges from 34 to 40 kD. A wide variety of cellular functions have been enumerated, including roles in endocytosis and exocytosis, cell proliferation and differentiation, apoptosis, and regulation of endothelial fibrinolysis.[14] Annexin A2 has a

fairly wide tissue distribution. It has been localized both intracellularly and bound to the plasma membrane in renal epithelial tubular cells in humans.[15-16] Renal annexin A2 expression has been shown to be upregulated after acute toxic renal insult and is postulated to be involved in the proliferation and repopulation of tubular epithelial cells.[15] It is also upregulated with oxidative stress; overexpression of annexin A2 secondary to oxidative stress may play a role in carcinogenesis.[17] Annexin A2 localized in the renal epithelial apical membrane binds calcium oxalate crystals and may play a role in the development of calcium oxalate nephroliths in humans.[16] Anti-annexin A2 antibodies are significantly correlated with thrombosis related to primary and SLE-associated antiphospholipid syndrome.[18-19] These antibodies precede disease and thus are believed to have a primary pathologic role. Anti-annexin A2 IgM antibodies have been also associated with severity of chorioamnionitis in pre-term infants.[20] A recent publication demonstrated a significant association between anti-annexin A2 antibodies and macular degeneration in cynomolgus monkeys, a research model for human macular degeneration.[21] Finally, annexin A2 autoantibody development has been associated with renal, lung, and gastric carcinomas and acute leukemia;[14,17,22-24] antibodies have been associated with increased hemorrhage and worsening prognoses.

Macrophage capping protein is an actin capping protein involved in regulating macrophage movement and fine-tuning conformational changes in these cells.[25] It is distributed primarily in the cytosol although it may have nuclear distribution in some tissues.[25-26] Studies evaluating MCP tissue distribution in humans are conflicting. One study suggests MCP is widely expressed[27] while another study states that MCP expression is limited to monocytes and the kidney;[25] positive splenic tissue analysis was postulated to result from its high density of macrophages.[25] Human MCP has been characterized as both 38 kD and 40 kD in size.[25,27] We could find no reports of anti-MCP antibody development in the literature.

Given its strictly intracellular distribution in living cells, endogenous MCP is unlikely to be accessible to the immune system. As antigen accessibility is considered the main determinant of an autoantibody's pathogenic potential, we consider it less likely that anti-MCP antibodies are associated with the development of disease. Further studies will be necessary to rule out this possibility.

Antibody bands were present in sera of all the cats in the study. Although the presence of antibody bands in the sera of intranasally vaccinated cats may appear to conflict with the previously published CRFK %ELISA results for the same samples, this discordance is not unexpected. Western blot immunoassays are typically more sensitive than ELISAs for the presence of antibodies. In the previously published study, antibody levels in sera were quantified by ELISA and categorized based on a %ELISA cutoff value. Antibody presence below the cutoff would not equate to a lack of antibody, merely a %ELISA value below the cutoff. When evaluated together, the results of the two studies suggest that CRFK protein presentation by antigen-presenting cells to the immune system occurs with intranasal administration but that the magnitude of the immune system's response to these proteins is much lower than when CRFK proteins are administered by injection.

This study validates the use of Western blot analysis to identify antibodies associated with CRFK cell protein inoculation. Immunodominant CRFK proteins have been identified that are highly conserved between species. Antibodies against one of these proteins, α-enolase, are recognized as a causative agent for immune-mediated nephritis. In addition, antibodies targeting α-enolase and annexin A2 are associated with other autoimmune syndromes in humans. Further research will be necessary to determine the distribution of these proteins throughout the body and whether development of antibodies is associated with the development of systemic disease in cats.

Footnotes

a. Irvine Scientific, Santa Ana, California.
b. T-175 flasks, Nalge Nunc International, Rochester, NY.
c. Steriflip®-GP 0.22 μm filter, Millipore Corporation, Billerica, Mass.
d. Prosep®-A Antibody Purification Kit, Millipore Corporation, Billerica, Mass.
e. NuPAGE® Bis-Tris gels, Invitrogen, Carlsbad, Calif.
f. NuPAGE® 4X LDS Sample Buffer, Invitrogen, Carlsbad, Calif.
g. NuPAGE® 10X Reducing Agent, Invitrogen, Carlsbad, Calif.
h. MultiMark® standards, Invitrogen, Carlsbad, Calif.
i. NuPAGE® 20X transfer buffer, Invitrogen, Carlsbad, Calif.
j. Invitrogen, Carlsbad, Calif.
k. Sigma-Aldrich Corporation, St. Louis, Missouri.
l. Bio-Rad Laboratories, Inc., Hercules, Calif.
m. Immunetics, Cambridge, Mass.
n. Kirkegaard and Perry Laboratories, Gaithersburg, MD.
o. Pierce Laboratories Inc., Rockford, IL.
p. Bio-Rad Gel Doc™ gel documentation system with Quantity One® software, Bio-Rad Laboratories, Inc., Hercules, Calif.
q. Trivalent intranasal/intraocular vaccine, Heska Corp, Loveland, Colo.
r. Felocell III, Pfizer Animal Health, NY.
s. PureVax, Merial, Athens, Ga.
t. Fel-O-Vax, Fort Dodge Animal Health, Overland Park, Kan.
u. Thermo Scientific LTQ linear ion trap, Waltham, Mass.
v. Thermo Scientific, Waltham, Mass.
w. Matrix Science, London, UK; version 2.1.04.
x. ThermoFinnigan, San Jose, CA; version 27, rev. 12.
y. Version Scaffold-01_06_06, Proteome Software Inc., Portland, OR.

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Table 1—Presence of immunodominant bands in CRFK hyperinoculated cats (n = 6) over time.

	CRFK	Wk 0	Wk 4	Wk 8	Wk 16	Wk 24	Wk 50	Wk 54
IBX1	10 μg	-	-	-	-	-	1,2,3,4	1,2,3,4
ABK4		-	4	4	2,4	2,4	2,4	2,4
ABI2	50 μg	-	-	-	-	-	2,4	2,4
ABI5		-	4	4	-	4	2,4	2,4
ABI1	50 μg (plus adjuvant)	-	-	-	-	-	4	-
QAM1		-	-	-	-	1	1	1

Where Band 1 = 35-37 kD, Band 2 = 37-39 kD, Band 3 = 41-43 kD, Band 4 = 46-49 kD.

Table 2—Presence of immunodominant bands in vaccinated cats (n = 8) over time.

	Vaccine	Wk 0	Wk 4	Wk 8	Wk 16	Wk 24	Wk 50	Wk 54
IBW3	1	-	4	2,3,4	-	-	2,3,4	2,4
ABK5		2,4	2,4	2,4	2,4	2	4	2,4
ABK2	2	-	4	-	-	-	2,4	2
IBW2		2	1,4	1	-	-	1,2	1,3
ABK3	3	4	4	4	4	2,4	2,4	4
ABI3		-	-	-	-	-	-	-
IBW1	4	4	4	4	-	2,4	2,4	2,4
QAM2		-	4	-	-	-	4	-

Where Band 1 = 35-37 kD, Band 2 = 37-39 kD, Band 3 = 41-43 kD, Band 4 = 46-49 kD.

Vaccine 1 = Trivalent intranasal/intraocular vaccine, Heska Corp, Loveland, Colo.

Vaccine 2 = Felocell III, Pfizer Animal Health, NYC, NY. Vaccine 3 = PureVax, Merial, Athens GA. Vaccine 4 = Fel-O-Vax, Fort Dodge Animal Health, Overland Park, KS.

Table 3—Alpha enolase amino acid sequence

MGGQKVTMSI	LKIHAREIFD	SRGNPTVEVD	LYTSKGLFRA	AVPSGASTGI	YEALELRDND
KTRYMGKGVS	KAVEHINKTI	APALVSKKVN	VVEQEKIDKL	MIKMDGTENK	SKFGANAILE
CPWLSARRGL	SRRGCPCGSH	AGNKLAMQEF	MILPVGAANF	KEAMRIGAEV	YHNLKNVIKE
KYGKDATNVG	DEGGFAPNIL	ENKEALELLK	NAIGKAGYTD	KVVIGMDIAA	SEFFRYITPD
QLADLYKSFI	RNYPVASFED	PFNQDDWEVW	QNFTASAGIQ	VVGDDLTVTN	PKQISKAVGK
KSCNCLLLKV	NQIGSVTESL	QACKLAQSNG	WGVMVSHRSG	ETKDTFIADL	VVGLCTGQVK
TGAPCRSERL	AKYNQILRIE	EELGSKAKFV	GRSFRNPLAK		

NCBI Sequence accession number gi|74007151. Shaded areas indicate 2 unique peptides, 2 unique spectra, 2 total spectra, 27/400 amino acids (6.7% coverage).

Table 4—Alpha enolase peptide sequencing information

Peptide sequence	Peptide identification probability	Parent ion mass	Charge	Best SEQUEST XCorr score	Best SEQUEST DCn score	Best Mascot Ion score	Best Mascot Identity score	Modifi-cations
VNQIGSV TESLQACK	95.00%	1633.46	2	3.39	0.526	79.9	38.5	Carbamido-methyl (+57)
YITPDQ LADLYK	95.00%	1439.41	2	2.44	0.291	38.5	38.6	-

Table 5—Macrophage capping protein amino acid sequence

MYTSLPQGGS	PFPGSVQDPG	LHVWRVEKLK	PVPVARENQG
VFFSGDSYLV	LHNGPEELSH	LHLWIGQQSS	RDEQGACAVL
AVHLNTLLGE	RPVQHREVQG	NESDLFMSYF	PRGLKYQEGG
VESAFHKTSP	GATAAPIKKL	YQVKGKKNIR	ATERALNWDN
FNTGECFILD	LGPNIFTWCG	GKSNILERNK	ARDLALAIRD
SERQGKAQVE	IVTDGEEPAE	MIQVLGPKPA	LKEGNPEEDL
TADRTNAQAA	ALYKVSDATG	QMNLTKVADS	SPFALELLLS
DDCFVLDNGL	CGKIYIWKGR	KANEKERQAA	LQVAEDFISR
MRYAPNTQVE	ILPQGRLSPI	FKQFFKDWK	

NCBI Sequence accession number gi|73980918|ref|XP_540197.2| (+1). Shaded areas indicate 2 unique peptides, 3 unique spectra, 3 total spectra, 25/349 amino acids (7.2% coverage).

Table 6—Macrophage capping protein peptide sequencing information

Peptide sequence	Peptide identification probability	Parent ion mass	Charge	Best SEQUEST XCorr score	Best SEQUEST DCn score	Best Mascot Ion score	Best Mascot Identity score	Modifications
QAALQV AEDFISR	95.00%	1447.04	2	3.09	0.59	74.7	39	-
YQEGGV ESAFHK	95.00%	1351.25	2	2.49	0.337	46.1	39.2	-

Table 7—Annexin A2 amino acid sequence

MSTVHEILCK	LSLEGDHSTP	PSAYGSVKAY	TNFDAERDAL
NIETAIKTKG	VDEVTIVNIL	TNRSNEQRQD	IAFAYQRRTK
KFIASALKSA	LSGHLETVIL	GLLKTPAQYD	ASELKASMKG
LGTDEDSLIE	IICSRTNQEL	QEINRVYKEM	YKTDLEKDII
SDTSGDFRKL	MVALAKGRRA	EDGSVIDYEL	IDQDARDLYD
AGVKRKGTDV	PKWISIMTER	SVCHLQKVFE	RYKSYSPYDM
LESIKKEVKG	DLENAFLNLV	QCIQNKPLYF	ADRLYDSMKG
KGTRDKVLIR	IMVSRSEVDM	LKIRSEFKRK	YGKSLYYYIQ
QDTKGDYQKA	LLYLCGGDD		

NCBI Sequence accession number gi|50950177,gi|50950177|ref|NP_001002961.1|. Shaded areas indicate 5 unique peptides, 5 unique spectra, 6 total spectra, 57/339 amino acids (17% coverage).

Table 8—Annexin A2 peptide sequencing information

Peptide sequence	Peptide identification probability	Parent ion mass	Charge	Best SEQUEST XCorr score	Best SEQUEST DCn score	Best Mascot ion score	Best Mascot identity score	Modifications
AYTNFDAER	95.00%	1086.49	2	2.05	0.49	52.3	39.8	-
LSLEGDHST PPSAYGSVK	95.00%	1844.90	3	2.62	0.419	39.3	37.8	-
QDIAFAYQR	95.00%	1111.55	2	2.45	0.385	64	38.9	-
TNQELQEINR	95.00%	1244.62	2	2.99	0.147	60.3	39.3	-
TPAQYDASELK	95.00%	1222.60	2	3.52	0.555	54.8	39.1	-

Figure 1—An 0.1% amido black protein stain of CRFK cell lysate on a PDVF membrane.

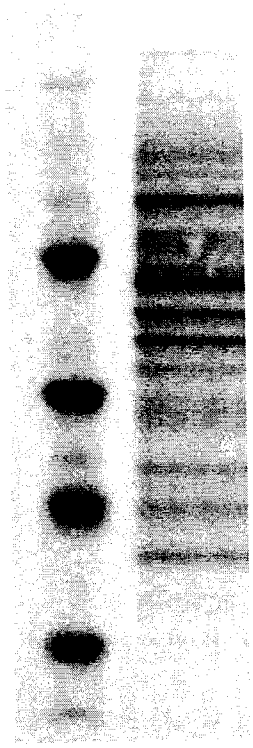


Figure 2—Western blot analysis of CRFK-hyperinoculated rabbit serum. Asterisks indicate bands of interest.

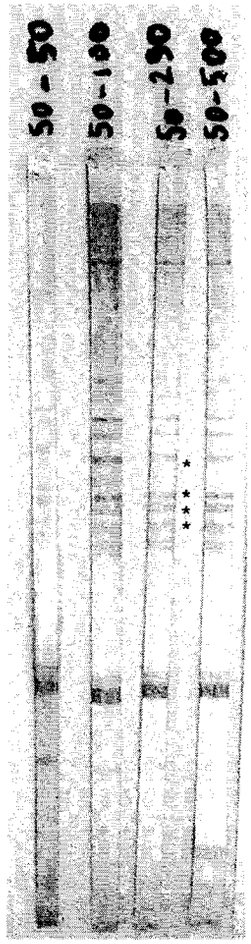
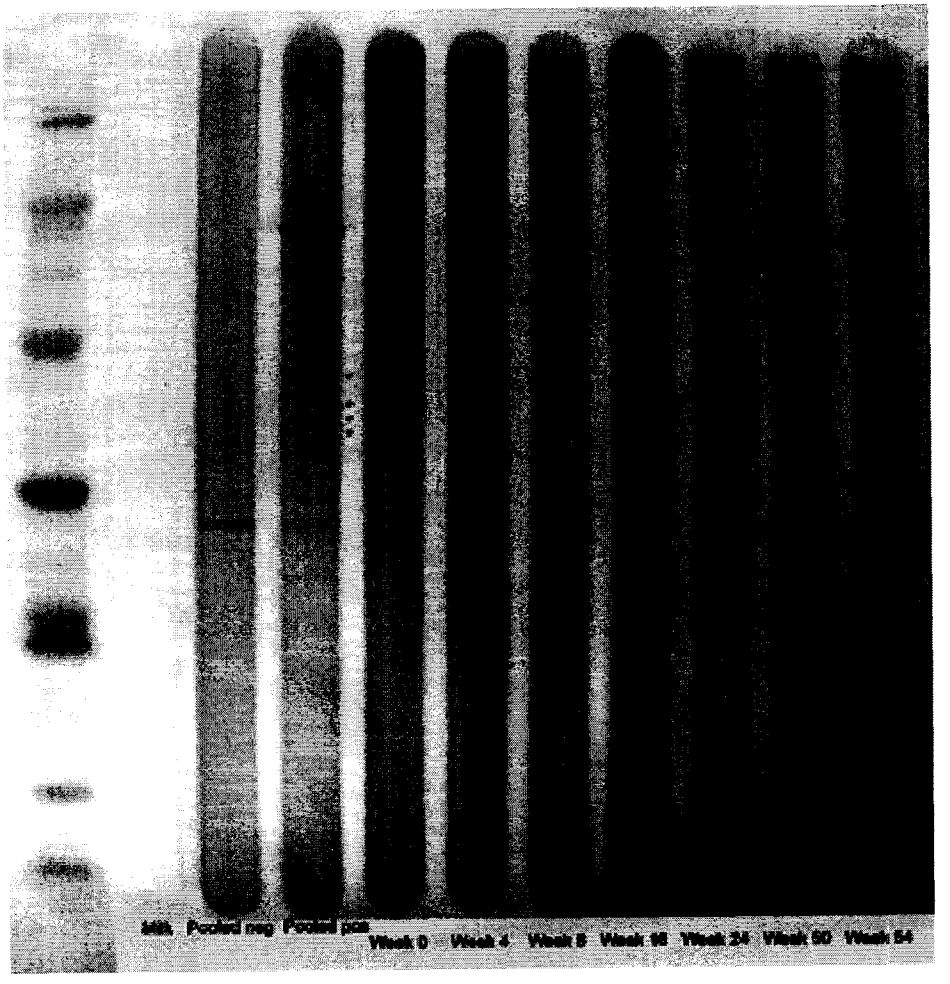


Figure 3—Western blot analysis of serum from a CRFK-hyperinoculated cat. Asterisks indicate bands of interest.



DETERMINATION OF THE DISTRIBUTION OF IMMUNODOMINANT CRANDELL REES FELINE KIDNEY (CRFK) CELL LINE ANTIGENS IN FELINE AND MURINE TISSUES

Whittemore J.C., Hawley J. R., Jensen W.A., and Lappin, M.R.

Objective—Apply a previously developed Western blot immunoassay for the immunodominant Crandell Rees Feline Kidney (CRFK) cell proteins α-enolase, annexin A2, and macrophage capping protein (MCP) to feline and murine tissues to map their distribution.

Sample Population—Tissues from 7 unowned cats with no gross abnormalities and one mouse euthanized for other reasons.

Procedure—Feline samples evaluated were brain, urinary bladder, ileocecal lymph node, gingiva, thyroid/parathyroid complex, adrenal gland, kidney, liver, duodenum, pancreas, heart, spleen, and lung. Murine samples evaluated were kidney, spleen, brain, liver, and pancreas. Samples were individually homogenized, sonicated, ultracentrifuged and the supernatants assayed for protein content. Pooled samples, adjusted for individual sample protein concentration, were made for each tissue and then were assessed by Western blot immunoassay.

Results—Several of the feline and murine tissues were positive for the immunodominant CRFK antigens. Tissues positive for all three bands were kidney, spleen, and brain. Antigen distribution by tissue was similar between species.

Conclusions and Clinical Relevance—This study validated the use of Western blot immunoassay analysis for the detection of immunodominant CRFK proteins in tissues. Tissue distribution was fairly consistent with previously described human distributions of α-enolase, annexin A2, and MCP. In humans, anti-α-enolase antibodies are directly nephritogenic. Anti-α-enolase and anti-annexin A2 antibodies have been associated with human autoimmune syndromes. Further research will be necessary to determine the clinical significance of these findings.

Introduction

Vaccines continue to be an important facet of preventative health programs for most companion animal species. Vaccine use in cats has led to a dramatic reduction in the prevalence and incidence of such catastrophic diseases as panleukopenia virus and rabies. However, like most other medical treatments and procedures, vaccination is not without risk. Negative links to vaccination in companion animals include injection-site fibrosarcomas,[1-4] development of autoimmunity,[5-6] immune-mediated thrombocytopenia and hemolytic anemia,[7] and nephritis.[8-9]

Significant progress has been made in the methodology of vaccine development, including the use of subunit vaccines and canarypox-vectored vaccines, to lessen the risks associated with vaccination. The majority of commercially available FVRCP vaccines, however, are generated from viruses grown using the Crandell Rees Feline Kidney (CRFK) cell line.[10-11] Following replication, the viruses are harvested for use and purified. Some contamination with cell proteins from the host cell line remains and is incorporated into the vaccine.

The CRFK cell line was developed in the 1960s by homogenization of naïve feline kidney tissue.[12] Inoculation of cats with CRFK proteins or FVRCP vaccinations according to a standard vaccination schedule has been associated with development of anti-CRFK and anti-renal tissue antibodies.[13] In addition, some cats developed lymphocytic-plasmacytic nephritis two weeks following CRFK booster inoculation.[14] Renal failure is very common in client-owned cats and the cause is usually not determined. Histologically, lymphocytic-plasmacytic infiltration is common, leading to concerns that interstitial nephritis may have an autoimmune etiology. In addition, cats are afflicted with many other syndromes with lymphocytic-plasmacytic infiltrates on histopathological examination including cholangiohepatitis, uveitis, pancreatitis, and inflammatory bowel disease.

We have isolated and identified three proteins that were determined by Western blot immunoassay to be immunodominant CRFK antigens.[15] These proteins, α-enolase, annexin A2, and macrophage capping protein (MCP) are highly conserved among species. The objectives of this study were to determine the distribution of these proteins in non-renal tissues and to evaluate cross-species antigen conservation and distribution by comparing distribution of these antigens in feline and murine tissues using a variation of a previously validated Western blot immunoassay.[a]

Materials and Methods

Tissue collection and preparation—

Feline tissues: Tissue samples were collected from 7 unowned cats whose bodies were presented after death for rabies testing. No gross abnormalities were identified in any of the cats and none were infected with rabies virus. Vaccine history was unknown. The cadavers were stored at 4°C until tissues were collected for use in this study. Four cats were altered (2 males, 2 females) and 3 were intact (all female). Six of the cats were young adults (1-3 years of age); one cat was approximately 5 months of age. Samples obtained for evaluation were brain (2), urinary bladder (6), ileocecal lymph node (6), gingiva (7), thyroid/parathyroid complex (7), adrenal gland (7), kidney (7), liver (7), duodenum (7), pancreas (7), heart (7), spleen (7), and lung (7). Samples were collected within 24 hours of presentation and frozen in phosphate buffered saline (PBS) at -70°C pending analysis.

After samples from all the cats had been collected, they were thawed at 20°C (room temperature). A 0.4 gm sample was collected from each tissue and placed in 0.7ml PBS in an individual lysing matrix tube.[b] Samples were homogenized using three 20 second cycles of homogenization alternating with one minute cooling in an ice bath. Following homogenization, samples were centrifuged for five minutes at 10,000 rpm.

The supernatant was collected and sonicated for 10 cycles of 1-minute sonication at 30% power alternating with one minute cooling in an ice bath. Sonicated samples were ultracentrifuged for 20 minutes at 14,000 rpm at 4°C. Samples were analyzed for protein content using the turbidimetric method with benzethonium chloride on an automated chemistry analyzer. Pooled samples, suspended in PBS, were made for each tissue to give a total protein concentration of 2.25 μg/μL.

Murine tissues: Samples from brain, kidney, liver, pancreas, and spleen were collected from a healthy mouse euthanized as part of another study. Each tissue was individually macerated with scissors in 0.5-3 mL of sterile phosphate buffered saline (PBS), followed by repeated aspiration through progressively smaller needles from 18-20 gauge. Tissues were then lysed using a sonicator on 30% power using one minute of lysis time cycled with one minute of cooling time while in an ice bath (20 cycles). The lysates were centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant of each sample was collected; the protein concentration was determined as described for feline tissues. Each tissue lysate, suspended in PBS, was adjusted to give a total protein concentration of 2 μg/μL.

Western blot immunoassay—The CRFK proteins were separated under reducing conditions on pre-cast 10% Bis-Tris mini-gels[c] with MOPS running buffer using a discontinuous buffer system using an adaptation of a previously reported assay for use with serum.[a] A total protein concentration of 6.5 μg of each pooled tissue lysate was mixed with the appropriate amount of sample buffer[d] and reducing agent,[e] denatured and loaded in a well on the gel along with a molecular weight standard.[f] After all samples were loaded, the gel was electrophoresed at 200V constant until the dye front reached the bottom of the gel. Once electrophoresis was complete, the membranes were prepared by premoistening with anhydrous methanol for 30 seconds, rinsing with ddH2O and soaking in transfer buffer,[g] prepared according to manufacturer's instructions with

20% methanol. The proteins were transferred to a polyvinylidene difluorure (PVDF) membrane[h] using the XCell II Blot Module[h] at 30V constant for 90 minutes. The membranes were air dried, placed in plastic bags and stored at -20°C.

The membranes were thawed at 20°C, premoistened with anhydrous methanol, rinsed with ddH2O and blocked with 1X NEH (250mM HEPES, 25mM EDTA, 750mM NaCl, 0.05%Triton X-100, 0.25% Gelatin, 3% BSA) for one hour on a rocker at 20°C. After the block was complete, the were membranes incubated for two hours at 20°C in anti-CRFK polyclonal rabbit antibodies diluted 1:50 in 0.01M Tris Base, 0.15M NaCl, 0.5% Tween 80 and 0.001M phenylmethyl-sulfonyl fluoride (PMSF) plus 10% nonfat milk (TNTP+10% milk). Following the primary antibody incubation, the membranes were washed three times for five minutes each in PBS-0.5% Tween 80 and incubated for one hour in horseradish peroxidase labeled goat anti-rabbit IgG[i] diluted 1:250 in TNTP+10% milk. After incubation with the secondary antibody, the membranes were washed as described above. The membranes were incubated in a 1:10 dilution of 4-chloro-1-naphthol/3,3'diamino benzidine tetrahydrochloride in peroxide substrate buffer (CN/DAB)[j] until color reaction was visible. The reaction was terminated by rinsing the membranes with ddH2O. Once dry, membranes were photographed using an image capture system.[k] Visible bands were plotted on a semi-log regression line of standards with known molecular weights to determine estimated molecular masses. Samples for which the rabbit anti-CRFK antibodies recognized a 47 kD antigen were considered to contain α-enolase.[15] Samples for which the rabbit anti-CRFK antibodies recognized a 40 kD antigen were considered to contain MCP, while samples recognizing a 38 kD antigen were considered to contain either annexin A2 or MCP since both were previously isolated from the 38 kD band.[15]

Results

Several of the feline and murine tissues were positive for one or more of the immunodominant CRFK antigens (Tables 1 and 2). Tissues that were positive for all three antigens were kidney, spleen, and brain. Tissues positive for zero or one band were duodenum, pancreas, and gingiva. The remaining tissues were positive for two of the three bands. Antigen distribution by tissue was fairly similar between the two species; the only disparate result was for pancreas.

Discussion

Autoantibody development secondary to vaccination has been previously reported in companion animals. Anti-thyroglobulin antibody development secondary to rabies vaccination has been documented in dogs for example.[6] Previous work by our laboratory has demonstrated anti-CRFK and anti-renal tissue extract antibody development in cats secondary to FVRCP vaccination and CRFK protein hyperinoculation.[13] In addition, some of the CRFK hyperinoculated control cats had lymphocytic-plasmacytic nephritis documented two weeks following booster inoculation,[14] and one of the original research cats was recently euthanized for kidney failure (JW, unpublished data 2007). This cat had severe lymphocytic-plasmacytic nephritis on necropsy.

Previously, we purified a CRFK protein lysate using polyclonal anti-CRFK rabbit antibodies. Using the previously developed Western blot immunoassay, we separated the remaining immunodominant proteins for isolation and sequencing. Three proteins were identified–α-enolase, annexin A2, and macrophage capping protein (MCP).[15] Their reported respective sizes are 47 kD, 38 kD, and 40/38 kD. All three of these proteins play important roles in cellular function and are highly conserved between species, so the presence of anti-CRFK antibody binding to murine tissue lysates was not surprising.

Alpha-enolase is a highly conserved protein known to have a wide distribution in humans.[16] In humans, the greatest concentrations of α-enolase are found in the kidney and thymus. It functions as a glycolytic enzyme in the cytoplasm and the membrane-bound protein acts as a plasminogen receptor in a variety of tissues.[16-18] The detection of α-enolase in extra-renal tissues of the cats and the mouse was not surprising. Given its wide range of functions and known human distribution, the negative results for feline pancreas and duodenum are curious. Although we cannot rule out the possibility that these tissues are devoid of α-enolase in the cat, we consider it more likely that these results are false negatives. Potential causes for false negative results include relative tissue dilution, incomplete protein extraction from the plasma membranes, or sampling error. The presence of α-enolase in murine pancreas and strong conservation of protein sequence and function across species support the likelihood that these were false negatives.

In humans, antibodies against α-enolase are associated with a variety of autoimmune diseases involving a large variety of organs including systemic lupus erythematosus (SLE), autoimmune-mediated retinopathy, autoimmune hepatitis and cholangitis, inflammatory bowel disease, severe asthma, premature ovarian failure, Hashimoto's encephalopathy, and membranous nephropathy.[16-24] Anti-α-enolase antibodies are not diagnostic for a particular autoimmune disease in humans, but they are associated with increasing mortality and can therefore be used prognostically.[16,21] It is currently unknown why humans develop anti-α-enolase antibodies. Anti-α-enolase antibodies have been shown to be directly nephritogenic in a number of human studies. They are also believed to play an active role in disease progression in other disease processes, though the mechanism behind this damage remains unclear. Possible mechanisms for tissue damage include immune-complex mediated cellular attack, complement mediated sublytic damage, inhibition of plasminogen binding leading to

derangement of the fibrinolytic system, and direct induction of apoptotic processes.[17-18] The high level of amino acid sequence and function conservation between species, accessibility of α-enolase to the immune system, and breadth of human associations between anti-α-enolase antibodies and autoimmune syndromes raise the possibility that FVRCP vaccination may be associated with feline immune-mediated disease in both renal and non-renal tissues through induction of anti-α-enolase antibodies.

There was fairly wide tissue binding of antibodies at the 38 and 40 kD positions. In previous work, we showed annexin A2 to migrate at 38 kD and macrophage capping protein (MCP) to migrate at both 38 kD and 40 kD using this assay.[15] Annexin A2 is a conserved calcium-binding protein.[25] It is important for a wide variety of cellular functions which include vesicle movement, cellular growth and apoptosis, and plasminogen activation.[25] Annexin A2 has a wide tissue distribution in people and exists in both intracellular and membrane-bound forms. Both forms are present in renal epithelial tubular cells in humans,[26-27] and annexin A2 is postulated to play an important role in renal tubular cell repair after acute toxic insult.[26] It is also upregulated in response to oxidative stress.[28] Annexin A2 localized in the renal epithelial apical membrane binds calcium oxalate crystals and may play a role in the development of calcium oxalate nephroliths in humans.[27]

Anti-annexin A2 antibodies have also been associated with autoimmune disorders in other species. They are believed to play a pathogenic role in the development of thrombosis in humans with primary and SLE-associated antiphospholipid syndrome.[28-30] Anti-annexin A2 IgM antibodies have been also associated with severity of chorioamnionitis in pre-term infants,[31] and anti-annexin antibodies have been associated with recurrent fetal loss. A significant association between anti-annexin A2 antibodies and macular degeneration in cynomolgus monkeys, a research model for human macular degeneration, has also been documented.[32]

Finally, annexin A2 autoantibody development has been associated with renal, lung, and gastric carcinomas and acute leukemia in humans;[25,28,33-35] antibodies have been associated with increased hemorrhage and worsening prognoses. Because antibody binding at 38 kD may reflect the presence of either annexin A2, MCP, or both proteins, we cannot definitively state which tissue binding correlates with each protein's tissue distribution although results from the human literature would suggest a wide tissue distribution for annexin A2.

Macrophage capping protein is part of a family of actin-binding proteins and is believed to play an important role in macrophage conformational and locomotive activities.[36] In the human literature, reports regarding the tissue distribution of MCP have conflicting results. The initial study by Yu and colleagues evaluating MCP mRNA distribution in tissues reported a wide distribution with highest concentrations in the kidney and lungs.[37] A later study evaluating both protein presence by Western blot and gene expression by Northern blot found that MCP was confined to macrophages and the kidney with possible low concentrations in neutrophils.[36] Authors of the latter study also noted positive results for the spleen that they hypothesized was secondary to its high number of resident macrophages. They postulate that monocytic contamination of tissues may have led to the wider results in Yu's study.

In our study we noted positive antibody binding at 40 kD consistent with MCP presence for kidney and spleen, but also for brain, liver, and lymph node. Since histologic evaluation of the tissues was not performed, it cannot be ruled out that these tissues were all highly infiltrated with monocytes. However, we consider this very unlikely given the normal gross appearance of the tissues and the fact that lysates from multiple animals were used to make the final pooled samples. As polyclonal rabbit antibodies were utilized for this experiment instead of monoclonal antibodies specific for MCP, it is possible that the antibodies were the result of cross-reactivity with other actin-binding

proteins present in those tissues. This is also unlikely since there is little similarity between the primary structure of the other actin binding proteins and MCP. Finally, it may be that cats have a distribution of MCP more consistent with the results of Yu and his colleagues. As MCP is localized to the cytoplasm and nucleus of cells,[36-38] it remains to be determined whether anti-MCP antibodies have clinical significance in the cat.

The high level of conservation of α-enolase, annexin A2, and MCP between species suggests that human monoclonal antibodies for them may be useful in further elucidating what role, if any, antibodies against these proteins have in stimulating disease in cats. Particularly, Northern and Southern blot analyses of protein expression and immunohistochemical analyses may be necessary to elucidate the potential role of anti-α-enolase, anti-annexin A2, and anti-MCP antibodies in the development of a variety of feline diseases.

Footnotes

a. Whittemore JC, Hawley JR, Lappin MR. Feline serum antibody responses to Crandell Rees Feline Kidney cell inoculations and characterization of target antigens (abst.). *Journal of Veterinary Internal Medicine* 2004;18:438.
b. Matrix A tubes, Qbiogene, Inc., MP Biologicals, Solon Ohio.
c. NuPAGE® Bis-Tris gels, Invitrogen, Carlsbad, Calif.
d. NuPAGE® 4X LDS Sample Buffer, Invitrogen, Carlsbad, Calif.
e. NuPAGE® 10X Reducing Agent, Invitrogen, Carlsbad, Calif.
f. MultiMark® standards, Invitrogen, Carlsbad, Calif.
g. NuPAGE® 20X transfer buffer, Invitrogen, Carlsbad, Calif.
h. Invitrogen, Carlsbad, Calif.
i. Kirkegaard and Perry Laboratories, Gaithersburg, MD.
j. Pierce Laboratories Inc., Rockford, IL.
k. Bio-Rad Gel Doc™ gel documentation system with Quantity One® software, Bio-Rad Laboratories, Inc., Hercules, Calif.

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Table 1—Distribution of CRFK immunodominant antigens as determined by Western blot immunoassay in a variety of feline tissues

	Kid	Sp	Br	Liv	Pan	UB	Gin	T/P	AG	Duo	Ht	LN	Lu
47 kD	X	X	X	X		X		X	X		X	X	X
40 kD	X	X	X	X								X	
38 kD	X	X	X			X	X	X	X		X		X

Note: X = Band present by Western blot immunassay analysis in the evaluated tissue pool. Kid kidney; Sp: spleen; Br: brain; Liv: liver; Pan: pancreas; UB: urinary bladder; Gin: gingiva; T/P: thyroid/parathyroid complex; AG: adrenal gland; Duo: duodenum; Ht: heart; LN: lymph node; Lu: lung.

Table 2—Distribution of CRFK immunodominant antigens as determined by Western blot immunoassay in a variety of murine tissues

	Kidney	Spleen	Brain	Liver	Pancreas
47 kD	X	X	X	X	X
40 kD	X	X	X	X	
38 kD	X	X	X		

Note: X = Band present by Western blot immunoassay in the evaluated tissue.

ANTIBODY RESPONSES TO CRANDELL REES FELINE KIDNEY (CRFK) CELL LINE EXTRACTS IN EXPERIMENTAL CATS AND ASSOCIATIONS BETWEEN CRFK ANTIBODY DEVELOPMENT AND BIOCHEMICAL ABNORMALITIES IN 1,477 PRIVATELY-OWNED CATS

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Background—Feline herpesvirus 1, calicivirus, and panleukopenia virus for use in FVRCP vaccines are commonly grown in Crandell Rees Feline Kidney (CRFK) cells. In a preliminary study, administration of injectable but not intranasal FVRCP vaccines induced CRFK antibody levels detectable by ELISA but small sample size precluded statistical comparisons.

Hypothesis—FVRCP vaccination is associated with development of anti-CRFK antibodies. Anti-CRFK antibodies are associated with biochemical abnormalities.

Animals—Part 1: 50 unvaccinated mixed sex kittens. Part 2: Serum from 1,477 privately-owned cats submitted for biochemical analysis.

Methods—Part 1: Kittens, divided into groups of 10, received one of the following on days 0, 28, and 56: an intranasal FVRCP vaccine or one of four SQ FVRCP vaccines. CRFK antibodies were assessed by ELISA in serum collected at multiple time-points. Vaccine group means were calculated and compared between groups. Part 2: Biochemical values were compared to CRFK %ELISA values by multiple logistic regression.

Results—Part 1: Significant differences were identified between groups at multiple post-inoculation time points. Part 2: There were significant negative associations between CRFK antibodies and glucose, creatinine, and ALP activity. A significant positive association was found for CRFK antibodies and bilirubin.

Conclusions and clinical importance—Administration of CRFK-containing FVRCP vaccines SQ induces a greater magnitude of CRFK antibody responses than administration of a FVRCP vaccine IN. Anti-CRFK antibody formation is significantly correlated with some biochemical abnormalities. Further studies evaluating the clinical significance of these results are necessary.

Introduction

Feline herpesvirus 1, calicivirus, and panleukopenia virus for use in feline vaccines (FVRCP) are commonly grown in Crandell Rees Feline Kidney (CRFK).[1-3] We previously showed that SQ, but not intranasal, intraocular (IN), administration of FVRCP vaccines that contain CRFK proteins induce CRFK antibody levels detectable by ELISA.[4] In that study, only two cats per group were assessed which precluded statistical comparisons between groups. In addition, a new formulation of the IN vaccine has been licensed in the United States[a]; whether CRFK antibodies are induced by administration of this vaccine is unknown.

We have isolated and identified three proteins that were determined by Western blot immunoassay to be immunodominant CRFK antigens. These proteins, α-enolase, annexin A2, and macrophage capping protein (MCP)[5] are widely distributed through feline tissues and highly conserved between species. In addition, antibodies against α-enolase and annexin A2 are associated with several autoimmune disorders in people.[6-10] Thus, it is possible that hyperinoculation of cats with CRFK proteins contaminated FVRCP vaccines may also stimulate a variety of immune-mediated diseases in cats which could lead to associated biochemical abnormalities. The potential of an association between biochemical abnormalities as biomarkers of organ dysfunction and CRFK antibody levels has yet to be prospectively evaluated.

The first objective of this study was to determine and compare the CRFK antibody responses of cats inoculated with commercially available FVRCP vaccines for IN or SQ administration that are known to contain CRFK proteins. The second objective was to evaluate for potential associations between CRFK antibody levels and biochemical biomarkers of organ dysfunction in 1,477 privately-owned cats.

Materials and Methods

Vaccination study:

Vaccines—Four injectable FVRCP vaccines[b-e] and the only trivalent, modified live, FVRCP vaccine for intranasal administration were [a] chosen for study.

Experimental cats—Fifty, eight-week old, mixed-sex, unvaccinated kittens were purchased from commercial vendors. The kittens were randomly divided into five groups of 10, gang-housed by group, and fed ad libitum. The kittens were observed daily throughout the study. The experimental design was reviewed and approved by the Colorado State University Animal Care and Use Committee in compliance with Federal regulations.

Experimental design—One of the following was administered to a group of kittens on days 0, 28, and 56: the FVRCP vaccine for IN administration or one of four FVRCP vaccines for SQ administration. Kittens administered one of the FVRCP vaccines[b] were adopted after day 185 of the study. Kittens in the other four groups were administered their respective vaccine as a booster on day 365. Blood for serum collection was collected by jugular venipuncture prior to inoculation and on days 67, 81, and 185, for kittens in all five groups as well as on days 365, 379, 393, and 421 for the four groups that remained for the duration of the study. Serum was saved frozen at -70°C until assayed.

CRFK ELISA—Antibodies against CRFK extracts were determined in each sample by use of a previously published ELISA protocol.[4] The mean absorbance value for the positive control sample, the negative control sample, and each test sample was calculated. The mean absorbance values were converted to %ELISA units by the following formula: test sample mean absorbance minus the negative control sample mean absorbance/positive control sample mean absorbance minus the negative control sample mean absorbance multiplied by 100.

Non-specific background ELISA absorbance values increase in kittens over time (Lappin MR, unpublished data, 2007). Thus, to determine the positive cutoff value for this portion of the study, sera from 14, seven to eight month old cats known to be herpesvirus 1, calicivirus, and panleukopenia naïve and never vaccinated were assayed.[11] These kittens were approximately the same age as the 50 vaccinated kittens at the day 185 post-inoculation period. Sera had been saved at -70°C until assayed in this study.

Data analysis—For vaccinated cat results, day 0 (pre-inoculation), 67, 81, 185, 365, 379, 393, and 421 group mean %ELISA results were log transformed (natural log (result + 1) prior to statistical analysis to normalize the residuals for the CRFK ELISA. A mixed model appropriate for a repeated measures experiment[f] was used to evaluate the fixed effects of vaccine group, time, and the time by group interaction. Effects were deemed statistically significant if p value < 0.05. If the interaction term was significant, within time differences between groups were evaluated when the overall group effect was detected. Otherwise, vaccine effects were evaluated through the main effect of the group. The CRFK %ELISA group mean plus two standard deviations from the 14 unvaccinated control cats at seven to eight months of age was used as the positive cutoff values for analysis of individual sample results.

Serosurvey of privately-owned cats:

Biochemical data—To evaluate for potential associations between biochemical abnormalities and CRFK antibody levels, residual serum samples were obtained from a commercial diagnostic laboratory[g] for 1,477 cats on which a biochemical profile had been performed. Results of assays for albumin, bilirubin, BUN, creatinine, calcium, chloride, cholesterol, globulin, glucose, phosphate, potassium, sodium, and total protein as well as activities of creatine kinase (CK), serum alkaline phosphatase (ALP), serum

alanine aminotransferase (ALT), and serum aspartate transferase (AST) were available for all cats included in the study. Age was available for 1,348 cases. Because samples were obtained from a commercial diagnostic laboratory in lieu of their disposal, additional information including signalment, vaccination history, and geographic distribution, was not available for the cats.

CRFK ELISA—CRFK ELISAs were performed as described for the vaccinated cats with the exception that, to compensate for the increased age range of cats in this subgroup, a different control group was used to establish the positive cutoff. Our laboratory maintains an extensive serum bank; feral cats of variable age from Mexico were considered the closest available unvaccinated control group for the study population with regard to antigenic exposure and age range. Sera, saved at -70°C until assayed in this study, from 32 feral cats from Mexico of variable age were assayed.

Data analysis—To evaluate the hypothesis that CRFK antibody development is associated with biochemical abnormalities, a stepwise logistic regression analysis was performed with the %ELISA value as the outcome variable. ELISA values were considered positive if the result exceeded the mean plus three standard deviations based on the results from the control population. This more aggressive cut-off was chosen since there were uncontrollable variables between the controls and study population that were not present for the vaccination portion of the study. Collinearity of the explanatory variables was evaluated prior to the evaluation of the association of antibodies and the biochemical markers. Assuming no collinearity of explanatory variables, the statistical model initially included age, albumin, ALP activity, ALT activity, bilirubin, BUN, creatinine, and glucose as covariates. Glucose, BUN, creatinine, bilirubin, ALP activity, and ALT activity were log transformed prior to the statistical analysis. A p value < 0.05 was considered significant. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for significant variables.

Results

Vaccination study—There were no differences in CRFK %ELISA values between vaccinated cat groups in the pre-inoculation samples (Figure 1). The group by time interaction was significant (p value < 0.001). Significant within time group effects (p value < 0.001) were detected at all post-vaccination time points except for day 185 (p value = 0.102). On all post-vaccination time points except for day 185, the four groups of cats administered FVRCP vaccines SQ had significantly higher CRFK %ELISA values than the cats administered the FVRCP vaccine IN. The three groups of cats that received booster FVRCP vaccines SQ after one year had significantly higher CRFK %ELISA values than the cats administered the FVRCP vaccine IN at all remaining time-points in the study. Over time, all of the cats vaccinated SQ, but only two of the cats vaccinated IN, exceeded the positive cutoff value in the CRFK ELISA (Table 1).

Serosurvey of privately-owned cats—Serum BUN was removed from the statistical model due to collinearity with creatinine. Age, ALT activity, and albumin were removed from the logistic regression model by stepwise regression as unrelated to CRFK %ELISA values. The likelihood of an increased glucose value, creatinine value, or ALP activity decreased with increasing CRFK %ELISA values, while the likelihood of an increased bilirubin increased with increasing CRFK %ELISA. After CRFK results were dichotomized based on the %ELISA cutoff, negative associations remained for increased glucose values (p value = 0.010, OR 0.691, 95% CI 0.521-0.914), increased creatinine values (p value = 0.002, OR 0.675, 95% CI 0.528-0.863), and increased ALP activity (p value < 0.001, OR 0.697, CI 0.589-0.824). Presence of an increased bilirubin value (p value < 0.001, OR 1.392, 95% CI 1.120-1.685) was still positively associated with CRFK %ELISA results after the CRFK results were dichotomized.

Discussion

Development of immune reactions against vaccine contaminants has been documented in humans and dogs.[12-14] For example, thyroglobulin autoantibody production has been documented in dogs after administration of vaccines that were thought to be contaminated with bovine thyroglobulin.[12] In addition, injectable administration of FVRCP vaccines has been associated with development of injection site sarcomas.[15] In humans, vaccination has been associated with Guillain-Barré syndrome, immune-mediated thrombocytopenia and hemolytic anemia, diabetes mellitus, and reactive arthritis,[16-18] though evidence for a causal relationship between vaccination and these diseases is lacking. Vaccine contaminants specifically have been associated with reactions including urticaria, eczema, granuloma formation, and macrophagic myofasciitis.[14,18] Results of these studies indicate that administration of vaccines may not always be 100% safe. Research is ongoing regarding the interplay between genetic factors, environmental triggers, and vaccination with regard to the development of autoimmune diseases. The multifactorial nature and relative rarity of autoimmune diseases complicate development of causality studies; definitive evidence for or against causal links between vaccination and autoimmune diseases remains lacking.

Results of this study document that in general, administration of CRFK lysate-containing FVRCP vaccines SQ induces greater CRFK antibody responses than administration of a CRFK protein-containing FVRCP vaccine administered IN after routine administration of a series of three vaccinations. All four CRFK lysate-containing FVRCP vaccines administered SQ induced greater CRFK antibody responses on days 67 and 81 than the FVRCP vaccine administered IN. The effect was also seen on day 365 and after booster vaccination on days 379, 393, and 421 for the three remaining FVRCP vaccines administered SQ. Results of this study confirm the trends noted in our

pilot study that only assessed two cats per vaccine group.[4] The difference in magnitude of responses between groups is most likely related to the route of administration. It is apparent that SQ administration of inactivated CRFK lysates results in greater uptake by antigen-presenting cells and greater humoral immune responses than when administered IN. It is also possible, but considered less likely, that the differences were because of differences in CRFK concentrations between vaccines.

Because the CRFK cell line was derived from feline tissues[3] and because lymphocytic plasmacytic interstitial nephritis resulting in renal failure is common in cats,[19-20] there is concern that administration of CRFK lysate contaminated vaccines may result in renal disease in some cats.[4] We have identified the immunodominant CRFK antigens as α-enolase, annexin A2, and macrophage capping protein (MCP).[5] Alpha-enolase is a highly conserved glycolytic enzyme that also functions as a membrane-bound plasminogen receptor in a variety of tissues.[6-7] Anti-α-enolase antibodies are known to be nephritogenic in humans. Derangements of both Th1 and Th2 cell function are believed to be involved.[8] In our preliminary study, vaccinated kittens were monitored for changes in serum biochemical panels, urinalyses, and microalbuminuria after vaccination or hypersensitization with CRFK lysates.[4] Kittens also were evaluated for histologic renal inflammation at the start of the study and six weeks after receiving booster inoculations at one year. Consistent abnormalities were not detected in the original study. All of the cats from the CRFK hyperinoculation groups and the intranasal vaccination cats were biopsied two weeks after receiving booster inoculations at two years of age. Neither cat receiving intranasal vaccinations developed nephritis, but three of the CRFK hyperinoculated cats developed histologic evidence of lymphocytic-plasmacytic nephritis following booster inoculation,[21] and one of these cats was later euthanized for kidney failure (Whittemore JW, unpublished data, 2007). This cat had severe lymphocytic-plasmacytic nephritis on necropsy. The presence of inflammation

two weeks after inoculation without inflammation six weeks after biopsy suggests that vaccination may lead to waxing and waning nephritis secondary to anamnestic antibody production. It is unknown whether this inflammation would be sufficient to generate clinically relevant renal dysfunction. The vaccinal group study cats were not available for comparison, so a definitive link between FVRCP vaccination and renal disease was not made.

Anti-α-enolase[6-8] and anti-annexin A2 antibodies[9-10] have also been associated with a wide variety of autoimmune diseases in humans, though the stimulus for development of these autoantibodies in humans remains unclear. Diseases with proven associations to α-enolase antibodies include systemic lupus erythematosus (SLE), autoimmune-mediated retinopathy, autoimmune hepatitis and cholangitis, inflammatory bowel disease, severe asthma, premature ovarian failure, Hashimoto's encephalopathy, and membranous nephropathy.[6-7,22-27] The wide spectrum of diseases associated with anti-α-enolase antibodies in humans limits their usefulness diagnostically, though they are useful prognostically since their presence is correlated with mortality.[15,22] Alpha-enolase autoantibodies in humans are believed to play an active role in the progression of immune-mediated diseases.[15-16] In addition, the portion of α-enolase recognized by autoantibodies may be important in determining their pathogenicity,[24] perhaps depending on whether the recognized sequence is generally accessible to the immune system. Possible mechanisms for antibody-associated tissue damage include immune-complex mediated cellular attack, complement mediated sublytic damage, inhibition of plasminogen binding leading to derangement of the fibrinolytic system, and direct induction of apoptotic processes.[16,19] Anti-annexin A2 antibodies are believed to play a pathogenic role in the development of thrombosis in human patients with primary and SLE-associated antiphospholipid syndrome.[9-10] Anti-annexin A2 IgM antibodies have been also associated with severity of chorioamnionitis in pre-term infants,[28] and anti-

annexin A2 antibodies have been correlated with recurrent fetal loss. A significant association between anti-annexin A2 antibodies and macular degeneration in cynomolgus monkeys, a research model for human macular degeneration, has also been documented.[29] Finally, annexin A2 autoantibody development has been associated with cases of renal, lung, and gastric carcinomas and acute leukemia in humans;[30-34] antibodies have been associated with increased hemorrhage and worsening prognoses. Given these findings, it raises the possibility that FVRCP vaccination may be associated with immune-mediated disease in renal and non-renal tissues through induction of anti-α-enolase and anti-annexin A2 antibodies.

In contrast, MCP is part of a family of actin-binding proteins and is believed to play an important role in macrophage conformational and locomotive activities.[35] As MCP is localized to the cytoplasm and nucleus of cells,[35-37] it remains to be determined whether anti-MCP antibodies have clinical significance in the cat or whether they are merely a marker of CRFK protein exposure. We could find no reports of anti-MCP antibody development in the literature.

There were a number of significant associations between CRFK antibodies and biochemical abnormalities in the serosurvey described here, none of which were detected in our short-term study of vaccinated cats or cats hyperinoculated with CRFK lysates. However, in that study, only small numbers of cats were used, prompting us to study the larger number of presumably vaccinated cats described here. It is possible that the significant associations described here are truly representative of privately-owned cats in general. However, some of the results warrant a discussion of potential sample bias. For example, while it is plausible that there is no association between antibodies against CRFK proteins and disease, it seems unlikely that presence of antibodies against this vaccine contaminant would protect against a significant disease syndrome based on our previous study showing interstitial nephritis in some CRFK

hyperinoculated cats.[21] The vaccination history of the cats in the serosurvey is unknown and so it is possible that selection bias may be playing a role in the results. Owners who bring their cats in for annual examinations and vaccinations may authorize wellness blood work, while owners that do not typically pay for prophylactic care may only bring in their cats when they are manifesting signs of illness. These less attentive owners will have had no opportunity *per se* to authorize wellness or screening blood work. Therefore, the proportion of regularly vaccinated cats in the population with biochemical abnormalities may be lower than that in the population with normal biochemical data. Simply put, the presence of CRFK antibodies may be an indirect marker of increased exposure to preventive health care and screening.

Another factor that may be influencing the results of this part of the study may relate to the CRFK antibody assay used. The CRFK lysates used in the ELISA described herein contain many proteins in addition to the immunodominant antigens α-enolase, annexin A2, and MAP. Thus, many different antibodies are being detected concurrently. If antibodies against some but not all CRFK proteins are associated with disease, the potential to detect associations between CRFK antibodies and biochemical abnormalities by use of this assay may be compromised. To further assess for associations between antibody responses against individual antigens and biochemical abnormalities, the sample set described here could be analyzed using Western blot immunoassay or antibody assays developed specifically against α-enolase, annexin A2, and MCP. In addition, in future studies, it would be optimal to have accurate vaccination histories for all cats.

There are other potential explanations for the results described here that relate to the varying mechanisms and manifestations of autoimmune disease. Persistent exposure to an autoantigen with development of autoantibodies is not, by itself, sufficient to create autoimmune disease.[38-39] In the presence of a persistent autoantigen, the

immune system may respond in one or more ways. First, the immune system may eliminate autoreactive Th0 cells or simply suppress their activity[40] and return to a non-autoimmune state. Second, Th0 cells may differentiate along a Th2 pathway and produce antibody. The production of autoantibody may or may not be associated with the development of disease. Part of this variability relates to the accessibility of the associated autoantigen by the immune system.[39] In some cases, antibody may persist without disease until unrelated or incidental damage leads to antigen exposure, at which time clinical signs or disease may develop.[38-39] Th2 cell-associated tissue damage may result from an antibody-associated lymphocyte attack on affected cells, cellular death secondary to sublytic complement binding, antibody-antigen complex deposition or a combination of these mechanisms. Development of a Th2 response to an autoantigen can even confer protective benefits, as has been demonstrated in experimental models for multiple sclerosis and diabetes mellitus.

Finally, cytokine effects and individual genetics may stimulate differentiation of autoreactive Th0 cells into Th1 cells, with damage to the individual mediated by cytotoxic T cells. Although antibody may still be produced, antibody levels may or may not be clinically relevant. A good example is human thyroiditis where neither the presence nor the severity of disease correlates with antibody levels.[41] In some cases, antibody levels may peak at the time of the onset of clinical signs of disease and then disappear thereafter. This has been demonstrated for humans affected by insulin dependent diabetes mellitus (IDDM).[39] In cases of cytotoxic lymphocytic disease, the disease may progress with intermittent relapsing signs, as constant antigen exposure maintains a hypersensitive state that may be boosted from time to time by further immunization or bursts of increased antigen exposure.

Development of autoimmunity also requires appropriate conditions of exposure, as well as a complex interplay of environmental and genetic factors. Concurrent

administration of adjuvants and infectious agents with self-proteins has been shown to be effective in stimulating the development of autoimmunity in laboratory animals.[38] These particular conditions of exposure are consistent with standard composition of FVRCP vaccines. Oral immunization of antigen has been shown to consistently result in a Th2 response, while injectable inoculation can lead to either Th1 or Th2 responses. Interestingly, a series of experiments has demonstrated that low level exposure to autoantigens or exposure to antigens of low avidity during thymic development may allow escape of high-affinity autoreactive T cells during intrathymic selection, while exposure to high levels of autoantigens inhibits the development of autoreactive Th0 cells.[42-43] Though propagation of autoreactive T cells is not sufficient by itself to cause autoimmune disease, concomitant or subsequent inflammation or infection may 'break' tolerance, leading to autoimmune disease. It has been postulated that development of a high affinity autoreactive T cell population may also stimulate a shift toward a Th1 phenotype, leading to greater destructive potential.[42] The FVRCP vaccine is given throughout normal thymic development, but it is currently unknown whether the concentration of CRFK proteins in FVRCP vaccines and their relative binding capability would fall above or below this stimulatory cutoff.

Individual host factors have been shown to play a large role in the development of autoimmune disease in humans. The association between MHC susceptibility alleles, specifically HLA alleles, and the development of IDDM[42-46] is probably the most compelling example of the importance of genetic factors. Similar associations have been demonstrated for a wide variety of human autoimmune diseases.[46] Genetically mediated aberrant cytokine expression has been associated with development of autoimmune disease in transgenic mice.[47] Similar associations may exist for naturally occurring autoimmune disease. It is likely that genetic factors play a similar role in feline autoimmune disease.

Given the multifactorial nature of autoimmunity, a variety of additional explanations for the negative association between CRFK antibody levels and creatinine, glucose, and ALP activity abnormalities exist. Firstly, CRFK immunodominant antigens may not generally be accessible to the immune system, so that circulating antibodies cannot bind to cause disease. This may be particularly true for anti-MCP antibodies as MCP has a cytosolic distribution. Once antigens become accessible, perhaps due to incidental damage, antibodies may aggressively complex with exposed antigen leading to depressed circulating antibody levels and perpetuating a cycle of tissue damage. In contrast, the positive association between bilirubin abnormalities and CRFK antibodies may suggest that CRFK antigens in the liver are more widely accessible to a more 'traditional' Th2 cell mediated attack.

Another hypothesis for the negative association between CRFK antibody levels and creatinine, glucose, and ALP activity abnormalities is that cats predisposed to mount a Th1 response to CRFK proteins develop cytotoxic lymphocytic disease while cats predisposed to mount primarily a Th2 response are actually protected from clinical disease. The particular proteins and epitopes recognized as well as the avidity of those interactions by individual cats may significantly affect the type of response generated as well as the potential for pathogenicity. As previously noted, which particular α-enolase epitopes are recognized by the immune system is relevant to the development of Hashimoto's encephalopathy in patients with Hashimoto's thyroiditis.[27] In humans, liver disease is associated with relatively low anti-α-enolase antibody titers while high titers are common with systemic diseases.[24] Genetic factors–affecting the avidity of intrathymic CRFK antigen binding, Th1 versus Th2 tendencies, or perhaps cytokine expression–may play a role, limiting the development of disease to a genetic subpopulation of cats. Finally, in humans, an individual's HLA alleles may play a role in

determining whether the subject develops anti-α-enolase antibody associated disease.[6] This may also be true for cats.

Although further work will be necessary to clarify the association between CRFK antibodies and disease, since FVRCP vaccines are not 100% safe, they should be administered no more often than indicated for each individual cat. The American Association of Feline Practitioners currently recommends that FVRCP vaccines be administered no more often than every three years after completion of a series of vaccinations as kittens and an one year booster.[48] Serological and challenge studies suggest that this recommendation is valid for most cats.[11,49-51] In addition, it would be prudent to use the safest and most efficacious vaccine available.

While challenge studies that directly compare the vaccines studied here have not been performed, indirect measurements of immune responses were completed in the kittens vaccinated in this study.[52] Cats administered the IN FVRCP vaccine had equivalent or greater FHV-1 cell mediated immune responses, concanavalin A induced lymphoblast transformation responses, and FHV-1, FCV, and FPV serological responses than cats administered SQ FVRCP vaccines. Since antigen injection may stimulate a Th1 response and results of this study suggest that antibody development may confer protective benefits, use of non-injectable vaccines as part of a tailored vaccination protocol may help ameliorate previously documented vaccinal risks without compromising vaccine-associated protection. Additional studies utilizing monoclonal antibodies for α-enolase, annexin A2, and MCP in general and targeted epitopes in particular will be necessary to further elucidate the potential for CRFK antibody-associated disease.

Footnotes

a. UltraNasal™, Heska Corporation, Loveland, Colo.
b. Fel-O-Vax LVK IV, Fort Dodge Animal Health, Overland Park, KS.
c. Fel-O-Vax PCT, Fort Dodge Animal Health, Overland Park, KS.
d. Felocell 3, Pfizer Animal Health, New York City, NY.
e. PureVax Feline 3, Merial, Athens GA.
f. The MIXED procedure, Statview for Windows v.5.0.1, SAS Institute, Inc., Cary, NC 27513.
g. Antech Diagnostics, Lake Success, New York.

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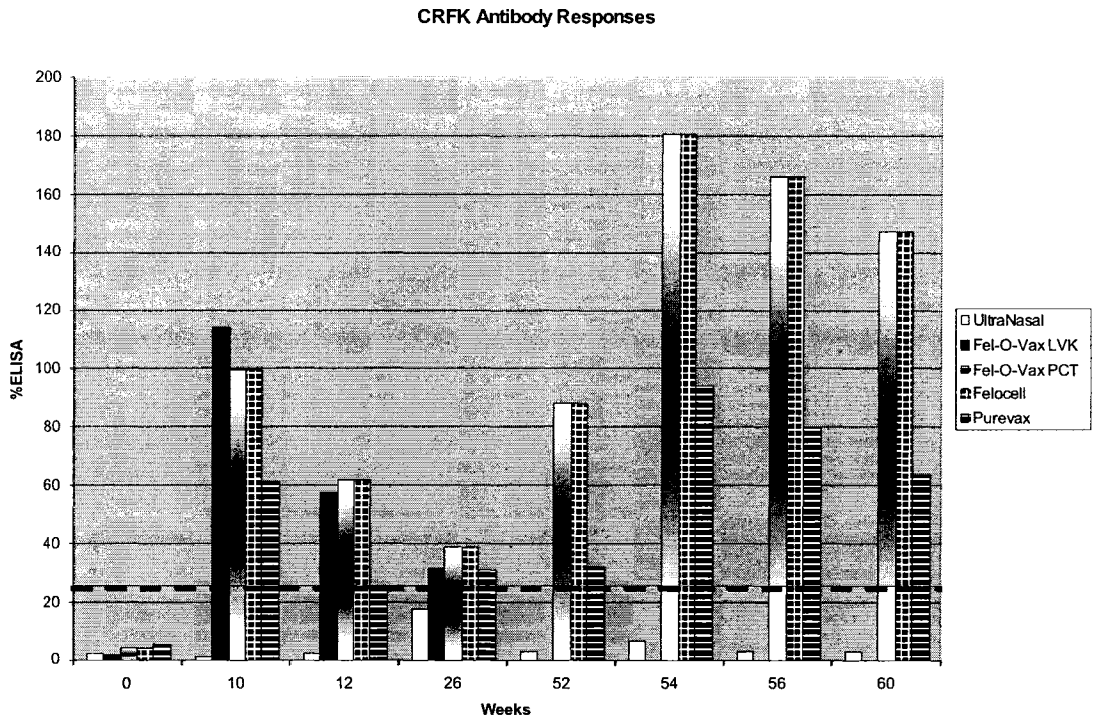

Figure 1—CRFK %ELISA results over time in the vaccination study group. Vaccinations were given on days 0, 28, and 56 of the study to all five groups and on day 365 for four groups. The dashed line is the positive cutoff as determined by the mean and 2 SD of 14, seven to eight month old, unvaccinated cats. The time by group interaction was significant ($P < 0.0001$). The vaccine for intranasal administration (UltraNasal, Heska Corporation, Loveland, Colo.) induced lower CRFK %ELISA results than all four SQ vaccines on all post-vaccination time points except for day 185 days. SQ #1 = Fel-O-Vax LVK IV, Fort Dodge Animal Health, Overland Park, KS; SQ #2 = Fel-O-Vax PCT, Fort Dodge Animal Health; SQ #3 = Felocell 3, Pfizer Animal Health, NYC, NY; SQ #4 = PureVax Feline 3, Merial, Athens, GA.

Table 1—Number of vaccinated cats positive for CRFK antibodies before and after administration of a FVRCP vaccine on days 0, 28, and 56 of the study to all five groups and on days 365, 379, 393, and 421 for four groups.

	Day 0	**Day 67**	**Day 81**	**Day 185**	**Day 365**	**Day 379**	**Day 393**	**Day 421**
Ultranasal	0	0	1	2	0°	1	0	0
Fel-O-Vax LVK	0	10	10	9	*	*	*	*
Felo-O-Vax PCT	0	10	10	10	10	10	10	10
Felocell 3	0	10	6	7	6	10	9	9
Purevax	0	10	9	4	7	10	10	9

There were 10 cats in each of the vaccine groups. The positive cutoff value (22 %ELISA) was determined by calculating the mean plus 2 standard deviation results of 14 unvaccinated, 7-8 month old kittens. The numbers listed are the numbers of cats in the group that exceeded the positive cutoff value on that sample date. ° = 1 sample was not available for this time point from this group. * = cats not available for the remainder of the study. See Figure 1 legend for complete vaccine information.

CONCLUSIONS

Our research documents that microalbuminuria is associated with underlying systemic disease in dogs without overt proteinuria. Dogs positive by the quantitative microalbuminuria assay are 2.3 times as likely to have systemic disease as dogs without microalbuminuria and are 2.7 times as likely to have neoplasia. Quantitative microalbuminuria assay results are also significantly associated with age, BUN, and hematuria. Dogs with positive semiquantitative microalbuminuria tests are 4.8 times as likely to have systemic disease as dogs without microalbuminuria and are 8.1 times as likely to have neoplasia. Semiquantitative microalbuminuria assay results are also significantly associated with age, BUN, and creatinine. Gender is not significantly associated with results of either microalbuminuria assay. In dogs without overt proteinuria, the semiquantitative microalbuminuria assay has the best combination of sensitivity and specificity for underlying disease. The urine albumin:creatinine ratio has very poor sensitivity for systemic disease in dogs without overt proteinuria and therefore is unlikely to be useful as a biomarker of systemic disease.

In cats, positive microalbuminuria results are also significantly associated with systemic disease. Cats with positive quantitative microalbuminuria results are 6.7 times as like to have underlying disease as cats without microalbuminuria. Quantitative microalbuminuria results are also significantly associated with BUN, pyuria, and hematuria. Cats with positive semiquantitative microalbuminuria assay results are 2.4 times as likely to have underlying disease. Positive semiquantitative microalbuminuria results are also significantly associated with serum creatinine, pyuria, and hematuria.

Although semiquantitative microalbuminuria is associated with age, this association is unlikely to be of clinical significance since the Odds Ratio is only 1.078. Gender is not significantly associated with results of either microalbuminuria assay. Results of neither microalbuminuria assay are associated with the presence of neoplasia but results of both are associated with having a disease diagnosis of renal or urinary disease. There were not enough cats with blood pressure measurements to evaluate for an association. In cats, the quantitative microalbuminuria assay has the best combination of sensitivity and specificity for underlying disease. As seen in the dog, the urine albumin:creatinine ratio has very poor sensitivity for systemic disease in cats and therefore is unlikely to be useful as a biomarker of systemic disease.

Prospective studies evaluating the predictive value of commonly performed screening tests–complete blood count, serum biochemical profile, urinalysis, T4, and systolic blood pressure measurement–without and with microalbuminuria results will be necessary to determine whether performance of microalbuminuria assays increases the diagnosis of underlying disease in dogs and cats.

Our results documents that CRFK protein inoculation is associated with autoantibody development measurable by Western blot immunoassay. In addition, we have shown that cats receiving commercially available FVRCP vaccines according to a standard vaccination schedule develop antibodies measurable using the validated Western blot immunoassay. Antibody development can occur as early as four weeks after vaccination. The number of antibody bands and density of those bands is greater after one year booster inoculation. Antibody bands develop against antigens that are 47 kD, 40 kD and 38 kD in size.

We have identified the immunodominant CRFK antigens to be α-enolase, annexin A2, and macrophage capping protein. Alpha-enolase is a highly conserved glycolytic enzyme approximately 47 kD in size found in many tissues but with highest

concentrations present in the kidney and thymus. It also is categorized as a shock protein because its expression is upregulated in some tissues, including the kidney, in response to hypoxia. Finally, α-enolase is found as a transmembrane protein in a wide variety of tissues including the kidney, where it functions as plasminogen receptor and contributes to fibrinolytic homeostasis. In humans, antibodies against α-enolase have been associated with a wide variety of systemic and localized autoimmune diseases, including membranous nephropathy, mixed cryoglobulinemia, systemic lupus erythematosus (SLE), Hashimoto's encephalopathy, autoimmune hepatitis and cholangitis, and severe asthma. Anti-α-enolase antibodies are recognized as directly nephritogenic in humans though the pathologic mechanisms involved remain undefined. In addition, anti-α-enolase antibodies are negatively correlated with survival and may be used prognostically. Differences in epitope recognition patterns, the avidity of antibody binding, and Th1 versus Th2 derangements all affect the type and severity of disease manifestations.

Results of our studies document a wide tissue distribution for α-enolase in cats, consistent with the human literature. The authors could find no previous reports in the literature of the tissue distribution of α-enolase in the cat. This is also the first report to the authors' knowledge of anti-α-enolase antibodies in cats.

Annexin A2 is a calcium-binding protein approximately 38 kD in size with both intracellular and membrane-bound distributions. It is important for a wide variety of cellular functions including vesicle movement, cellular proliferation, differentiation and apoptosis, and plasminogen activation. It is highly conserved between species and has a wide tissue distribution in people. Both the intracellular and membrane-bound forms are present in renal epithelial tubular cells in humans, and annexin A2 may play an important role in renal tubule repair.

Anti-annexin A2 antibodies have been associated with autoimmune disorders in other species. They are believed to play a pathogenic role in the development of thrombosis in human patients with primary and SLE-associated antiphospholipid syndrome. Anti-annexin A2 antibodies have been also associated with severity of chorioamnionitis in pre-term infants and with recurrent fetal loss. A significant association between anti-annexin A2 antibodies and macular degeneration in cynomolgus monkeys, a research model for human macular degeneration, has also been documented. Finally, in humans annexin A2 autoantibody development has been associated with cases of renal, lung, and gastric carcinomas and acute leukemia; antibodies have been associated with increased hemorrhage and worsening prognoses. This is the first report to the authors' knowledge of anti-annexin A2 antibodies in cats.

Macrophage capping protein (MCP) is also a highly conserved protein. It is classified as an actin binding protein and plays an important role in regulating macrophage locomotion and conformational changes. Macrophage capping protein is primarily confined to the cytoplasm with limited expression in the nucleus of cells. It is present in greatest concentrations in monocytes; human studies conflict regarding its extra-monocytic tissue distribution though reports agree regarding a high concentration in the kidney. Results of our studies document a wide tissue distribution for MCP. Although monocytic contamination of the tissues cannot be ruled out, we consider it unlikely given the normal gross appearance of tissues and the number of individual cats utilized to generated the pooled tissue lysates.

Macrophage capping protein has been reported at sizes ranging from 38 to 45 kD, though most reports categorize it as 38-41 kD in size. Differences in reported sizes may related to differences in experimental techniques, though the presence of two distinct sizes in one report suggests that differences in post-translation modifications may also contribute to the varying size reports. The authors could find no reports in the

human or animal literature of anti-MCP antibody development or association with immune-mediated disease. Given MCP's strictly intracellular distribution, the authors consider it likely that anti-MCP antibodies are a marker of CRFK protein inoculation and less likely that they have pathogenic potential.

Results of our studies conflict regarding the development of anti-CRFK antibodies after intranasal vaccination. Antibody bands were present in the sera of intranasally vaccinated cats by Western blot immunoassay but previously published CRFK %ELISAs for the same samples were low. This discordance is not particularly surprising, since Western blot immunoassays are typically more sensitive than ELISAs for antibody presence. In the previously published study, antibody levels in sera were quantified by ELISA and categorized based on a %ELISA cutoff value. Antibody presence below the cutoff would not equate to a lack of antibody, merely a %ELISA value below the cutoff. When evaluated together, the results of the two studies suggest that CRFK protein presentation by antigen-presenting cells to the immune system occurs with intranasal administration but that the magnitude of the immune system's response to these proteins is much lower than when CRFK proteins are administered by injection.

Our studies showed significant associations between anti-CRFK antibody levels and biochemical abnormalities. There was a significant positive correlation between CRFK %ELISA values and serum bilirubin levels. There were also significant negative associations between CRFK %ELISA values and serum creatinine, ALP activity, and glucose. Because polyclonal antibodies were utilized in this experiment, we cannot parse out the effect of anti-α-enolase, anti-annexin A2, and anti-MCP antibodies. The positive association between bilirubin and anti-CRFK antibodies is not unexpected given human associations between anti-α-enolase antibodies and autoimmune hepatitis and cholangitis. The negative associations between anti-CRFK antibodies and creatinine, ALP activity, and glucose suggest that development of Th2 response to CRFK proteins

confers protective benefit, though other potential explanations cannot be ruled out at this time.

Given the known associations between anti-α-enolase and anti-annexin A2 antibodies with immune-mediated disease in humans as well as results of our studies to date, limiting feline exposure to CRFK proteins is prudent. Elimination of FVRCP vaccines from prophylactic care is not reasonable or wise, and the authors are not aware of any commercial FVRCP vaccines free of CRFK protein contamination. Therefore, to limit CRFK protein inoculation, we would recommend limiting FVRCP vaccination to the minimum frequency necessary given an individual cat's risk factors and published vaccination guidelines. In addition, administering intranasal vaccines instead of injectable vaccines may decrease total CRFK protein administration, decrease anti-CRFK antibody development, and steer any autoimmune response toward a Th2 response that may confer protective benefits over a Th1 response.

Given the breadth of human data on autoimmune disease associated with anti-α-enolase antibodies, the high prevalence of anti-CRFK antibodies in vaccinated cats, and the recent increase in a variety of immune-mediated diseases in cats, future research in this area is warranted. Immunohistochemical analysis of normal and affected feline tissues using anti-α-enolase, anti-annexin A2, and anti-MCP monoclonal antibodies will help to separate the potential roles antibodies against these three proteins may have in various feline immune-mediated diseases. Focused serologic studies utilizing monoclonal antibodies may also help elucidate the potential associations between anti-CRFK antibodies and disease. Finally, analyses of Th1 and Th2 cell function in affected cats may help better determine the potential etiologies of a variety of diseases. Research in all these areas may also provide valuable insight into novel therapeutic and preventative options in cats.