Perinuclear antineutrophil cytoplasmic autoantibodies in dogs infected with various vector-borne pathogens and in dogs with immune-mediated hemolytic anemia

Anna E. Karagianni, DVM, MRes; Laia Solano-Gallego, DVM, PhD; Edward B. Breitschwerdt, DVM; Frédéric P. Gaschen, Dr med vet, Dr habil; Michael J. Day, BVMS, PhD, DSc; Michele Trotta, DVM, PhD; Barbara Wieland, Dr med vet, PhD; Karin Allenspach, Dr med vet, PhD

Objective—To determine the prevalence of perinuclear antineutrophil cytoplasmic autoantibodies (pANCA) in dogs with confirmed or suspected immune-mediated hemolytic anemia (IMHA) or dogs infected with various vector-borne pathogens, including Rickettsia rickettsii, Bartonella henselae, Bartonella vinsonii subsp berkholffii, Ehrlichia canis, Borrelia burgdorferi, and Leishmania infantum.

Animals—55 dogs with confirmed or suspected IMHA, 140 dogs seroreactive for vector-borne pathogens, and 62 healthy dogs and dogs seronegative for vector-borne pathogens.

Procedures—Samples were allocated to subgroups on the basis of the health status of the dogs and the degree of seroreactivity against various vector-borne pathogens. Serum samples were tested retrospectively via indirect immunofluorescence assay to determine pANCA status.

Results—26 of 55 (47%) dogs with confirmed or suspected IMHA and 67 of 140 (48%) dogs seroreactive for vector-borne pathogens had positive results when tested for pANCA. Serum samples with the highest antibody concentrations against L infantum antigen had the highest proportion (28/43 [65%]) that were positive for pANCA. One of 20 (5%) dogs seronegative for tick-borne pathogens and 8 of 22 (36%) dogs seronegative for L infantum had positive results for pANCA. One of 20 (5%) healthy dogs had serum antibodies against pANCA.

Conclusions and Clinical Relevance—pANCA were detected in a high percentage of dogs with IMHA and vector-borne infectious diseases. Therefore, pANCA may be a relatively nonspecific marker for dogs with inflammatory bowel disease, although they could represent a biomarker for immune-mediated diseases and infections. (Am J Vet Res 2012;73:1403–1409)
anol-fixed neutrophils is considered the most accurate assay for the detection of ANCA in dogs and humans with IBD.13–15

Only a few studies9,11,13,14 have been conducted to investigate the role of pANCA in diseases of dogs. Investigators in these studies have focused primarily on chronic enteropathies with or without protein-losing nephropathy. Similar to results for human patients, the presence of pANCA in the serum of dogs is associated with IBD.13–15 It has also been suggested that pANCA are associated with food-responsive disease in dogs and could become a useful noninvasive clinical marker for the diagnosis of chronic enteropathy in this subgroup of dogs.11

Various other immune-mediated diseases in humans, including a spectrum of inflammatory and infectious diseases, may be associated with positive results for pANCA testing.16 For example, pANCA have been identified in human patients with IMHA that developed concurrently with other autoimmune diseases.16–18 Although pANCA have been reported in humans with various infectious diseases, only a limited number of studies19–21 have been conducted to address pANCA status in human patients with specific infections, such as infections with Mycobacterium spp. In case reports,22–24 human patients with toxocariasis, visceral leishmaniasis, and endocarditis caused by Bartonella quintana and Bartonella henselae had positive results when tested for pANCA. There are limited data regarding the prevalence of pANCA in dogs with infectious diseases. In 1 study,14 investigators determined that the specificity of pANCA in dogs with IBD was only 76%, compared with the specificity for dogs with other inflammatory and infectious disorders. However, the dogs in that study14 were not infected with a specific pathogen. Infectious disorders caused by the vector-borne pathogens B henselae, Bartonella vinsonii subsp berkhoffii, Ehrlichia canis, and Leishmania infantum are associated with an immune-mediated pathogenesis.25–27 Investigators in a previous study25 found that dog sera reactive for B vinsonii subsp berkhoffii, L infantum, and E canis were likely to have positive results for ANA. Hence, pANCA might have a similar pattern. Thus, determining pANCA status in certain dog populations with defined exposure to various infectious organisms (such as vector-borne diseases), and in association with specific autoimmune diseases (such as IMHA), would provide clinically important diagnostic information regarding the usefulness of pANCA as a marker of disease for IBD as well as for infectious and immune-mediated inflammatory disease in dogs. Therefore, the objective of the study reported here was to investigate the prevalence of pANCA in dogs with IMHA and various vector-borne infectious diseases.

Materials and Methods

Dogs—To evaluate the diagnostic accuracy of pANCA for IMHA and vector-borne infectious diseases, serum samples were obtained from dogs in which IMHA was diagnosed or suspected clinically, dogs in which vector-borne infections were confirmed or suspected, and control dogs. Owner consent was not necessary because the samples were collected for diagnostic reasons in each case and the rest of the volume was stored and used retrospectively in the present study. The study was approved by the Royal Veterinary College’s Ethics Committee.

Dogs in which IMHA was diagnosed or suspected clinically

Serum samples17 from 16 dogs with primary IMHA, as determined after examination at the University of London Royal Veterinary College Queen Mother Hospital, were used in the study. These dogs were all females, except for 1 male, and 13 of 16 were neutered. Mean ± SD age was 8.1 ± 2.66 years (range, 2.11 to 13.40 years). The diagnosis of IMHA required that the dogs were anemic (PCV < 37%), had positive results for a saline (0.9% NaCl) solution agglutination test, and had positive results for a direct Coombs’ test or moderate to marked spherocytosis on a peripheral blood smear.28

Thirty-nine dogs were studied clinically of having IMHA by the attending veterinarian. Serum samples from these dogs were obtained from the University of Bristol Clinical Immunology Diagnostic Laboratory at the School of Veterinary Sciences. Mean ± SD age of 36 dogs was 6.3 ± 3.39 years (range, 4 months to 15 years); the age of 3 dogs was not known. Twenty dogs were females, and 17 were males; sex of 2 dogs was not known. These 39 dogs were classified into dogs with positive (n = 19) and negative (20) results of a Coombs’ test. Further clinical information was not available for these dogs.

Dogs in which vector-borne disease was confirmed or suspected

Ninety-seven serum samples from dogs of various breeds were submitted to the Vector-Borne Diseases Diagnostic Laboratory at North Carolina State University by veterinarians who suspected infection with a tick-borne pathogen; these sera were found to be seroreactive for vector-borne pathogens. Information was not available regarding sex, age, and clinical status or final diagnosis. All samples were tested via IFA for seroreactivity for R rickettsii, B henselae, B vinsonii subsp berkhoffii, and E canis.15,20 An antibody titer ≥ 1:64 was considered indicative that the dog was seroreactive. Dogs with titers between 1:64 and 1:128 were classified as low seroreactors, and dogs with titers ≥ 1:256 were considered as medium to high seroreactors. Enzyme-linked immunosorbent assays16–19 were used to detect antibodies against B burgdorferi C6 peptide in the serum samples.30

Dogs seroreactive for L infantum included 43 dogs of various breeds with clinical signs and clinicopathologic abnormalities compatible with leishmaniasis and with high antibody titers against L infantum. Serum samples were submitted to the Laboratorio Veterinario Privato San Marco in Padova, Italy. An in-house quantitative ELISA was used to test all samples, as described elsewhere.31 Sera from these dogs were not tested for exposure to other vector-borne pathogens.

Control dogs

Serum samples were included from 20 dogs of unknown breeds that were suspected by the attending veterinarian of being infected with a tick-borne disease but in which antibodies against the defined vector-
borne pathogens were not detected. Serum samples were tested by personnel at the Vector-Borne Diseases Diagnostic Laboratory. All of these dogs were seronegative for antigens of the tick-borne pathogens *R rickettsii*, *B henselae*, *B vinsonii* subsp *berkhoffii*, *E canis*, and *B burgdorferi*.

Sick dogs in which leishmaniasis was clinically suspected but antibodies against *L infantum* were not detected comprised 22 dogs of various breeds tested at the Laboratorio Privato Veterinario San Marco and found to be seronegative for *L infantum*. These dogs were not tested for exposure to other vector-borne pathogens, and no clinical information was available for these dogs.

Blood samples were collected from 20 dogs (13 males and 7 females) of various breeds during a general health screen at the Queen Mother Hospital. Mean ± SD age was 6.5 ± 4.37 years (range, 1.0 to 14.2 years). Residual serum samples were used as a healthy control group for the study. Nine dogs had orthopedic problems, 1 was admitted for castration, and 10 were healthy with no clinical signs. None of these dogs had clinical evidence of infectious or autoimmune diseases.

**pANCA assay**—Detection of pANCA via IFA and isolation of granulocytes was performed as described elsewhere. Briefly, prepared IFA slides were overlaid with thawed canine serum (dilutions 1:10 and 1:20), incubated for 1 hour, and then washed. Each slide contained a positive serum sample (1:10 dilution) and a negative control sample. Fluorescein isothiocyanate–labeled secondary antibody (1:100 dilution) was added, and the slides were incubated again (1 hour in a humid chamber at 22°C) and then washed. Fluorescence microscopy was used to evaluate the results. Slides were examined separately by 2 investigators (KA and AEK) who were not aware of the identity of dogs from which the serum samples were obtained. For samples with uncertain results, tests were repeated until samples were clearly identified as having positive or negative results. Samples with perinuclear immunofluorescence in the neutrophils were considered positive for pANCA, and samples with intranuclear or atypical labeling for pANCA were considered negative for pANCA (Figure 1).

**Statistical analysis**—The χ² or Fisher exact test was used to identify significant differences in pANCA prevalence.

![Figure 1](image-url) —Photomicrographs indicating the typical perinuclear staining pattern of canine granulocytes in a sample obtained from a dog with positive results (A) and a dog with negative results (B) for pANCA. Indirect immunofluorescence staining; bar = 100 µm.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. (%) of dogs with positive results for pANCA</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs in which IMHA was diagnosed or suspected clinically (n = 55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMHA diagnosed (n = 16)</td>
<td>11 (69)</td>
<td>46–92</td>
</tr>
<tr>
<td>IMHA suspected; positive result for Coombs’ test (n = 19)</td>
<td>6 (32)</td>
<td>11–52</td>
</tr>
<tr>
<td>IMHA suspected; negative result for Coombs’ test (n = 20)</td>
<td>9 (45)</td>
<td>23–67</td>
</tr>
<tr>
<td>Dogs confirmed or suspected of having vector-borne infections (n = 140)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinically suspected and seroreactive for ≥1 tick-borne diseases including <em>Rickettsia rickettsii</em>, <em>Bartonella henselae</em>, <em>Bartonella vinsonii</em> subsp <em>berkhoffii</em>, <em>Ehrlichia canis</em>, and <em>Borrelia burgdorferi</em> infection (n = 97)</td>
<td>29 (40)</td>
<td>30–50</td>
</tr>
<tr>
<td>Clinically suspected and seroreactive for <em>Leishmania infantum</em> (n = 43)</td>
<td>28 (65)</td>
<td>51–79</td>
</tr>
<tr>
<td>Control dogs (n = 62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs clinically suspected but seronegative for tick-borne pathogens (n = 20)</td>
<td>1 (5)</td>
<td>0–25</td>
</tr>
<tr>
<td>Dogs clinically suspected but seronegative for <em>L infantum</em> (n = 22)</td>
<td>8 (38)</td>
<td>20–57</td>
</tr>
<tr>
<td>Healthy dogs (n = 20)</td>
<td>1 (5)</td>
<td>0–25</td>
</tr>
</tbody>
</table>

Table 1—Summary of pANCA results for serum samples obtained from several groups of dogs.
lence between the disease groups and the control groups. An OR was then calculated to measure the strength of the association. Significance was set at values of \( P < 0.05 \). Data analysis was performed with statistical software.\(^7\)

**Results**

The percentage of pANCA-positive dogs in each group and subgroup was summarized (Table 1). Among the dogs in which IMHA was diagnosed or suspected clinically, the prevalence of pANCA-positive dogs (11/16) was highest in the subgroup of dogs in which IMHA was confirmed. Dogs with IMHA were 16.27 times (95% CI, 4.11 to 54.34; \( P < 0.001 \)) as likely to have positive results for pANCA as were the healthy control dogs. There was no association between positive results for pANCA and positive or negative results for the Coombs’ test for dogs suspected of having IMHA.

Of the 140 dogs seroreactive for \( \geq 1 \) vector-borne disease pathogen, 67 (48%) had positive results for pANCA. Of the 97 dogs seroreactive for \( R. rickettsii, B. henselae, B. vinsonii subsp. berkhoffii, \) or \( E. canis, 39 \) (40%) had positive results for pANCA. Of the 43 dogs seroreactive for \( L. infantum, 28 \) (65%) had positive results for pANCA (Table 1).

A significant association was detected between a positive pANCA result and seroreactivity for any vector-borne pathogen evaluated in the study. Dogs seroreactive for any vector-borne pathogen were 17.4 times (95% CI, 2.27 to 133.85; \( P < 0.001 \)) as likely to have positive results for pANCA as were healthy control dogs or dogs suspected by the attending veterinarian of being infected with a tick-borne pathogen. The OR was 4.7 (95% CI, 1.72 to 12.96; \( P = 0.03 \)) when the dogs seroreactive for a tick-borne pathogen were compared with the dogs suspected by the attending veterinarian of being infected with a tick-borne pathogen and healthy control dogs.

To further investigate potential associations between pANCA and infection, dogs seroreactive for a tick-borne pathogen were allocated into subgroups on the basis of the antibody titer against vector-borne pathogens. For the 30 dogs with moderate to high IFA antibody titers against \( B. henselae \) or \( E. canis \) antigens, there was a stronger association with positive results for pANCA (Table 2).

Of the 43 dogs infected with \( L. infantum \), 28 (65%) had positive results for pANCA. There was a significant \( (P < 0.001) \) association between a positive pANCA status and \( L. infantum \) seroreactivity (OR, 35.5; 95% CI, 4.32 to 291.49), compared with results for healthy dogs (Table 2). Compared with dogs in which leishmaniasis was suspected but antibodies against \( L. infantum \) were not detected, seroreactive dogs were significantly \( (P = 0.027) \) more likely (OR, 3.28; 95% CI, 1.12 to 9.54) to have positive results for pANCA. Compared with healthy control dogs, dogs seronegative for \( L. infantum \) were significantly \( (P = 0.022) \) more likely to be seroreactive for pANCA (OR, 6.16; \( P = 0.022; 95\% \) CI, 1.43 to 26.49).

**Discussion**

Perinuclear ANCA have been described in association with various inflammatory and immune-mediated gastrointestinal diseases in dogs.\(^9,\)\(^13,\)\(^14\) To the authors’ knowledge, the present study represents the first attempt to investigate pANCA status in dogs exposed to specific pathogens and in dogs with IMHA. A large proportion of dogs (67/140 [48%]) that were seroreactive for various pathogens in the present study also had positive results for pANCA. Moreover, 11 of 16 of dogs with confirmed IMHA also had positive results for pANCA. There was a significant association between positive results for pANCA and a diagnosis of IMHA or exposure to tick- or sandfly-transmitted infectious diseases.

Authors of several case reports\(^32–34\) have described pANCA seroreactivity in association with human autoimmune or immune-mediated diseases, including IMHA, glomerulonephritis, systemic lupus erythematosus, and IBD. The results of the study reported here suggested that pANCA production could also be associated with autoimmune disorders or vector-borne infectious diseases in dogs. There is a need for future studies with well-characterized serum samples from various dog populations with complete clinical and historical data to define the diagnostic specificity and sensitivity of pANCA in dogs. Interestingly, the highest pANCA prevalence was found in dogs with stringent inclusion criteria for IMHA. In contrast, there was no significant associa-

<table>
<thead>
<tr>
<th>Vector-borne pathogens</th>
<th>No. (%) of dogs with positive results for pANCA</th>
<th>OR*</th>
<th>95% CI</th>
<th>( P ) value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroreactivity for at least 1 vector-borne pathogen (n = 140)</td>
<td>67 (48)</td>
<td>17.4</td>
<td>2.27–133.85</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Seroreactivity for &gt; 1 pathogen (n = 51)</td>
<td>21 (41)</td>
<td>13.3</td>
<td>1.65–107.18</td>
<td>0.003</td>
</tr>
<tr>
<td>Seroreactivity for at least 1 tick-borne pathogen (n = 97)</td>
<td>39 (40)</td>
<td>4.7</td>
<td>1.72–12.96</td>
<td>0.003</td>
</tr>
<tr>
<td>Bartonella only (n = 15)</td>
<td>5 (33)</td>
<td>9.5</td>
<td>0.97–92.83</td>
<td>0.064</td>
</tr>
<tr>
<td>Ehrlichia only (n = 12)</td>
<td>5 (42)</td>
<td>13.6</td>
<td>1.34–137.45</td>
<td>0.018</td>
</tr>
<tr>
<td>Leishmania (n = 43)</td>
<td>28 (65)</td>
<td>35.5</td>
<td>4.32–291.49</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Rickettsia, medium to high titers (n = 9)‡</td>
<td>3 (23)</td>
<td>9.5</td>
<td>0.826–109.24</td>
<td>0.076</td>
</tr>
<tr>
<td>Bartonella, medium to high titers (n = 17)†</td>
<td>9 (53)</td>
<td>21.4</td>
<td>2.31–197.79</td>
<td>0.001</td>
</tr>
<tr>
<td>Ehrlichia, medium to high titers (n = 13)†</td>
<td>8 (62)</td>
<td>30.4</td>
<td>3.05–303.36</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Healthy control dogs were the referent group. †Values were considered significant at \( P < 0.05 \). ‡Medium to high titers were defined as titers \( \geq 1:256 \).
tion between pANCA reactivity and the outcome of a Coombs’ test. The prevalence of pANCA in Coombs’ test–positive and –negative dogs was 6 of 19 (32%) and 9 of 20 (45%), respectively. Because of a lack of sensitivity, the Coombs’ test will yield negative results for dogs with IMHA and positive results for dogs with bartonellosis. Therefore, the Coombs’ test–positive and –negative dogs included in the present study, although suspected of having primary IMHA, could have been assigned incorrectly to a group or could have had underlying infectious or neoplastic disease. No other clinicopathologic information was available on these dogs, and a positive result for a Coombs’ test by itself is not diagnostic of IMHA, so it is not possible to draw any further conclusions regarding these dogs.

Mechanisms by which an infectious agent could induce autoimmune reactivity have been described. For example, pANCA cross-reactivity with colonic bacteria has been reported in humans with ulcerative colitis. In particular, pANCA appear to cross-react with both Bacteroides spp (Bacteroides cacae and Bacteroides thetaiotaomicron) and Escherichia coli. In addition, bacterial permeability-increasing protein, an endogenous antimicrobial protein directed against gram-negative bacteria, appears to target pANCA antigens. Moreover, bacterial permeability-increasing protein has affinity for endotoxins of gram-negative bacteria, which might explain the reason that pANCA develop in humans and dogs infected with these enteric pathogens. Neutrophils are the first inflammatory cell types recruited in any infection, so it is possible that antibodies against neutrophils are produced in association with many acute infections that develop in human and other animal patients. Moreover, it is thought that superantigens are involved in the pathogenesis of pANCA-associated IBD in humans because superantigens stimulate the immune system greatly and may ultimately lead to autoantibody production, including pANCA.

In the present study, serologic responses against several vector-borne gram-negative bacteria (Rickettsia spp, B. henselae, B. vinsonii subsp berkhoffii, E canis, and B. burgdorferi) were investigated. Gram-negative bacteria can be a target antigen of pANCA. Moreover, dogs were seroreactive, it is difficult to ascertain whether all of the dogs had an active infection at the time blood samples were collected because there was no clinicopathologic information available for these dogs. However, it is likely that these dogs had been exposed to the specified pathogens at some time point before collection of blood samples; therefore, we speculate that the formation of pANCA was likely associated with these vector-borne infectious diseases.

Another theory supports defective apoptosis as a cause of pANCA development in patients with autoimmune and inflammatory disorders. Ineffective apoptosis or defective removal of cell debris by neutrophils and macrophages might lead to production of pANCA because substances that are normally intracellular are released and detected as antigens by the immune system. A better understanding of the immunopathogenesis of pANCA in a spectrum of inflammatory and infectious diseases would benefit both canine and human medicine. Dogs and humans share the same environment and similar or identical genetic diseases, so it is conceivable that dogs could be used to investigate environmental and genetic factors that influence pANCA induction, as described in human patients. Similar to the results of the present study, it was reported by other investigators that dogs seroreactive for Bartonella spp, E canis, and L infantum develop another type of autoantibody (ie, ANA). It is speculated that dogs infected with these vector-transmitted pathogens may develop persistent infection within the systemic circulation and therefore may be more prone to develop ANA. On the basis of results of that study, this likelihood may also be evident in the production of pANCA. Interestingly, dogs that were seroreactive for > 1 organism were more likely to develop ANA, compared with results for dogs that were seroreactive for only 1 pathogen. Similar results were found in the present study, in which there was a higher prevalence of pANCA in samples with medium to high antibody titers and coinfections, compared with results for samples with reactivity for single pathogens. Interestingly, the highest pANCA prevalence among all vector pathogen groups was found in the dogs seroreactive for L infantum. This may be explained by the fact that most dogs in this group had strong antibody responses against L infantum; alternatively, some of these dogs may have been coinfected with other vector-borne pathogens.

In several studies have reported a spectrum of clinical signs similar to those seen for IMHA, immune-mediated thrombocytopenia, endocarditis, vasculitis, and nonseptic neutrophilic polyarthritis in dogs infected with various Bartonella spp. In Bartonella-infected dogs, it is also possible that the pathogen triggered a secondary autoimmune response that can include the production of pANCA, as has been described in humans with endocarditis attributable to Bartonella infection. Although the common pathway or pathways have yet to be definitively characterized, it is likely that many intravascular infections induce an immune response that results in the production of a spectrum of autoantibodies (ie, antinuclear, antinuclear, and antinuclear) have been found in sera of dogs with ehrlichiosis and leishmaniosis. Analysis of results of the present study suggested that pANCA are an additional autoantibody that can be found in a high percentage of dogs with these infections. Because of deposition of immune complexes, glomerulonephritis, uveitis, polyarthritis, and neutrophilic vasculitis are common diagnoses in dogs persistently infected with vector-borne organisms. The extent to which deposition of immune complexes is linked to the development of ANA and pANCA needs further investigation.

In the present study, we investigated the prevalence of pANCA in healthy dogs, dogs with IMHA, and dogs that were seroreactive for selected vector-borne pathogens. We detected pANCA in a high percentage of dogs with IMHA and vector-borne infectious diseases; therefore, pANCA may be a relatively nonspecific marker for dogs with IBD. The low prevalence of pANCA in healthy dogs is similar to results reported in a previ-
ous study. This may imply that although pANCA have poor diagnostic specificity, the development of pANCA in a dog may represent a biomarker for systemic illness. Consequently, positive results for pANCA in an individual dog should be interpreted with caution because pANCA are associated with chronic enteropathies as well as with immune-mediated and vector-borne infectious diseases. Further prospective studies are needed to define the diagnostic utility of pANCA in dogs with immune-mediated and infectious diseases.

References

a. Provided by Robert Goggs, Queen Mother Hospital, Royal Veterinary College, University of London, Hatfield, Hertfordshire, AI9 7TA, England.
b. SNAP3Dx, IDEXX Laboratories Inc, Westbrook, Me.
c. SNAP4Dx, IDEXX Laboratories Inc, Westbrook, Me.
e. SPSS, version 18.0, SPSS Inc, Chicago, Ill.