Serum concentrations of the third component of complement in healthy dogs and dogs with protein-losing nephropathy

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Objective—To develop a method for determining the concentration of the third component of complement (C3) in canine serum, to establish a reference range for C3 in healthy dogs, and to evaluate dogs with protein-losing nephropathy (PLN) to determine whether PLN is associated with decreased serum C3 concentrations.

Animals—30 healthy dogs and 49 dogs with PLN.

Procedures—Serum samples were obtained from healthy dogs at the time of examination, whereas serum samples were obtained from dogs with PLN at the time of diagnosis. All samples were frozen at –70°C until analyzed. Serum C3 concentrations were determined by use of a sandwich ELISA. Concentrations were expressed as the number of dilutions in which C3 could be detected.

Results—C3 was detectable in healthy control dogs (range, 1,920,000 to 15,400,000 dilutions; median, 9,600,000 dilutions). This represented a range of four 2-fold serum dilutions. In addition, C3 was detectable in dogs with PLN (range, 1,460,000 to 30,070,000 dilutions; median, 7,680,000 dilutions), which represented a range of six 2-fold serum dilutions. There was no significant difference in C3 concentrations between the 2 groups.

Conclusions and Clinical Relevance—C3 is a critical part of the immune defense system that has not been extensively examined in veterinary medicine. An ELISA was developed for measuring C3 concentrations, and a reference range for healthy dogs was established. Significant decreases in C3 concentrations were not detected in any dog with PLN. Additional studies will be required to definitively determine the importance of serum C3 concentrations in PLN. (*Am J Vet Res* 2006;67:1105–1109)

The complement cascade is a fundamental part of the host immune system that comprises > 20 serum proteins, many of which are proenzymes.[1] A functioning complement system is essential for immune function because it can eliminate invading microorganisms via lysis, opsonization, agglutination, chemotaxis, modulation of antibody production, and stimulation of local inflammatory responses.[1–3] Dogs with deficiencies in C3 are predisposed to pneumonia, sepsis, pyometra, and renal amyloidosis.[4]

Abbreviations

C3	Third component of complement
PLN	Protein-losing nephropathy

The complement cascade can be activated by 3 mechanisms. In the classical pathway, the formation of antigen-antibody complexes exposes a reactive site on the antibody that directly activates complement.[1,3] The alternative and lectin pathways rely on products of microbial cell membranes to attract and then activate initiating proteins in the complement cascade.[3] Regardless of which of these 3 pathways is initiated, the end result is the activation of C3 and the common terminal pathway.[3] The C3 is the most abundant complement protein and is generally considered to play an essential role in all complement cascade activities.[2,4] Once the complement pathway is initiated, each subsequent step is amplified, and a relatively small stimulation can result in a substantial, immunologically important response.[1]

Protein-losing nephropathy is a common renal syndrome characterized by the loss of serum proteins through the glomeruli.[5,6] It is generally accepted that glomerulonephritis and amyloidosis are responsible for virtually all PLNs in dogs.[7,8] Differentiating between these disease processes requires microscopic examination of specially stained renal tissues, although glomerulonephritis is more common.[8] In amyloidosis, chronic stimulation of macrophages leads to production of interleukin-1, interleukin-6, and tumor necrosis factor with resulting hepatic production of amyloid precursors that have a β-pleated sheet configuration.[7] These precursors polymerize in the blood and then become trapped in the glomerular mesh. The β-pleated sheet configuration prevents enzymatic degradation.[9] In glomerulonephritis, antigen-antibody complexes stimulate an immune response that damages the glomeruli. These complexes, which are trapped in the glomerular basement membrane, may form via antigen-antibody complexes within the circulation, or they may form within the kidney as a result of foreign antigens that have been deposited within the glomeruli.[10] Both of these are believed to be secondary to underlying disease processes that provide chronic antigenic

Received October 21, 2005.
Accepted January 16, 2006.
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Presented in part at the American College of Veterinary Internal Medicine Annual Meeting, Minneapolis, June 2004.
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stimulation. Such processes include infectious diseases, inflammatory reactions, neoplasia, and adverse drug reactions. In addition, genetic predispositions have been reported.[11–14]

After extensive testing to determine underlying causes, most cases of PLN in dogs are eventually classified as idiopathic.[5] In humans, the routine use of light microscopy, electron microscopy, and immunohistochemical staining combined with large amounts of clinical data has allowed for substantial refinement of the types of glomerular diseases and their underlying causes.[7,15,16] This degree of sophistication is lacking in veterinary medicine in which biopsy specimens are much less commonly obtained and electron microscopy and immunohistochemical staining are rarely performed.[17] This likely explains the large number of idiopathic cases of PLN in veterinary medicine.

In humans, complement concentrations are often combined with histologic evaluations to differentiate among potential underlying causes of glomerulonephritis.[15,16,18] Although reductions in C3 concentrations have been associated with the acute phase of systemic lupus erythematosus, acute poststreptococcal exposure, and membranoproliferative glomerulonephritis types I and II, other causes of PLN in humans are not generally associated with changes in complement concentrations.[15,16,18–20] For example, a young person who has acute renal failure, proteinuria, and a reduction in C3 concentrations would be evaluated further for evidence of a hemolytic streptococcal infection, a common cause of PLN and decreased C3 concentrations in children.[18,19] Such complement screening tests are routinely used and assist physicians in narrowing the list of differential diagnoses, developing a diagnostic plan, and understanding the clinical course of the disease.[16,18] The reduction in serum C3 concentrations in the acute phase of these disease conditions is associated with activation of the classical pathway by immune complexes and consumption of C3 proceeding at a rate greater than the rate of hepatic production.[21–23] Serum C3 concentrations in affected patients are often < 20% of the concentration for clinically normal humans.[20,24]

Despite the essential role that complement plays in the host defense mechanism, the ability to quantify specific components of complement in canine serum is not readily available. The authors are aware of only 1 study[5] in which complement concentrations were evaluated in dogs with PLN. In that study, investigators measured C3 concentrations in 8 dogs with glomerulonephritis. Although the investigators found no reduction in C3 concentrations in those 8 dogs, the method used to measure C3 concentrations and the source of the reference range was not explained.[5] Gel diffusion experiments conducted by personnel in our laboratory group have revealed weak and inconsistent interactions between purified human C3 and antibodies specific for canine C3. This suggests that for an ELISA or other antibody-dependent quantification methods to be used for C3 detection, species-specific antibodies are essential.

The goal of the study reported here was to develop an assay to measure C3 concentrations in dogs and establish a reference range. To our knowledge, there are no established reference ranges for serum concentrations of C3 in dogs, and a standard with a known canine C3 concentration is not available.[25] In addition, we evaluated C3 concentrations in dogs with PLN to determine whether PLN in dogs is commonly associated with decreases in C3 concentrations.

Materials and Methods

Animals—Healthy dogs of assorted breeds and ages were identified from animals at the Foster Hospital for Small Animals at Tufts Cummings School of Veterinary Medicine during routine examinations. In addition, dogs of faculty and staff were also used in the study. Signed owner consent was obtained for each dog.

Dogs with a known history of antigenic stimulation, such as vaccination or infection, during the preceding 4 months were excluded from the study. Additionally, each dog was minimally restrained, and 4 mL of blood was collected via jugular or cephalic venipuncture. A urine sample was collected by use of bladder catheterization, cystocentesis, or midstream catch during natural voiding. Samples were immediately submitted to a veterinary diagnostic laboratory for urinalysis, serum biochemical analysis, and a CBC. In addition, all dogs were screened for serologic evidence of exposure to *Borrelia burgdorferi*, *Dirofilaria immitis*, and *Ehrlichia canis*.[a] Remaining blood was allowed to clot at 21°C. Samples were then centrifuged, split into aliquots, and stored at –70°C for subsequent analysis. Dogs with abnormalities detected during physical examination or on the basis of a CBC, serum biochemical analysis, urine analysis, or screening for infectious diseases were removed from the study, and their owners were informed of the abnormality.

On the basis of the aforementioned criteria, 30 healthy dogs of 10 breeds comprised the control group. This group consisted of 9 German Shepherd Dogs, 7 Labrador Retrievers, 5 mixed-breed dogs, 2 Newfoundlands, 1 Golden Retriever, 1 Briard, 1 Rottweiler, 1 Siberian Husky, 1 Akita, 1 American Pit Bull Terrier, and 1 Pomeranian. Dogs ranged from 2 to 12 years of age (mean, 5.6 years) and were selected to develop a reference range.

Dogs examined at the Foster Hospital for Small Animals at Tufts Cummings School of Veterinary Medicine between July 2002 and July 2004 in which PLN was diagnosed were included in the study. These dogs were part of a larger study on PLN in dogs. For inclusion, a dog had to have proteinuria without bacteria, debris, WBCs, or blood contamination of the urine.[17,26] Proteinuria was defined as a urine protein-to-urine creatinine ratio > 1.0.[27] Any dog in which a specific underlying cause for the proteinuria was determined was excluded from the study. Routine diagnostic testing included abdominal ultrasonography and serologic screening to detect evidence of exposure to *D immitis*, *B burgdorferi*, and *Ehrlichia* spp. Dogs that had positive results for exposure to *B burgdorferi* when tested by use of western blots, an ELISA conducted at a diagnostic laboratory, or in-office test kits were not excluded from the study.

On the basis of the aforementioned criteria, 49 dogs of 10 breeds comprised the PLN group. This group consisted of 20 Labrador Retrievers, 11 Golden Retrievers, 4 mixed-breed dogs, 2 Border Collies, 1 Lhasa Apso, 1 Siberian Husky, 1 Doberman Pincher, 1 Cairn Terrier, 1 Cocker Spaniel, 1 German Shepherd Dog, 1 Saint Bernard, 1 Dalmatian, 1 Airedale, 1 Brittany, 1 Great Dane, and 1 Vizsla. Dogs ranged from 6 months to 10 years of age (mean, 5.6 years). Thirty-nine of these dogs had positive results for serologic testing to detect exposure to *B burgdorferi*. When a potential candidate was identified, written informed owner consent was

obtained, and a blood sample was collected, processed, and stored as previously described. Renal tissues from 10 dogs with PLN were available and examined by a veterinary pathologist.

Measurement of C3 concentrations—Serum concentrations of C3 were determined by use of ELISA techniques,[25,28] with modifications. Microtiter plates[b] were coated (50 µL/well) with affinity-purified goat anti-canine C3[c] diluted 1:1,000 in PBS solution. Plates were incubated for 3 hours at 37°C. Wells were then emptied, and uncoated protein binding spots were blocked by the addition of 200 µL of 5% nonfat dry milk[d] in PBS solution with 0.1M NaN_3. Plates were again incubated for 3 hours at 37°C. Plates were then washed with buffered washing solution (10mM Tris, 0.15M NaCl, and 0.1% Tween 20 [pH, 7.2]). One hundred microliters of a 1:25,000 dilution of each serum sample was applied in duplicate to each of 2 wells. Serial 1:2 dilutions were added to wells across the plates that contained blocking solution. Serum was added to all rows of wells, except 2 rows that were used as control samples, to which only blocking solution was added. Those 2 rows were used to control for nonspecific background absorbance.

Plates were incubated for 3 hours at 37°C and then washed with buffered washing solution. After addition (50 µL/well) of a 1:100 dilution of rabbit anti-canine C3 antiserum,[e] plates were incubated for another 3 hours at 37°C and then washed with buffered washing solution. Alkaline phosphatase–labeled goat anti-rabbit IgG[f] (1:1,000 dilution) was added to each well, and plates were again incubated for 3 hours at 37°C. After incubation, plates were washed, and a solution (1 mg/mL) of *p*-nitrophenyl phosphate[g] in carbonate substrate buffer was added to each well (50 µL/well).

Absorbance was measured at 450 nm. The C3 titer (maximum dilution at which the ELISA could detect C3) for each dog was determined from the lowest dilution with an absorbance 2 or more times greater than the mean background absorbance for the plates. Median value for duplicates of each sample was recorded.

Statistical analysis—On the basis of results for the Kolmogorov-Smirnov test for normality, it was determined that the number of dilutions at which serum concentrations of C3 could be detected was not normally distributed in the control or PLN groups. Raw C3 data were adjusted by use of logarithmic transformation. Adjusted serum C3 titers for the control and PLN groups were compared by use of a Student *t* test. Ages of the healthy dogs and dogs with PLN were found to be normally distributed (Kolmogorov-Smirnov test) and were compared by use of a Student *t* test. Statistical analysis was performed by use of a commercially available statistical program.[h]

Results

Mean ± SD age of dogs in the control group was 5.5 ± 3.1 years, whereas mean age of dogs with PLN was 5.7 ± 2.0 years. Age did not differ significantly ($P = 0.91$) between groups.

We were able to detect C3 in serum obtained from healthy control dogs (range, 1,920,000 to 15,400,000 dilutions; median, 9,600,000 dilutions). This represented a range of four 2-fold serum dilutions. We were also able to detect C3 in serum obtained from dogs with PLN (range, 1,460,000 to 30,070,000 dilutions; median, 7,680,000 dilutions). This represented a range of six 2-fold serum dilutions (**Figure 1**). There was not a significant ($P = 0.313$) difference in C3 concentrations between the control dogs and dogs with PLN.

Renal tissues from 10 dogs with PLN were available for microscopic examination. Membranoproliferative glomerulonephritis was the veterinary pathologist's diagnosis for all tissue samples.

Discussion

In the study reported here, we developed a technique for measuring C3 concentrations, established a reference range, and then examined samples obtained from dogs with evidence of PLN to determine whether they had abnormal serum concentrations of C3. The C3 is the most abundant protein in the complement cascade and is recognized as having a central role in complement function. In humans, C3 concentrations are typically low during the acute stage of specific types of glomerulonephritis. In addition, a congenital deficiency in C3 has been associated with the development of PLN in dogs.[4] Despite this, a significant difference in C3 concentrations was not detected between the healthy dogs and dogs with PLN.

None of the dogs had a reduction in C3 concentrations that would be similar to that for humans with PLN secondary to acute lupus, poststreptococcal exposure, or membranoproliferative glomerulonephritis types I and II. The decrease in C3 concentrations in membranoproliferative glomerulonephritis types I and II develops despite differences in pathogenesis for the 2 conditions. Type I glomerulonephritis is characterized by deposition of immune complexes in the mesangium and subendothelial space, whereas type II is a complement-based autoimmune disorder that affects primarily children and young adults.

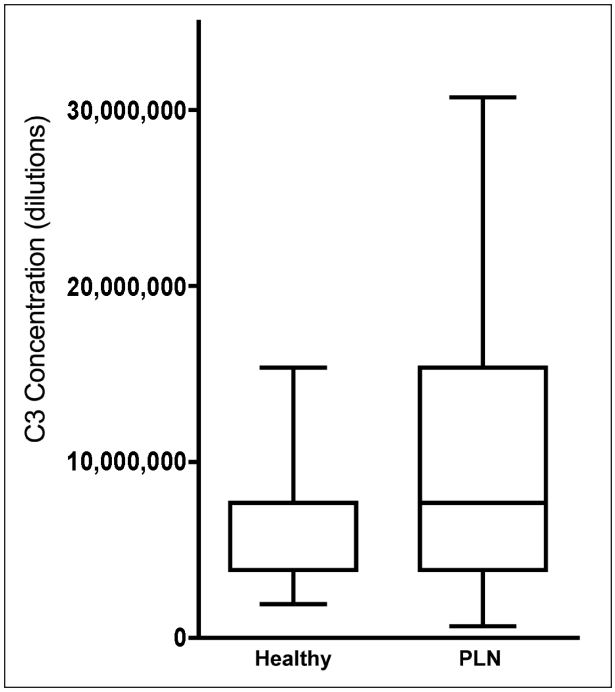

Figure 1—Box-and-whisker plots of serum dilutions at which C3 could be detected in control dogs and dogs with PLN. The boxes represent values from the first to the third quartiles, whereas whiskers extend from the lowest to the highest values. The horizontal line in the box for the PLN dogs represents the median value. The median value for the healthy dogs equals the value for the third quartile and is therefore not visible.

Protein-losing nephropathy is not a disease; rather, it is a syndrome caused by 2 conditions (ie, glomerulonephritis and amyloidosis). Furthermore, in veterinary medicine, glomerulonephritis has historically been used as a nonspecific term to describe a number of antigen-antibody diseases that affect the glomeruli. This aggregation has hindered the understanding of the mechanisms that can lead to various forms of glomerulonephritis as well as possible treatments for those conditions. To better define the role of C3 in glomerular disease would require a larger study with specific inclusion criteria that require obtaining a renal biopsy specimen. By use of a combination of light microscopy, electron microscopy, and immunohistochemical staining, the type and stage of renal disease could be specifically determined. In this context, the role of C3 in glomerular disease could be better understood. However, the primary focus of the study reported here was to develop an assay to measure concentrations of C3 in canine serum and establish a reference range. Therefore, renal biopsy specimens were not a requirement for inclusion in the study.

One problem encountered while developing the technique was the lack of a positive control sample. At the time this study was conducted, there was no source for standardized, purified canine C3. In our experience, use of gel diffusion revealed poor reactivity between antibodies against canine C3 and purified human C3. Therefore, we did not believe it was appropriate to use an ELISA based on human antibodies to detect canine C3. In addition, human C3, which is commercially available, could not be used as a surrogate control sample in this study. Nevertheless, results provided by the ELISA developed for this study proved to be repeatable. In addition, a study[28] in which investigators used various methods to quantify C3 in dogs was able to detect C3 at dilutions virtually identical to those for our control dogs. Thus, our reference range is in agreement with that reported in another study[28] of dogs.

The role of *B burgdorferi* as a causative agent of PLN is controversial. We are aware of only a single study[29] in which investigators have evaluated this relationship. In that study, investigators found a putative link between infection with *B burgdorferi* and development of a specific glomerular lesion. Within the general canine population, a substantial number of dogs have positive results when serologic tests are conducted to detect exposure to *B burgdorferi*; however, these dogs do not have clinical signs of PLN.[30–32] Dogs experimentally infected with *B burgdorferi* can develop orthopedic-related signs; however, they do not develop renal disease.[33] This apparent incongruity could be attributable to differences in the virulence of *B burgdorferi* found in various geographic areas, differences in host responses for specific breeds of dogs, the amount of time required for glomerular lesions to develop, or coinfection with another unidentified infectious agent. The large number of dogs in the study reported here that had positive results for serologic tests to detect exposure to *B burgdorferi* could have been attributable to the endemic nature of borreliosis in the New England area. Alternatively, it could have been the cause of the glomerular disease in these dogs. Nevertheless, none of the dogs in the study had significant reductions in C3 concentrations, regardless of their exposure to *B burgdorferi*.

The complement cascade is a critical and perhaps overlooked component of the immune system in dogs. When functioning correctly, it can help eliminate invading microbes through lysis, opsonization, agglutination, chemotaxis, modulation of antibody production, and stimulation of local inflammatory responses. Therefore, complement plays a critical role in a wide range of infectious and inflammatory conditions. Despite this, measuring the concentration of C3 in canine serum has rarely been attempted. In the study reported here, we developed a method for measuring the concentration of C3 in canine serum and established a reference range. Concentrations of C3 in dogs with PLN did not differ significantly from concentrations detected in the control group. However, to fully evaluate the role of C3 in PLN would require serial measurements of C3 concentrations in a larger number of dogs from which renal biopsy specimens could be obtained and examined by use of various histologic techniques.

a. Canine SNAP 3Dx test, IDEXX Laboratories, Westbrook, Me.
b. Costar 9018, Corning Costar Corp, Acton, Mass.
c. A40-109A, Bethyl Labratories, Montgomery, Tex.
d. Carnation instant nonfat dry milk, Nestlé USA, Wilkes-Barre, Pa.
e. A40-107, Bethyl Labratories, Montgomery, Tex.
f. A-0418, Sigma-Aldrich, St Louis, Mo.
g. N1891, Sigma-Aldrich, St Louis, Mo.
h. GraphPad Prism, version 4.0 for Macintosh, GraphPad Software Inc, San Diego, Calif.

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