Acta vet. scand. 1999, *40*, 205-212.

Canine Antinuclear Antibodies: Comparison of Immunofluorescence Staining Patterns and Precipitin Reactivity

By *H. Hansson*[1] and *A. Karlsson-Parra*[2]

[1]Department of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden and [2]Haugesund County Hospital, Haugesund, Norway.

Hansson H, Karlsson-Parra A: Canine antinuclear antibodies: Comparison of immunofluorescence staining patterns and precipitin reactivity. Acta vet. scand. 1999, 40, 205-212. – The occurrence of antinuclear antibodies (ANAs) against several specific nuclear antigens is clearly associated with certain systemic rheumatic disorders in human patients. Determination of ANAs on a routine basis, usually by indirect immunofluorescence (IIF) technique, has therefore become an important diagnostic tool. The subdividing of positive ANA-sera into different nuclear IIF staining patterns often gives clues to antibody specificity. The present investigation aimed at studying whether such subgroups of staining patterns in IIF ANA positive canine sera may represent certain specific ANAs that can be verified by standard methods used for specificity determination in humans.

The presence of precipitating antibodies, determined by the Ouchterlony immunodiffusion (ID) technique, was found to be strictly associated with a positive IIF ANA, exhibiting a speckled staining pattern without any chromosomal reactivity. None of the sera with chromosomal reactivity contained precipitating antibodies. Among the ID positive serum samples, different antigenic reactivities were detected, represented by different ID subgroups. Only 1 of the 4 main subgroups obtained by ID showed identity with any of the common and disease-associated human ANA specificities, exhibiting anti-RNP reactivity. One of these serum samples concomitantly exhibited precipitating antibodies against the Sm antigen.

dog; immunodiffusion; anti-RNP.

Introduction

Antinuclear antibodies (ANAs) constitute a heterogeneous population of autoantibodies directed against various nuclear antigens. The serological determination of ANA has become well-established in the disease classification of human patients with connective tissue disease (*Tan* 1989, *Tomer et al.* 1993, *von Mühlen & Tan* 1995), while the usefulness of ANA determination in canine autoimmunity is still mainly restricted to the diagnosis of systemic lupus erythematosus (*Bennett & Kirkham* 1987; *Costa et al.* 1984, *Lewis et al.* 1965).

Indirect immunofluorescence (IIF) microscopy is by far the most widely used technique for routine ANA screening in man as well as in animals, and the importance of antigenic substrate has become apparent. In order to avoid spurious results in apparently healthy animals, the establishment of a significant ANA cut-off level, excluding 95% of normal dog sera, has proved to

be of importance when evaluating the ANA test (*Hansson et al.* 1996). The appropriate cut-off level must be interpreted by each laboratory, due to interlaboratory variation and the composition of both patient and appropriate control populations (*McCarty & Rice* 1980).

In our hands, a high prevalence of positive ANA was found at low serum dilutions among healthy dogs when using cryopreserved sections of rodent organs (rat liver sections) as ANA substrate. This problem was significantly reduced using monolayers of human tissue culture cells (the epithelial cell line HEp-2) as ANA substrate. No reactivity against nuclei of HEp-2 cells was observed in any of the normal dog sera analysed at a dilution of 1/25. Thus, when using this substrate and this dilution as cut-off level, it was confirmed that ANA determination is of diagnostic value in diseased dogs, and the association between ANA positivity and connective tissue disease, CTD, is increased (*Hansson et al.* 1996). Moreover, the HEp-2 cells were found not only to be superior to rat liver cryostat sections due to the low reactivity with normal sera, but also provided a considerably facilitated and more consistent ANA fluorescence pattern discernment. This kind of substrate also allows further subdividing of positive reactivity into different groups, according to the IIF staining pattern (*Hansson et al.* 1996, *McCarty & Rice* 1980). The fluorescence patterns may, thereby, give clues to the specific ANA reactivity as shown in human patients. This may be exemplified by antibodies against double-stranded (ds) DNA, ribonucleoprotein (RNP), centromeric antigen and the proliferating cell nuclear antigen (PCNA) (*McCarty et al.* 1984, *Tan* 1989).

As new ANA specificities were characterized, the notion has progressively emerged that particular autoimmune disorders are associated with certain profiles or certain specificities of ANA. This may be exemplified by antibodies against the Smith (Sm) antigen, ds DNA and PCNA being strongly associated with systemic lupus erythematosus (SLE), the anti-Jo1 antibody being strongly associated with dermatomyositis, and the anti-RNP antibody associated with SLE and mixed connective tissue disease (MCTD) (*McCarty et al.* 1984, *Sharp et al.* 1972, *Tomer et al.* 1993). The knowledge of different ANA specificities and their associations to certain syndromes in humans is related to defined clinical criteria, improved methods of specific ANA determination and the use of optimal substrates in routine ANA screening by IIF.

Since a number of autoantibodies appears to be of diagnostic and/or prognostic value in the evaluation of different systemic rheumatic diseases in humans, secondary testing of IIF ANA positivity is now highly recommended in order to determine ANA specificity. A number of assays are available for such determination. One of the most commonly used methods is the Ouchterlony immunodiffusion (ID) test, which is easy to perform and interpret (*Nakamura et al.* 1985).

In a recent study, using HEp-2 cells as substrate, IIF ANA positive canine sera could be subdivided into at least 2 major different subgroups of fluorescence patterns (*Hansson et al.* 1996). This prompted us to study whether distinct staining patterns indicate certain ANA-specificities. We chose *Crithidiae luciliae* as substrate for anti-dsDNA determination by IIF microscopy and the Ouchterlony technique for determination of ANAs reacting with extractable nuclear antigens.

Materials and methods

Sera

All 62 canine sera comprised in the present study were collected by practising veterinarians throughout Sweden from privately owned dogs

with a suspected autoimmune disease. These serum samples were sent to 1 of 2 laboratories conducting the ANA test in Sweden. The sera were stored at –20 °C and reevaluated by IIF ANA using Hep-2 cells as substrate. All the sera were positive at the dilution ≥ 1/100 (*Hansson et al.* 1996).

Indirect immunofluorescence

The ANA IIF tests were performed as described earlier (*Hansson et al.* 1996). In brief, this included monolayers of human epitheloid (HEp-2) cells fixed on glass slides (Immuno Concepts, Sacramento, CA) as substrate.

In the first step, the substrate was incubated with a 1/25 dilution of serum for 30 min. Anti-canine IgG (Fab + Fc) labelled with fluorescein isothiocyanate (FITC) (Nordic; Tilburg, The Netherlands) was diluted 1/100 in phosphate-buffered saline (PBS) and used in the second incubation step for 30 min. The last washing step incorporated a counterstain (Evans Blue) added to the washing buffer (PBS). The slides were examined by fluorescence microscopy.

In order to detect antibodies against double stranded (ds) DNA the flagellate *Crithidia luciliae* was used as substrate (Immuno Concepts, Sacramento, CA) (*Aarden et al.* 1975). This analysis was performed by IIF with ANA positive serum samples displaying a positive chromosomal fluorescence staining in mitotic cells.

Immunodiffusion

The immunodiffusion technique used in this study is the double diffusion technique of Ouchterlony (*Ouchterlony* 1953). The 62 ANA positive sera and 24 serum samples from healthy, privately owned, dogs were analysed with the commercially available Auto I.D. Autoantibody Test System using lyophilized mammalian nuclear antigen (Immuno Concepts, Sacramento, CA). After incubation for 24-48 h at room temperature, nuclear reactivity was identified as lines of precipitation formed in the agarose gel. Positivity was determined by a visible precipitation line between the canine serum sample added to 1 of 6 peripheral wells in the agar plate and the nuclear antigen substrate added to the central well.

Specific human antisera reactive to SSA (Ro), SSB (La), Sm, RNP, Jo1 and Scl 70 were used in order to determine the specificity of precipitating dog sera. This was investigated by studying the precipitation line of the reference serum with the precipitation line of the canine serum sample (using the same nuclear antigen substrate in the central well and the canine and human sera in adjacent, peripheral wells). An identical reactivity was defined as a continuous precipitation line between the adjacent serum sample wells and the central well containing the nuclear extract.

Results

Indirect immunofluorescence

Sixty-two ANA positive sera were earlier analysed and subgrouped according to the fluorescence staining pattern in the chromosomal regions of the substrate (*Hansson et al.* 1996). The 51 sera displaying a negative chromosomal reactivity in mitotic cells were reanalysed as to subtypes of nuclear staining patterns in non-mitotic cells (Table 1). Thirty-two of these serum samples exhibited a fine speckled nuclear fluorescence pattern with an almost homogeneous appearance. Seven sera showed a grainy speckled nuclear pattern. The remaining 12 serum samples constituted a third subgroup exhibiting a combination of fine and grainy speckles, thus giving rise to a mixed speckled staining pattern. The 11 sera displaying a positive chromosomal reaction in mitotic cells, all with a homogeneous nuclear staining pattern of non-mitotic cells, were further analysed for anti-dsDNA. However, no anti-dsDNA reactivity was found among these serum samples.

Table 1. 62 ANA positive canine sera, subgrouped according to indirect immunofluorescence (IIF) staining patterns in the chromosomal regions of mitotic cells, correlated to precipitin reactivity obtained by immunodiffusion (ID). All ID positive sera were among the IIF ANA positive serum samples with a negative chromosomal reactivity (speckled staining pattern).

	IIF ANA positive			
	Chromosal pos.	Chromosomal neg.		
	Homogenous	Fine speckled	Grainy speckled	Mixed speckled
ID pos.	0	14	4	7
ID neg.	11	18	3	5
Total	11	32	7	12

Immunodiffusion

A clearly demonstrated precipitin line was formed in 25 out of the 62 serum samples investigated. Testing these ID-positive sera for identity against each other, defined as a continuous (identical) precipitation line between adjacent serum sample wells and the central well containing the nuclear extract, gave rise to 4 different subgroups. Each subgroup thus presented identical precipitin lines within the group (Table 2).

One subgroup consisted of sera from 12 dogs, where 10 different breeds were represented. In the second subgroup, serum samples from 6 dogs all of different breeds were found. One of these serum samples showed more than one type of precipitating antibodies, giving rise to 2 precipitin lines. One of these lines was identical to the precipitin line shown by the other sera in the subgroup. The third subgroup comprised serum samples from another 6 dogs, 2 of them showing partial identity (like the serum sample described above) with the others. Five of these dogs were German Shepherds. The last subgroup consisted of serum from one dog, a Whippet.

None of the 24 control sera exhibited any precipitin reactivity.

Fluorescence patterns versus precipitin reactions obtained by immunodiffusion

As seen in Table 1, all precipitin positive dog sera were among the IIF ANA positive serum samples exhibiting a negative chromosomal reactivity in mitotic cells. However, no clear-cut correlation was obtained between different subgroups of staining patterns in non-mitotic cells and certain subgroups of immunodiffusion (Table 2).

Among the 26 precipitin negative sera that concomitantly displayed a chromosomal negative reactivity by IIF, 22 serum samples were from German Shepherds.

ANA specificity as determined by immunodiffusion

In one of the subgroups displaying precipitin reactivity (ID subgroup nr 2), all 6 serum samples produced precipitin lines of identity with the anti-RNP human reference sera. One of these serum samples in addition exhibited an anti-Sm precipitin line. None of the other precipitin positive dog sera showed any precipitin lines of identity with specific human antisera reactive to SSA (Ro), SSB (La), Sm, RNP, Jo1 or Scl 70.

Clinical signs versus IIF ANA and ID results

Patient charts were accessible in 57 out of the 62 IIF ANA-positive dogs. As described in the earlier study (*Hansson et al.* 1996), musculoskeletal disorders mainly accompanied by stiffness and pain while moving or intermittent lameness involving multiple limbs were the dominating clinical signs (79%). Other clinical signs were skin disorders (5 dogs), mainly involving ulcerations and crusting, non-specific depression (3 dogs), anaemia (3 dogs) and megaoesophagus (1 dog). One dog had cystin-

Table 2. Subgrouping of 25 canine sera with precipitating antibodies: Fluorescence patterns (fine, grainy or mixed speckled pattern) versus different precipitin (ID) reactions. No clear-cut correlation was obtained between different subgroups of staining patterns and certain ID subgroups. Only one of the ID subgroups, no. 2, showed identity with any of the common and disease-associated human ANA specificities, the RNP antigen.

ID pos.	Fine speckled	Grainy speckled	Mixed speckled
ID subgroup No. 1	8	0	4
ID subgroup No. 2	0	3	3
ID subgroup No. 3	6	0	0
ID subgroup No. 4	0	1	0
Total	14	4	7

Table 3. Dominating clinical signs among the 62 ANA positive dogs correlated to IIF ANA staining patterns (chromosomal reactivity in mitotic cells) and precipitin (ID) reactions. Musculoskeletal disorders were evenly distributed and were the clearly dominating clinical signs among the dogs in different IIF ANA as well as in ID groups.

Clinical signs	IIF ANA Chromosomal pos.	IIF ANA Chromosomal neg.	
	ID neg.	ID pos.	ID neg.
Musculoskeletal	5	20	19
Skin disorders	2	0	3
Anaemia	2	0	0
Other	1	3	2
No data	1	2	2
Total	11	25	26

uria and had been treated with the drug Thiola 250 mg (containing 2-mercaptopropionylglycine, closely related to D-penicillamine). This dog was clinically healthy at the time of the ANA test.

Musculoskeletal disorders were evenly distributed and were the clearly dominating clinical signs among the dogs in the different IIF ANA as well as in the ID groups (Table 3). Thus, no specific clinical sign was dominating in a special ID subgroup.

Discussion

We have recently shown that when using the tissue culture HEp-2 cell substrate, IIF ANA positive canine sera could be subdivided into at least 2 major subgroups of fluorescence patterns (*Hansson et al.* 1996). This prompted us to study whether such subgroups may represent different specific ANAs as determined by the commercially available ID technique.

The presence of precipitating antibodies was found to be strictly associated with a positive IIF ANA exhibiting a speckled staining pattern without any chromosomal reactivity. However, the precipitin reactivity did not seem to be directly correlated to any of the 3 ANA IIF subpatterns of speckled nuclear staining of non-mitotic cells. All the ANA positive sera displaying a chromosomal reactivity with a homogeneous staining pattern were found negative by immunodiffusion. None of these chromosomal reactive sera, however, displayed any anti-dsDNA reactivity using *Crithidia luciliae* as substrate, which is in concordance with earlier investigations (*Monier et al.* 1980, *Thoburn et al.* 1972). In more recent studies, canine sera reacting with chromosomal antigens usually present anti-histone antibodies (*Monestier et al.* 1995, *Monier et al.* 1992, *Brinet et al.* 1988). In sharp contrast to the serum samples displaying a chromosomal reactivity, nearly half (25 out of 51) of the IIF ANA positive speckled sera, without chromosomal staining, were positive by ID. A number of the canine sera with a speckled staining pattern did not, however, present precipitating antibodies detectable with the ID technique. Experience of human patients indicates a similar condition, in which certain autoantibody specificities do not present precipitin lines. The processing technique of

extractable nuclear antigens (ENAs) for the ID antigenic mixture is known to exclude certain reactivities from the antigenic extracts.

Another finding of interest was that the precipitating sera were restricted as to specificity, as only 4 different major subgroups were demonstrated. No association between ANA IIF subpatterns and the different ID subgroups was, however, found.

One of the ID subgroups consisted of nearly half of the ID positive sera, all of them exhibiting a common but unidentified specificity. Moreover, 5 out of 6 precipitin positive German Shepherd sera also exhibited a common but unidentified specificity. Only 1 of the 4 subgroups obtained by ID showed identity with any of the well-defined and clinically important human ANA specificities. These 6 canine sera exhibited anti-RNP reactivity, with one of them concomitantly showing precipitating antibodies against the Sm antigen. A serum sample may contain more than one type of precipitating antibodies. In this study, 3 of the sera in 2 different ID subgroups not only showed identity with the other sera in their subgroup, but also exhibited precipitating autoantibodies directed towards another autoantigen.

The patient charts from the majority of the dogs with IIF ANA revealed that the clinical signs made an autoimmune systemic disease very probable. However, there was no specific clinical sign that was found to dominate a certain IIF ANA staining pattern or a special ID group/subgroup, as the majority of the dogs all showed signs of a rheumatic disorder with the musculoskeletal changes described above. In human patients, the autoimmune systemic diseases or syndromes are known to be interrelated and may have many overlapping clinical features. This might be the case also in dogs, which would possibly explain why we could not clinically separate the dogs in the different groups. Thus, according to the clinical findings and the IIF ANA positivity, the ID negative canine autoantibodies still reflect an autoimmune disorder, although the autoantibody specificity remains to be detected.

Taken together, in the search for canine ANA specificities that can be determined by immunodiffusion, it appears to be of particular interest to know whether the nuclear reactivity obtained by ANA screening with immunofluorescence technique displays chromosomal reactivity or not. Among the ID positive canine serum samples, different autoantigenic reactivities are detected (represented by different ID subgroups), but with a majority of the sera not reacting against common disease-associated autoantigens in the human system. This may reflect difficulties in interactions of precipitating antibodies between the two different species. Generation of immunoprecipitates not only requires lattice formation between F(ab)′2 parts of the antibodies and the relevant antigen but also Fc-Fc interactions between adjacent immune complexes (*Moller & Steensgaard* 1979). A lack of such Fc-Fc interaction thus might explain why precipitating antibodies of one species do not necessarily form a precipitin line of identity when tested against a precipitating antibody of another species, even though the antibodies present the same antigenic specificity. Another explanation might be that canine ANA of a certain antigen specificity preferably recognise other epitopes than those recognised by corresponding human antibodies. However, as the canine sera produced precipitin lines of identity using human Sm/RNP-specific serum as reference, we believe that the differences in precipitin line formation reflect dog-specific subgroups of autoantigenic reactivities as well as dog-specific subgroups of systemic autoimmune diseases.

Further investigations of canine sera as to the relationship between individual ANA staining pattern and specific reactivity using additional

techniques (for example CIE, counter immuno electrophoresis, or western blot), may lead to a better understanding of ANA in dogs. These kinds of investigations may also provide further information on whether associations exist between specific ANA and certain diseases similar to those seen in human patients.

Acknowledgements

This study was supported by grants from Agria Insurance Company, Stockholm, Sweden and the Swedish Kennel Club, Stockholm, Sweden.

References

Aarden LA, de Groot ER, Feltkamp TE: Immunology of DNA: III. Chrithidia luciliae, a simple substrate for the determination of anti-DS-DNA with immunofluorescence technique. Ann. N.Y. Acad. Sci. 1975, *254,* 505-515.

Bennett D, Kirkham D: The laboratory identification of serum antinuclear antibody in the dog. J. Comp. Path. 1987, *97,* 523-539.

Brinet A, Fournel C, Faure JR, Venet C, Monier JC: Anti-histone antibodies (ELISA and immunoblot) in canine lupus erythematosus. Clin. Exp. Immunol. 1988, *74* (1), 105-109.

Costa O, Fournel C, Lotchouang E, Monier JC, Fontaine M: Specificities of antinuclear antibodies detected in dogs with systemic lupus erythematosus. Vet. Imm. Immpath. 1984, *7,* 369-382.

Hansson H, Trowald-Wigh G, Karlsson-Parra A: Detection of antinuclear antibodies by indirect immunofluorescence in dog sera: Comparison of rat liver tissue and human epithelial-2 cells as antigenic substrate. J. Vet. Int. Med. 1996, *10,* 199-203.

Lewis RM, Schwartz R, Henry WB: Canine systemic lupus erythematosus. Blood 1965, *25,* 417-438.

McCarty GA, Rice JR: Characterization and comparison of commercially available antinuclear antibody kits using single pattern index sera. J. Rheumatol. 1980, *7,* 339-347.

McCarty GA, Valencia DW, Fritzler MJ: ANA standardization and interpretation. In: McCarty GA, Valencia DW and Fritzler MJ (Editors): Antinuclear Antibodies, Contemporary Techniques and Clinical Application to Connective Tissue Diseases. Oxford University Press, New York, 1984, pp. 54-69.

Moller NP, Steensgaard J: Fc-mediated immune precipitation. II. Analysis of precipitating immune complexes by rate-zonal ultracentrifugation. Immunology 1979, *38* (3), 641-648.

Monestier M, Novick KE, Karam ET, Chabanne L, Monier JC, Rigal D: Autoantibodies to histone, DNA and nucleosome antigens in canine systemic lupus erythematosus. Clin. Exp. Immunol. 1995, *99* (1), 37-41.

Monier JC, Dardenne M, Rigal D, Costa O, Fournel C, Lapras M: Clinical and laboratory features of canine lupus syndromes. Arthritis Rheum. 1980, *23,* 294-301.

Monier JC, Ritter J, Caux C, Chabanne L, Fournel C, Venet C, Rigal D: Canine Systemic Lupus Erythematosus II: Antinuclear antibodies. Lupus 1992, *1* (5), 287-293.

Nakamura RM, Peebles CL, Rubin RL, Molden DP, Tan EM: Antibodies to nuclear antigens (ANA): Advances in laboratory tests and significance in systemic rheumatic diseases (Second Edition). American Society of Clinical Pathologists Press 1985.

Ouchterlony Ö: Antigen-antibody reactions in gels, IV. Types of reactions in coordinated systems of diffusion. Acta Path. Microbiol. Scand. 1953, *32,* 231.

Sharp GC, Irwin WS, Tan EM, Gould RD, Holman HR: Mixed connective tissue disease – an apparently distinct rheumatic disease syndrome associated with a specific antibody to extractable nuclear antigen (ENA). Am. J. Med. 1972, *52,* 148-159.

Tan EM: Antinuclear antibodies: Diagnostic markers for autoimmune diseases and probes for cell biology. Adv. Immunol. 1989, *44,* 93-151.

Thoburn R, Hurvitz AL, Kunkle HG: A DNA-binding protein in the serum of certain mammalian species. Proc. Nat. Acad. Sci. 1972, *69,* 3327.

Tomer Y, Buskila D, Shoenfeld Y: Pathogenic significance and diagnostic value of lupus autoantibodies. In Arch. Allergy Immunol. 1993, *100,* 293-306.

von Mühlen CA, Tan EM: Autoantibodies in the diagnosis of systemic rheumatic diseases. Semin. Arthritis Rheum. 1995, *24* (5), 323-358.

Sammanfattning

Antinukleära antikroppar hos hund: En jämförande studie mellan immunfluorescensmönster och precipiterande antikroppar.

Förekomst av antinukleära antikroppar (ANA) mot ett flertal specifika nukleära antigen är starkt associerat till vissa reumatiska systemsjukdomar hos människa. Som en viktig del i diagnostiken av dessa sjukdomar utförs därför rutinmässigt, med en indirekt immunfluorescensteknik (IIF), den sk ANA-testen. Baserat på utseendet på immunfluorescensmönstret har man kunnat dela in de ANA-positiva patientproverna i undergrupper, som ofta kan kopplas till en specifik antikroppsreaktivitet. Vanligtvis behöver man dock gå vidare med ytterligare undersökningar för att kunna verifiera den exakta antikroppsspecificiteten.

Målsättningen med denna studie var att undersöka om olika fluorescensmönster vid positiv IIF ANA hos hund representerar olika specifika antikroppsreaktiviteter som kan påvisas med standardtekniker som används inom humandiagnostiken. Förekomst av precipiterande antikroppar, undersökta med immunodiffusionsteknik (ID) enligt Ouchterlony, visade sig vara starkt associerat med en positiv IIF ANA med ett kornigt fluorescensmönster utan samtidig kromosomal reaktivitet. Inga sera med kromosomal reaktivitet gav upphov till precipiterande antikroppar. Bland de ID-positiva serumproverna kunde i sin tur olika undergrupper av precipiterande antikroppar påvisas. Endast en av de fyra undergrupper som erhölls vid ID visade identitet med någon av de vanliga sjukdomsassocierade humana ANA-specificiteterna. Denna grupp uppvisade en specifik RNP (ribonucleoprotein) reaktivitet, där ett av serumproverna dessutom gav upphov till en samtidig Sm (Smith) reaktivitet.

(Received October 20, 1998; accepted March 31, 1999).

Reprints may be obtained from: H. Hansson, Department of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences, Box 7037, S-75007 Uppsala, Sweden. E-mail: Helene.Hansson@kirmed.slu.se, tel: +46-18-671468, fax: +46-18-673534.