

# Immune-Mediated Fever in the Dog. Occurrence of Antinuclear Antibodies, Rheumatoid Factor, Tumor Necrosis Factor and Interleukin-6 in Serum

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**Øvrebø Bohnhorst J, Hanssen I, Moen T: Immune-mediated fever in the dog. Occurrence of antinuclear antibodies, rheumatoid factor, tumor necrosis factor and interleukin-6 in serum. Acta vet. scand. 2002, 43, 165-171.** – Contents of antinuclear antibodies (ANA), rheumatoid factor (RF), tumor necrosis factor (TNF- $\alpha$ ) and interleukin-6 (IL-6) were measured in serum from 20 dogs with immune-mediated fever. Seven out of 20 patients were ANA positive, 1 out of 20 was positive to antibodies against extractable nuclear antigens (ENA), 1 out of 20 was positive to antibodies against deoxyribonucleoproteins (DNP), 2 out of 13 were RF positive and none out of 20 patients had antibodies against native DNA in the serum. TNF- $\alpha$  was not detected in any serum of 15 dogs with immune-mediated fever, while 10 out of 13 presented with elevated IL-6. The results varied between patients, but the IL-6 level was high in most of them. This indicates a role for IL-6 in the pathogenesis of immune-mediated fever in most cases.

**dog; immune; fever; antinuclear antibodies; rheumatoid factor; tumor necrosis factor; interleukin-6.**

## Introduction

Fever of unknown origin (FUO) is not an uncommon problem in clinical canine medicine. In human medicine the criteria which define FUO are (1) prolonged fever of more than 3 weeks duration associated with non-specific signs of illness such as lethargy, anorexia and weight loss, (2) temperature at least 0.83°C above normal on several occasions and (3) diagnosis still uncertain after one week of hospitalization and routine laboratory tests (Petersdorf & Beeson 1961, Vickery & Quinnell 1977, Wolf & Dinarello 1979).

These criteria are not directly applicable to canine medicine, but may serve as useful guidelines to exclude fever of shorter duration associated with infections. The definition of FUO in

dogs was expanded by Dunn & Dunn 1998 to include dogs with temperature in excess of 40°C on more than one occasion.

In humans the relative frequencies of causes of FUO are well established (Petersdorf & Beeson 1961, Jacoby & Swartz 1973), with the cases being divided as follows: infection 40%, neoplasia 20%, immune-mediated 15%, miscellaneous 20% and 5% remaining undiagnosed.

In canine medicine the relative frequency of different causes of FUO varies between studies. The distribution suggested by Feldman (1980) was infections 40%, neoplasia 20%, immune-mediated disease 20%, miscellaneous conditions 10% and undiagnosed (true FUO) 10%. Bennet (1995) claimed that infection is ac-

counting for 50%, immunemediated disease 40% and neoplasia 10% of cases with diagnosed causes of FUO.

Dunn & Dunn (1998) sorted 101 dogs with FUO into 6 main groups with the following result: infections 16%, neoplasia 9.5%, immune-mediated disease 22%, miscellaneous conditions 11.5%, primary bone marrow abnormalities 22%, and true FUO 19%.

In the present study we have investigated the occurrence of selected auto antibodies and 2 inflammatory mediators, interleukin-6 (IL-6) and tumour necrosis factor (TNF- $\alpha$ ) in serum from dogs with fever that we eventually diagnosed as immune-mediated.

The criteria for being included into the material was fever above 39.9°C that did not respond to 2 successive treatments with different antibiotics, but to a subsequent prednisolone cure.

## Materials and methods

### Dogs

The material consisted of 20 dogs of 13 different breeds. All dogs were brought to the clinic within 3 days after they became ill. They were submitted to general clinical examinations, and separated into 3 groups based on clinical signs (Table 1). One group showed only fever, while the others who showed muscle pain and muscle and joint-pain, respectively. Dogs included in the latter 2 groups were stiff and lame. Pain could commonly be located in the *M. triceps brachii* and *M. quadriceps femoris*, and in the elbow, carpal, stifle or tarsal joints. Neck stiffness and pain were also observed, but not as single sign. The fever was measured to lie between 39.9 and 41.4°C.

Blood specimen was drawn from a cephalic vein and serum was prepared and kept frozen at -20°C till the analyses were performed. The patients were first treated with phenoxymethylpenicillin and secondly trimetoprim /sulphadiazin in adequate doses, 2-3 days with each,

without clinical effect. Then they got prednisolone, starting with 1mg/kg/day and tapered down during a period of 2-3 weeks.

Forty-four healthy dogs of 7 different breeds and 2 mongrels taken to the clinic for vaccination, served as controls. The breeds represented were border collie (12), bullmastif (1), cavalier King Charles spaniel (1), English setter (3), golden retriever (1), Gordon setter (21), Irish soft coated wheaten terrier (2) and riesenschнауzer (1). The sex distribution was 22 female and 22 male, and the mean age was 4 year, ranging between 1 and 12.

### Serum analyses

The methods applied for detection of canine auto antibodies were locally modified variants of routine human diagnostical techniques and established as part of a C. Sc. Dissertation (unpublished). The techniques were worked out by use of a collection of 500 sera from dogs of different breeds with a variety of symptoms of mainly rheumatic, autoimmune and febrile disease conditions and with 45 sera from healthy dogs as controls. The sera were partly collected locally and partly provided by Kjerstin Thoren-Tolling and Solveig Knagenhjelm, The Norwegian College of Veterinary medicine, Oslo, Norway.

Antinuclear antibodies (ANA) were detected by use of the indirect immunofluorescence (IIF) technique using Hep-2 cells fixed in alcohol as antigen substrate (Miller et al. 1985).

The cells were cultivated in the laboratory and dispersed into Terasaki plates for application in the test. The sera were screened for ANA reactivity at a 1:20 dilution in PBS and the reaction visualised by a FITC conjugated Fc-specific goat anti-dog IgG (Cappel research Products, Durham, NC) at dilution 1:60. The serum dilution 1:20 was chosen on the basis of positive reactions in 70/230 sera (31.3%) from dogs mainly with signs of systemic disease and 0/45

sera from healthy controls, both groups comprising different breeds. This corresponds well with what has been published by *Hansson et al.* 1996.

Screening for antibodies against extractable nuclear antigens (ENA) was done by 2 methods displaying partly overlapping results. One technique used immunoelectrophoresis in agarose gel with calf thymus antigen (Calf thymus acetone powder 60 mg/ml, Pel-Freez, Rogers, AR). Litex agarose gel (FMC Bio Products, Rockland, ME) and barbiturate buffer (0.05 M, pH 8.6) were used for electrophoresis and 10  $\mu$ l of ENA reagent applied to 20  $\mu$ l of undiluted canine serum. The electrophoresis was run for 45 min using 120V and 44mA. Antibodies against ENA bind to antigen and create a visible band of precipitation in the gel. The other method used was an ELISA anti ENA screening kit (Quanta Lite™ Inova Diagnostics INC. St. Louis, MO) which is composed of 6 purified autoantigens, all well characterised in human diagnostics: SSA, SSB, RNP, Sm, Scl-70 and JO-1. The ELISA kit was modified for application with canine sera by using a rabbit antidog IgG peroxidase conjugate diluted 1:25000 (Sigma Chemical Co. St. Louis, MO), but otherwise following the procedure described for the kit which implies a serum dilution of 1:100. By the electrophoresis method 41 out of 141 patient sera (29%) were tested as positive whereas the result was 0/45 in the controls. Correspondingly the ELISA method gave 44 positive out of 129 sera (34.1%) and 1/45 in the controls: Positive reactions to all 6 specific ENA antigens could be detected among the positive ENA sera (unpublished).

One technique was established for detecting antibodies to chromatin (DNP) using ELISA kit with purified antigen (Novamed Ltd. Jerusalem, Israel) and applying the same adaptation for canine sera as for the ENA ELISA kit. As substrate for detecting antibodies to native

DNA by IIF was utilized a protozoon, *Crithidia luciliae* (*Aarden et al.* 1975). The crithidia were cultivated in the laboratory and dispersed onto slides to be used in IIF. Like in the human variant a serum dilution of 1:10 was applied. The anti DNP test gave 53 positives out of 142 patient sera tested (37.3%) and 1/45 controls. The anti DNA method gave no positive reaction in any sera tested, which seems to correspond well with findings of other investigators (*Hansson et al.* 1999, *Monier et al.* 1980, *Thoburn et al.* 1972).

The activity of IL-6 was determined using IL-6 dependent mouse hybridoma cell line B13.29 clone B9 (*Aarden et al.* 1987, *HogenEsch et al.* 1995, *Carter et al.* 1999). Serial dilutions of the test sample were incubated for 72 h with IL-6 dependent cells. Viability was measured in a colorimetric assay with a tetrazolium salt (Sigma Chemical Co., St. Louis, MO) (*Mosmann* 1983). rIL-6 (*Brakenhoff et al.* 1987) was included as a standard. The detection limit of assay was 15-20 pg IL-6/ ml serum.

TNF- $\alpha$  was determined by cytotoxic effect on the fibrosarcoma cell line WEHI 164 clone 13 (*Espevik & Nissen-Meyer* 1986, *Hogen Esch et al.* 1995, *Carter et al.* 1999).

rTNF- $\alpha$  (Biogen, Cambridge, MA and BASF/Knoll, Ludwigshafen, FRG) were included as standard. The detection limit of the assay was 2-3 pg TNF- $\alpha$ /ml serum. An antiserum to rTNF (Neutralizing capacity, 600ng rTNF- $\alpha$ / ml) (*Espevik & Nissen-Meyer* 1986) completely neutralized the TNF- $\alpha$  activity in the serum samples.

## Results

All patients responded to prednisolone therapy by abolition of clinical signs. A 4 months old female Irish setter in the muscle and joint group was euthanized because of 2 relapses, and that the owner preferred to start with another and healthy pup. This Irish setter became ill for the

Table 1. Antinuclear antibodies (ANA), antibodies against extractable nuclear antigens (anti ENA), rheumatoid factor (RF), and of interleukin-6 (IL-6) in serum from dogs with immune-mediated fever.

Groups	Breed, years/sex(m,f)	ANA	Anti ENA	RF	Il-6 pg/ml
Fever	Bearded collie 1/f	+	-	-	<25
	German shepherd,1/m	-	-	-	653
	German shorthaired pointer,1/m	-	-	nt	<25
	Large poodle 13/m	+	-	nt	478
Fever and Muscle pain	Breton 3/f	-	-	+	nt
	English setter 2/m	+	-	-	nt
	English setter 2/m	+	-	nt	nt
	Flatcoated retriev.9/f	+	-	-	nt
	Flatcoated retriev.1/f	-	-	nt	6280
	German shepherd 6/f	-	-	+	<25
	German shorthaired pointer,7/m	-	-	-	396
	Irish setter,1/f	-	-	nt	578
	Irish wolf hound, 9/m	-	-	nt	nt
	Irish wolf hound, 2/?	-	-	nt	nt
	Kleiner Münsterl. 5/f	-	-	-	8265
	Schiller dog 1/m	-	-	-	1033
Fever, muscle And joint pain	Berner Sennen, 7/f	+	+	-	nt
	Border collie, 1/m	+	-	-	880
	Irish setter, 1/f	-	-	-	>12500
	Schiller dog, 1/f	-	-	-	5288

Nt means not tested.

first time a few days after parvo virus vaccination. Clinical signs that could be related to canine leucocyte adhesion deficiency (*Trowald-Wigh et al.* 2000) were not observed.

The same happened to a 4 months old male German short-haired pointer, but he restituted completely after one prednisolone cure. He was vaccinated later on and after 2 years he has still not shown any relapses. No triggering event or reason could be revealed for the other patients, and none of them has presented signs later on related to this history.

The results of the ANA, anti ENA, RF and IL-6 analyses are presented in Table 1.

Seven out of 20 patients were ANA positive, 1 out of 20 was anti ENA positive on both tests, 2

out of 13 was RF positive and 10 out of 13 patients presented elevated IL-6 values.

*In the fever group (4 dogs)* 1 bearded collie and 1 large poodle were ANA positive. The poodle showed elevated IL-6 level, while the other did not. One out of 2 ANA negative German shepherds presented elevated IL-6 level, and the other did not.

*In the fever and muscle pain group (12 dogs)* 2 English setters and 1 flatcoated retriever tested positive for ANA, and 1 breton and 1 German shepherd were RF positive. Only the German shepherd was tested for IL-6 and she was normal. Another 5 dogs in this group were tested for IL-6, and all of them showed elevated IL-6 values: a 7 year old male German short-haired

pointer, a 5 year old female kleiner Münsterländer, a female Irish setter, a male Schiller dog and a female flatcoated retriever. The latter 3 dogs were all in their first year of life.

In the fever, muscle and joint pain group (4 dogs) 1 Berner Sennen and 1 border collie tested positive for ANA and the Berner Sennen was also anti ENA positive. Serum from the Berner Sennen was not tested for IL-6 activity, but the other 3 dogs were tested and presented all elevated IL-6 activity.

Antibodies against native DNA were not detected in any serum of 20 dogs with fever, and antibodies against DNP was only found in serum of one out of 12 dogs. This dog was a two-year-old male English setter in the fever and muscle pain group. He was also ANA positive.

All controls were negative for antibodies against native DNA and DNP. Sera from 18 controls and 15 patients were examined for TNF- $\alpha$ . All sera were negative.

### Discussion

Infection seems unlikely to be the reason for the fever in this material. The clinical findings, the fact that no patients responded to antibiotic therapy, that many of them were ANA positive and all responded to immune suppressive doses of prednisolone, indicate that the fever and other clinical signs in these dogs were immune-mediated. This is further supported by that none of the patients during the past 2 years since the study was ended has shown evidence of another reason for the fever.

The sex distribution in the present material was even, and except for the Schiller dogs, a breed which is uncommon in the present practice, no breed seemed to be over represented. *Dunn & Dunn* (1998) found a high percentage of springer spaniels, German shepherd dogs and border collies, less than one year old and familiarly related, showing immune-mediated fever

and muscle and joint pain. The present study did not catch more than one border collie, but before the study started several young, familiarly related dogs of this breed had been observed in this practice with fever and muscle and joint pain. It is, however, evident that 9 out of 20 dogs in our material were 1 year or younger.

*Bennet & May* (1995) have defined criteria for diagnosis of immune-based arthropathies of dogs. The polyarthritis/polymyositis syndrome (PM) is defined by presence of non-erosive polyarthritis, chronic active myositis in at least 2 muscle biopsies and negative for antinuclear antibodies. We find it difficult to fit our patients into this system. From a clinical point of view it seems rational to classify most of them as having polyarthritis/polymyositis syndrome, but the fact that many of them are ANA positive, without showing signs of systemic lupus erythematosus, exclude them from any group.

It may be relevant that our patients had only some few days disease history before they were examined and treated, and thus might have developed additional signs if treatment had been delayed. The patients of *Bennet & Kelly* (1987) comprising 2 springer spaniels, 2 cavalier King Charles spaniels, 1 cocker spaniel and 1 whippet and sorted into the PM category had been sick for several weeks before they got their diagnosis.

The present study did not demonstrate anti DNA in serum from any patient. This is in accordance with earlier investigations (*Hansson & Karlsson-Parra* 1999) examining sera from dogs with musculoskeletal disorders.

The 2 inflammatory mediators, IL-6 and TNF- $\alpha$  are known to contribute in local inflammatory response and can have systemic effects. TNF- $\alpha$  was not detected in serum of any patient. *HogenEsch et al.* (1995) studying juvenile polyarteritis syndrom (JPS), an idiopathic febrile disease affecting primarily beagles be-

tween 3 and 18 months, found markedly elevated IL-6 during acute episodes of the disease, but no TNF- $\alpha$ . The disease follows a remittant course with episodes of clinical disease and disease-free intervals. Clinical signs include fever ( $>40^{\circ}\text{C}$ ), weight loss and severe neck pain. Sick dogs improved dramatically upon treatment with corticosteroids and the clinical improvement was accompanied by a decrease in IL-6 activity. Withdrawal of corticosteroid treatment caused reappearance of clinical signs and high serum IL-6 within few days. These results support a role for IL-6 in the pathogenesis of JPS. An inherited recurrent fever of unknown origin with renal amyloidosis in Chinese Shar-pei dogs was also associated with elevated IL-6 (Rivas et al. 1992).

In our material 7/20 dogs were ANA positive, 1/20 was anti ENA positive, 2/13 were RF positive and 10/13 presented with elevated IL-6.

Comparing the results of the individual patients (Table 1), it is evident that they differ much and do not present uniform patterns neither within nor between the groups. Among 9 dogs tested for all parameters, one was ANA and another was RF positive only, while a third was ANA positive and showed elevated IL-6 level. Six dogs presented elevated IL-6 only, which indicate a role for IL-6 in the pathogenesis of most cases in this study. This and the diagnostic significance of this factor in immune-mediated fever in dogs are aspects that should be looked at in the future.

One breton and one German shepherd dog were RF positive, but none of them presented signs of rheumatoid arthritis. Detection of RF in canine sera by latex agglutination technique is commonly used in screening, but the test is not specific for diagnosing rheumatoid arthritis.

Circulating immune complexes produced in other inflammatory diseases may to some extent bind to the IgG coated latex particles (Thoren-Tolling 1991).

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### Sammendrag

*Immunmediert feber hos hund. Forekomst av antinukleære antistoffer, reumatoid faktor, tumor nekrose faktor og interleukin-6 i serum.*

Innhold av antinukleære antistoffer (ANA), reumatoid faktor (RF), tumor nekrose faktor (TNF- $\alpha$ ) og interleukin-6 (IL-6) ble målt i serum fra 20 hunder med immun-mediert feber. Sju av 20 pasienter var ANA positive, 1 av 20 var positive for antistoffer mot ekstraherbare kjerneantigener (ENA), 1 av 12 var positiv for antistoffer mot deoxynukleoprotein (DNP), 2 av 13 var RF positive og ingen av disse 20 pasientene hadde antistoffer mot nativt DNA. TNF- $\alpha$  ble ikke påvist i serum fra noen av de 15 pasientene som ble undersøkt, mens 10 av 13 hadde forhøyet IL-6. Resultatene var forskjellige for de enkelte pasientene, men for de fleste var IL-6 forhøyet. Det indikerer at IL-6 er en faktor i patogenesen ved de fleste tilfeller av immun-mediert feber.

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